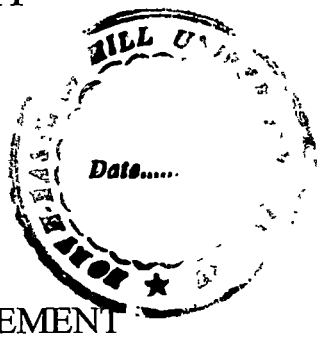
STUDY OF MOLECULAR CHANGES IN TUMOR SUPPRESSOR GENES DURING CARCINOGENESIS

(ABSTRACT)

BY

YASHMIN CHOUDHURY

DEPARTMENT OF BIOCHEMISTRY



SUBMITTED

IN PARTIAL FULFILMENT OF THE REQUIREMENT

OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOCHEMISTRY

NORTH-EASTERN HILL UNIVERSITY

SHILLONG, INDIA

MARCH, 2009

Carcinogenesis is a multistage process driven by carcinogen-induced genetic or epigenetic damage in cells that gain a selective growth advantage and undergo clonal expansion as a result of the activation of proto-oncogenes and/or inactivation of tumor suppressor genes. Animal model studies reveal that the process of carcinogenesis has three distinct stages, namely, initiation, promotion and progression. Initiation involves the exposure of normal cells to chemical, physical or microbial carcinogens that cause a genetic change (s) producing an irreversibly altered cell with both an altered responsiveness to the microenvironment and a selective clonal expansion advantage when compared to the surrounding normal cells. During promotion, the initiated cell undergoes selective clonal expansion leading to the appearance of a benign lesion or preneoplastic focus. The probability of conversion of an initiated cell to malignancy is substantially increased by further exposure to DNA-damaging agents which may activate protooncogenes and/or inactivate tumor suppressor genes leading to the progression of benign lesions to malignant cancers. Thus, the transition between the different stages of carcinogenesis is driven by environmental and endogenous factors and involves different biochemical mechanisms and genetic elements.

Studies indicate that the effect of a carcinogen or a mutagen may be transmitted transgenerationally either when exposure occurs *in utero* or when parents (most frequently fathers) are exposed prior to mating, leading to an increased risk of cancer in the progeny. Experimental evidence suggest that following the exposure of germ cells to a mutagen/ carcinogen, an initiating event could be inherited by subsequent generations and revealed after postnatal exposure to a mutagen/ carcinogen or non-genotoxic agent. Thus, the postnatal environment is crucial, as exposure to various environmental factors which may enhance the progression to cancer.

Tumor suppressor genes are genes whose loss-of-function releases the constraint on cell growth and is therefore tumorigenic. They normally function as physiologic barriers against clonal expansion or genomic mutability and are able to hinder the growth and metastasis of cells driven to uncontrolled proliferation, and are thus vulnerable sites for DNA damage. Tumor suppressor genes have been classified into two main types- 'gatekeepers" and "caretakers". Gatekeepers are genes that directly regulate the growth of tumors by inhibiting growth or promoting death through regulation of the cell-cycle.

The most widely studied gatekeeper tumor suppressor gene is the p53 gene, which is mutated in over fifty percent of human cancers. Caretakers are genes that maintain genomic integrity through their role in maintaining error-free DNA repair. Inactivation of a caretaker gene therefore does not promote tumor initiation directly. Instead it leads to genetic instability which results in increased mutation in all genes, including gatekeepers. Examples of caretaker genes are the breast cancer and ovarian cancer associated *BRCA1* and *BRCA2* genes.

Betel nut (BN), *Areca catechu L.* is a commonly used masticatory which is consumed by over 600 million individuals world-wide. The habit of BN chewing is believed to be strongly associated with cancers of the mouth, oropharyngeal cavity, and upper parts of the digestive tract in humans. The genotoxic and cytotoxic effects of BN powder, aqueous extract of betel nut (AEBN), its primary alkaloid, arecoline, and/or their nitroso derivatives, have been reported. AEBN was previously found to induce strand breaks in DNA of mouse kidney cells, unscheduled DNA synthesis (UDS) in Hep-2 cells *in vitro* and enhanced rate of cell proliferation. Teratogenic effects of chronic BN and arecoline exposures have also been reported in mice and rats. Arecoline was reported to cause general developmental retardation of zebrafish embryos predominantly due to a general cytotoxic effect induced by depletion of intracellular thiols. Furthermore, arecoline was reported to induce abnormality in the shape of sperm heads and unscheduled DNA synthesis (UDS) in the early spermatid stages of Swiss albino mice, and, to induce micronuclei formation in fetal mouse blood after transplacental exposure. Previous studies in Swiss Albino mice have shown that AEBN or arecoline induced DNA damage, affected cell cycle characteristics and induced qualitative changes in mice liver high mobility group (HMG) proteins similar to that induced by a hepatocarcinogen, diethylnitrosamine (DEN), leading to the development of preneoplastic nodules in the liver.

In the light of the above information, Swiss Albino mice chronically exposed to AEBN were selected as a model to study the combined response of the *p53*, *Brca1* and *Brca2* tumor suppressor genes in BN induced carcinogenesis. Further, the study also aimed to determine if BN has a transgenerational carcinogenic effect. For this purpose, the study was designed taking the significance of postnatal carcinogen exposure into consideration. Male and female 6 week old Swiss Albino mice were exposed to aqueous extract of

betel nut (AEBN) in drinking water at a dose of 2 mg ml^{-1} for 24 weeks. These mice are referred to as the previously unexposed P generation mice. The transgenerationally exposed mice received AEBN before and during mating. The F1 generation was raised by allowing P generation mice exposed to AEBN for 6 weeks to breed. Similarly, the F2 and F3 generations were raised from AEBN-exposed F1 and F2 mice, respectively.

It was observed that exposure to AEBN severely impaired the ultrastructure of the nucleus, endoplasmic reticulum and mitochondria, with a significant reduction in mitochondrial index from the P through F3 generations, indicating a progressive loss of apoptosis. It was observed that the different stages of AEBN- induced carcinogenesis in P generation Swiss Albino mice involved alterations in the levels of the p53, Brca1 and Brca2 tumor suppressor proteins in comparison to control level. Exposure to AEBN resulted in immediate upregulation of p53 protein to 2-2.5 fold that of age-matched control after 6-8 weeks of exposure, and upregulation of Brca1 and Brca2 proteins to 1.4 fold the age-matched control after 2 weeks of exposure. Subsequently, the p53 protein declined to control level, and the Brca1 and Brca2 proteins to 70 % that of the control after 16 weeks of exposure, concomitant with the appearance of preneoplastic nodules in the liver. In contrast, in the transgenerationally exposed mice, the level of p53 protein remained largely invariant in comparison to control, and the levels of Brca1 and Brca2 proteins declined rapidly below control level, without recording an initial increase. The appearance of preneoplastic nodules of the liver was significantly advanced, developing in F1 mice after 8 weeks, in F2 mice after 6 weeks and in F3 mice after 4 weeks of exposure, exhibiting progressively increasing genomic instability. DNA sequence analyses revealed no mutations in exons 5 and 7 the *p53* gene, and the amplified segment (nucleotides 1-257) of the *Brca2* gene in P, F1, F2 and F3 mice. A missense mutation (G→C) was observed in exon 11 of the *Brca1* gene in F1, F2 and F3 mice, but not in P mice. It can, therefore, be concluded that the *p53, Brca1* and *Brca2* tumor suppressor genes are intrinsically involved in the process of BN-induced carcinogenesis in mice, as well as in the transgenerational transmission of carcinogenic risk following AEBN exposure. The p53, Brca1 and Brca2 responses were abrogated in the mice exposed transgenerationally to AEBN.

Biomarkers are anatomic, physiologic, biochemical, or molecular parameters associated with the presence and severity of specific disease states. They are detectable and

measureable by a variety of methods including physical examination, laboratory assays and medical imaging. As an important biological indicator of cancer status and progression for the physiological state of the cell at a specific time, biomarkers represent powerful tools for monitoring the course of cancer and gauging the efficacy and safety of novel therapeutic agents. Malignant transformation involves alterations in protein expression with subsequent clonal proliferation of the altered cells. These alterations can be monitored at the protein level, both qualitatively and quantitatively. Protein signatures in cancer thus provide valuable information that may be an aid to more effective diagnosis, prognosis, and response to therapy. As part of the present study, we have investigated the levels of p53, BRCA1 and BRCA2 proteins in the peripheral blood lymphocytes (PBL) of patients suffering form breast cancer, cervix cancer and head and neck cancers, in comparison to levels in PBL of healthy volunteers, by slot-blot analysis, with the objective of determining whether their levels in PBL can be used as a biomarker of cancer.

The study of the p53, BRCA1 and BRCA2 tumor suppressor proteins is especially relevant because they are intricately involved not only in the process of carcinogenesis, but also in the response to various modes of cancer therapy. The presence of a p53 missense mutation indicated by an overabundance of p53 protein is considered as an unfavorable prognostic factor for various cancers, such as cancer of the breast, lung and gastric cancer. It has been reported that p53 can regulate the sensitivity to cancer therapies by affecting the expression of drug targets, the access of drugs to intracellular targets, and the response to DNA damage. The two major types of microtubule-interfering agents are the vinca alkaloids and the taxanes, and, mutations in the *p53* gene were reported to simultaneously increase the sensitivity to taxanes and decrease the sensitivity to vinca alkaloids. Mutation of p53 has also been reported to cause resistance to cisplatin. DNA-damaging drugs cause DNA double strand breaks either directly or indirectly, and it is widely accepted that the absence of *BRCA1* expression leads to hypersensitivity of cells to DNA-damage based chemotherapy using cisplatin and etoposide. The presence of *BRCA1* has also been reported to promote an increase in sensitivity to antimicrotubule agents.

In comparison to controls, the level of p53 protein was found to be upregulated in all cases of breast cancer (170 %) and cervix cancer (290 %). On an average, p53 protein

was also found to be upregulated in head and neck cancers (170 %). However, the level of p53 varied with the site of cancer among the head and neck cancer cases. Cancers of the tongue and base of tongue, lower lip, buccal mucosa, pre-epiglottis and epiglottis, pyriform sinus and external mouth cavity (class I) exhibited a level close to that of controls, while cancers of the oesophagus, larynx, vocal chord, throat and nasopharynx, and secondary cancer of neck nodes (class II) exhibited elevated levels. The levels of both BRCA1 and BRCA2 proteins were downregulated in PBL of breast cancer patients. The level of BRCA1 was 82 %, and the level of BRCA2 87 % that in PBL of respective controls. In contrast, an upregulation of BRCA1 to 184 % in cervical cancer, and 140 % in head and neck cancer was observed in comparison to control levels. BRCA2 was also upregulated to 180 % in cervical cancer, and 150 % in head and neck cancer in comparison to controls.

In light of the available information, the cancer of cervix and class I of head and neck cancer cases studied are likely to show resistance to cisplatin, doxorubicin and vinblastine as they had elevated level of p53 protein. The same cancers also had elevated BRCA1 leading to resistance to DNA-damage based chemotherapy using agents like cisplatin. Thus, these cancers are likely to respond better to taxanes. Class II cancers of the head and neck region displayed control level of p53, which would indicate increased sensitivity to cisplatin, doxorubicin and vinblastine. These cancers, however, also had an elevated level of BRCA1 suggesting resistance to DNA-damaging agents. Thus, if the p53 protein level or BRCA1 protein level are taken individually as a biomarker, they give rise to a conflict with regards to the mode of therapy which is likely to be effective. Similarly, the breast cancer cases studied displayed elevated level of p53 indicating resistance to cisplatin, doxorubicin and vinblastine, while their BRCA1 levels were downregulated, suggesting increased sensitivity to cisplatin and other DNA-damage-inducing cheomotherapeutic agents. Thus, in these cases too, the p53 level or the BRCA1 level taken individually, cannot serve as a biomarker to assess an effective mode of therapy. It, therefore, follows that while the level of a tumor suppressor protein in PBL can serve as a good biomarker, a combination of more than one tumor suppressor would permit a more comprehensive understanding of the potential response of a cancer to different therapeutic agents, rather than the use of a single tumor suppressor.

STUDY OF MOLECULAR CHANGES IN TUMOR SUPPRESSOR GENES DURING CARCINOGENESIS

BY

YASHMIN CHOUDHURY

DEPARTMENT OF BIOCHEMISTRY

SUBMITTED

IN PARTIAL FULFILMENT OF THE REQUIREMENT

OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOCHEMISTRY

NORTH-EASTERN HILL UNIVERSITY

SHILLONG, INDIA

MARCH, 2009

The North-Eastern Hill University

March, 2009

I, **Yashmin Choudhury**, hereby declare that the subject matter of this thesis is of the record done by me, that the content of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree to any other University / Institute.

This is being submitted to the North-Eastern Hill University for the degree of **Doctor of Philosophy** in **Biochemistry**.

Y Choudhury 23/3/2009

(YASHMIN CHOUDHURY)

Candidate

23/3/09

(PROF. A. ALAM)

Head

Department of Biochemistry

23/02/09

(PROF. R.N. SHARAN)

Supervisor

ACKNOWLEDGEMENT

As I take the final steps towards the completion of this thesis, I gratefully remember all those who have been instrumental in helping me reach this goal.

First and foremost, I thank the Almighty for His grace and blessings.

Words are inadequate to express my gratitude to my supervisor, Prof. R. N. Sharan. I feel privileged to have worked under his excellent guidance. He has always encouraged free expression of ideas while demanding the highest quality of work, and has the quality of being both kind and critical. From him I have learnt not only the intricacies of research, but also the requirement of untiring effort that goes into making a researcher.

I would like to thank Prof A. Alam, the head of the department of Biochemistry for allowing me to use the facilities in the department. I would also like to sincerely thank all the teachers in the department, Prof R. Lalthantluanga, Prof. A. N. Rai, Prof. R. Sharma, Dr. A. K. Singh, Dr. D. Syiem, Dr. M. B Syiem, Dr. L. Kma, Dr P. Ambasht and Dr S. Bhan for their encouragement and support. Several of them have been my teachers during the Masters course in Biochemistry, and they shaped my interest in research.

I record with gratitude the invaluable help extended to me by the non-teaching staff of the department, Smt. Kharkongor, Puspadi, Mr M. B. Singh, Mr B. Medhi, Bah Bani, Veronica and very specially Pynkup for helping me with innumerable tasks with the computer, and teaching me in the process.

I am grateful to Rennie for sharing with me the samples he collected from B. Barooah Cancer Institute, Guwahati, without which this thesis would be incomplete.

I thank the heads of the Department of Botany and the Department of Zoology for allowing me to use the facilities in their departments when required. I am grateful to Prof A. Chatterjee, Department of Biotechnology and Bioinformatics, for permitting me

to use the thermal cycler, and his students Avdhesh, Gitanjali and Biplob. I am grateful to Prof A. K. Mishra and Prof N .K. Churungoo for permitting me to use the gel-documentation systems, and to Cressida, Shiny, Nabanita, David, Meena, Mipeshwari and Jubanri. I am also indepted to Bah Proforma for his invaluable help with histological studies.

I am grateful to the Head, Sophisticated Analytical Instrumentation Facility, NEHU, to Jos, and to Smt B. Dkhar for lending excellent support and technical expertise for the transmission electron microscope studies. I am also grateful to the Bioinformatics Centre, NEHU, for provided help for literature survey.

The Radiation and Molecular Biology Unit of the department of Biochemistry is now a second home to me, and all its members are close to my heart. I will miss the warmth, the laughter, the hard work, odd hours and "working" holidays. I am grateful to Pathaw for introducing me to the world of mice. I will always cherish the intense scientific and non-scientific discussions with Dr Meriyani and Dr Chaitali, and their guidance as seniors. I am grateful to Rennie, Dr Sanghamitra, Banri, Khet, Dannis and Judap for their warmth and all their help. I am reluctant to leave the lab because I will miss all of you.

I would like to acknowledge the help received from my seniors in the department Dr Nonibala, Rajkumar, Khup, Ashima, Dr Nakhuru, and my friends Danswrang, Thomas, Piyali, Daniel, Lux, Preeticia, Kit, Norna and Jignasa. The times shared with all of you will always be remembered. I will especially miss the tea-times.

I am grateful to the Principal, St. Edmund's College, Shillong, and the teachers of the department of Biochemistry in the college, Dr A. Debroy, Dr O. Kyndiah, Dr J. Wahlang and Dr H. Ranhotra, where I was employed during the initial months of my research, for their encouragement and guidance.

I am grateful to the Principal, St. Anthony's College, Shillong, Fr. I. Warpakma, where I am currently employed for his encouragement and keen interest in the progress of my research work. I will always be indebted to my seniors and colleagues in the departments of Biotechnology and Bioinformatics of the college, Dr M. A. Laskar, Dr

H. G. Lyngdoh, Dr L. I. Singha, Ivee, Saiba, Thyan, Morgen, Inda, Phiba and Dr Meriyani. They have always ensured that I could devote sufficient time to my research, and have encouraged me to persevere in spite of the constraints of time and other difficulties. I sincerely feel I would have been unable to reach the completion of my work without their support. Words are insufficient to thank them for this. I thank all my students for co-operating with me, and enabling me to handle the pressures of research along with my teaching assignments. I also fondly remember Mitali.

My parents and family have played a vital role in my pursuits, and their support and encouragement have given me the strength to complete my work. I cherish the love, blessings, prayers and endurance of my parents, and the faith and affection of my brother and sister. I fondly remember my grandfather and know his blessings are always with me.

I am grateful to Eros for his support and encouragement. Thank you for teaching me to believe in myself.

I would like to thank my friends Dr Malabika, Sucharita, Farhana, Parvati, Amlana and Sushila for their patience and good wishes. I will be failing in my duties if I do not acknowledge the contribution of all my teachers through the years, who have shaped me personally and professionally. I would like to thank all of them.

Financial assistance received from UGC in the form of JRF and SRF is duly acknowledged.

Yashmin Choudhury

TABLE OF CONTENTS

ABBREVIATIONS

% C	Percent crosslinker
% T	Percent total monomer
°C	Degree Celsius
µg	Microgram
µg μl^{-1}	Microgram per microlitre
µl	Microlitre
µm	Micron
Å	Ångström
AAEBN	Acetic acid extract of betelnut
Abl	Abelson murine leukemia viral oncogene homolog 1
AEBN	Aqueous extract of betelnut
AGE	Agarose Gel Electrophoresis
AIP1	Actin interacting protein 1
ALL1	Acute lymphoblastic leukemia 1
AR	Aromatase
Asp	Aspartate
ATM	Ataxia telangiectasia mutated
ATR	Ataxia-telangiectasia mutated and rad3-related
BAP1	BRCA1-associated protein-1
BARD1	BRCA1-associated RING domain 1
BAX	BCL2-associated X protein
bcl2	B-cell lymphoma 2
Bcr	Breakpoint cluster region
bp	Base pair
BQ	Betel quid
BRCA1	Breast cancer 1, early onset
Brca1	Murine breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
Brca2	Murine breast cancer 2, early onset
BRCT	BRCA1 C-terminal
BSNA	Betel nut specific nitrosamines
BUBR1	BUB1 (budding uninhibited by benzimidazoles 1)-related protein kinase
CA	Chromosomal Aberration
Ca^{2+}	Calcium ion
CAF1	Chromatin assembly factor I
CBP	CREB *(cAMP response element binding)* binding protein
Cdc	Cell division cycle

CDKN2A	Cyclin dependent kinase 2a
c-erbB	Cellular erythroblastic leukemia oncogene homolog 2
CHAF1,CHAF2	Chromatin assembly factor 1 gene, Chromatin assembly factor 2 gene
CHEK2	CHK2 checkpoint homolog (*S. pombe*)
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CHO	Chinese hamster ovary
cM	centi- Morgan
c-myc	Cellular myelocytomatosis oncogene
COOH-	Carboxy
COX-2/PTGS2	Cyclooxygenase-2/ Prostaglandin-endoperoxide synthase 2
CpG	Cytosine-phosphate-guanine
CSFs	Colony-stimulating factors
c-sis	Simian sarcoma viral oncogene homolog
CtIP	CtBp (*C-terminal binding protein*)-interacting protein
CYP17	Cytochrome P450, subfamily XVII (steroid 17-alpha-hydroxylase)
Cys	Cysteine
Dbl	MCF2 cell line derived transforming sequence (oncogene)
DEN	Diethylnitrosamine
DNA	Deoxyribonucleic acid
E1A	Early region 1A
E6	Early protein 6
E7	Early protein 6
EEBN	Ethanol extract of betelnut
EGFR	Epidermal growth factor receptor
ESCC	Esophageal squamous cell carcinoma
F1	First filial generation
F2	Second filial generation
F3	Third filial generation
FANCG	Fanconi anemia complementation group G
Fas/APO1	TNF (tumor necrosis factor) receptor superfamily, member 6/ Apo-1 antigen
FGF	Fibroblast growth factor
Fig.	Figure
FIHT	Fragile histidine triad
Fos	v-fos FBJ murine osteosarcoma viral oncogene homolog
g	Centrifugal force
g	Gram
G1	Gap 1

G1/S	Gap 1/Synthesis
G2	Gap 2
G2/M	Gap 2/ mitosis
GADD45	Growth arrest and DNA damage-inducible gene
GCR	Gross chromosomal arrangement
GDF15/MIC-I	Growth differentiation factor 15/ Macrophage inhibitory cytokine 1
GEF	GTPase exchange factors
GK	Gingival keratinocyes
GSH	Glutathione
H & E	Hematoxylin and eosin
H4	Histone 4
HADC	Histone deacetylase
hBUBR1	Human BUBR1
HEBN	Hydrochloric acid extract of betelnut
Hep2	Cell line established from human laryngeal carcinoma
HGF	Human gingival fibroblasts
HMG	High mobility group
hMLH	Human *Mut L* homolog
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HR	Homologous recombination
H-*ras*	Harvey-retrovirus associated DNA sequence
hsp 70	Heat shock protein 70
human KB epithelial cell	cell line derived from a human carcinoma of the nasopharynx
ICR mice	Imprinting Control Region mice
IGF-1	Insulin-like growth factor 1
IGF-2	Insulin-like growth factor 2
IGF-BP	Insulin-like Growth Factor Binding Protein
IGF-BP3	Insulin-like Growth Factor Binding Protein 3
IgG	Immunoglobulin G
int-I	Integrase
kDa	Kilo Dalton
KILLER/ DR5	Tumor necrosis factor receptor superfamily, member 10b /Death receptor 5
Ki-*ras*/ K-*ras*	Kirsten-retrovirus associated DNA sequence
KRAB	Kruppel-associated box
KS3/fgf4	Fibroblast growth factor 4
LINE	Long interspersed nuclear element
LOH	Loss of heterozygosity

M	Molar
MAP4	Microtubule-associated protein
mdm2	Murine double minute 2
MER	Medium reiteration frequency repeats
mg	Milligram
mg ml^{-1}	Milligram per millilitre
min	Minute
ml	Millilitre
MLL	Mixed lineage leukemia
mm	Millimetre
mM	Millimolar
MNPA	Methylnitrosaminopropionaldehyde
MNPN	3-methylnitrosaminopropionitrile
Mos	v-mos Moloney murine sarcoma viral oncogene homolog
MRE11	Meiotic recombination 11 homolog
MW	Molecular weight
N	Normal
NBS-1	Nijmegen breakage syndrome 1 (nibrin)
NER	Nucleotide excision repair
ng	Nanogram
NGC	N-nitosoguvacine
NGCO	N-nitrosoguvacoline
NHEJ	Nonhomologous end joining
NLS	Nuclear localization signal
nm	Nanometre
N-myc	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived
N-*ras*	Neuroblastoma retrovirus associated DNA sequence viral (v-ras) oncogene
NT	Nucleotide
O/N	Overnight
–OH	Hydroxyl group
OSCC	Oral squamous cell carcinoma
OSF	Oral submucous fibrosis
P	Previously unexposed generation
p14ARF/$p16^{INK4a}$	Cyclin-dependent kinase inhibitor 2A
p21	Cyclin-dependent kinase inhibitor 1A
p300	E1A binding protein p300
p53	Tumor suppressor protein p53
PBL	Peripheral blood lymphocytes

PCNA	Proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
PDGF	Platelet derived growth factor
Pidd	p53-induced protein with a death domain
PIG3	Tumor protein p53 inducible protein 3
pim-I	Pesticin immunity protein-I
pmol	Picomole
PTEN	Phosphatase and tensin homolog
raf-I	v-raf-1 murine leukemia viral oncogene homolog 1
RB	Retinoblastoma
RER	Rough endoplasmic reticulum
RHA	RNA helicase A
RNase	Ribonuclease
ROS	Reactive oxygen species
RPA	Replication protein A
RT	Room temperature
SC	Spleen cells
SCC	Squamous cell carcinoma
SCE	Sister chromatid exchange
SD	Standard Deviation
SDS-PAGE	Sodium Docecyl Sulphate-Poly Acrylamide Gel Electrophoresis
Sec	Second
Ser	Serine
–SH	Thiol group
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
SSA	Single-strand annealing
STK11/LKB1	Serine/threonine kinase 11
SW1/SNF	Switch/ Sucrose non fermentable (chromatin remodeling complex)
TEM	Transmission Electron Microscope
TFIIH	Transcription Factor II H
TGF-α	Transforming growth factor, alpha
U	Unit
U μl^{-1}	Unit per microlitre
UDS	Unscheduled DNA synthesis
UV	Ultraviolet
V	Volts
v /v	Volume/volume
vav	vav 1 guanine nucleotide exchange factor

VEGFR1, VEGFR2 and VEGFR3	Vascular endothelial growth factor receptor 1, 2, 3
w/v	Weight/volume
Wee-1	WEE 1 homolog 1 (S. pombe)
WH	Whole homogenate
X	Times
XRCC9	*X*-ray *R*epair *C*ross *C*omplementing
ZBRK1	Zinc finger protein 350
χ^2	Chi-square

INTRODUCTION

1.1. AN OVERVIEW OF CANCER

Cancer is an umbrella term covering a plethora of conditions characterized by unscheduled and uncontrolled cellular proliferation. It can develop in almost any mammalian organ giving rise to a wide array of clinical outcomes (Ponder, 2001). Cancer cells differ from normal cells in various aspects. Some of the earliest differences to be discovered were in their patterns of enzymatic activity. In the 1920's, Otto Warburg found that there was a general trend towards an increased rate of glycolysis in tumor cells. The conversion of glucose to lactic acid in the presence of oxygen is called the "Warburg effect" or aerobic glycolysis (Gatenby and Gillies, 2004; Kim and Dang, 2006). Experimental evidence for the enhanced glycolysis in cancer cells was further affirmed by the widespread clinical success of positron electron microscopy using fluorodeoxyglucose (Czernin and Phelps, 2002). The enzymatic cascades for oxidative metabolism are present in the mitochondria, and it has therefore been suggested that the disruptions of oxidative metabolism seen in malignant tumors may be the result of damage to mitochondria (Ruddon, 2007). The mitochondria also play a key role in apoptosis and alterations in these mitochondria-mediated events have been reported in cancer cells.

The "deletion hypothesis" of cancer proposed in 1947 by Miller and Miller suggested that carcinogenesis resulted from a permanent alteration or loss of protein essential for control of growth (Ruddon, 2003). In 1958, Potter proposed the "feedback deletion hypothesis" which suggested that the proteins lost during carcinogenesis may be involved in the feedback control of enzyme systems required for cell division. In this hypothesis, Potter postulated that "repressors" crucial to the regulation of genes involved in cell proliferation are lost or inactivated by the action of oncogenic agents on the cell, either by interacting with DNA to block repressor-gene transcription, or by reacting directly with repressor proteins and inactivating them. This prediction anticipated the discovery of tumor-suppressor proteins, such as p53 and RB, by about 25 years (Ruddon, 2003). In 1977, Weber formulated the "molecular concept of cancer" which states that "the biochemical strategy of the genome in neoplasia could be identified by elucidation of the pattern of gene expression as revealed in the activity, concentration, and isozyme aspects of key enzymes and their linking with neoplastic transformation and progression." A number of enzyme activities that Weber

and others have found to be altered in malignant cells are those involved in nucleic acid synthesis and catabolism. In general, the key enzymes in the *de novo* and salvage pathways of purine and pyrimidine biosynthesis are increased and the opposing catabolic enzymes are decreased during malignant transformation and tumor progression (Ruddon, 2003). Hanahan and Weinberg have suggested that cancer is the result of six essential alterations in cell physiology namely self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. These capabilities are acquired by and common to most human tumors, and have been referred to as the "Hallmarks of Cancer" (Hanahan and Weinberg, 2000).

Various distinguishing phenotypic alterations of cells transformed *in vitro* and cancer cells growing *in vivo* have been identified (Ruddon, 2003). These characteristics are not exclusive, and some may be seen both in transformed cells in culture, as well as in tumors growing *in vivo* in experimental animals or patients. The important properties of cancer cells *in vivo* are as follows: -

a. Activation of proto-oncogenes or increased expression of oncogene protein products.
b. Loss of tumor suppressor gene function.
c. Alterations in DNA methylation patterns.
d. Genetic imprinting errors that lead to overproduction of growth-processing substances (eg, IGF-2).
e. Increased or unregulated production of growth factors (eg TGF-α), tumor angiogenesis factors, and hematopoietic growth factors (eg, CSFs, interleukins).
f. Genetic instability leading to progressive loss of regulated cell proliferation, increased invasiveness, and increased metastatic potential.
g. Alteration in enzyme patterns- Malignant cells have increased levels of enzymes involved in nucleic acid synthesis and produce higher levels of lytic enzymes (eg, proteases, collagenases, glycosidases).
h. Production of onco-developmental gene products- Many cancers produce increased amounts of oncofetal antigens (eg, carcinoembryonic antigen), placental hormones (eg, human chorionic gonadotropin), or placental-fetal type isoenzymes (eg, placental alkaline phosphatase).

i. Ability to avoid the antitumor immune response of the host.

According to a statistical estimate of the global cancer burden, there were 10.9 million new cases of cancer, 6.7 million cancer-related deaths, and 24.6 million persons living with cancer in the year 2002. The most prevalent cancer in the world was breast cancer with 4.4 million survivors within a period of five years after diagnosis, while the other commonly diagnosed cancers were lung cancer (1.35 million) and colorectal cancer (1 million), and the most common causes of cancer death were lung cancer (1.18 million deaths), stomach cancer (700,000 deaths), and liver cancer (598,000 deaths). Striking variations were observed in the risk of different cancers by geographic area, most of which were due to exposure to known or suspected risk factors related to lifestyle or environment (Parkin *et al.*, 2005).

1.2. CARCINOGENESIS

Carcinogenesis is a multistage process driven by carcinogen-induced genetic or epigenetic damage in cells that gain a selective growth advantage and undergo clonal expansion as a result of the activation of proto-oncogenes and/or inactivation of tumor suppressor genes (Harris, 1991). Animal model studies reveal that the process of carcinogenesis has three distinct stages, namely, initiation, promotion and progression (Pitot *et al.*, 1991) (Fig. 1.1)

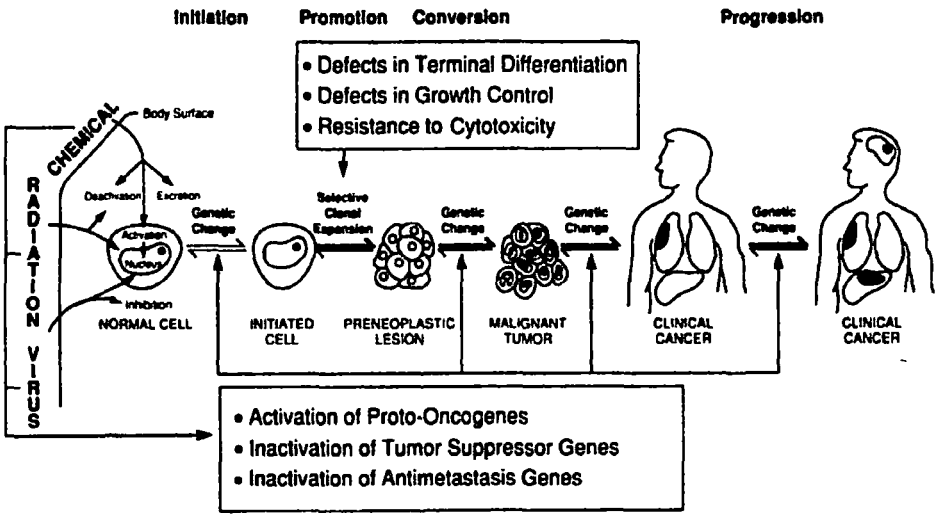


Figure 1.1. Carcinogenesis is a multistage process involving multiple genetic and epigenetic changes in proto-oncogenes, tumor suppressor genes, and anti-metastasis genes (Harris, 1991).

Initiation involves the exposure of normal cells to chemical, physical or microbial carcinogens that cause a genetic change (s) producing an irreversibly altered cell with both an altered responsiveness to the microenvironment and a selective clonal expansion advantage when compared to the surrounding normal cells (Harris, 1991). During promotion, the initiated cell undergoes selective clonal expansion leading to the appearance of a benign lesion or preneoplastic focus. Promotion involves at least some epigenetic factors that selectively influence the proliferation of the initiated cell and enhances the probability of accumulation of additional genetic damage including endogenous mutations in the expanding population of neoplastic cells. The probability of conversion of an initiated cell to malignancy is substantially increased by further exposure to DNA-damaging agents which may activate protooncogenes and/or inactivate tumor suppressor genes leading to the progression of benign lesions to malignant cancers. The necessity for a malignant cell to acquire multiple, heritable alterations at independent genetic loci may account for the long latency period of cancer (Barrett, 1993). Malignant cells continue to exhibit phenotypic alterations such as abnormal structure and number of chromosomes, gene amplification and gene expression (Harris, 1991; Barrett, 1993). Thus, the transition between the different stages of carcinogenesis is driven by environmental and endogenous factors and involves different biochemical mechanisms and genetic elements (Weinstein, 1988).

Genomic stability ensures cellular homeostasis and genetic continuity in multicellular organisms. However, cellular genomes are continuously exposed to endogenous and environmentally induced damage that can alter the informational integrity of cellular DNA. Neoplastic cells typically possess numerous genetic alterations, namely, point mutation, gene amplification, chromosomal rearrangement and aneuploidy. It has therefore been suggested that the acquisition of genomic instability is an early step in the process of carcinogenesis, and neoplasia may, in fact, represent a delayed phenotypic manifestation of genomic instability. Since genomic instability can predispose a cell to accumulate stable DNA mutations at a greater rate than normal, the efficacy of the DNA repair mechanisms in a cell is of paramount importance, and studies suggest that genetic predisposition to cancer generally involves dysregulation of cellular processes that directly or indirectly affect the cell's ability to repair damaged DNA as observed in patients suffering from Xeroderma pigmentosum, ataxia telangiectasia, Fanconi anemia and Bloom Syndrome (Coleman and Tsongalis, 1995).

Most, if not all, types of cancers may arise spontaneously due to the low level of spontaneous errors during DNA replication and normal cell division (Barrett, 1993). It has therefore been hypothesized that cell proliferation may itself be carcinogenic, and some carcinogens which fail to produce a detectable mutagenic activity may operate exclusively by increasing cell proliferation. Cell proliferation may influence carcinogenesis by a number of mechanisms, as detailed in Table 1 (Barrett, 1993).

Table 1: Mechanisms by which chemicals affecting cell proliferation might influence carcinogenesis

Serial No.	*Mechanism*
1	Increase fixation and expression of premutagenic DNA lesions
2	Increase the number of initiated cells occurring spontaneously during cell replication
3	Increase the number of spontaneous initiated cells by blocking cell death/ elimination
4	Increase the number of initiated cells by perturbing checkpoints in the cell cycle leading to mutagenic events
5	Increase the rate of neoplastic progression by previous four mechanisms
6	Promote clonal expansion of initiated cells

Today, it is widely accepted that epigenetic patterns are profoundly altered in neoplasia, and major disruption of DNA methylation, histone modification and chromatin organization play a crucial role in human cancer (Jones and Baylin, 2002; Esteller, 2006). Two apparently contrasting phenomena of DNA methylation coexist in the cancer cell: hypermethylation of CpG islands located in the promoters of tumor suppressor genes such as $p16^{INK4a}$, *BRCA1* and *hMLH* (Esteller, 2006); and, hypomethylation predominantly in repetitive and endoparasitic sequences of DNA which has been linked to the generation of chromosomal instability (Esteller, 2006). Histones are another key player in epigenetics. Generally, the acetylation of histones marks active, transcriptionally competent regions, whereas hypoacetylated histones are found in transcriptionally inactive euchromatic or heterochromatic regions (Egger *et al.*, 2004). Histone methylation can be a marker for both active and inactive regions of chromatin. Human tumors commonly undergo an overall loss of monoacetylation of lysine 16 and trimethylation of lysine 20 in the tail of histone H4 (Fraga *et al.*, 2005).

1.3. TRANSGENERATIONAL CARCINOGENESIS

Studies pertaining to the exposure of germ cells to radiation and chemical mutagens and the consequent adverse effects such as cancer, malformation and abortion etc in the offspring derived from these exposed germ cells, are referred to as "Transgenerational Carcinogenesis and Teratogenesis", "Paternal Toxicology" or "Male-mediated Developmental Toxicology". The preconceptional exposure of females has also been reported to induce such effects in the offspring (Nomura, 2008). The suggestion that prezygotic exposure to a carcinogen or mutagen may lead to an increased risk of cancer in the progeny is derived from experimental and epidemiological observations (Tomatis, 1994). Such transgenerational transmission of the effect of a carcinogen may occur either when exposure occurs *in utero* or when parents (most frequently fathers) are exposed prior to mating (Yamasaki *et al.*, 1992). The experimental evidence for transgenerational transmission of an increased risk of cancer was obtained using various approaches as listed in Table 2.

Table 2. Transgeneration effect of carcinogens: experimental evidence (adapted from Tomatis, 1994)

1. Treatment during pregnancy and follow-up for several generations		
Agent	Species (strain)	Tumors observed in descendants
DMBA	Mouse (Swiss, MA)	Various sites in F1-F2
NMUt	Rat (WKA)	Various sites in F1-F3
NMU	Rat (BD)	Tumors of nervous tissue in F1-F2
ENU	Rat (BD)	Tumors of kidney, CNS, mammary gland in F1-F3
DES	Mouse (CD-1)	Uterine and adenocarcinomas in F2 females
DEN	Hamster	Respiratory tract tumors in F1. No effect in F2-F3
BaP	Mouse (A)	Increased multiplicity in lung tumors in F1-F5
DES	Mouse (CBA)	Uterine sarcomas, ovarian tumors in F2 females
2. Treatment of males prior to mating with untreated females		
Agent	Species (strain)	Tumors observed in descendants
X-rays, urethane	Mouse (ICR)	Lung tumors in F1-F3 (ovarian tumors, leukemia)
ENU	Rat (BD)	Tumors of nervous tissue in F1
ENU	Mouse (AKR/B6)	Multiple intestinal neoplasia
Neutron irradiation	Mouse (C3H)	Liver tumors in F1

3. Initiation and promotion			
Agent	Species (strain)	Treatment	Tumors observed in descendants
X-rays	Mouse (ICR)	Male parent prior to mating + urethane in F1-F2	Multiplicity of lung tumors

DMBA	Mouse (SHR)	DMBA to pregnant mothers + TPA in F1-F2	Skin tumors
X-rays	Mouse (SHR)	Total body irradiation of males before mating + urethane to F1	Multiplicity of lung tumors
DMBA	Mouse (SHR)	DMBA in pregnant mothers + TPA in F1 and F2	Skin tumors

BaP-benz[α]pyrene, **DEN**-N-nitrosodiethylamine, **DES**-diethylstilbestrol, **DMBA**–7, 12-dimethylbenz[α]anthracene, **ENU**-ethylnitrosourea, **NMU**-methylnitrosourea, **NMUt**-nitrosomethylurethane, **TPA**-12-O-tetradecanoylphorbol-13-acetate

The first attempt to amplify the effect of *in utero* or transplacental exposure to a carcinogen by postnatal exposure to another carcinogen was made by Goerttler *et al.* in 1980. After exposure *in utero* to DMBA (Table 2), exposed offspring were painted with a tumor promoting agent TPA, when an increased number of skin tumors were found to be induced (Goerttler *et al.,* 1980). The transplacental initiation-postnatal promotion protocol was subsequently used in various experimental models which verified that *in utero* exposure to a carcinogen resulted in the initiation of carcinogenesis, leading to an increased incidence of cancer in the offspring following postnatal exposure to a promoting agent (Napalkov *et al.,* 1987; Yamasaki *et al.,* 1987; Diwan *et al.,* 1989, 1992 and 1995; Rice *et al.,* 1989; Loktionov *et al.,* 1992; Waalkes *et al.,* 2004). Similarly, Nomura (Nomura, 1983) and Vorobtsova and Kitaev (Vorobtsova and Kitaev, 1988) reported that large clusters of lung tumor nodules developed when the offspring of X-irradiated mice were postnatally exposed to urethane (Table 2). These results suggest that following the exposure of germ cells to a mutagen/ carcinogen, an initiating event could be inherited by subsequent generations and revealed after postnatal exposure to a mutagen/ carcinogen or non-genotoxic agent (Tomatis *et al.,* 1992). Extrapolation of this information to humans may have some important implications as it suggests that while *in utero* exposure may lead to initiation of carcinogenesis in some cells, tumor formation will result only if there is exposure to a tumor promoter later in life (Autrup, 1993). Thus, the postnatal environment is crucial, as we are exposed throughout our lifetime to various environmental factors which may enhance the progression to cancer (Tomatis *et al.,* 1992).

Epidemiological evidence of a transgenerational effect of carcinogens is mainly derived from studies on childhood cancer, in relation to parental occupational exposures to chemicals, or occupational and non-occupational exposure to radiation

(Tomatis, 1994). These studies mostly consider paternal exposure because it is often impossible to distinguish between pre- and post-conception maternal exposure (Tomatis, 1994). Data supporting an association between occupational exposure and childhood cancer is listed in Table 3.

Table 3. Transgeneration effect of carcinogens: epidemiological evidence (adapted from Tomatis, 1994)

1. Studies on total cancers			
Paternal occupation	**Agent (s) involved**	**Tumor types**	**Odds ratio**
Motor vehicle mechanics, machinists	Hydrocarbons, lead	Leukemia	1.2-2.5
Metal workers, farmers	?	Kidney tumors	2.5-5.0
Paper and pulp industry	?	Brain tumors	2.8-4.6
2. Case control studies			
Paternal occupation	***Agent (s) involved***	***Tumor types***	***Odds ratio***
Motor vehicle mechanics Involving exposure to exhaust fumes Service station attendants Manufactures of machines, aircraft	? ? ? Chlorinated solvents, cutting oils	All leukemias	2.0-2.4
Mechanics Service station attendants Welders, machinists	Lead, hydrocarbons Aromatic hydrocarbons Metals	Wilms' tumor	4.0-7.5
Aircraft industry Electricians Involving exposure to electromagnetic fields	Solvents, paints, low frequency fields	Brain tumors	2.0-8.0
Welders, machinists	Metals	Retinoblastoma	1.6
Aircraft production, service station attendants	Chlorinated solvents, cutting oils, aromatic hydrocarbons	Seminomas	2.0-5.3
Involving exposure to metals	Metals	Hepatoblastoma	3.0
3. Exposure to radiations			
Preconception paternal exposure	**Total dose**	**Tumors in progeny**	**Odds ratio**
X-rays	1- >21 diagnostic films	Leukemia	1.31 (RR)
Radiation-related occupations	Unknown	Bone tumors Wilms' tumors	5.35 2.48
Industrial exposure radiation	Unknown	Tumors of CNS	1.7-2.1
Nuclear plant	100 mSv	Childhood leukemia	8.4
Various occupations	Exposure to radionuclides	All cancers Leukemia	2.70 2.75
Occupational exposure of fathers to radiation	0.1-10 mSv	No association found	-
Occupational exposure of fathers to radiation	>0.1mSv	No association found	-
Preconception maternal exposure to X-rays	1- >21 diagnostic films	Leukemia	1.7 (RR)
Preconception maternal exposure to X-rays	Unknown	Childhood cancers	2.6
Preconception exposure of both parents (atomic bomb)	0.37-3.8 Sv	No increase in the incidence of cancer in the first 2 decades of life	-

RR- Relative Risk or Risk Ratio, Sv- Sievert, mSv-millisievert

The sensitivity of germ cells to mutagens varies, depending on the stage of development, and studies reveal that male germ cells are more sensitive to X-rays and chemical mutagens in the post-spermatogonial stages, than spermatogonial germ cells (Tomatis *et al.,* 1992). This varying sensitivity of germ cells at different stages of maturation to carcinogens may depend upon the expression of specific target genes. The protooncogenes *raf-I, Ki-ras* and *fos* are found to be predominantly expressed in the spermatogonia stage, whereas *mos, abl, int-I, pim-I* and *N-ras* are expressed in the post-meiotic haploid spermatids (Tomatis et al., 1992). Studies suggest that induced germ cell changes causing tumors in the offspring of irradiated and urethane-exposed ICR and N5 mice do not correlate with translocations and gross chromosomal changes, but the possibility that smaller genetic changes may be involved has not been excluded (Nomura, 2006). The *ras, mos* and/or *abl* oncogenes were found to be amplified in undifferentiated multiple tumors and lymphocytic leukemia in the offspring of X-irradiated N5 mice. Some tumors were also found to contain the *mos* and *cot* oncogenes, and, *p53* mutations were detected in a glioma of X-irradiated N5 progeny. Known and new oncogenes were therefore found to be activated in the tumors of the descendants of X-irradiated N5 parents (Nomura, 2006). The possibility that mutant tumor suppressor genes can be transmitted through germ cells has been shown in humans in case of the Li-Fraumeni syndrome which is a syndrome involving predisposition to cancer that is caused by germinal mutation of the *p53* gene, and the hereditary form of retinoblastoma which is due to a point mutation in the *RB* gene (Tomatis *et al.,* 1992). Thus, while experimental evidence is still limited, the inactivation of tumor suppressor genes appears to play a vital role in the transgenerational transmission of cancer risk.

1.4. GENES INVOLVED IN CARCINOGENESIS

The importance of genetic alterations in somatic cells in the development of cancer was predicted by Bauer in 1928 (Sugimura *et al.,* 1992). The first evidence that cancer arises from somatic genetic alterations subsequently came from studies of Burkitt's lymphoma. Transfection experiments have shown that mouse fibroblasts undergo transformation when transfected *in vitro* with human DNA. It has also been found that transgenic mice which carry an activated oncogene from a human tumor develop

cancers that resemble the human tumor. These cancers, however, appear only after a latent period of time suggesting that alterations in other genes must occur before progression to neoplasia may occur, and that activation of the oncogene is necessary, but insufficient for the development of overt cancer (Croce, 2008). A current paradigm is that the multistage process through which a cancer develops reflects the progressive acquisition of mutations in at least two classes of genes-dominant activating mutations in growth enhancing genes (oncogenes) and inactivating recessive mutations in growth inhibitory genes (tumor suppressor genes) (Weinstein, 2000). These multiple genetic alterations in carcinomas are presumably caused by DNA damage due to exposure to endogenous factors and multiple kinds of environmental agents and xenobiotics, and, the alterations show some specificity depending on the organ of origin, histology, and clinical stage of the cancer (Sugimura *et al.*, 1992). Increased emphasis on the role of genetic predisposition in cancer began in the 1980s, and population-based epidemiological studies showed that genetic effects might account for a substantial fraction of cancer incidence (Balmain, 2003).

1.4.1. ONCOGENES

Oncogenes result from the inappropriate activation of proto-oncogenes, and their products can lead to the transformation of a normal cell. Proto-oncogenes were initially identified as the transduced genes of acute transforming retroviruses (viral oncogenes, *v-onc*), and a number of them have cellular counterparts (cellular oncogenes, *c-onc*) (Anderson, 1992). The proteins encoded by proto-oncogenes play a crucial role in cellular growth and differentiation, and in apoptosis of a normal cell. They are expressed during processes of regulated growth such as embryogenesis, wound healing, regeneration of damaged liver, and stimulation of cell mitosis by growth factors. The generation of an oncogene may involve gain-of-function mutation, gross DNA rearrangements such as translocation and gene amplification leading to constitutive activation or overexpression of a proto-oncogene, or a failure to turn off expression of the proto-oncogene at the appropriate time. The oncogenes subsequently produce proteins which function as transcription factors, chromatin remodelers, growth factors, growth factor receptors, signal transducers and apoptosis regulators (Croce, 2008).

a. Transcription factors

Chromosomal rearrangements frequently lead to the activation of transcription-factor genes in lymphoid cancers, such as the development of Burkitt's lymphoma due to the three variant translocations of the *c-myc* gene from 8q24 to 14q32, 22q11 and 2p12 leading to its juxtaposition with immunoglobulin loci (Croce, 2008). Oncogene activation of the *c-myc* gene may also occur through retroviral insertion and gene amplification.

b. Chromatin remodelers

Modifications in the degree of compaction of chromatin play a critical role in the control of gene expression, replication and repair, and chromosome segregation (Croce, 2008). In acute lymphocytic leukemia and acute myelogenous leukemia, the *ALL1* (*MLL* for mixed lineage leukemia) gene is found to be interrupted in the vast majority of translocations and inversions involving the chromosome band 11q23, and is involved in at least the following variant translocations: t(4;11), t(9;11), t(6;11), t(11;19), t(1;11), t(10;11), t(11;16), t(11;17), t(11;23) and t(X;11) with different partner genes (Cimino *et al.,* 1998). These fusions result in chromatin remodeling by altering the recruitment of proteins such as the SW1/SNF complex by ALL1 which alter chromatin structure in an ATP-dependent fashion, and by fusion with genes such as *CBP* and *p300* which encode histone acetylase activity (Redner *et al.,* 1999).

c. Growth factors

The constitutive activation of a growth factor can lead to malignant transformation. The oncogene *c-sis* codes for the β chain of the platelet derived growth factor (PDGF), which can induce proliferation of various cell types. Overexpression of PDGF leads to transformation of fibroblasts containing PDGF receptors. Similarly, the *KS3* gene which is a member of the fibroblast growth factor (FGF) family is constitutively expressed in Kaposi's sarcoma (Croce, 2008).

d. Growth factor receptors

Aberrant growth stimulation by growth factors due to alterations in the growth factor receptors is observed in various cancers. In squamous cell carcinomas, a deletion of the ligand-binding domain of the epidermal growth factor receptor (EGFR), a transmembrane protein with tyrosine kinase activity, causes constitutive activation of

the receptor in the absence of ligand binding. The activated receptor subsequently phosphorylates tyrosine residues in the intracellular domain of the receptor, providing interaction sites for cytoplasmic proteins having the SRC homology domain, which leads to altered signaling in many pathways. Gene amplification and activating mutations in the EGFR gene, *c-erbB*, also occur in lung and breast cancer and gastrointestinal stromal tumors. Similarly, constitutive activation of the vascular epidermal growth factor receptors VEGFR1, VEGFR2 and VEGFR3 stimulates angiogenesis in a variety of cancers (Croce, 2008).

e. Signal transducers

Many oncogenes encode members of signal transduction pathways. They fall into two main categories: non-receptor protein kinases and guanosine-triphosphate-binding proteins. Non-receptor protein kinases are of further two types- tyrosine kinases such as *src, lck* and *abl* and serine/ threonine kinases such as *mos*, *raf* and *pim1*. The guanosine-triphosphate-binding proteins may be membrane-associated G proteins such as *ras* and *braf* and GTPase exchange factors (GEF) such as *vav* and *dbl*. The *src* gene is constitutively activated in colon carcinoma, while chromosomal translocation of the *abl* gene t (9; 22) leads to its fusion with the *bcr* gene to produce an *abl-bcr* fusion gene which encodes constitutively activated tyrosine kinase activity, leading to chronic myeologenic leukemia (CML). The *mos* and *raf* genes are constitutively activated in sarcoma, and the *pim1* gene in T-cell lymphoma, respectively. The H-*ras*, K-*ras* and N-*ras* genes code for membrane-bound G-proteins which are involved in mediating the transfer of extracellular information necessary for cell growth and regulation to the nucleus. Point mutations inhibit the GTPase function, thereby leading to constitutively active H-*ras* protein in colon, lung and pancreas carcinomas, K-*ras* protein in acute myeloid leukemia (AML), thyroid carcinoma, melanoma, colon and lung carcinoma, and N-*ras* protein in carcinoma and melanoma (Croce, 2008).

f. Apoptosis regulators

The bcl2 protein localizes to mitochondria and increases cell survival by inhibiting apoptosis. In follicular lymphomas, the translocation of the *bcl2* gene from chromosome 14 to chromosome 18 juxtaposes it to the enhancer element of immunoglobulin genes, leading to deregulation of expression and inhibition of apoptosis. The *mdm2* oncogene has also been found to be amplified in sarcomas, and it

leads to inhibition of apoptosis by formation of a complex between the MDM2 protein and p53 tumor suppressor protein, thereby targeting the p53 protein for degradation (Croce, 2008).

1.4.2. TUMOR SUPPRESSOR GENES

Tumor suppressor genes are genes whose loss-of-function releases the constraint on cell growth and is therefore tumorigenic. They thus have an effect opposite to oncogenes. Tumor suppressor genes are vulnerable sites for critical DNA damage because they normally function as physiologic barriers against clonal expansion or genomic mutability and are able to hinder the growth and metastasis of cells driven to uncontrolled proliferation by oncogenes (Harris and Hollstein, 1993). Loss of tumor-suppressor function can occur through damage to the genome by mutation, chromosomal rearrangement and nondisjunction, gene conversion, imprinting or mitotic recombination. Tumor suppressor activity can also be neutralized by interaction with other cellular proteins or viral oncoproteins (Harris and Hollstein, 1993). Several familial cancers have been shown to be associated with a loss of function of a tumor suppressor gene, as listed in Table 4.

Table 4. Tumor suppressor genes implicated in various familial cancers in humans (adapted from King, 2008)

Serial No.	*Tumor suppressor gene*	*Familial cancer syndrome*	*Function*	*Chromosomal location*	*Tumor types observed*
1	***p53***	Li-Fraumeni syndrome	Cell cycle regulation, apoptosis	17p13.1	Brain tumors, sarcomas, leukemia, breast cancer
2	***RB***	Familial retinoblastoma	Cell cycle regulation	13q14.1-q14.2	Retinoblastoma, osteogenic sarcoma
3	***WT1***	Wilms tumor	Transcriptional regulation	11p13	Pediatric kidney cancer
4	***NF1*** (protein-neurofibromin 1)	Neurofibromatosis type 1	Catalysis of *Ras* inactivation	17q11.2	Neurofibromas, sarcomas, gliomas
5	***NF2*** (protein- merlin or neurofibromin 2)	Neurofibromatosis type 2	Linkage of cell membrane to actin cytoskeleton	22q12.2	Schwann cell tumors, astrocytomas, meningiomas, ependymonas

6	***APC***	Familial Adenomatous Polyposis	Signaling through adhesion molecules to nucleus	5q21-q22	Colon cancer
7	***TSC1*** (protein-hamartin)	Tuberous sclerosis 1	Forms complex with TSC2 protein, inhibits signaling to downstream effectors of mTOR (mammalian Target Of Rapamycin) signaling pathway	9q34	Facial angiofibromas
8	***TSC2*** (protein-tuberin)	Tuberous sclerosis 2	Forms complex with TSC1	16p13.3	Benign growths (hamartomas) in many tissues, astrocytomas, rhabdo-myosarcomas
8	***DCC***	Deleted in Colorectal Carcinoma	Transmembrane receptor involved in axonal guidance via netrins	18q21.3	colorectal cancer
9	***BRCA1***	Familial Breast Cancer	Cell cycle control, controlling protein degradation, DNA damage repair, and transcriptional regulation; interacts with Rad51 in DNA repair	17q21	Breast and ovarian cancer
10	***BRCA2***	Familial Breast Cancer	Transcriptional regulation of genes involved in DNA repair and homologous recombination	13q12.3	Breast and ovarian cancer
11	***PTEN***	Cowden syndrome	Phospho-inositide 3-phosphatase, protein tyrosine phosphatase	10q23.3	Gliomas, breast cancer, thyroid cancer, head & neck squamous carcinoma
13	***MSH2***	Hereditary Nonpolyposis Colon Cancer type 1, HNPCC1	DNA mismatch repair	2p22-p21	Colon cancer

14	***MLH1***	Hereditary Nonpolyposis Colon Cancer type 2, HNPCC2	DNA mismatch repair	3p21.3	Colon cancer
15	***CDH1*** (protein - E-cadherin)	Familial diffuse-type gastric cancer	cell-cell adhesion protein	16q22.1	Gastric cancer, lobular breast cancer
16	***VHL***	von Hippel-Lindau syndrome	Regulation of transcription elongation through activation of a ubiquitin ligase complex	3p26-p25	Renal cancers, hemangio-blastomas, pheochromo-cytoma, retinal angioma
17	$p16^{INK4a}$ also called ***CDKN2A***(protein-cyclin-dependent kinase inhibitor 2A)	Familial Melanoma	cell-cycle regulation	9p21	Melanoma, pancreatic cancer, others
18	***PTCH*** (protein – patched)	Gorlin Syndrome	Transmembrane receptor for sonic hedgehog (shh), involved in early development through repression of action the Smoothened (SMO) receptor	9q22.3	Basal cell skin carcinoma
19	***MEN1***	Multiple Endocrine Neoplasia Type 1	Intrastrand DNA crosslink repair	11q13	parathyroid and pituitary adenomas, islet cell tumors, carcinoid

According to Knudson's "two-hit" hypothesis based on studies of retinoblastoma, the inactivation of a tumor suppressor gene by mutation requires two inactivating "hits" or mutations. In the dominantly inherited form of familial cancers, one mutation is inherited by the germinal cells while the second occurs in somatic cells. In non-hereditary forms, both mutations are somatic (Knudson, 1971). In addition, a tumor suppressor gene may be inactivated by loss of heterozygosity (LOH) as reported for example in case of *PTCH* (Lu *et al.,* 2000), *BRCA1* and *BRCA2* (Silva et al., 1999), *p53*, *RB* and *PTEN* (Bettendorf *et al.,* 2008) tumor suppressor genes; or, by epigenetic silencing through hypermethylation of promoters in case of *RB*, APC, *MLH1*, *VHL*, *CDNK2A*, *CDKN2B* (Jones and Laird, 1999), *BRCA1* and *STK11* (Jones and Baylin, 2002). Thus, accordingly, Knudson's "two-hit" hypothesis may be revised to

accommodate the inactivation of the first active allele of a tumor suppressor gene by localized mutation or transcriptional repression through DNA methylation, and the second hit, that is, inactivation of the second allele by LOH or transcriptional silencing, in addition to mutation (Fig. 1.2).

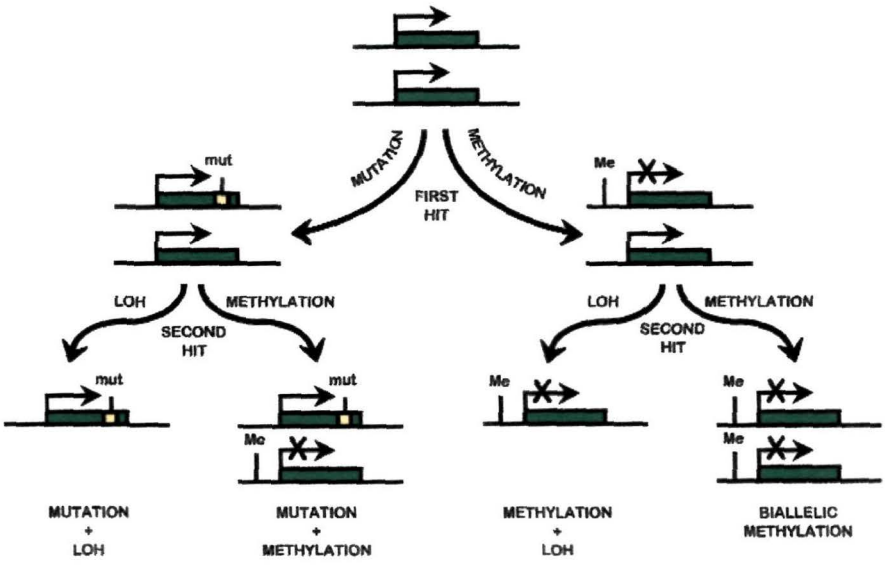


Figure 1.2. Revised form of Knudson's "two hit" hypothesis explaining the inactivation of tumor suppressor genes (adapted from Jones and Laird, 1999).

1.5. CLASSES OF TUMOR SUPPRESSOR GENES

Since tumor suppressor genes function in the prevention of tumorigenesis, it is evident that they are required not only to control cellular proliferation, but also to main the stability of the genome. Kinzler and Vogelstein (Kinzler and Vogelstein, 1997) have accordingly classified tumor suppressor genes into "gatekeepers" and "caretakers".

Gatekeepers are genes that directly regulate the growth of tumors by inhibiting growth or promoting death through regulation of the cell-cycle. Inactivation of these genes is rate-limiting for the initiation of a tumor, and both maternal and paternal copies must be altered for tumor development. Predisposed individuals inherit one mutant copy of a gatekeeper gene so they require an additional somatic mutation to initiate neoplasia. Tumors develop sporadically in individuals who do not have a germline mutation when both copies of the gatekeeper gene become mutated somatically. Because the probability of acquiring a single somatic mutation is exponentially greater than the probability of acquiring two such mutations, people with a hereditary mutation of a gatekeeper gene are at a much greater risk (greater than 1000 fold) of developing

tumors than the general population (Kinzler and Vogelstein, 1997). The most widely studied gatekeeper tumor suppressor gene is the p53 gene. Other examples are the *RB*, *VHL*, *NF1* and *APC* genes.

Caretakers are genes that maintain genomic integrity through their role in maintaining error-free DNA repair. Inactivation of a caretaker gene therefore does not promote tumor initiation directly. Instead it leads to genetic instability which results in increased mutation in all genes, including gatekeepers. In dominantly inherited cancer-predisposition syndromes of the caretaker type, patients inherit a single mutant caretaker gene from the affected parent. Thus, three subsequent mutations usually required to initiate cancer: mutation of the normal caretaker allele inherited from the unaffected parent, followed by mutation of both copies of a gatekeeper gene. Because three mutations are required, the risk of cancer in affected families is only 5-50 folds higher than the general population. Moreover, the frequency of occurrence of sporadic cancers due to mutations in caretaker genes would be low because four mutations- two in the caretaker alleles, and, two in gatekeeper alleles would be required. Some examples of caretaker genes are the genes involved in nucleotide-excision repair which are mutated in xeroderma pigmentosum, the *ATM* gene which is mutated in ataxia telangiectasia, and the breast cancer and ovarian cancer associated *BRCA1* and *BRCA2* genes (Kinzler and Vogelstein, 1997).

1.6. p53 AS A TUMOR SUPPRESSOR GENE

The *p53* gene was discovered in 1979 as a gene coding for a 53 kDa nuclear phosphoprotein bound to the large T antigen of the simian virus 40 (SV40) DNA virus (Lane and Crawford, 1979; Linzer and Levine, 1979). Since it was known that the T antigen is essential for both the initiation and maintenance of the transformed state in SV40-infected cells, p53 was thought to be a transforming oncogene. This view was further corroborated by the finding that p53 is overexpressed in methylcholanthrene-induced sarcoma, leukemias, virus-transformed cell lines and spontaneously transformed fibroblasts (DeLeo *et al.*, 1979). Studies also demonstrated that cloned murine p53 cDNA could immortalize cells thereby leading to their susceptibility to transformation by the *ras* oncogene (Jenkins *et al.*, 1984), and that p53 co-operated with the activated *Ha-ras* oncogene to transform normal embryonic cells (Eliyahu *et*

al., 1984). However, in 1984 itself Maltzman and Czyzyk demonstrated that p53 production is stimulated and p53 protein undergoes post-translational stabilization in non-transformed cells upon ultraviolet irradiation. It was observed that only mutant *p53* could co-operate with *ras* during cellular transformation, and wild-type *p53* could in fact inhibit transformation induced by the combined effect mutant *p53* with E1A antigen or *ras*. It was also demonstrated that colorectal carcinomas in which one allele of the *p53* gene is lost, also harbored mutations in the remaining allele, a characteristic hallmark of loss of tumor suppressor function according to Knudson's hypothesis. This pattern was found to be true for other cancers, and it was observed that introduction of wild-type *p53* resulted in suppression of growth of human colorectal cell lines (reviewed by Velculescu and El-Deiry, 1996). Thus, by 1989 there was substantial evidence to support the role of wild-type *p53* as a tumor suppressor gene rather than an oncogene.

Mutations in the *p53* gene have been shown to occur in 50-55 percent of human cancers (Hollstein *et al.,* 1991). The p53 mutation spectrum differs among cancers of the colon, lung, esophagus, breast, liver, brain, reticuloendothelial tissues and hemopoietic tissues. There is a clear link between the exposure to aflatoxin B1 leading to typical GC to TA transversion at the third base of codon 249 of the *p53* gene, and liver cancer (Staib *et al.,* 2003). Both qualitative and quantitative data link G to T mutations at CpG sites in the *p53* gene to lung cancer (Vähäkangas, 2003). In breast cancer, a slight excess in deletions and mutations affecting AT base pairs have been observed, while in esophageal cancer, insertions and AT to TA transversion mutations predominate. In stomach and colorectal cancers GC to AT transition mutations in the *p53* gene are reported to be dominant (Cetin-Atalay and Ozturk, 2000).

1.6.1. p53 structure

The *p53* gene is located in humans on chromosome 17 at location 17p13.1, and in mice on chromosome 11 at location 11 B2-C; 11 39.0 cM (available at www.ncbi.nlm.nih.gov/sites/entrez). The gene is composed of 11 exons, the first of which is non-coding. Exons 5 through 9 of the *p53* gene contain conserved sequence blocks. The wild-type *p53* gene codes for a protein of 393 amino acids which is composed of three distinct structural and functional domains: (i) a N-terminus containing

an amino-terminal transactivation domain (residues 1-42) which is required for transactivation activity and interacts with various transcription factors including acetyltransferases and MDM2 (murine double minute 2); and, a proline-rich region with multiple copies of the PXXP sequence (residues 61-94, where X is any amino acid). The proline-rich region plays a role in p53 stability regulated by MDM2, wherein p53 becomes more susceptible to degradation mediated by MDM2 if this region is deleted (ii) a central core domain (residues 102-292) constitutes the DNA-binding domain which is required for sequence-specific DNA binding. The consensus sequence contains two copies of the 10-bp motif 5'-PuPuPuC (A/T)-(T/A) GPyPyPy-3', separated by 0-13 bp (iii) a C-terminal region (residues 301-393) containing an oligomerization domain (residues 324-355), a strongly basic carboxyl terminal regulatory domain (residues 363-393), a nuclear localization signal sequence (NLS) and nuclear export signal sequences (NES). The basic C-terminus of p53 functions as a negative regulatory domain and has also been implicated in induction of cell death. The central domain of p53 is its most highly conserved region, not only when p53 is compared with its homologs from *Drosophila* and *Caenorhabditis elegans*, but also as compared with its mammalian family members, p63 and p73. In fact, structural studies of p53 have revealed that majority of p53 mutations found in various cancers are missense mutations that are mostly located in this central DNA-binding domain (Fig. 1.3) (reviewed in Bai and Zhu, 2006).

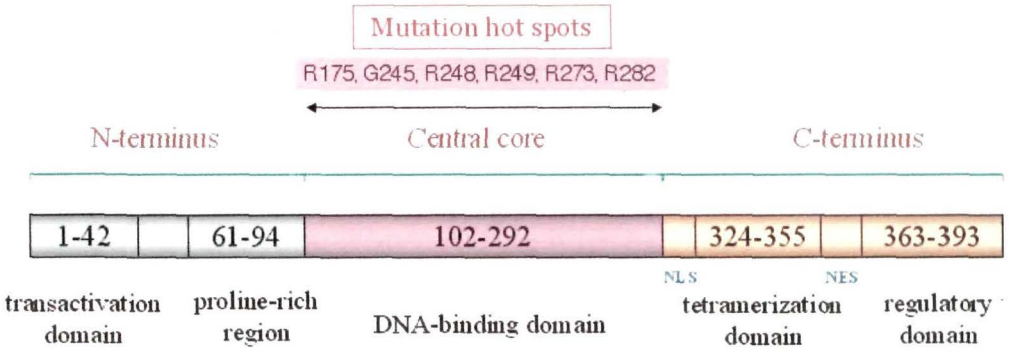


Figure 1.3. Schematic representation of the structure of p53 protein (adapted from Bai and Zhu, 2006)

1.6.2. p53 functions

In normal unstressed cells, p53 is an unstable protein with a half-life ranging from 5 to 30 mins, and is present at very low cellular levels (Levine, 1997). Following various intracellular and extracellular stimuli, such as DNA damage (induced by various factors

including ionizing radiation, UV radiation, exposure to cytotoxic or chemotherapeutic agents and viruses), heat shock, hypoxia, and oncogene overexpression, wild-type p53 is activated and emerges as a pivotal regulatory protein which triggers diverse biological responses, both at the level of a single cell as well as in the whole organism. p53 activation involves an increase in overall p53 protein level by inhibition of MDM2-mediated degradation, as well as qualitative changes in the protein through extensive post-translational modifications including phosphorylation and acetylation, thus resulting in activation of p53-targeted genes (reviewed in Bai and Zhu, 2006) (Fig. 1.4).

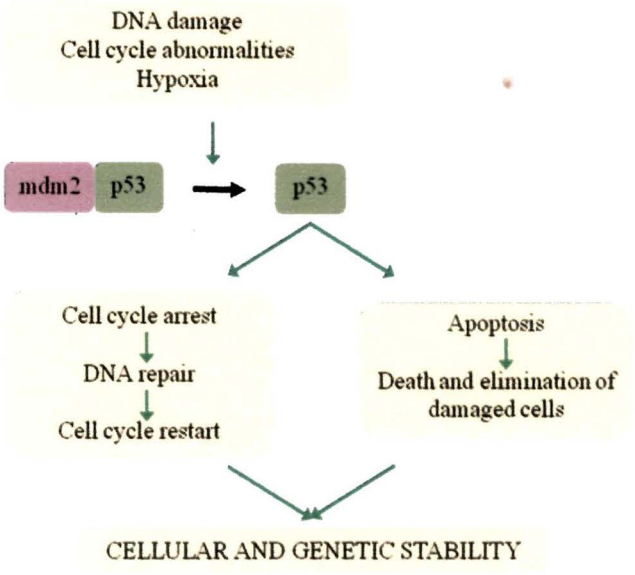


Figure 1.4. Tumor-suppressive functions of p53 (adapted from Wikipedia-p53 pathways)

a. Role of p53 as a transcription factor

The p53 protein exerts its tumor suppressive effect essentially by binding to DNA in a sequence-specific manner and regulating the transcription of gene products involved in growth arrest, DNA repair, apoptosis, and the inhibition of angiogenesis. p53-mediated growth arrest results from the p53-dependent transactivation of p21 and GADD45 p53 regulates DNA repair pathways by the transcriptional upregulation of p21, GADD45, and the p48 xeroderma pigmentosum protein. p53-induced apoptosis is proposed to be mediated by the transactivation of BAX, Fas/APO1, KILLER/DR5, IGF-BP3, AIP1, Pidd, and the p53-inducible genes. In addition, the p53-dependent transcriptional upregulation of thrombospondin-1, glioblastoma-derived angiogenesis inhibiting factor and hypoxia-inducible factor-1 has been implicated in p53-mediated regulation of angiogenesis. Another key target of p53 transcriptional upregulation is MDM2. The MDM2 protein promotes the rapid degradation of the p53 protein, thus forming an

autoregulatory feedback loop that tightly regulates p53 protein levels. p53 also mediates transcriptional repression of proteins implicated in diverse signaling pathways. These proteins include BRCA1, Bcl-2, Wee-1, c-fos, interleukin-6, topoisomerase I, MAP4, presenilin, hsp 70, stathmin, cyclin B1, Cdc2, and the insulin-like growth factor-1 receptor. p53-dependent transcriptional repression may result from p53 binding to the basal transcriptional machinery, thus prohibiting its interaction with other promoters. p53 transcriptional repression may also be mediated by its association *in vivo* with histone deacetylases and the transcriptional corepressor mSin3a (reviewed in Stewart and Pietenpol, 2001).

b. Role of p53 in regulation of cell cycle checkpoints

p53 plays a critical role during the DNA damage-induced G1/S cell cycle checkpoint. After exposure of cells containing wild-type p53 to genotoxic agents, p53 is activated and transcriptionally upregulates the Cdk inhibitor, p21. p21 then binds to, and inactivates cyclin-Cdk complexes that mediate G1 phase progression, resulting in pRB hypophosphorylation, E2F sequestration, and cell cycle arrest at the G1/S transition (Fig. 1.5). p53-dependent induction of p21 also results in an S phase checkpoint response, as p21 binds to the proliferating cell nuclear antigen (PCNA) and prevents PCNA from mediating recognition of the DNA primer template complex, thus inhibiting the elongation step in DNA replication (reviewed in Stewart and Pietenpol, 2001).

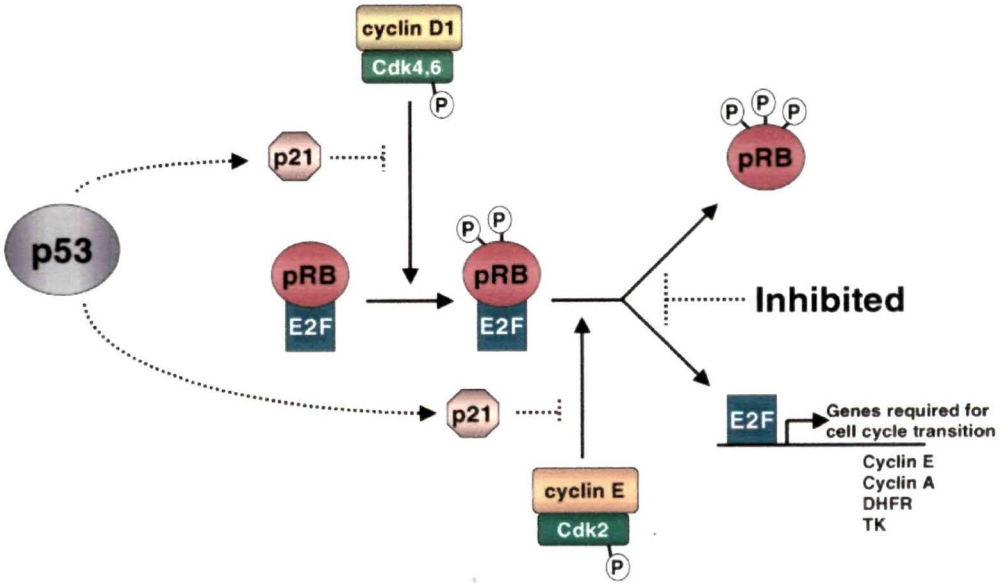


Figure 1.5. Role of p53 in G1/S checkpoint. (adapted from Stewart and Pietenpol, 2001).

During S and G2 phases, cells accumulate the cyclin B1-Cdc2 complex in an inactive form due to inhibitory phosphorylations by Weel and Myt1 kinases. The conversion of Cdc2 from an inactive to active form is mediated by the Cdc25C phosphatase. Mitotic events are stimulated by active cyclin B1-Cdc2 complexes. In response to genotoxic stress, the Chk1 and Chk2 kinases are activated and phosphorylate Cdc25C. This phosphorylation promotes the interaction of Cdc25C with 14-3-3 adaptor proteins and inhibits the ability of Cdc25C to activate the cyclin B1-Cdc2 complex, resulting in cell cycle arrest at the G2/M transition. After DNA damage, activated Chk1 and Chk2 also phosphorylate p53, resulting in stabilization and activation of the protein. p53-dependent signaling contributes to maintenance of the G2 cell cycle arrest by upregulating the 14-3-3*ó* protein that binds to Cdc2 and sequesters the kinase in the cytoplasm. p53-dependent transcription also elevates the Cdk inhibitor p21, which binds to cyclin-Cdk complexes to reduce the level of phosphorylation of pRB. Hypophosphorylated pRB remains bound to E2F, preventing E2F from mediating the biosynthesis of cyclin B1 and Cdc2 (Fig. 1.6). The p53-induced increase in level of expression of GADD45 also results in G2 arrest since GADD45 directly inhibits the cyclin B1-Cdc2 complex (reviewed in Stewart and Pietenpol, 2001).

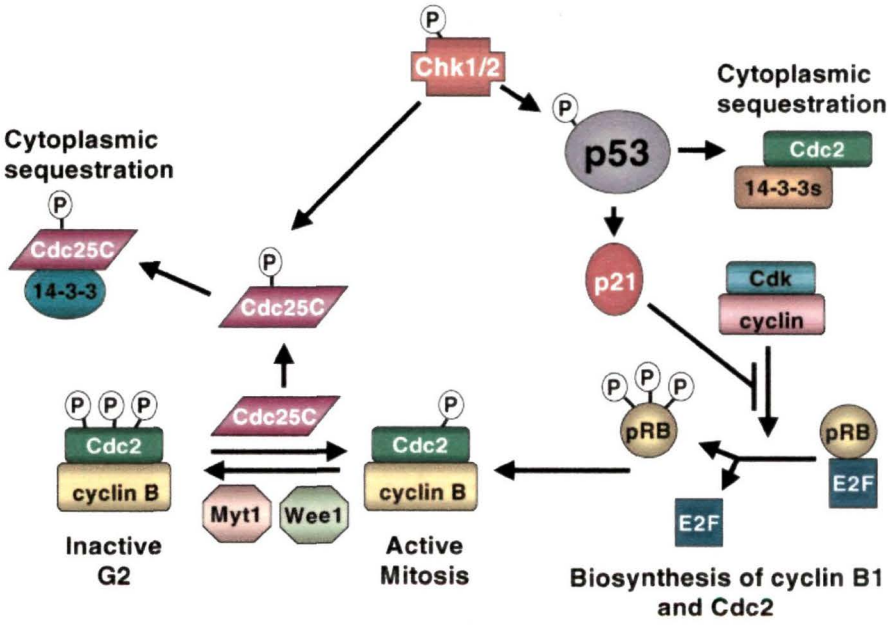


Figure 1.6. Role of p53 in G2 checkpoint. (adapted from Stewart and Pietenpol, 2001).

c. Role of p53 in DNA repair

After genotoxic stress, p53 modulates DNA repair through multiple mechanisms, including sequence specific transactivation and direct interaction with components of the repair machinery. p53 sequence-specific transactivation-dependent DNA repair occurs in part by transactivation of p21. p21 specifically inhibits PCNA-mediated DNA replication while allowing PCNA-regulated DNA repair. p53 also induces expression of GADD45 which not only binds to PCNA but also stimulates DNA excision repair *in vitro* and inhibits the entry of cells into the S phase. p53 sequence-specific transactivation of the xeroderma pigmentosum *p48* gene results in expression of the p48 protein which functions in global genomic repair of UV-induced cyclobutane pyrimidine dimers. p53 also directly interacts with proteins that function in DNA repair pathways. Most of these proteins are members of the TFIIH complex that initiates basal transcription of RNA polymerase II and couples transcription with nucleotide excision repair (NER). p53 binds to the Cockayne syndrome B repair helicase and replication protein A (RPA), a trimeric protein complex that functions in DNA replication, homologous recombination, and NER. Besides, p53 may also be involved in DNA repair through direct interaction with DNA. Association of the carboxy terminus of p53 with single-stranded DNA ends facilitates the binding of the p53 core domain to DNA and helps in recruiting different repair factors. p53 also possesses intrinsic 3' → 5' exonuclease activity that is associated with the core domain of the protein. This exonuclease activity may play an important role in p53-mediated repair, as tumor-derived p53 mutants are exonuclease-deficient and cells expressing such mutant p53 are defective in global NER (reviewed in Stewart and Pietenpol, 2001). It has been reported that endogenous p53, phosphorylated at Ser15 (p53Ser15), accumulates as discrete, dose-dependent and chromatin-bound foci within 30 minutes following induction of DNA breaks or DNA base damage, constituting a biologically distinct subpool of p53Ser15 phosphoform which colocalizes and coimmunoprecipitates with γ-H2AX at sites of DNA-double strand breaks as part of the initial genome surveillance complex (Al Rashid *et al.*, 2005).

d. Role of p53 in apoptosis

The p53 apoptotic target genes can be divided into two groups; the first group encodes proteins that act through receptor-mediated signaling, and the second group encodes proteins that regulate apoptotic effector proteins. p53-dependent transactivation of IGF-

BP3 induces apoptosis by blocking IGF-1 survival signaling to the IGF-1 receptor (IGF-1R). When combined with p53-dependent repression of the IGF-1R, p53 signaling results in a highly efficient block of this survival pathway. p53 also mediates apoptosis through activation of the Fas/APO1/CD95 (Fas) and KILLER/DR5 death receptors. Early cell changes that occur during apoptosis are associated with mitochondrial changes mediated by members of the Bcl-2 family of proteins, including antiapoptotic Bcl-2 and pro-apoptotic BAX proteins. BAX facilitates the release of the apoptosis-inducing factor (AIF) and cytochrome *c* from the mitochondria, thus activating the caspase cascade. p53 inhibits expression of the anti-apoptotic Bcl-2 protein, which normally blocks apoptosis by preventing the release of AIF and cytochrome *c* from the mitochondria. p53 may also mediate mitochondrial apoptotic signaling through the elevation of the level of reactive oxygen species through PIG3 and PIG8 induction. p53 may also mediate apoptosis by sequence-specific transactivation-independent mechanisms (Fig. 1.7).

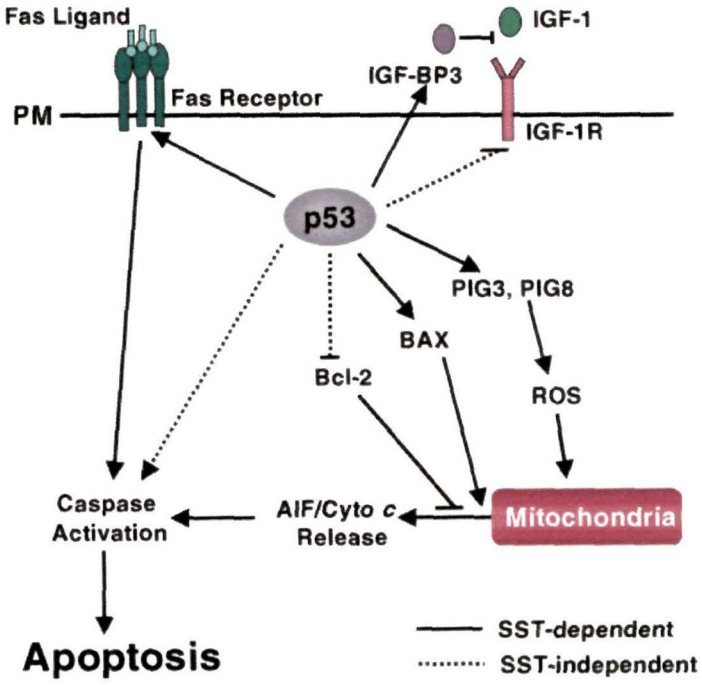


Figure 1.7. p53-mediated apoptotic signaling. (adapted from Stewart and Pietenpol, 2001).

It can thus be concluded that wild-type p53 functions essentially as a "gatekeeper" tumor suppressor (Levine, 1997). As a result of missense mutations occurring in the central DNA-binding domain, the mutated p53 gene encodes a full-length protein incapable of transactivating its target genes. In addition to this loss of function, mutant

p53 can have a dominant negative effect over wild-type p53 and/or gain-of-function activity independently of the wild-type protein. In fact, it has been shown that p53 missense mutants markedly reduce the binding of wild-type p53 to the p53 responsive element in the target genes of p21, MDM2, and PIG3 thereby abrogating the growth suppression activity of wild-type p53 (Willis *et al.*, 2004).

1.7. BRCA1 AND *BRCA2* AS TUMOR SUPPRESSOR GENES

The breast cancer-associated *BRCA1* and *BRCA2* genes are located in humans on chromosome 17 at position 17q21 and chromosome 13 at position 13q12.3 respectively. The murine homologs of these genes, that is, *Brca1* and *Brca2*, are located on chromosome 11 at 11 D; 11 60.5 cM, and on chromosome 5 at 5 G3; 5 84.0 cM (available at www.ncbi.nlm.nih.gov/sites/entrez). The *BRCA1* gene was identified by positional cloning methods as the gene responsible for increasing susceptibility to breast and ovarian cancer by (Miki *et al.*, 1994). The gene encompasses 24 exons in approximately 81 kb of genomic DNA and codes for a protein of 1863 amino acid residues (Oesterrich and Fuqua, 1999). *BRCA1* mutations are thought to account for about 45 % of families with high breast cancer risk, and greater than 80 % of families with high risk of early onset breast and ovarian cancers (Nadeau *et al.*, 2000). A second gene associated with susceptibility to breast cancer, the *BRCA2* gene, was subsequently identified by linkage analysis (Wooster *et al.*, 1995). The gene consists of 27 exons spanning 70 kb of genomic DNA and encodes a protein of 3418 amino acid residues.

1.7.1. BRCA1 and BRCA2 structure

Although the breast and ovarian cancer phenotypes associated with mutation in *BRCA1* and *BRCA2* are similar, the genes are not detectably related by sequence. However, the two genes share striking genomic parallels, one common feature being both have extremely large central exons, that is, exon 11 which encodes more than 50 percent of the respective proteins. Comparison of the amino acid sequence of human BRCA1 and BRCA2 proteins with their respective murine homologs indicates that the proteins share approximately 60 percent identity (Welsch *et al.*, 2000). The genomic regions of both BRCA1 and BRCA2 contain very high densities of repetitive DNA elements that may contribute to genetic instability. The *BRCA1* gene contains 42 % *Alu* sequences and 5 % non-*Alu* repeats. The *BRCA2* gene contains 47 % repetitive DNA, consisting

of 20 % *Alu* sequences and 27 % LINE and MER repetitive DNA. The presence of such repeats may be responsible for the large deletions in and around the *BRCA1* and *BRCA2* genes observed in both inherited and sporadic breast and ovarian cancers, due to homologous recombination between the repeat sequences (Welsch and King, 2001).

The BRCA1 and BRCA2 polypeptides have distinct domains which are involved in their myriad functions. The N-terminal RING finger of BRCA1 interacts with BRCA1-associated RING domain 1 (BARD1) protein, which recruits BRCA1 to the nucleus, and with the deubiquitinating enzyme, BRCA1-associated protein-1 (BAP1). BRCA1 also has two nuclear localization signals (NLS) and nearby regions which interact with p53, c-myc, pRb, RAD50, MRE11 and NBS-1. A domain within BRCA1 (amino acids 758-1064) interacts with RAD51. The BRCA1 C-terminal (BRCT) repeats interact with BRCA2, histone deacetylase (HADC) 1 and 2, RNA helicase A (RHA), p300/CBP and the CtBp-interacting protein (CtIP). The N-terminus of BRCA2 interacts with p300/CBP-associated factor (P/CAF) which has histone acetylase activity. Four of the eight BRC repeats of BRCA2 interact with RAD51. Besides, BRCA2 also has a second RAD51 interaction domain and a NLS at the C-terminus (Welsch *et al.,* 2000) and Fig. 1.8 (Scully and Livingston, 2000).

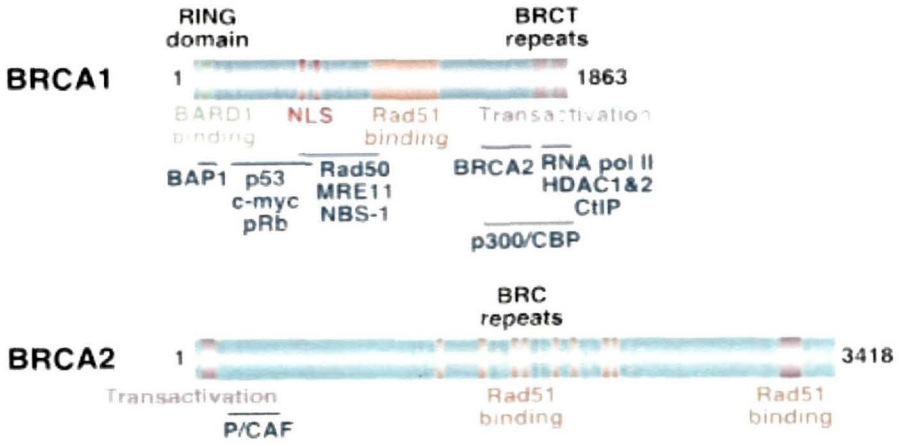


Figure 1.8. Structure of the BRCA1 and BRCA2 polypeptides. (adapted from Scully and Livingston, 2000)

1.7.2. BRCA1 and BRCA2 functions

Initial evidence linking *BRCA1* and *BRCA2* functions to cancer predisposition came from the observation that cells deficient in the murine *BRCA2* homolog sustain spontaneous aberrations in chromosome structure. Chromosome painting also revealed

gross chromosomal arrangements (GCRs) such as translocations or deletions as well as fusions that encompass multiple, non homologous chromosomes. Similar structural aberrations were also observed in *BRCA1*-deficient mouse cells, as well as *BRCA1*- and *BRCA2*- deficient human cells. Collectively, these findings established that *BRCA* genes are essential for preserving chromosome structure, suggesting that in their role as tumor suppressors, they behave as "caretakers" by suppressing genomic instability (reviewed in Venkitaraman, 2002).

Biochemical, genetic and cytological studies have revealed multiple functions for BRCA1 and BRCA2. The BRCA proteins have been found to be involved in control of homologous recombination (HR) and double-strand break (DSB) repair in response to DNA damage (Welsch and King, 2001). In mammalian cells, DSBs can be repaired by *nonhomologous end joining* (NHEJ), potentially an error-prone process in which nucleotide alterations are tolerated at the sites of rejoining, or by *recombination* between homologous DNA sequences (HR) which is error-free when the exchange is between identical sister chromatids (or homologous chromosomes). A third mechanism, *single-strand annealing* (SSA), is also initiated by homologous pairing, except that unlike in HR the homology is between short stretches of stranded DNA (ssDNA) at staggered DSBs, and pairing precedes religation, not strand exchange. SSA is error-prone because sequence information can be lost or rearranged when ends overlapping by as little as approximately 30 bp are unsuitably joined (Fig. 1.9) (Venkitaraman, 2002).

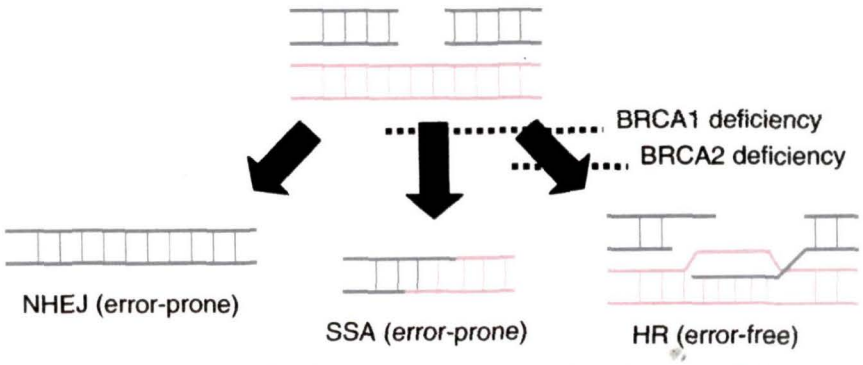


Figure 1.9. Different routes of DSB repair (adapted from Venkitaraman, 2002).

Substantial evidence indicates a role for both BRCA1 and BRCA2 in HR. Both BRCA1 and BRCA2 can interact directly or indirectly with RAD51, the eukaryotic homolog of Rec A, which catalyses strand exchange during homology directed repair of DNA

DSBs by gene conversion. BRCA1 binds to numerous cellular proteins *in vivo* and appears to have multiple functions depending on the cellular context (Fig. 1. 10).

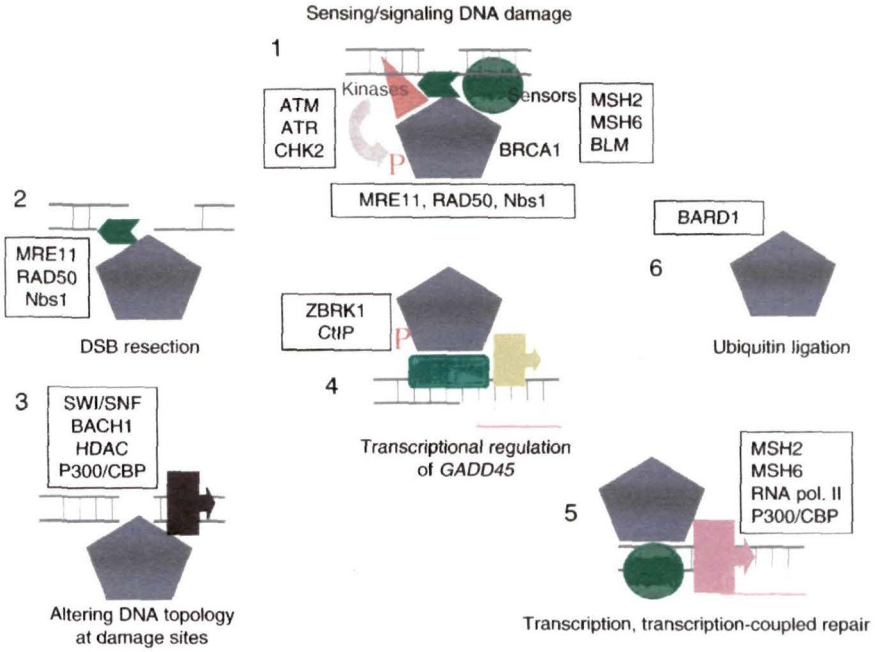


Figure 1. 10. Protein-protein interactions of BRCA1 (adapted from Venkitaraman, 2002)

a. Role of BRCA1 in DSB repair

Sites of DNA damage are marked within minutes by phosphorylation of a histone species, H2A-X, which spreads over a region spanning thousands of bases around the lesion, allowing chromatin remodeling to occur to facilitate access of the repair machinery. BRCA1 is an early migrant to sites of H2A-X phosphorylation. BRCA1 is involved in various protein-protein interactions relevant to DNA repair. It interacts with the MRE11/RAD50/Nbs1 complex, known to participate in DSB repair with MRE11 encoding a nuclease activity which resects flush DSB ends to generate ssDNA tracts, which are substrates for HR. BRCA1 may also have local activities at DSB sites through its interaction with enzymes that alter chromatin structure. It interacts with SWI/SNF proteins that remodel chromatin, with regulators of histone acetylation/ deacetylation such as HDAC and DNA helicases including BLM and BACH1, in order to alter DNA topology at the site of a lesion. BRCA1 also functions by regulating the expression of *GADD45*, a tumor suppressor gene that is also a downstream target of the p53 pathway. *GADD45* transcription is normally suppressed by a corepressor complex in which BRCA1 associates with the KRAB domain transcription factor ZBRK1. After ionizing irradiation, phosphorylation of BRCA1 by ATM relieves *GADD45* repression.

In this way, BRCA1 processes signals through ATM to achieve transcriptional regulation of *GADD45* in response to DSB (Venkitaraman, 2002).

b. Role of BRCA1 in cell cycle checkpoint

BRCA1 is rapidly phosophorylated after DNA damage in dividing cells by the ATM, ATR and CHK2 kinases. Phosphorylation of BRCA1 by each of these kinases is activated by distinct stimuli and targeted to distinct clusters of serine residues- ATM and CHK2 phosphorylate BRCA1 after ionizing radiation, whereas ATR is more specifically activated after UV-irradiation or replication arrest (Venkitaraman, 2002). BRCA1 then induces p53-independent G1/S- arrest by interacting with hypophosphorylated pRb or by p21-induction (Deng, 2006). BRCA1 may also induce p53-dependent G1/S arrest, and a study demonstrated that the BRCA1-BARD1 complex is required for ATM/ATR-mediated phosphorylation of p53 at Ser-15 following IR or UV radiation-induced DNA damage (Deng, 2006). Further, it was observed that phosphorylated BRCA1 is involved in the S-phase cell cycle checkpoint, and, BRCA1 mediates G2/M arrest via its interaction with CHK1 and CHK2 (Deng, 2006).

c. Role of BRCA1 in transcription-coupled repair

BRCA1 is also reported to show a role in transcription-coupled repair presumably via base-excision repair. In fact, BRCA1 associates with the mismatch repair proteins MSH2 and MSH6 which have been implicated in transcription-coupled repair (Wang *et al.,* 2000). BARD1, a protein which interacts with BRCA1 through the N-terminal RING domain (Fig. 1.8), has been implicated in the control of RNA processing following DNA damage. Normally messenger RNAs must be endonucleolytically cleaved at their 3' end prior to polyadenylation. This reaction is found to be strongly but inhibited following DNA damage. Inhibition is dependent upon BARD1 and possibly upon complex formation with BRCA1 and the polyadenylation factor CstF50. The ubiquitin ligase activity of the BARD1-BRCA1 complex may be involved in inhibition, targeting the proteins that carry out RNA processing for degradation (Venkitaraman, 2002).

d. Role of BRCA1 in apoptosis

Wild-type BRCA1 can promote ionizing radiation-induced apoptosis in breast and ovarian cancer cell lines. This function of BRCA1 is dependent on the H-Ras, MEKK4, JNK, Fas ligand/Fas interactions, and caspase-9 activation and is independent of p53 status (Thangaraju *et al.,* 2000). Another study has identified BARD1 as a regulator of BRCA1-induced apoptosis. It was demonstrated that BARD1
reduced BRCA1-dependent apoptosis by a mechanism involving nuclear sequestration, and regulation of apoptosis by BARD1 was reduced by BRCA1 cancer mutations that disrupt ubiquitin ligase function (Fabbro *et al.,* 2004).

e. Role of BRCA2 in DSB repair

BRCA2 binds directly with RAD51 primarily through the approximately 40 amino acid BRC motifs in BRCA2, eight repeats of which are well conserved in sequence and spacing from mammals to birds. The interaction involves a substantial proportion of the total cellular pool of each protein. RAD51 has a catalytic activity central to HR. It coats ssDNA to form a nucleoprotein filament that invades and pairs with a homologous DNA duplex, initiating strand exchange between the paired DNA molecules. Evidence suggests that BRCA2 works directly to regulate the availability and activity of RAD51 in this key reaction, consistent with the high stoichiometry of their binding. According to the proposed model (Fig. 1.11), DSBs activate signaling mechanisms (1) and are then resected (2) by exonuclease activity to generate ssDNA tracts. RAD51 is loaded onto the ssDNA (3) to form a nucleoprotein filament that mediates homologous pairing (4) followed by strand extension (5), exchange, and repair (6). In this model, phosphorylation by DNA damage signaling kinases such as ATM or ATR triggers transition of an inactive BRCA2- RAD51 (purple-green) complex to an active complex at the site of damage. At later stages in HR, dephosphorylation may permit the removal of RAD51 from nucleoprotein filaments by BRCA2 binding. Thus, there may be cycling between active and inactive states at the sites of repair (Venkitaraman, 2002).

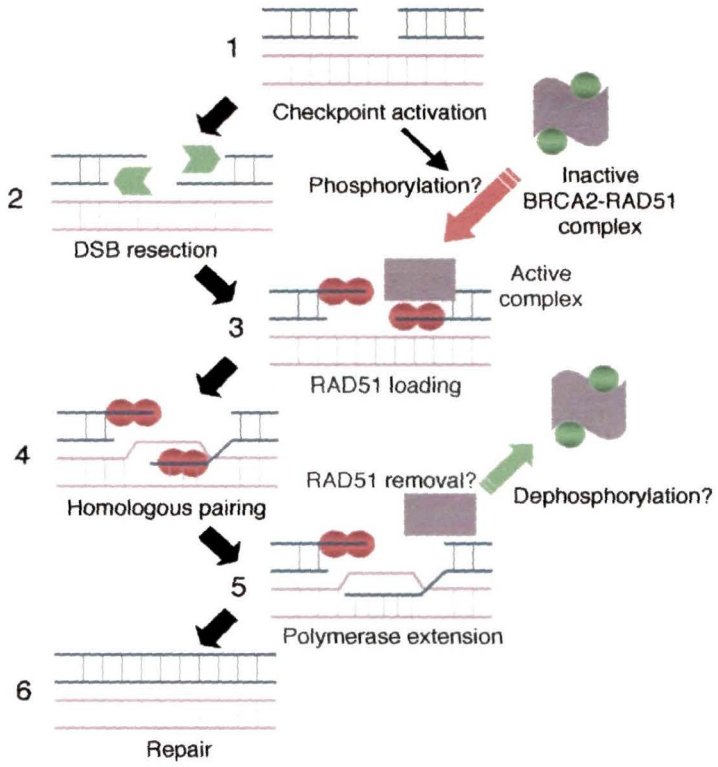


Figure 1.11. Hypothetical model for BRCA2 function in HR (adapted from Venkitaraman, 2002)

Although RAD51 co-localizes with both BRCA1 and BRCA2 in S-phase nuclei and in cells treated with DNA damaging agents, the majority of BRCA1 does not physically associate with BRCA2 and RAD51. Moreover, most of the proteins that have been shown to interact with BRCA1 fail to associate with BRCA2, and *vice versa.* Such exclusivity supports the argument that these two tumor suppressors form distinct complexes *in vivo*, although it is also likely that these complexes may interact in terms of their repair functions (Liu and West, 2002).

One report suggests a potential role for BRCA2 in mitotic checkpoint through its phosphorylation by human BUBR1, hBUBR1 (Futamura *et al.*, 2001).

1.8. ROLE OF BETEL NUT AND ITS CONSTITUENTS IN CARCINOGENESIS

Areca nut is the seed of fruit of a tropical palm, *Areca catechu* L. It forms the most basic ingredient of a variety of widely used social and habitual masticatory products, which are often wrapped in the leaf of another tropical creeper, *Piper betle* L., commonly known as the betel leaf. Hence, the *Areca* nut is more commonly known as

betel nut (BN) (Warnakulasuriya, 2002). The use of BN is deeply ingrained in various socio-cultural and religious practices across the globe. BN is believed to be used by both men and women across all age groups and social classes though in some societies the latter predominate (Warnakulasuriya, 2002). *Areca* nut is normally harvested as unripe (green) or ripe (orange/red) fruit from the *Areca* palm. The *Areca* fruits may be sun dried for several weeks, fibrous shells removed and the hard, dry nuts are ready for use. Alternatively, the ripe *Areca* fruits are boiled for several hours in an aqueous solution containing the bark of the plant *Eugenia jambolana*, jaggery or brown sugar, and various edible oils, to 'cure' it. The cured fruits are sun dried for several weeks, fibrous shell removed and very hard, brown nuts are ready to use. In contrast, unripe or partly ripe *Areca* fruits are freshly picked, fibrous shells removed and the relatively soft nuts are masticated. Occasionally, the fruits can be cured by burying them into moist pits for one to two weeks for fermentation (maturation) before deshelling and use. Such raw and wet variety of BN of the north-eastern part of India is locally called '*kwai*' or '*tambul*'. The BN is either consumed alone or with a wide variety of region and socio-culture specific additives, as betel quid (BQ). In latter case, dry variety of BN is usually wrapped along with slaked lime (calcium oxide and calcium hydroxide or slacked lime) and catechu (*Acacia catechu*) without or with a host of additives, which may also include a variety of tobacco products, perfumes, stimulants, etc., in a piece of betel leaf. The wet variety of BN is usually masticated with slaked lime wrapped in a betel leaf and occasionally supplemented with chewing tobacco (IARC, 1985; Sharan, 1996; Warnakulasuriya, 2002). In India, most habitual chewers of BQ add tobacco, while in some countries, such as Papua New Guinea and China, tobacco is not added. Betel leaf is perishable and the preparation of BQ is somewhat complex. Hence, over the past three decades, commercial BQ substitutes - flavored and sweetened dry mixture of *Areca* nut, catechu and slaked lime with tobacco (*gutkha*) or without tobacco (*pan masala*), have become increasingly popular (Nair *et al.,* 2004; Asotra and Sharan, 2008).

The constituents of BN include carbohydrates, crude fiber, fats, polyphenols, alkaloids, tannins, proteins, ash and water. Trace amounts of fluorine, sapogenein, and free amino acids have also been reported in some forms. The relative amounts of these constituents are highly variable in raw/wet or dry variety of BN. Geographical and climatic conditions of growth of the *Areca* palm tree and the methods of curing BN also

contribute to the observed variation in the constituents (Sharan, 1996). The primary constituents of BN which have been implicated in its role as a carcinogen are as follows:-

(a) Alkaloids: Alkaloids are reduced pyridines. BN primarily contains four alkaloids that are biologically relevant, of which arecoline (1, 2, 4, 5, - tetrahydro-1-methyl-pyridinecarboxylic acid; molecular weight 155. 19 Da) is the most abundant. Other alkaloids such as arecaidine (1, 2, 5, 6, - tetrahydro-1-methyl-3-pyridinecarboxylic acid; molecular weight 141. 17 Da), guvacine (methyl ester of arecaidine), guvacoline (methyl ester of guvacine) and arecolinidine are also present in small to very small amounts especially in the raw/wet variety of BN (Sharan, 1996).

(b) Polyphenols and tannins: The main polyphenols of betel nut are catechin, flavanoids, flavan-3:4-diols, leucocyanidins and hexahydroxyflavans. When oxidized in the presence of lime, these give the characteristic red color to the saliva, teeth and lips of BQ masticator. The predominant tannin of BN is gallotannic acid. In addition, minor amounts of gallic acid, D-catechol, and phiobatannin are also present (Sharan, 1996).

(c) Betel nut specific nitrosamines (BSNA): Nitrosamine derivatives are produced from each of the four major BN alkaloids, that is, arecoline, arecaidine, guvacine and guvacoline by nitrosation of the alkaloids in dried stored nuts, in the mouth and especially in the acidic milieu of the stomach in the presence of nitric oxide generated by bacterial action (Wary and Sharan, 1991; Boucher and Mannan, 2002). The major biologically relevant nitrosamines of BN, also called betel nut specific nitrosamines (BSNA), are 3-methylnitrosaminopropionaldehyde (MNPA), 3-methylnitrosaminopropionitrile (MNPN), N-nitosoguvacine (NGC) and N-nitrosoguvacoline (NGCO), and have been found in the saliva of betel-quid users (IARC, 1985; Nair *et al.,* 1985). Of these, MNPA was reported to be the most potent on a molar basis, decreasing both survival and thiol content of cultured human buccal epithelial cells and causing significant formation of DNA single strand breaks (Sundqvist *et al.,* 1989).

(d) Reactive oxygen species (ROS): Aqueous extracts of *Areca* nut and catechu were found to be capable of generating superoxide anion and hydrogen peroxide at pH

greater than 9 (Nair *et al.,* 1987). While saliva was found to inhibit both O_2 and H_2O_2 formation from BQ ingredients, ROS are formed in the alkaline chewing mixture within the saliva of a chewer due to the addition of slaked lime (Stich and Anders, 1989).

Today, there is sufficient evidence that *Areca* nut or BN as well as BQ without or with tobacco is carcinogenic to humans (Sharan, 1996; IARC, 2004). BQ without tobacco causes oral cancer, while BQ with tobacco causes cancers of the oral cavity, pharynx and oesophagus (IARC, 2004). A causal association between tobacco and BQ chewing habits and oral mucosal diseases such as leukoplakia, oral submucous fibrosis (OSF) and oral cancer has been established, and heavy users have a significantly increased mortality rate. An increased incidence of local tumors was observed in mice after subcutaneous injection of aqueous extracts of BQ without tobacco. Local tumors were produced in mice and local mesenchymal tumors in rats following subcutaneous injection of aqueous extracts of betel nut (AEBN). In hamsters, administration of *Areca* nut and application of its aqueous or dimethyl sulphoxide extracts to the cheek-pouch mucosa resulted in squamous cell carcinomas (SCC) of the cheek pouch and carcinomas of the forestomach (IARC 1985). While BQ has various components (IARC, 1985; Sharan, 1996; Warnakulasuriya, 2002), a study on Syrian hamsters revealed that BN fibre and cold aqueous extract are the major components of BQ that may promote carcinogenesis in the hamster buccal pouch, leading to tumor formation. AEBN has been shown to induce conformational changes in mouse liver high mobility group (HMG) proteins similar to that induced by a hepatocarcinogen, diethylnitrosamine (DEN), leading to the development of preneoplastic nodules in the liver (Pariat and Sharan, 1998 a & b).

Different extracts of BN such as, AEBN, acetic acid extract (AAEBN), HCl extract (HEBN) and ethanol extract (EEBN) as well as arecoline showed different extents of cytostatic and cytotoxic effects on Hep2 cells *in vitro*, with arecoline, HEBN and EEBN being the most potent (Sharan and Wary, 1992). AEBN was also found to reduce GSH levels, induce chromosomal aberrations (CA) and delay cell kinetics in mouse bone marrow cells with the induction of sister chromatid exchange (SCE) probably involving p53-dependant changes in cell proliferation (Kumpawat *et al.,* 2003). Frequency of SCE was elevated in mouse bone marrow cells, when mice were

exposed to the AEBN and its tannin (Panigrahi and Rao, 1989). AEBN also induced DNA strand breaks and enhanced cell proliferation in mouse kidney cells *in vitro* (Wary and Sharan, 1988a). Aqueous extracts of BQ without tobacco were reported to induce mutations in *Salmonella typhimurium* but not in Chinese hamster V79 cells, and do not induce micronuclei in Swiss mice (IARC, 1985). AEBN was reported to induce mutations in *Salmonella typhimurium* and in Chinese hamster V79 cells besides inducing gene conversion in *Saccharomyces cerevisiae* as well as CA in CHO cells. AEBN also induced micronuclei in bone marrow cells of Swiss mice while BN tannin fraction induced gene conversion in *Saccharomyces cerevisiae* (IARC, 1985). Arecoline, AEBN, AAEBN, HEBN and EEBN induced variable levels of dose-dependent unscheduled DNA synthesis (UDS) in Hep2 cells *in vitro* (Sharan, 1996). Ames test using *Salmonella typhimurium* strain TA 1535 revealed that arecoline was nonmutagenic, AEBN and HEBN were weak mutagens, and AAEBN and EEBN were strong mutagens suggesting that the mutagenic potential of alkaloids (arecoline) is significantly enhanced by other constituents of BN (Sharan, 1994; Balachandran and Sharan, 1995; Sharan, 1996). Aqueous extracts of processed and unprocessed varieties of BN were reported to be fetotoxic in Swiss albino mice leading to death, enhanced resorption and reduced weight of fetuses. Other abnormalities such as hematomas, curved tails, abnormal ribs and delay in skeletal maturity were also reported (Sinha and Rao, 1985a).

Global gene expression profiling in human gingival fibroblasts (HGF) exposed to arecoline revealed that four genes related to maintenance of genome stability and DNA repair were repressed by arecoline, namely *FANCG* also known as *XRCC9* that encodes a tumor suppressor capable of correcting CA, *CHAF1* and *CHAF2* that encode for chromatin assembly factor I (CAF1), and *BRCA1* which is implicated in DNA damage response and DNA repair, with *BRCA1* being repressed in a dose-dependant manner. *COX-2/ PTGS2* which is involved in cancer initiation and progression was overexpressed in HGF cells. *HSP4A1* and *DNAAJA1* which belong to the *HSP70* family of stress-induced proteins are also upregulated by arecoline in a dose-dependent manner. GDF15/ MIC-1 were also induced following arecoline exposure (Chiang *et al.*, 2007). Arecoline was found to inhibit growth of human KB epithelial cells in a dose- and time-dependent manner, and caused cell cycle arrest in late-S and G2/M phases by inducing cyclin Bl, Wee 1, and phosphorylated cdc2 protein levels whereas it declined

p21 protein expression in KB cancer cells. At the same time, arecoline also induced p21, but decreased cdc2 and cyclin B1 protein levels in primary human gingival keratinocyes (GK), thus suggesting that differential regulation of S and/or G2/M cell cycle-related proteins in the GK and KB cells play a crucial role in different stages of BQ-mediated carcinogenesis (Lee *et al.,* 2006). Arecoline was reported to induce abnormality in the shape of sperm heads and unscheduled DNA synthesis (UDS) in the early spermatid stages of Swiss albino mice (Sinha and Rao, 1985 b), and, to induce micronuclei formation in fetal mouse blood after transplacental exposure (Sinha and Rao, 1985c). Arecoline was reported to cause general developmental retardation of zebrafish embryos predominantly due to a general cytotoxic effect induced by depletion of intracellular thiols (Chang *et al.,* 2001).

The *p53* gene is known to be mutated in a variety of human cancers, and an accumulation of p53 protein acts as an important indicator of the presence of mutant p53 protein (Harris and Hollstein 1993). However, reports pertaining to *p53* mutation status of cancers associated with BN chewing have been widely contradicting. A study of Sri Lankan subjects with histologically confirmed oral squamous cell carcinoma (OSCC) and the habit of BN chewing with tobacco revealed low expression of p53 protein (Ranasinghe *et al.,* 1993). However, a similar study in BN and tobacco associated OSCC from Southern India showed nuclear p53 staining and p53 expression, indicating that carcinogens derived from tobacco and BN chewing may induce p53 mutations (Kuttan *et al.,* 1995). BQ chewers in Taiwan exhibited significantly higher incidence of *p53* gene mutations than non-chewers in esophageal squamous cell carcinoma (ESCC). The A: T to G: C transition and G: C to T: A transversion were the prevalent spectrum of *p53* gene mutations and alcohol consumption could enhance this peculiar spectrum of *p53* mutation in ESCC, suggesting that *p53* might be an important molecular target of BQ carcinogens in the development of ESCC in Taiwanese (Goan *et al.,* 2005). Another study on patients of OSCC in Taiwan revealed that G: C to A: T transitions were the predominant mutations in the *p53* gene associated with BQ and tobacco use (Hsieh *et al.,* 2001). Mutations in the *p53* gene were also frequent in OSCC specimens from Sri Lanka obtained from BQ chewers, the mutations being clustered significantly in exon 5 of the *p53* gene, and including small deletions and inclusions besides point mutations. These results indicate that exon 5 of the *p53* gene could be one of the specific targets for some BQ ingredients, and betel-quid chewing may be a

critical environmental factor in the development of OSCC (Chiba *et al.,* 1998). A study of potentially malignant oral lesions (leukoplakia) and OSCCs associated with BQ consumption in Northern India revealed a good correlation between p53 missense mutations, p53 antibodies and p53 protein accumulation in matched potentially malignant and malignant oral lesions (Ralhan *et al.,* 2001). Alternatively, the incidence of *p53* mutations was reported to be infrequent or absent in oral premalignant lesions and OSCC in subjects chewing betel quid with tobacco (Kannan *et al.,* 1999) and without tobacco (Thomas *et al.,* 1994; IARC 2004).

1.9. HEAD AND NECK CANCER

Head and neck cancer is the term given to a variety of malignant tumors that develop in the oral cavity, pharynx, paranasal sinuses, nasal cavity, larynx and salivary glands etc (www.oncologychannel.com/headneck/index.shtml). Squamous-cell carcinoma of the head and neck is a heterogeneous disease with distinct patterns of presentation and behavior. More than 50 percent of these cancers arising in the oropharynx, particularly in the palatine tonsils and the base of the tongue, contain oncogenic human papillomavirus (HPV) DNA. Head and neck squamous cell carcinoma (HNSCC) is strongly associated with alcohol and tobacco consumption. Molecular data provide evidence that the carcinogens found in these substances have a causal role in that the prevalence of *p53* mutations are significantly greater in cancers in patients who smoke and drink alcohol than in those in patients who abstain from these substances. Laryngeal, hypopharyngeal, and floor-of-mouth cancers rarely develop in patients who do not smoke, whereas the lateral border of the tongue is a common site for head and neck cancer in the nonsmoking group. Patients with primary cancers who often smoke and drink heavily may present with multiple precancerous and cancerous lesions, so-called field cancerization, throughout the upper aerodigestive tract. Secondary cancers develop in nonsmokers and smokers with similar frequency, but patients with second primary HNSCCs are much more likely to have abused tobacco and alcohol (Forastiere *et al.,* 2001). Occupational risk factors include nickel refining, woodworking, and exposure to textile fibers. Dietary factors may also play a part, and epidemiologic data suggest a protective role of dietary carotenoids, and an inverse association between the consumption of fruits and vegetables and the incidence of head and neck cancer (Vokes *et al.,* 1993). Betel quid and "bidi" smoking is also a major risk factor in Southeast

Asia. HNSCC remains a significant cause of morbidity and mortality, with approximately 540,000 new cases annually worldwide and 271,000 deaths accounting for a mortality of 50 percent (Perez-Ordoñez *et al.*, 2006).

It is generally accepted that HNSCC arises from a common premalignant progenitor followed by outgrowth of clonal populations associated with cumulative genetic alterations and phenotypic progression to invasive malignancy. These genetic alterations result in inactivation of tumor suppressor genes and activation of proto-oncogenes by deletions, point mutations, promoter methylation, and gene amplification.

Loss of chromosomal region 9p21 is found in 70–80 % of cases, thus representing the most common genetic alteration seen in squamous dysplasia and HNSCC. Loss of heterozygosity (LOH) of 9p21 appears to be an early event in squamous neoplasia of the head and neck and has been found in preneoplastic lesions, including 30 percent of cases of squamous hyperplasia. The CDKN2A gene locus found in chromosome 9p21 encodes two different transcripts, p16 and p14ARF, which are responsible for G1 cell cycle regulation and MDM2 mediated degradation of p53. p16 is often inactivated in HNSCC through homozygous deletion, by promoter methylation, and less commonly by point mutations. Loss of chromosome region 3p is another common early genetic event in squamous dysplasias and invasive HNSCC. Investigators have identified at least four distinct regions of allelic loss, that is, 3p14, 3p21, 3p22, 3p24, and 3p26. 3p14 contains the fragile histidine triad gene or FIHT, a putative tumor suppressor gene, which has been found to be inactivated by exonic deletions in a small percentage of HNSCC. LOH of 17p and point mutations of the *p53* gene are seen in approximately 50 percent of HNSCC cases. Most of these mutations appear to occur late in the progression from epithelial dysplasia to invasive carcinoma. Amplification of 11q13 and overexpression of cyclin D1 is seen in 30–60 percent of HNSCC cases and has been associated with an increased rate of lymph node metastases and overall poor prognosis.

In HNSCC, microsatellite instability has been identified in only a small subset of preinvasive lesions of the head and neck. Gene expression microarrays suggest that most of transcriptional alterations in head and neck carcinogenesis occur during the transition from normal mucosa to premalignant lesions rather than in the transformation

from premalignant lesion to invasive carcinoma (Perez-Ordoñez *et al.,* 2006). While amplification of oncogenes is not normally observed in head and neck carcinomas in the Western world, one study found amplification of H-ras (17 %), N-ras (30 %), N-myc (39 %) and c-myc (17 %) oncogenes in Indian patients with oral cancer, these results being explained by the specific smoking habits and betel nut chewing in India (Field, 1995).

Evaluation of DNA ploidy in oral leukoplakia allows the identification of gross genomic alterations and has been shown to be a useful tool in identifying lesions with a high risk of malignant transformation. A study revealed that patients with aneuploid leukoplakia have a 98 percent rate of developing primary oral carcinoma, an 81 percent rate of recurrent or second primary tumors, and a 76 percent risk of death from disease despite negative histology of the surgical margins (Perez-Ordoñez *et al.,* 2006).

1.10. BREAST CANCER

Breast cancer is the most frequently diagnosed cancer in women. In the United States alone an estimated 182,460 new cases of invasive breast cancer and 67,770 new cases of in situ breast cancer were expected to occur among women in 2008, and about 1,990 new cases were expected in men. Gender is a crucial risk factor for breast cancer. Risk is also increased by inherited genetic mutations in the BRCA1 and BRCA2 genes, a personal or family history of breast cancer, high breast tissue density, biopsy-confirmed hyperplasia, and high-dose radiation to the chest, typically related to a medical procedure. Reproductive factors that increase risk include a long menstrual history, never having children, recent use of oral contraceptives, and having the first child after the age of 30. Some potentially modifiable factors that increase risk include being overweight or obese after menopause, use of postmenopausal hormone therapy (especially combined estrogen and progestin therapy), physical inactivity, and consumption of alcohol (American Cancer Society, 2008). Breast cancer in men is a rare disease, accounting for 1% of all breast cancer cases. Major genetic factors associated with an increased risk of breast cancer for men include BRCA2 mutations, which are believed to account for the majority of inherited breast cancer in men, Klinefelter syndrome, and a positive family history. Suspected genetic factors include *AR* gene mutations, CYP17 polymorphism, Cowden syndrome, and CHEK2.

Epidemiologic risk factors for male breast cancer include disorders relating to hormonal imbalances such as obesity, testicular disorders and radiation exposure. Suspected epidemiologic risk factors include prostate cancer, prostate cancer treatment, gynecomastia, occupational exposures such as exposure to electromagnetic fields, polycyclic aromatic hydrocarbons, and high temperatures and dietary factors such as meat and alcohol intake (Weiss *et al.,* 2005).

At least 5 percent of all breast cancer cases are thought to result from a hereditary predisposition to the disease. Women who inherit loss-of-function mutations in one allele of either the *BRCA1* or *BRCA2* gene have an up to 85 percent risk of breast cancer by the age of 70. Carriers of mutations in these genes are also at elevated risk of cancer in the ovary, pancreas and prostrate (Kerr and Ashworth, 2001). Susceptibility alleles in other genes, such as *p53, PTEN,* and *STK11/LKB1,* are even less common causes of breast and ovarian cancer.

An association between the risk of breast cancer and persistently elevated blood levels of estrogen has been found consistently in many studies. These support the hypothesis that cumulative, excessive exposure to endogenous estrogen across a woman's life span contributes to and may be a causal factor in breast cancer. Several endocrine-associated risk factors are regularly associated with an increased relative risk of breast cancer in postmenopausal women. One of these factors is obesity, which is probably related to an increased production of estrogen by aromatase activity in breast adipose tissue. Another factor is an elevated blood level of endogenous estrogen. An increased relative risk is also associated with higher-than-normal blood levels of androstenedione and testosterone, androgens that can be directly converted by aromatase to the estrogens estrone and estradiol, respectively. Elevated urinary levels of estrogens and androgens are also associated with an increased risk of breast cancer in postmenopausal women. The estrogen 3, 4-quinone formed as a result of metabolism of estrogen by cytochrome P450 enzymes can form unstable adducts with adenine and guanine in DNA, leading to depurination and mutation *in vitro* and *in vivo* (Yager and Davidson, 2006).

Because *BRCA1* and *BRCA2* are expressed in a broad spectrum of tissues and cell types it is not clear how loss of their functions can lead to tissue and gender-specific cancers. The breast and ovary are estrogen-responsive tissues with estrogen metabolites thus

acting as tissue-specific carcinogens. Moreover the breast and ovarian epithelia proliferate rapidly under the influence of estrogens, the progeny of this proliferative burst being retained in the breast lobules and ovarian inclusion cysts. A cancer-predisposing mutation, such as in p53 in a lobular precursor cell, accompanied by loss of BRCA mutations, could thus increase the risk of breast and ovarian cancer (Scully and Livingston, 2000; Sowter and Ashworth, 2005). Ghosh *et al.* have proposed that BRCA1 inhibits a breast-cancer associated promoter of the aromatase gene in human adipose stromal cells (ASCs). Thus a role of BRCA1 in modulating estrogen biosynthesis may also contribute to its tissue-specific tumor suppressor function (Ghosh *et al.*, 2007).

1.11. CERVIX CANCER

Cancer of the uterine cervix is the second-most common cancer in the world, but is the most prevalent cancer in Indian women, posing a major public health problem. In India, about 100,000 women develop this cancer annually (Das *et al.*, 2000). In 2003, there was an estimated annual global incidence of 500,000 cases of cervical cancer, of which India contributed 100,000, that is, one-fifth of the world burden. The number of cervical cancer deaths in women in India is projected to increase to 79,000 by the year 2010 (Bobba and Khan, 2003). The cancers that develop from uterine cervix are of two types: (i) squamous cell carcinomas, which develop from squamous epithelium, cover mostly visible part of cervix; and (ii) adenocarcinomas, which arise from glandular lining of endocervical canal. About 85 to 90 percent of cervical cancers are squamous cell carcinomas and the rest 10 to 15 percent are adenocarcinomas. Over 90 percent of these cancers are diagnosed at advanced stages, the majority of which are presented at 35–64 years of age. Squamous cell carcinoma (SCC) is preceded by well-recognized epithelial changes, the precancerous lesions which may progress to malignancy, or persist, or regress to normalcy. The distinct premalignant or dysplastic changes are generally detected by a simple exfoliated cytological screening, the 'Pap-test'30. The precancerous lesions may take a few months to several years (10–15 years) to progress to the stage of invasive cervical cancer. Therefore, early diagnosis of premalignant cervical lesions plays a pivotal role in controlling cervical cancer. Several epidemiological studies the world over have concluded that infection of human papillomavirus (HPV), particularly of high-risk HPV types 16 and 18, is the primary

risk factor for cervical cancer (Das *et al.,* 2000). IARC studies also reported the contribution of additional factors to the risk of cervical cancer in HPV carriers. Among HPV-positive women, any use of oral contraceptives was associated with a significant increase in risk- use for less than 5 years was not related to cervical cancer but the risk increased significantly for 5 to 9 years of use and for 10 or more years. HPV-positive women who reported 7 or more full- term pregnancies had a 4-fold increased risk of cervical cancer as compared to similar HPV-positive women that were nulliparous. Smoking was associated with a 2-fold statistically significant increased risk of cervical cancer with a significant dose response (Monsonego *et al.,* 2004).

The high-oncogenic-risk HPV types associated with invasive cervical cancer produce two oncoproteins, designated E6 and E7, which interact with endogenous cell cycle regulatory proteins, including p53 and Rb. The E6 protein is able to bind to host p53 causing inactivation of its function through the mechanism of ubiquitin-dependent degradation. The interaction of virally derived and endogenous cellular proteins converges in deregulation of cell cycle progression and appears to be critical for the development of cervical cancers. However, the development of cervical cancer is a multistep process that cannot be explained simply by infection with specific types of HPV. One additional event that appears to play a role in tumor progression is integration of HPV DNA into the host genome. One study reported that the extent of p53 dysfunction caused by HPV depends on the status of a proline (Pro) or arginine (Arg) polymorphism at codon 72 of p53. It was demonstrated that an individual homozygous for the Arg allele had about a seven times higher risk of developing cervical cancer than a patient homozygous for Pro (Storey *et al.,* 1998). Homozygosity for p53 Arg was also reported as a potential risk factor for cervical cancer in South African (Pegoraro *et al.,* 2002) and Chinese populations (Min-min *et al.,* 2006). However, other studies have reported that the polymorphism does not contribute to an increased risk of cervical cancer in Japanese (Yamashita *et al.,* 1999), and South Indian populations (Pillai *et al.,* 2002). Thus, the association of p53 polymorphism at codon 72 and HPV- induced cervical cancer remains unclear.

1.12. BIOMARKERS OF CANCER

Biomarkers are anatomic, physiologic, biochemical, or molecular parameters associated with the presence and severity of specific disease states. They are detectable and measureable by a variety of methods including physical examination, laboratory assays and medical imaging (www.biomarkers.org). As an important biological indicator of cancer status and progression for the physiological state of the cell at a specific time, biomarkers represent powerful tools for monitoring the course of cancer and gauging the efficacy and safety of novel therapeutic agents. They can have tremendous therapeutic impact in clinical oncology, especially if the biomarker is detected before clinical symptoms or enable real-time monitoring of drug response. Malignant transformation involves alterations in protein expression with subsequent clonal proliferation of the altered cells. These alterations can be monitored at the protein level, both qualitatively and quantitatively. Protein signatures in cancer thus provide valuable information that may be an aid to more effective diagnosis, prognosis, and response to therapy (Cho, 2007). Alterations in *p53* expression and level of the p53 protein are commonly observed in various cancers. Thus studies have focused on the use of p53 as a biomarker of cancer. Immunocytochemical staining of cytologic specimens, exfoliated cells, needle-biopsy specimens and fixed tissues with anti-p53 antibody showing nuclear accumulation is used as a biomarker of cancer. The overabundance of p53 protein indicates p53 mutation and is an unfavorable prognostic factor of lung cancer and bladder cancer etc. In fact, the determination of serum levels of p53 protein has been proposed as a convenient and useful non-invasive screening test for early detection of hepatocellular carcinoma (HCC) (Pich, 1998). Use of BRCA1 as a biomarker of cancer has also been reported. Immunohistochemical staining with anti-BRCA1 antibodies showing reduced or undetectable expression of BRCA1 in sporadic breast cancer is considered as a biomarker of breast cancer (Wilson *et al.,* 1999; Yoshikawa *et al.,* 1999). BRCA1 protein is also downregulated in ovarian cancer (Zheng *et al.,* 2000) and in sporadic pancreatic adenocarcinoma (Beger *et al.,* 2004).

1.13. AIMS AND OBJECTIVES

While the role of BN and its various constituents in carcinogenesis have been well documented, there is no report on the response of the *p53*, *BRCA1* and *BRCA2* tumor suppressor genes in the same model of BN exposure and/or the same cases of betel-

induced cancer in humans. There is also no report on the effect of transgenerational exposure to BN. Since the *p53*, *BRCA1* and *BRCA2* genes play a critical role in the prevention of transformation of a normal cell to a cancer cell (§ 1.6. and § 1.7.), alterations in the levels of these tumor suppressor proteins are likely to be intrinsically involved in the process of BN-induced carcinogenesis. Moreover, considering the prevalence of the habit of BN chewing in various populations (§ 1.8.), it is relevant to examine the result of transgenerational exposure to BN. Thus, in the present study the effect of chronic and transgenerational exposure to BN with respect to response/s of the *p53*, *BRCA1* and *BRCA2* genes were determined. The specific aims and objectives of this study were as follows:-

1.13.1. To understand the combined roles of the *p53*, *Brca1* and *Brca2* tumor suppressor genes in carcinogenesis upon chronic exposure of Swiss Albino mice to aqueous extract of betel nut (AEBN), by determining the effect on

- **a.** liver, spleen and peripheral blood lymphocytes (PBL) of mice;
- **b.** alterations in ultrastructure of cell organelles in liver;
- **c.** alterations in levels of p53, Brca1 and Brca2 proteins in liver, spleen and PBL;
- **d.** occurrence of mutations in relevant regions of *p53*, *Brca1* and *Brca2* genes. Exons 5 and 7 of the *p53* gene were selected for the study because these regions are constituents of the region of the *p53* gene which code for it DNA binding domain which contains the hotspots of mutation in *p53* (§ 1.6.). Exon 11 of the *Brca1* gene was selected because it codes for the RAD51 interaction domain of the Brca1 protein which is vital for its role in DNA repair. Similarly, exon 27 of the *Brca2* gene was selected because it also codes for the second RAD51 interaction domain, as well as the nuclear localization signal of the Brca2 protein (§ 1.7.).

1.13.2. To determine whether transgenerational exposure to betel nut may alter the susceptibility to cancer, and, to understanding the combined roles of the *p53*, *Brca1* and *Brca2* tumor suppressor genes in mice exposed transgenerationally to AEBN by determining the effect on all parameters employed for the chronic exposure study (§ 1.13.1 [a] through [d]).

Previous studies on the role of the *p53* and *BRCA1* genes as biomarkers of cancer have been limited to the use these genes individually, using samples obtained through invasive techniques such as biopsies. Given the intrinsic role played by these genes in carcinogenesis, the current study also sought to determine the usefulness of alterations in the levels of p53, BRCA1 and BRCA2 tumor suppressor proteins as biomarkers of cancer using a blood-based assay by:

1.13.3. Determining the alterations in levels of p53, BRCA1 and BRCA2 proteins in PBL of patients suffering from head and neck, cervix and breast cancer in comparison to normal controls. The above mentioned cancers were selected for the study because of their high incidence in India (Bobba and Khan, 2003).

MATERIALS & METHODS

2.1. CHEMICALS

2.1.1. General chemicals

All chemicals used were of analytical grade and were used without further purification. Histopaque 1077 & 1083, ethylenediamine tetraacetic acid (EDTA), 2-mercaptoethanol, phenylmethanesulfonyl fluoride (PMSF), sodium azide (NaN_3), triton X-100, sodium dodecyl sulfate (SDS), hexadecyltrimethyl- ammonium bromide (CTAB), N-lauroyl sarcosine, ammonium persulfate (APS), proteinase K, ribonuclease (RNase), acrylamide, N,N'-methylene bisacrylamide, N,N,N'N'-tetramethylenediamine (TEMED) and bovine albumin fraction V (BSA) were from Sigma Chemical Company, St. Louis, USA. India ink was procured from Rotring Zeichentusche Drawing Ink, Hamburg, Germany. RPMI-1640 was from HyClone, Logan, Utah. 5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium (BCIP/NBT), Tween-20, Protein A CL-agarose beads and agarose were from Bangalore Genei Pvt. Ltd., Bangalore, India. Isoamyl alcohol, methanol, tris base (hydroxymethyl) methylamine, glycine, potassium chloride, sucrose, acetic acid glacial, isopropyl alcohol and D-glucose were from Qualigens Fine Chemicals, Mumbai, India. Coomassie brilliant blue G250, phenol saturated, chloroform and heparin were from Sisco Research Laboratories (SRL) Pvt. Ltd, Mumbai, India. Ortho-phosphoric acid, sodium acetate, D-sorbitol and calcium chloride were from s.d fine-chem limited, Mumbai, India. Sodium hydroxide, di-sodium hydrogen phosphate and sodium dihydrogen phosphate were from Merck, Mumbai, India. Dehydrated alcohol was from Bengal Chemicals and Pharmaceuticals Ltd, Kolkata, India. Ethidium Bromide was from Merck, Germany. BIOTAQ Red DNA Polymerase was from Bioline, Randolph, USA.

2.1.2. Water

Water was deionized using Elix 10 water purification system, Millipore. All solutions were prepared with this water. However, the solutions for transmission electron microscope (TEM) studies were prepared using double distilled water.

2.1.3. Protein molecular weight markers and DNA markers

Protein molecular weight markers and 100 bp DNA ladder were procured from Bangalore Genei Pvt. Ltd., Bangalore, India.

2.1.4. Kits and primers

DNA Amplification Reagent Kit and Gel Extraction Kit were purchased from Bangalore Genei Pvt. Ltd., Bangalore, India. Primers were synthesized by Hysel India Pvt. Ltd., New Delhi, India.

2.1.5. Membrane and antibodies

Nitrocellulose membrane (NCM) was from Sigma Chemical Company, St. Louis, USA.

Primary antibodies: Anti-p53 and anti-actin were obtained from Sigma Chemical Company, St. Louis, USA. Anti-BRCA1 and anti-BRCA2 were procured from Santacruz Biotechnologies, USA.

Secondary antibodies: Alkaline- phosphatase labeled donkey anti-sheep IgG was from Sigma Chemical Company, St. Louis, USA. Alkaline phosphatase labeled goat anti-rabbit IgG was from Bangalore Genei Pvt. Ltd., Bangalore, India.

2.2. INSTRUMENTS

The following instruments were routinely used:

Lyolab 3000 (Heto-Holten A/S, Allerød, Denmark); Laboratory Centrifuges 3K3 (Sigma Chemical Company, St. Louis, USA) R8C Laboratory Centrifuge and tissue homogenizer (Remi Motors, Bombay, India); UV-Vis Spectrophotometer 119 (Systronics, India); Bio-Dot SF Microfiltration Apparatus, Mini-Protean II Electrophoretic Cell, Mini Trans-Blot Electrophoretic Transfer Cell, and Mini transilluminator (Bio-Rad, USA); Thermal Cycler (Applied Biosystems, USA); HP Scanjet 7400C; Kodak digital camera; Olympus BX60 brightfield microscope; 100 CXII Transmission electron microscope (Jeol, Tokyo, Japan).

2.3. COLLECTION OF HUMAN BLOOD SAMPLES

Blood samples were collected from patients at the B. Barooah Cancer Institute, Guwahati, as well as from healthy volunteers without a history of cancer who served as controls, with informed consent, and data was collected in response to a questionnaire. Patients included in the study were those suffering from breast cancer, cervical cancer and head and neck cancers at various sites namely the tongue and base of tongue, buccal mucosa, pre-epiglottic region and epiglottis,

pyriform sinus, external mouth cavity, oesophagus, larynx, vocal chord, throat, nasopharynx, and patients with secondary cancer of the neck lymph nodes.

2.4. EXPERIMENTAL ANIMALS

Six week old male and female inbred Swiss Albino mice weighing 25 ± 1 g were used. The mice were housed in polycarbonate cages with husk bedding in a well ventilated animal room maintained at 25 °C. Five mice were housed in one cage, and male and female mice were maintained separately, except when mice were to be bred for the transgenerational studies. A strict coding system was employed to maintain the different generations of mice separately.

2.5. PREPARATION OF CARCINOGEN

The carcinogen used for all experiments was aqueous extract of betel nut (AEBN), which was prepared as follows.

2.5.1. Materials required

1. Betel nut: The betel nut was procured from the local market, and is a raw, unprocessed form of betel nut (*Kwai im*) commonly used as a masticatory in Meghalaya, and various parts of North-East India.
2. Millipore water
3. Whatman Filter Paper No. 1

2.5.2. Methodology

Aqueous extract of betel nut (AEBN) was prepared as previously described (Wary and Sharan, 1988). Whole betel nuts were shelled, ground coarsely with the help of a mortar and pestle, and weighed. 250 ml of Millipore water was added to every 100 g of ground betel nut, and the suspension was left overnight (O/N) at room temperature (RT). The following day, the extract was filtered through Whatman Filter Paper No 1. The filtrate was collected in a lyophilization flask and kept to freeze at -80 °C. The frozen filtrate was then lyophilized. The lyophilized powder thus obtained was AEBN. Approximately 2 g of AEBN was obtained per 100 g of ground betel nut.

2.6. ADMINISTRATION OF CARCINOGEN AND EXPERIMENTAL DESIGN

Six week old male and female mice were exposed to AEBN at a dose of 2 mg ml^{-1} in drinking water, *ad libitum,* in chronic as well as transgenerational exposure regimens. Conversely, control mice were provided drinking water without AEBN. Both exposed and control mice were provided with pelleted laboratory mouse feed (Amrut laboratories, Pune, India) *ad libitum.* The experimental designs followed for chronic and transgenerational exposure regimens were as detailed in Fig. 2.1, and are briefly described below.

2.6.1. Chronic exposure

Six week old male and female mice were chronically exposed to AEBN at a dose of 2 mg ml^{-1} in drinking water up to 24 weeks. These mice are referred to as the P generation exposed mice. The exposed as well as age-matched control mice were sacrificed at regular intervals, that is, after 2, 4, 6, 8, 10, 12, 16, 20 and 24 weeks of exposure by cervical dislocation. However, when blood was to be drawn, mice were sacrificed under chloroform anesthesia.

2.6.2. Transgenerational exposure

The transgenerational exposure experiment was initiated after completion of the chronic exposure regimen. Male and female mice of the P generation, which had been exposed to AEBN in drinking water for 6 weeks, were allowed to breed by maintaining one male and four female mice per cage with standard food pellet and drinking water containing AEBN *ad libitum.* The offspring of the exposed P generation mice constituted the F1 generation exposed mice. Post-weaning, i.e. at 6 weeks of age, the F1 mice were separated from their parents, male and female mice being kept separately, and were maintained on AEBN drinking water for a period to 24 weeks. The F2 and F3 generations were similarly raised from F1 and F2 mice, respectively. Age-matched unexposed control mice of the P generation were also bred in parallel, and their offspring served as age-matched controls for the F1 exposed mice. Respective controls for the F2 and F3 generations were also raised similarly. A strict coding system was followed to maintain the F1, F2 and F3 generations and their respective controls. AEBN exposed F1, F2 and F3 mice, as well

as their respective age-matched controls were sacrificed in groups of 5 mice at intervals of 4, 6, 8, 12, 16 and 24 weeks for monitoring alterations in p53 protein level; and, at intervals of 2, 6, 8, 12, 16 and 24 weeks for monitoring alterations in Brca1 and Brca2 protein levels. Mice were sacrificed by cervical dislocation. However, when blood was to be drawn they were sacrificed under chloroform anesthesia.

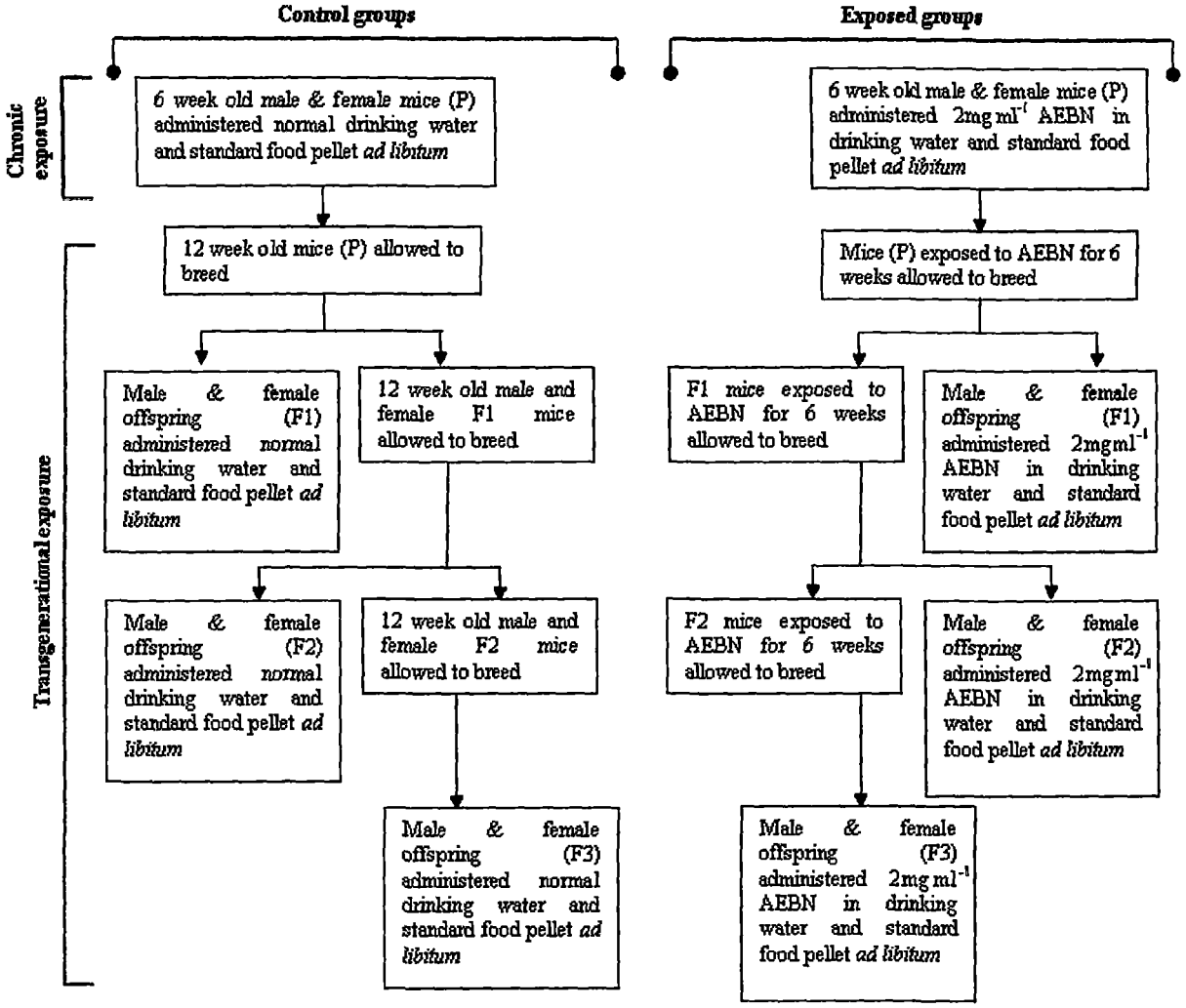


Figure 2.1. Experimental designs of chronic and transgenerational exposure of Swiss Albino mice exposed to AEBN in drinking water at a dose of 2 mg ml^{-1}.

2.7. HISTOLOGICAL EXAMINATION

Histological sections of liver from preneoplastic nodules of AEBN exposed P, F1, F2 and F3 mice and corresponding regions of livers of age-matched control mice were

prepared according to the method described by Ratcliffe (1983), with some modifications.

2.7.1. Materials required

1. Fixative: The fixative used was 10 % neutral buffered formalin. The pH of 10 % formalin was adjusted to neutral pH with normal saline (0.097 g NaCl/ litre of Millipore water).
2. Stains: [a] Harris hematoxylin was used directly as supplied by the manufacturer and not processed further
   [b] 1 % (v/v) aqueous solution of eosin
3. Graded series of alcohol: 50 %, 70 %, 90 % and absolute (95 %) ethanol
4. Acetone
5. Xylene
6. Paraffin wax
7. Egg albumin
8. Acid water/ ethanol mixture: Mixture of 1 % HCl in water and 70 % ethanol in the ratio 1: 1 (v /v)
9. DPX mountant
10. Microtome
11. Glass slides
12. Cover slips

2.7.2. Methodology

Sections of liver tissue were prepared through a series of steps described as follows: -

2.7.2.1. **Fixing:** - Liver tissue was cut into 2 mm thick pieces and fixed in 10 % formalin O/N. The following day the tissue sections were washed in tap water.

2.7.2.2. **Dehydration:** - Tissue sections were dehydrated through different grades of ethanol as follows- (a) 70 % ethanol for 12 hours (b) 90 % ethanol for 12 hours (c) 2 changes of 100 % ethanol for 2 hours each. This was followed by washing with 2 changes of acetone for 1 hour each and 2 changes of xylene for 45 min each.

2.7.2.3. **Embedding:** - The tissue sections were embedded in paraffin wax maintained at 58-60°C in an oven, with 3 changes of wax for 1 hour each.

2.7.2.4. **Block making:** - Tissue sections were transferred to paper moulds containing paraffin wax maintained at 60-62°C. The wax was allowed to cool at RT.

2.7.2.5. **Sectioning:** - Tissue blocks were cut into 5 -7 μm thick sections with the help of a microtome, transferred to albumin coated glass slides, and left to dry at RT.

2.7.2.6. **Deparaffinization:** - Tissue sections were deparaffinized by pressing the glass slides over a hot plate till the paraffin just melts. The slodes are immediately dipped in xylene and kept immersed for 20 min.

2.7.2.7. **Drying and rehydration:** - The glass slide was blotted dry and then transferred through serial dilutions of ethanol, i.e., 100 % ethanol for 1 min, 90 % ethanol for 1 min, 70 % ethanol for 1 min, and 50 % ethanol for 1 min. This was followed by immersion in tap water for 2 min.

2.7.2.8. **Staining:** - Staining was performed following a stepwise procedure. The sections were first stained with Harris hematoxylin for 10 min, and dipped in acid water/ ethanol mixture, followed immediately by washing under running tap water for 10 min. At this stage the sections were viewed under a microscope for staining of nuclei, and staining with hematoxylin was repeated for 5 min if the nuclei were not distinct. Sections stained with hematoxylin were dipped in acid water/ alcohol mixture and removed immediately. The sections were then stained with 1 % aqueous solution of eosin for 30 sec. These sections are referred to as H & E stained sections.

2.7.2.9. **Dehydration:** - The H & E stained sections were dehydrated through serial grades of ethanol, i.e. 70 % ethanol for 1 min, 90 % ethanol for 1 min and absolute alcohol for 1 min.

2.7.2.10. **Mounting:** - The H & E stained sections were dried in an incubator maintained at 37° C and dipped in xylene for 5 min. A drop of DPX mountant was then taken on a cover slip and pressed firmly over the sections, taking care to avoid trapping air bubbles. Excess DPX was wiped off, and the slides were left to dry O/N at RT.

2.7.2.11. **Imaging:** - The H & E sections were viewed and photographed with the help of Olympus Brightfield Microscope at a magnification of 400 X.

2.8. TRANSMISSION ELECTRON MICROSCOPE (TEM) STUDIES

Regions of preneoplastic nodules in livers of P, F1, F2 and F3 mice exposed to AEBN for 24 weeks, and the corresponding regions of the livers of age-matched controls were examined under transmission electron microscope (TEM) at Sophisticated Analytical Instrumentation Facility, NEHU, Shillong.

2.8.1. Materials required

1. Cacodylate buffer (0.2 M): - This buffer had the following components:

[a] Sodium cacodylate	21.4 g
[b] 1 N HCl	3.45 ml
[c] Double distilled water	

The above components were dissolved in double distilled water, and the final volume adjusted to 500 ml.

2. Ten percent paraformaldehyde solution: - This solution had the following components:

[a] Paraformaldehyde	100 g
[b] Double distilled water	100 ml
[c] 1 N NaOH	

Paraformaldehyde was dissolved in double distilled water by heating to 60-65° C. The solution was then cleared by adding a few drops of 1 N NaOH.

3. Twenty five percent glutaraldehyde: - This solution had the following components:

[a] Glutaraldehyde	25 g
[b] Double distilled water	100 ml

Glutaraldehyde was dissolved in double distilled water to prepare the solution.

4. Primary fixative: - The primary fixative used was Karnovsky's fixative, and it had the following components:

[a] 0.2 M cacodylate buffer	50 ml
[b] 10 % paraformaldehyde	20 ml
[c] 25 % glutaraldehyde	10 ml
[d] Double distilled water	20 ml

The above components were mixed to prepare the fixative.

5. Secondary fixative: - the secondary fixative used was 1 % OsO_4 in water.
6. Cacodylate buffer (0.1 M): - this buffer was prepared by mixing 0.2 M cacodylate buffer with an equal volume of double distilled water.
7. Graded series of acetone: 30 % aetone, 50 % acetone, 70 % acetone, 80 % acetone, 90 % acetone, 95 % acetone, 100 % acetone and dry acetone
8. Propylene oxide

9. Embedding medium: - The embedding medium consisted of the following components:

[a] Araldite CY2121	10 ml
[b] Dodecyl succinic anhydride (DDSA)	10 ml
[c] 2, 4, 6-tri (dimethylaminomethylphenol) (DMP-30)	0.4 ml
[d] Dibutyl phthalate	1 ml

The above components were mixed to prepare the embedding medium.

10. BEEM embedding capsule
11. Uranyl acetate stain

2.8.2. Methodology

Samples were prepared for TEM studies following standard protocol described by Hayat (1985) with some modifications, described as follows:-

***2.8.2.1.* Fixing:** - Liver tissue was cut into 1 mm thick pieces and fixed in Karnovsky's fixative for 4 hours.

***2.8.2.2.* Washing:** - The tissue pieces were washed by immersion in 0.1 M cacodylate buffer for 10 min, followed by centrifugation at 10,000 x *g* for 1 min. This washing step was repeated thrice.

***2.8.2.3.* Secondary fixation:** - Post fixation of tissue pieces was performed in 1 % OsO_4 for 1 hour.

***2.8.2.4.* Clearing:** - The tissue pieces were cleared in propylene oxide with 2 changes for 30 min each at RT.

***2.8.2.5.* Infiltration:** - Embedding of tissue pieces was performed using a mixture of embedding medium and propylene oxide in the ratio 1:3, O/N at RT. The next day the the tissue pieces were transferred to a mixture of embedding medium and propylene oxide in the ratio 1:1 for 1 hour at RT, mixture of embedding medium and propylene oxide in the ratio 3:1 for 1 hour at RT, kept for 1 hour in vacuum, and then transferred to pure embedding medium at 50 °C. The tissue pieces are then placed in embedding BEEM capsules, covered with pure embedding medium, and left at 50° C for 12 hours.

***2.8.2.6.* Polymerization:** - The tissue pieces are maintained in the BEEM embedding capsule at 60° C for 24-48 hours.

2.8.2.7. **Sectioning:** - 60-90 nm thick sections are cut with the help of an ultra-microtome.

2.8.2.8. **Staining:** - The sections are stained with uranyl acetate for 30-120 min at RT, as described by Terzakis (1968).

2.8.2.9. **Imaging:** - The sections were viewed and photographed at different magnifications ranging from 5000 X to 40, 000 X.

2.9. SEPARATION OF PERIPHERAL BLOOD LYMPHOCYTES

2.9.1. Peripheral blood lymphocytes (PBL) were separated from whole blood samples collected from human cancer patients, control volunteers, mice exposed to AEBN and age-matched control mice by the method of Bøyum (1968a & 1968b) and as previously described (Kma and Sharan, 2006) with some modifications.

2.9.2. Materials required

1. Balanced salt solution (BSS) pH 7.6, consisted of the following components:-

[a] anhydrous glucose	5.5 mM
[b] $CaCl_2$	5 mM
[c] $MgCl_2$	0.098 mM
[d] KCl	5.4 mM
[e] Tris- Cl	145 mM

   The above chemicals were dissolved in Millipore water, and the pH of the solution was adjusted with concentrated HCl before making up the necessary volume with water.
2. Heparin solution: - 4 mg ml^{-1} solution of heparin sodium salt in Millipore water
3. Histopaque 1077 for recovery of viable mononuclear cells from whole human blood.
4. Histopaque 1083 for recovery of viable mononuclear cells from whole mouse blood.
5. RPMI-1640 medium: - RPMI-1640 obtained in powder form was dissolved in the required volume of Millipore water as per the manufacturer's recommendations, and sterilized by autoclaving before use.

6. Siliconized glass centrifuge tubes and Pasteur pippettes: - Glass centrifuge tubes and Pasteur pippettes were siliconized in order to retard clotting of blood. For this the tubes were by coated with a thin layer of Sigmacote used as supplied by the manufacture without further dilution, and allowed to dry.

2.9.3. Methodology

The collected blood was mixed with a small volume of heparin in a siliconized centrifuge tube. The heparinized blood was then mixed with an equal volume of BSS. Taking the blood-BSS mixture and Histopaque in the ratio 4:3 (v/v), the mixture was carefully layered over Histopaque 1083 or Histopaque 1077 depending upon whether the blood sample was obtained from humans or mice, respectively. Following centrifugation (400 x *g*) for 40 min at RT, the lymphocyte layer formed at the interphase was recovered using a siliconized Pasteur pipette, and washed twice with 3 volumes of BSS (100 x *g*) for 10 min at room temperature. The lymphocytes were then resuspended in 1 ml of RPMI-1640 and stored at -80 °C till further use.

2.10. PREPARATION OF LYSATE OF PERIPHERAL BLOOD LYMPHOCYTES

The recovered PBL were lysed to obtain a PBL lysate using the method of Rosenberg (1996) with some modifications, as previously described (Kma, 2003).

2.10.1. Materials required

1. Phosphate buffered saline (PBS) pH 7.4: - This buffer consisted of the following components:

[a] NaCl	140 mM
[b] KCl	2 mM
[c] Na_2HPO_4	10 mM
[d] KH_2PO_4	1 mM

The above chemicals were dissolved in the required volume of Millipore water. The pH recorded was approximately 7.4, and the pH was not adjusted further. The buffer was kept refrigerated at 4 °C.

2. Cell lysis buffer: - This buffer consisted of the following components:

[a] Tris- Cl, pH 8.0	20 mM
[b] NaCl	10 mM
[c] Triton X-100	0.5 %
[d] EDTA	5 mM
[e] $MgCl_2$	3 mM
[f] PMSF	10 mM

To prepare 100 ml of Tris-Cl buffer, 0.24 g of Tris base was dissolved in Millipore water, the pH was adjusted to 8 with concentrated HCl, and the final volume was raised to 100 ml with Millipore water. The remaining chemicals were dissolved in the appropriate volume of Tris-Cl buffer. PMSF was prepared as a 100 mM stock solution in isopropanol by heating to 60 °C until the PMSF flakes had dissolved completely. The required volume was added to the cell lysis buffer, and the buffer was stored refrigerated at 4 °C.

3. Microcentrifuge tubes of 1.5 ml capacity

2.10.2. Methodology

For lysis of lymphocytes, RPMI-1640 was removed by centrifuging (250 x *g*) for 10 min at 4 °C, and the supernatant discarded. The pellet was washed once with 1 ml of cold PBS by centrifuging (200 x *g*) for 10 min at 4 °C, and the supernatant again discarded. The pellet was resuspended in 1 ml of cold cell lysis buffer and kept at -20 °C for 30 min, followed by centrifugation (5000 x *g*) for 15 min at 4 °C. The supernatant was collected and its protein content determined by the method of Bradford (1976) as described in § 2.13.

2.11. PREPARATION OF WHOLE HOMOGENATE OF LIVER

A 10 % (w/v) whole homogenate (WH) of liver of AEBN-exposed and age-matched control mice was prepared using the method of Rosenberg (1996) with some modifications.

2.11.1. Materials required

1. Cell extract buffer: - This buffer consisted of the following components:

[a] Tris-Cl, pH 7.5	100 mM
[b] Sucrose	250 mM

[c] NaCl	100 mM
[d] EDTA	3 mM
[e] 2-mercaptoethanol	10 mM
[f] PMSF	1 mM

To prepare 100 ml of Tris-Cl buffer, 1.21 g of Tris base was dissolved in Millipore water, the pH was adjusted to 7.5 with concentrated HCl, and the final volume was raised to 100 ml with Millipore water. PMSF was prepared as described in § 2.10.1. The above chemicals were dissolved in the required volume of Tris-Cl, and the cell extract buffer was stored refrigerated at 4 °C.

2. Phosphate buffered saline (PBS) pH 7.4: - This buffer was prepared as described in § 2.10.1.
3. Centrifuge tubes of 10 ml capacity

2.11.2. Methodology

For preparation of the WH of liver, 0.15 g of tissue was taken from each liver. The tissue was cut into fine pieces and washed with an excess of cold PBS by centrifugation (200 x *g*) for 10 min at 4 °C. The liver pieces were then homogenized in 1.5 ml cold cell extract buffer by 30 strokes of a tissue homogenizer kept in ice. The homogenate was centrifuged (800 x *g*) for 10 min at 4 °C. The supernatant was collected and its protein content determined by the method of Bradford (1976) as described in § 2.13.

2.12. PREPARATION OF WHOLE HOMOGENATE OF SPLEEN CELLS

A WH of spleen cells (SC) of AEBN-exposed and age-matched control mice was prepared using the method described in § 2.11.

2.12.1. Materials required

Materials required were as listed in § 2.11.1.

2.12.2. Methodology

The WH of SC was prepared by following, the methodology described in § 2.11.2 and homogenizing the spleen recovered from each mouse in 2 ml of cell extract

buffer. The supernatant was collected and its protein content determined by the method of Bradford (1976) as described in § 2.13.

2.13. QUANTIFICATION OF PROTEIN

The total protein content of PBL lysate, and WH of liver and SC were estimated by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.13.1. Materials required

1. Solution of BSA in water at a concentration of 1 mg ml^{-1}
2. Stock solution of Bradford reagent: - This reagent consisted of the following components:

   [a] Coomassie brilliant blue (CBB) G-250 100 mg

   [b] 95 % ethanol 50 ml

   [c] 85 % orthophosphoric acid 100 ml

   The CBB was completely dissolved in ethanol and then mixed with orthophosphoric acid. This solution was stored refrigerated in a dark bottle at 4 °C
3. Working solution of Bradford reagent: - The following materials were required for preparing 100 ml of working solution:

   [a] Stock solution of Bradford reagent 15 ml

   [b] Millipore water 85 ml

   [c] Whatmann filter paper No. 1

   Working solution was prepared by mixing the stock solution with water just before use. This solution was filtered through Whatmann filter paper No 1.
4. Millipore water

2.13.2. Methodology

For preparation of standard solutions, varying volumes of BSA solution from 10-100 µl were taken in different test tubes, and the final volume of each made up to 100 µl with Millipore water. The amount of BSA in each tube varied from 10 µg to 100 µg i.e. the concentration varied form 0.1 µg μl^{-1} to 1 µg μl^{-1}. 5 ml of working solution was added to each tube. A blank solution was prepared by adding 5 ml of working solution to 100 µl of Millipore water. Test samples were prepared in

triplicate in the same way by taking 30 µl of PBL lysate and 5 µl each of WH of liver and SC. The absorbance of standard as well test samples was read at 595 nm against the blank solution, with the help of a spectrophotometer. A standard calibration curve was plotted by plotting concentration of standard solutions versus the corresponding absorbance at 595 nm, and, this curve was used to determine the concentration of protein in the test samples.

2.14. SLOT BLOTTING OF PROTEIN

Slot blotting was performed to facilitate accurate densitometric analysis of expression levels of the proteins of interest, following the method previously described (Sharan *et al.*, 2005) with some modifications.

2.14.1. Materials required

1. Bio-Dot SF Microfiltration Apparatus
2. NCM pore size 0.45 µm.
3. Whatman filter paper No 1.
4. Tris buffered saline (TBS) pH 7.5: - This buffer consisted of the following components

[a] Tris-Cl, pH 7.5	10 mM
[b] NaCl	500 mM

To prepare 100 ml of Tris-Cl buffer, 0.12 g of Tris base was dissolved in 100 ml of Millipore water, the pH was adjusted to 7.5 with concentrated HCl, and the final volume was adjusted to 100 ml with Millipore water. TBS buffer was prepared by dissolving the required amount of NaCl in Tris-Cl buffer. The buffer was stored at RT.

2.14.2. Methodology

After estimation of total protein, samples were prepared for slot blotting by serial dilution of PBL lysate or WH of liver and SC with Millipore water to obtain a concentration of 400 ng total protein per 100 µl of sample. The samples were boiled for 3-4 min to heat inactivate endogenous alkaline phosphatase activity, as the secondary antibody used was labeled with alkaline phosphatase. NCM was cut to the required size and soaked in TBS at RT for 20 min, taking care to avoid touching

the membrane with bare fingers.Three sheets of Whatman filter paper, cut to appropriate size were also soaked in TBS. The apparatus was assembled by placing the NCM over the filter paper. Equal volumes of the samples (100 µl containing 400 ng total protein) was loaded into each slot. Each sample was applied in 4-5 replicates along successive slots in one column. Care was taken to ensure that sample in all the slots dried simultaneously, following which the membrane was washed once with TBS. The apparatus was then disassembled, and the membrane removed to be used for immunoprobing or staining with India ink.

2.15. IMMUNOPRECIPITATION OF SELECTED PROTEINS

The Brca1 and Brca2 proteins were not detectable by Western blotting of PBL lysate and WH of liver and SC. The proteins were, therefore, immunoprecipitated from lysate and WH containing 150 mg of total protein, following the method described by Rosenberg (1996) with some modifications.

2.15.1. Materials required

1. Specific primary antibody: anti-BRCA1 and anti-BRCA2 antibodies.
2. Suspension of Protein A-CL agarose beads: this was used directly from the bottle as supplied.
3. PMSF (100 mM): this solution was prepared as described in § 2.10.1.
4. Immunoprecipitation buffer: - This buffer consisted of the following components:

[a] Tris-Cl, pH 8	50 mM
[b] NaCl	500 mM
[c] EDTA	5 mM
[d] NaN_3	0.02 %
[e] Triton X-100	0.5 %
[f] SDS	0.1 %

To prepare 100 ml of Tris-Cl buffer, 0.6 g of Tris base was dissolved in Millipore water, the pH was adjusted to 8 with concentrated HCl, and the final volume was raised to 100 ml with Millipore water. The immunoprecipitation buffer was prepared by dissolving the remaining chemicals in Tris-Cl, and stored refrigerated at 4°C.

5. Micropipette tips cut 5 mm from the end
6. Laemmli buffer (5 X) : - This buffer consisted of the following components:

[a] 0.5 M Tris-Cl, pH 6.8	1 ml
[b] Glycerol	0.8 ml
[c] 10 % (w/v) glycerol	1.6 ml
[d] 2-mercaptoethanol (used directly from the bottle as supplied)	0.4 ml
[e] 0.05 % (w/v) bromophenol blue	0.2 ml
[f] Millipore water	4 ml

The buffer was prepared by mixing the above chemicals and stored refrigerated at 4°C. The buffer was diluted to 2 X strength with Millipore water just before use.

7. Boiling water bath

2.15.2. Methodology

The Brca1 and Brca2 proteins were immunoprecipitated from PBL lysate and WH of liver and SC containing equal amount (150 mg) of total protein. Seven µl of supplied anti-BRCA1 and anti-BRCA2 antibodies containing 3.5 µg of the respective antibody, and PMSF to a final strength of 10 mM were added to the lysate/WH, and mixed well, followed by incubation at 4 °C O/N. The next day, 40 µl of Protein A-CL agarose bead suspension was added to the lysate/WH with the help of cut tips to prevent lysis of the beads. After thorough mixing, the lysate/WH was incubated at 4 °C for 2 hours. The Protein A-CL agarose beads were recovered by centrifugation (18000 x *g*) for 3 min at 4 °C, and the supernatant discarded. The recovered beads were then washed thrice with cold immunoprecipitation buffer by centrifugation (18000 x *g*) for 3 min at 4 °C. The washed beads were resuspended in 40 µl of 2 X Laemmli buffer and incubated in a boiling water bath for 5 min, followed by centrifugation (18000 x *g*) for 3 min. The supernatant was collected and used directly for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.16. SEPARATION OF PROTEINS BY SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

Proteins in PBL lysate and WH of liver and SC, as well as immunoprecipitated proteins, were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using the Laemmli buffer system, as described by Laemmli (Laemmli, 1970).

2.16.1. Materials required

1. Mini-Protean II Electrophoretic Cell
2. Monomer stock solution (30 % T, 2.67 % C) : - The following materials were required for the preparation of this reagent:

   [a] Acrylamide 29.2 g

   [b] N, N'-bis-methyleneacrylamide 0.8 g

   [c] Millipore water

   [d] Whatmann filter paper No. 1

   The acrylamide and N, N'-bis-methyleneacrylamide were dissolved in Millipore water. The solution was then filtered through Whatmann filter paper No. 1 and stored refrigerated in a dark bottle at 4 °C
3. Tris-Cl, pH 8.8 1.5 M

   To prepare100 ml of Tris-Cl buffer, 18.15 g of Tris base was dissolved in 100 ml of Millipore water, the pH was adjusted to 8.8 with concentrated HCl, and the final volume was raised to 100 ml with Millipore water.
4. Tris-Cl, pH 6.8 0.5 M

   To prepare 100 ml of Tris-Cl buffer, 6 g of Tris base was dissolved in 100 ml of Millipore water, the pH was adjusted to 6.8 with concentrated HCl, and the final volume was raised to 100 ml with Millipore water.
5. SDS solution in water 10 % (w/v)

   To prepare 100 ml of SDS solution, 10 g of SDS was dissolved in 100 ml of Millipore water by constant stirring and warming to 37 °C.
6. APS solution in water 10 % (w/v)

   To prepare 1 ml of APS solution, 1 g of APS was dissolved in 10 ml of Millipore water. This solution was prepared immediately before use

7. N, N. N', N'-tetramethylenediamine (TEMED): this was used directly from the bottle as supplied.
8. Laemmli buffer (5 X) prepared as described in § 2.15.1
9. Electrode buffer/ running buffer (5 X): - This buffer consisted of the following components:

[a] Tris base	9 g
[b] Glycine	43.2 g
[c] SDS	3 g
[d] Millipore water	600 ml

The above chemicals were dissolved in Millipore water to obtain a 5 X electrode buffer. This buffer was diluted to 1 X with Millipore water before use.

10. Boiling water bath

2.16.2. Gel preparation

A discontinuous gel system comprising of a resolving gel and a stacking gel was used. The percentage of the resolving gel varied with the size of protein to be separated. Accordingly, a 10 % gel was used for separation of actin and p53 proteins (MW 42 kDa and 53 kDa respectively), 7 % gel was used for separation of Brca1 protein (MW 220 kDa) and 5 % gel was used for separation of Brca2 protein (MW 380 kDa). A stacking gel of 4 % was used in all cases. The gels were prepared as detailed in Table 5.

Table 5: Reagents required for preparation of resolving and stacking gel for SDS-PAGE

Stock reagent	*Resolving gel* (5 %)	*Resolving gel* (7 %)	*Resolving gel* (10 %)	*Stacking gel* (4 %)
Monomer solution	1.67 ml	2.33 ml	3.33 ml	1.33 ml
Tris-Cl buffer pH 8.8, 1.5 M	2.5 ml	2.5 ml	2.5 ml	.....
Tris-Cl buffer pH 6.8, 0.5 M	.....	.....	.....	2.5 ml
Millipore water	5.65 ml	5 ml	4.2 ml	6.1 ml
SDS 10 % *	100 µl	100 µl	100 µl	100 µl
APS 10 % *	50 µl	50 µl	50 µl	50 µl
TEMED *	5 µl	5 µl	5 µl	5 µl

* These components were added after a mixture of the remaining components was degassed using a vacuum pump.

2.16.3. Assembly of electrophoresis apparatus

The glass plates of the electrophoretic cell were assembled using 0.75 mm thick spacers. The resolving gel mixture was poured in between plates, taking care to avoid trapping air bubbles, and leaving a space of 2 cm above the gel. The gel was overlaid with water and left undisturbed for 30 min to polymerize. Once the resolving gel had polymerized, the overlaying layer of water was poured off, and the stacking gel mixture was poured over the resolving gel. A comb of 0.75 mm thickness was then inserted into the stacking gel, again taking care to avoid trapping air bubbles. The stacking gel was left undisturbed for 40 min to polymerize.

2.16.4. Sample preparation

2.16.4.1. **For separation of actin protein:** Samples used for separation of actin protein were PBL lysate and WH of liver and SC. The samples were diluted serially to obtain an equal amount of 150 µg of protein in a final volume of 20 µl, and mixed with 5 X Laemmli buffer in the ratio 4:1, i.e. with 5 µl of Laemmli buffer. The mixtures were incubated in a boiling water bath for 5 min, and then loaded into the gel.

2.16.4.2. **For separation of p53 protein:** Samples used for separation of p53 protein were PBL lysate and WH of liver and SC. The samples were diluted serially to obtain an equal amount of 150 µg of protein in a final volume of 20 µl, and mixed with 5 X Laemmli buffer in the ratio 4:1, i.e. with 5 µl of Laemmli buffer. The mixtures were incubated in a boiling water bath for 5 min, and then loaded into the gel.

2.16.4.3. **For separation of Brca1 and Brca2 proteins:** Samples used for separation of Brca1 and Brca2 proteins were the immunoprecipitates obtained after immunoprecipitation of the proteins from the PBL lysate and WH of liver and SC. The immunoprecipitates were directly loaded into the gel.

2.16.5. Electrophoresis conditions

Electrophoresis was performed at a constant voltage and different durations for different proteins to be separated, as listed in Table 6.

Table 6: Electrophoresis conditions for separation of different proteins by SDS-PAGE

Serial No	*Protein*	*Resolving gel percentage (%)*	*Voltage ($V\ cm^{-1}$)*	*Duration of electrophoresis (min)*
1	actin	10	25	60
2	p53	10	25	60
3	Brca1	7	25	45
4	Brca1	5	25	45

2.17. TRANSFER OF PROTEIN BY WESTERN BLOTTING

Following separation by SDS-PAGE, proteins were transferred to nitrocellulose membrane by Western Blotting using the method described by Towbin (1979), with some modifications.

2.17.1. Materials required

1. Mini Trans-Blot Electrophoretic Transfer Cell
2. NCM pore size 0.45 µm
3. Whatmann filter paper No. 1
4. Transfer buffer (Towbin buffer, pH 8.3): - This buffer consisted of the following components:

[a] Tris base	25 mM
[b] Glycine	192 mM
[c] Methanol	20 % (v/v)
[d] Millipore water	

The above chemicals were mixed in appropriate volume of Millipore water to prepare the buffer. The resulting pH of the buffer was determined to be approximately 8.3, and was not adjusted further. The buffer was stored refrigerated at 4 °C.

2.17.2. Methodology

On completion of SDS-PAG electrophoresis, the gel was equilibrated in Towbin buffer for 15 min. The NCM and three sheets of Whatmann filter paper were cut to the required size and also soaked in Towbin buffer for 15 min. The gel sandwich was assembled within the gel holder cassette of the apparatus as per manufacture's instructions. Transfer was performed in chilled Towbin buffer under the conditions as detailed in Table 7.

Table 7: Conditions of transfer of different proteins by Western blotting

Serial No	*Protein*	*Voltage (V)*	*Duration of transfer*
1	actin	100	1 hour
2	p53	100	1 hour
3	Brca1	20	O/N
4	Brca1	20	O/N

2.18. INDIA INK STAINING OF SLOT BLOT AND WESTERN BLOT

Protein blots can be stained for total protein with India ink, in order to ascertain equal loading of protein sample for further quantitative studies. The method previously standardized in the laboratory (Kma, 2003; Sharan *et. al,* 2005) was followed for staining of slot-blots or Western blots with India ink, with a few modifications.

2.18.1. Materials required

1. Phosphate buffered saline (PBS) buffer, pH 7.4: This buffer was prepared as described in § 2.10.1.
2. Phosphate buffered saline- Tween-20 (PBST)/ Tween solution: This solution consisted of 0.3 % Tween-20 (v/v) in PBS buffer, pH 7.4 prepared as described in § 2.10.1. To prepare 1 litre of PBST solution, 3 ml of Tween-20 was dissolved in 1 litre of PBS buffer by continuous stirring.
3. Staining solution: The staining solution was a 0.2 % solution of India ink in PBST, and 100 ml of staining solution was prepared by mixing 200 µl of India ink with 100 ml of PBST solution by continuous stirring. India ink was used directly from the bottle as supplied by the manufacturer. This solution was prepared immediately before use.

2.18.2. Methodology

On completion of slot blot and/or Western blot, the membrane was stained with India ink for total protein using the following procedure:

2.18.2.1. **Washing at RT:** The membrane was washed by completely immersing it in 10 ml of PBST solution, with shaking on a rocker at medium speed. Washing was performed with three changes of PBST solution for 30 min each at RT.

2.18.2.2. **Washing at 37 °C:** The membrane was washed by completely immersing it in 10 ml of PBST solution, with mild shaking. Washing was performed with three changes of PBST solution for 30 min each at 37 °C.

2.18.2.3. **Staining:** The membrane was immersed in 10 ml of staining solution and incubated at 37 °C for 1-2 hours.

2.18.2.4. **Destaining:** On completion of staining, the membrane was destained at RT with 2-3 changes of PBST solution, for 10 min each, by shaking on a rocker at medium speed.

2.19. IMMUNOPROBING OF SLOT BLOT AND WESTERN BLOT

Following slot-blotting and Western blotting, and after ascertaining equal loading of total protein by India ink staining, the slot-blots and Western blots were immunoprobed with specific antibody using the method standardized in the laboratory (Kma, 2003; Sharan *et al.*, 2005), with some modifications.

2.19.1. Materials required

1. Tris buffered saline (TBS) buffer, pH 7.5: This buffer was prepared as described in § 2.14.1
2. Tween-20- tris buffered saline (TTBS) solution: This solution consisted of 0.05 % Tween-20 (v/v) in TBS buffer. To prepare 1 litre of TTBS solution, 50 µl of Tween-20 was dissolved in 1 litre of TBS buffer by continuous stirring.
3. Blocking solution (BS): BS was a 5 % solution of fat free milk in TBS buffer. To prepare 100 ml of BS, 100 ml of TBS buffer was warmed slightly and 5 g of fat free milk was dissolved in it by continuous stirring. This solution was prepared immediately before use.
4. Primary antibody solution: Primary antibody solution was prepared just before use. It consisted of BS in which the primary antibody was diluted as detailed in Table 8.
5. Secondary antibody solution: Secondary antibody solution was prepared just before use. It consisted of BS in which the secondary antibody was diluted as detailed in Table 8.
6. BCIP/NBT solution: This was used directly from the bottle as supplied by the manufacturer.

2.19.2. Methodology

On completion of slot blot and/or Western blot, the membrane was immunoprobed for p53, Brca1 or Brca2 protein using the following procedure: -

2.19.2.1. **Blocking:** The NCM was blocked by immersing in 10 ml BS, and kept shaking on a rocker at medium speed for 45 min.

2.19.2.2. **Incubation in primary antibody solution:** The BS was discarded and 10 ml of fresh BS was added. Primary antibody was added to the BS at dilutions as described in Table 8, followed by shaking on a rocker at medium speed for 20 min. The NCM was then incubated O/N in primary antibody solution at 37 °C, in a water-bath.

2.19.2.3. **Washing:** The primary antibody solution was discarded, and the membrane washed with 20 ml of TTBS for 10 min by shaking on a rocker at medium speed.

2.19.2.4. **Incubation in secondary antibody solution:** After washing with TTBS, the membrane was immersed in 10 ml of BS, and alkaline-phosphatase labeled secondary antibody was added at dilutions as described in Table 8 followed by shaking on a rocker at medium speed for 20 min. The NCM was then incubated in secondary antibody solution for 2 hours at 37 °C, in a water-bath.

2.19.2.5. **Washing:** The secondary antibody solution was discarded, and the membrane washed with 20 ml of TTBS for 10 min by shaking on a rocker at medium speed.

2.19.2.6. **Washing:** The TTBS was discarded, and the membrane washed with 20 ml of TBS for 10 min, by shaking on a rocker at medium speed.

2.19.2.7. **Developing:** The TBS was discarded. Appropriate volume of BCIP/NBT solution was added to cover the NCM, and then incubated at 37 °C until optimum color developed. It usually took for 10-15 min.

Table 8: Primary and secondary antibody dilutions used for immunoprobing following slot blotting

Serial No.	*Primary antibody*	*Dilution used for immunoprobing slot-blot (volume added in 10 ml of BS)*	*Dilution used for immunoprobing Western blot (volume added in 10 ml of BS)*	*Secondary antibody*	*Dilution used for developing slot-blot and Western blot (volume added in 10 ml of BS)*
1	**Anti- p53 raised in sheep.** Supplied stock of 1 mg μl^{1} was diluted with PBS to obtain a working dilution of 10 $\mu g\ \mu l^{-1}$, by diluting 0.1 µl of antibody stock to final volume of 10 µl just before use	1: 50,000 (20 µl of working solution)	1: 5000 (200 µl of working solution)	**Donkey anti-sheep IgG-Alkaline phosphatase conjugate** Supplied stock was used directly as supplied	1: 15, 000 (0.7 µl)
2	**Anti-BRCA1 polyclonal antibody raised in rabbit** Supplied stock of 200 $\mu g\ ml^{-1}$ was used directly as supplied	1: 2000 (5 µl of supplied stock)	1: 1000 (10 µl of supplied stock)	**Goat anti-rabbit IgG- Alkaline phosphatase conjugate** Supplied stock was used directly as supplied	1: 10, 000 (1 µl)
3	**Anti-BRCA2 polyclonal antibody raised in rabbit** Supplied stock of 200 $\mu g\ ml^{-1}$ was used directly as supplied	1: 2000 (5 µl of supplied stock)	1: 1000 (10 µl of supplied stock)	**Goat anti-rabbit IgG- Alkaline phosphatase conjugate** Supplied stock was used directly as supplied	1: 10, 000 (1 µl)
4	**Anti-actin polyclinal antibody raised in rabbit** Supplied stock of 0.8 $mg\ ml^{-1}$ was used directly as supplied	1: 250 (40 µl of supplied stock)	1: 150 (66 µl of supplied stock)	**Goat anti-rabbit IgG- Alkaline phosphatase conjugate** Supplied stock was used directly as supplied	1: 10, 000 (1 µl)

2.20. DOCUMENTATION AND DENSITOMETRIC ANALYSIS OF SLOT BLOT AND WESTERN BLOT

Following staining with India ink for total protein and immunoprobing with specific antibody, the slot blots and Western blots were digitized using HP Scanjet 7400C, and the image was used for densitometric analysis with KDS-1D software (Kodak).

The software measured the pixel density of the selected area which was one slot, or a Western blot band, in terms of net intensity, i.e. the intensity of the selected area after subtraction of background intensity.

2.21. PREPARATION OF MOUSE GENOMIC DNA

Genomic DNA was prepared from regions of preneoplastic nodulation in livers of P, F1, F2, and F3 mice, which had been exposed to AEBN for 24 weeks, from corresponding regions of livers of age-matched control mice, and from solid tumors, which developed in F1 and F3 mice. Preparation of DNA was according to the method described by Ausubel *et al.*, (1995), with some modifications which were incorporated from the method described by Tel-zur *et al.*, (1999) to increase the yield of DNA.

2.21.1. Materials required

1. Extraction buffer: This buffer consisted of the following components:

[a] Tris-Cl, pH 8	100 mM
[b] Sorbitol	350 mM
[c] EDTA	5 mM
[d] Millipore water	

EDTA was prepared as a 500 mM stock solution by dissolving 14.61 g of EDTA in Millipore water and adjusting the pH to 8 with pellets of NaOH. The final volume was then raised to 100 ml with Millipore water. To prepare 100 ml of Tris-Cl buffer, 1.21 g of Tris base was dissolved in Millipore water, the pH was adjusted to 8 with concentrated HCl, and the final volume was raised to 100 ml with Millipore water. To prepare 100 ml of extraction buffer, 6.38 g of sorbitol and 1 ml of EDTA stock were dissolved in 100 ml of Tris-Cl buffer. The extraction buffer was sterilized by autoclaving and stored at RT.

2. High-salt CTAB buffer: This buffer consisted of the following components:

[a] Tris-Cl, pH 8	50 mM
[b] NaCl	4 M
[c] CTAB	1.8 %

[d] EDTA, pH 8 25 mM

The Tris-Cl buffer and EDTA were prepared by diluting the stock solutions used for preparation of extraction buffer to the required molarity. To prepare 100 ml of high-salt CTAB buffer, 23.38 g of NaCl and 1.8 g of CTAB were dissolved in a solution containing 50 ml of 100 mM Tris-Cl, pH 8 and 5 ml of EDTA (500 mM) pH 8, by constant stirring and warming in a water bath to 60 °C, since CTAB does not dissolve at RT. The final volume was made up to 100 ml with Millipore water. The buffer was sterilized by autoclaving and stored at RT. If CTAB formed a precipitate, the buffer was warmed at 60 °C until CTAB went into solution again, before use.

3. Proteinase K solution 20 mg ml^{-1}

This was prepared by dissolving 20 mg of Proteinase K in 1 ml of sterilized Millipore water in a sterile microcentrifuge tube. The Proteinase K solution was divided into small aliquots and stored refrigerated at -20 °C.

4. RNase solution 5 mg ml^{-1}

This was prepared by dissolving 5 mg of DNase free ribonuclease A in 1 ml of sterilized Millipore water in a sterile microcentrifuge tube. The RNase solution was divided into small aliquots and stored refrigerated at -20 °C.

5. Sodium acetate solution, pH 5.2 3 M

To prepare 100 ml of sodium acetate solution, 26.4 g of sodium acetate was dissolved in Millipore water and the pH was adjusted to 5.2 with glacial acetic acid. The final volume was made up to 100 ml with Millipore water. This solution was sterilized by autoclaving and was stored at RT.

6. Sarkosyl 30 %

This was prepared by dissolving 30 g of N-lauroyl sarcosine in 100 ml of sterilized Millipore water in a sterile bottle.

7. SDS solution in water 10 %

This was prepared by dissolving 10 g of SDS in 100 ml of sterilized Millipore water in a sterile bottle. The solution was stored at RT. If SDS precipitated out, the solution was incubated at 37 °C until SDS went into the solution again, before use.

8. Mixture of chloroform and isoamyl alcohol in the ratio 24 :1 (v/v)
9. Mixture of phenol and chloroform in the ratio 1:1 (v/v)
10. Isopropanol (100 %): This was stored refrigerated at -20 °C until use.
11. Absolute alcohol (95 % ethanol): This was stored refrigerated at -20 °C until use.
12. Ethanol (70 %): This was stored refrigerated at -20 °C until use.
13. Chloroform (100 %): This was stored refrigerated at -20 °C until use.
14. Sterilized Millipore water
15. Pre-chilled mortar, pestle and spatula
16. Sterilized centrifuge tubes of 20 ml, 10 ml and 1.5 ml capacity
17. Sterilized micropipette tips of 5 ml, 1 ml and 200 µl capacity

2.21.2. Methodology

The region of tissue to be used for DNA isolation (liver or solid tumors) weighing 0.5 g was cut into pieces and frozen O/N at -80 °C. The frozen tissue was quickly ground to a fine powder with the help of a pre-chilled mortar and pestle and scooped into a sterilized 20 ml centrifuge tube with the help of a pre-chilled spatula. Five ml of extraction buffer, 0.5 % SDS (250 µl from a 10 % stock solution of SDS) and 0.1 mg ml^{-1} Proteinase K (25 µl from a 20 mg ml^{-1} stock solution of Proteinase K) were added to the powdered tissue, the contents of the tube were mixed well by vortexing, and the tube was incubated at 55 °C for 1 hour. After 1 hour of incubation, 3.5 ml of high salt-CTAB buffer and 300 µl of 30 % Sarkosyl were added to the tube, the contents of the tube were mixed well by vortexing, and incubation at 55 °C was continued for another 90 min. The mixture was extracted with an equal volume, i.e. 8.5 ml of chloroform isoamyl alcohol mixture, by centrifugation at 10000 x *g* for 10 min at RT. The supernatant was transferred to a sterilized centrifuge tube of 10 ml capacity, and mixed with $2/3^{rd}$ volume of cold absolute isopropanol and $1/10^{th}$ volume of sodium acetate solution. The contents of the tube were mixed well by gentle inversion, and centrifuged at 10000 x *g* for 20 min at 4 °C. The supernatant was

decanted, and the pellet was washed with excess of 70 % ethanol by centrifugation at 12000 x *g* for 5 min at 4 °C. The pellet was dried in an incubator at 37 °C. When the pellet had turned transparent, and the ethanol had completely evaporated, the pellet was dissolved in 200 µl of sterilized Millipore water and 10 µl of RNase solution was added to the solution. The solution was mixed well by gently tapping the centrifuge tube, followed by incubation at 37 °C for 40 min. After incubation, the contents of the centrifuge tube were transferred to a sterilized microcentrifuge tube of 1.5 ml capacity, and extracted with an equal volume of phenol: chloroform mixture by centrifugation at 12000 x *g* for 10 min at RT. The clear upper phase was transferred to a fresh tube by pipetting carefully so as to avoid mixing it with the lower phase. The upper phase was extracted with an equal volume of cold chloroform by centrifugation at 12000 x *g* for 10 min at RT. The clear upper phase was again transferred to a fresh tube and extracted with two volumes of absolute ethanol mixed with $1/10^{th}$ volume of sodium acetate solution. The mixture was kept for 30 min to O/N at -20 °C. The DNA was pelleted out by centrifugation at 12000 x *g* for 15 min at 4 °C. The pellet was washed with 1 ml of 70 % ethanol by centrifugation at 12000 x *g* for 5 min at 4 °C. The pellet was then dried in an incubator maintained at 37 °C, till the pellet was transparent and the ethanol had evaporated completely. The pellet was dissolved in 30-40 µl of sterilized autoclaved water, depending upon the amount of DNA which had precipitated out. The quantity and purity of DNA were determined spectrophotometrically as described in § 2.22. The isolated DNA was subjected to 0.8 % agarose gel electrophoresis (AGE) as described in § 2.24.

2.22. QUANTIFICATION OF DNA

DNA was quantified, and its purity was assessed by the spectrophotometric absorbance method (Sambrook and Russell, 2001), using spectrophotometer. The quantity of DNA was calculated using the relationship that one unit absorbance at 260 nm is equivalent to 50 µg ml^{-1} of DNA. The purity of a DNA sample was assessed by calculating the ratio of absorbance at 260 nm and 280 nm (A_{260}:A_{280}). A ratio between 1.8 and 2 was taken as an indicator of pure DNA.

2.22.1. Materials required

1. Sterilized Millipore water

2. Sterilized microcentrifuge tubes of 1.5 ml capacity
3. Sterilized micropipette tips

2.22.2. Methodology

DNA sample was prepared by diluting 2 µl of isolated DNA to a final volume of 1000 µl with sterilized Millipore water in a sterile microcentrifuge tube. The absorbance of this sample at 260 nm and 280 nm was recorded spectrophotometrically against a blank of sterile Millipore water. The concentration of DNA in µg μl^{-1} and the A_{260}: A_{280} ratio was calculated.

2.23. AMPLIFICATION OF SELECTED REGIONS OF MOUSE TUMOR SUPPRESSOR GENES USING POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) was used for amplification of selected regions of the mouse *p53*, *Brca1* and *Brca2* genes. The standard protocol described by Sambrook and Russell (2001) was followed for this purpose.

2.23.1. Materials required

1. DNA Amplification Kit (Bangalore Genei): This kit comprised of the following components:

   1.1. Amplification buffer/ Taq polymerase buffer 10 X

   This buffer had the following composition:

[a] Tris-Cl, pH 9	100 mM
[b] KCl	500 mM
[c] $MgCl_2$	15 mM
[d] Gelatin	0.1 %

   1.2. Deoxyribonucleotide (dNTP) mix 10 mM

   This kit was stored refrigerated at -20°C.

2. Red taq DNA polymerase (BIOTAQ) 1 U μl^{-1}
3. Primers 100 U μl^{-1}
4. Sterilized Millipore water
5. Sterilized PCR tubes 200 µl capacity

6. Sterilized micropipette tips

2.23.2. Designing and preparation of primers

Primers were designed using the Genamics Expression 1.1 software and Genefisher online primer design tool (http://bibiserv.techfak.uni-bielefeld.de/genefisher/). For amplification of exons 5 and 7 of the *p53* geneprimers were designed to anneal to the intronic regions flanking each exon. Exon 11 of the *Brca1* gene was very large in size (1386 bp) and for the purpose of amplification, it was divided into five overlapping regions including segments of the flanking introns, in order to ensure accurate sequencing of smaller sized amplicons as shown in Fig. 2.2. Amplification of various regions of the *Brca2* gene did not yield satisfactory products, with the exception of a segment of exon 27 of the gene, which was amplified using one flanking, and one exonic primer. Details of the primers used for amplification of all target regions are listed in Table 9. Primers supplied in lyophilized form were resuspended in required volume of sterilized Millipore water to obtain a final concentration of 100 pmol μl^{-1} and were stored refrigerated at -20 °C.

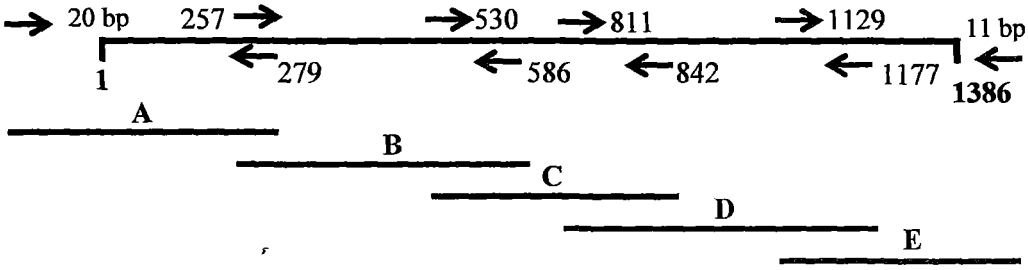


Figure 2.2. Experimental design for amplification of exon 11 of the mouse *Brca1* gene to yield 5 overlapping amplicons A through E. The arrows indicate forward primer (→) and reverse primer (←) for each amplicon respectively.

Table 9. Primer sequences used for amplification of target regions

Serial No	*Gene/ Target region*	*Primer (Site of annealing)*	*Annealing temperature (°C)*	*Amplicon size (bp)*
1	*p53*/exon 5	Forward 5'- ATCGTTACTCGGCTTGTCCC-3' (29 bp upstream of exon 5) Reverse 5'- TAACCCCACAGGCGGTGTT-3' (10 bp downstream of exon 5)	56.5	262
2	*p53*/exon 7	Forward 5'- TAGTGAGGTAGGGAGCGACTT-3' (26 bp upstream of exon 5) Reverse 5'- CTGGGGAAGAAACAGGCTAAC-3' (24 bp downstream of exon 5)	54.8	202
3	*Brca1/* exon 11 region A (NT 1-260)	Forward 5'- AGTCCTGGAACGCTCACA-3' (20 bp upstream of exon 11) Reverse 5'- CAGTTCCTTTGAGGGCACA-3' (NT 261-279)	55.5	317
4	*Brca1/* exon 11 region B (NT 280-586)	Forward 5'- ACTCTGTGCCCTCAAAGGA-3' (NT 257-279) Reverse 5'- CCTCTGTCAGAGGTTTCCTA-3' (NT 567-586)	55.5	330
5	*Brca1/* exon 11 region C (NT 550-842)	Forward 5'- CAGTTTCTCCCATCACCTCA-3' (NT 530-548) Reverse 5'- ACAGGAACACTTTGCTGACA-3' (NT 823-842)	55.5	313
6	*Brca1/* exon 11 region D (NT 811-1158)	Forward 5'- CAGCCTGGTGTCTGTCA -3' (NT 811-827) Reverse 5'- TGGAGTCGCTCTCTTCTGA-3' (NT 1159-1177)	58.5	367
7	*Brca1/* exon 11 region E (NT 1129 -1386)	Forward 5'- CTCCACAGAGCGTCTAGGA-3' (1129-1158) Reverse 5'- CAGAGCATCTTGGATCCTCA-3' (NT 1376-11 bp downstream of exon 11)	52	268
8	*Brca2/* segment of exon 27 (NT 1- 257)	Forward 5'- TCCACACCGAACAAAGACC-3' (89 bp upstream of exon 27) Reverse 5'- TATTTCGTGCCACAGCTCC-3' (NT- 238-257 of exon 27)	52.7	365

2.23.3. Preparation of PCR reaction mix

In order to perform a PCR reaction for amplification of a target region, different components were taken in the volumes listed in Table 10. The PCR mix was prepared in a sterile hood by mixing all the components well in a sterile PCR tube. The contents of the tube were briefly spun down by centrifugation (10000 x *g* for 30 sec at RT), and the tube was then transferred to a thermal cycler in order to perform the PCR reaction.

Table 10. Preparation of PCR reaction mix for amplification of target regions

Serial No.	*Gene/ Target region*	*Component*	*Volume (µl)*	*Final volume(µl)*	*Final concentration*
1	*p53*/exon 5	10 X amplification buffer dNTP mix Forward primer Reverse primer Taq DNA polymerase DNA sample Sterile Millipore water	1.5 0.9 0.4 0.4 0.5 1-2 10.3-9.3	15	1 X 9 mM 40 pmol 40 pmol 0.5 U 0.5 µg .....
2	*p53*/ exon 7	10 X amplification buffer dNTP mix Forward primer Reverse primer Taq DNA polymerase DNA sample Sterile Millipore water	1.5 0.9 0.6 0.6 0.6 1-2 9.8-8.8	15	1 X 9 mM 60 pmol 60 pmol 0.6 U 0.5 µg .....
3	*Brca1*/ exon 11 region A	10 X amplification buffer dNTP mix Forward primer Reverse primer Taq DNA polymerase DNA sample Sterile Millipore water	2.5 1.5 1 1 1 1-2 17-16	25	1 X 15 mM 100 pmol 100 pmol 1 U 0.5 µg .....
4	*Brca1*/ exon 11 region B	10 X amplification buffer dNTP mix Forward primer Reverse primer Taq DNA polymerase DNA sample Sterile Millipore water	2.5 1.5 1 1 1 1-2 17-16	25	1 X 15 mM 100 pmol 100 pmol 1 U 0.5 µg
5	*Brca1*/ exon 11 region C	10 X amplification buffer dNTP mix Forward primer Reverse primer Taq DNA polymerase DNA sample Sterile Millipore water	2.5 1.5 1 1 1 1-2 17-16	25	1 X 15 mM 100 pmol 100 pmol 1 U 0.5 µg .....
6	*Brca1*/ exon 11 region D	10 X amplification buffer dNTP mix Forward primer Reverse primer Taq DNA polymerase DNA sample Sterile Millipore water	2.5 1.5 1 1 1 1-2 17-16	25	1 X 15 mM 100 pmol 100 pmol 1 U 0.5 µg .....

7	*Brca1/* exon 11 region E	10 X amplification buffer dNTP mix Forward primer Reverse primer Taq DNA polymerase DNA sample Sterile Millipore water	2.5 1.5 1 1 1 1-2 17-16	25	1 X 15 mM 100 pmol 100 pmol 1 U 0.5 µg .....
8	*Brca2/* segment of exon 27	10 X amplification buffer dNTP mix Forward primer Reverse primer Taq DNA polymerase DNA sample Sterile Millipore water	2.5 1.5 1.0 1.0 1.0 1-2 17-16	25	1 X 15 mM 100 pmol 100 pmol 1.0 U 0.5 µg .....

2.23.4. Thermal cycling conditions

The thermal cycling conditions used for amplification of each target region were programmed into the thermal cycler. These thermal cycling conditions are listed in Table 11. For amplification of the selected region of *Brca2* gene, a touchdown PCR protocol was used, in order to minimize non-specific annealing of primers.

Table 11. Thermal cycling conditions used for amplification of different target regions

Serial No.	*Gene/Target region*	*Step/Temperature/ time*	*No. of cycles*
1	*p53/* exon 5	Initial denaturation/95 °C/ 3 min	1
		Denaturation/94 °C/ 1 min Annealing/56.5 °C/ 1 min Extension/72 °C/ 1 min	35
		Final extension/72 °C/ 7 min	1
2	*p53/* exon 7	Initial denaturation/95 °C/ 3 min	1
		Denaturation/94 °C/ 1 min Annealing/54.8 °C/ 1 min Extension/72 °C/ 1 min	30
		Final extension/72 °C/ 7 min	1
3	*Brca1/* exon 11 region A	Initial denaturation/95 °C/ 3 min	1
		Denaturation/94 °C/ 1 min Annealing/55.5 °C/ 1 min Extension/72 °C/ 1 min Final extension / 72 °C/ 7 min	35
4	*Brca1/* exon 11 region B	Initial denaturation/95 °C/ 3 min	1
		Denaturation/94 °C/ 1 min Annealing/55.5 °C/ 1 min Extension/72 °C/ 1 min	30
		Final extension/72 °C/ 7 min	1

5	*Brca1*/ exon 11 region C	Initial denaturation/95 °C/ 3 min	1
		Denaturation/94 °C/ 1 min Annealing/55.5 °C/ 1 min Extension/72 °C/ 1 min	30
		Final extension/72 °C/ 7 min	1
6	*Brca1*/ exon 11 region D	Initial denaturation/95 °C/ 3 min	1
		Denaturation/94 °C/ 1 min Annealing/58.5 °C/ 1 min Extension/72 °C/ 1 min	30
		Final extension/72 °C/ 7 min	1
7	*Brca1*/ exon 11 region E	Initial denaturation/95 °C/ 2 min	1
		Denaturation/95 °C/ 1 min Annealing/52 °C/ 1 min Extension/72°C/ 1 min	30
		Final extension/72 °C/ 7 min	1
8	*Brca2*/ segment of exon 27 **(Touchdown PCR)**	Initial denaturation/95°C/ 2 min	1
		Denaturation/95°C/ 30 sec Annealing/59.7°C/ 30 sec decrement in temperature by 0.5 °C per cycle Extension/72°C/ 40 sec	15
		Denaturation/95°C/ 30 sec Annealing/52.7°C/ 30 sec Extension/72°C/ 40 sec	20
		Final extension/72 °C/5min	1

2.24. AGAROSE GEL ELECTROPHORESIS OF DNA AND PCR-AMPLICONS

Agarose gel electrophoresis (AGE) was performed according to standard protocol (Ausubel *et al.,* 1995), which was adapted in the laboratory (Odyuo and Sharan, 2005). The procedure is described below.

2.24.1. Materials required

1. Tris-acetate EDTA (TAE) buffer, pH 8 : - This buffer had the following composition:

[a] Tris-acetate, pH 8	40 mM
[b] EDTA, pH 8	1 mM

The buffer was prepared and stored as a stock solution of 50 X, at RT. To prepare 1 litre of 50 X TAE buffer, 242 g of Tris base, 57.1 ml of glacial acetic acid and 100 ml of 500 mM EDTA, pH 8 (§ 2.21.1) were dissolved and the final volume made up to 1 litre with Millipore water. This stock solution was diluted to 1 X with Millipore water before use.

2. Sample loading solution: - This solution had the following composition:

[a] Sucrose 40 % (w/v)

[b] Bromophenol blue 0.25 %

To prepare 10 ml of loading solution, 4 g of sucrose and 20 mg of bromophenol blue were dissolved in 10 ml of Millipore water. This solution was stored refrigerated at 4°C.

3. Agarose
4. Ethidium bromide (EB) solution 10 mg ml^{-1}

   This solution was used directly from the bottle as supplied by the manufacturer
5. 100 bp DNA ladder

   This was diluted in the ratio 1:3 with sterile Millipore water immediately before use. For one AGE, 1 µl of the ladder solution supplied by the manufacture was mixed with 3 µl of water.

2.24.2. Gel preparation: -

The percentage of agarose gel used varied with the size of the DNA sample to be electrophoresed. A 0.8 % gel was used for electrophoresis of genomic DNA, and 2 % gel for electrophoresis of PCR amplicons. Accordingly, the gel was prepared by taking 0.8 g or 2 g of agarose in 100 ml of 1 X TAE buffer, and heating in a microwave oven with frequent swirling until the agarose flakes had completely dissolved, and, a clear solution was obtained. The agarose solution was allowed to cool to 40°C, and was poured into a gel casting tray, following which a comb of appropriate size was inserted into the gel. Care was taken to avoid trapping air bubbles within the gel. The gel was left undisturbed at RT for 20 min, in order to polymerize. The comb was then removed, and the gel was submerged in 1 X TAE buffer in an electrophoresis tank.

2.24.3. Sample preparation: -

For AGE of genomic DNA, a constant amount of 3 µg of each DNA sample was loaded in the agarose gel. For AGE of PCR amplicon, a constant volume of 5 µl of each PCR product was loaded in the agarose gel. The 100 bp DNA ladder was used for determination of size of PCR amplicon. Sample was prepared for electrophoresis by mixing the appropriate volume of DNA solution, PCR product or 100 bp DNA

solution with $1/10^{th}$ volume of sample loading solution. This mixture was then directly loaded into the wells of the submerged agarose gel.

2.24.4. Electrophoresis: -

The samples loaded into wells of the submerged agarose gel were subjected to electrophoresis at constant voltage as listed in Table 12, using a Mupid gel electrophoresis apparatus (Japan).

Table 12. Electrophoresis conditions for AGE of different samples

Serial No.	*Sample for electrophoresis*	*Gel percentage (%)*	*Voltage (V)*	*Time (min)*
1	Genomic DNA	0.8	100	45
2	PCR amplicon	2	50	90

2.24.5. Staining: -

EB is intercalated between adjacent nucleotides of a nucleic acid, and fluoresces under UV-irradiation, thereby enabling the nucleic acids to be visualized. Thus, on completion of electrophoresis, the agarose gels were stained with a 0.3 μg ml^{-1} solution of EB in 1 X TAE buffer for 10 min, with gentle shaking on a rocker, followed by destaining with Millipore water for 5-10 min in order to remove excessive stain and obtain a clear background.

2.25. IMAGING AND ANALYSIS OF AGAROSE GEL ELECTROPHEROGRAM

The EB-stained agarose gels were visualized and photographed on a mini transilluminator with UV illumination, using a Kodak DC120 camera. The digitized images were analysed using KDS-1D software, with the help of which the size of the PCR amplicons were determined.

2.26. ELUTION OF DNA BAND FROM AGAROSE GEL

Amplification of the targeted region of exon 27 of the *Brca2* gene yielded multiple amplification products, including the required product of 365 bp. The 365 bp band was therefore eluted out from the gel. The procedure is described below.

2.26.1. Materials required

1. Gel Extraction kit (Bangalore Genei) : - This kit had the following components:
   - [a] Glass solution
   - [b] Sodium iodide solution
   - [c] Wash buffer
2. Sterilized Millipore water
3. Sterilized microcentrifuge tubes of 1.5 ml capacity
4. Sterilized blade

2.26.2. Methodology: -The following steps were followed as per instructions provided with the kit:

2.26.2.1. The DNA band was excised from the gel with the help of the sterilized blade, and the gel piece was weighed. Assuming the specific gravity of the gel to be 1, 2.5 volumes of sodium iodide solution was then added to the gel, in a microcentrifuge tube.

2.26.2.2. The gel was solubilized by incubation in a water bath at 55 °C for 7-8 min, and thoroughly mixing the contents, until the agarose gel was completely dissolved.

2.26.2.3. The glass solution was vortexed until it formed a homogenous mixture, and 20 µl was then added to the solubilized sample. The contents were mixed thoroughly and left at RT for 10 min, with occasional mixing.

2.26.2.4. The mixture was centrifuged at 15000 x *g* for 30 sec, and the supernatant was discarded.

2.26.2.5. The pellet was washed twice by adding 250 µl of wash buffer and vortexing, followed by centrifuging at 15000 x *g* for 30 sec. The supernatant was discarded each time.

2.26.2.6. After the final washing step, the tube was kept at 55 °C for 10 min in a water bath, in order to remove all traces of wash buffer.

2.26.2.7. The pellet was resuspended in 30-40 µl of sterile water by mild vortexing, and incubation at 55 °C for 5 min in a water bath, in order to elute the DNA.

2.26.2.8. The suspension was centrifuged at 15000 x *g* for 30 sec, and the supernatant was collected in a fresh tube.

2.26.2.9. The elution of the desired band was verified by subjecting part of the supernatant to AGE alongside a 100 bp DNA ladder as molecular weight marker.

2.27. DIRECT DNA SEQUENCING OF PCR AMPLICONS

Following PCR amplification and AGE of PCR amplicons, the amplicons whose sizes were determined to be equal to the expected size were lyophilized, and sequenced using ABI's AmpliTaq FS dye terminator cycle sequencing chemistry (Bangalore Genei Pvt Ltd, India).

2.28. ANALYSES OF DNA SEQUENCE

Following direct DNA sequencing of PCR amplicons, the correct identity of the DNA sequences was established by database search using the BLASTN tool (Altschul *et al.,* 1997). In order to screen for mutation, the sequences from AEBN-exposed samples and control samples were compared by multiple alignment using the Multalin tool (Corpet, 1988). Since the sequencing electropherograms showed poor signal in the terminal 10-20 NT of each sequence, alignment data corresponding to these regions was excluded from the analysis.

2.29. NUCLEOTIDE SEQUENCE SUBMISSION

The nucleotide sequences obtained by direct DNA sequencing were submitted to the GenBank database.

2.30. MOLECULAR MODELING OF PREDICTED PROTEIN SEQUENCES

The wild-type and mutant nucleotide sequences obtained by direct DNA sequencing of exon 11 of the *Brca1* gene were translated into the respective amino acid sequences using the Translate tool available at the ExPASy Proteomics server (www.expasy.ch/tools/#translate). The amino acid sequences thus obtained were used to generate models of the corresponding region of the Brca1 protein in order to determine the effect of the observed mutation on protein conformation. The molecular models were constructed and analysed using Swiss-Pdb Viewer version 4.0 (www.expasy.ch/sprot/).

2.31. DATA AND STATISTICAL ANALYSES

For experiments with human samples, slot-blotting of each sample was performed thrice, with each slot-blot comprising 5 replicates of the sample. Thus, data presented is mean ± SD of a total of 15 replicates for each sample. Significance of differences in levels of p53, Brca1 and Brca2 protein between controls and cancer patients were analysed using Student's t-test. For experiments with mice, each experiment was repeated at least thrice, such that the total number of exposed and control mice at each data point were 15 ± 1. All data presented are the mean ± SD of 3 independent experiments, each with 4-5 replicates in case of slot-blotting. Significance of differences in levels of p53, Brca1 and Brca2 protein and relative organ weights in exposed and age-matched controls were analysed using Student's t-test. Significance of difference in the period of exposure after which preneoplastic nodules developed in livers of chronically and transgenerationally exposed mice, as well as the difference in the number of nodules were analysed using χ^2- test with Yates' correction. Significance of development of various anomalies in transgenerationally exposed mice in comparison to chronically exposed mice, and also between F1, F2 and F3 generations was analysed using 2x2 contingency χ^2-test.

RESULTS

3.1. GENERAL AND MICROSCOPICAL OBSERVATIONS

Mice were exposed to AEBN at a dose of 2 mg ml^{-1} in drinking water, following a chronic and transgenerational exposure protocol (§ 2.6.), for a period up to 24 weeks. The chronically exposed P generation mice and their transgenerationally exposed progeny (F1, F2 and F3 mice) were monitored throughout the period of exposure for signs of illness, including alteration in body weight. Following sacrifice, the internal organs were carefully observed for alterations and weighed. The mice did not show any signs of illness throughout the treatment period in P generation. Similarly, no congenital malformations were observed in F1, F2 or F3 progeny of parents exposed to AEBN, indicating an absence of teratogenicity following AEBN exposure.

3.1.1. Alteration in body weight, relative organ weight and organs of mice upon chronic and transgenerational exposure to AEBN

Careful examination of organs revealed that AEBN exposure predominantly affected the liver and to some extent the spleen. The relative body and organ weights of AEBN exposed mice were evaluated in comparison to respective age-matched controls (Table 14). The body weights of exposed mice did not vary significantly from that of controls except for some fluctuations in F3 generation mice. The relative weights of the liver and spleen, however, showed more definitive trends, recording a tendency to increase significantly upon AEBN exposure and with progression of generations. In the P generation, exposure to AEBN initially led to significant decline in relative weight of liver and spleen up to 8 weeks of exposure, followed by increase in relative weight after 10 weeks of exposure onwards. In the following generations, a significant increase in relative weight of liver and spleen was recorded after 6 weeks of exposure to AEBN onwards, and this increase was most pronounced in F3 generation (Table 14).

Table 14. Details of number of mice used and alterations in body weight and relative organ weight upon chronic and transgenerational exposure to 2mg ml^{-1} AEBN in drinking water for different periods of time

Generation with time in weeks		*Control*				*Exposed*			
		No. mice (M/F)	Final body weight (g ± SD)	Relative organ weight (g/ 100 g bodyweight ± SD)		No. mice (M/F)	Final body weight (g ± SD)	Relative organ weight (g/ 100 g bodyweight ± SD)	
				liver	*spleen*			*liver*	*spleen*
P	2	15(7/8)	30 ± 2.8	4.3 ± 0.20	0.52 ± 0.03	15(6/9)	30 ± 2.3	4.4 ± 0.24	0.38 ±0.10ΔΔΔ
	4	14(7/7)	26 ± 3.0	5.0 ± 0.23	0.54 ± 0.19	14(7/7)	25 ± 3.3	4.8 ± 0.24 Δ	0.60 ± 0.24
	6	15(6/9)	29 ± 2.3	4.7 ± 0.14	0.45 ± 0.17	15(7/8)	28 ± 7.3	4.4 ± 0.39 ΔΔ	0.53 ± 0.11
	8	14(7/7)	30 ± 3.2	5.1 ± 0.44	0.50 ± 0.17	15(8/7)	28 ± 5.3	4.4 ± 0.76 ΔΔ	0.58 ± 0.25
	10	15(6/9)	30 ± 2.4	5.0 ± 0.32	0.63 ± 0.23	15(7/8)	30 ± 0.5	5.3 ± 0.94***	0.51 ± 0.20
	12	14(7/7)	34 ± 4.9	4.8 ± 0.06	0.51 ± 0.15	15(8/7)	31±2.2ΔΔΔ	5.3 ± 0.10	0.58 ± 0.16
	16	15(7/8)	35 ± 2.3	4.3 ± 0.66	0.40 ± 0.06	15(7/8)	34 ± 3.7	4.6 ± 1.03	0.32 ± 0.06***
	20	16(7/9)	31 ± 1.7	5.0 ± 0.49	0.49 ± 0.16	15(7/8)	30 ± 2.8	4.9 ± 0.31	0.85 ± 0.17***
	24	16(8/8)	30 ± 5.1	5.0 ± 0.30	0.53 ± 0.07	16(8/8)	31 ± 3.5	5.7 ± 0.73**	0.86 ± 0.10***
F1	4	14(7/7)	31 ± 2.3	4.0 ± 0.03	0.37 ± 0.03	15(8/7)	33 ± 1.2**	4.3 ± 0.52*	0.53 ± 0.09***
	6	15(8/7)	31 ± 4.1	4.4 ± 0.03	0.56 ± 0.03	15(8/7)	26 ±3.0ΔΔΔ	4.6 ± 0.16***	0.70 ± 0.08***
	8	15(8/7)	30 ± 0	5.1 ± 0.23	0.43 ± 0.07	15(7/8)	33 ± 1.8***	5.3 ± 1.18	0.64 ± 0.24**
	12	14(7/7)	34 ± 4.9	4.8 ± 0.06	0.51 ± 0.15	15(7/8)	31 ± 2.8	5.1 ± 1.36	0.65 ± 0.42
	16	14(7/7)	28 ± 2.0	5.1 ± 1.14	0.57 ± 0.21	14(7/7)	31 ± 3.1	5.7 ± 0.46	0.46 ± 0.065Δ
	24	15(8/7)	29 ± 1.2	5.1 ± 0.07	0.34 ± 0.07	16(9/7)	36 ± 6.9	6 6 ± 1.28***	1.20 ± 1***
F2	4	15(7/8)	26 ± 3.0	5.0 ± 0.23	0.54 ± 0.19	14(7/7)	25 ± 3.3	4.8 ± 0.24Δ	0.60 ± 0.24
	6	15(9/6)	23 ± 0.7	4.3 ± 0.33	0.54 ± 0.17	15(7/8)	27 ± 1.1***	5.0 ± 0.45***	0.70 ± 0.15*
	8	15(8/7)	32 ± 3.5	5.1 ± 0.44	0.44 ± 0.06	14(7/7)	31 ± 1.2	5.9 ± 0.20***	0.39 ±0.03ΔΔΔ
	12	15(8/7)	37 ± 2.7	4.3 ± 0.30	0.54 ± 0.22	15(7/8)	31 ± 1.2ΔΔΔ	5.0 ± 0.33***	0.68 ± 0.16
	16	15(6/9)	30 ± 2.0	4.8 ± 0.10	0.50 ± 0.14	15(6/9)	31 ± 1.7	5.3 ± 0 32***	1.13 ± 0.52***
	24	15(8/7)	32 ± 2.1	4.5 ± 1.29	0.42 ± 0.43	17(8/9)	33 ± 2.6	5.2 ± 0.09*	0.88 ± 0.27 **
F3	4	16(8/8)	27 ± 1.7	4.8 ± 0.56	0.44 ± 0.15	15(8/7)	30 ± 2.0***	4.6 ± 0.07	0.65 ± 0.03***
	6	15(8/7)	28 ± 0.5	4.8 ± 0.14	0.46 ± 0.18	15(7/8)	23 ± 6.4ΔΔ	6.1 ± 0.03***	0.77 ± 0.09***
	8	15(8/7)	31 ± 0.4	5.2 ± 0.13	0.52 ± 0.03	15(7/8)	27 ± 4.2ΔΔΔ	6.3 ± 0.56***	0.63 ± 0.19***
	12	14(6/8)	37 ± 2.7	4.3 ± 0.03	0.54 ± 0.22	15(8/7)	34 ± 4.1Δ	5.5 ± 0.27***	0.57 ± 0.12
	16	15(7/8)	31 ± 3 5	4.7 ± 0.10	0.49 ± 0.03	14(6/8)	35 ± 3.1**	5.8 ± 0.89***	0.65 ± 0.09***
	24	15(7/8)	33 ± 6.1	5.4 ± 0.92	0.49 ± 0.09	20(9/11)	34 ± 3.3	6.7 ± 1.36***	0.47 ± 0.06Δ

M/F – No of males/ No of females
* significant increase at P < 0 05 in comparison to age-matched control group
** significant increase at P < 0 01 in comparison to age-matched control group
*** significant increase at P < 0 001 in comparison to age-matched control group
Δ significant decrease at P < 0 05 in comparison to age-matched control group
ΔΔ significant decrease at P < 0 01 in comparison to age-matched control group
ΔΔΔ significant decrease at P < 0 001 in comparison to age-matched control group

3.1.1.1. Development of preneoplastic nodules of the liver

In P generation mice, liver nodules (Fig. 3.1 a) appeared after 16 weeks of exposure to AEBN, primarily in the right and caudate lobes of the liver (Fig. 3.1 c; arrow) and were confirmed to be preneoplastic by histological examination (§ 3.1.1.2). These nodules were well developed after 24 weeks of exposure (Fig. 3.1 e; arrow). Transgenerational exposure to AEBN, however, led to advancement in the period of appearance of liver nodules in subsequent generations in comparison to the P generation. Nodules were observed after 8 weeks of exposure in F1 mice, 6 weeks of exposure in F2 mice and 4 weeks of exposure in F3 mice (Figs. 3 g, 3 i

and 3 k respectively). These nodules were also confirmed to be preneoplastic by histological examination (§ 3.1.1.2). While 1-2 nodules per liver were observed in P generation mice, 3-4 nodules developed in the liver of F1, F2 and F3 mice after 24 weeks of exposure. However the increase in multiplicity of nodules was not found to be significant using χ^2- test with Yates' correction.

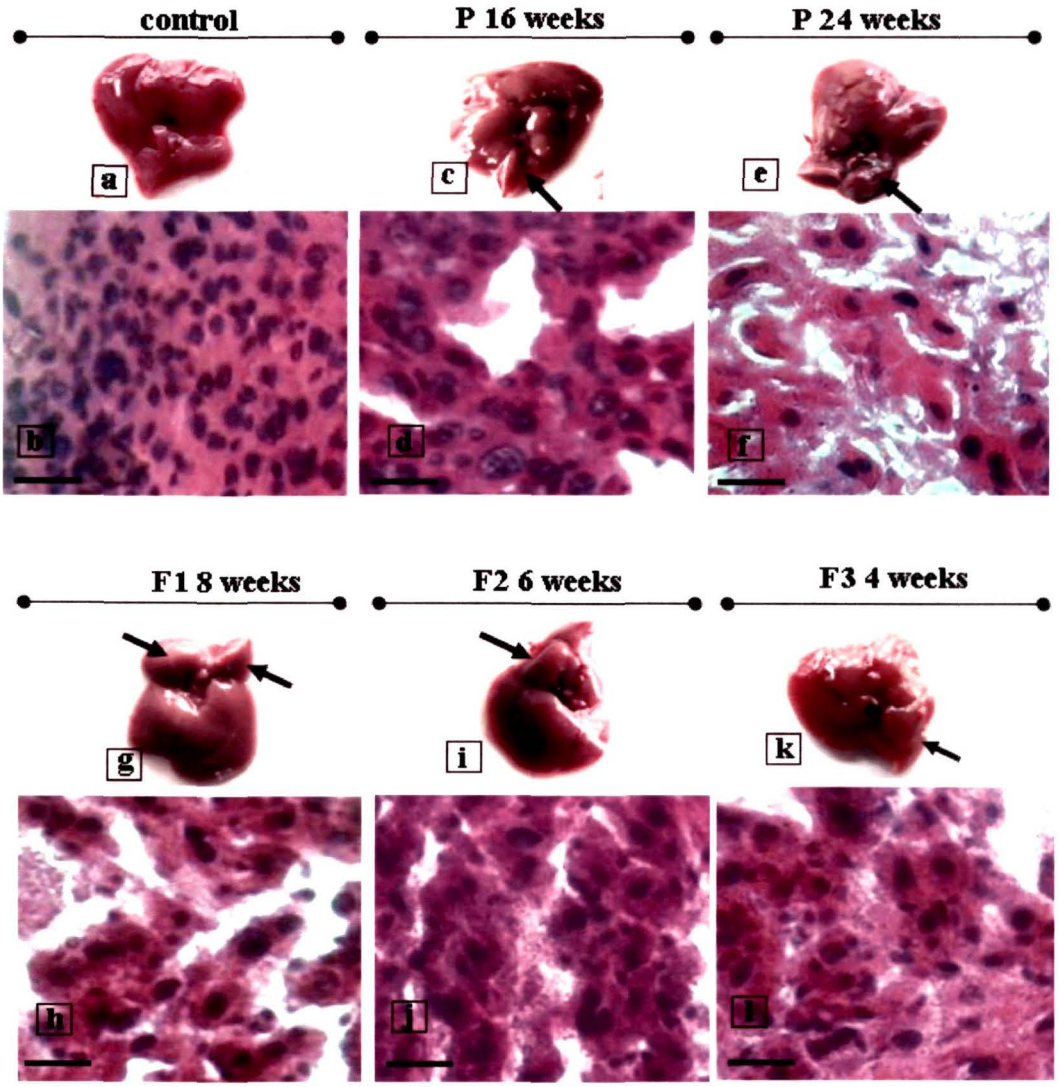


Figure 3.1. Preneoplastic nodule formation in the liver of Swiss Albino mice exposed to AEBN drinking water (2 mg ml^{-1}) and their respective H & E stained histological sections at magnification X 400 (bar = 20 μm). Normal, control liver (**a**) and histological section of normal liver (**b**); Nodule formation (arrow) in livers of P (**c**), F1 (**g**), F2 (**i**) and F3 (**k**) mice after 16, 8, 6 and 4 weeks, respectively, of exposure to AEBN drinking water – their respective histological sections are shown in **d, h, j** and **l.** Well developed nodule (arrow) in liver of P generation mice after 24-week exposure (**e**) and its histological section (**f**).

The progressive advancement in the period of appearance of liver preneoplastic nodules in transgenerationally exposed mice, in comparison to P generation mice, was found to be significant using χ^2- test with Yates' correction (Table 15).

Table 15. Development of preneoplastic nodules in liver of mice chronically and transgenerationally exposed to 2mg ml^{-1} AEBN in drinking water

Generation	Period of exposure after which preneoplastic nodules developed in liver (weeks)
P	16
F1	8*
F2	6**
F3	4***

* significant increase at $P < 0.05$ in comparison to P generation
** significant increase at $P < 0.01$ in comparison to P generation
*** significant increase at $P < 0.001$ in comparison to P generation

3.1.1.2. Histological observations

The nodules which developed in liver of AEBN exposed mice (§ 3.1.1.1.) were confirmed to be preneoplastic by histological examination (Fig. 3.1). The H & E stained sections of liver nodules exhibited a number of alterations in comparison to corresponding regions of normal liver. In liver of control mice, the cells were uniformly shaped and were closely attached with one another in a regular arrangement (Fig. 3.1 b). In contrast, the cells of liver nodules developing in P generation mice after 16 weeks of AEBN exposure (Fig. 3.1 c) were distorted in shape, had enlarged nuclei, and also displayed loss of attachment from neighbouring cells (Fig. 3.1 d), which are features of transformed cells. Histological examination of the well developed liver nodules after 24 weeks of exposure (Fig. 3.1 e) revealed irregularly shaped cells with enlarged nuclei, and more pronounced loss of attachment (Fig. 3.1 f), exhibiting a trabecular pattern which is a characteristic feature of hepatocellular carcinoma (Narama *et al.*, 2003). The liver nodules developing in F1, F2 and F3 mice after exposure to AEBN for 8 weeks (Fig. 3.1 g), 6 weeks (Fig. 3.1 i) and 4 weeks (Fig. 3.1 k), respectively, also exhibited irregularly shaped cells with enlarged nuclei, and loss of attachment (Figs. 3 h, j and l respectively), and were thus confirmed to be preneoplastic.

3.1.1.3. Development of other anomalies in transgenerationally exposed mice

Mice exposed transgenerationally to AEBN were found to develop various anomalies other than preneoplastic nodules of the liver (Fig. 3.2 and Table 16). The development of these anomalies was however not found to be significant in

comparison to chronically exposed mice or between successive generations of transgenerationally exposed mice, using the 2X2 contingency χ^2-test.

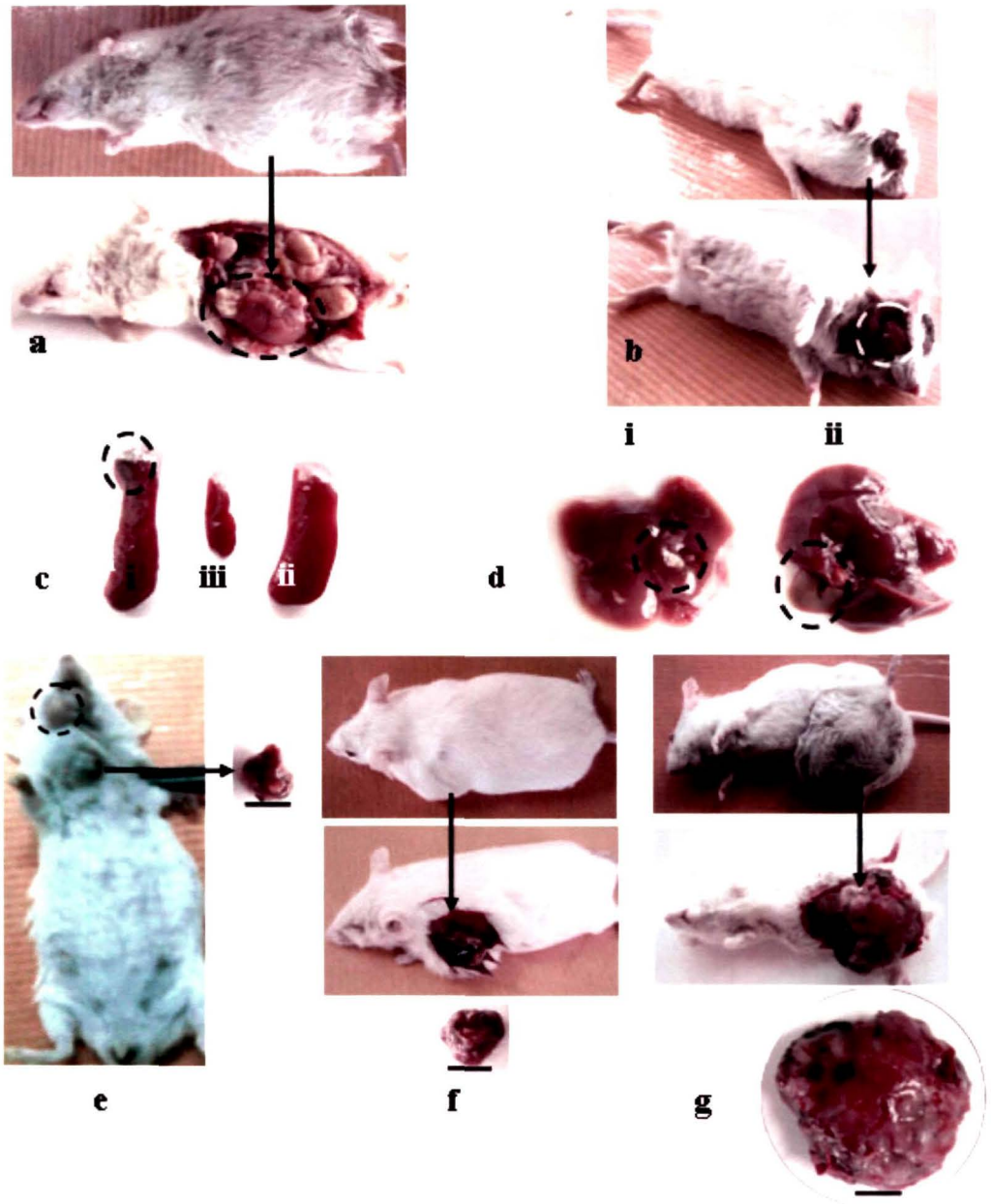


Figure 3.2. Various anomalies observed in Swiss Albino mice exposed transgenerationally to AEBN in drinking water (2 mg ml^{-1}). 24-week exposed F2 mouse showing pus-filled sac in gastrointestinal tract (circle) (**a**), enlarged neck node (circle) (**b**) or enlarged spleen (**c**) with protrusion (circle) (**i**) or without protrusion (**ii**); spleen of corresponding age-matched F2 generation control mouse is shown in (**iii**) (**d**) shows necrotic areas (circle) in liver of F2 mice after 12 (**i**) or 24 (**ii**) week exposure (**e**) shows a 24-week exposed F1 mouse with pus-filled sac on the mandible (circle) and solid tumor (7.5 mm in diameter) originating from epithelium of chest (**f**) shows a 24-week exposed F3 mouse with solid tumor (9.3 mm in diameter) originating from skin epithelium of left forearm (**g**) shows a 24-week exposed F3 mouse with solid tumor (30 mm in diameter) originating from epithelium of stomach. (For **e**, **f**, and **g** scale bar = 7.5 mm).

Table 16. Anomalies/ alterations observed in mice transgenerationally exposed to 2 mg ml^{-1} AEBN in drinking water

Type of anomaly	Generation	present(+)/ absent (-)	Site	Week of exposure	Incidence[a] (%)
Solid tumor	F1+	+	**Chest epithelium** tumor diameter = 7.5 mm tumor load* = 2.59	24	1/16 (6.25)
	F2	-	-	-	0/17 (0)
	F3	+	**Skin epithelium** tumor diameter = 9.3 mm tumor load* = 3.40	24	2/20 (10)
	F3	+	**Stomach epithelium** tumor diameter = 30 mm tumor load* = 50.64	24	-
Pus-filled sacs	F1+	+	Right mandible	24	1/16 (6.25)
	F2	+	Gastrointestinal	24	1/17 (5.88)
	F3+++	+	Neck	24	1/20 (5)
Enlarged nodes	F1	-	-	-	0/16 (0)
	F2	+	Neck	24	1/17 (5.88)
	F3+++	+	Neck	24	1/20 (5)
Necrosis of liver	F1	-	-	-	0/16 (0)
	F2	+	Liver	12	1/15 (6.67)
	F2++	+	Liver	24	1/17 (5.88)
	F3	+	Liver	24	1/20 (5)
Spleen protrusion	F1	-	-	-	0/16 (0)
	F2++	+	Spleen	24	1/17 (5.88)
	F3	-	-	-	0/20 (0)

[a] No. of mice with anomaly/ Total no. of mice exposed to AEBN for the same period of time.
+ indicates anomalies developing in the same F1 mouse.
++ indicates anomalies developing in the same F2 mouse.
+++ indicates anomalies developing in the same F3 mouse.
* g/ 100 g body weight.
Using 2X2 contingency χ^2-test, development of these anomalies was not significant in comparison to chronically exposed mice or between successive generations of transgenerationally exposed mice.

3.1.2. Transmission electron microscope (TEM) studies

Ultrastructural alterations in liver nodules of P, F1, F2 and F3 mice after 24 weeks of AEBN exposure, were studied using TEM. Various alterations in cellular organelles were observed in these nodules in comparison to corresponding regions of liver of age-matched control mice.

3.1.2.1. Alterations in nucleus

The hepatocytes of control mice were found to contain one well-defined and spherical nucleus per hepatocyte, with an intact nuclear envelope, distinct nucleoli, and uniformly distributed heterochromatin (Fig. 3.3 I-a). However, in P generation mice, a significant number of cells of the liver nodules were observed to be binucleated (Fig. 3.3 I-b), the nuclei were distorted in shape with coarse heterochromatin aggregates (Fig. 3.3 I-c), were enlarged (Fig. 3.3 I-d), had disrupted nuclear envelopes (Fig. 3.3 I-d & e; arows) and had enlarged nucleoli with a distinct clear zone apparently separating the nucleoli from a surrounding layer of condensed chromatin (Fig. 3.3 I-f & inset). The liver nodules of transgenerationally exposed mice also exhibited the nuclear deformities observed in the P generation mice. In addition the liver nodules of F1 and F3 mice exhibited small nuclei with lack with heterochromatin and inconspicuous nucleoli (Figs. 3.3 I-g & i), while the liver nodules of F2 mice exhibited nucleus with dispersed heterochromatin (Fig. 3.3 I-h).

3.1.2.2. Alterations in rough endoplasmic reticulum (RER)

The RER in the hepatocytes of control mice were found to be an extensive, well-stacked system of membranes with ribosomes attached along the surface appearing like fine particles (Fig. 3.3 II-a). In comparison, the RER of the liver nodules of the P generation mice were less extensive with diffused stacking (Fig. 3.3 II-b), and, the membrane was also disrupted at several places (Fig. 3.3 II-b & c; arrows). The RER continued to remain sparse, with diffuse stacking, in the liver nodules of F1 mice (Fig. 3.3 II-d; arrow), F2 mice (Fig. 3.3 II-e; arrow) and F3 mice (Fig. 3.3 II-f; arrow).

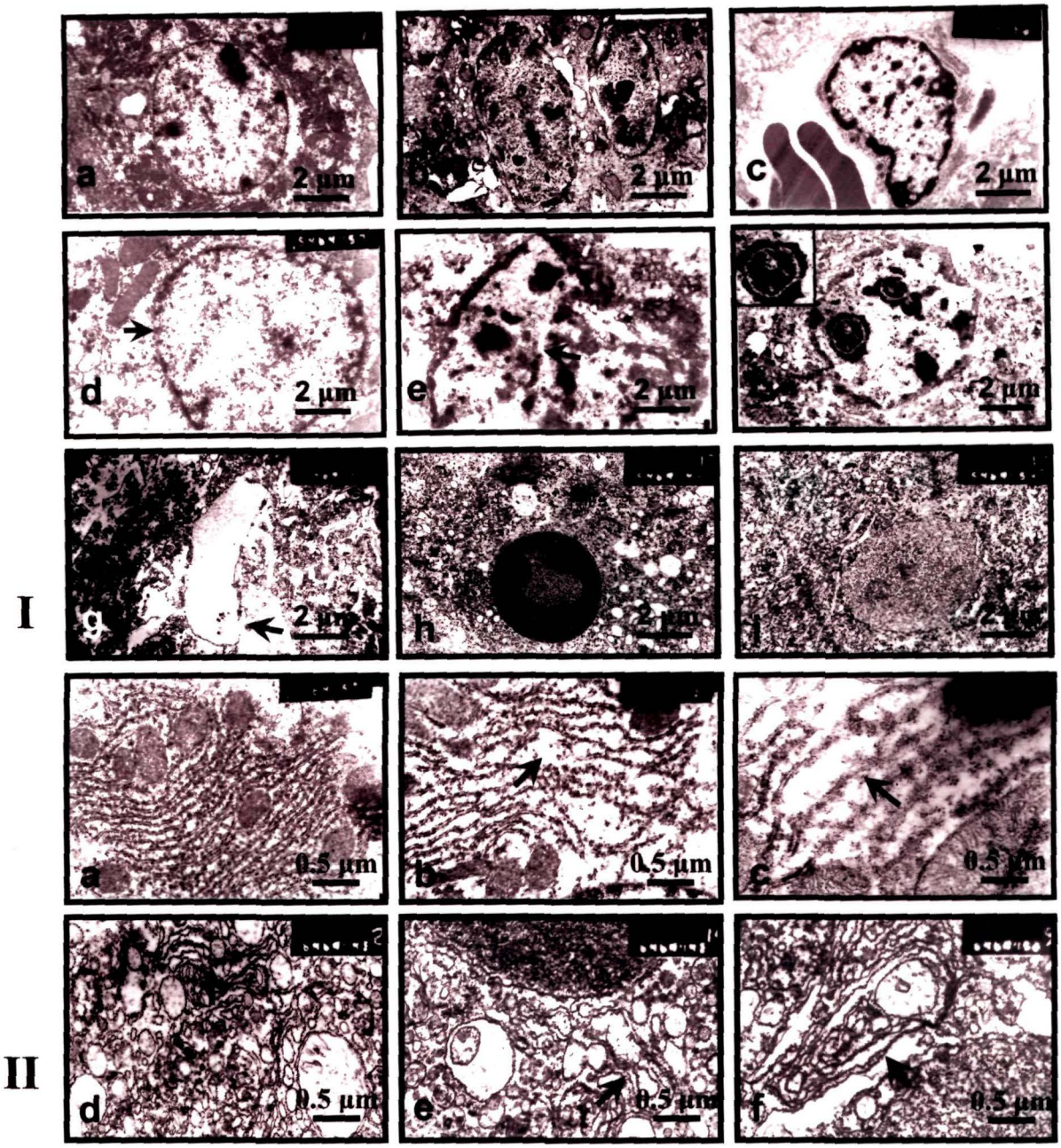


Figure 3.3. Ultrasructural changes in (I) nucleus and (II) rough endoplasmic reticulum (RER) of liver of mice exposed chronically and transgenerationally to AEBN for 24 weeks. (**I**) **a**- control; **b, c, d, e & f**- P; **g**- F1; **h**- F2 and **i**- F3. (**II**) **a**-control; **b & c**- P; **d**- F1; **e**- F2 and **f**- F3.

3.1.2.3. Alterations in mitochondria

The mitochondria of the hepatocytes of control mice were observed to be bound by a double-layered membrane, and had an extensive network of cristae (Fig. 3.4 I-a). In contrast, the mitochondria of liver nodules of P, F1, F2 and F3 generation mice exhibited extensive cristolysis (Figs. 3.4 I-b through e), and the mitochondrial membrane was also disrupted in some instances (Figs. 3.4 I-b & d; arrows). The mitochondria were also found to progressively decline in size, as well as number from

the P through F3 generations (Figs. 3.4 I-b through e & II-b through e) in comparison to control (Figs. 3.4 I-a & II-a).

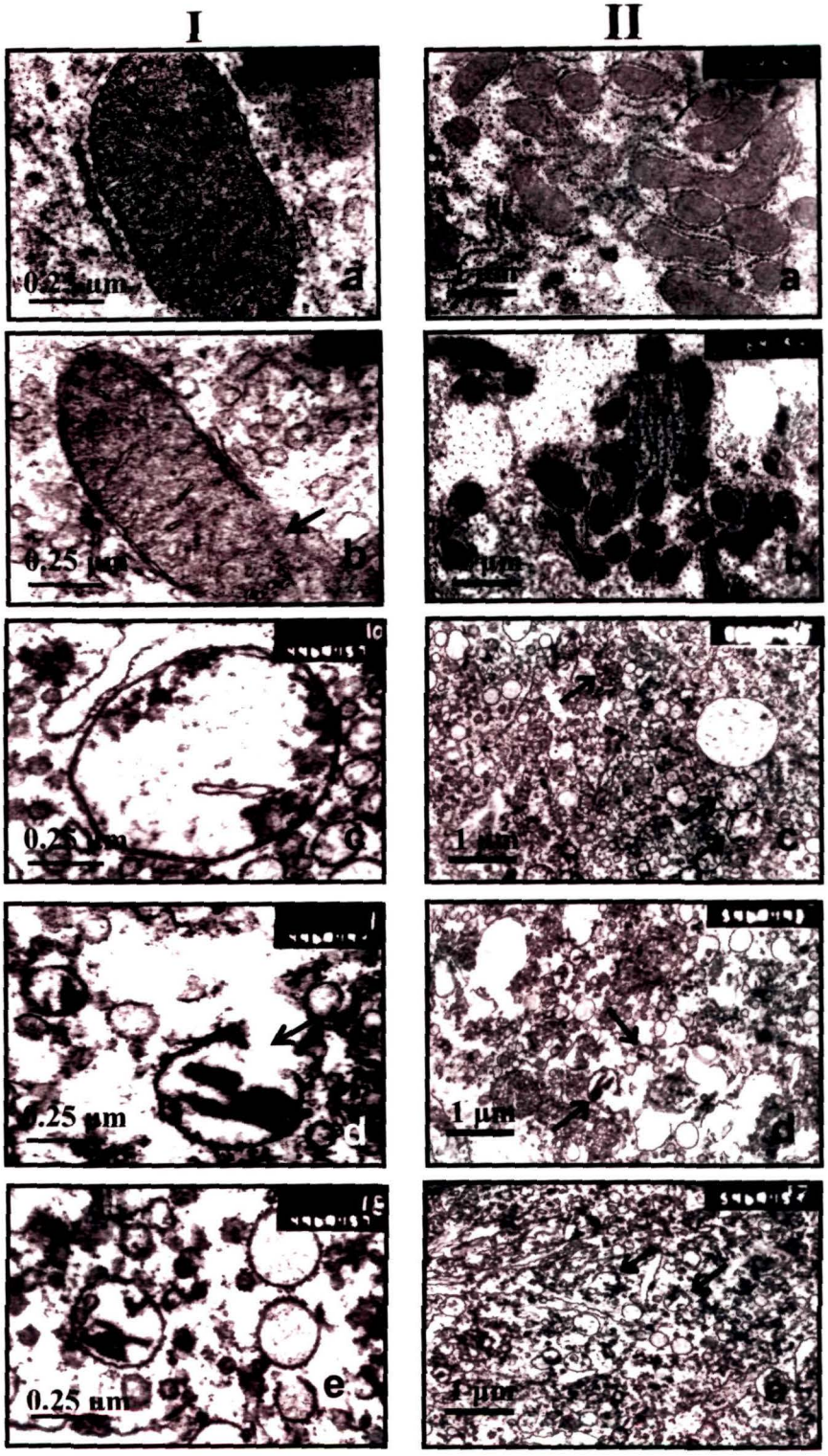


Figure 3.4. Ultrastructural changes in the mitochondria of mice exposed chronically and transgenerationally to AEBN for 24 weeks at 2 mg ml^{-1} showing alterations in (**I**) size of mitochondria and (**II**) number of mitochondria, (**I**) **a**- control; **b**- P; **c**- F1; **d**-F2 and **e**- F3. (**II**) **a**- control; **b**- P; **c**- F1; **d**-F2 and **e**- F3 (arrows indicate mitochondria). (**I**) b & d- arrows indicate disrupted mitochondrial membrane.

The mitochondrial index was determined as an indicator of mitochondrial function by determining the product of the total number and area of mitochondria, and was found to decline progressively from P through F3 generations (Fig. 3.5)

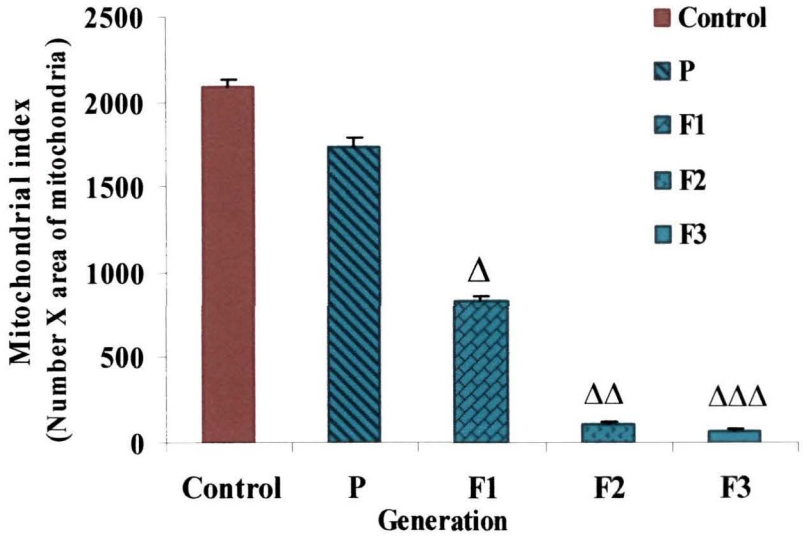


Figure 3.5. Bar diagram showing alteration in mitochondrial index of liver of mice exposed chronically (P) and transgenerationally (F1, F2 & F3) to AEBN, in comparison to liver of control (C) mice. Δ indicates significant decrease at $P<0.05$, ΔΔ at $P<0.01$ and ΔΔΔ at $P<0.001$.

3.2. SLOT-BLOT ANALYSIS OF EXPRESSION OF P53, BRCA1 AND BRCA2 PROTEINS UPON EXPOSURE OF MICE TO AEBN

3.2.1. Chronic exposure

In order to elucidate the molecular mechanisms involved in various stages of AEBN-induced carcinogenesis in mice, the P generation mice were exposed to AEBN at a dose of 2 mg ml^{-1} in drinking water for a period up to 24 weeks, and were sacrificed at regular intervals (§ 2.1.), following which liver, SC and PBL were used for analysis of alteration in expression levels of p53, Brca1 and Brca2 tumor suppressor proteins. For each experiment, slot-blotting was performed for 4-5 replicates which were subsequently immunoprobed with specific antibody, while a replica slot-blot was stained with India ink for total protein. In the following sections, only one replicum slot-blot for each data-point has been shown for the purpose of representation.

3.2.1.1. p53 tumor suppressor protein

3.2.1.1.1. in liver: Densitometric analysis of India ink-stained slot-blots (Fig. 3.6; panel A-II) and blots immunoprobed with anti-actin antibody (Fig. 3.6; panel A-III) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein. Immunoprobing with anti-p53 (Fig. 3.6; panel A-I) revealed significant changes in the level of p53 protein in the liver of AEBN exposed mice of P generation in comparison to age-matched controls. Upon quantification of slot-blots and normalization for equal loading of total protein, a progressive upregulation of p53 protein was observed beginning 2 weeks of exposure and recording a 2.5-fold increase at 6 weeks (Fig. 3.6 B). Downregulation of p53 began from 8 weeks of exposure reaching the control level at 16 weeks concomitant with the appearance of preneoplastic nodules in the liver (Fig. 3.1). Subsequently, the level of p53 in the livers of exposed mice was maintained at control level up to 20 weeks after which it was significantly below the control level (80 %) at 24 weeks (Figs. 3.6 A & B). Level of p53 was determined in preneoplastic nodules as well as the adjoining regions of the livers of mice exposed to AEBN for 16, 20 and 24 week and was found to be comparable.

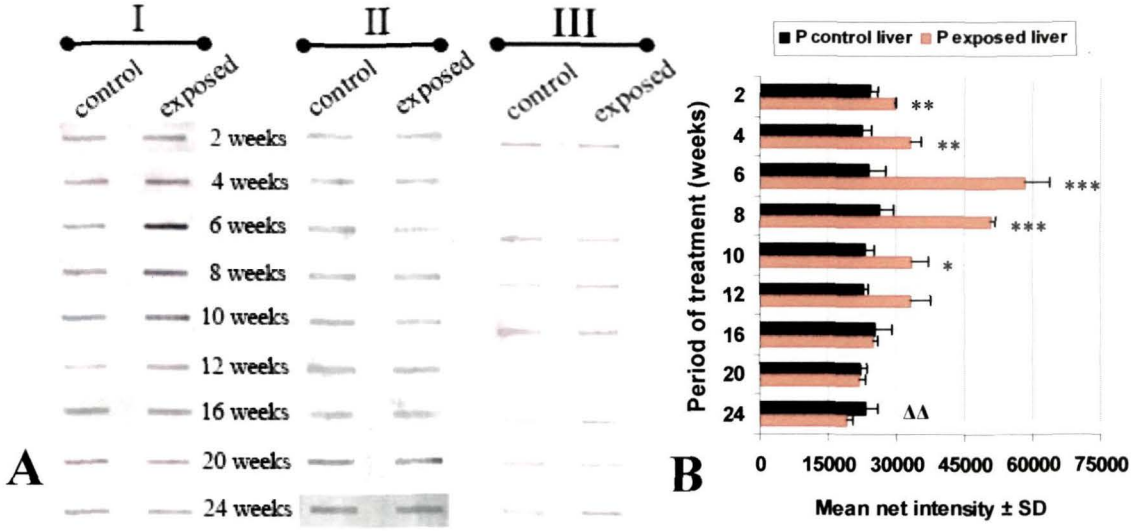


Figure 3.6. Slot-blot analysis for alterations in level of p53 protein in liver of P generation AEBN exposed mice. **A**- panel **I** slot-blots immunoprobed with anti-p53 antibody for p53 protein, **A**- panel **II** slot-blots stained with India ink for total protein, serving as loading control and **A**- panel **III** slot-blots immunoprobed with anti-actin antibody for actin protein as loading control; **B**- Densitometric plot (% of age-matched controls; mean ± SD) of the level of p53 protein expression in liver as obtained by densitometric analysis of the immunoprobed slot-blots (A-I) after normalization for equal protein loading (A-II & III). * indicates significant increase at $P < 0.05$, ** indicates significant increase at $P < 0.01$, *** indicates significant increase at $P < 0.001$ and ΔΔ indicates significant decrease at $P < 0.01$

In order to determine whether the observed effect of AEBN was specific to the liver, we also studied the changes in the level of p53 protein in the SC (§ 3.2.1.1.2.) and PBL (§ 3.2.1.1.3.).

3.2.1.1.2. in spleen cells (SC): Densitometric analysis of India ink-stained slot-blots (Fig. 3.7; panel A-II) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein. The levels of p53 protein in the SC (Fig. 3.7 A-I & B) of the exposed mice were largely found to mirror those in the liver (Fig. 3.6). As in the liver, the highest level of p53 protein in SC, i.e. 2-fold that of age-matched control level was also recorded at 6 weeks following AEBN exposure (Fig. 3.7 A-I & B).

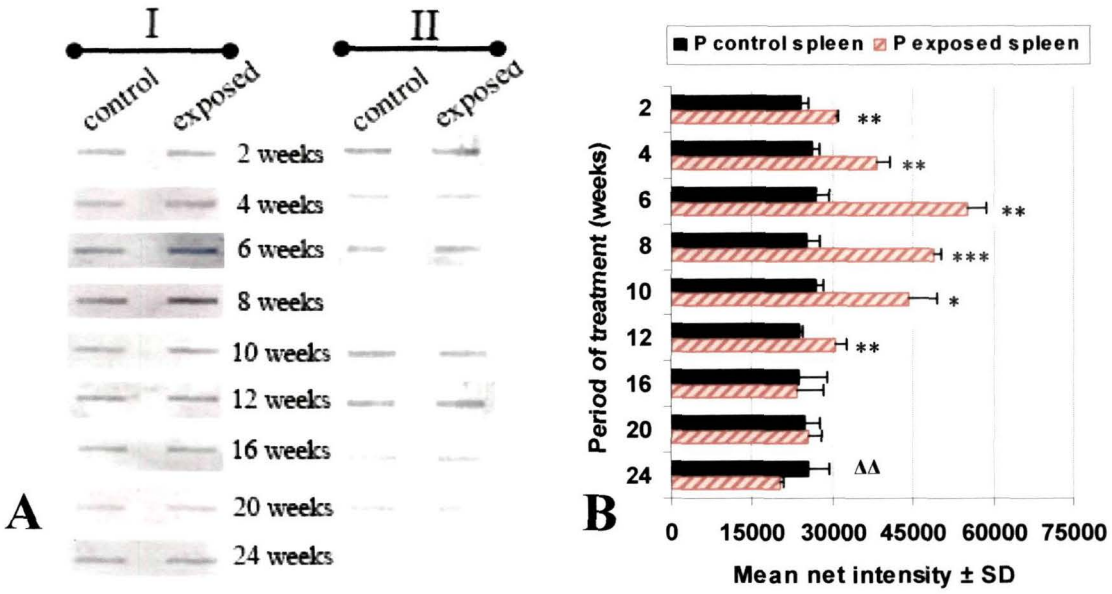


Figure 3.7. Slot-blot analysis for alterations in level of p53 protein in SC of P generation AEBN exposed mice. **A**- panel **I** slot-blots immunoprobed with anti-p53 antibody for p53 protein, **A**- panel **II** slot-blots stained with India ink for total protein, serving as loading control, **B**- Densitometric plot (% of age-matched controls; mean ± SD) of the level of p53 protein expression in liver as obtained by densitometric analysis of the immunoprobed slot-blots (A-I) after normalization for equal protein loading (A-II). * indicates significant increase at $P < 0.05$, ** indicates significant increase at $P < 0.01$, *** indicates significant increase at $P < 0.001$, Δ indicates significant decrease at $P < 0.05$ and ΔΔ indicates significant decrease at $P < 0.01$.

3.2.1.1.3. in peripheral blood lymphocytes (PBL): Densitometric analysis of India ink-stained slot-blots (Fig. 3.8; panel A-II) and blots immunoprobed with anti-actin antibody (Fig. 3.8; panel A-III) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein. As in case of SC, the levels of p53 protein in the PBL (Fig. 3.8 A-I & B)

of the exposed mice were largely found to mirror those in the liver (Fig. 3.6) In case of PBL, however, the highest level of p53 protein was recorded after 4 weeks of AEBN exposure, and was found to be 1.9-fold that of age-matched control level (Fig. 3.8 A-I & B).

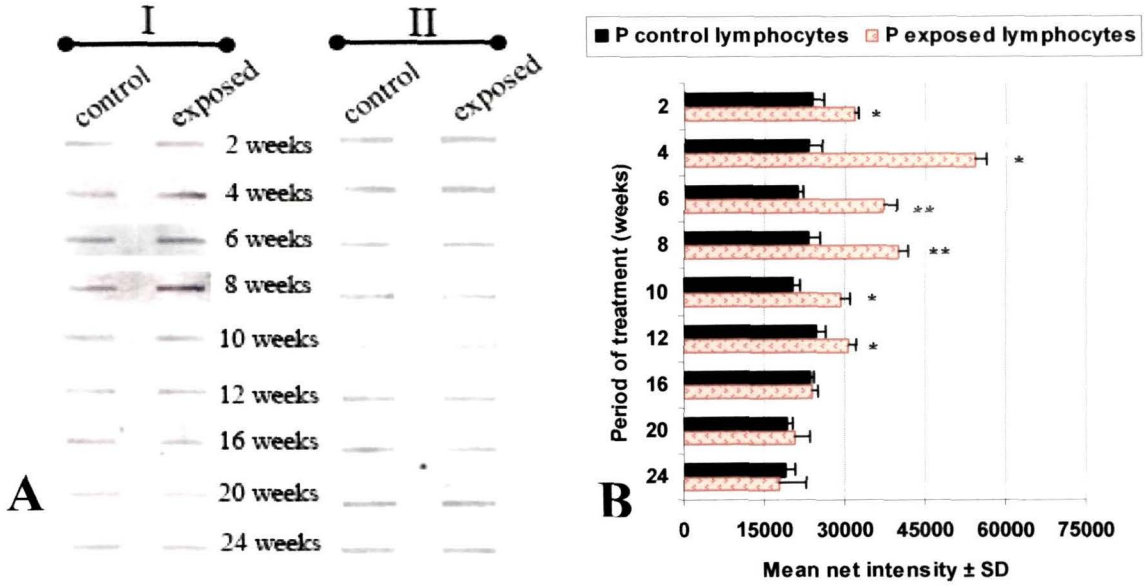


Figure 3.7. Slot-blot analysis for alterations in level of p53 protein in PBL of P generation AEBN exposed mice. **A**- panel **I** slot-blots immunoprobed with anti-p53 antibody for p53 protein, **A**- panel **II** slot-blots stained with India ink for total protein, serving as loading control, **B**- Densitometric plot (% of age-matched controls; mean ± SD) of the level of p53 protein expression in liver as obtained by densitometric analysis of the immunoprobed slot-blots (A-I) after normalization for equal protein loading (A-II). * indicates significant increase at $P < 0.05$ and ** indicates significant increase at $P < 0.01$.

3.2.1.2. Brca1 tumor suppressor protein

3.2.1.2.1. in liver: Densitometric analysis of India ink-stained slot-blots (Fig. 3.9; panel A-II) and blots immunoprobed with anti-actin antibody (Fig. 3.9; panel A-III) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein. Immunoprobing with anti-BRCA1 revealed significant alterations in the level of Brca1 protein in the liver of AEBN-exposed mice, in comparison to age-matched controls (Fig. 3.9 panel A-I & B). Exposure to AEBN led to an immediate Brca1 response, with significant elevation in Brca1 protein to 1.3-fold the level of age-matched controls after 2 weeks of exposure. The level of Brca1 protein declined to control level after 4 weeks of exposure, followed by a continuous decline to 66 % that of control level after 16 weeks of exposure when preneoplastic nodules appeared in the liver. After

24 weeks of exposure, the level of Brca1 protein in the preneoplastic nodules, as well as adjoining regions of liver was found to be 50 % that of age-matched control level.

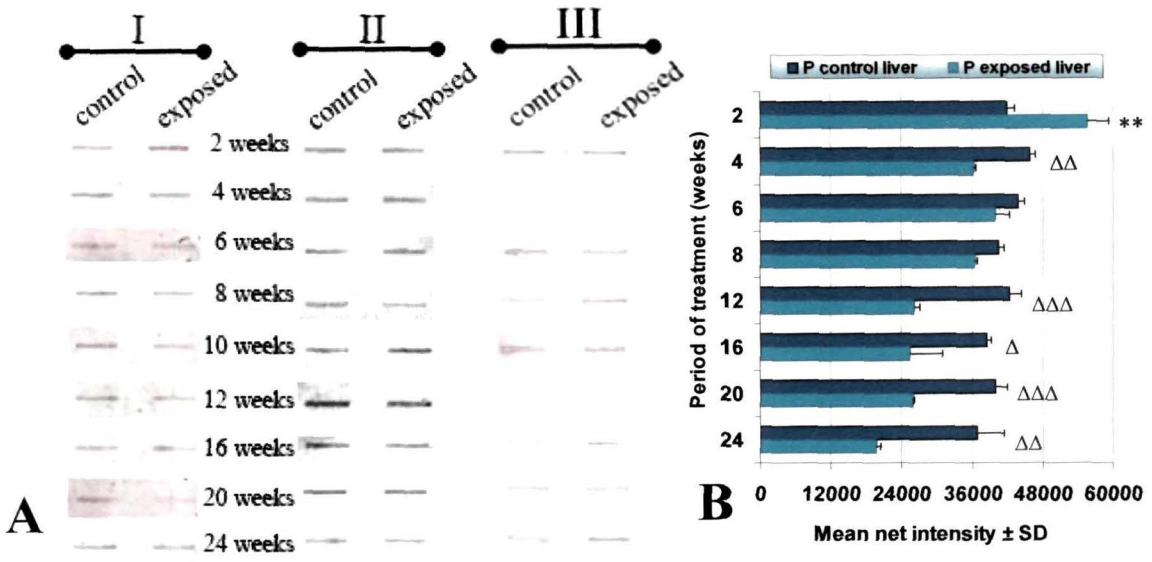


Figure 3.9. Slot-blot analysis for alterations in level of Brca1 protein in liver of P generation AEBN exposed mice. **A**- panel **I** slot-blots immunoprobed with anti-p53 antibody for p53 protein, **A**- panel **II** slot-blots stained with India ink for total protein, serving as loading control, **A**- panel **III** slot-blots immunoprobed with anti-actin antibody for actin protein as loading control, **B**- Densitometric plot (% of age-matched controls; mean ± SD) of the level of p53 protein expression in liver as obtained by densitometric analysis of the immunoprobed slot-blots (A-I) after normalization for equal protein loading (A-II & III). ** indicates significant increase at $P < 0.01$, ΔΔ indicates significant decrease at $P < 0.01$ and ΔΔΔ indicates significant decrease at $P < 0.001$.

3.2.1.2.2. in spleen cells (SC): Densitometric analysis of India ink-stained slot-blots (Fig. 3.10; panel A-II) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein. As in case of p53 protein, the pattern of alterations in level of Brca1 protein observed in liver of AEBN-exposed mice (Fig. 3.9), were largely mirrored by the SC (Fig. 3.10 panel A-I & B). Thus, exposure to AEBN resulted in significant elevation of Brca1 protein to 1.3-fold the level of age-matched controls after 2 weeks of exposure. The level of Brca1 protein subsequently declined, reaching 50 % that of age-matched control level after 24 weeks of exposure (Fig. 3.10 panel A-I & B).

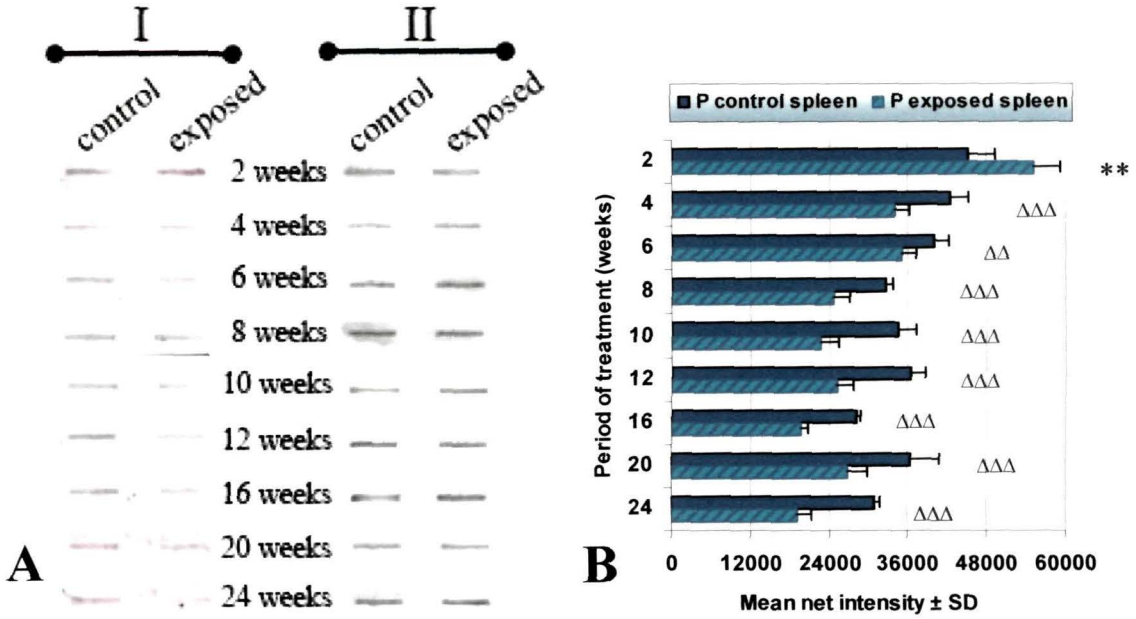


Figure 3.10. Slot-blot analysis for alterations in level of Brca1 protein in SC of P generation AEBN exposed mice. **A-** panel **I** slot-blots immunoprobed with anti-p53 antibody for p53 protein, **A-** panel **II** slot-blots stained with India ink for total protein, serving as loading control, **B-** Densitometric plot (% of age-matched controls; mean ± SD) of the level of p53 protein expression in liver as obtained by densitometric analysis of the immunoprobed slot-blots (A-I) after normalization for equal protein loading (A-II). ** indicates significant increase at $P < 0.01$, Δ indicates significant decrease at $P < 0.05$, ΔΔ indicates significant decrease at $P < 0.01$ and ΔΔΔ indicates significant decrease at $P < 0.001$.

3.2.1.2.3. in peripheral blood lymphocytes (PBL): Densitometric analysis of India ink-stained slot-blots (Fig. 3.11; panel A-II) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein. As in the SC (Fig. 3.10), the level of Brca1 protein was significantly elevated to 1.3-fold the age-matched control level after 2 weeks of exposure, followed by rapid decline to 50 % the age-matched control level after 24 weeks of exposure (Fig. 3.11 panel A-I & B).

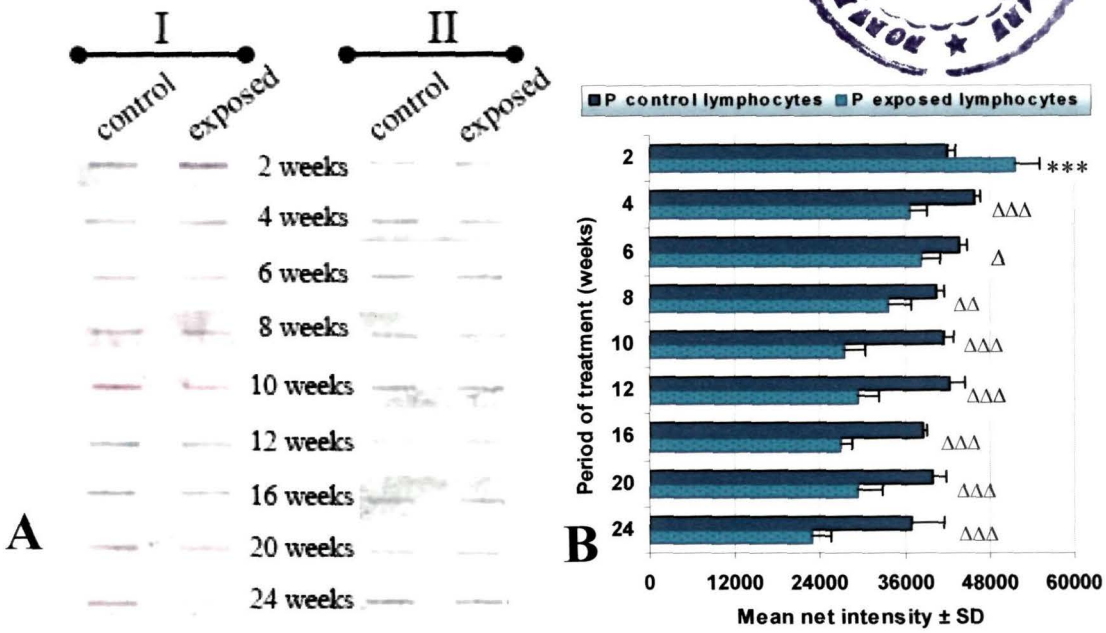


Figure 3.11. Slot-blot analysis for alterations in level of Brca1 protein in PBL of P generation AEBN exposed mice. **A**- panel **I** slot-blots immunoprobed with anti-p53 antibody for p53 protein, **A**- panel **II** slot-blots stained with India ink for total protein, serving as loading control, **B**- Densitometric plot (% of age-matched controls; mean ± SD) of the level of p53 protein expression in liver as obtained by densitometric analysis of the immunoprobed slot-blots (A-I) after normalization for equal protein loading (A-II). *** indicates significant increase at $P < 0.001$, Δ indicates significant decrease at $P<0.05$, ΔΔ indicates significant decrease at $P < 0.01$ and ΔΔΔ indicates significant decrease at $P < 0.001$.

3.2.1.3. Brca2 tumor suppressor protein

3.2.1.3.1. in liver: Densitometric analysis of India ink-stained slot-blots (Fig. 3.12, panel A-II) and blots immunoprobed with anti-actin antibody (Fig. 3.12; panel A-III) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein. Immunoprobing with anti-BRCA2 revealed significant changes in the level of Brca2 protein in the liver of AEBN-exposed mice, in comparison to age-matched controls (Fig. 3.12 panel A-I & B). Exposure to AEBN led to an immediate Brca2 response, with significant elevation in Brca2 protein to 1.4-fold the level of age-matched controls after 2 weeks of exposure. The level of Brca1 protein declined to control level after 4 weeks of exposure, followed by a continuous decline to 70 % that of control level only after 6 weeks of exposure. After 24 weeks of exposure, the level of Brca1 protein in the preneoplastic nodules, as well as adjoining regions of liver was found to be 50 % that of age-matched control level.

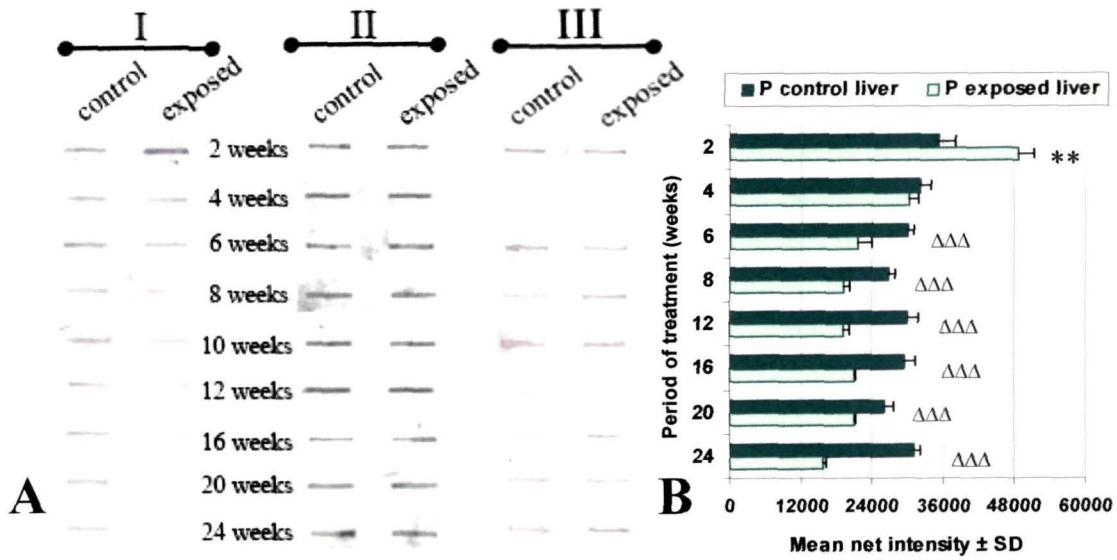


Figure 3.12. Slot-blot analysis for alterations in level of Brca2 protein in liver of P generation AEBN exposed mice. **A**- panel **I** slot-blots immunoprobed with anti-p53 antibody for p53 protein, **A**- panel **II** slot-blots stained with India ink for total protein, serving as loading control, **A**- panel **III** slot-blots immunoprobed with anti-actin antibody for actin protein as loading control, **B**- Densitometric plot (% of age-matched controls; mean ± SD) of the level of p53 protein expression in liver as obtained by densitometric analysis of the immunoprobed slot-blots (A-I) after normalization for equal protein loading (A-II & III). ** indicates significant increase at $P < 0.01$ and ΔΔΔ indicates significant decrease at $P < 0.001$.

3.2.1.3.2. in spleen cells cells (SC): Densitometric analysis of India ink-stained slot-blots (Fig. 3.13, panel A-II) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein. Immunoprobing with anti-BRCA2 revealed significant alterations in the SC of mice exposed to AEBN, in comparison to age-matched controls (Fig. 3.13 panel A-I and B). After 2 weeks of exposure, the level of Brca2 protein was elevated significantly to 1.2-fold that of age-matched control level. A significant decline in Brca2 protein level upon AEBN exposure was observed only after 20 and 24 weeks of exposure when it was 70 % that of age-matched control level (Fig. 3.13 panel A-I and B).

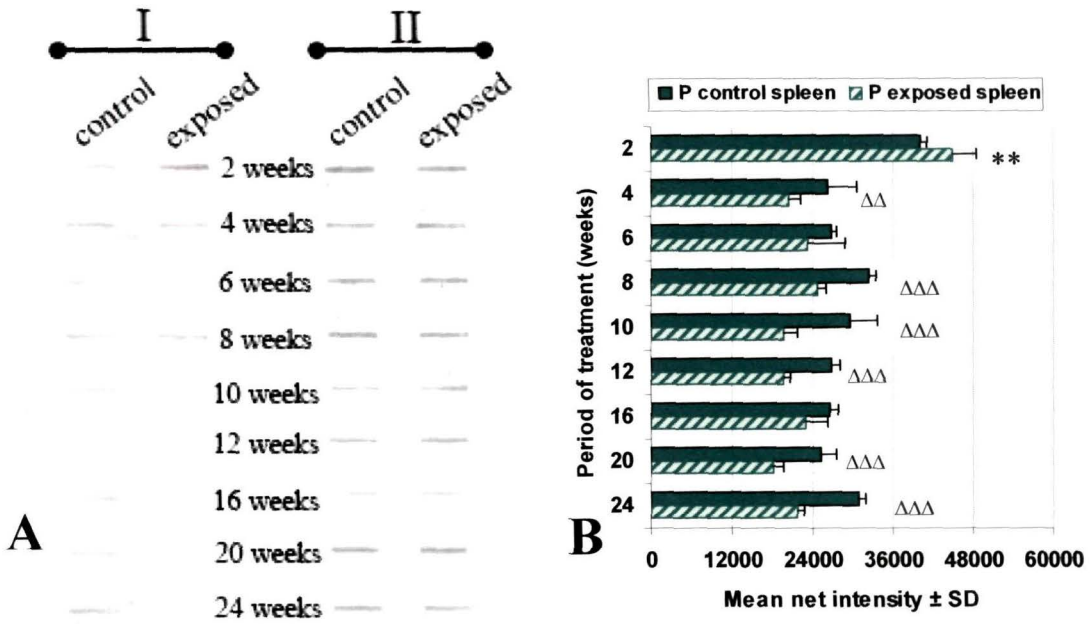


Figure 3.13. Slot-blot analysis for alterations in level of Brca2 protein in SC of P generation AEBN exposed mice. **A**- panel **I** slot-blots immunoprobed with anti-p53 antibody for p53 protein, **A**- panel **II** slot-blots stained with India ink for total protein, serving as loading control, **B**- Densitometric plot (% of age-matched controls; mean ± SD) of the level of p53 protein expression in liver as obtained by densitometric analysis of the immunoprobed slot-blots (A-I) after normalization for equal protein loading (A-II). ** indicates significant increase at $P < 0.01$, ΔΔ indicates significant decrease at $P < 0.01$ and ΔΔΔ indicates significant decrease at $P < 0.001$

3.2.1.3.3. in peripheral blood lymphocytes (PBL): Densitometric analysis of India ink-stained slot-blots (Fig. 3.14, panel A-II) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein. Immunoprobing with anti-BRCA2 revealed significant alterations in the level of Brca2 protein in the PBL of AEBN-exposed mice, in comparison to age-matched controls (Fig. 3.14 panel A-I & B). After 2 weeks of exposure the level of Brca2 protein was elevated significantly to 1.2-fold that of age-matched control level (Fig. 3.14 panel A-I & B). However like in the liver (Fig. 3.12) the level of Brca2 protein in the PBL also declined to 70 % that of age-matched control level only after 8 weeks of exposure, and reached 60 % of the age-matched control level after 24 weeks of exposure. Thus the alterations in level of Brca2 protein in PBL of P generation exposed mice mirrored those in the liver.

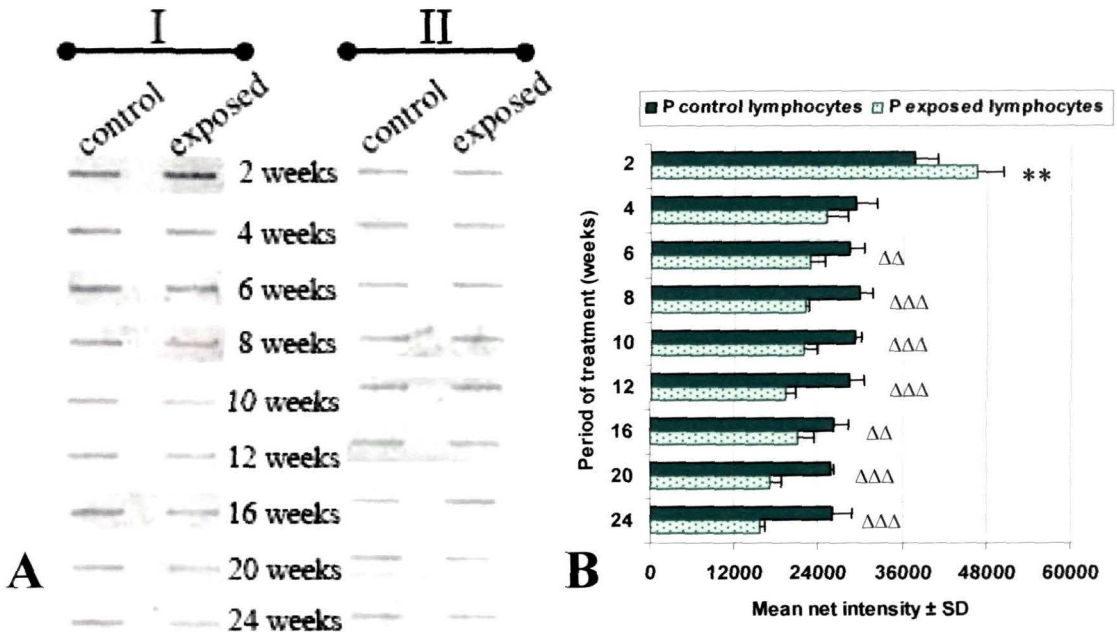


Figure 3.14. Slot-blot analysis for alterations in level of Brca2 protein in PBL of P generation AEBN exposed mice. **A-** panel **I** slot-blots immunoprobed with anti-p53 antibody for p53 protein, **A-** panel **II** slot-blots stained with India ink for total protein, serving as loading control, **B-** Densitometric plot (% of age-matched controls; mean ± SD) of the level of p53 protein expression in liver as obtained by densitometric analysis of the immunoprobed slot-blots (A-I) after normalization for equal protein loading (A-II). ** indicates significant increase at $P<0.01$, ΔΔ indicates significant decrease at $P < 0.01$ and ΔΔΔ indicates significant decrease at $P < 0.001$.

3.2.2. Transgenerational exposure

The effect of prenatal exposure to AEBN on susceptibility to AEBN-induced cancer was determined by exposing mice to AEBN using a transgenerational exposure protocol (§ 2.6.). The development of preneoplastic nodules of the liver was found to be significantly advanced in progeny exposed prenatally to AEBN (F1, F2 and F3 generations), in comparison to previously unexposed mice (P generation) (§ 3.1.). Thus, prenatal exposure to AEBN apparently enhanced susceptibility to cancer. In order to explain this observation in light of alterations in tumor suppressor genes, and taking into consideration alterations in the same genes induced by chronic exposure to AEBN (§ 3.2.1), the liver, SC and PBL of transgenerationally exposed mice were examined as in case of chronic exposure.

3.2.2.1. p53 tumor suppressor protein

Slot-blot analysis of p53 protein level in AEBN-exposed P generation mice exhibited significant alterations after 4, 6, 8, 12, 16 and 24 weeks of exposure to AEBN (§ 3.2.1.1.). Hence the effect of transgenerational exposure to AEBN upon expression level of p53 protein was evaluated only for these periods. In contrast to P generation mice, the level of p53 protein in mice exposed transgenerationally to AEBN remained invariant throughout the exposure period, in comparison to age-matched controls, for F1, F2 as well as F3 generations.

3.2.2.1.1. ***F1 generation:*** Densitometric analysis of India ink-stained slot-blots for liver, SC and PBL of AEBN-exposed F1 mice (Figs. 3.15 A, C & E; panel II) and blots for liver immunoprobed with anti-actin antibody (Fig. 3.15 A.; panel A-III) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein. The level of p53 protein in liver of AEBN exposed mice of F1 showed a significant decline to 75 % that of age-matched control level, only after 24 weeks of exposure. At all periods of investigation prior to this, the level remained unaltered in comparison to control level (Fig. 3.15 A & B). The tendency of p53 protein to remain at control level was also observed in SC of F1 mice (Fig. 3.15 C & D). The PBL of F1 mice showed significant increase in level of p53 protein in comparison to age-matched controls after 4 weeks and 8 weeks of exposure to AEBN (Fig. 3.15 E & F). These increases, however, did not follow a trend as in case of the P generation (Fig. 3.8), and were apparently sporadic.

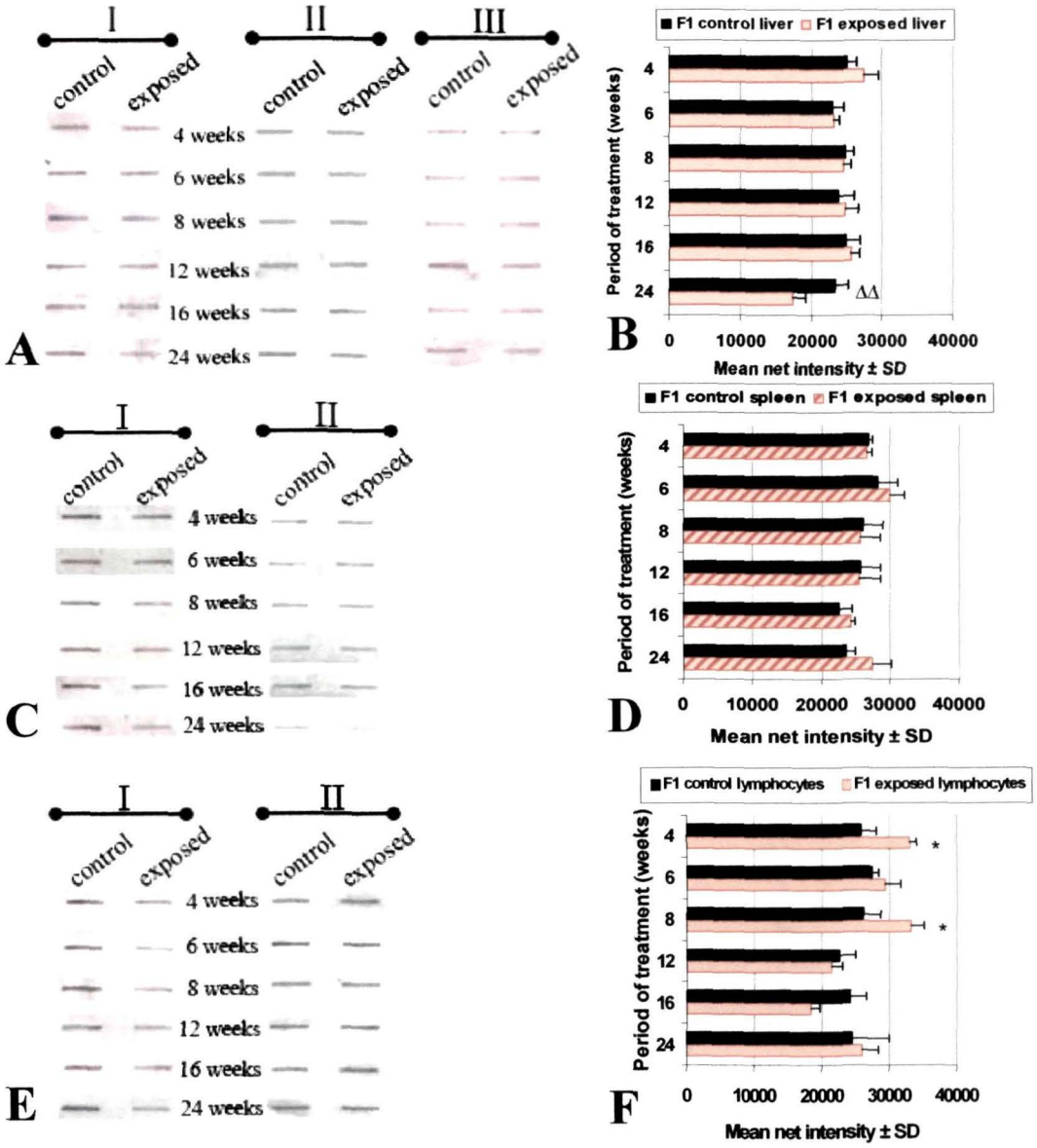


Figure 3.15. Slot-blot analysis for alterations in level of p53 protein in **A**-liver, **C**- SC and **E**-PBL of F1 generation AEBN exposed mice. panel **I** -slot-blots immunoprobed with anti-p53 antibody for p53 protein, panel **II**- slot-blots stained with India ink for total protein and panel **III**- slot-blots immunoprobed with anti-actin antibody, serving as loading controls; **B, D & F**- Densitometric plots (% of age-matched controls; mean ± SD) of the level of p53 protein expression in liver, SC and BL respectively obtained by densitometric analysis of the immunoprobed slot-blots (A-I, C-I & D-I) after normalization for equal protein loading (A-II & III, C-II & D-II). * indicates significant increase at $P < 0.05$ and ΔΔ indicates significant decrease at $P < 0.001$.

3.2.2.1.2. ***F2 generation:*** Densitometric analysis of India ink-stained slot-blots for liver, SC and PBL of AEBN-exposed F2 mice (Figs. 3.16 A, C & E; panel II) and blots for liver immunoprobed with anti-actin antibody (Fig. 3.16 A.; panel A-III) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein. The level of p53 protein in liver of AEBN exposed mice F2 remained unaltered in comparison to

control level throughout the period of investigation, with the exception of sporadic significant increase after 4 weeks of exposure, and decline after 16 weeks of exposure (Fig. 3.16 A & B). The tendency of p53 protein to remain at control level was also observed in SC (Fig. 3.16 C & D) and PBL (Fig. 3.16 E & F) of F3 mice, again with exception of sporadic decline after 4 and 8 weeks of exposure in SC and 16 weeks of exposure in PBL.

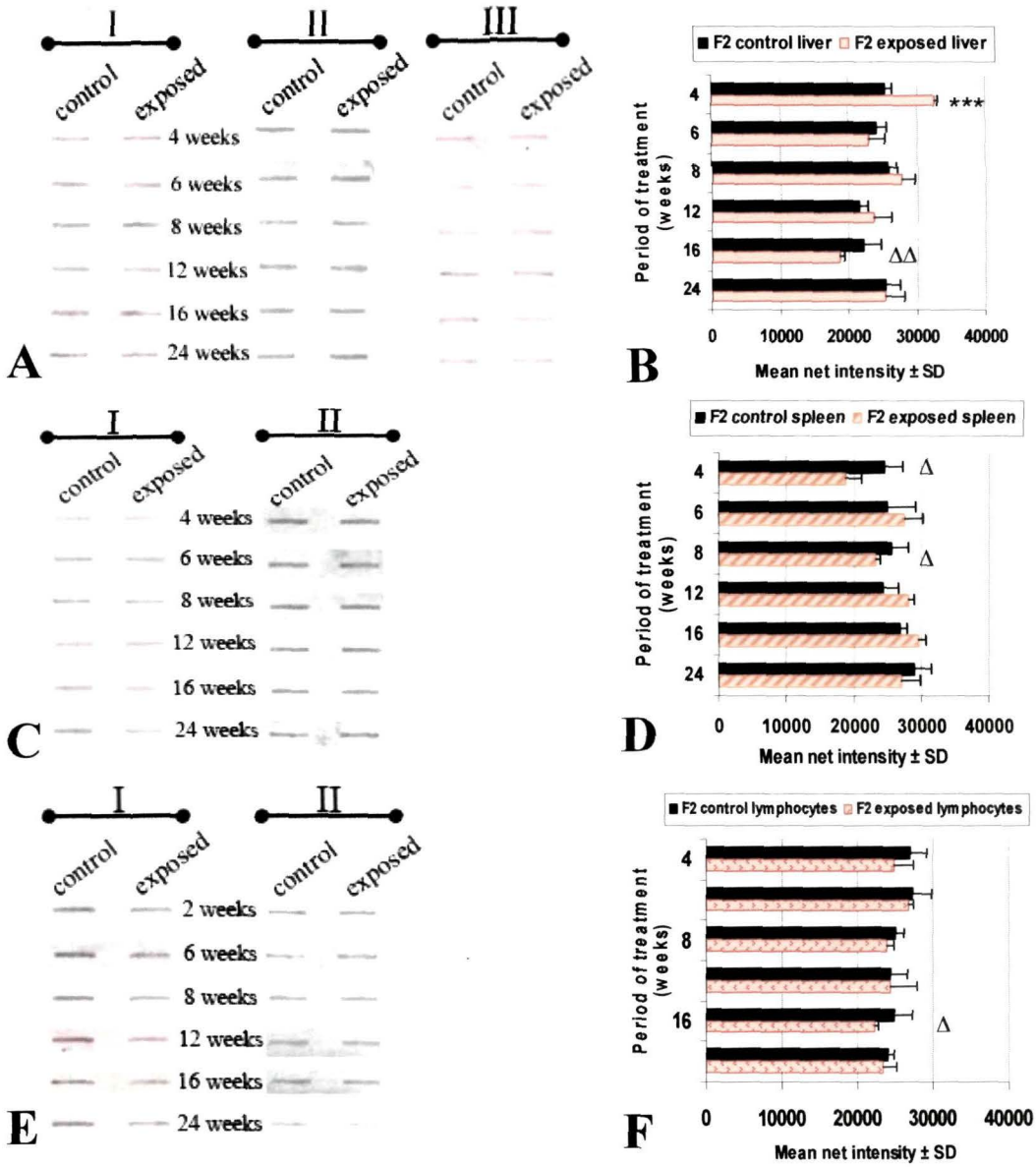


Figure 3.16. Slot-blot analysis for alterations in level of p53 protein in **A**-liver, **C**- SC and **E**-PBL of F2 generation AEBN exposed mice. panel **I** -slot-blots immunoprobed with anti-p53 antibody for p53 protein, panel **II**- slot-blots stained with India ink for total protein and panel **III**- slot-blots immunoprobed with anti-actin antibody, serving as loading controls; **B, D & F**- Densitometric plots (% of age-matched controls; mean ± SD) of the level of p53 protein expression in liver, SC and BL respectively as obtained by densitometric analysis of the immunoprobed slot-blots (A-I, C-I & D-I) after normalization for equal protein loading (A-II & III, C-II & D-II). *** indicates significant increase at $P < 0.001$, Δ indicates significant decrease at $P < 0.05$ and ΔΔ indicates significant decrease at $P < 0.01$.

3.2.2.1.3. ***F3 generation*****:** Densitometric analysis of India ink-stained slot-blots for liver, SC and PBL of AEBN-exposed F2 mice (Figs. 3.17 A, C & E; panel II) and blots for liver immunoprobed with anti-actin antibody (Fig. 3.17 A.; panel A-III) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein.

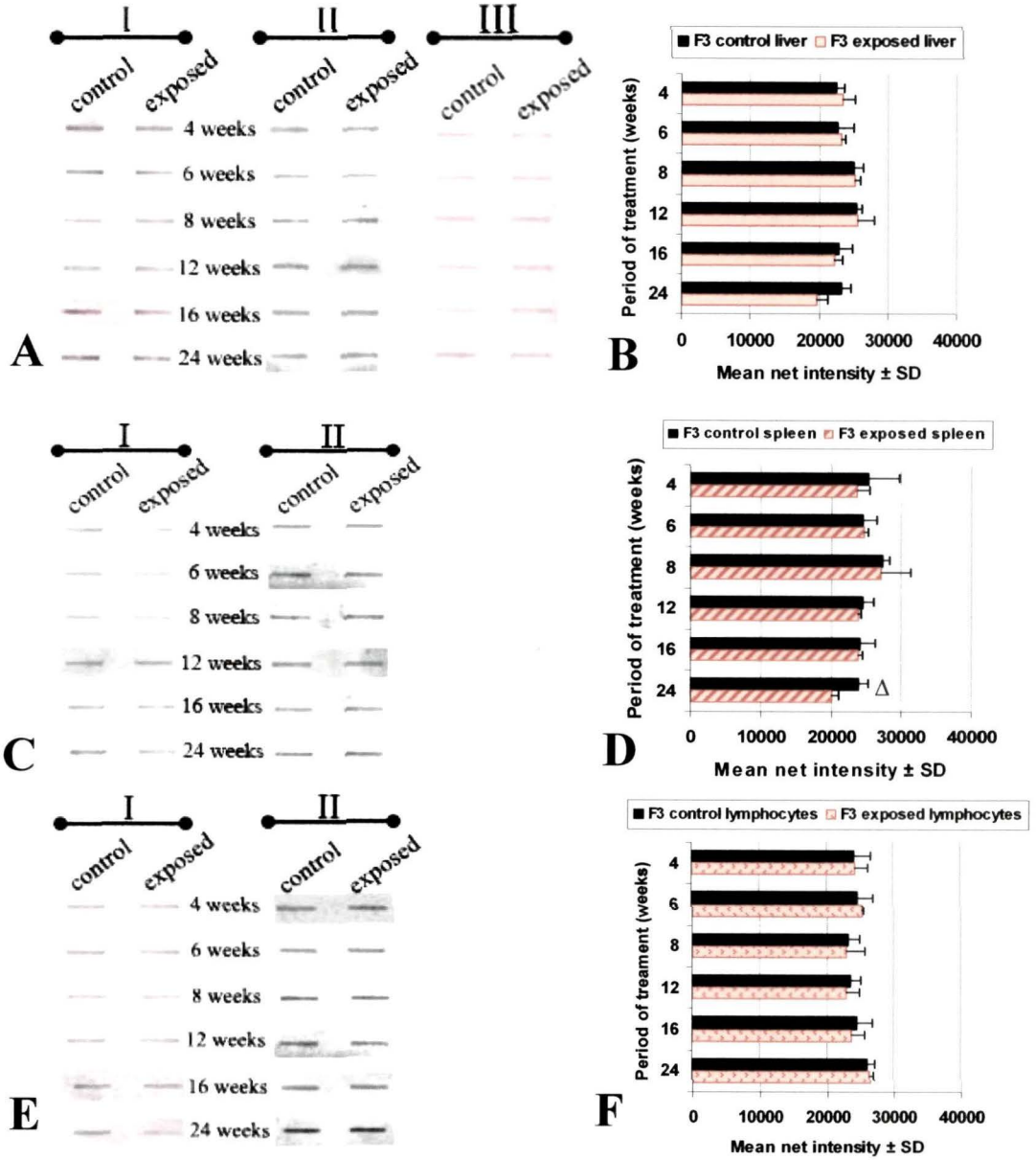


Figure 3.17. Slot-blot analysis for alterations in level of p53 protein in **A**-liver, **C**- SC and **E**-PBL of F3 generation AEBN exposed mice. panel **I** -slot-blots immunoprobed with anti-p53 antibody for p53 protein, panel **II**- slot-blots stained with India ink for total protein and panel **III**- slot-blots immunoprobed with anti-actin antibody, serving as loading controls; **B, D & F**- Densitometric plots (% of age-matched controls; mean ± SD) of the level of p53 protein expression in liver, SC and BL respectively as obtained by densitometric analysis of the immunoprobed slot-blots (A-I, C-I & D-I) after normalization for equal protein loading (A-II & III, C-II & D-II). Δ indicates significant decrease at $P < 0.05$.

The level of p53 protein in liver of AEBN exposed F3 mice remained unaltered in comparison to control level throughout the period of investigation (Fig. 3.17 A & B). The tendency of p53 protein to remain at control level was also observed in SC (Fig. 3.17 C & D) and PBL (Fig. 3.17 E & F) of F3 mice, again with exception of sporadic decline after 24 weeks of exposure in SC.

3.2.2.2. Brca1 tumor suppressor protein

Slot-blot analysis of Brca1 protein level in AEBN-exposed P generation mice exhibited significant alterations after 2, 6, 8, 12, 16 and 24 weeks of exposure to AEBN (§ 3.2.1.2.). Hence the effect of transgenerational exposure to AEBN upon expression level of Brca1 protein was evaluated only for these periods. In contrast to P generation mice, the level of Brca1 protein in mice exposed transgenerationally to AEBN did not show an initial elevation in comparison to age-matched controls after 2 weeks of exposure, and the decline in level in comparison to age-matched control was also more rapid. As in the P generation, an age-dependent decline in level of Brca1 protein in control mice was also observed.

3.2.2.2.1. ***F1 generation:*** Densitometric analysis of India ink-stained slot-blots for liver, SC and PBL of AEBN-exposed F1 mice (Figs. 3.18 A, C & E; panel II) and blots for liver immunoprobed with anti-actin antibody (Fig. 3.18 A.; panel A-III) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein. The level of Brca1 protein in liver of AEBN exposed mice was 77 % that of age-matched control level after 2 weeks of exposure, and rapidly declined to 70 % that of age-matched control level after 8 weeks of exposure (Fig. 3.18 A & B), concomitant with appearance of preneoplastic nodules in the liver (Fig. 3.1 g). Similarly, in SC, the level of Brca1 protein was 80 % that of age-matched control level after 2 weeks of exposure, and rapidly declined to 70 % that of age-matched control level after 8 weeks of exposure (Fig. 3.18 C & D) and in PBL the level of Brca1 protein was 74 % that of age-matched control level after 2 weeks of exposure, and rapidly declined to 70 % that of age-matched control level after 8 weeks of exposure (Fig. 3.18 E & F).

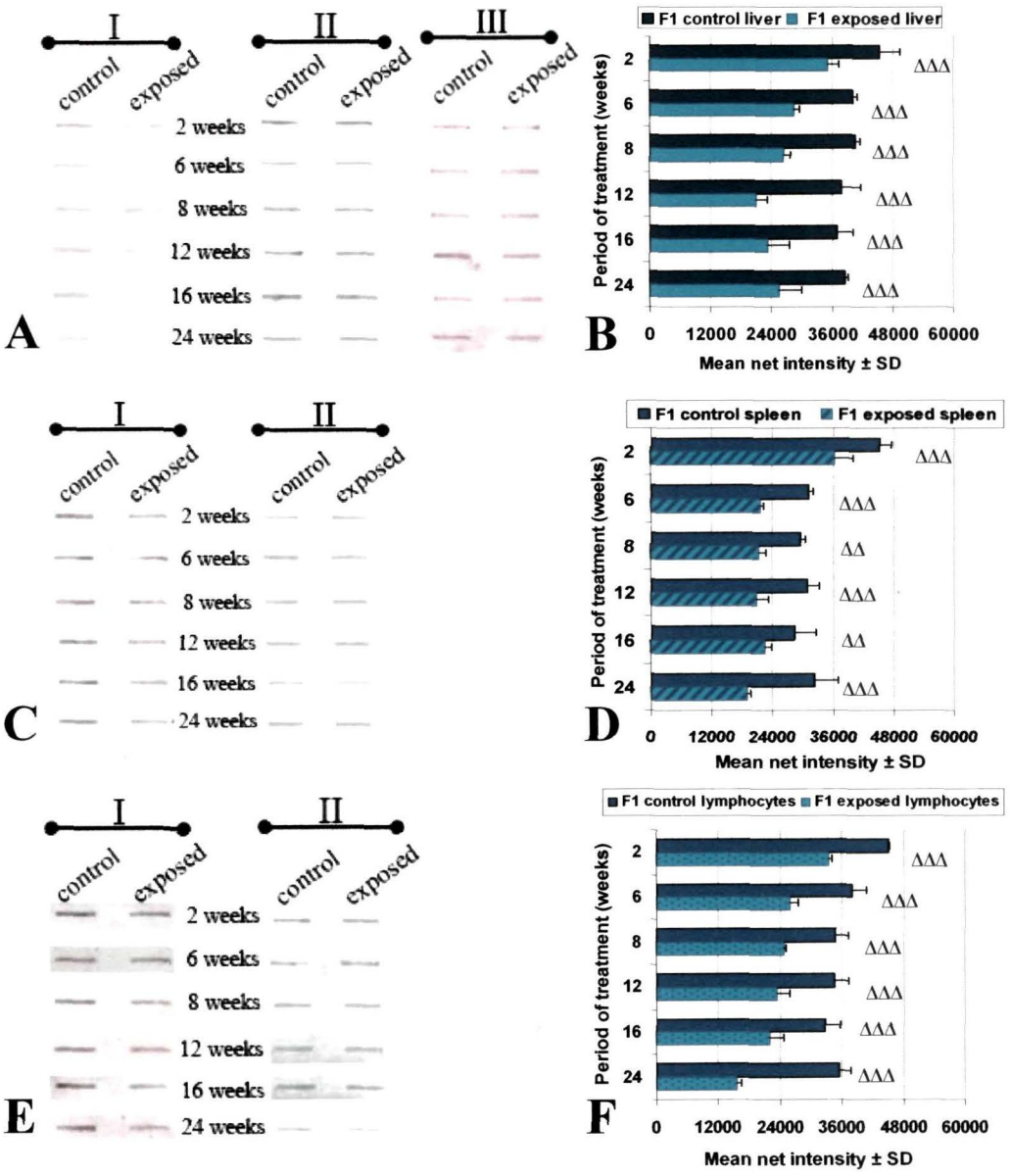


Figure 3.18. Slot-blot analysis for alterations in level of Brca1 protein in **A**-liver, **C**- SC and **E**-PBL of F1 generation AEBN exposed mice. panel **I** -slot-blots immunoprobed with anti-p53 antibody for p53 protein, panel **II**- slot-blots stained with India ink for total protein and panel **III**- slot-blots immunoprobed with anti-actin antibody, serving as loading controls; **B, D & F**- Densitometric plots (mean ± SD) of the level of p53 protein expression in liver, SC and BL respectively as obtained by densitometric analysis of the immunoprobed slot-blots (A-I, C-I & D-I) after normalization for equal protein loading (A-II & III, C-II & D-II). ΔΔ indicates significant decrease at $P < 0.01$ and ΔΔΔ indicates significant decrease at $P < 0.001$.

*3.2.2.2.2. **F2 generation:*** Densitometric analysis of India ink-stained slot-blots for liver, SC and PBL of AEBN-exposed F2 mice (Figs 3.19 A, C & E; panel II) and blots for liver immunoprobed with anti-actin antibody (Fig. 3.19 A.; panel A-III) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein.

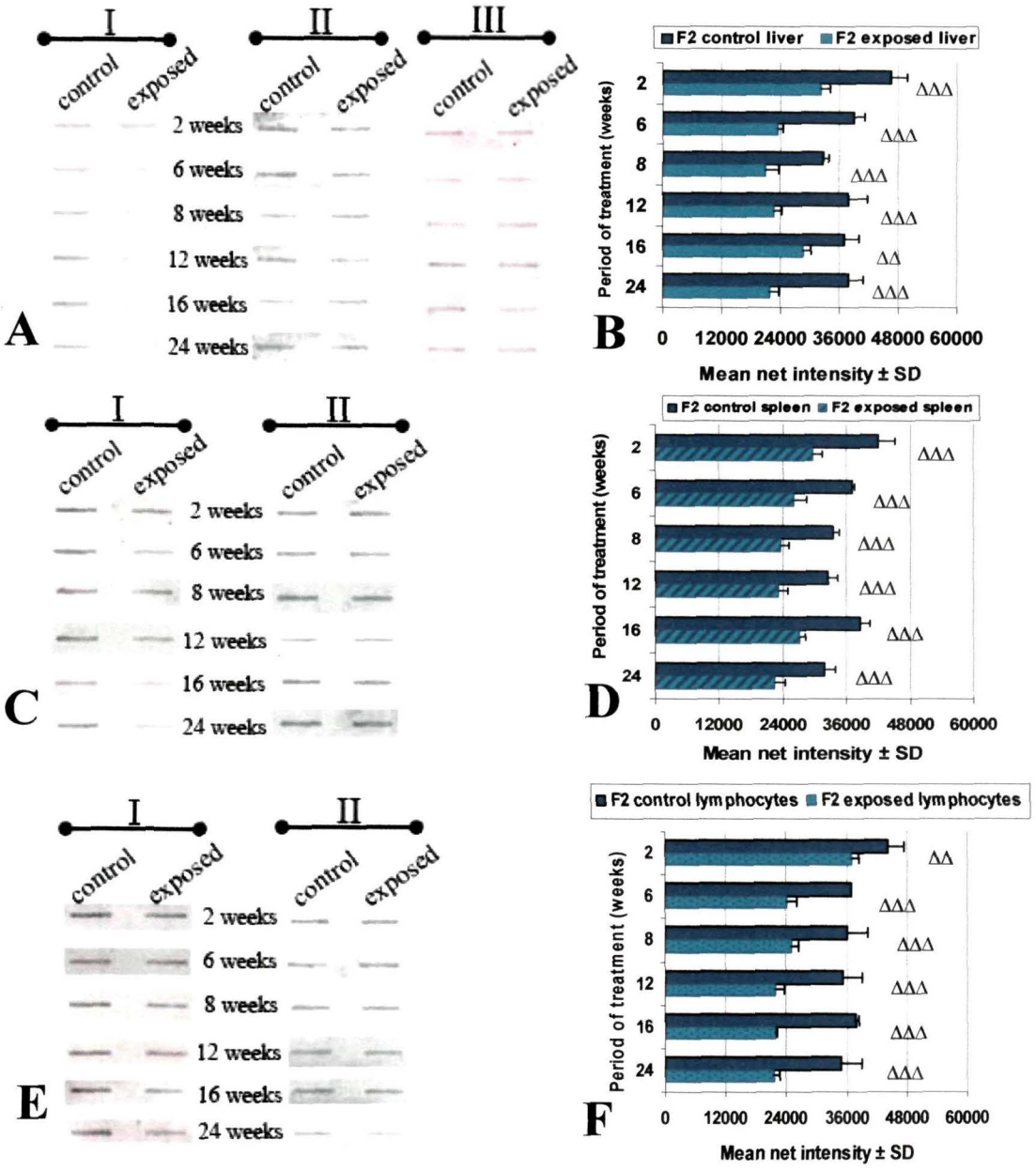


Figure 3.19. Slot-blot analysis for alterations in level of Brca1 protein in **A**-liver, **C**- SC and **E**-PBL of F2 generation AEBN exposed mice. panel **I** -slot-blots immunoprobed with anti-p53 antibody for p53 protein, panel **II**- slot-blots stained with India ink for total protein and panel **III**- slot-blots immunoprobed with anti-actin antibody, serving as loading controls; **B, D & F**- Densitometric plots (mean ± SD) of the level of p53 protein expression in liver, SC and BL respectively as obtained by densitometric analysis of the immunoprobed slot-blots (A-I, C-I & D-I) after normalization for equal protein loading (A-II & III, C-II & D-II). ΔΔ indicates significant decrease at $P < 0.01$ and ΔΔΔ indicates significant decrease at $P < 0.001$.

The level of Brca1 protein in liver of AEBN exposed mice was 70 % that of age-matched control level after 2 weeks of exposure, and rapidly declined to 62 % that of age-matched control level after 6 weeks of exposure (Fig. 3.19 A & B), concomitant with appearance of preneoplastic nodules in the liver (Fig. 3.1 i). Similarly, in SC, the level of Brca1 protein was 77 % that of age-matched control level after 2 weeks of exposure, and rapidly declined to 70 % that of age-matched control level after 6 weeks of exposure (Fig. 3.19 C & D) and in PBL the level of Brca1 protein was 84 % that of age-matched control level after 2 weeks of exposure, and rapidly declined to 67 % that of age-matched control level after 6 weeks of exposure (Fig. 3.19 E & F).

3.2.2.2.3. ***F3 generation:*** Densitometric analysis of India ink-stained slot-blots for liver, SC and PBL of AEBN-exposed F3 mice (Figs. 3.20 A, C & E; panel II) and blots for liver immunoprobed with anti-actin antibody (Fig. 3.20 A.; panel A-III) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein. The level of Brca1 protein in liver of AEBN exposed mice was 77 % that of age-matched control level after 2 weeks of exposure, and rapidly declined to 61 % that of age-matched control level after 6 weeks of exposure (Fig. 3.20 A & B), following appearance of preneoplastic nodules in the liver after 4 weeks of exposure (Fig. 3.1 k). Similarly, in SC, the level of Brca1 protein was 83 % that of age-matched control level after 2 weeks of exposure, and rapidly declined to 65 % that of age-matched control level after 6 weeks of exposure (Fig. 3.20 C & D) and in PBL the level of Brca1 protein was 70 % that of age-matched control level after 2 weeks of exposure, and rapidly declined to 64 % that of age-matched control level after 6 weeks of exposure (Fig. 3.20 E & F).

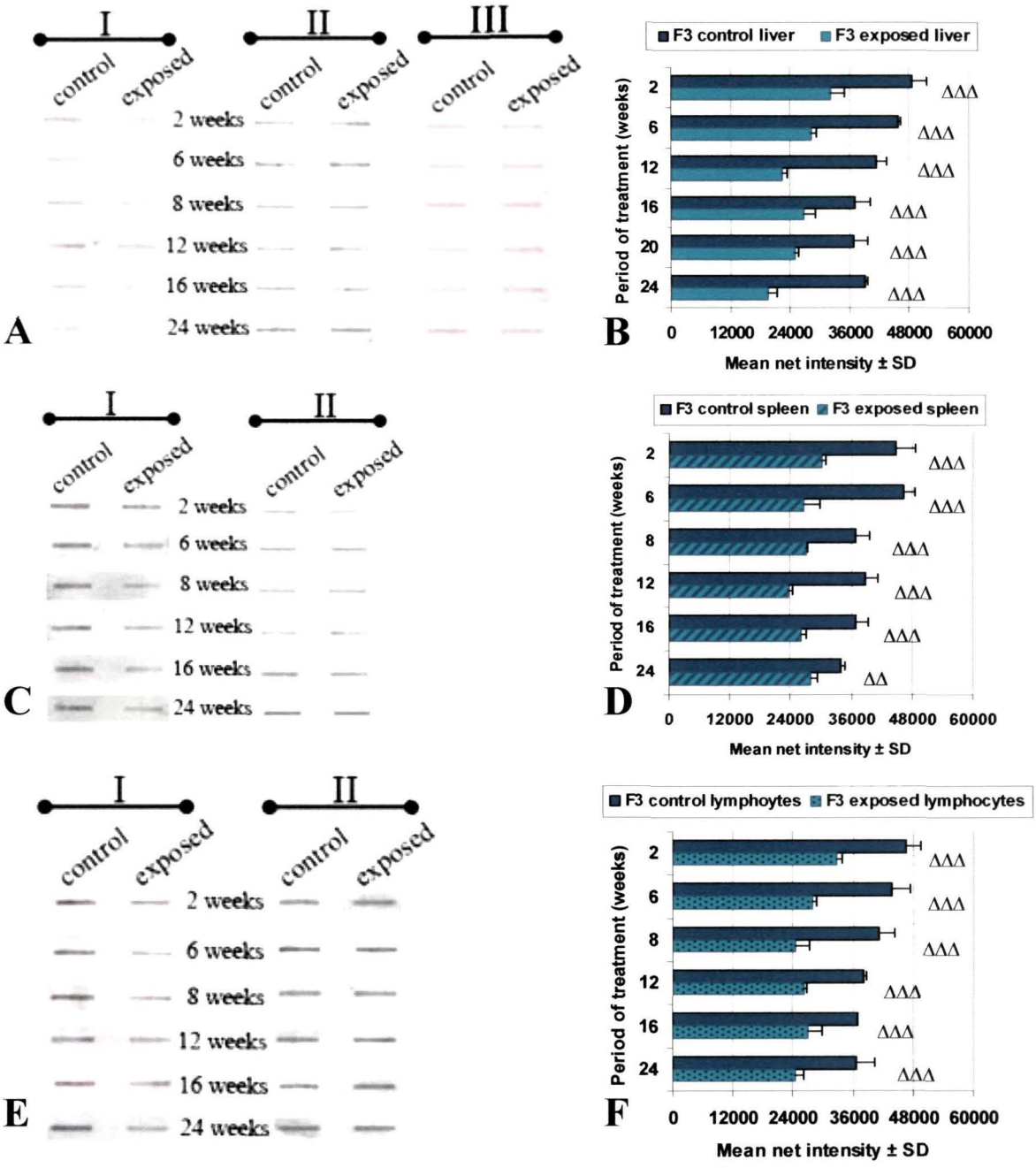


Figure 3.20. Slot-blot analysis for alterations in level of Brca1 protein in **A**-liver, **C**- SC and **E**-PBL of F3 generation AEBN exposed mice. panel **I** -slot-blots immunoprobed with anti-p53 antibody for p53 protein, panel **II**- slot-blots stained with India ink for total protein and panel **III**- slot-blots immunoprobed with anti-actin antibody, serving as loading controls; **B, D & F**- Densitometric plots (mean ± SD) of the level of p53 protein expression in liver, SC and BL respectively as obtained by densitometric analysis of the immunoprobed slot-blots (A-I, C-I & D-I) after normalization for equal protein loading (A-II & III, C-II & D-II). ΔΔΔ indicates significant decrease at $P < 0.001$.

3.2.2.3. Brca2 tumor suppressor protein:

Slot-blot analysis of Brca2 protein level in AEBN-exposed P generation mice exhibited significant alterations after 2, 6, 8, 12, 16 and 24 weeks of exposure to AEBN (§ 3.2.1.3.). Hence the effect of transgenerational exposure to AEBN upon expression level of Brca1 protein was evaluated only for these periods. In contrast to P generation mice, the level of Brca1 protein in mice exposed transgenerationally to AEBN did not show an initial elevation in comparison to age-matched controls after 2 weeks of exposure, and the decline in level in comparison to age-matched control was also more rapid. As in the P generation, age-dependent decline in level of Brca2 protein in control mice was also observed.

3.2.2.3.1. ***F1 generation:*** Densitometric analysis of India ink-stained slot-blots for liver, SC and PBL of AEBN-exposed F1 mice (Figs. 3.21 A, C & E; panel II) and blots for liver immunoprobed with anti-actin antibody (Fig. 3.21 A.; panel A-III) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein. The level of Brca2 protein in liver of AEBN exposed mice was 80 % that of age-matched control level after 2 weeks of exposure, and remained at 80 % that of age-matched control level after 8 weeks of exposure when preneoplastic nodules were observed in the liver (Fig. 3.1 g). However, this was preceded by a decline to 67 % that of control level after 6 weeks of exposure (Fig. 3.21 A & B). In SC, the level of Brca2 protein was 74 % that of age-matched control level after 2 weeks of exposure, and rapidly declined to 65 % that of age-matched control level after 8 weeks of exposure (Fig. 3.21 C & D) and in PBL the level of Brca2 protein was 74 % that of age-matched control level after 2 weeks of exposure, and rapidly declined to 70 % that of age-matched control level after 8 weeks of exposure (Fig. 3.21 E & F).

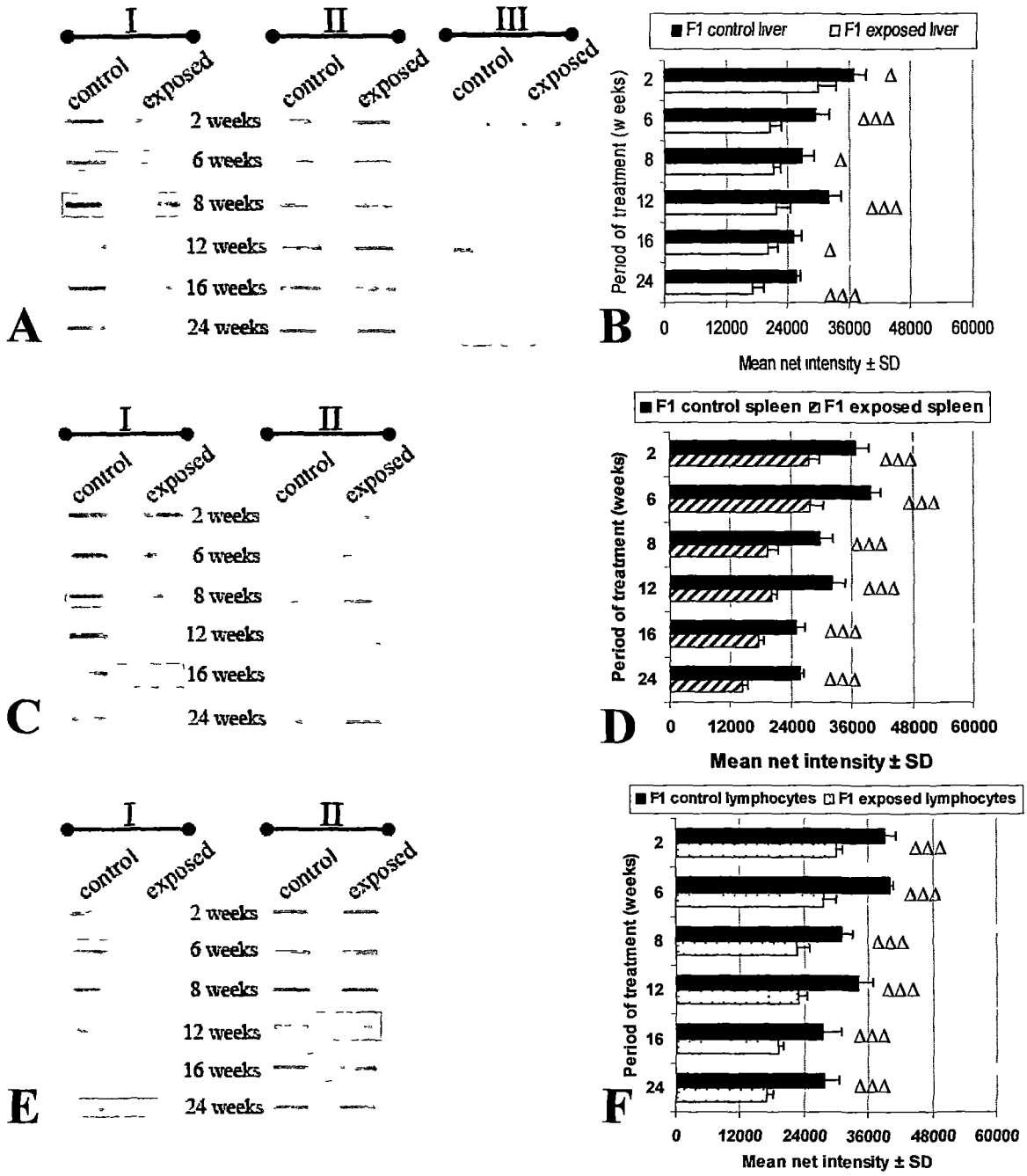


Figure 3.21. Slot-blot analysis for alterations in level of Brca2 protein in **A**-liver, **C**- SC and **E**-PBL of F1generation AEBN exposed mice. panel **I** -slot-blots immunoprobed with anti-p53 antibody for p53 protein, panel **II**- slot-blots stained with India ink for total protein and panel **III**- slot-blots immunoprobed with anti-actin antibody, serving as loading controls; **B, D & F**- Densitometric plots (mean ± SD) of the level of p53 protein expression in liver, SC and BL respectively as obtained by densitometric analysis of the immunoprobed slot-blots (A-I, C-I & D-I) after normalization for equal protein loading (A-II & III, C-II & D-II). Δ indicates significant decrease at $P < 0.05$, ΔΔ indicates significant decrease at $P < 0.01$ and ΔΔΔ indicates significant decrease at $P < 0.001$.

3.2.2.3.2. ***F2 generation:*** Densitometric analysis of India ink-stained slot-blots for liver, SC and PBL of AEBN-exposed F2 mice (Figs. 3.22 A, C & E; panel II) and blots for liver immunoprobed with anti-actin antibody (Fig. 3.22 A.; panel A-III) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein.

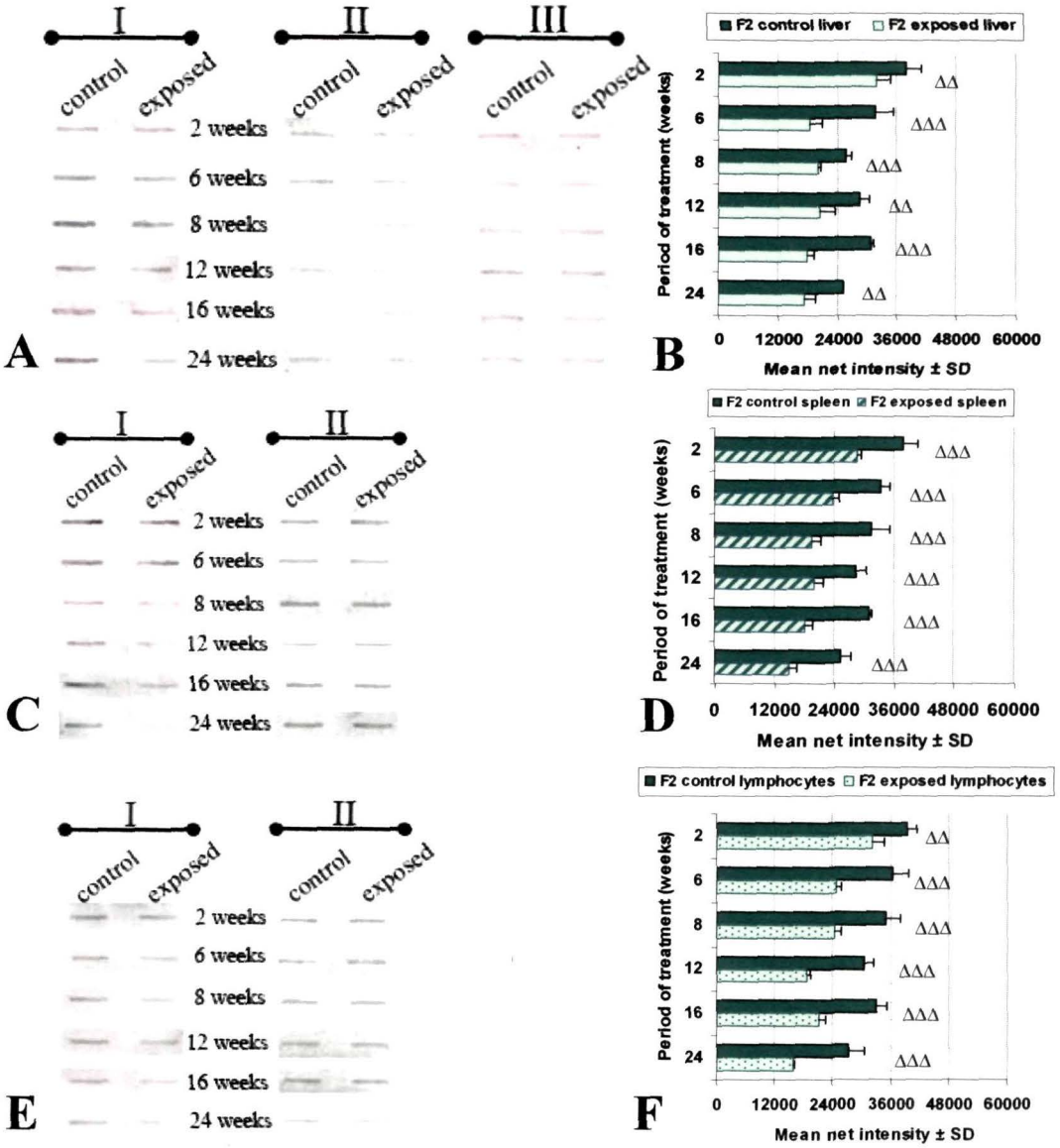


Figure 3.22. Slot-blot analysis for alterations in level of Brca2 protein in **A**-liver, **C**- SC and **E**-PBL of F2 generation AEBN exposed mice. panel **I** -slot-blots immunoprobed with anti-p53 antibody for p53 protein, panel **II**- slot-blots stained with India ink for total protein and panel **III**- slot-blots immunoprobed with anti-actin antibody, serving as loading controls; **B, D & F**- Densitometric plots (mean ± SD) of the level of p53 protein expression in liver, SC and BL respectively as obtained by densitometric analysis of the immunoprobed slot-blots (A-I & III, C-I & D-I) after normalization for equal protein loading (A-II, C-II & D-II ΔΔ indicates significant decrease at $P < 0.01$ and ΔΔΔ indicates significant decrease at $P < 0.001$.

The level of Brca2 protein in liver of AEBN exposed mice was 87 % that of age-matched control level after 2 weeks of exposure, and rapidly declined to 65 % that of age-matched control level after 6 weeks of exposure (Fig. 3.22 A & B), concomitant with appearance of preneoplastic nodules in the liver (Fig. 3.1 i). In SC, the level of Brca2 protein was 76 % that of age-matched control level after 2 weeks of exposure, and rapidly declined to 70 % that of age-matched control level after 6 weeks of exposure (Fig. 3.22 C & D). Similarly, and in PBL the level of Brca2 protein was 86 % that of age-matched control level after 2 weeks of exposure, and rapidly declined to 78 % that of age-matched control level after 6 weeks of exposure (Fig. 3.22 E & F).

3.2.2.3.3. ***F3 generation:*** Densitometric analysis of India ink-stained slot-blots for liver, SC and PBL of AEBN-exposed F3 mice (Figs. 3.23 A, C & E; panel II) and blots for liver immunoprobed with anti-actin antibody (Fig. 3.23 A.; panel A-III) did not show significant differences in net intensity of control and AEBN exposed samples, thus confirming loading of equal amounts of protein. The level of Brca2 protein in liver of AEBN exposed mice was 82 % that of age-matched control level after 2 weeks of exposure, and rapidly declined to 68 % that of age-matched control level after 6 weeks of exposure (Fig. 3.23 A & B), following the appearance of preneoplastic nodules in the liver after 4 weeks of exposure (Fig. 3.1 k). Similarly, in SC, the level of Brca1 protein was 83 % that of age-matched control level after 2 weeks of exposure, and rapidly declined to 62 % that of age-matched control level after 6 weeks of exposure (Fig. 3.23 C & D). In PBL the level of Brca2 protein was 74 % that of age-matched control level after 2 weeks of exposure, and and rapidly decline to 58 % that of age-matched control level after 8 weeks of exposure, to AEBN (Fig. 3.23 E & F).

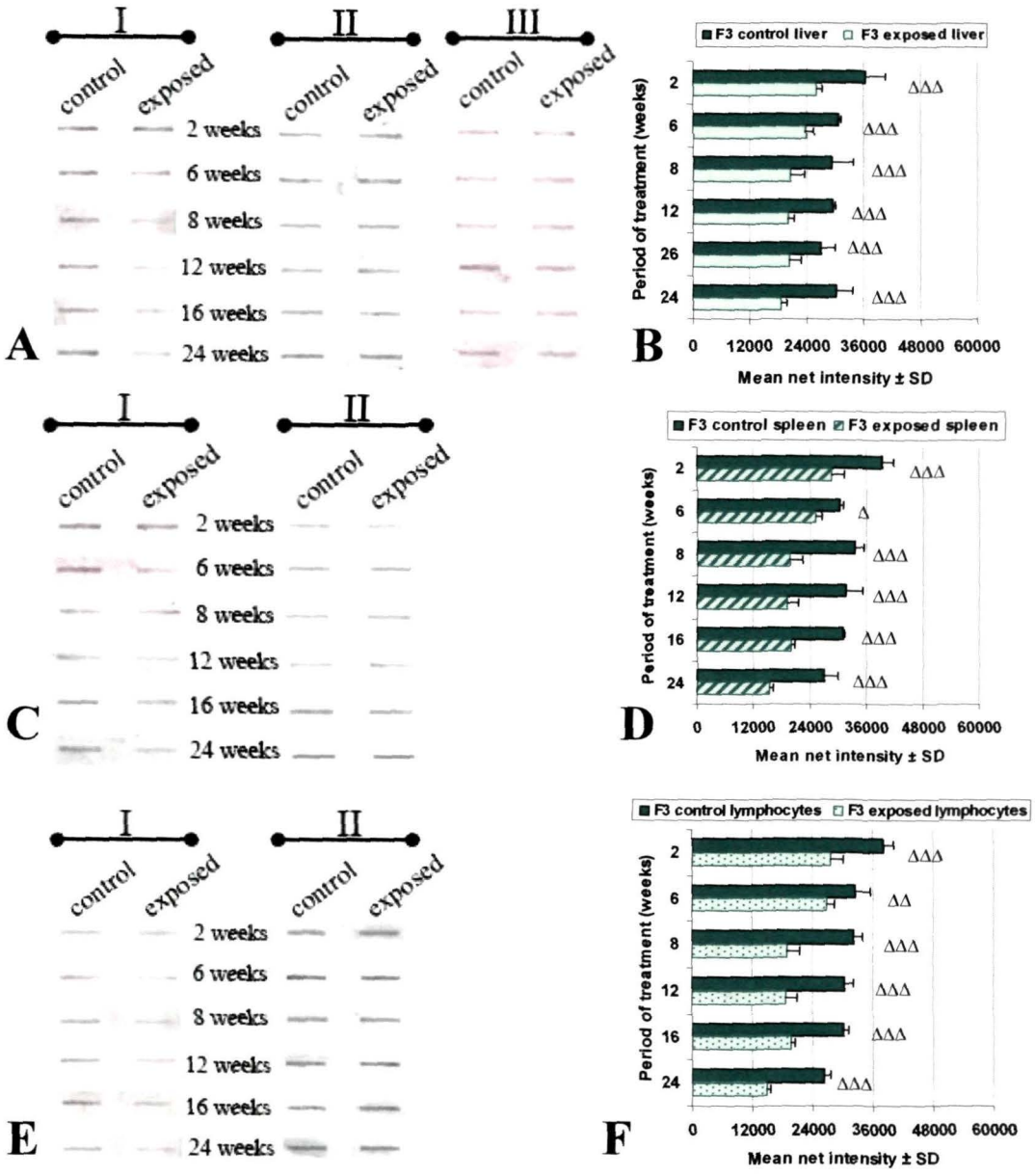


Figure 3.23. Slot-blot analysis for alterations in level of Brca2 protein in **A**-liver, **C**- SC and **E**-PBL of F3 generation AEBN exposed mice. panel **I** -slot-blots immunoprobed with anti-p53 antibody for p53 protein, panel **II**- slot-blots stained with India ink for total protein and panel **III**- slot-blots immunoprobed with anti-actin antibody, serving as loading controls; **B, D & F**- Densitometric plots (mean ± SD) of the level of p53 protein expression in liver, SC and BL respectively as obtained by densitometric analysis of the immunoprobed slot-blots (A-I, C-I & D-I) after normalization for equal protein loading (A-II & III, C-II & D-II). ΔΔΔ indicates significant decrease at $P < 0.001$.

3.3. COMPARISON OF EXPRESSION OF P53, BRCA1 AND BRCA2 PROTEINS UPON CHRONIC AND TRANSGENERATIONAL EXPOSURE OF MICE TO AEBN

Based on the results obtained by slot-blot analysis (§ 3.2.), a comparison was made between the relative expression of the p53, Brca1 and Brca2 proteins in the chronically exposed P generation mice, and the different generations of transgenerationally exposed mice, that is, the F1, F2 and F3 mice. The level of protein in the liver, SC and PBL of AEBN-exposed mice was expressed in terms of percent of the age-matched control level. The observations made were as follows.

3.3.1. p53 tumor suppressor protein

The exposure of P generation mice to AEBN was found to induce a significant elevation in the level of p53 protein in the liver, SC and PBL to 2-2.5 folds the age-matched control level immediately after exposure to AEBN, that is, within 4-6 weeks (Fig. 3.24 A, B & C). Subsequently, the level of p53 protein declined, reaching age-matched control level after 16 weeks of exposure, concomitant with the appearance of pre-neoplastic nodules in the liver (Fig. 3.1 c & d). However, in the transgenerationally exposed mice, the p53 protein remained largely invariant from the control level throughout the period of exposure of AEBN, in the liver, SC as well as PBL (Fig. 3.24 A, B & C). Thus, there was a marked difference in the p53 response in transgenerationally exposed mice, in comparison to the chronically exposed mice.

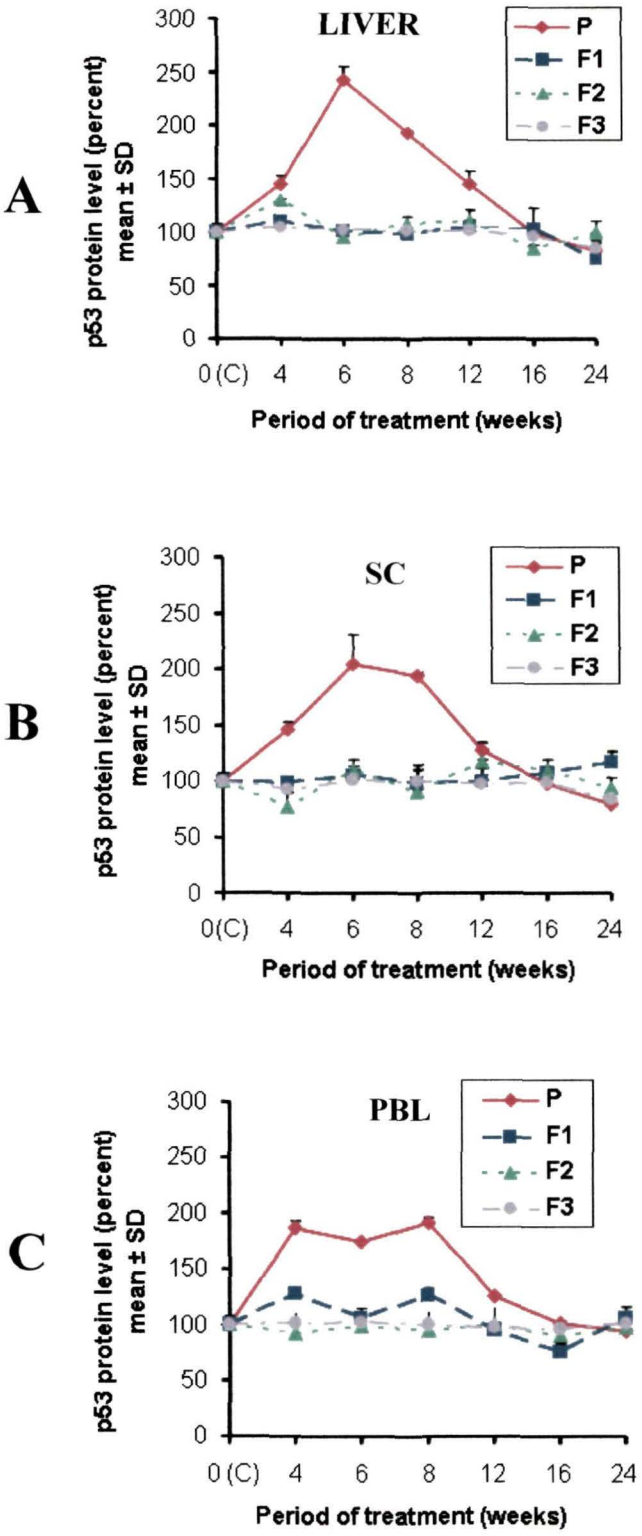


Figure 3.24. Graphical representation of expression of p53 protein in P, F1, F2, and F3 generation mice after exposure to AEBN (% age-matched control; mean ± SD) as determined by slot-blot analysis. **A**-liver, **B**-SC and **C**-PBL

3.3.2. Brca1 tumor suppressor protein

Exposure of P generation mice to AEBN resulted in immediate elevation in level of Brca1 protein, followed by decline to 70 ± 5 % of age-matched control level after 16 weeks of exposure in the liver, SC and PBL (Fig. 3.25 A, B & C), concomitant with the appearance of preneoplastic nodules of the liver (Fig. 3.1 c & d).

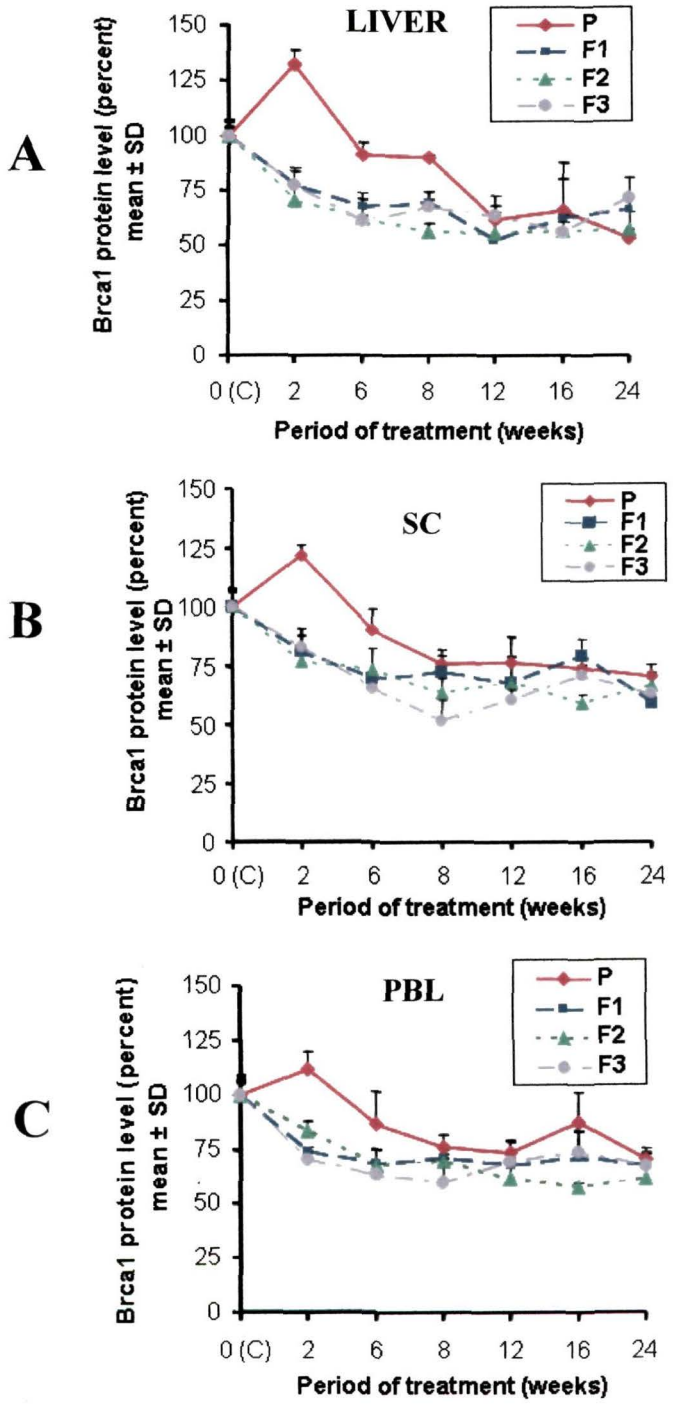
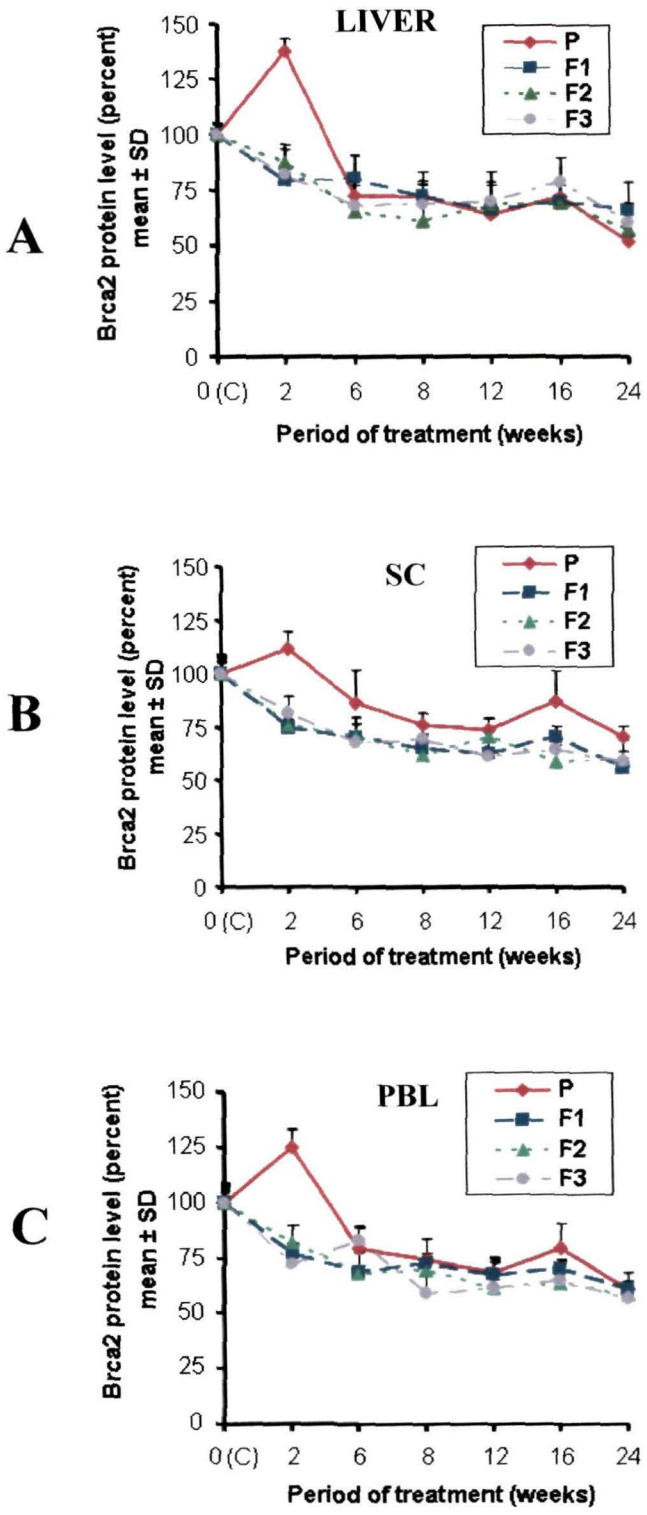


Figure 3.25. Graphical representation of expression of Brca1 protein in P, F1, F2, and F3 generation mice after exposure to AEBN (% age-matched control; mean ± SD) as determined by slot-blot analysis. **A**-liver, **B**-SC and **C**-PBL

However, the level of Brca1 in F1, F2 and F3 generations declined to 77 ± 5 % below age-matched control level after 2 weeks of exposure, followed by rapid decline to 65 ± 5 % below age-matched control level after 8 weeks of exposure in F1 and 6 weeks of exposure in F2 and F3 mice, concomitant with appearance of preneoplastic nodules after 8 weeks of exposure in F1 (Fig. 3.1 g & h), 6 weeks of exposure in F2 (Fig. 3.1 i & j) and 4 weeks of exposure in F3 mice (Fig. 3.1 k & l) respectively.

3.3.3. Brca2 tumor suppressor protein:

Exposure of P generation mice to AEBN resulted in elevation in level of Brca2 protein, followed by decline to 70 % that of age-matched control level after 16 weeks of exposure (Fig. 3.26 A, B & C), concomitant with the appearance of preneoplastic nodules of the liver (Fig. 3.1 c & d). However, the level of Brca1 in F1, F2 and F3 generations declined to 80 ± 5 % below age-matched control level after 2 weeks of exposure, followed by rapid decline to 65 ± 5 % below age-matched control level after 8 weeks of exposure in F1 mice and 6 weeks of exposure in F2 and F3 mice (Fig. 3.26 A, B & C), concomitant with appearance of preneoplastic nodules after 8 weeks of exposure in F1 (Fig. 3.1 g & h), 6 weeks of exposure in F2 (Fig. 3.1 i & j) and 4 weeks of exposure in F3 mice (Fig. 3.1 k & l) respectively.



'igure 3.26. Graphical representation of expression of Brca2 protein in P, F1, F2, and F3 generation nice after exposure to AEBN (% age-matched control; mean ± SD) as determined by *slot-blot* nalysis. **A**-liver, **B**-SC and **C**-PBL

3.4. WESTERN BLOT ANALYSIS OF EXPRESSION OF P53, BRCA1 AND BRCA2 PROTEINS UPON EXPOSURE OF MICE TO AEBN

Western Blot analysis was performed inorder to verify the results obtained by slot-blot analysis of expression of p53, Brca1 and Brca2 proteins upon chronic and transgenerational exposure of mice to AEBN.

3.4.1. p53 tumor suppressor protein:

As observed by slot-blot analysis (§ 3.2), Western blot analysis confirmed that exposure of P generation mice to AEBN resulted in rapid upregulation of level of p53 protein in the liver in comparison to age-matched control level after 6-8 weeks of exposure, followed by decline to, or below age-matched control after 16 weeks of exposure (Fig. 3.27). In contrast, transgenerational exposure of mice to AEBN failed to induce an elevation of p53 protein level, and the level of p53 protein remained at, or below control level for F1, F2 and F3 generations. Loading of equal amounts of protein for all samples was verified by staining replica blots with India ink, for total protein and immunoprobing with anti-actin antibody (Fig. 3.27)

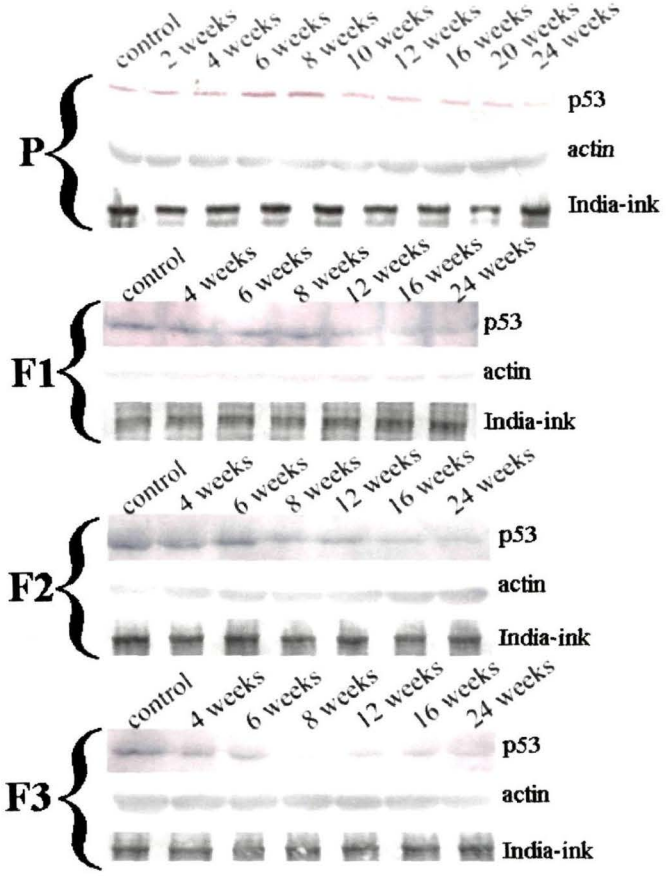


Figure 3.27. Western blot analysis for alterations in level of p53 protein in the liver of P, F1, F2 and F3 mice after exposure to AEBN.

Western blot analysis also confirmed that the p53 response in the SC (Fig. 3.28) and PBL (Fig. 3.29) of P, F1, F2 and F3 mice mirrored the responses observed in the liver. Loading of equal amounts of protein for all samples was verified by staining replica blots with India ink (Figs. 3.28 & 3.29).

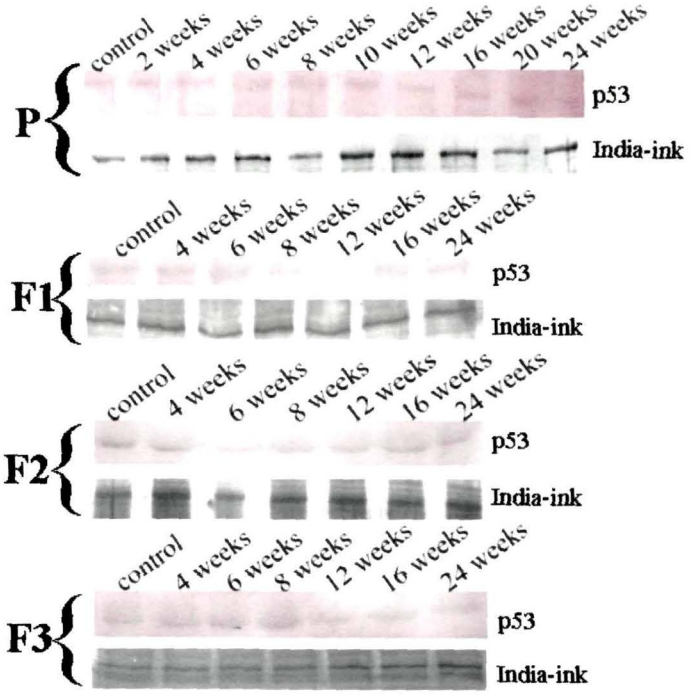


Figure 3.28. Western blot analysis for alterations in level of p53 protein in the SC of P, F1, F2 and F3 mice after exposure to AEBN.

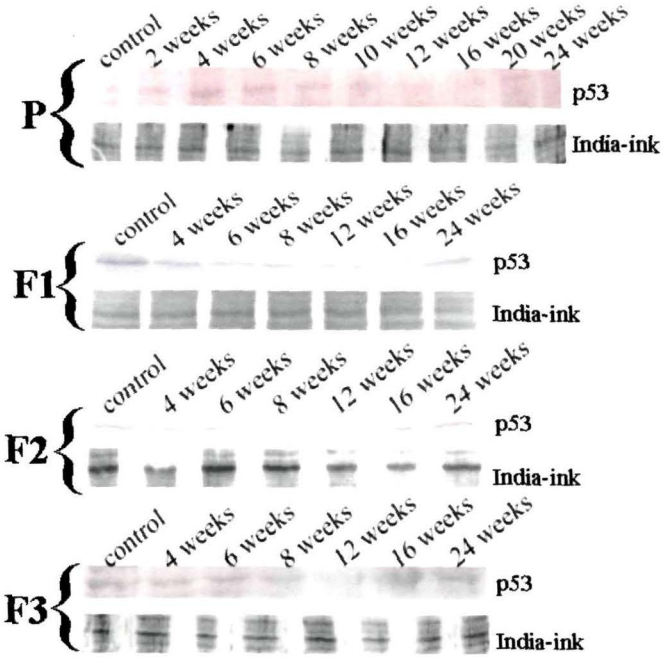


Figure 3.29. Western blot analysis for alterations in level of p53 protein in the PBL of P, F1, F2 and F3 mice after exposure to AEBN.

3.4.2. Brca1 tumor suppressor protein:

As observed by slot-blot analysis (§ 3.2.1.2. & § 3.2.2.2), Western blot analysis confirmed that exposure of P generation mice to AEBN resulted in rapid upregulation of level of Brca1 protein in the liver in comparison to age-matched control level after 2 weeks of exposure, followed by decline below age-matched control level, reaching 70 % control level after 16 weeks of exposure (Fig. 3.30). In contrast, transgenerational exposure of mice to AEBN resulted in a decline in level of Brca1 protein below control level after 2 weeks of exposure onwards, followed by a rapid decline to 70 % that of control level after 6-8 weeks of exposure, unlike in P generation mice (Fig. 3.30). Loading of equal amounts of protein for all samples was verified by staining replica blots with India ink, for total protein and immunoprobing with anti-actin antibody (Fig. 3.30)

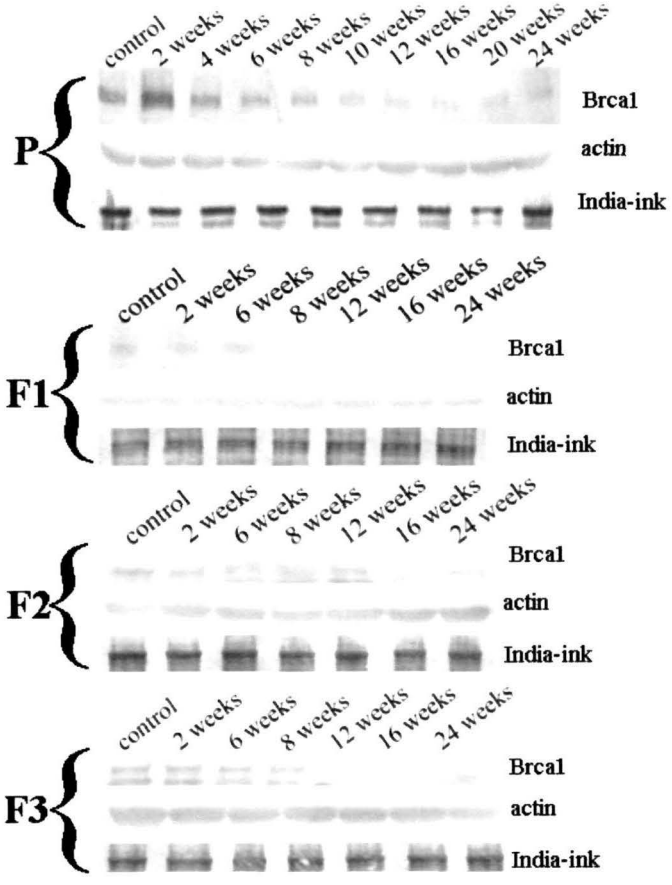


Figure 3.30. Western blot analysis for alterations in level of Brca1 protein in the liver of P, F1, F2 and F3 mice after exposure to AEBN.

Western blot analysis also confirmed that the p53 response in the SC (Fig. 3.31) and PBL (Fig. 3.32) of P, F1, F2 and F3 mice mirrored the responses observed in

the liver. Loading of equal amounts of protein for all samples was verified by staining replica blots with India ink (Figs. 3.31 & 3.32).

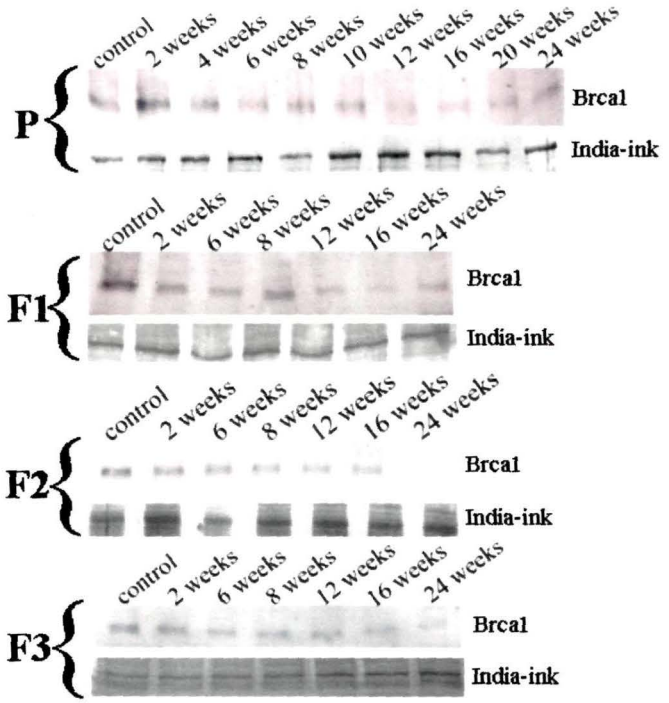


Figure 3.31. Western blot analysis for alterations in level of Brca1 protein in the SC of P, F1, F2 and F3 mice after exposure to AEBN.

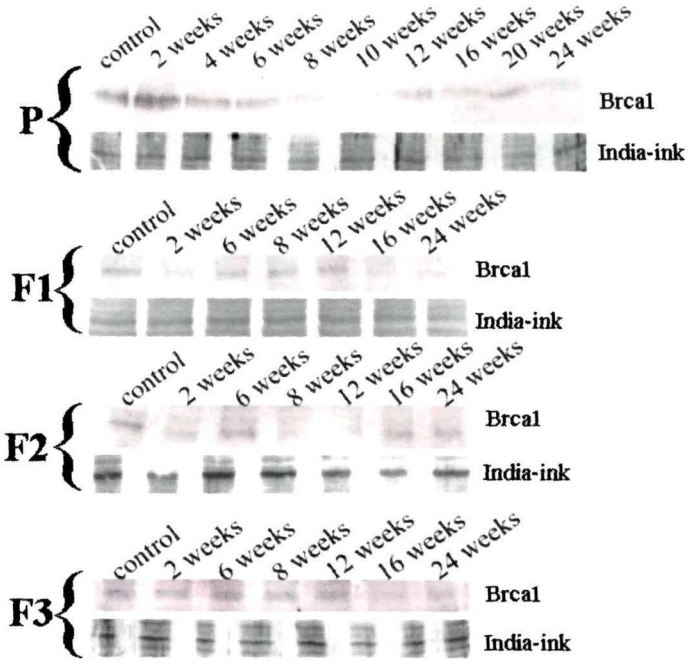


Figure 3.32. Western blot analysis for alterations in level of Brca1 protein in the PBL of P, F1, F2 and F3 mice after exposure to AEBN.

3.4.3. Brca2 tumor suppressor protein:

As observed by slot-blot analysis (§ 3.2.1.3. & § 3.2.2.3), Western blot analysis confirmed that exposure of P generation mice to AEBN resulted in rapid upregulation of level of Brca2 protein in the liver in comparison to age-matched control level after 2 weeks of exposure, followed by decline below age-matched control level, reaching approxiamtely 70 % control level after 16 weeks of exposure (Fig. 3.33). In contrast, transgenerational exposure of mice to AEBN resulted in a decline in level of Brca2 protein below control level after 2 weeks of exposure onwards, followed by a rapid decline to 70 % that of control level after 6-8 weeks of exposure, unlike in P generation mice (Fig. 3.33). Loading of equal amounts of protein for all samples was verified by staining replica blots with India ink, for total protein and immunoprobing with anti-actin antibody (Fig. 3.33).

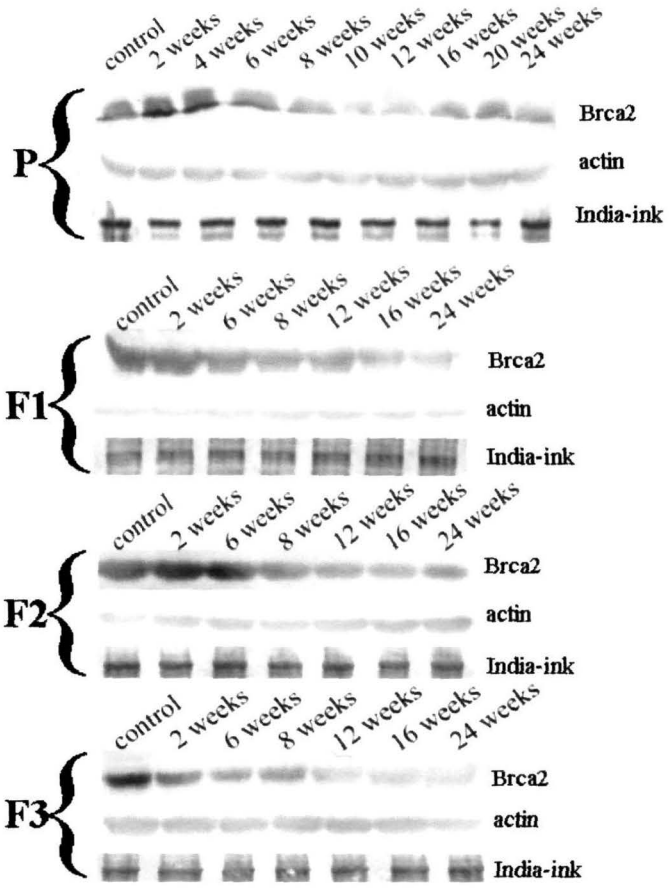


Figure 3.33. Western blot analysis for alterations in level of Brca2 protein in the liver of P, F1, F2 and F3 mice after exposure to AEBN.

Western blot analysis also confirmed that the p53 response in the SC (Fig. 3.31) and PBL (Fig. 3.32) of P, F1, F2 and F3 mice mirrored the responses observed in

the liver. Loading of equal amounts of protein for all samples was verified by staining replica blots with India ink (Figs. 3.34 & 3.35).

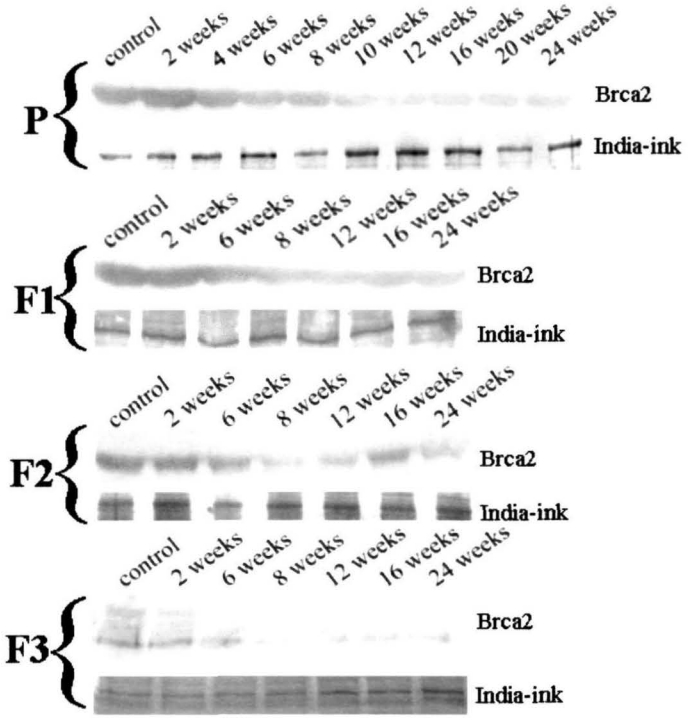


Figure 3.34. Western blot analysis for alterations in level of Brca1 protein in the SC of P, F1, F2 and F3 mice after exposure to AEBN.

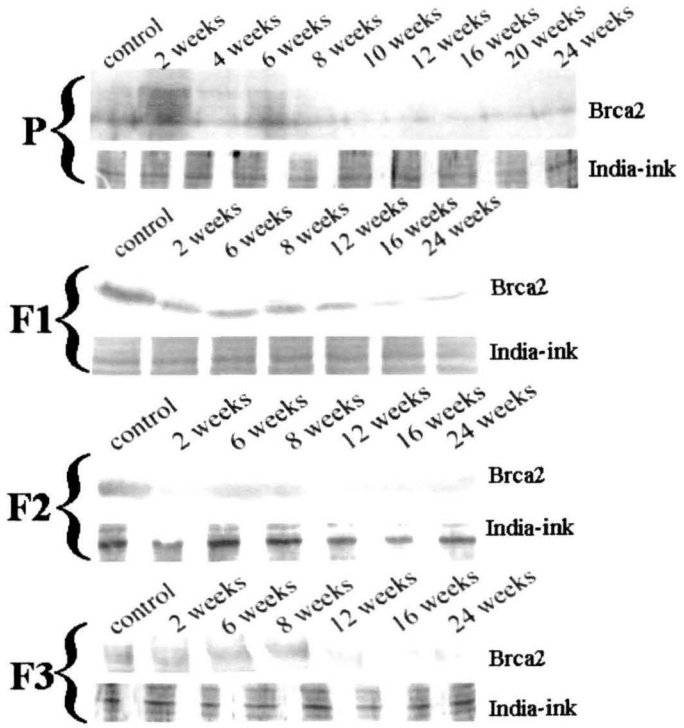


Figure 3.35. Western blot analysis for alterations in level of Brca1 protein in the PBL of P, F1, F2 and F3 mice after exposure to AEBN.

3.5. PCR-AMPLIFICATION AND DIRECT DNA SEQUENCING OF SELECTED REGIONS OF TUMOR SUPPRESSOR GENES

Selected regions of the mouse p53, Brca1 and Brca2 genes were amplified from DNA isolated from liver nodules of mice exposed to AEBN chronically and transgenerationally for 24 weeks, as well as from solid tumors developing in F1 and F3 mice. The regions were also amplified from DNA isolated from corresponding regions of liver of age-matched control mice, which served as controls for this experiment. Following amplification, the amplicons were sequenced by direct DNA sequencing. DNA sequencing was performed for each sample using the forward and reverse primers independently. A nucleotide change was considered to be significant only when complementary changes were observed in corresponding postions of a sample following sequencing by both primers.

3.5.1. p53 tumor suppressor gene

3.5.1.1. Exon 5: Fig. 3.36 shows the electropherogram of the amplified 262 bp fragment obtained by amplifying exon 5 of the mouse p53 gene.

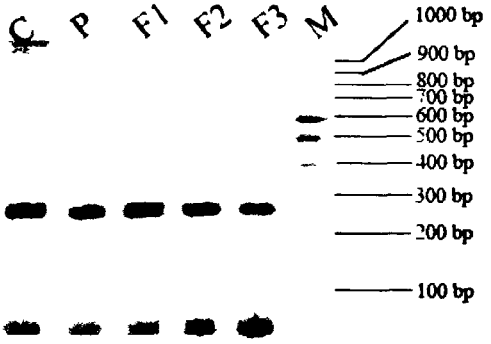


Figure 3.36. Electropherogram of the amplicons of exon 5 of the mouse p53 gene. Lanes 1, 2, 3, 4 and 5 are the profiles of amplicons obtained from unexposed control (C), exposed P generation, exposed F1 generation, exposed F2 generation and exposed F3 generation samples, respectively. Lane M is the DNA molecular size marker (100 bp DNA ladder).

Upon analysis of the electropherogram by KDS1 software, no significant difference was observed in size of the amplicons of exposed samples in comparison to control. Direct DNA sequencing of the amplicons (Genbank accession # EF570972, Appendix I), followed by alignment and analysis of the sequences using Multalin software revealed no significant changes in the sequence of amplicons of exposed samples in comparison to control (Fig. 3.37).

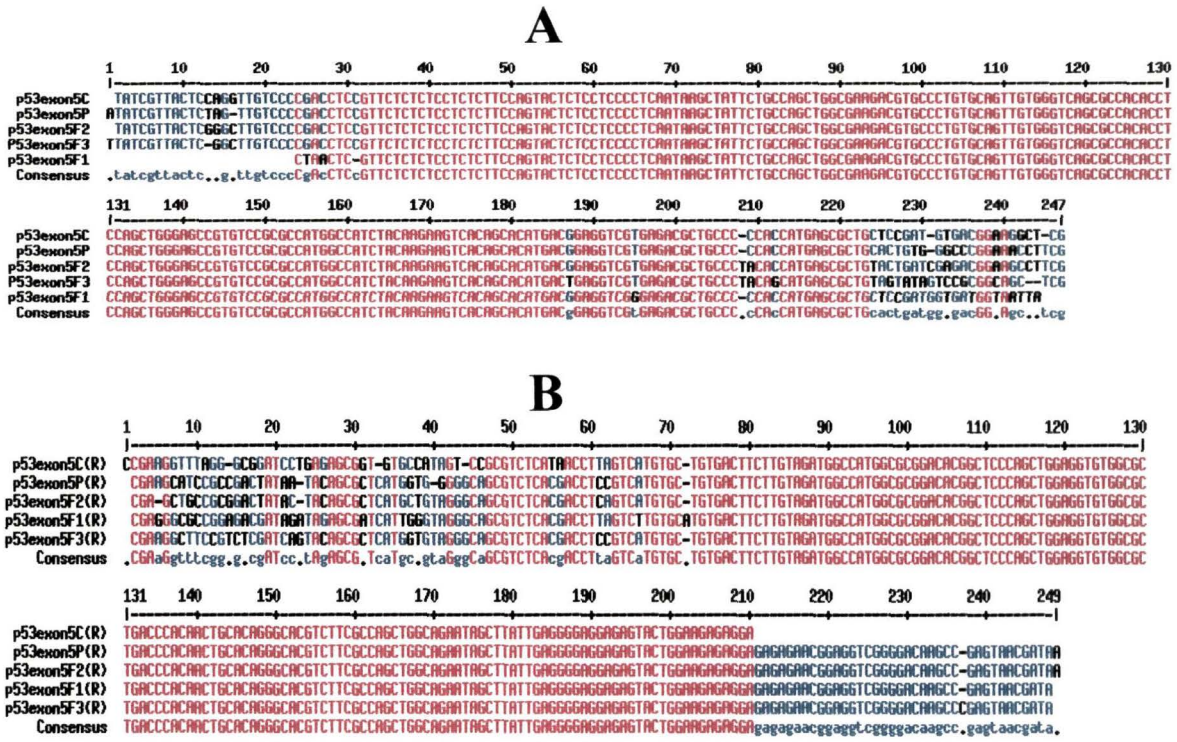


Figure 3.37. Alignment of sequences obtained after direct DNA sequencing of amplicons of exon 5 of the mouse p53 gene with (**A**) forward primer and (**B**) reverse primer.

In the obtained alignment (Fig. 3.37), the nucleotide changes observed towards the beginning and end of the sequences (blue) were not considered significant as they could be the result of errors in sequencing. Nucleotide changes were also observed in position 187 in the amplicon from F3 sample and position 195 in the sample from F1 sample, but the complementary changes were not observed in the corresponding positions when the samples were sequenced with the reverse primer. Hence, these nucleotide changes were not considered significant. Thus it can be concluded that chronic as well as transgenerational exposure of mice to AEBN did not induce mutation in exon 5 of the mouse p53 gene.

3.5.1.2. Exon 7: Fig. 3.38 shows the electropherogram of the amplified 202 bp fragment obtained by amplifying exon 7 of the mouse p53 gene.

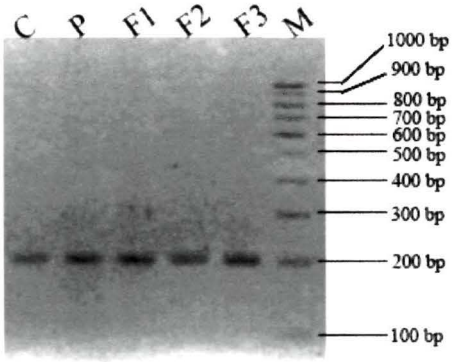


Figure 3.38. Electropherogram of the amplicons of exon 7of the mouse p53 gene. Lanes 1, 2, 3, 4 and 5 are the profiles of amplicons obtained from unexposed control (C), exposed P generation, exposed F1 generation, exposed F2 generation and exposed F3 generation samples, respectively. Lane M is the DNA molecular size marker (100 bp DNA ladder).

Upon analysis of the electropherogram by KDS1 software, no significant difference was observed in size of the amplicons of exposed samples in comparison to control. Direct DNA sequencing of the amplicons (Genbank accession # EF634061, Appendix II), followed by alignment and analysis of the sequences using Multalin software revealed no significant changes in the sequence of amplicons of exposed samples in comparison to control (Fig. 3.39).

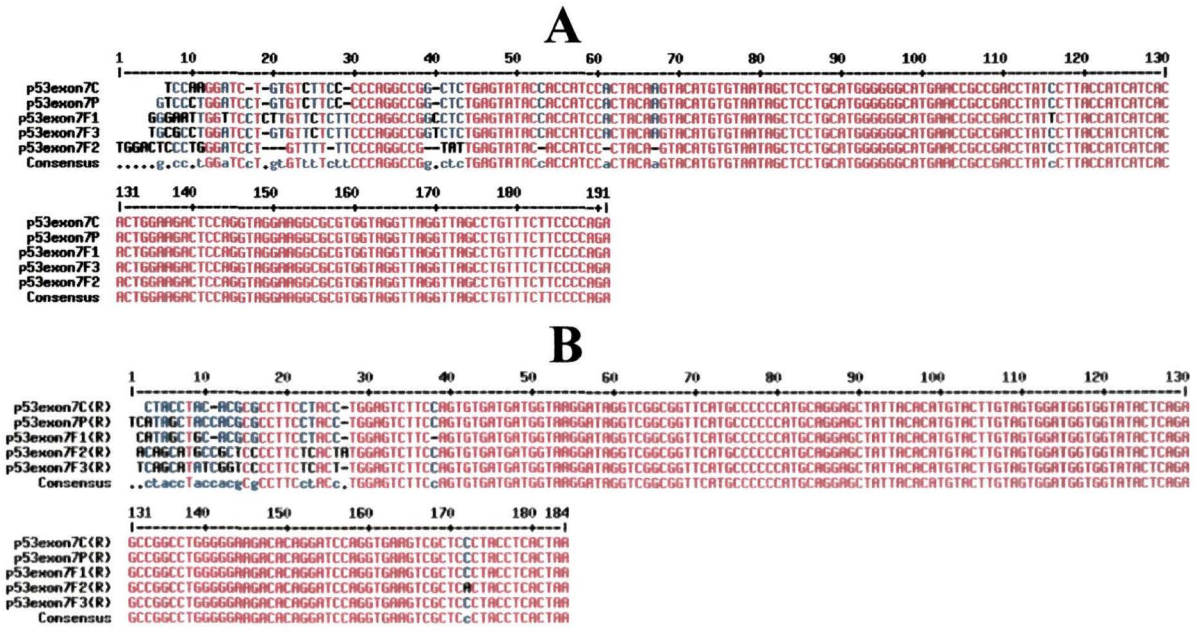


Figure 3.39. Alignment of sequences obtained after direct DNA sequencing of amplicons of exon 7 of the mouse p53 gene with (**A**) forward primer and (**B**) reverse primer.

In the obtained alignment (Fig. 3.39), the nucleotide changes observed towards the beginning the sequences (blue) were not considered significant as they could be the result of errors in sequencing. A nucleotide change was also observed in position 116 in the amplicon from F1 sample, but the complementary change was not observed in the corresponding position when the sample was sequenced with the reverse primer. Hence, this nucleotide change was not considered significant. Thus it can be concluded that chronic as well as transgenerational exposure of mice to AEBN did not induce mutation in exon 7 of the mouse p53 gene.

3.5.2. Brca1 tumor suppressor gene

Exon 11 of the mouse *Brca1* gene was selected for this study. The exon is 1386 bp long, and was divided into five overlapping regions which were amplified and sequenced individually in order to ensure accuracy of sequencing (§ 2.32.2.).

3.5.2.1. Region A: Fig. 3.40 shows the electropherogram of the amplified 317 bp fragment obtained by amplifying region A of exon 11 of the mouse *Brca1* gene.

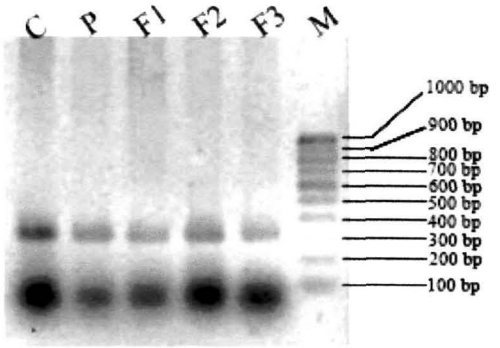


Figure 3.40. Electropherogram of the amplicons of region A of exon 11 of the mouse *Brca1* gene. Lanes 1, 2, 3, 4 and 5 are the profiles of amplicons obtained from unexposed control (C), exposed P generation, exposed F1 generation, exposed F2 generation and exposed F3 generation samples, respectively. Lane M is the DNA molecular size marker (100 bp DNA ladder).

Upon analysis of the electropherogram by KDS1 software, no significant difference was observed in size of the amplicons of exposed samples in comparison to control. Direct DNA sequencing of the amplicons followed by alignment and analysis of the sequences using Multalin software revealed no significant changes in the sequence of amplicons of exposed samples in comparison to control (Fig. 3.41).

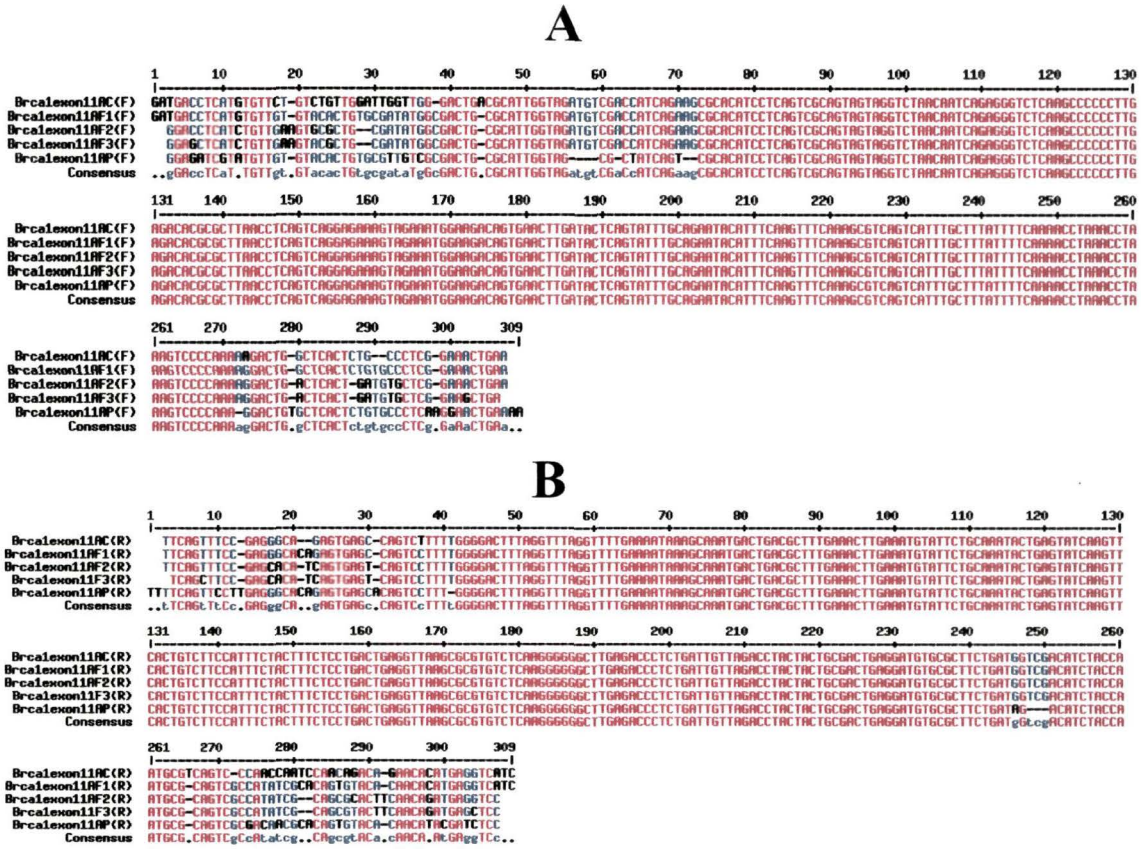


Figure 3.41. Alignment of sequences obtained after direct DNA sequencing of amplicons of region A of exon 11 of the mouse *Brca1* gene with (**A**) forward primer and (**B**) reverse primer.

In the obtained alignment (Fig. 3.41), the nucleotide changes observed towards the beginning and end of the sequences (blue) were not considered significant as they could be the result of errors in sequencing. Nucleotide changes were also observed in positions 56-72 in the amplicon from P sample, but the complementary changes were not observed in the corresponding positions when the sample was sequenced with the reverse primer. Hence, these nucleotide changes were not considered significant. Thus it can be concluded that chronic as well as transgenerational exposure of mice to AEBN did not induce mutation in region A of exon 11 of the mouse *Brca1* gene.

3.5.2.2. Region B: Fig. 3.42 shows the electropherogram of the amplified 330 bp fragment obtained by amplifying region B of exon11 of the mouse *Brca1* gene.

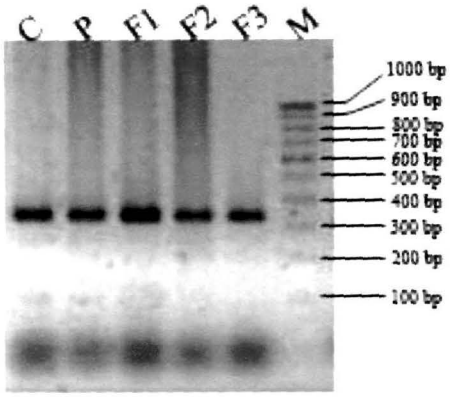


Figure 3.42. Electropherogram of the amplicons of region B of exon 11 of the mouse *Brca1* gene. Lanes 1, 2, 3, 4 and 5 are the profiles of amplicons obtained from unexposed control (C), exposed P generation, exposed F1 generation, exposed F2 generation and exposed F3 generation samples, respectively. Lane M is the DNA molecular size marker (100 bp DNA ladder).

Upon analysis of the electropherogram by KDS1 software, no significant difference was observed in size of the amplicons of exposed samples in comparison to control. Direct DNA sequencing of the amplicons followed by alignment and analysis of the sequences using Multalin software revealed a significant change in the sequence of amplicons of samples F1, F2 and F3 in comparison to control. In the obtained alignment (Fig. 3.43), the nucleotide changes observed towards the beginning and end of the sequences (blue) were not considered significant as they could be the result of errors in sequencing. Nucleotide changes observed in positions 66 and 89 in the amplicons were not considered significant as the complementary changes were not observed in the corresponding positions when the sample was sequenced with the reverse primer. However, the nucleotide change (G→C) at position 180 in transgenerationally exposed samples F1, F2 and F3 in comparison to control C, and chronically exposed sample P was confirmed by a complementary (C→G) change in the corresponding position i.e. at position 134 when the samples was sequenced with the reverse primer (Fig. 3.43 B.).

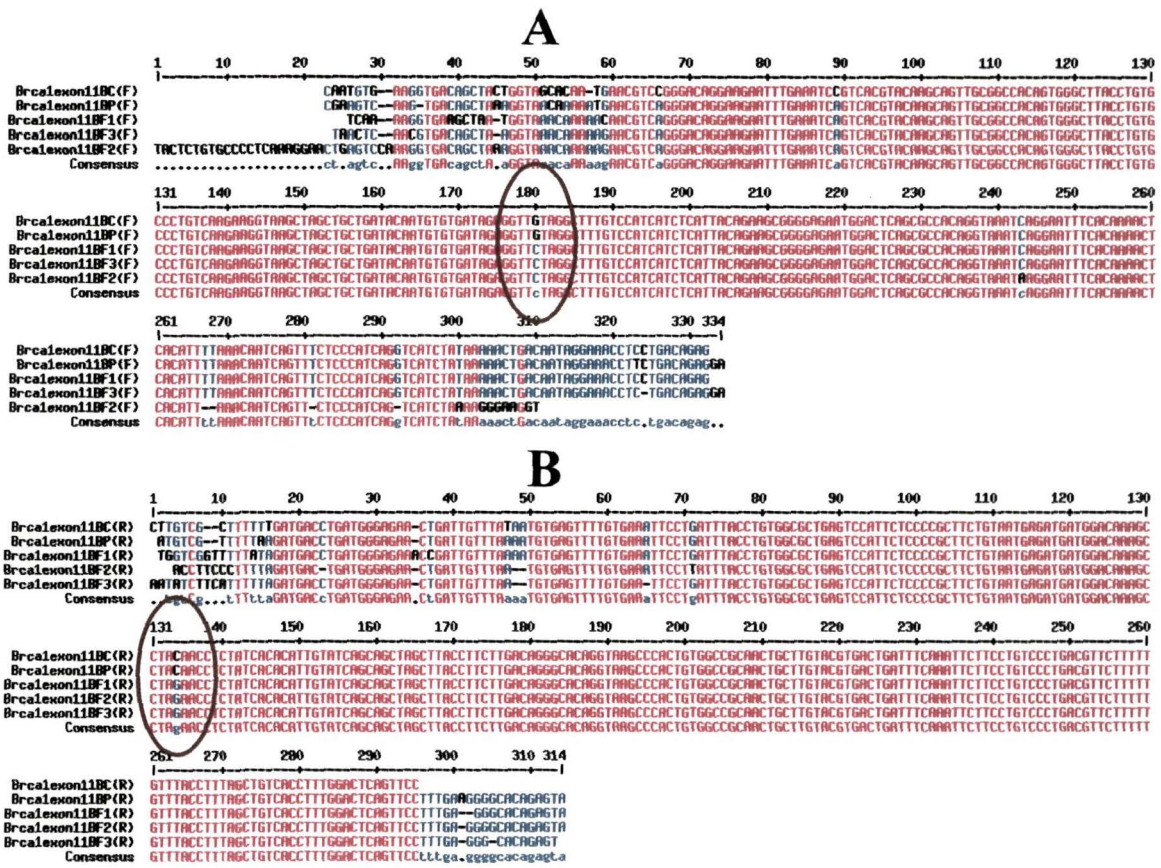


Figure 3.43. Alignment of sequences obtained after direct DNA sequencing of amplicons of region B of exon 11 of the mouse *Brca1* gene, with (A) forward primer and (B) reverse primer showing the G→C mutation in the forward strand and the complementary C→G mutation in the reverse strand (circles)

The consensus nucleotide sequences obtained for control and transgenerationally exposed samples were translated into the corresponding amino acid sequence. The amino acid substitution corresponding to the mutation was found to be the missense mutation cysteine (Cys) → serine (Ser) (TGT→TCT, Fig. 3.44). Thus, the results indicate that transgenerational exposure of mice to AEBN induces mutation in region B of exon 11 of the mouse *Brca1* gene.

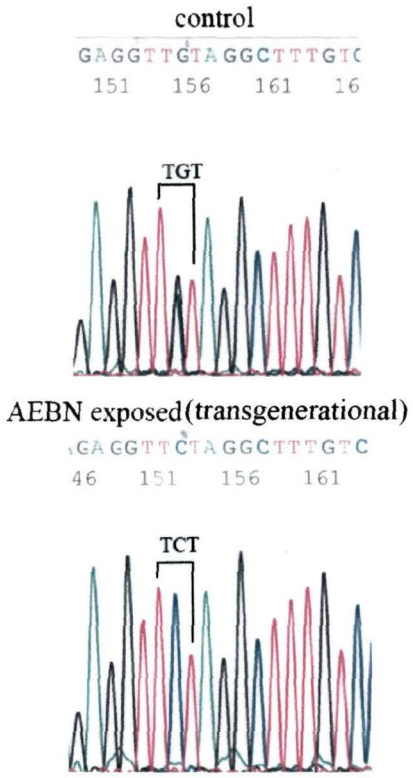


Figure 3.44. Sequencing electropherograms of control and AEBN exposed samples, showing the TGT→TCT mutation in exon 11 of the *Brca1* gene of mice exposed transgenerationally to AEBN. The corresponding amino acid substitution is Cys→Ser.

3.5.2.3. Region C: Fig. 3.45 shows the electropherogram of the amplified 313 bp fragment obtained by amplifying region C of exon11 of the mouse *Brca1* gene.

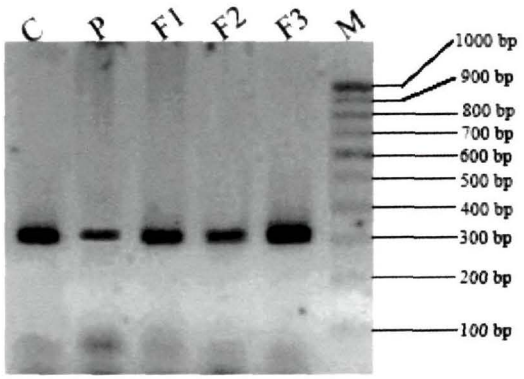


Figure 3.45. Electropherogram of the amplicons of region C of exon 11 of the mouse *Brca1* gene. Lanes 1, 2, 3, 4 and 5 are the profiles of amplicons obtained from unexposed control (C), exposed P generation, exposed F1 generation, exposed F2 generation and exposed F3 generation samples, respectively. Lane M is the DNA molecular size marker (100 bp DNA ladder).

Upon analysis of the electropherogram by KDS1 software, no significant difference was observed in size of the amplicons of exposed samples in comparison to control. Direct DNA sequencing of the amplicons followed by alignment and analysis of

the sequences using Multalin software revealed no significant changes in the sequence of amplicons of exposed samples in comparison to control (Fig. 3.46).

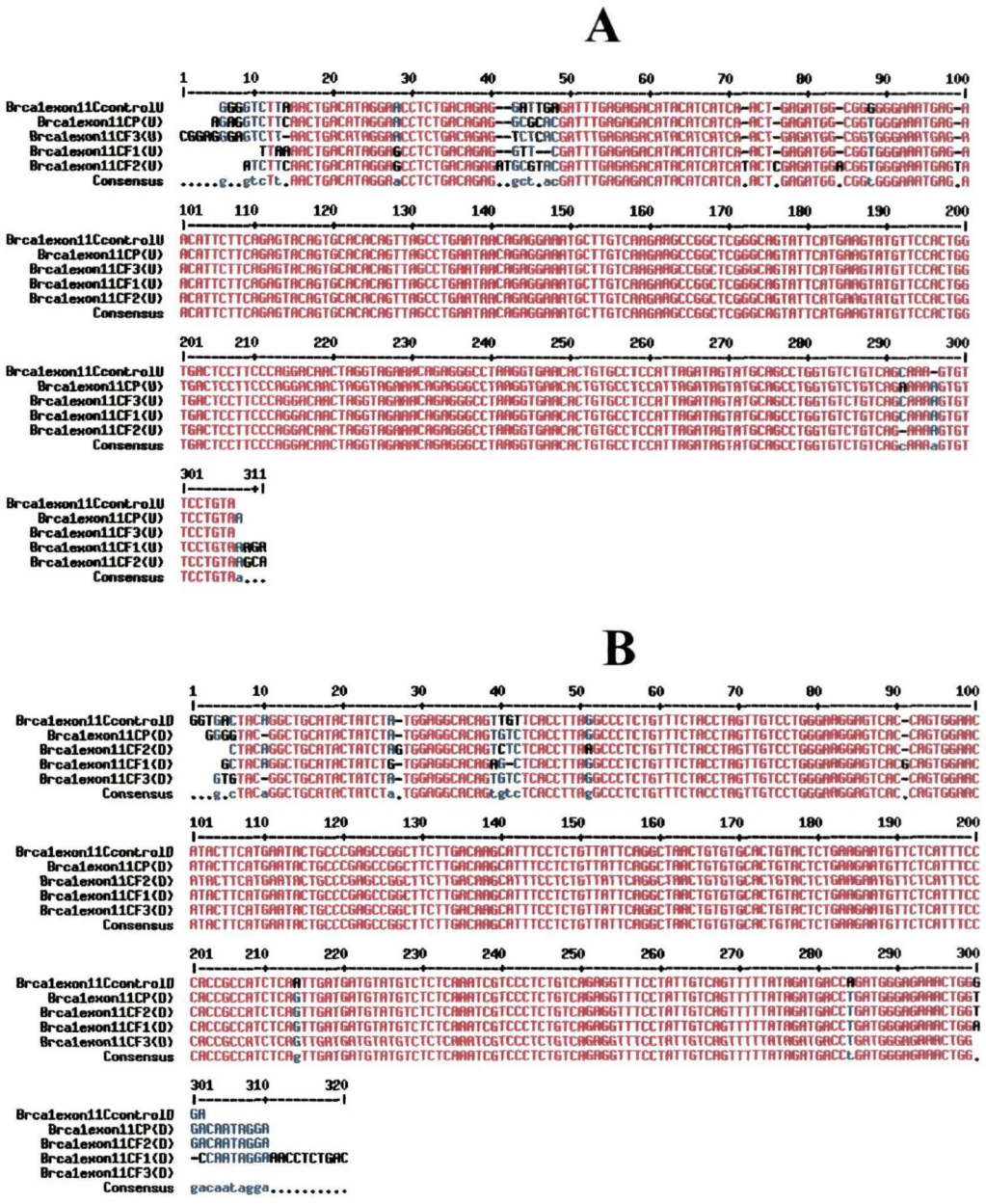


Figure 3.46. Alignment of sequences obtained after direct DNA sequencing of amplicons of region C of exon 11 of the mouse *Brca1* gene, with (A) forward primer and (B) reverse primer.

In the obtained alignment (Fig. 3.46), the nucleotide changes observed towards the beginning and end the sequences (blue) were not considered significant as they could be the result of errors in sequencing. Thus it can be concluded that chronic as well as transgenerational exposure of mice to AEBN did not induce mutation in region C of exon 11 of the mouse *Brca1* gene.

3.5.2.4. Region D: Fig. 3.47 shows the electropherogram of the amplified 367 bp fragment obtained by amplifying region D of exon11 of the mouse *Brca1* gene.

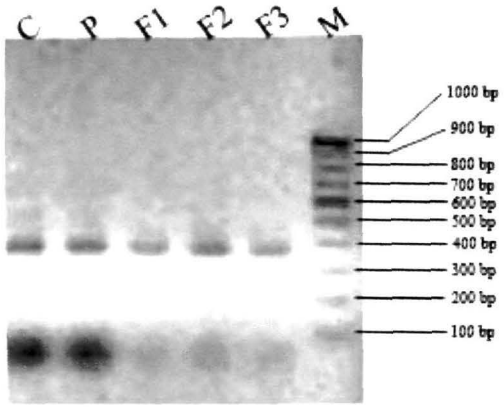


Figure 3.47. Electropherogram of the amplicons of region D of exon 11 of the mouse *Brca1* gene. Lanes 1, 2, 3, 4 and 5 are the profiles of amplicons obtained from unexposed control (C), exposed P generation, exposed F1 generation, exposed F2 generation and exposed F3 generation samples, respectively. Lane M is the DNA molecular size marker (100 bp DNA ladder).

Upon analysis of the electropherogram by KDS1 software, no significant difference was observed in size of the amplicons of exposed samples in comparison to control. Direct DNA sequencing of the amplicons followed by alignment and analysis of the sequences using Multalin software revealed no significant changes in the sequence of amplicons of exposed samples in comparison to control (Fig. 3.48).

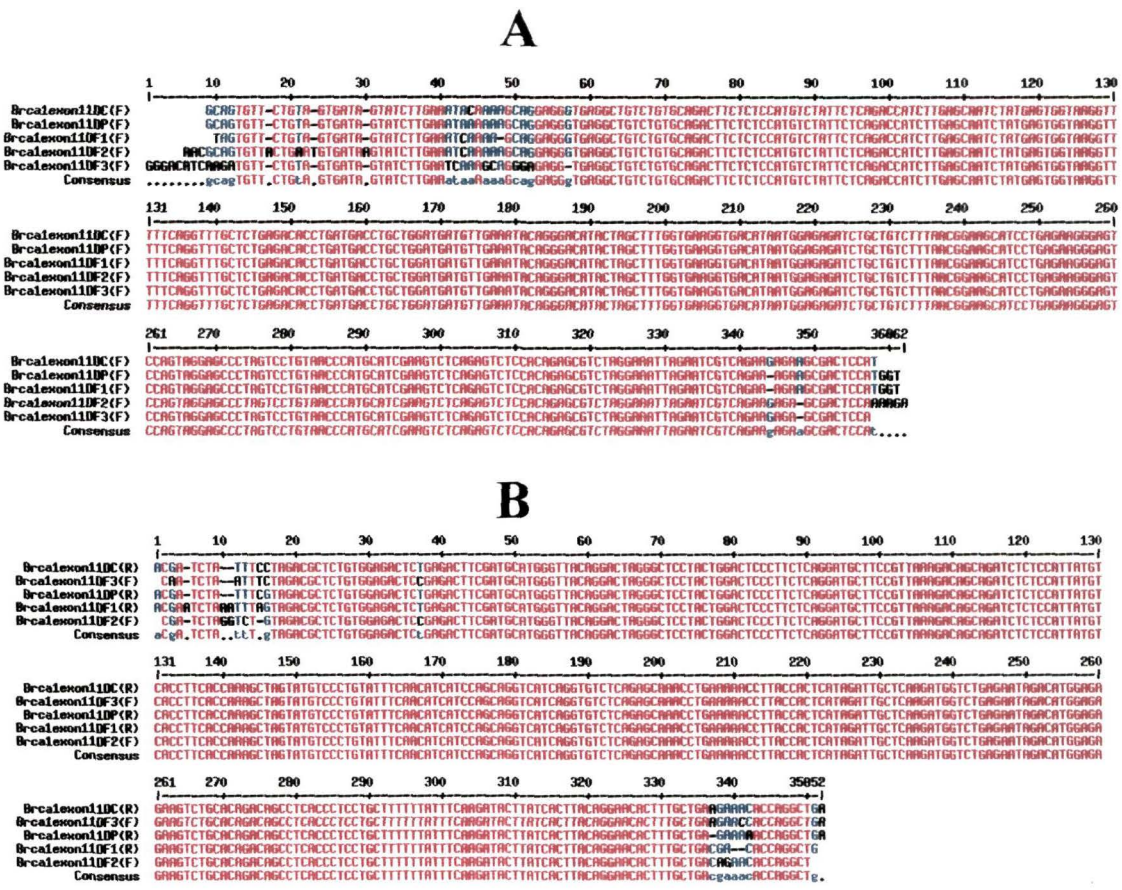


Figure 3.48. Alignment of sequences obtained after direct DNA sequencing of amplicons of region D of exon 11 of the mouse *Brca1* gene, with (**A**) forward primer and (**B**) reverse primer.

In the obtained alignment (Fig. 3.48), the nucleotide changes observed towards the beginning and end the sequences (blue) were not considered significant as they could be the result of errors in sequencing. Thus it can be concluded that chronic as well as transgenerational exposure of mice to AEBN did not induce mutation in region D of exon 11 of the mouse *Brca1* gene.

3.5.2.5. Region E: Fig. 3.49 shows the electropherogram of the amplified 268 bp fragment obtained by amplifying region D of exon11 of the mouse *Brca1* gene.

well as transgenerational exposure of mice to AEBN did not induce mutation in region E of exon 11 of the mouse *Brca1* gene.

Thus, while the nucleotide sequence of exon 11 of the *Brca1* gene the P generation mice was similar to that of control, that is the wild-type sequence (GenBank Accession # FJ497232, Appendix III), a G→C transversion mutation was found to occur in the transgenerationally exposed F1, F2 and F3 mice (GenBank Accession # FJ589202, Appendix IV).

3.5.3. Brca2 tumor suppressor gene

Fig. 3.51 shows the electropherogram of the amplification products obtained by amplifying a segment of exon 27 of the mouse *Brca2* gene. Upon analysis of the electropherogram by KDS1 software, no significant difference was observed in size of the amplicons of exposed samples in comparison to control. The desired 365 bp amplification product was eluted out of the gel and re-amplified, and the re-amplification product was sequenced.

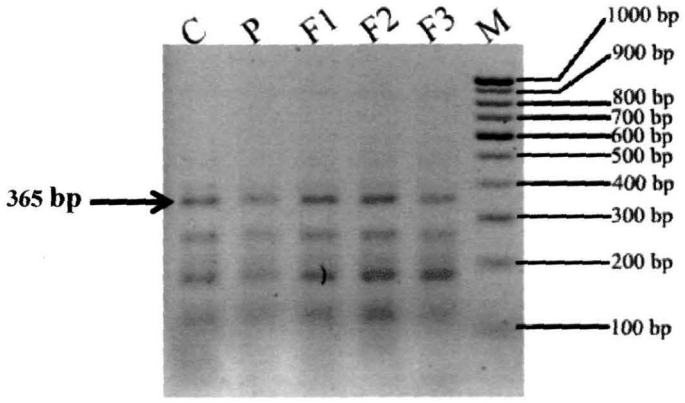


Figure 3.51. Electropherogram of the amplicons of a segment (NT 1-257) of exon 27 of the mouse *Brca2* gene. Lanes 1, 2, 3, 4 and 5 are the profiles of amplicons obtained from unexposed control (C), exposed P generation, exposed F1 generation, exposed F2 generation and exposed F3 generation samples, respectively. Lane M is the DNA molecular size marker (100 bp DNA ladder).

Direct DNA sequencing of the amplicons (Genbank accession # FJ825143) followed by alignment and analysis of the sequences using Multalin software revealed no significant changes in the sequence of amplicons of exposed samples in comparison to control (Fig. 3.52).

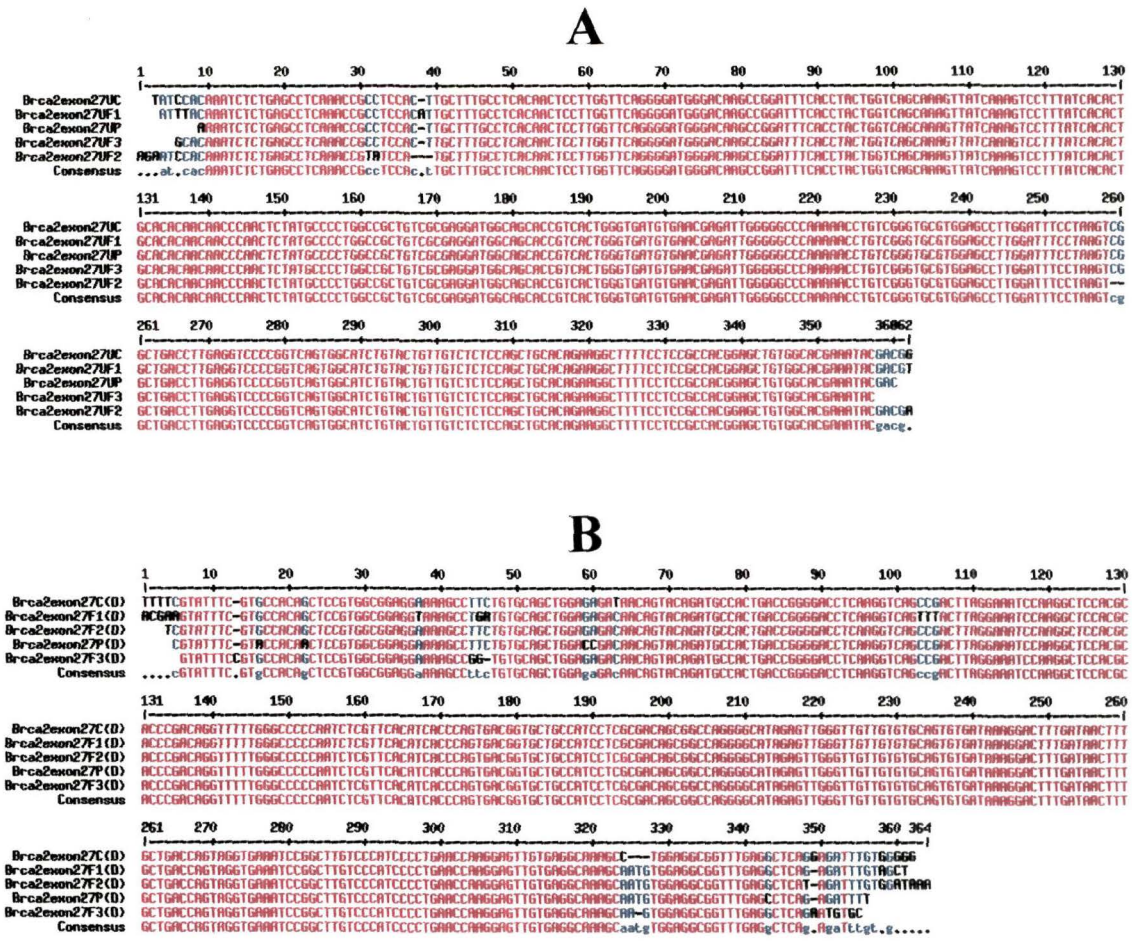


Figure 3.52. Alignment of sequences obtained after direct DNA sequencing of amplicons of a segment (NT 1-257) of exon 27 of the mouse *Brca2* gene, with (**A**) forward primer and (**B**) reverse primer.

In the obtained alignment (Fig. 3.52), the nucleotide changes observed towards the beginning and end the sequences (blue) were not considered significant as they could be the result of errors in sequencing. Nucleotide changes were also observed in positions 259 and 260 in the amplicon from F2 sample, but the complementary changes were not observed in the corresponding positions when the sample was sequenced with the reverse primer. Hence, these nucleotide changes were not considered significant. Thus it can be concluded that chronic as well as transgenerational exposure of mice to AEBN did not induce mutation in the amplified region of the mouse *Brca2* gene.

However, in rotamer 2, the bond length was found to be altered from 2.19 Å in the wild-type protein to 2.34 Å in the mutant protein (Fig. 3.55).

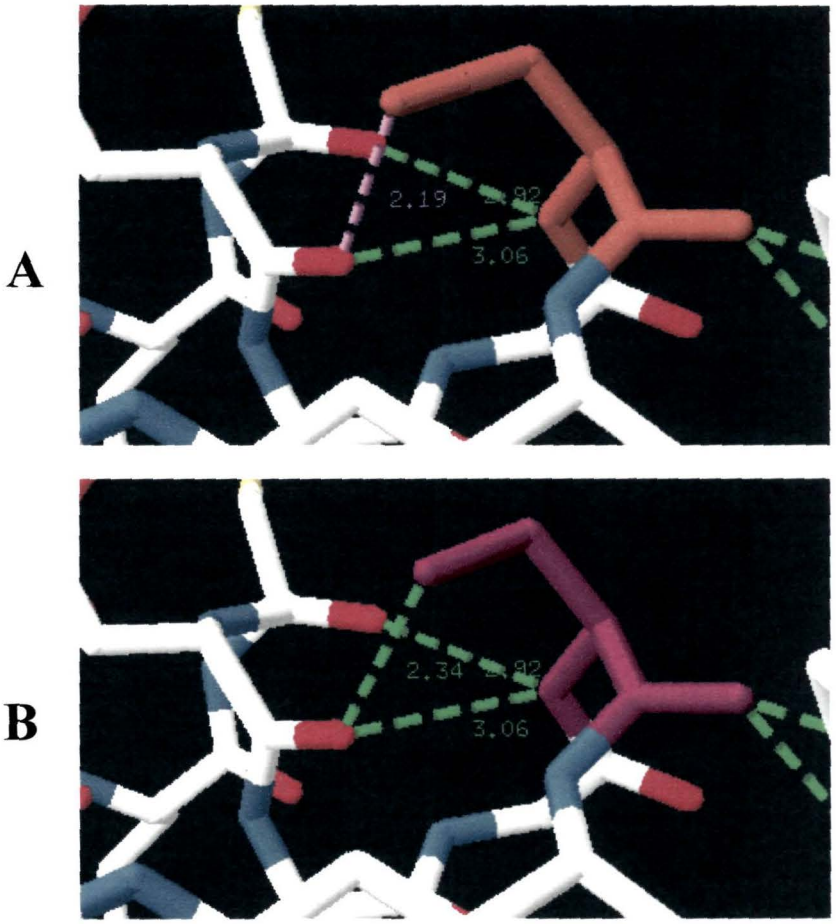


Figure 3.55. Difference of H-bond length between –SH group of Cys 41 in wild-type (**A**) and –OH group of Ser 41 in mutant (**B**) with the backbone oxygen of Asp 38 in rotamer 2.

The bond angle in rotamer 2 was also found to be altered from 66.07° in the wild-type protein to 70.70° in the mutant protein (Fig. 3.56).

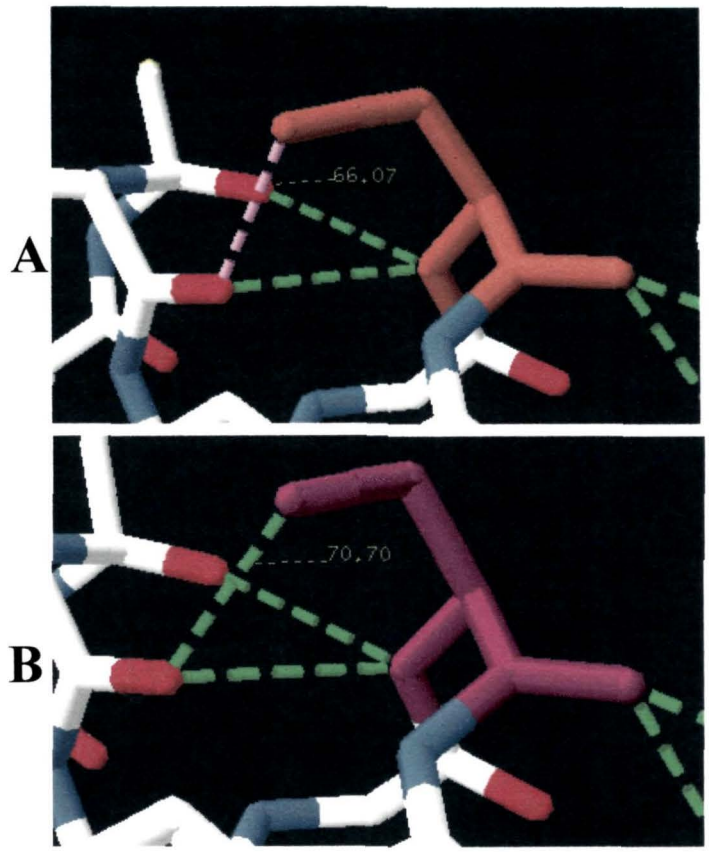


Figure 3.56. Difference of H-bond angle between –SH group of Cys 41 in wild-type (**A**) and –OH group of Ser 41 in mutant (**B**) with the backbone oxygen of Asp 38 in rotamer 2.

In the third rotamer, the –SH group of Cys in the wild-type protein was not found to be involved in hydrogen bonding with the backbone oxygen of the neighboring Cys 37, while the –OH group of Ser formed a hydrogen bond with the backbone oxygen of the Cys 37 residue (Fig. 3.57).

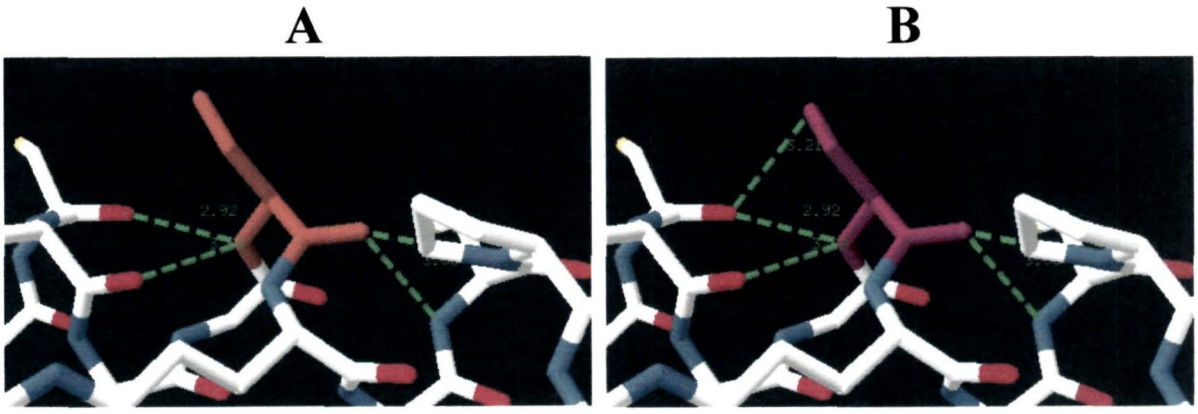


Figure 3.57. Difference in H-bonding of the –SH group of Cys 41 in wild-type (**A**) and the –OH group of Ser 41 in mutant (**B**) with the backbone oxygen of Cys 37 in rotamer 3.

3.6. SLOT-BLOT ANALYSIS OF EXPRESSION OF P53, BRCA1 AND BRCA2 PROTEINS IN SELECTED HUMAN CANCERS

Slot-blot analysis was used to study the expression of P53, Brca1 and Brca2 proteins in BL of patients suffering from various cancers, in comparison to the levels of expression of these proteins in samples obtained from healthy volunteers who served as controls for this study. The slot-blot analysis of the proteins in cancer and control samples was performed independently, unlike in samples from control and AEBN exposed mice (§ 3.2.) which were performed simultaneously.

3.6.1. p53 tumor suppressor protein

Densitometric analysis of India ink stained slot-blots of control and cancer samples did not reveal significant differences between the two, thus confirming equal loading of protein. However densitometric analysis of immunoprobed slot-blots revealed differences in level of expression of P53 protein between different cancers and controls.

3.6.1.1. Head and neck cancer: On the average, P53 protein was found to be upregulated in head and neck cancers (1.7 fold) in comparison to control (Fig. 3.58).

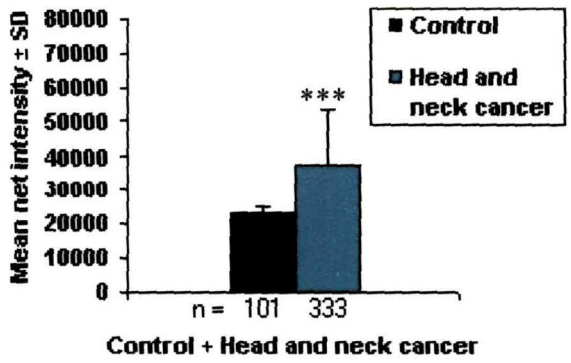


Figure 3.58. Densitometric plot (mean ± SD) showing upregulation of p53 protein in PBL of patients suffering from head and neck cancer in comparison to control. n- number of samples; *** indicates significant increase at $P < 0.001$.

However the level of p53 protein varied with the site of cancer among head and neck cancers (Figs. 3.59 & 3.60). Cancers of the tongue and base of tongue, lower lip, buccal mucosa, pre-epiglottis and epiglottis, pyriform sinus and external mouth cavity exhibited a level close to that of controls, while cancers of the oesophagus,

larynx, vocal chord, throat and nasopharynx and secondary cancer of neck nodes exhibited elevated levels (Figs. 3.59 & 3.60).

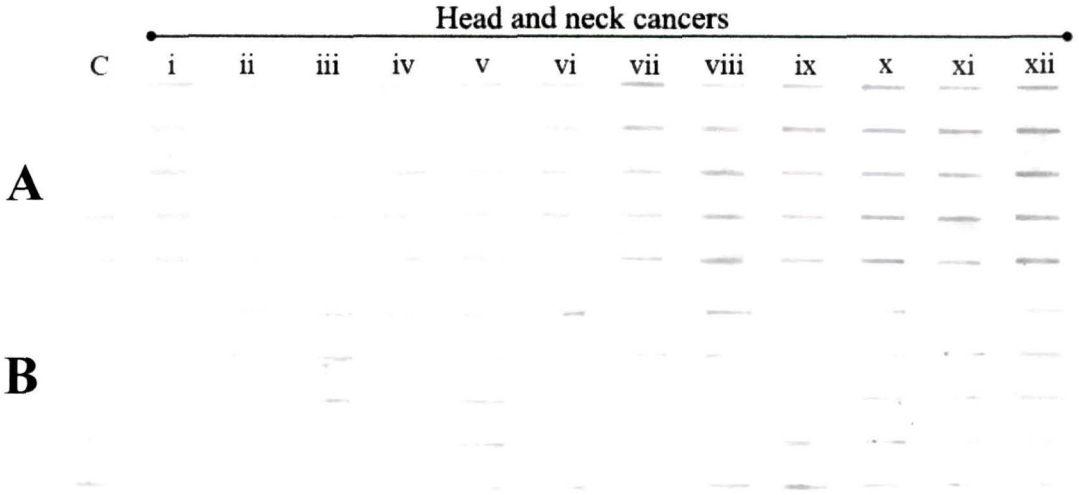


Figure 3.59. Slot-blot analysis of expression of p53 protein in head and neck cancers at various sites, in comparison to control. **A**- slot-blots immunoprobed with anti-p53. **B**- replica slot-blots stained with India ink for total protein. **C**-control, **i**-tongue and base of tongue, **ii**-lower lip, **iii**-buccal mucosa, **iv**-pre-epiglottic region/ epiglottis, **v**-pyriform sinus, **vi**-external mouse cavity, **vii**-oesohagus, **viii**-larynx, **viii**-secondary neck node, **ix**-vocal chord, **x**-throat, **xi**-nasopharynx

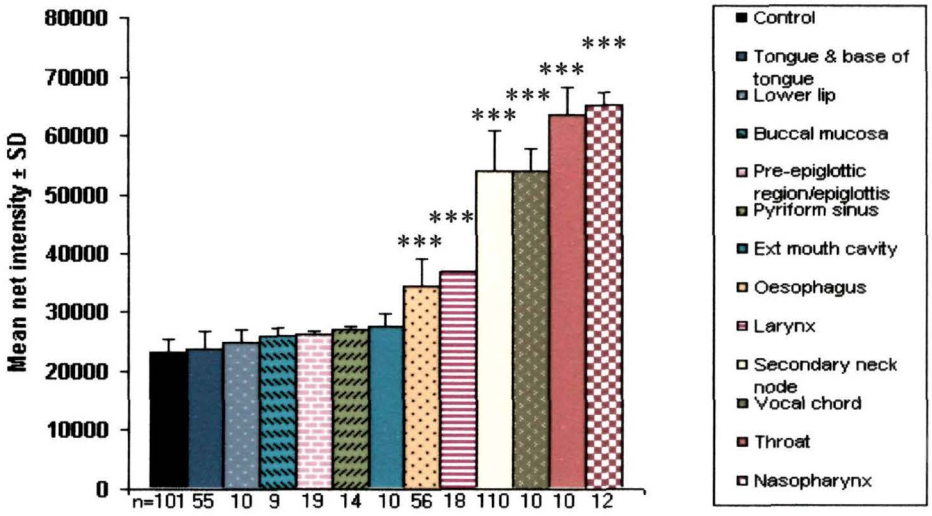


Figure 3.60. Densitometric plot (mean ± SD) showing variation in expression of p53 protein in PBL of patients suffering from head and neck cancer at various sites in comparison to control. n- number of samples; *** indicates significant increase at $P < 0.001$.

3.6.1.2. Breast cancer: P53 protein was found to be upregulated in all cases of breast cancer studied (1.7 fold) in comparison to control (Figs. 3.61 A & B).

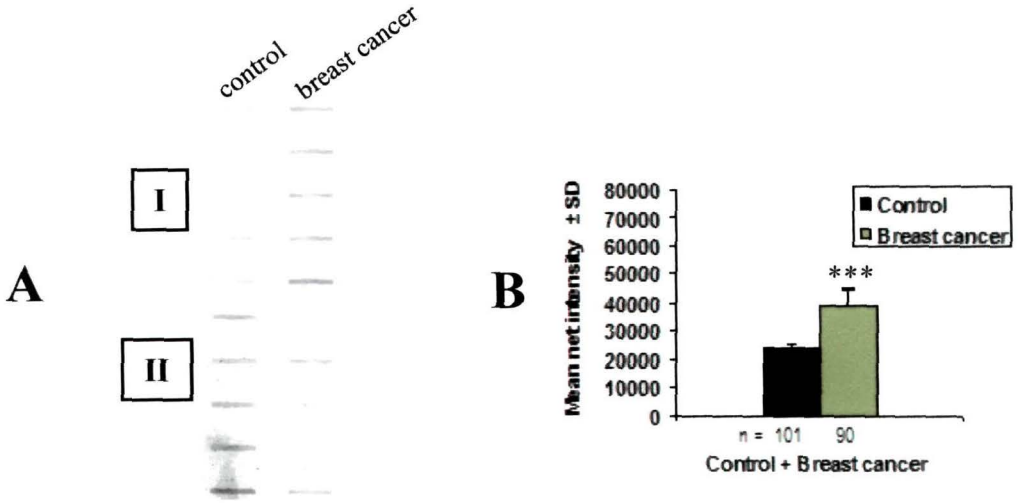


Figure 3.61. Upregulation in expression of p53 protein in PBL of patients suffering from breast cancer in comparison to control. **A**- slot-blots immunoprobed with anti-p53 antibody (I) and stained with India ink for total protein (II). **B**-densitometric plot (mean ± SD) obtained . n- number of samples; *** indicates significant increase at $P < 0.001$.

3.6.1.3. Cervix cancer: P53 protein was found to be upregulated in all cases of cervix cancer studied (2.9 fold) in comparison to control (Figs. 3.62 A & B).

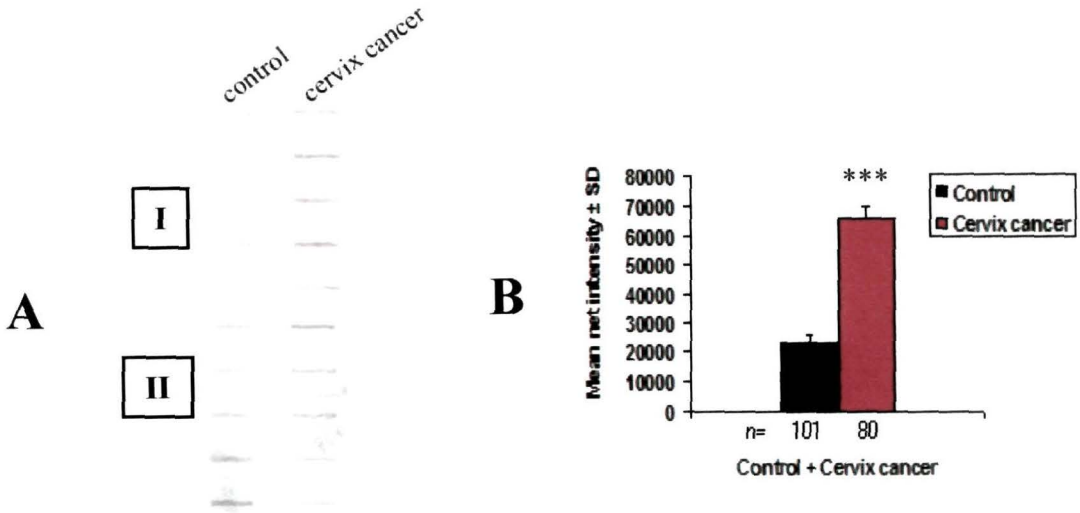


Figure 3.62. Upregulation in expression of p53 protein in PBL of patients suffering from cervix cancer in comparison to control. **A**- slot-blots immunoprobed with anti-p53 antibody (I) and stained with India ink for total protein (II). **B**-densitometric plot (mean ± SD) obtained. n- number of samples; *** indicates significant increase at $P < 0.001$.

3.6.2. Brca1 tumor suppressor protein

Densitometric analysis of India ink stained slot-blots of control and cancer samples did not reveal significant differences between the two, thus confirming equal loading of protein. However densitometric analysis of immunoprobed slot-blots

revealed differences in level of expression of Brca1 protein between different cancers and controls.

3.6.2.1. Head and neck cancer: Brca1 protein was found to be upregulated in all cases of head and neck cancer studied, however the average increase (1.4 fold) was non-significant in comparison to control (Fig. 3.63 A & B).

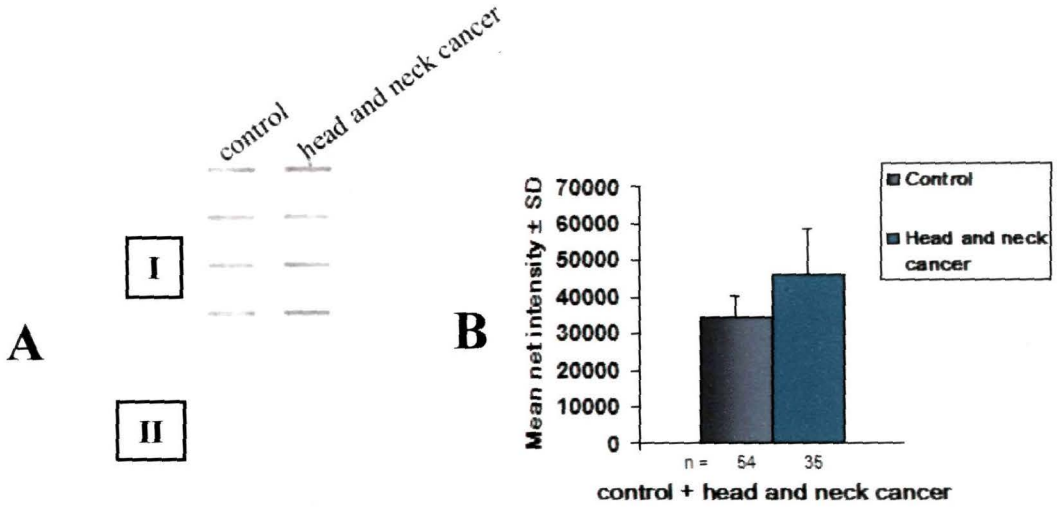


Figure 3.63. Upregulation in expression of Brca1 protein in PBL of patients suffering from head and neck cancers in comparison to control. **A**- slot-blots immunoprobed with anti-Brca1 antibody (I) and stained with India ink for total protein (II). **B**-densitometric plot (mean ± SD) obtained. n- number of samples.

3.6.2.2. Breast cancer: Brca1 protein was found to be significantly downregulated in all cases of breast cancer studied (82 %) in comparison to control (Fig. 3.64 A & B).

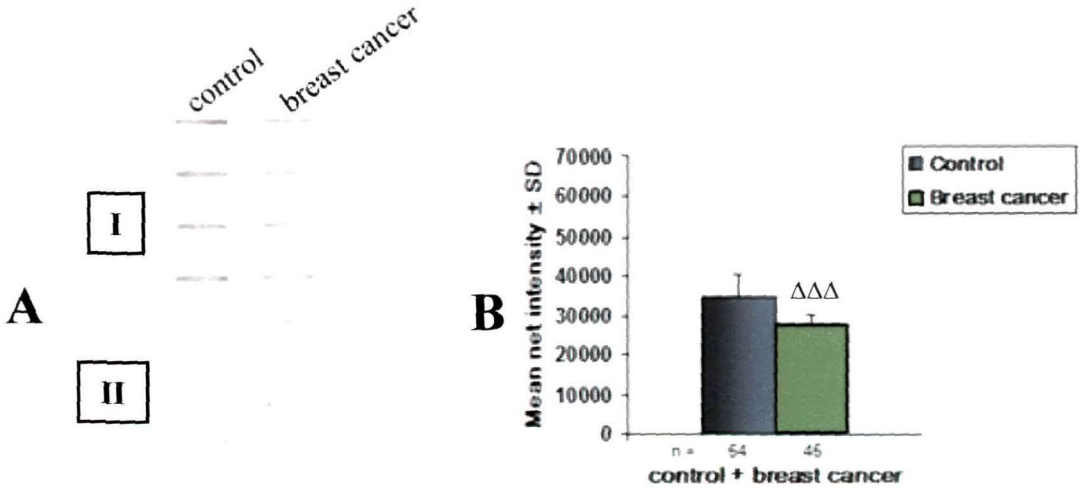


Figure 3.64. Downregulation in expression of Brca1 protein in PBL of patients suffering from breast cancer in comparison to control. **A**- slot-blots immunoprobed with anti-Brca1 antibody (I) and stained with India ink for total protein (II). **B**-densitometric plot (mean ± SD) obtained. n- number of samples; ΔΔΔ indicates significant decrease at $P < 0.001$.

3.6.2.3. Cervix cancer: Brca1 protein was found to be significantly upregulated in all cases of breast cancer studied (1.8 fold) in comparison to control (Fig. 3.65 A & B).

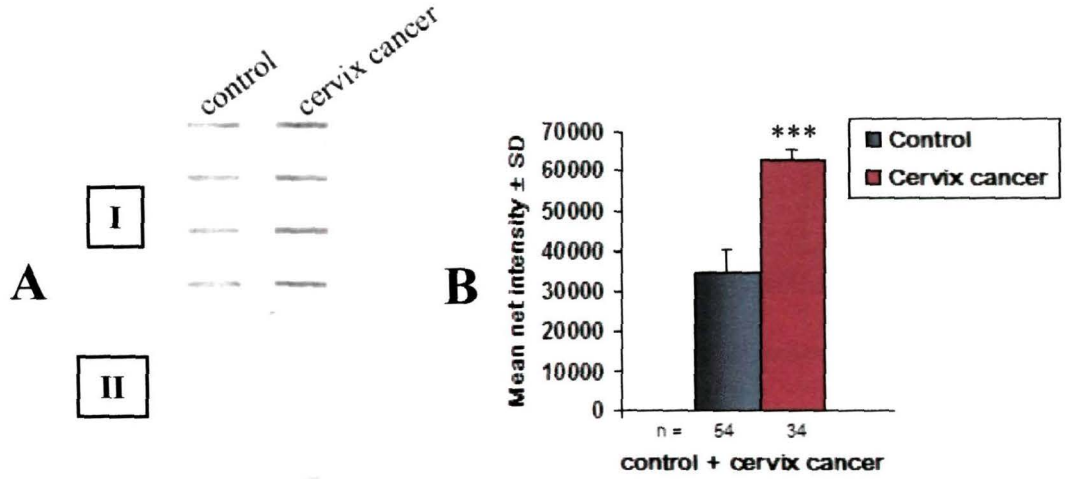


Figure 3.65. Upregulation in expression of Brca1 protein in PBL of patients suffering from head and neck cancers in comparison to control. **A**- slot-blots immunoprobed with anti-Brca1 antibody (I) and stained with India ink for total protein (II). **B**-densitometric plot (mean ± SD) obtained. n- number of samples; *** indicates significant increase at $P < 0.001$.

3.6.3. Brca2 tumor suppressor protein

Densitometric analysis of India ink stained slot-blots of control and cancer samples did not reveal significant differences between the two, thus confirming equal loading of protein. However densitometric analysis of immunoprobed slot-blots revealed differences in level of expression of Brca2 protein between different cancers and controls.

*3.6.3.1. **Head and neck cancer:*** Brca2 protein was found to be upregulated in all cases of head and neck cancer studied, however the average increase (150 %) was non-significant in comparison to control (Fig. 3.66 A & B).

3.6.3.3. Cervix cancer: Brca2 protein was found to be significantly upregulated in all cases of breast cancer studied (1.8 fold) in comparison to control (Fig. 3.68 A & B).

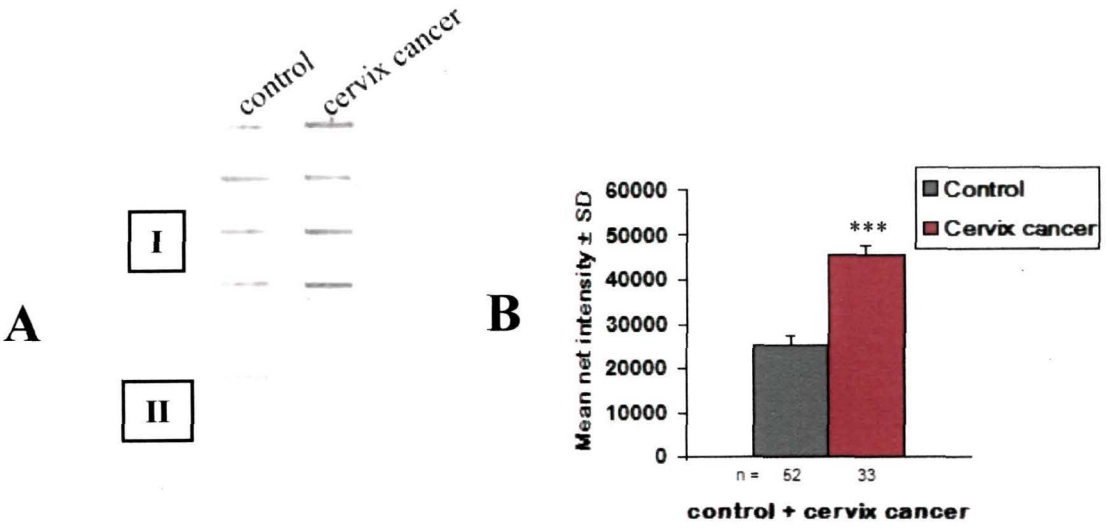


Figure 3.68. Upregulation in expression of Brca2 protein in PBL of patients suffering from cervix cancer in comparison to control. **A**- slot-blots immunoprobed with anti-Brca2 antibody (I) and stained with India ink for total protein (II). **B**-densitometric plot (mean ± SD) obtained. n- number of samples; *** indicates significant increase at $P < 0.001$.

DISCUSSION

The carcinogenicity of betel nut (BN) is well documented, and, the habit of BN chewing is strongly associated with various cancers in humans (Sharan, 1996). Tumor suppressor genes contribute significantly towards the suppression of tumorigenesis, and have been classified into two categories-"gatekeepers" and "caretakers" based on the difference in their mode of functioning (Kinzler and Vogelstein, 1997). The *p53* gene is a critical tumor suppressor gene known to be mutated in a variety of human cancers (Hollstein *et al.,* 1991), and its involvement in BN associated cancer in different human populations has been widely studied (Ranasinghe *et al.,* 1993; Thomas *et al.,* 1994; Kuttan *et al.,* 1995; Chiba *et al.,* 1998; Kannan *et al.,* 1999; Ralhan *et al.,* 2001; Hsieh *et al.,* 2001; Goan *et al.,* 2005). Since cancer is a multistep, multifactorial disorder involving several discrete genetic alterations (Pitot *et al.,* 1991), it is therefore likely that BN-induced carcinogenesis involves the loss of function of more than one critical tumor suppressor gene. One objective of this study was thus to elucidate the dose-dependent response of the "gatekeeper" p53 protein and the "caretaker" BRCA1 (murine Brca1) and BRCA2 (murine Brca2) proteins during chronic exposure to BN, beginning with onset of exposure and culminating ultimately in the discernable development of cancer.

The carcinogenic potential of the aqueous extract of betel nut (AEBN) is well established (Wary and Sharan, 1988; Sharan and Wary, 1992; Sharan, 1996) and previous studies in Swiss Albino mice have shown that AEBN induced DNA damage, affected cell cycle characteristics and induced qualitative changes in mice liver high mobility group (HMG) proteins similar to that induced by the hepatocarcinogen, diethylnitrosamine (DEN), leading to the development of preneoplastic nodules in the liver (Wary and Sharan, 1988; Pariat and Sharan, 1998). Swiss Albino mice chronically exposed to AEBN were therefore selected as a model for this study.

The effect of a carcinogen may be transmitted multigenerationally or transgenerationally by *in utero* exposure or by exposure of parents prior to mating (Tomatis *et al.,* 1992). The habit of BN chewing is inherent in the social milieu of various populations, and approximately 600 million individuals worldwide are exposed to it (Sharan, 1996). The frequency of transgenerational exposure to BN, therefore, may be expected to be high. There is, however, no report on the effect of transgenerational

exposure to BN on carcinogenic risk. Thus, this study also aimed to determine if BN has a transgenerational carcinogenic effect. For this purpose, the study was designed taking the significance of postnatal carcinogen exposure into consideration (Mohr *et al.,* 1991; Tomatis *et al.,* 1992; Nomura *et al.,* 2006). The progeny of parents exposed to AEBN were, therefore, exposed to the same dose of AEBN as their parents, and subsequently assessed for increased susceptibility to cancer. Three generations of progeny were examined in this manner.

The *p53*, *Brca1* and *Brca2* tumor suppressor genes were investigated for mutations in critical areas of the genes, under both chronic as well as transgenerational exposure regimens. Exon 5 and exon 7 of the *p53* gene were selected for the study because they are part of the region that codes for the DNA-binding domain of the *p53* protein (Bai and Zhu, 2006). Moreover, exon 7 of the p53 gene is reported to be frequently mutated in hepatocellular carcinoma in humans (Staib *et al.,* 2003). Exon 11 of the *Brca1* gene was selected because it codes for the vital RAD51 interaction domain of the Brca1 protein (Welsch *et al.,* 2000), and deletion of exon 11 of murine *Brca1* has been reported to cause impaired DNA damage (Huber *et al.,* 2001). Similarly, exon 27 of the *Brca2* gene was selected because it codes for the COOH- terminus of the Brca2 protein which directly interacts with RAD51 and contains a nuclear localization signal. Deletion of exon 27 of the *Brca2* gene was reported to result in a significantly increased overall tumor incidence in mice (McAllister *et al.,* 2002), and to cause hypersensitivity to DNA crosslinks, increased chromosomal instability, and reduced life span in mice (Donoho *et al.,* 2003). Studies of mouse embryonic stem cells expressing Brca2 gene which lacked exon 27 also revealed two fold, and five to six fold reduction in homologous recombination repair (Lu *et al.,* 2005).

The body weight and organ weight are important indicators of the health status of laboratory mice (Culleré-Ullman and Foltz, 1999). Mice exposed chronically and transgenerationally to AEBN, as well as the age-matched control mice were therefore monitored regularly for consumption of food and water. Body weight and relative weight of liver and spleen of the sacrificed mice were determined (Table 14). No significant change was observed in the body weight of the AEBN-exposed mice belonging to the chronically exposed P generation, or the transgenerationally exposed F1, F2 and F3 generations, in comparison to the respective age-matched controls

throughout the exposure periods. However, significant alterations were observed in the relative weight of both liver and spleen. The relative weight of liver of the P generation mice declined significantly with respect to that of age-matched control mice from 4 weeks to 8 weeks of exposure to AEBN, indicating retardation in cellular proliferation and growth of the liver, during this period. The decline in relative weight was also observed in the spleen of P generation mice exposed to AEBN for 2 weeks. Subsequently, the relative weight of both liver and spleen of AEBN-exposed mice increased in comparison to the relative weight of these organs in the age-matched control mice, recording the greatest increase after 24 weeks of exposure. It thus appears that exposure of P generation mice to AEBN resulted in an inhibition of cellular proliferation during the early periods of exposure, followed by uncontrolled and excessive cellular proliferation on prolonged exposure to AEBN, which resulted in increase in relative organ weight of AEBN-exposed mice in comparison to respective age-matched controls.

In contrast, in the F1, F2 and F3 mice exposed transgenerationally to AEBN, significant increase in relative weight of both liver and spleen in comparison to the age-matched control mice was observed immediately after exposure to AEBN for 2 weeks or 4 weeks (Table 14), and was maintained almost during the entire period of exposure. Thus, transgenerational exposure of mice to AEBN appeared to induce uncontrolled cellular proliferation in the liver and spleen from the onset of exposure onwards, and the suppression of growth during the initial period of exposure of P generation mice, was not observed in this case.

Exposure of mice to AEBN was found to induce the formation of preneoplastic nodules in the liver as reported earlier (Wary and Sharan, 1988; Pariat and Sharan, 1998). The period of appearance of preneoplastic nodules was found to be significantly advanced in mice exposed transgenerationally to AEBN, in comparison to P generation mice (Table 15). The P generation mice developed nodules after 16 weeks of exposure to AEBN, primarily in the right and caudate lobes of the liver (Fig. 3.1 c & d). However, the F1 mice developed nodules after 8 weeks of exposure (Fig. 3.1 g & h), the F2 mice (Fig. 3.1 i & j) after 6 weeks of exposure and the F3 mice after 4 weeks of exposure to AEBN (Fig. 3.1 k & l). The frequency of nodulation also increased from 1-2 nodules per liver of the P generation mice, to 3-4 nodules per liver of the the F1, F2 and F3

congestion of misfolded proteins (Shiraishi *et al.,* 2006). The extensive disruption of the ER observed upon chronic exposure to AEBN (Fig. 3.3 II b & c), could lead to calcium release from the ER lumen. Aberrant Ca^{2+} regulation in the ER may cause protein unfolding because of the Ca^{2+}-dependent nature of the molecular chaperones Grp78, Grp94, and calreticulin (Xu *et al.,* 2005) Prolonged stress resulting in the inappropriate folding of proteins has been reported to result in apoptosis of cells (Shiraishi *et al.,* 2006). The mice exposed to AEBN under the chronic or transgenerational exposure regimes are subject to cellular strain for an extensive postnatal period of 24 weeks during which extensive cellular injury is incurred as revealed by TEM studies. It is thus expected that the exposed and damaged cells may proceed to apoptosis. However, data pertaining to the weight and gross morphology of the organs of AEBN-exposed mice (Table 14 and Fig 3.1.) suggests excessive proliferation of cells rather than cell death. These results may perhaps be explained by the significant alterations observed in the ultrastructure of mitochondria (Fig. 3.4 b through e). The hepatocytes of control mice had abundant mitochondria with an intact double-layered membrane which encompassed an extensive network of cristae (Figs. 3.4 II-a and I-a). However, the hepatocytes of P generation mice exposed chronically to AEBN exhibited fewer and smaller mitochondria, with irregular arrangement of cristae and cristolysis (Figs. 3.4 I-b and II- b). The mitochondria of transgenerationally exposed mice showed extensive cristolysis with electron-lucent matrix, and progressive decline in size (Figs. 3.4 I c through e) and number (Fig. 3.4 II-c through e) of mitochondria. Such alterations have been reported in the mitochondria of human astrocytic tumors (Morillo-Arismendi and Catellano-Ramirez, 2008). It has also been reported that the mitochondria of rapidly growing tumors tend to be fewer in number, smaller and have fewer cristae (Modica-Napolitano and Singh, 2002). The mitochondrial index, calculated as a measure of mitochondrial function, was found to decrease progressively and significantly from the P through the F3 generation, in comparison to control (Fig. 3.5.), thus indicating a progressive loss of mitochondrial function. One important function of the mitochondria is to act as an integrating sensor of multiple death insults by releasing cytochrome *c* into the cytosol, where it triggers caspase activation (Evan and Vousden, 2001). Cytochrome *c* is enriched in the intermembrane space encased by the cristae membranes, and the regulated opening of cristae junctions might be important for its relocalization during apoptosis (Detmer and Chan, 2007). A gross reduction of mitochondrial index, and concomitant cristolysis

would therefore compromise this essential function, possibly leading to evasion of apoptosis, which is characteristic of cancer. Further, the progressive decline in mitochondrial index indicates a progressive loss of apoptosis from the P through F3 generations, thereby resulting in an increased risk of cancer in mice exposed transgenerationally to AEBN.

In multigeneration/ transgenerational carcinogenesis, it is assumed that heritable changes induced in germ cells will be present in all somatic cells of offspring. Therefore, multiple tumors may be expected to occur more frequently in multigeneration cancer than in sporadic cases. It has, in fact, been suggested that tumor multiplicity is a multigeneration effect of carcinogens (Tomatis *et al.,* 1992). In addition to the enhancement in period of appearance of preneoplastic nodules of the liver, and the increase in multiplicity of these nodules, the mice exposed transgenerationally to AEBN developed various anomalies which were not seen in the chronically exposed mice. These anomalies comprised solid tumors, pus-filled sacs, enlarged neck nodes, necrosis of the liver and spleen deformities including enlargement and protrusion of spleen (Fig. 3.2 & Table 16). The propensity of transgenerationally exposed mice to develop these anomalies, as well as their greater susceptibility to cancer in comparison to chronically exposed mice may be regarded as a manifestation of alterations induced due to transgenerational exposure, which, in this study, includes exposure of parents prior to mating as well as prenatal and pre-weaning exposure of offsprings.

Chronic exposure to AEBN was found to induce an immediate response of the p53, Brca1 and Brca2 tumor suppressors with respect to the cellular levels of these vital proteins. The level of the p53 protein was elevated in comparison to age-matched control level after 2 weeks of exposure to AEBN onwards, recording a 2.5-fold increase in the liver after 6 weeks of exposure (Fig. 3.6). A 2-fold increase in the level of the p53 protein was also recorded in the spleen cells (SC) after 8 weeks of exposure (Fig. 3.7) and in the peripheral blood lymphocytes (PBL) after 4 weeks of exposure to AEBN in comparison to the respective age-matched controls (Fig. 3.8). Similarly, the levels of both Brca1 and Brca2 proteins were elevated to about 1.4-fold the age-matched controls in the liver after 2 weeks of exposure to AEBN (Figs. 3.9 & 3.12, respectively). The levels of Brca1 and Brca2 proteins were also elevated after 2 weeks

of exposure to approximately 1.3-fold the age-matched control levels in the spleen (Figs. 3.10 & 3.13, respectively) and approximately 1.4-fold the age-matched control levels in the PBL (Figs. 3.11 & 3.14, respectively). These results indicate that exposure to AEBN elicits an immediate protective effect by inducing a rise in the level of the tumor suppressor proteins above the basal level maintained in the age-matched control mice. One consequence of such a rise in the level of the p53 protein would be the arrest of cells which have incurred AEBN-induced damage, the suppression of cellular proliferation, and the likely induction of apoptosis of cells with irreparable damage (Stewart and Pietenpol, 2001; Moll and Petrenko, 2003). Elevation of the levels of Brca1 and Brca2 proteins would be expected to evoke a similar response, in addition to facilitating the DNA repair processes (Welsch *et al.,* 2000; Scully and Livingston, 2000; Venkitaraman, 2002). Thus, the decline in the relative weight of the liver and the spleen observed during the early periods of exposure to AEBN (Table 14) may be explained on the basis of the tumor-suppressive responses elicited during these periods.

Continued exposure to AEBN, however, resulted in a decline in the cellular level of p53, Brca1 and Brca2 tumor suppressor proteins. The level of p53 protein gradually declined after 6 weeks of exposure in the liver (Fig. 3.6), 8 weeks of exposure in the SC (Fig. 3.7) and 6 weeks of exposure in the PBL (Fig. 3.8), reaching the age-matched control level after 16 weeks of exposure to AEBN (Figs. 3.6, 3.7 & 3.8). The decline of p53 protein in the liver, SC and PBL of AEBN-exposed mice was thus concomitant with the appearance of preneoplastic nodules in the liver (Fig. 3.1 c & d) and an increase in the relative weight of the liver of AEBN-exposed mice (Table 14). The rapid upregulation of p53 protein by various types of stress prevents the proliferation of cells carrying damaged DNA with potentially oncogenic mutations (Moll and Petrenko, 2003). Thus, loss either of the ability to activate p53 or of p53 function is an important step in carcinogenesis (Evan and Vousden, 2001). The inability to maintain upregulated status of p53 in response to DNA damage may lead to carcinogenesis by disruption of p53-mediated cell cycle arrest and/or apoptosis of damaged cells. The gradual, dose-dependent downregulation of p53 could thus be viewed as an adaptive mechanism by which cells evade the growth suppressive activities of p53 and continue to survive under conditions of continuing AEBN exposure. Further exposure to AEBN beyond 16 weeks did not upregulate p53, indicating a lack of p53 response. The preneoplastic nodules observed after 24 weeks of exposure were larger than those formed after 16

weeks of exposure, and the level of p53 in the liver was significantly lower than that after 16 weeks (Figs. 3.1 e & f, and 3.6). These findings may be explained on the premise that maintenance of p53 level at or below control level allows cells to undergo uncontrolled proliferation, leading to the development and subsequent enlargement of preneoplastic nodules as well as an overall increase in the relative weight of the liver (Table 14). The cause of decline in the cellular level of the p53 protein was not investigated in this study. It may be the result of ubiquitin-mediated proteasomal degradation due to overexprerssion of the MDM2 protein, as reported in some instances of BN-associated cancer (Shwe *et al.,* 2001). This aspect may be the subject of future investigations.

The levels of both Brca1 and Brca2 proteins were found to decline below age-matched control level after 4 weeks of exposure onwards. The level of Brca1 was 70 % that of age-matched control level in the liver, SC and PBL after 16 weeks of exposure to AEBN (Figs. 3.9, 3.10 and 3.11), and the corresponding level of Brca2 protein was approximately 60 % that of age-matched control level (Figs. 3.12, 3.13 & 3.14). After 24 weeks of exposure to AEBN both Brca1 and Brca2 proteins declined to approximately 50 % that of age-matched control level in the liver (Figs. 3.9 & 3.12), SC (Figs. 3.10 & 3.13) and PBL (Figs. 3.11 & 3.14) of AEBN-exposed mice. Thus, continued exposure to AEBN resulted in a rapid decline in the levels of both Brca1 and Brca2 proteins below the basal level maintained in age-matched control mice. The pattern of alteration in cellular levels of Brca1 and Brca2 proteins is, therefore, nearly identical. This observation may be explained on the basis of a previous report that *Brca1* and *Brca2* are coordinately regulated (Rajan *et al.,* 1996).

The Brca1 and Brca2 proteins are required for the preservation of genomic stability (Venkitaraman, 2002). A decline in the levels of these proteins in the event of DNA damage would, therefore, compromise the efficiency of DNA repair and probably result in erroneous repair of damaged DNA (Wang *et al.,* 2000; Venkitaraman, 2002; Powell and Kachnic, 2003), leading to instability of the genome. In fact, it has been reported that cells deficient in the murine homolog of *BRCA2* sustain spontaneous aberrations in chromosome structure; gross chromosomal aberrations have also been reported in Brca1-deficient mouse cells, and in *BRCA1* or *BRCA2* deficient human cancer cells (Venkitaraman, 2002). Thus, the ability of AEBN to induce a decline in the cellular

level of the Brca1 and Brca2 proteins, would explain the previously reported induction of chromosomal aberrations and sister chromatid exchange upon exposure to AEBN (Kumpawat *et al.,* 2003). The resultant genomic instability would also contribute to the appearance and development of preneoplastic nodules of the liver, as reported earlier (Wary and Sharan, 1988; Pariat and Sharan, 1998), and also observed in the current study. Moreover, the high frequency of binucleate cell formation induced by exposure to AEBN (Fig. 3.3 I b) may be attributed to the decline in cellular level of Brca2 protein, which leads to delayed or defective cytokinesis, as previously reported (Daniels *et al.,* 2004).

Global gene expression profiling in human gingival fibroblasts (HGF) exposed to arecoline revealed that *BRCA1* was repressed in a dose-dependent manner (Chiang *et al.,* 2007). A previous study reported that the treatment of cultured cells with the DNA-damaging agents adriamycin and mitomycin C resulted in an initial upregulation of BRCA1 RNA as well as protein, followed by a reduction to below basal levels (MacLachlan *et al.,* 2000). It was suggested that BRCA1 participates in the accumulation of p53 during the early periods of DNA-damage, and subsequently, p53 may be responsible for the reduction of BRCA1 to or below basal level after the initial treatment by repressing BRCA1 at its promoter (MacLachlan *et al.,* 2000). Another study revealed that adriamycin and mitomycin C repress BRCA2 promoter activity in a dose- and time-dependent manner by inhibiting binding of an upstream stimulatory factor protein complex to the promoter, and also reduce BRCA2 mRNA and protein levels by altering the stability of both BRCA2 mRNA and protein. Both these processes require the presence of wild-type p53 (Wu *et al.,* 2003). The alterations in Brca1 and Brca2 levels observed in the current study are, thus, consistent with these previous reports (MacLachlan *et al.,* 2000; Wu *et al.,* 2003).

Mutations in the *p53* gene are observed in a variety of cancers in mice, but are rarely found in murine liver tumors, suggesting an alternate route of p53 inactivation in murine hepatocarcinogenesis (Jaworski *et al.,* 2005). Amplification and sequence analysis of the DNA samples obtained from liver preneoplastic nodules of chronically exposed mice indicate that the exons 5 and 7 of the *p53* gene were not mutated under the chronic exposure regimen (Appendices I and II and Figs. 3.37 & 3.39) respectively. Similarly, mutations in exon 11 of the *BRCA1* gene have been widely reported in breast

and ovarian cancers (Risch *et al.,* 2001; Ciernikovά *et al.,* 2003; Byrski, 2003; Valarmathi *et al.,* 2004; Muller *et al.,* 2004; Tommasi *et al.,* 2005 and Saxena *et al.,* 2006). However, exon 11 of the murine *Brca1* gene (Appendix III and Figs. 3.41, 3.43, 3.46, 3.48 and 3.50), and a segment of exon 27 of the murine *Brca2* gene (Fig. 3.52) were not found to be mutated after chronic exposure to AEBN. Thus, AEBN induced carcinogenesis in Swiss Albino mice does not involve mutations of exons 5 and 7 of the *p53* gene, exon 11 of the *Brca1* gene, and exon 27 of the *Brca2* gene, though we cannot exclude the possibility of mutations occurring in other regions of the genes that have not been included in this study. It, thus, appears that upon chronic exposure to AEBN, wild-type p53 mediates the decline in the levels of Brca1 and Brca2 proteins, by a mechanism as previously described (MacLachlan *et al.,* 2000; Wu *et al.,* 2003).

AEBN-induced carcinogenesis, therefore, proceeds through a series of distinct responses of the p53, Brca1 and Brca2 proteins. The immediate response triggered by exposure to AEBN is the upregulation of the Brca1 and Brca2 proteins, as well as the p53 protein, evoking both "caretaker" as well as "gatekeeper" tumor suppressor response against DNA damage induced by AEBN. The initiation of carcinogenesis involves continued upregulation of the p53 protein in a dose- or exposure period dependent fashion, while the Brca1 and Brca2 proteins are concomitantly downregulated to below basal level. At this stage, "caretaker" response is thus, abrogated, probably leading to genomic instability, but "gatekeeper" response continues to be maintained. Subsequently, the p53 protein is downregulated to control level, while Brca1 and Brca2 proteins continue to be suboptimal. Final progression to cancer requires loss of both "caretaker" as well as" gatekeeper" responses, with the maintenance of p53 protein at or below control level, and Brca1 and Brca2 proteins well below control level (Fig. 4.1). Under such circumstances, the cells would have accumulated significant damage due to chronic exposure to AEBN and compromised DNA repair, and would also be able to evade cell-cycle arrest and/or apoptosis, thereby progressing to cancer.

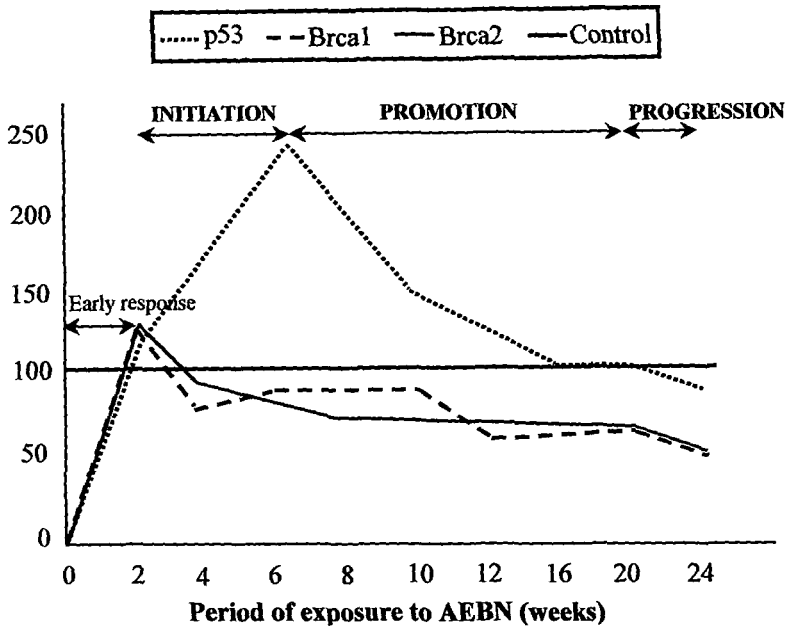


Figure 4.1. Representative line diagram showing alterations in the cellular levels of p53, Brca1 and Brca2 proteins upon chronic exposure of Swiss Albino mice to AEBN, in comparison to the levels in age-matched controls. The alterations are hypothesized to correspond to a multistage model of AEBN-induced carcinogenesis (Levels of the tumor suppressor proteins in all age-matched control mice are taken as 100 percent, and experimentally derived data has been plotted).

Identical patterns of alteration in levels of p53, Brca1 and Brca2 proteins were also observed in the SC and PBL of chronically exposed mice with noticeable increase in the relative weight of the spleen (Table 14), indicating uncontrolled proliferation of splenic cells upon AEBN exposure. The results obtained from studies with the SC and PBL support our oft-expressed contention that AEBN is a general, rather than tissue-specific carcinogen (IARC, 1985; Sharan, 1996; Pariat and Sharan, 1998).

The chronic exposure study revealed that one mechanism of AEBN-induced carcinogenesis in Swiss Albino mice primarily involves alterations in the cellular level of the p53, Brca1 and Brca2 tumor suppressor proteins. It was, therefore, of interest to determine whether transgenerational exposure to AEBN modulated carcinogenic risk by the same mechanism. The levels of the p53, Brca1 and Brca2 tumor suppressor proteins were, therefore, determined in transgenerationally exposed mice for the periods of treatment after which significant alterations were observed in the chronic exposure regimen. The responses of the p53, Brca1 and Brca2 tumor suppressors elicited by transgenerational exposure to AEBN were found to be in striking contrast to those elicited by chronic exposure.

It was observed that the cellular level of the p53 protein in transgenerationally exposed mice remained largely invariant in comparison to age-matched control level throughout the period of postnatal exposure to AEBN. This observation was true for the liver, SC and PBL of F1 (Fig. 3.15), F2 (Fig. 3.16) as well as F3 mice (Fig. 3.17). Unlike in the chronic exposure study, the level of the p53 protein was not upregulated throughout the period of exposure. It thus emerges that the protective response evoked by an elevation of the p53 protein above the basal level observed in the chronic exposure study was completely abrogated during transgenerational exposure to AEBN, as reported earlier (Choudhury and Sharan, 2009). Similarly, the Brca1 and Brca2 responses were also drastically altered. The levels of both these proteins were again not elevated during the entire period of exposure, and instead declined rapidly below age-matched control level from the second week of postnatal exposure to AEBN onwards. This observation was again true for the liver, SC and PBL of F1 (Figs. 3.18 & 3.21), F2 (Figs. 3.19 & 3.22) and F3 mice (Figs. 3.20 & 3.23). Thus, as in case of the p53 protein, the protective response elicited by an upregulation in the levels of the Brca1 and Brca2 proteins, which functioned during the early period of the chronic exposure regimen was also lost during transgenerational exposure to AEBN.

The rapid decline in the cellular level of Brca1 and Brca2 proteins of the F1, F2 and F3 mice below basal level from the very onset of postnatal exposure to AEBN, would severely compromise the repair of AEBN-induced DNA damage in these mice. Such a shortcoming would lead to increasing genomic instability, causing the cells to accumulate damage with continued exposure to AEBN. The cumulative damage should, in turn, impair the chances of survival of the cells by inducing their cell cycle arrest, or alternately cell death via apoptosis. However, the complete abrogation of the p53 response to AEBN, and maintenance of p53 protein at control level, would, at best induce a p53-mediated "gatekeeper" response at the basal level, which would be insufficient to deal with the increasing gamut of cells with damaged DNA. This would result in uncontrolled proliferation of cells as well as inefficient cell death, leading to the early and significant increase in the relative weight of the liver and spleen observed in the F1, F2 and F3 mice (Table 14). Moreover, while preneoplastic nodules of the liver developed in P generation mice after 16 weeks of exposure, they developed in F1 mice after 8 weeks, F2 mice after 6 weeks and in F3 mice after 4 weeks of exposure to

AEBN (Figs. 3.1 g, i & k). Thus, the development of preneoplastic nodules of the liver was significantly advanced in exposed F1 mice by 50 %, F2 mice by 62.5 % and F3 mice by 75 % of the period of AEBN exposure required for initiation of nodulation in exposed P generation mice (Table 15). Mice exposed transgenerationally to AEBN also exhibited an increase in the frequency of nodulation in comparison to P generation mice. These differences between P mice and transgenerationally exposed mice reflect an increased predisposition of transgenerationally exposed mice to cancer, and may be attributed to an early onset of uncontrolled cell proliferation in transgenerationally exposed mice, thereby enabling progression of carcinogenesis to a greater degree than in the P generation mice.

A comparison was drawn between the pattern of alteration in the levels of p53 (Fig. 3.24), Brca1 (Fig. 3.25) and Brca2 (Fig. 3.26) proteins in the liver, SC and PBL of P, F1, F2 and F3 mice during postnatal exposure to AEBN, in terms of percentage of the age-matched control level. It appears that the first exposure event of the previously unexposed P generation is crucial, and a memory of this exposure is subsequently transmitted to the future generations. Previous studies have shown that following the exposure of germ cells to a mutagen or carcinogen, an initiating event could be inherited by subsequent generations and revealed after postnatal exposure to mutagens, carcinogens or non-genotoxic agents (Nomura, 1983; Tomatis *et al.*, 1992). With respect to AEBN, the pattern of p53, Brca1 and Brca2 response during AEBN-induced carcinogenesis as elucidated by the chronic exposure study is vital in this regard. The chronic exposure study reveals that the p53 protein is upregulated to elicit tumor-suppression during the initiation of AEBN-induced carcinogenesis, while the Brca1 and Brca2 proteins are downregulated below control level; the promotion stage involves downregulation of p53 protein to control level, and the Brca1 and Brca2 proteins continue to be downregulated further below control level (Fig. 4.1). Since AEBN is a general carcinogen capable of affecting various tissues, it is likely that exposure of P generation parental mice causes an alteration in p53, Brca1 and Brca2 protein levels of their germ cells. The previously reported genotoxic potential of arecoline in mouse germ cells (Sinha and Rao, 1985a; Sinha and Rao, 1985b) supports this assumption. The P generation mice were exposed to AEBN for 6 weeks prior to mating to raise the F1 mice. Thus, AEBN-induced carcinogenesis would have been initiated during the 6 weeks of exposure prior to mating (Wary and Sharan, 1988; Wary and Sharan, 1991),

and the initiating event could be inherited by F1 progeny through the germ cells. Subsequent exposure of the F1 progeny to AEBN postnatally would immediately induce promotion followed by progression, leading to the observed 50 % advancement in the period of preneoplastic nodule appearance from the P to the F1 generation (Table 15). Similarly, the germ cells of the F1 and F2 mice would inherit a promoting event, hence, the progressive advancement of period of preneoplastic nodule development from F1 to F2 generations, and from F2 to F3 generations (Table 15). Keeping in view the reported transplacental effect of arecoline (Sinha and Rao, 1985b) another plausible explanation for the observed reults would be the transplacental exposure of the F1 fetus to AEBN, or, components derived from AEBN, leading to the initiation of AEBN-induced carcinogenesis at the fetal stage, and consequently an advancement in the period of preneoplastic nodule appearance in the successive generations of transgenerationally exposed mice.

PCR amplification and analysis of the DNA sequences thus obtained revealed that exons 5 and 7 of the *p53* gene (Figs. 3.37 & 3.39; Appendices I and II), and the targeted segment of exon 27 of the *Brca2* gene (Fig. 3.52; Genbank accession # FJ825143) continued to remain unmutated in the F1, F2 and F3 mice exposed transgenerationally to AEBN. However, exon 11 of the *Brca1* gene was found to be mutated in the solid tumors as well as liver nodules developing in mice exposed transgenerationally to AEBN (Fig. 3.43; Appendices III and IV). Exon 11 of the *Brca1* gene codes for the RAD51-interaction domain of the BRCA1 protein which is pivotal in its role in DNA repair (Welsch *et al.,* 2000; Huber *et al.,* 2001). The mutation observed was the base-substitution mutation G→C, with the corresponding codon change TGT → TCT (Fig. 3.44) and the resultant amino acid-substitution cysteine (Cys) 41 → serine (Ser) 41 (Appendix IV). Molecular modeling was used to study the bonding/s of the thiol (-SH) group of Cys, and the hydroxy (-OH) group of Ser, with the neighboring amino acids in the Brca1 protein. The Cys and Ser residues assumed three different orientations known as rotamers (Figs. 3.53 through 3.57). The hydrogen bonding specificities were found to be altered between the wild-type and mutant proteins in rotamers 2 and 3 of the model as summarized in Table 17.

Table 17: Alterations in hydrogen bonding specificities between wild-type and mutant Brca1 proteins as revealed by molecular modeling

Rotamer	Protein	H-bond	Bond length	Bond angle
2	Wild-type	Cys41- Asp38	2.19 Å	66.07°
	Mutant	Ser41-Asp38	2.34 Å	70.70°
3	Wild-type	Cys41- Cys37	Bond absent	
	Mutant	Ser41-Cys37	Bond present	

In rotamer 2, the bond length (Fig. 3.54) and bond angle (Fig. 3.57) were altered between the wild-type and mutant proteins (Table 17), and in rotamer 3 a hydrogen bond was formed only by the -OH group of Ser in the mutant protein, and not the –SH group of Cys in the wild-type (Fig. 3.56 and Table 17). These alterations suggest a conformational change in the RAD51-interaction domain of the mutant Brca1 protein, which could possibly alter its interaction with RAD51, and hence, its role in DNA repair. It is, therefore, likely that the mutation in exon 11 of the *Brca1* gene observed in mice exposed transgenerationally to AEBN could contribute to a disruption of Brca1 function in these mice. The mutation would also be transmitted by the germ cells of the mice to the future generations, resulting in the transmission of carcinogenic risk.

It can, thus, be hypothesized that the abrogated tumor-suppressor responses of the p53, Brca1 and Brca2 proteins, as well as mutation of exon 11 of the *Brca1* gene are responsible for the increased predisposition to cancer of mice exposed prenatally/ transgenerationally to AEBN, when they are postnatally challenged with the same dose of AEBN as the previously unexposed P generation mice.

In the chronic exposure as well as transgenerational exposure studies, alterations in the levels of the p53, Brca1 and Brca2 tumor suppressor proteins were determined by slot-blotting in order to ensure accuracy of quantitative densitometry analysis. However, slot-blotting involves the blotting of total cellular protein, and it was essential to verify whether the results obtained indeed pertained to the respective proteins in question. Thus, Western blotting was performed for the p53, Brca1 and Brca2 proteins (Figs. 3.27 through 3.35). The alterations observed by Western blotting were found to agree with those determined by slot-blotting. Thus, the results obtained by slot-blotting could

be verified by Western blotting. It was also essential to support the results obtained for p53, Brca1 and Brca2 proteins by the use of appropriate and sensitive loading control/s. The use of India-ink for staining total protein, and, as a loading control has been reported previously (Sharan *et al.*, 2005; Kma and Sharan, 2006). India-ink was, therefore, used as a loading control for the slot-blotting and Western-blotting experiments performed in this study. AEBN-induced carcinogenesis was primarily manifested by the appearance of preneoplastic nodules of the liver, which was closely associated with crucial alterations in the levels of p53, Brca1 and Brca2 proteins in the liver. Thus, these alterations were verified by employing the use of actin as a loading control, in addition to the India-ink.

The use of the *p53*, *BRCA1* and *BRCA2* tumor suppressor genes as biomarkers of various cancers in humans have been widely reported (Harris and Hollstein, 1993; Pich, 1998; Wilson *et al.*, 1999; Yoshikawa *et al.*, 1999; Zheng *et al.*, 2000; Beger *et al.*, 2004). The methods currently used include serological tests, genetic analysis and immunohistological detection in cytologic specimens, exfoliated cells, needle-biopsy specimens and fixed tissues (Harris and Hollstein, 1993; Pich, 1998). Most of these methods involve an invasive procedure of obtaining samples for the relevant tests. Results obtained through our study with mice indicate that the levels of the p53, Brca1 and Brca2 proteins were altered from the early stages of carcinogenesis onwards, and varied as the cancer progressed. While the cancer primarily affected the liver, the alterations in the levels of these tumor suppressor proteins were also reflected by the PBL. It was evident that alterations in the levels of the p53, Brca1 and Brca2 proteins in the PBL of mice could serve as an indicator of the progress of a cancer. The use of PBL seperated from whole blood ensures that the method is minimally invasive, while the slot-blotting technique employed is fairly rapid. Therefore, it was of interest to determine whether a similar approach could be used for detecting alterations in the levels of p53, BRCA1 and BRCA2 proteins in cancer patients. This would raise the possibility of potentially developing a rapid, blood-based technique for the use of the p53, BRCA1 and BRCA2 tumor suppressor proteins as biomarkers of different human cancers.

Our results show significant upregulated level of p53 protein in PBL of all breast cancer patients (Fig. 3.61), cervix cancer patients (Fig. 3.62) and on average, for all

head and neck cancer cases studied (Figs. 3.59 and 3.60). However, the head and neck cancers displayed variability in the level of p53 protein, concurrent with the site of the cancer. Cancer at some sites in the head and neck cancer category resulted in an elevated level of p53 protein in comparison to healthy controls, indicating an upregulation of p53 in these patients (Figs. 3.59 & 3.60). Though not experimentally verified, cancers showing an increase in p53 protein in comparison to control level may involve mutation of the p53 gene, as accumulation of p53 protein has been reported to be consistent with mutant forms of p53 (Harris and Hollstein, 1993). However, patients included in our study with head and neck cancer at other sites showed control levels of p53 (Figs. 3.59 and 3.60). All these patients presented with advanced cases, suggesting an alternate route of p53 inactivation in these cases, such as MDM2 overexpression (Shwe *et al.,* 2001) or loss of heterozygosity. We observed a downregulation of BRCA1 protein in comparison to control, only in PBL of breast cancer patients (Fig. 3.64), while patients with head and neck cancer (Fig. 3.63) and cervix cancer showed an elevation of BRCA1 (Fig. 3.65). A similar pattern of response was also observed in case of BRCA2 protein, with downregulation of BRCA2 in PBL of breast cancer patients (Fig. 3.67), and elevation in head and neck cancer (Fig. 3.66) and cervix cancer (Fig. 3.68). Our results, therefore, indicate a tissue-specific response of the BRCA1 and BRCA2 proteins. The alterations observed in this study pertaining to the levels of p53, BRCA1 and BRCA2 proteins in different human cancers, in comparison to the respective control level may thus be summarized as follows (Table 18).

Table 18: Summary of alterations in the levels of P53, BRCA1 and BRCA2 proteins observed in different cancers

Cancer	P53	BRCA1	BRCA2
Head & neck (class I)	↑	↑	↑
Head & neck (class II)	↔	↑	↑
Breast	↑	↓	↓
Cervix	↑	↑	↑

↔ indicates invariant from control level
↑ indicates upregulation in comparison to control level
↓ indicates downregulation in conparison to control level

The study of the p53, BRCA1 and BRCA2 tumor suppressor proteins is especially relevant because they are intricately involved not only in the process of carcinogenesis, but also in the response to various modes of cancer therapy. The presence of a p53 missense mutation indicated by an overabundance of p53 protein is considered as an unfavorable prognostic factor for various cancers, such as cancer of the breast, lung and gastric cancer (Harris and Hollstein, 1993). It has been reported that p53 can regulate the sensitivity to cancer therapies by affecting the expression of drug targets, the access of drugs to intracellular targets, and the response to DNA damage. The two major types of microtubule-interfering agents are the *Vinca* alkaloids, including vinorelbine and vincristine, which act to destabilize microtubules, and the taxanes paclitaxel and docetaxel which promote microtubule polymerization and stabilization, ultimately leading to the disruption of mitosis and subsequent apoptosis. Mutations in the *p53* gene were reported to simultaneously increase the sensitivity to taxanes and decrease the sensitivity to vinca alkaloids by transcriptional regulation of MAP4 and stathmin which change the polymerization dynamics of tubulin, thereby, affecting the binding of drugs to microtubules (Hait and Yang, 2006). p53 also regulates the expression of multidrug resistance protein-1 (MRP1), a transporter that mediates the sensitivity to *Vinca* alkaloids and anthracycline drugs such as doxorubicin. p53 mutation increased the expression of MRP1, thereby producing significant resistance to vinblastine and doxorubicin. Mutation of p53 has also been reported to cause resistance to cisplatin (Perego *et al.,* 1996). DNA-damaging drugs cause DNA double strand breaks either directly or indirectly, and it is widely accepted that the absence of *BRCA1* expression leads to hypersensitivity of cells to DNA-damage based chemotherapy. Thus, abrogation of *BRCA1* protein expression in HBL100 breast cancer cells resulted in increased sensitivity to both cisplatin and etoposide. The presence of *BRCA1* has been reported to promote an increase in sensitivity to antimicrotubule agents (James *et al.,* 2007). Tumors that carry BRCA1 mutations have frequently been found to also carry p53 mutations. Moreover, mouse embryos with a conditional knockout of the *Brca1* gene and with wild-type *p53* genes die via a p53-dependent mechanism suggesting that loss of p53 function is required for a cell to tolerate loss of BRCA1 function, an observation that may be important when considering the response of cancer cells to DNA-damaging drugs (Kennedy *et al.,* 2004).

In light of the available information, the cancer of cervix and some sites (class I) of head and neck cancer studied (Table 18) had elevated level of p53 protein that is an indicator of resistance to cisplatin, doxorubicin and vinblastine. The same cancers also had elevated BRCA1 leading to resistance to DNA-damage based chemotherapy using agents like cisplatin. Thus, these cancers are likely to respond better to taxanes. Cancers of other sites of the head and neck region (class II) displayed control level of p53, which would indicate increased sensitivity to cisplatin, doxorubicin and vinblastine. These cancers, however, also had an elevated level of BRCA1 suggesting resistance to DNA-damaging agents. Thus, if the p53 protein level or BRCA1 protein level are taken individually as a biomarker, they give rise to a conflict with regards to the mode of therapy which is likely to be effective. Similarly, the breast cancer cases studied displayed elevated level of p53 indicating resistance to cisplatin, doxorubicin and vinblastine, while their BRCA1 levels were downregulated, suggesting increased sensitivity to cisplatin and other DNA-damage-inducing cheomotherapeutic agents. Thus, in these cases too, the p53 level or the BRCA1 level taken individually, cannot serve as a biomarker to assess an effective mode of therapy. It, therefore, follows that while the level of a tumor suppressor protein in PBL can serve as a good biomarker, a combination of more than one tumor suppressor would permit a more comprehensive understanding of the potential response of a cancer to different therapeutic agents, rather than the use of a single tumor suppressor.

The following conclusions can thus be drawn from the current study:-

Features of the study of chronic exposure of Swiss Albino mice to AEBN

1. Chronic exposure of Swiss Albino mice (P generation) to AEBN at a dose of 2 mg ml^{-1} in drinking water resulted in the formation of preneoplastic nodules in the liver after 16 weeks of exposure.

2. AEBN-induced carcinogenesis intricately involved coordinated alterations in the cellular level of p53, Brca1 and Brca2 tumor suppressor proteins. The p53 protein was upregulated to 2.5-fold over the age-matched control level after 6-8 weeks of exposure and Brca1 and Brca2 proteins were upregulated to 1.4 fold over the age-matched

control level after 2 weeks of exposure, indicating an initial protective response against the DNA damage induced by AEBN exposure.

3. Subsequently, the p53 protein declined to control level, and Brca1 and Brca2 proteins declined below control level indicating a loss of tumor suppressor functions.

4. The appearance of preneoplastic nodules of the liver was concomitant with the decline of p53 protein to control level, and the Brca1 and Brca2 proteins below control level indicating a loss of the protection tendered by these tumor suppressors.

5. Exons 5 and 7 of the *p53* gene, exon 11 of the *Brca1* gene and the amplified segment of exon 27 of the *Brca2* gene were not mutated in the liver nodules formed after 24 weeks of exposure to AEBN indicating that the loss of the function of these tumor suppressors upon chronic exposure to AEBN does not involve mutation of these critical regions of the gene

6. The liver nodules formed after 24 weeks of exposure to AEBN exhibit extensive disruption of the integrity of subcellular organelles, namely the nucleus, endoplasmic reticulum and mitochondria.

7. Thus, chronic exposure to AEBN led to carcinogenesis due to

- ***a.*** loss of control over cellular proliferation through loss of p53 function; and
- ***b.*** genomic instability because of loss of Brca1 and Brca2 functions.

8. Alterations in the levels of p53, Brca1 and Brca2 proteins observed in the liver were mirrored by the SC and the PBL.

Features of the study of transgenerational exposure of Swiss Albino mice to AEBN

1. The responses of p53, Brca1 and Brca2 tumor suppressors in F1, F2 and F3 mice subjected to transgenerational exposure to AEBN differed from that in P generation mice subjected to chronic exposure to AEBN.

2. In F1, F2 and F3 generations, the level of p53 protein remained at control level throughout the exposure period, while the levels of Brca1 and Brca2 proteins rapidly declined below control level after 2 weeks of exposure to AEBN without recording an initial increase.

3. The appearance of preneoplastic nodules of the liver was significantly advanced in transgenerationally exposed mice, appearing in F1 mice after 8 weeks of exposure, in F2 mice after 6 weeks of exposure, and in F3 mice after 4 weeks of exposure to AEBN. At these periods, the p53 protein was maintained at control level, while the Brca1 and Brca2 proteins were well below control level, as in the chronic exposure regime.

4. F1, F2 and F3 mice developed various anomalies including solid tumors which were observed in the P generation mice.

5. Exons 5 and 7 of *p53* gene, and the amplified segment of exon 27 of the *Brca2* gene were not mutated upon transgenerational exposure to AEBN in both liver nodules as well as solid tumors.

6. Exon 11 of *Brca1* gene was, however, mutated in F1, F2 and F3 mice. The mutation observed was a G→C transversion mutation.

7. Liver nodules of transgenerationally exposed mice exhibited extensive alteration of the ultrastructure of subcellular organelles. The mitochondrial index progressively decreased from P, F1, F2 through F3 generations, and also in comparison to control indicating progressive loss of apoptosis.

8. Transgenerational exposure to AEBN, thus, led to predisposition to cancer manifested by early appearance of liver preneoplastic nodules, loss of apoptosis and development of anomalies including solid tumors.

9. This predisposition to cancer was caused by abrogation of p53 response, and increasing genomic instability due to progressively rapid decline of Brca1 and Brca2 proteins, as well as mutation of *Brca1* gene.

10. Alterations in the levels of p53, Brca1 and Brca2 proteins observed in the liver were also mirrored by the SC and the PBL.

Features of the study of p53, BRCA1 and BRCA2 proteins as a potential biomarkers of cancer

1. Determination of levels of p53, BRCA1 and BRCA2 proteins in PBL as a biomarker of cancer can provide a fast, non-invasive, blood based test for the clinical management of cancer.

2. A combination of all three tumor suppressors is more effective as a biomarker of cancer, rather than the use of a single tumor suppressor.

REFERENCES

Al Rashid, S.T., Dellaire, G., Cuddihy, A., Jalali, F., Vaid, M., Coackley,C., Folkard, M., Xu, Y., Chen, B.P.C., Chen, D.J., Lilge, L.,Prise, K.M., Jones D.P.B., and Bristow,R.G. (2005) Evidence for the direct binding of phosphorylated p53 to sites of DNA breaks *in vivo, Cancer Research,* **65**, 10810-10821.

Altschul, Stephen, F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., Lipman, D. J., (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Research,* **25**, 3389-3402.

American Cancer Society. *Cancer Facts & Figures 2008*, Atlanta: American Cancer Society; 2008.

Anderson, M. W., Reynolds, S. H., Ming, Y. and Maronpot, R. M. (1992), *Environmental Health perspectives*, **98**, 13-24.

Asotra, K. and Sharan, R.N. (2008) Health hazards of chewing Areca nut, betel quid and Gutkha. *TRDRP Newsletter*, **10**, 6-8. (also in "Burning Issues" available online at www.trdrp.org/publications/Newsletters.asp)

Ausubel, F., Brent, R., Kinston, R. E., Moore, D. D., Seidman, J. G., Struhl, K. and Smith, J. A. (1995). **Short Protocols in Molecular Biology**, John Wiley & Sons, USA.

Autrup, H. (1993) Transplacental transfer of genotoxins and transplacental carcinogenesis, *Environmental Health Perspectives Supplements,* **101**, 33-38.

Bai, L. and Zhu, W-G. (2006) p53: Structure, functions and therapeutic applications, *Journal of Cancer Molecules*, **2**, 141-153.

Balachandran, B., and Sharan, R. N. (1995) Induction of mutations by different extracts of betel nut and radiation: Their implication in Carcinogenesis. In, **Radiation Research** 1895-1995, Hagen, U., Jung, H., and Streffer, C. (eds) pp 165, vol. 1, Würzburg, Germany.

Balmain, A., Gray, J. and Ponder, B. (2003) The genetics and genomics of cancer, *Nature Genetics supplement*, **33**, 238-244.

Barrett, J.C. (1993) Mechanisms of multistep carcinogenesis and carcinogen risk assessment, *Environmental Health Perspectives*, **100**, 9-20.

Beger, C., Ramadani, M., Meyer, S., Leder, G., Krüger, M., Welte, K., Gansauge, F. and Beger, H.G. (2004) Down-regulation of BRCA1 in chronic pancreatitis and sporadic pancreatic adenocarcinoma, *Clinical Cancer Research*, 10, 3780-3787.

Bettendorf, O., Schmidt, H., Staebler, A., Grobholz, R., Heinecke, A., Boecker, W., Hertle, L. and Semjonow, A. (2008) Chromosomal imbalances, loss of heterozygosity, and immunohistochemical expression of TP53, RB1, AND PTEN in intraductal cancer, intraepithelial neoplasia, and invasive adenocarcinoma of the prostrate, *Genes Chromosomes Cancer*, **47**, 565-72.

Bobba, R. and Khan, Y. (2003) Cancer in India-an overview, GOR Vol.5 No.4.

Boucher, B. J., and Mannan, N. (2002) Metabolic effects of the consumption of *Areca catechu, Addiction Biology*, **7**, 103-110.

Bøyum, A. (1968) Isolation of leucocytes from human blood-further observations. (Paper II),*Scandinavian Journal of Clinical Laboratory Investigation 21 Supplement*, **97**, 31-50.

Bøyum, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. (Paper IV), *Scandinavian Journal of Clinical Laboratory Investigation 21 Supplement*, **97**, 77-89.

Bradford, M. M., 1976. A rapid and sensitive method for quantification of microgram quantities of proteins using the principle of dye binding, *Analytical Biochemistry*, **72**, 248-254.

Byrski, T. (2003) Germline mutation of BRCA1 gene in Polish families with strong aggregation of breast and/or ovarian cancer based on coding sequence ananlysis using the SSCP method, *Annales Academiae Medicae Stetinensis*, **49**, 27-43.

Califano, J., van der Riet, P., Westra, W., Nawroz, H., Clayman, G., Piantadosi, S. *et al.* (1996) Genetic progression model for head and neck cancer: implications for field cancerization, *Cancer Research*, **56**, 2488–92.

Cano, A., Calasanz, M.J., Colomer, D., Piris, M.A., Ahn, N., Imhof, A., Caldas, C., Jenuwein, T. and Esteller, M. (2005) Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer, *Nature Genetics*, **37**, 391-400.

Center for Biomarkers in Imaging. www. biomarkers.org

Cetin-Atalay and Ozturk, M. (2000) p53 mutations as fingerprints of environmental carcinogens, *Pure and Applied Chemistry*, **72**, 995-999.

Chang, B., Liao, M., Kuo, M. Y., and Chen, C. (2001). Developmental toxicity of arecoline, the major alkaloid in betel nuts, in zebrafish embryos, *Birth Defects Research, Part A*, **70**, 28-36.

Chiang, S., Jiang, S., Wang, Y., Chiang, H., Chen, P., Tu, H., Ho, K., Tsai, Y., Chang, I., and Ko, Y. (2007) Characterization of arecoline-induced effects on cytotoxicity in normal human gingival fibroblasts by global gene expression profiling, *Toxicological Science*, **100**, 66-74.

Chiba, I., Muthumala, M., Yamazaki, Y., Zaman, A. U., Iizuka, T., Amemiya, A., Shibata, T., Kashiwazaki, H., Sugiura, C., and Fukuda, H. (1998) Characteristics of mutations in the *p53* gene of oral squamous-cell carcinomas associated with betel-quid chewing in Sri Lanka, *International Journal of Cancer*, **77**, 839-842.

Cho, W. C. S. (2007) Contribution of oncoproteomics to cancer biomarker discovery, *Molecular Cancer*, **6**, 25.

Choudhury, Y. and Sharan, R.N. (2009) Altered p53 response and enhanced transgeneration transmission of carcinogenic risk upon exposure of mice to betel nut, *Environmental Toxicology and Pharmacology*, **27**, 127-138.

Ciernikova, S., Tomka, M., Sedláková, O., Reinerová, M., Stevurková, V., Kovác, M., Cente, M., Ilenciková, D., Bella, V. and Zajac, V. (2003) The novel exon 11 mutation of BRCA1 gene in a high-risk family, *Neoplasma*, **50**, 403-407.

Cimino, G., Rapanotti, M. C., Sprovieri, T. and Elia, L. (1998) ALL1 gene alterations in acute leukemia: biological and clinical aspects, *Haematologica*, **83**, 350-357.

Coleman, W.B. and Tsongalis, G.J. (1995) Multiple mechanisms account for genomic instability and molecular mutation in neoplastic transformation, *Clinical Chemistry*, **41/5**, 644-657.

Corpet, F. (1988) Multiple sequence alignment with hierarchical clustering, *Nucleic Acids Research*, **16**, 10881-10890.

Croce, C. M. (2008) Molecular origins of cancer –Oncogenes and cancer. *New England Journal of medicine,* **358**, 502-511.

Culleré- Ullman, M.H. and Foltz, C.J. (1999) Body condition scoring: A rapid and accurate method for assessing health status in mice, *Laboratory Animal Science*, **49**, 319-323.

Czernin, J. and Phelps, M.E. (2002) Positron emission tomography scanning: current and future applications, *Annual Review of Medicine*, **53**, 89-112.

Daniels, M.J., Wang, Y., Lee, M. and Venkitaraman, A.R. (2004) Abnormal cytokinesis in cells deficient in the breast cancer susceptibility protein BRCA2, *Science*, **306**, 876-879.

Das, B.C., Gopalkrishna, V., Hedau, S. and Katiyar, S. (2000) Cancer of the uterine cervix and human papillomavirus infection, *Current Biology*, **78**, 52-63.

Deleo, A.B., Jay, G., Apella, E., Dubois, G.C., Law, L.W., and Old, L.J. (1979) Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse, *Proceedings of the National Academy of Science, U.S.A*, 1979, **76**, 2420-2424.

Deng, C-X (2006) BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution, *Nucleic Acids Research*, **34**, 1416-1426.

Detmer, S.A. and Chan, D.C. (2007) Functions and dysfunctions of mitochondrial dynamics, *Nature Reviews Molecular Cell Biology*, **8**, 870-879.

Diwan, B. A., Anderson, J. M., Ward, L. M., Henneman, J. R. and Rice, J. M. (1995) Transplacental carcinogenesis by cisplatin in F344/NCr rats: promotion of kidney tumors by postnatal administration of sodium barbital. *Toxicology and applied pharmacology,* **132**, 115-121.

Diwan, B. A., Kasprzak, K. S., and Rice, J. M. (1992) Transplacental carcinogenic effect of nickel (II) acetate in the renal cortex, renal pelvis and adenohypophysis of F3477/ NCr rats, *Carcinogenesis.* **13**, 1351-1357.

Diwan, B. A., Ohshima, M., and Rice, J. M. (1989) Effect of postnatal administration of tumour-promoting barbiturates on the development of tumours initiated by prenatal exposure of fetal rats and mice to N-alkylnitrosoureas. In: **Perinatal and Multigeneration Carcinogenesis.** Napalkov, N. P., Rice, J. M., Tomatis, L. and Yamasaki, H. (eds.), *IARC Scientific Publication,* **96**, 75-80.

Donoho, G., Brenneman, M.A., Cui, T.X., Donoviel, D., Vogel, H., Goodwin, E.H., Chen, D.J. and Hasty, P. (2003) Deletion of Brca2 exon 27 causes hypersensitivity to DNA crosslinks, chromosomal instability, and reduced life span in mice, *Genes Chromosomes Cancer*, **36**, 317-331.

Egger, G., Liang, G., Aparicio, A. and Jones, P.A. (2004) Epigenetics in human disease and prospects for epigenetic therapy, *Nature*, **429**, 457-464.

Eliyahu, D., Raz, A., Gruss, P., Givol, D. and Ore, M. (1984) Participation of p53 cellular tumour antigen in transformation of normal embryonic cells, *Nature*, **312**, 646-649.

Esteller, M. (2006) Epigenetics provides a new generation of oncogenes and tumour-suppressor genes, *British Journal of Cancer*, **94**, 179-183.

Evan, G. I. and Vousden,K. H. (2001) Proliferation, cell cycle and apoptosis in cancer, *Nature*, **411**, 342-348.

Fabbro, M., Schuechner, S., Au, W.W.Y. and Henderson, B.R. (2004) BARD1 regulates BRCA1 apoptotic function by a mechanism involving nuclear retention, *Experimental Cell Research*, **298**, 661-673.

Field, J.K. (1995) The role of oncogenes and tumour-suppressor genes in the aetiology of oral, head and neck squamous cell carcinoma, *Journal of the Royal Society of Medicine*, **88**, 35P-39P.

Forastiere, A., Koch, W., Trotti, A. and Sidransky, D. (2001) Head and Neck cancer, *The New England Journal of Medicine*, **345**, 1890-1900.

Fraga, M.F., Ballestar, E., Villar-Garea, A., Boix-Chornet, M., Espada, J., Bonaldi, T., Haydon, C., Ropero, S., Petrie, K., Iyer, N.G., Pérez-Rosado, A., Calvo, E., Lopez, J.A.,

Futamura, M., Arakawa, H., Matsuda, K., Katagiri, T., Saji, S., Miki, Y. and Nakamura, Y. (2000) Potential role of BRCA2 in mitotic checkpoint after phosphorylation by hBUBR1, *Cancer Research*, **60**, 1531-1535.

Gatenby, R.A. and Gillies, R.J. (2004) Why do cancers have high aerobic glycolysis? *Nature Reviews Cancer*, **4**, 891-899.

Ghosh, S., Lu, Y., Katz, A., Hu, Y., and Li, R. (2007) Tumor suppressor BRCA1 inhibits a breast cancer- associated promoter of the aromatase gene (CYP19) in human

adipose stromal cells, *Ameriacan Journal of Physiology, Endocrinology and Metabolism*, **292**, E246-E252.

Goan, Y. G., Chang, H. C., Hsu, H. K., Chou, Y. P., and Cheng, J. T. 2005. Risk of *p53* gene mutation in esophageal squamous cell carcinoma and habit of betel quid chewing in Taiwanese, *Cancer Science*, **96**, 758-765.

Goerttler, K., Loehrke, H., Schweizer, J., and Hease, B. (1980). Two-stage skin carcinogenesis by systemic initiation of pregnant mice with 7, 12-dimethylbenz[α]anthracene during gestation days 6-20 and postnatal promotion of the F1-generation with the phorbol ester 12- tetradecanoylphorbol-13-acetate, *Journal of Cancer Research and Clinical Oncology*, **98**, 267-275.

Hait, W.N. and Yang, J-M. (2006) The individualization of cancer therapy: the unexpected role of p53, *Transactions of the American Clinical and Climatological Association*, **117**, 85-101.

Hanahan, D and Weinberg, R. A. (2000) The hallmarks of cancer, *Cell*. **100**, 57-70.

Harris, C.C. (1991) Chemical and physical carcinogenesis: Advances and perspectives for the 1990s, *Cancer Research*, **51**, 5023s-5044s.

Harris, C.C. and Hollstein, M. (1993) Clinical implications of the p53 tumor suppressor gene. *New England Journal of Medicine*. 329, 1318-1327.

Hayat, M. A. (1985) **Basic techniques for Transmission Electron Microscopy**, Academic Press Inc, U.S.A.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C.C. (1991) p53 mutations in human cancers, *Science*, **253**, 49-53.

Hsieh, L. L., Wang, P. F., Chen, I. H., Liao, C. T., Wang, H. M., Chen, M. C., Chang, J.T., and Cheng, A. J. (2001) Characterisics of mutations of the *p53* gene in oral

squamous cell carcinoma associated with betel quid chewing and cigarette smoking in Taiwanese, *Carcinogenesis*, **22**, 1497-1503.

http://bibiserv.techfak.uni-bielefeld.de/genefisher/

http://en.wikipedia.org/wiki/P53

Huber, L.J., Yang, T.W., Sarkisian, C.J., Master, S.R., Deng, C-X., and Chodosh, L.A. (2001) Impaired DNA damage response in cells expressing an exon 11-deleted murine Brca1 variant that localizes to nuclear foci, *Molecular and Cellular Biology*, **21**, 4005-4015.

IARC (1985) Tobacco habits other than Smoking; Betel quid and Areca-nut chewing; and some related nitrosamines, In *Monographs on the evaluation of carcinogenic risks to humans*, **37**, 263.

IARC (2004) International agency for Research on Cancer (IARC) Summaries & Evaluations: Betel-quid and areca-nut chewing, **85**, 39.

James, C.R., Quinn, J.E., Mullan, P.B., Johnston, P.G. and Harkin, P., (2007). BRCA1, a potential predictive biomarker in the treatment of breast cancer, *The Oncologist*, **12**, 142-150.

Jaworski, M., Hailfinger. S., Buchmann. A., Herhenhahn, M., Hollstein, M., Ittrich, C. and Schwarz, M., (2005). Human p53 knock-in (hupki) mice do not differ in liver tumor response from their counterparts with murine p53, *Carcinogenesis*, **26**, 1829-1834.

Jenkins, J.R., Rudge, K. and Currie, G.A. (1984) Cellular immortalization by a cDNA clone encoding the transformation associated phosphoprotein p53, *Nature*, **312**, 651-654.

Jones, P. A., and Baylin, S. B. (2002) The fundamental role of epigenetic events in cancer, *Nature Reviews*, **3**, 415-428.

Jones, P.A. and Laird, P.W. (1999) Cancer epigenetics comes of age, *Nature Genetics,* **21**, 163-167.

Kannan, K., Munirajan, A. K. and Krishnamurthy, J. (1999) Low incidence of p53 mutation in betel quid and tobacco chewing-associated oral squamous carcinoma from India, *International Journal of Oncology*, **15**, 1133-1136.

Kennedy, R.D., Quinn, J.E., Mullan, P.B., Johnston, P.G. and Harkin, D.P. (2004). The role of BRCA1 in the cellular response to chemotherapy. *Journal of the National Cancer Institute*, **22**, 1659-1668.

Kerr, P. and Ashworth, A. (2001) New complexities for BRCA1 and BRCA2. *Current Biology*, **11**, R668-R676.

Kim, J. and Dang, C.V. (2006) Cancer's molecular sweet tooth and the Warburg effect, *Cancer Research,* **66**, 8927-8929.

King, M.W. (2008) www.themedicalbiochemistrypage.org

Kinzler, K.W. and Vogelstein, V. (1997) Gatekeepers and Caretakers, *Nature*, **386,** 761-763.

Kma L. 2003. Ph.D thesis entitled Study of molecular characteristics of chromosomes during chemically induced cellular transformation. p30.

Kma L. and Sharan, R.N. (2006) *In vivo* exposure of Swiss albino mice to chronic low dose of dimethylnitrosamine (DMN) lowers poly-ADP-ribosylation (PAR) of bone marrow cell and blood lymphocyte proteins, *Molecular and Cellular Biochemistry*, **288**, 143-149.

Knudson, A. G. (1971) Mutation and cancer: Statistical study of Retinoblastoma, *Proceedings of National Academy of Sciences, USA*, **68**, 820-823.

Kumpawat, K., Deb, S., Ray, S., and Chatterjee, A. (2003) Genotoxic effect of raw betel-nut extract in relation to endogenous glutathione levels and its mechanism of action in mammalian cells, *Mutation Research*, **538**, 1-12.

Kuttan, N. A. A., Rosin, M. P., Ambika, K., Priddy, R. W., Bhakthan, N. M. G., and Zhang, L. (1995) High prevalence of expression of p53 oncoprotein in oral carcinomas from India associated with betel and tobacco chewing, *European Journal of Cancer*, **31**, 169-173.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, **227**, 680-385.

Lane, D.P. and Crawford, L.V. (1979) T antigen is bound to a host protein in SV40 transformed cells, *Nature*, **278**, 261-263.

Lee, P., Chang, M., Chang, W., Wang, T., Wang, Y., Hahn, L., Ho, Y., Lin, C., and Jeng, J. (2006) Prolonged exposure to arecoline arrested human KB epithelial cell growth: Regulatory mechanisms of cell cycle and apoptosis, *Toxicology*, **220**, 81-89.

Levine, A.J. (1997) p53, the cellular gatekeeper of growth and division, *Cell*, **88**, 323-331.

Linzer, D.I. and Levine, A.J. (1979) Characterization of a 54 K Dalton cellular SV40 tumour antigen present in SV40-transformed cells cells and unifected embryomal carcinoma cells, *Cell*, **17**, 43-52.

Liu, Y. and West, S.C. (2002) Distinct functions of BRCA1 and BRCA2 in double-strand break repair, *Breast Cancer Research*, **4**, 9-13.

Loktionov, A., Popovich, I., Zabezhinski, M, Martel, N., Yamasaki, H., and Tomatis, L. (1992) Transplacental and transgeneration carcinogenic effect of 7, 12-dimethylbenz[α]anthracene: relationship with ras oncogene activation, *Carcinogenesis*, **13**, 19-24.

Lu, H., Guo, X., Meng, X., Liu, J., Allen, C., Wray, J., Nickoloff, J.A. and Shen, Z. (2005) The BRCA2-interacting protein BCCIP functions in RAD51 and BRCA2 focus formation and homologous recombinational repair, *Molecular and Cellular Biology*, **25**, 1949-1957.

Lu, X., Nikaido, T., Toki, T., Zhai, Y. L., Kita, N., Konishi, I. and Fujii, S. (2000) Loss of heterozygosity among tumor suppressor genes in invasive and in situ carcinoma of the uterine cervix, *International Journal of Gynecological Cancer,* **10**, 452-458.

MacLachlan, T.K., Dash, B.C., Dicker, D.T. and El-Deiry, W.S. (2000) Repression of BRCA1 through a feedback loop involving p53, *The Journal of Biological Chemistry*, **275**, 31869-31875.

Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M., Ding, W. et al (1994) A strong candidate for breast and ovarian cancer susceptibility gene BRCA1, *Science*, **266**, 66-71.

Min-min, H., Ming-rong, X., Ze-yi, C., Kai-xuan, Y. and Zhi-lin, S. (2006). Analysis of p53 codon 72 polymorphism and its association with human papillomavirus 16 and 18 E6 in Chinese cervical lesions, *International Journal of Gynecological cancer*, **16**, 2004-2008.

Modica-Napolitano, J. and Singh, K.K. (2002) Mitochondria as targets for detection and treatment of cancer. *Expert Reviews in Molecular Medicine*, 1-19.

Moll, U. M. and Petrenko. O. (2003). The MDM2-p53 interaction. *Molecular Cancer Research*, **1**, 1001-1008.

Monsonego, J., Bosch, F. X., Coursaget, P., Cox, J.T., Franco, E., Frazier, I., Sankaranarayanan, R., Schiller, J., Singer, A., Wright, T., Kinney, W., Meijer, C. and Linder, J. (2004) Cervical cancer control, priorities and new directions, *International Journal of Cancer*, **108**, 329-333.

Morillo-Arismendi, G.J. and Catellano-Ramirez, A.V. (2008) Ultrastructural mitochondrial pathology in human astrocytic tumors: potentials implications pro-therapeutics strategies, *Journal of Electron Microscopy*, **57**, 33-39.

Muller, D., Bonaiti-Pellié, C., Abecassis, J., Stoppa-Lyonnet, D. and Fricker, J.P. (2004) BRCA1 testing in breast and/or ovarian cancer families from northeastern France identifies two common mutations with a founder effect, *Familial Cancer*, **3**, 15-20.

Nadeau, G., Boufaied, N., Moisan, A., Lemieux, K.M., Cayanan, C., Monteiro, A.N.A. and Gaudreau.L. (2000) BRCA1 can stimulate gene transcription by a unique mechanism, *EMBO Reports*, **3**, 260-265.

Nair U, Bartsch H, Nair J. (2004) Alert for an epidemic of oral cancer due to use of the betel quid substitutes gutkha and pan masala: a review of agents and causative mechanism, *Mutagenesis,* **19**, 251-262.

Nair, J., Ohshima, H., Friesen, M., Croisy, A., Bhide, S. V., and Bartsch, H. (1985) Tobacco-specific and betel nut-specific N-nitroso compounds: occurrence in saliva and urine of betel quid chewers and formation *in vitro* by nitrosation of betel quid, *Carcinogenesis*, **6**, 295-303.

Nair, U.J., Floyd, R. A., Nair, J, Bussachini, V., Friesen, M. and Bartsch, H. (1987) Formation of reactive oxygen species and of 8-hydroxydeoxyguanosine in DNA in vitro with betel quid ingredients, *Chemico- Biological Interactions*, **63**, 157-169.

Napalkov, N., Likhachev, A., Anisimov, V., Loktionov, A., Zabezhinski, M., Ovsyannikov, A., Wahrendorf, J., Becher, H. and Tomatis, L. (1987) Promotion of skin tumors by TPA in the progeny of mice exposed prenatally to DMBA, *Carcinogenesis,* **8**, 381-385.

Narama, I., Imaida, K., Iwata, H., Nakae, D., Nishikawa, A. and Harada, T. (2003) A review of nomenclature and diagnostic criteria for proliferative lesions in the liver of

rats by a working group of Japanese Society of Toxicologic Pathology, *Journal of Toxicology and Pathology*, **16**, 1-17.

Nomura, T. (2006) Transgenerational effects of radiation and chemicals in mice humans, *Journal of Radiation Research,* **47**, B83-B97.

Nomura, T. (2008) Transgenerational effects from exposure to environmental toxic substances, *Mutation Research/ Reviews in Mutation Research*, **659**, 185-193.

Nomura. T. (1983) X-ray induced germ-line mutation leading to tumors. Its manifestation in mice given urethane post-natally, *Mutation Research,* **121**, 59-65.

Odyuo, M. M. and Shran, R. N. (2005) Differential DNA strand breaking abilities of °OH and ROS generating radiomimetic chemicals and γ-rays: Study of plasmid, pMTa4, in vitro. Free Radical Research, **39**, 499-505.

Oesterrich, H. and Fuqua, S.W. (1999) Tumor suppressor genes in breast cancer, *Endocrine-Related Cancers,* **6**, 405-419.

Panigrahi, G. B., and Rao, A. R. (1989) Study of the genotoxicity of the total aqueous extract of betel nut and its tannin, *Carcinogenesis,* **7**, 37-39.

Pariat, T. and Sharan, R. N. (1998a) Betel nut and diethylnitrosamine induced conformational changes in the liver HMG proteins *in vivo*. In, **Trends in Radiation and Cancer Biology**, Sharan, R. N. (ed) pp 162-165, vol. 29. International co-operation bilateral seminars series, Forschungszentrum Juelich GmbH, Germany.

Pariat, T. and Sharan, R. N. (1998b) Qualitative change in mice liver HMG proteins after low dose chronic administration of aqueous extract of betel nut and diethynitrosamine, *Hepatology Research*, **12**, 177-185.

Parkin, D. M., Bray, F., Ferlay, J. and Pisani, P. (2005) Global Cancer Statistics, 2002, *CA: A Cancer Journal for Clinicians,* **55**, 74-108.

Pegoraro, R.J., Rom, L., Lanning, P.A., Moodley, M., Naiker, S. and Moodley, J. (2002) P53 codon 72 polymorphism and human papillomavirus type in relation to cervical cancer in South African women, *International Journal of Gynecological Cancer*, **12**, 383-388.

Perego, P., Giarola, M., Righetti, S.C., Supino, R., Caserini, C., Delia, D., Pierotti, M.A., Miyashita, T., Reed, J.C. and Zunino, F. (1996) Association between cisplatin resistance and mutation of *p53* gene and reduced Bax expression in ovarian carcinoma cell systems, *Cancer Research*, **56**, 556-562.

Perez-Ordoñez, B., Beauchemin, M. and Jordan, R.C.K. (2006) Molecular biology of squamous cell carcinoma of the head and neck, *Journal of Clinical Pathology*, **59**, 445-453.

Pich, A. (1998) p53 expression, proliferative activity and prognosis in cancer, *The Cancer Journal*, **11**, 223-227.

Pillai, M.R., Sreevidya, S., Pollock, B.H., Jayaprakash, P.G. and Herman, B. (2002) Polymorphism at codon 72 of p53, human papillomavirus, and cervical cancer in South India, *Journal of Cancer Research and Clinical Oncology*, **128,** 627-631.

Pitot, H.C., Neveu, M. J., Hully, J. H., Sargent, L., Paul, D. and Nicholson, B. (1991) Gene activation and deactivation during multistage hepatocarcinogenesis in rat. In, **Chemical Carcinogenesis 2: Modulating Factors**, Plenum Press, New York.

Ponder, B.A.J. (2001) Cancer Genetics, *Nature*, **411**, 336-411.

Powell, S.N. and Kachnic, L.A. (2003) Roles of BRCA1 and BRCA2 in homologous recombination, DNA replication fidelity and the cellular response to ionizing radiation, *Oncogene*, **22**, 5784-5791.

Rajan, J.V., Wang, M., Marouis, S.T. and Chodosh, L.A. (1996) Brca2 is coordinately regulated with Brca1 during proliferation and differentiation in mammary epithelial cells, *Proceedings of National Academy of Sciences, USA*, **93**, 13078-13083.

Ralhan, R., Agarwal, S., Nath, N., Mathur, M., Wasylyk, B., and Srivastava, A. (2001) Correlation between p53 gene mutations and circulating antibodies in betel- and tobacco-consuming North Indian population, *Oral Oncology*, **37**, 243-250.

Ranasinghe, A. W., Warnakulasuriya, K. A. A. S., and Johnson, N. W. (1993) Low prevalence of expression of p53 oncoprotein in oral carcinomas from Sri Lanka associated with betel and tobacco chewing, *European Journal of Cancer*, **29**, 147-150.

Ratcliffe N. A. (1983) **Practical Illustrated Histology**, Macmillan Press, p 23-24.

Redner, R.L., Wang, J., and Liu, J.M. (1999), Chromatin modeling and leukemia: new therapeutic approaches, *Blood*, **94**, 417-428.

Rice, J. M., Rehm, S., Donovan, P. J. and Perantoni, A. O. (1989) Comparitive transplacental carcinogenesis by directly acting and metabolism dependent alkylating agents in rodents and nonhuman primates. In: **Perinatal and Multigeneration Carcinogenesis.** Napalkov, N. P., Rice, J. M., Tomatis, L. and Yamasaki, H. (eds.), *IARC Scientific Publication*, **96**, 17-34.

Risch, H.A., McLaughlin, J.R., Cole, D.E.C., Rosen, B., Bradley, L., Kwan, E., Jack, E., Vesprini, D.J., Kuperstein, G., Abrahamson, J.L.A., Fan, I., Wong, B. and Narod, S.A. (2001) Prevalence and penetrance of germline BRCA1 and BRCA2 mutations in a population series of 649 women with ovarian cancer, *The American Journal of Human Genetics*, **68**, 700-710.

Rosenberg, I. M. 1996.Getting started with protein purification. In, **Protein Analysis and Purification, Bench top Techniques**, Birkhäuser, Boston, 99-109.

Ruddon, R.W. (2003) Biochemistry of cancer-Historical perspectives. In, **Cancer Medicine**, United States BC Decker Inc., 2003.

Ruddon, R.W. (2007) The Biochemistry and Cell Biology of Cancer. In, **Cancer Biology**, Oxford University Press, USA. pp 118.

Sambrook, J. and Russell, D. W. (2001). **In, Molecular Cloning: A laboratory manual.** New York, Cold Spring Harbor Laboratory Press.

Saxena, S., Chakraborty, A., Kaushal, M., Kotwal, S., Bhatanager, D., Mohil, R.S., Chintamani, C., Aggarwal, A.K., Sharma, V.K., Sharma, P.C., Lenoir, G., Goldgar, D.E. and Szabo, C.I. (2006) Contribution of germline BRCA1 and BRCA2 sequence alterations to breast cancer in Northern India, *BMC Medical Genetics*, **7**, 75.

Scully, R. and Livingston, D.M. (2000) In search of the tumour-suppressor functions of BRCA1 and BRCA2, *Nature*, **408**, 429-432.

Sharan, R. N. (1994) Biochemical investigation of carcinogenic potency of betel nut (*Kwai*) of north-east India. In, **Oral Oncology**, pp. 190-193, vol. III, New Delhi: Mac Millan India Ltd.

Sharan, R. N., and Wary, K. K. (1992) Study of unscheduled DNA synthesis following exposure of human cells to arecoline and extracts of betel nut *in vitro*, *Mutation Research,* **278**, 271-276.

Sharan, R. N., Devi, B. J., Humtsoe, J. O., Kma, L., 2005. Detection and quantification of poly-ADP-ribosylated cellular proteins of spleen and liver tissues of mice in vivo by slot and Western blot immunoprobing using polyclonal antibody against mouse ADP-ribose polymer, *Molecular and Cellular Biochemistry*, **278**, 213-221.

Sharan, R.N. (1996) Association of betel nut with carcinogenesis, *The Cancer Journal,* **9**, 13-19.

Shiraishi, H., Okamoto, H., Yoshimura,A. and Yoshida,H. (2006) ER-stress induced apoptosis and caspase-12 activation occurs downstream of mitochondrial apoptosis involving Apaf-1, *Journal of Cell Science*, **119**, 3958-3966.

Shwe, M., Chiguchi, G., Yamada, S., Nakajima, T., Maung, K.K, Takagi, M., Amagasa, T. and Tsuchida, N. (2001) P53 and MDM2 co-expression in tobacco and

betel-chewing associated oral squamous cell carcinomas, *Journal of Medical and Dental Science*, **48**, 113-119.

Silva, J.M., Gonzalez, R., Provencio, M., Dominguez, G., Garcia, J.M., Gallego, I, Palacios, J., España, P. and Bonilla, F. (1999) Loss of heterozygosity in BRCA1 and BRCA2 markers and high-grade malignancy in breast cancer, *Breast Cancer Research and Treatment*, **53**, 9-17.

Sinha, A., and Rao, A. R. (1985a) Embryotoxicity of betel nuts in mice, *Toxicology*, **37**, 315-326.

Sinha, A., and Rao, A. R. (1985b). Induction of shape abnormality and unscheduled DNA synthesis by arecoline in the germ cells of mice, *Mutation Research*, **158**, 189-192.

Sinha, A., and Rao, A. R. (1985c). Transplacental micronucleus inducing ability of arecoline, a betel nut alkaloid in mice, *Mutation Research,* **158**, 193-194.

Sowter, H.M. and Ashworth, A. (2005) BRCA1 and BRCA2 as ovarian cancer susceptibility genes, *Carcinogenesis,* **26**, 1651–1656.

Staib, F., Hussain, S.P., Hofsth, L.J., Wang, X.W. and Harris, C.C. (2003) TP53 and liver carcinogenesis, *Human Mutation,* **21**, 201-216.

Stewart, Z.A. and Pietenpol, J.A. (2001) p53 signaling and cell cycle checkpoints, *Chemical Research in Toxicology*, **14**, 243-263.

Stich HF and Anders F. (1989) The involvement of reactive oxygen species in oral cancers of betel quid/tobacco chewers, *Mutation Research*, 214, 47-61.

Storey, A., Thomas, M., Kalita, A., Harwood, C., Gardiol, D., Mantovani, F., Breuer, J., Leigh, I.M., Matlashewski, G. and Banks, L. (1998) Role of a p53 polymorphism in the development of human papillomavirus-associated cancer, *Nature*, **393**, 229-234.

Sugimura, T., Terada, M., Yokota, J., Hirohashi, S., and Wakabayashi, K. (1992) Multiple genetic alterations in cancer, *Environmental Health perspectives*, **98**, 5-12.

Sundqvist, K., Liu, Y., Nair, J., Bartsch, H., Arvidson, K., Grafström, R.C. (1989) Cytotoxic and genotoxic effects of areca nut-related compounds in cultured human buccal epithelial cells, *Cancer Research,* **49**, 5294-5298.

Tel-zur, N., Abbo, S., Myslabodski, D. and Mizrahi, Y. (1999) Modified CTAB procedure for DNA isolation from epiphytic cacti of the genera *Hylocereus* and *Selenicereus* (Cactaceae), *Plant Molecular Biology Reporter*, **17**, 249-254.

Terzakis, J.A. (1968) Uranyl acetate a stain and a fixative, Journal of Ultrastructural Research, **22**, 168.

Thangaraju, M., Kaufmann, S.H., and Couch, F.J. (2000) BRCA1 facilitates stress-induced apoptosis in breast and ovarian cancer cell lines, *Journal of Biological Chemistry*, **275**, 33487-33496.

Thomas, S., Brennan, J., and Martel, G. (1994) Mutations in the conserved regions of p53 are infrequent in betel-associated oral cancers from Papua New Guinea, *Cancer Research*, **54**, 3588-3593.

Tomatis, L. (1994) Transgeneration Carcinogenesis: A review of the experimental and epidemiological evidence, *Japanese Journal of Cancer Research*, **85**, 443-454.

Tomatis, L., Narod, S. and Yamasaki, H. (1992) Transgeneration transmission of carcinogenic risk, *Carcinogenesis,* **13**, 145-151.

Tommasi, S., Crapolicchio, A., Lacalamita, R., Bruno, M., Monaco, A., Petroni, S., Schittulli, F., Longo, S., Digennaro, M., Calistri, D., Mangia, A. and Paradiso, A. (2005) BRCA1 mutations and polymorphisms in a hospital-based consecutive series of breast cancer patients from Apulia, Italy, *Mutation research*, **578**, 395-405.

Towbin, J., Staehelin, T., Gordon, J. (1979). Electrophorectic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, *Proceedings of National Academy of Sciences, USA*, **76,** 4350-4354.

Vähäkangas, K. (2003) TP53 mutations in workers exposed to occupational carcinogens. *Human Mutation*, **21**, 240-251.

Valarmathi, M.T., Sawhney, M., Deo, S. S., Shukla, N. K. and Das, S. N. (2004) Novel germline mutations in the BRCA1 and BRCA2 genes in Indian breast and breast-ovarian cancer families, *Human Mutation*, **23**, 205.

Velculescu, V.E. and El-Deiry, W.S. (1996) Biological and clinical importance of the p53 tumor suppressor gene, *Clinical Chemistry,* **42**, 858-868.

Venkitaraman, A.R. (2002) Cancer susceptibility and the functions of BRCA1 and BRCA2, *Cell*, **108**, 171-182.

Vokes, E.E., Weichselbaum, R.R., Lippman, S.M. and Hong, W.K. (1993) Head and Neck Cancer, *The New England Journal of Medicine,* **328**, 184-194.

Vorobtsova, I. and Kitaev. E. (1988) Urethane-induced lung adenomas in the first-generation progeny of irradiated male mice, *Carcinogenesis,* **9**, 1931-1934.

Waalkes, M. P., Ward, J. M. and Diwan, B. A. (2004) Induction of tumors of the liver, lung, ovary and adrenal in adult mice after brief maternal gestational exposure to inorganic arsenic: promotional effects of postnatal phorbol ester exposure on hepatic and pulmonary, but not dermal cancers, *Carcinogenesis,* **25**, 133-141.

Wang, H., Zeng, Z-C, Bui, T-A, DiBiase, S.J., Qin, W., Xia, F., Powell, S.N. and Iliakis, G. (2000) Nonhomologous end-joining of ionizing radiation-induced DNA double-starnded breaks in human tumor cells deficient in BRCA1 or BRCA2, *Cancer Research*, **61**, 270-277.

Wang, Q., Zhang, H., Fishel, R. and Greene, M.I. (2000) BRCA1 and cell signaling. *Oncogene*, **19**, 6152-6158.

Warnakulasuriya, S. (2002) Areca nut use: an independent risk factor for oral cancer, *British Medical Journal*, **324**, 799–800.

Wary, K. K. and Sharan, R. N. (1988) Aqueous extract of betel-nut of North-East India induces DNA strand breaks and enhances rate of cell proliferation *in vitro*. *Journal of Cancer Research and Clinical Oncology*, **114**, 579-582

Wary, K. K., and Sharan, R. N. (1991) Cytotoxic and cytostatic effects of arecoline and sodium nitrite on human cells *in vitro*, *International Journal of Cancer*, **47**, 396-400, 1991.

Weinstein, I.B. (1988) The origins of human cancer: molecular mechanisms of carcinogenesis and their implications for cancer prevention and treatment-Twenty-seventh G. H. A. Clowes Memorial Award Lecture, *Cancer Research*, **48**, 4135-4143.

Weinstein, I.B. (2000) Disorders in circuitry during multitage carcinogenesis: the role of homeostasis, *Carcinogenesis*, **21**, 857-864.

Weiss, J.R., Moysich, K.B. and Swede, H. (2005) Epidemiology of male breast cancer, *Cancer Epidemiology, Biomarkers and Prevention*, **14**, 20-26.

Welsch, P.L. and King, M-C. (2001) BRCA1 and BRCA2 and the genetics of breast and ovarian cancer, *Human Molecular Genetics*, **10**, 705-713.

Welsch, P.L., Owens, K.N. and King, M-C (2000) Insights into the functions of BRCA1 and BRCA2, *Trends in Genetics*, **16**, 69-74.

Willis, A., Jung, E. J., Wakefield, T., and Chen, X. (2004) Mutant p53 exerts a dominant negative effect by preventing wild-type p53 from binding to the promoter of its target genes, *Oncogene*, **23**, 2330-2338.

Wilson, C.A., Ramos, L., Villaseñor, M.R., Anders, K.H., Press, M.F., Clarke, K., Karlan, B., Chen, J.J., Scully, R., Livingston, D., Zuch, R.H., Kanter, M.H., Cohen, S., Calzone, F.J. and Slamon, D.J. (1999) Localization of human BRCA1 and its loss in high-grade, non-inherited breast carcinomas, *Nature Genetics*, **21**, 236-240.

Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N., Gregory, S., Gumbs, C. and Micklem, G. (1995) Identification of the breast cancer susceptibility gene BRCA2, *Nature*, **378**, 762-763.

Wu, K., Jiang, S-W and Couch, F.J. (2003) p53 mediates repression of the BRCA2 promoter and down-regulation of BRCA2 mRNA and protein levels in response to DNA damage, *The Journal of Biological Chemistry*, **278**, 15652-15660.

www.expasy.ch/sprot/

www.expasy.ch/tools/#translate

www.ncbi.nlm.nih.gov/sites/entrez

www.oncologychannel.com/headneck/index.shtml

Xu, C., Bailly-Maitre, B. and Reed, J.C. (2005) Endoplasmic reticulum stress: cell life and death decisions. *Journal of Clinical Investigation,* **115**, 2656–2664.

Yager, J.D., and Davidson, N.E. (2006) Estrogen carcinogenesis in breast cancer, *New England Journal of Medicine*, **354**, 270-282.

Yamasaki, H., Hollstein, M., Martel, N., Cabral, J. R. P., Galendo, D. and Tomatis, L. (1987) Transplacental induction of a specific mutation in fetal Ha-ras and its critical role in post-natal carcinogeensis, *International Journal of Cancer*, **40**, 818-822.

Yamasaki, H., Loktionov, A., and Tomatis, L. (1992) Perinatal and multigeneration effect of carcinogens: possible contribution to determination of cancer susceptibility, *Environmental Health Perspectives,* **98**, 39-43.

Yamashita, T., Yaginuma, Y., Saitoh, Y., Kawai, K., Kurakane, T., Hayashi, H. and Ishikawa, M. (1999) Codon 72 polymorphism of p53 as a risk factor for patients with human papillomavirus-associated squamous intraepithelial lesions and invasive cancer of the uterine cervix, *Carcinogenesis*, **20**, 1733-1736.

Yoshikawa, K., Honda, K., Inamoto, T., Shinohara, H., Yamauchi, A., Suga, K., Okuyama, T., Shinmada, T., Kodama, H., Noguchi, S., Gazdar, A.F., Yamaoka, Y. and Takahashi, R. (1999) Reduction of BRCA1 protein expression in Japanese sporadic breast carcinomas and its frequent loss in BRCA1-associated cases, *Clinical Cancer Research*, **5**, 1249-1261.

Zheng, W., Luo, F., Lu, J.J., Baltayan, A., Press, M.F., Zhang, Z-F. and Pike, M.C. (2000) Reduction of BRCA1 expression in sporadic ovarian cancer, *Gynecologic Oncology*, **3**, 294-300.

Zink, D., Fischer, A.H. and Nickerson, J.A. (2004) Nuclear structure in cancer cells, *Nature Reviews Cancer*, **4**, 677-687.

PUBLICATIONS

Environmental Toxicology and Pharmacology 27 (2009) 127–138

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Altered p53 response and enhanced transgenerational transmission of carcinogenic risk upon exposure of mice to betel nut

Yashmin Choudhury, Rajeshwar N. Sharan *

Radiation and Molecular Biology Unit, Department of Biochemistry, North-Eastern Hill University, Umshing, Shillong 793022, India

ARTICLE INFO

Article history:
Received 27 May 2008
Received in revised form 13 August 2008
Accepted 10 September 2008
Available online 23 September 2008

Keywords:
Aqueous extract of betel nut (AEBN)
p53
Exons 5 and 7
Transgenerational carcinogenesis, Swiss albino mice

ABSTRACT

Alteration of p53 protein level, and possible mutation of the *p53* gene during carcinogenesis in mice exposed chronically (P) and transgenerationally to 2 mg/ml aqueous extract of betel nut (AEBN) in drinking water, were studied. Exons 5 and 7 of the *p53* gene were not mutated under both chronic and transgenerational exposure, but, p53 protein response was altered. In P mice, p53 protein was initially upregulated in comparison to age-matched controls, reaching 2.5 folds in the liver after 6 weeks of exposure. Subsequently, p53 protein declined to control level after 16 weeks, with concomitant preneoplastic nodulation of the liver. After 24 weeks, p53 protein was below control level, and preneoplastic nodules were well-developed. The level of p53 protein in transgenerationally exposed mice remained invariant in comparison to age-matched controls. Liver nodulation was significantly advanced, developing in F1 mice after 8 weeks, F2 mice after 6 weeks and F3 mice after 4 weeks of exposure. Anomalies not observed in P mice, developed in transgenerationally exposed mice, albeit, non-significantly. Thus, AEBN exposure enhanced transgeneration transmission of carcinogenic risk.

1. Introduction

Betel nut (BN), *Areca catechu* L. is a commonly used masticatory which is consumed by over 600 million individuals world-wide (Sharan, 1996). The habit of BN chewing is believed to be strongly associated with cancers of the mouth, oropharyngeal cavity, and upper parts of the digestive tract in humans (Sharan, 1996; IARC, 1985, 2004). The genotoxic and cytotoxic effects of BN powder, aqueous extract of betel nut (AEBN), its primary alkaloid, arecoline, and/or their nitroso derivatives, have been reported (Sharan and Wary, 1992; Wary and Sharan, 1988, 1991; Saikia et al., 1999). AEBN was previously found to induce strand breaks in DNA of mouse kidney cells (Sharan and Wary, 1992), unscheduled DNA synthesis (UDS) in Hep-2 cells *in vitro* and enhanced rate of cell proliferation (Wary and Sharan, 1991). Teratogenic effects of chronic BN and arecoline exposures have also been reported in mice and rats (Sharan, 1996). Arecoline was reported to cause general developmental retardation of Zebra fish embryos predominantly due to a general cytotoxic effect induced by depletion of intracellular thiols (Chang et al., 2001). Furthermore, arecoline was reported to induce abnormality in the shape of sperm heads and UDS in the early spermatid stages of Swiss albino mice (Sinha and Rao, 1985a) and, to induce micronuclei formation in fetal mouse blood after transplacental exposure (Sinha and Rao, 1985b). Experimental and epidemiological observations indicate that prezygotic exposure to a carcinogen or mutagen may lead to an increased risk of cancer in the progeny (Tomatis, 1994; Tomatis et al., 1992). Various studies have reported an increase of tumor incidence in the offsprings of parents exposed to carcinogens prior to conception (Newbold et al., 1998; Mohr et al., 1999; Nomura, 2006). Studies also suggest that while a susceptibility to cancer is inherited by the initial exposure of germ cells, the postnatal environment plays a crucial role in the subsequent development of cancer in the offspring, and the consequences of germ cell exposure are revealed by postnatal exposure of the offspring to a carcinogen (Tomatis et al., 1992; Mohr et al., 1999; Nomura, 2006). In this regard, while the carcinogenicity of BN is well documented (Sharan, 1996; IARC, 1985, 2004; Sharan and Wary, 1992; Wary and Sharan, 1988, 1991; Saikia et al., 1999) it is not known if prenatal exposure to BN can lead to a predisposition to cancer.

The *p53* gene is a critical tumor suppressor gene known to be mutated in a variety of human cancers (Hollstein et al., 1991). In normal unstressed cells, p53 is an unstable protein with a half-life ranging from 5 to 30 min and is present at low cellular levels (Levine, 1997). Stressful conditions including exposure to DNA damaging agents, hypoxia, UV, nucleotide depletion or oncogene activation lead to p53 stabilization, resulting in a rapid increase in the level of p53 in the cell (Lohram and Vousden, 1999). p53 then induces cell-cycle arrest at the G1/S or G2 checkpoints allowing the cell to repair DNA damage, with p53 itself modulating DNA repair

* Corresponding author. Tel.: +91 364 272 2121; fax: +91 364 255 0108.
E-mail addresses: rnsharan@nehu.ac.in, rnsharan@gmail.com (R.N. Sharan).

1382-6689/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.etap.2008.09.003

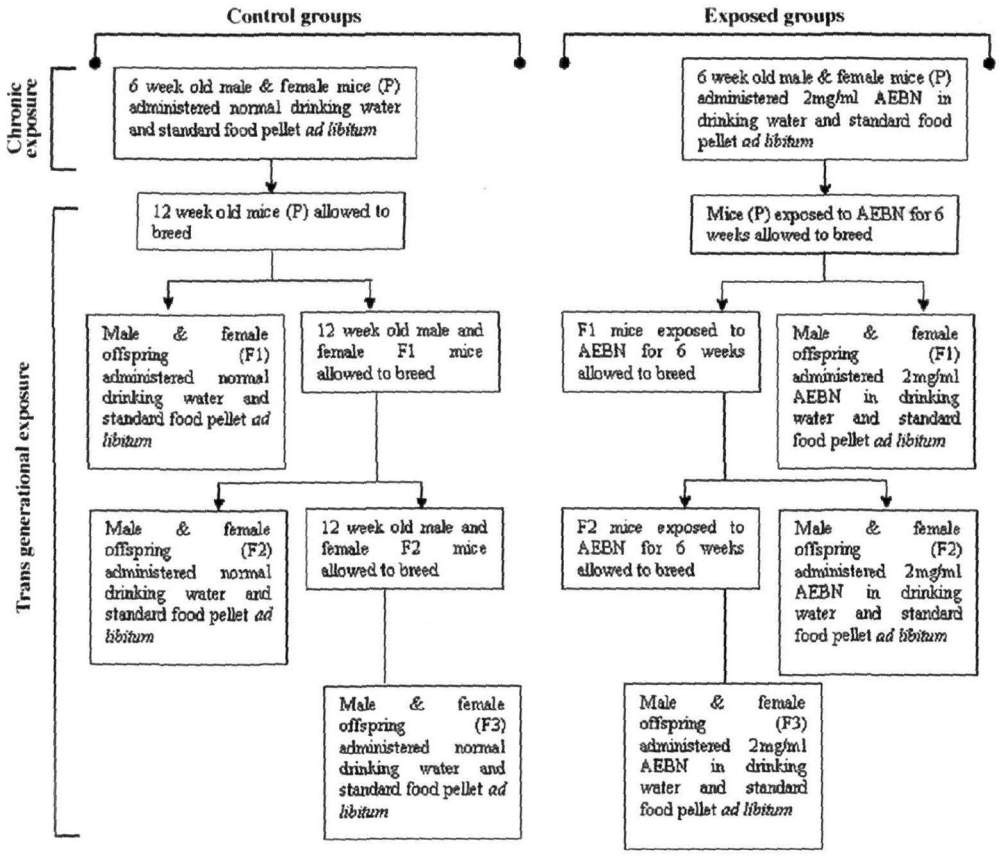


Fig. 1. Experimental designs of chronic and transgenerational exposure of Swiss Albino mice to AEBN drinking water (2 mg/ml).

through multiple mechanisms including sequence-specific trans-activation and direct interaction with components of the repair machinery (Stewart and Pietenpol, 2001). Alternatively, p53 may trigger apoptosis (Stewart and Pietenpol, 2001). Thus, the p53 signaling pathway plays a vital role in the prevention of cancer, and is consequently also the most commonly subverted pathway in tumorigenesis (Stewart and Pietenpol, 2001).

Cancer being a multistep disorder (Pitot et al., 1989), one objective of this study was to elucidate the dose-dependent response of the p53 protein during long-term chronic exposure to BN, beginning with onset of exposure and culminating ultimately in the discernable development of cancer. The carcinogenic potential of AEBN is well established (Sharan, 1996; Sharan and Wary, 1992; Wary and Sharan, 1988), and previous studies in Swiss Albino mice have shown that AEBN or arecoline induced DNA damage, affected cell cycle characteristics and induced qualitative changes in mice liver high mobility group (HMG) proteins similar to that induced by a hepatocarcinogen, diethylnitrosamine (DEN), leading to the development of preneoplastic nodules in the liver (Wary and Sharan, 1988; Pariat and Sharan, 1998). Swiss Albino mice chronically exposed to AEBN were therefore selected as a model for this study, which was aimed to ascertain if mutations of the *p53* gene are involved in AEBN induced carcinogenesis in mice. Further, the study also aimed to determine if BN has a transgenerational carcinogenic effect. For this purpose, the study was designed taking the significance of postnatal carcinogen exposure into consideration (Tomatis et al., 1992; Mohr et al., 1999; Nomura, 2006). The progeny of parents exposed to AEBN were, therefore, exposed to the same dose of AEBN as their parents, and subsequently assessed for increased susceptibility to cancer. Three generations of progeny were examined in this manner.

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade and were used without further purification. Nitrocellulose membrane, specific antibody (anti-p53) raised in sheep against a GST fusion protein of human p53 corresponding to amino acids 1–393 and reactive in human, mouse and rat, was obtained from Sigma Chemical Company, St. Louis, MO, USA (Catalog No. P4235), secondary antibody (alkaline-phosphatase labeled donkey anti-sheep IgG) was obtained from Sigma Chemical Company, St. Louis, MO, USA (Catalog No. A 5187), 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) and DNA Amplification Reagent Kit were from Bangalore Genei Pvt. Ltd., Bangalore, India. PCR primers were supplied by Hysel India Pvt. Ltd., New Delhi, India.

2.2. Experimental animals

Six-week-old inbred male and female Swiss Albino mice weighing 25 ± 1 g were used. The mice were housed in polycarbonate cages with husk bedding in a well-ventilated animal room maintained at 25 °C, with five mice per cage. Male and female mice were maintained in separate cages except for transgenerational breeding. Standard mouse pellet and drinking water with or without AEBN were provided *ad libitum*. All experiments were conducted according to the guidelines of the Institutional Ethics Committee for animal experimentation.

2.3. Carcinogen exposure protocol and experimental design

(a) *Chronic exposure regimen.* AEBN, prepared as described earlier (Wary and Sharan, 1988), was administered to the mice at a concentration of 2 mg/ml of drinking water in a chronic oral exposure protocol for a period up to 24 weeks (Fig. 1). The amount of drinking water consumed by each mouse per day was approximately 7 ml, i.e. the amount of AEBN consumed by each mouse was approximately 14 mg per day, and remained invariant throughout the exposure period. These mice have been henceforth referred to as the parental (P) generation exposed mice. Age-matched mice maintained on drinking water without AEBN served as controls. Exposed and control mice were sacrificed in groups of five mice at intervals of 2, 4, 6, 8, 10, 12, 16, 20 and 24 weeks by cervical dislocation except when blood was to be drawn when they were killed under chloroform anesthesia.

(b) *Transgenerational exposure regimen.* The transgenerational exposure experiment was initiated after completion of the chronic exposure regimen. The experimental design for the transgenerational experiment is shown in Fig. 1. Briefly, male and female mice of the P generation, which had been exposed to AEBN drinking water for 6 weeks, were allowed to breed by maintaining one male mouse and four female mice per cage with standard food pellet and drinking water containing AEBN *ad libitum*. The mother mice continued to receive drinking water with AEBN throughout the period of pregnancy, and during the postnatal period before weaning of offspring. The offspring of the exposed P generation mice formed the F1 generation exposed mice. Post-weaning, i.e. at 6 weeks of age, the F1 mice were separated from their parents, male and female mice being maintained separately, and were maintained on AEBN drinking water for a period to 24 weeks, as in case of AEBN exposed P generation mice. The F2 and F3 generations were similarly raised from F1 and F2 mice, respectively. Age-matched unexposed control mice of the P generation were also allowed to breed in parallel, and their offspring served as age-matched controls for the F1 exposed mice. Respective controls for the F2 and F3 generations were also raised similarly. A strict coding system was followed to maintain the F1, F2 and F3 generations and their respective controls. AEBN exposed F1, F2 and F3 mice, as well as their respective age-matched controls were sacrificed in groups of five mice at intervals of 4, 6, 8, 12, 16 and 24 weeks by cervical dislocation, except when blood was to be drawn when they were killed under chloroform anesthesia.

Each experiment was repeated at least three times, such that the total number of exposed and control mice at each data point were 15 ± 1. All exposed and control mice used for both chronic and transgenerational experiment regimens were carefully monitored throughout the treatment regimen for any visible sign of ailment. The weight of both exposed and control mice was recorded prior to sacrifice, following which the liver and spleen were excised and weighed, and whole blood was collected for isolation of blood lymphocytes. Other organs such as the lungs and the gastrointestinal tract were also carefully observed for any macroscopically visible changes. Mice with visible anomalies were sacrificed immediately, and the affected regions excised and studied. However, the periods of investigation for p53 protein expression in the transgenerational exposure study were determined taking into consideration the periods during which significant alterations were observed between the exposed and control groups of the chronic exposure regimen.

2.3.1. Histological examination

Histological examination was performed to confirm the macroscopic observation of initiation of preneoplastic nodule formation in the liver of mice exposed to AEBN in both the chronic exposure as well as transgenerational exposure regimens. In addition, preneoplastic nodules of the liver formed at the termination of treatment in the chronic exposure regimen were also examined. Segments of liver from the affected area, as well as corresponding regions of liver of age-matched control mice were fixed in 10% neutral formalin, embedded in paraffin and cut into 5–7 μm thick sections which were processed routinely for hematoxylin–eosin staining.

2.4. Preparation of whole homogenates of liver, spleen cells, enlarged lymph nodes, pus-filled sacs and solid tumors

Whole homogenates were prepared following the method of Rosenberg (Rosenberg, 1996) with some modifications. Briefly, a 10% (w/v) whole homogenate of liver was prepared using 1.5 ml of the cell extract buffer (0.1 M Tris–HCl pH 7.5, 0.25 M sucrose, 0.1 M NaCl, 3 mM EDTA, 10 mM 2-mercaptoethanol and 1 mM PMSF) and 0.15 g of liver. A whole homogenate of spleen cells was prepared using 1 ml of the buffer and one whole spleen. Similarly, 10% (w/v) whole homogenates of the enlarged lymph nodes, pus-filled sacs and solid tumors were also prepared as for whole homogenate of liver. The homogenate was centrifuged ($800 \times g$) for 10 min at 4 °C and the supernatant collected. The protein content of the supernatant was determined by the method of Bradford (Bradford, 1976) using BSA as a standard.

2.5. Preparation of blood lymphocyte sample

Blood lymphocytes were isolated as previously described (Kma and Sharan, 2006) and lysed with cell lysis buffer (20 mM Tris–HCl pH 8.0, 10 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 3 mM $MgCl_2$ and 10 mM PMSF) at −20 °C for 30 min followed by centrifugation ($5000 \times g$) for 15 min at 4 °C. The supernatant was collected and its protein content determined by the method of Bradford (Bradford, 1976) using BSA as a standard.

2.6. Slot-blot and Western-blot immunoprobing

Equal quantities of protein (400 ng for slot and 150 μg for Western blots) were slot or Western blotted onto 0.45 μm nitrocellulose membrane using Bio-Dot SF Microfiltration Apparatus or Mini-Protean II Electrophoretic Cell & Trans-Blot Electrophoretic Transfer Cell (Bio-Rad), respectively, as previously described (Sharan et al., 2005). The blots were immunoprobed as well as stained with India ink. Briefly, for immunoprobing, the nitrocellulose membrane was incubated with anti-p53 overnight at 37 °C at a dilution of 1:50,000 for slot-blot and 1:5000 for Western blot. The secondary antibody incubation was at a dilution of 1:15,000 for 2 h at 37 °C. Color development was done using BCIP/NBT at 37 °C for approximately 10 min. For slot-blotting, each experimental set comprised 4–5 replicate slot-blots of control and exposed samples, which were immunoprobed and a replica blot was stained with India ink for total protein.

Table 1
Primers used for amplification of selected regions of mouse *p53* gene.

Amplified region (size)	Sequence (5′ → 3′)	Strand	T_a (°C)
Exon 5 (264 bp)	ATC GTT ACT CGG CTT GTC CC	Sense	56.5
	TAA CCC CAC AGG CGG TGT T	Antisense	
Exon 7 (212 bp)	TAG TGA GGT AGG GAG CGA CTT	Sense	54.8
	CTG GGG AAG AAA CAG GCT AAC	Antisense	

2.7. DNA extraction, PCR amplification, direct DNA sequencing and sequence analysis

DNA was extracted from the preneoplastic nodules of livers of chronically and transgenerationally exposed mice after 24-week exposure to AEBN, and the corresponding regions of livers of age-matched controls, as well as from solid tumors of transgenerationally exposed mice, using proteinase-K and CTAB (Ausubel et al., 1995). The concentration of isolated DNA was estimated by recording the absorbance at 260 nm. Primers were designed for the intronic regions flanking exons 5 and 7 of the *p53* gene (Table 1). PCR amplifications were performed in a 15 μl reaction mixture containing 0.5 μg genomic DNA, 1X Taq DNA polymerase buffer, 1.5 mM $MgCl_2$, 90 μM of dNTPs, 0.5 U Taq polymerase and 40 pmol of each primer for exon 5 of *p53* gene. For exon 7, 60 pmol of each primer was used. Amplification was carried out using 2720 Thermal Cycler (Applied Biosystems) for 30 cycles under optimized conditions (95 °C for 3 min followed by cycle of denaturation at 94 °C for 1 min, annealing (see Table 1) for 1 min and extension at 72 °C for 1 min). Final extension was conducted at 72 °C for 7 min. The amplified products were lyophilized (Heto Lyolab 3000, Heto-Holten A/S, Allerød, Denmark) and sequenced by direct nucleotide sequencing using ABI's AmpliTaq FS dye terminator cycle sequencing chemistry (Bangalore Genei Pvt. Ltd., India). The nucleotide sequences thus obtained were analyzed with BLASTN (Altschul et al., 1997) and Multalin (Corpet, 1988).

2.8. Imaging and densitometric analysis

Hematoxylin–eosin stained sections were photographed using Olympus BX60 brightfield microscope at 400× magnification. Immunoprobed and India ink stained slot- or Western-blotted nitrocellulose membrane were digitized (HP Scanjet 7400C) for densitometric analysis using KDS-1D software (Kodak). Agarose gels of PCR products were photographed on a Bio-Rad mini transilluminator using a Kodak digital camera.

2.9. Statistical analyses

All data presented are the mean ± S.D. of three independent experiments each with 4–5 replicates. The significance of differences in levels of p53 protein and relative organ weights in exposed and age-matched controls were analyzed using Student's *t*-test. The significance of difference in period of exposure after which preneoplastic nodules developed in livers of chronically and transgenerationally exposed mice was analyzed using χ^2-test with Yates' correction. The significance of development of various anomalies in transgenerationally exposed mice in comparison to chronically exposed mice, and also between F1, F2 and F3 generations was analyzed using 2×2 contingency χ^2-test.

3. Results

3.1. General and histological observations

No difference was observed in the amount of food pellet and drinking water, with or without AEBN, consumed by the control and exposed mice in both chronic as well as transgenerational exposure regimens. The mice did not show any signs of illness throughout the treatment period in P generation. No significant change in number of offspring and gender ratio was observed in F1, F2 or F3 progeny of parents exposed to AEBN. Similarly, no congenital malformations were observed in F1, F2 or F3 progeny of parents exposed to AEBN, indicating an absence of teratogenicity following AEBN exposure. Careful examination of organs revealed that AEBN exposure predominantly affected the liver (Fig. 2) and to some extent spleen,

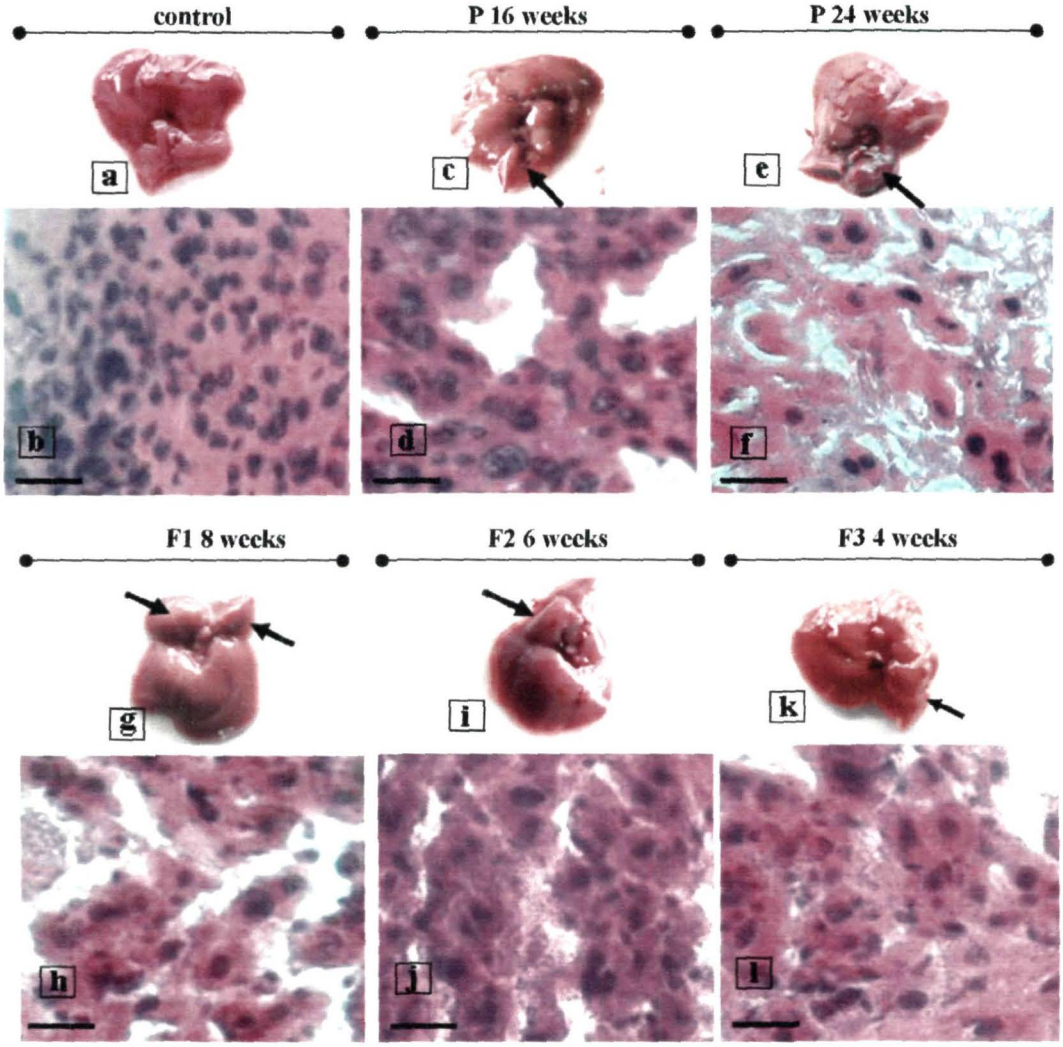


Fig. 2. Preneoplastic nodule formation in the liver of Swiss Albino mice exposed to AEBN drinking water (2 mg/ml) and their respective hematoxylin–eosin stained histological sections at magnification 400× (bar = 20 μm). Normal, control liver (a) and histological section of normal liver (b); nodule formation (arrow) in livers of P (c), F1 (g), F2 (i) and F3 (k) mice after 16, 8, 6 and 4 weeks, respectively, of exposure to AEBN drinking water—their respective histological sections are shown in (d), (h), (j) and (l). Well-developed nodule (arrow) in liver of P generation mice after 24-week exposure (e) and its histological section (f).

and lesions or alterations were not observed in other organs. The number of mice used at each data point, body weight, and relative weight of liver and spleen are detailed in Table 2.

In P generation mice liver (Fig. 2a), nodules appeared after 16 weeks of exposure primarily in the right and caudate lobes of the liver (Fig. 2c; arrow) and were confirmed to be preneoplastic by histological examination (Fig. 2d). While the liver cells of control mice were regularly arranged and attached (Fig. 2b), the cells of the preneoplastic nodules lacked regular attachment and also exhibited enlarged nuclei. The nodules were well developed after 24 weeks of exposure (Fig. 2e; arrow) and histological examination revealed irregularly shaped cells with enlarged nuclei displaying pronounced loss of attachment (Fig. 2f). Exposure of the progeny to drinking water with AEBN at the same dose as their parents post-weaning, however, led to advancement in the period of appearance of liver nodules in subsequent generations in comparison to the P generation. Nodules were observed after 8 weeks of exposure in F1 mice, 6 weeks of exposure in F2 mice and 4 weeks of exposure in F3 mice (Fig. 2g, i, and k, respectively, and Table 3). These nodules also exhibited irregularly shaped cells with enlarged nuclei and loss of regular attachment with neighboring cells (Fig. 2h, j and l, respectively). The incidence of preneoplastic nodule formation in the liver was 100% in the P as well as F1 through F3 generation mice. However, the frequency of nodulations progressively increased from P generation onwards. While 1–2 nodules per liver were observed after 16 weeks or more of AEBN exposure in P generation mice, 3–4 nodules developed in the liver of F1, F2 and F3 mice after 24 weeks of exposure. No sex associated difference was observed in the frequency of nodule development. No nodule development was observed in age-matched control mice of P, F1, F2 and F3 generations throughout the duration of the experiments.

The relative body and organ weights of exposed mice were evaluated in comparison to respective age-matched controls (Table 2). The body weights of exposed mice did not vary significantly from that of controls except for some fluctuations in P generation mice. The relative weights of liver and spleen, however, showed more definitive trends. Their weights, which fluctuated in P generation, recorded a tendency to increase significantly upon AEBN exposure and with progression of generations. Increase in relative weights of these two organs was most pronounced in F3 generation (Table 2).

Table 2
Number of mice used, alterations in body weight and relative organ weight upon chronic and transgenerational exposure to 2 mg/ml AEBN in drinking water for different periods of time in comparison to the appropriate controls.

Generation with period of AEBN exposure in weeks	Control				Exposed			
	No. of mice (*M*/*F*)	Final body weight (g ± S.D.)	Relative organ weight (g/100 g body weight ± S.D.)		No. of mice (*M*/*F*)	Final body weight (g ± S.D.)	Relative organ weight (g/100 g body weight ± S.D.)	
			Liver	*Spleen*			*Liver*	*Spleen*
P								
2	15 (7/8)	30 ± 2.8	4.3 ± 0.20	0.52 ± 0.03	15 (6/9)	30 ± 2.3	4.4 ± 0.24	0.38 ± 0.10$^{\Delta\Delta\Delta}$
4	14 (7/7)	26 ± 3.0	5.0 ± 0.23	0.54 ± 0.19	14 (7/7)	25 ± 3.3	4.8 ± 0.24$^{\Delta}$	0.60 ± 0.24
6	15 (6/9)	29 ± 2.3	4.7 ± 0.14	0.45 ± 0.17	15 (7/8)	28 ± 7.3	4.4 ± 0.39$^{\Delta\Delta}$	0.53 ± 0.11
8	14 (7/7)	30 ± 3.2	5.1 ± 0.44	0.50 ± 0.17	15 (8/7)	28 ± 5.3	4.4 ± 0.76$^{\Delta\Delta}$	0.58 ± 0.25
10	15 (6/9)	30 ± 2.4	5.0 ± 0.32	0.63 ± 0.23	15 (7/8)	30 ± 0.5	5.3 ± 0.94***	0.51 ± 0.20
12	14 (7/7)	34 ± 4.9	4.8 ± 0.06	0.51 ± 0.15	15 (8/7)	31 ± 2.2$^{\Delta\Delta\Delta}$	5.3 ± 0.10	0.58 ± 0.16
16	15 (7/8)	35 ± 2.3	4.3 ± 0.66	0.40 ± 0.06	15 (7/8)	34 ± 3.7	4.6 ± 1.03	0.32 ± 0.06***
20	16 (7/9)	31 ± 1.7	5.0 ± 0.49	0.49 ± 0.16	15 (7/8)	30 ± 2.8	4.9 ± 0.31	0.85 ± 0.17***
24	16 (8/8)	30 ± 5.1	5.0 ± 0.30	0.53 ± 0.07	16 (8/8)	31 ± 3.5	5.7 ± 0.73**	0.86 ± 0.10***
F1								
4	14 (7/7)	31 ± 2.3	4.0 ± 0.03	0.37 ± 0.03	15 (8/7)	33 ± 1.2**	4.3 ± 0.52*	0.53 ± 0.09***
6	15 (8/7)	31 ± 4.1	4.4 ± 0.03	0.56 ± 0.03	15 (8/7)	26 ± 3.0$^{\Delta\Delta\Delta}$	4.6 ± 0.16***	0.70 ± 0.08***
8	15 (8/7)	30 ± 0	5.1 ± 0.23	0.43 ± 0.07	15 (7/8)	33 ± 1.8***	5.3 ± 1.18	0.64 ± 0.24**
12	14 (7/7)	34 ± 4.9	4.8 ± 0.06	0.51 ± 0.15	15 (7/8)	31 ± 2.8	5.1 ± 1.36	0.65 ± 0.42
16	14 (7/7)	28 ± 2.0	5.1 ± 1.14	0.57 ± 0.21	14 (7/7)	31 ± 3.1	5.7 ± 0.46	0.46 ± 0.065$^{\Delta}$
24	15 (8/7)	29 ± 1.2	5.1 ± 0.07	0.34 ± 0.07	16 (9/7)	36 ± 6.9	6.6 ± 1.28***	1.20 ± 1***
F2								
4	15 (7/8)	26 ± 3.0	5.0 ± 0.23	0.54 ± 0.19	14 (7/7)	25 ± 3.3	4.8 ± 0.24$^{\Delta}$	0.60 ± 0.24
6	15 (9/6)	23 ± 0.7	4.3 ± 0.33	0.54 ± 0.17	15 (7/8)	27 ± 1.1***	5.0 ± 0.45***	0.70 ± 0.15*
8	15 (8/7)	32 ± 3.5	5.1 ± 0.44	0.44 ± 0.06	14 (7/7)	31 ± 1.2	5.9 ± 0.20***	0.39 ± 0.03$^{\Delta\Delta\Delta}$
12	15 (8/7)	37 ± 2.7	4.3 ± 0.30	0.54 ± 0.22	15 (7/8)	31 ± 1.2$^{\Delta\Delta\Delta}$	5.0 ± 0.33***	0.68 ± 0.16
16	15 (6/9)	30 ± 2.0	4.8 ± 0.10	0.50 ± 0.14	15 (6/9)	31 ± 1.7	5.3 ± 0.32***	1.13 ± 0.52***
24	15 (8/7)	32 ± 2.1	4.5 ± 1.29	0.42 ± 0.43	17 (8/9)	33 ± 2.6	5.2 ± 0.09*	0.88 ± 0.27**
F3								
4	16 (8/8)	27 ± 1.7	4.8 ± 0.56	0.44 ± 0.15	15 (8/7)	30 ± 2.0***	4.6 ± 0.07	0.65 ± 0.03***
6	15 (8/7)	28 ± 0.5	4.8 ± 0.14	0.46 ± 0.18	15 (7/8)	23 ± 6.4$^{\Delta\Delta}$	6.1 ± 0.03***	0.77 ± 0.09***
8	15 (8/7)	31 ± 0.4	5.2 ± 0.13	0.52 ± 0.03	15 (7/8)	27 ± 4.2$^{\Delta\Delta\Delta}$	6.3 ± 0.56***	0.63 ± 0.19***
12	14 (6/8)	37 ± 2.7	4.3 ± 0.03	0.54 ± 0.22	15 (8/7)	34 ± 4.1$^{\Delta}$	5.5 ± 0.27***	0.57 ± 0.12
16	15 (7/8)	31 ± 3.5	4.7 ± 0.10	0.49 ± 0.03	14 (6/8)	35 ± 3.1**	5.8 ± 0.89***	0.65 ± 0.09***
24	15 (7/8)	33 ± 6.1	5.4 ± 0.92	0.49 ± 0.09	20 (9/11)	34 ± 3.3	6.7 ± 1.36***	0.47 ± 0.06$^{\Delta}$

M/*F*—Number of males/number of females mice.
* Significant increase at $P<0.05$ in comparison to age-matched control group.
** Significant increase at $P<0.01$ in comparison to age-matched control group.
*** Significant increase at $P<0.001$ in comparison to age-matched control group.
$^{\Delta}$ Significant decrease at $P<0.05$ in comparison to age-matched control group.
$^{\Delta\Delta}$ Significant decrease at $P<0.01$ in comparison to age-matched control group.
$^{\Delta\Delta\Delta}$Significant decrease at $P<0.001$ in comparison to age-matched control group.

Table 3
Development of preneoplastic nodules in liver of mice chronically and transgenerationally exposed to 2 mg/ml AEBN in drinking water.

Generation	Period of exposure after which preneoplastic nodules developed in liver (weeks)
P	16
F1	8*
F2	6**
F3	4***

* Significant difference from P generation at $P < 0.05$.
** Significant difference from P generation at $P < 0.01$.
*** Significant difference from P generation at $P < 0.001$.

3.2. Cellular level of p53

The level of p53 protein in control and AEBN exposed mice was monitored by slot-blotting in order to facilitate accurate densitometric quantification and confirmed by Western blotting. The India ink stained slot-blots (Fig. 3; panel A-II) and Western blots (Fig. 3C-India ink stained) did not show significant differences in net intensity of control and AEBN exposed samples upon densitometric analysis, thus confirming the loading of equal amounts of protein. Immunoprobing with anti-p53 (Fig. 3; panels A-I and C-anti-p53 immunoprobed) revealed significant changes in the level of p53 protein in the liver of AEBN exposed mice of P generation in comparison to age-matched controls. Upon quantification of slot-blots and

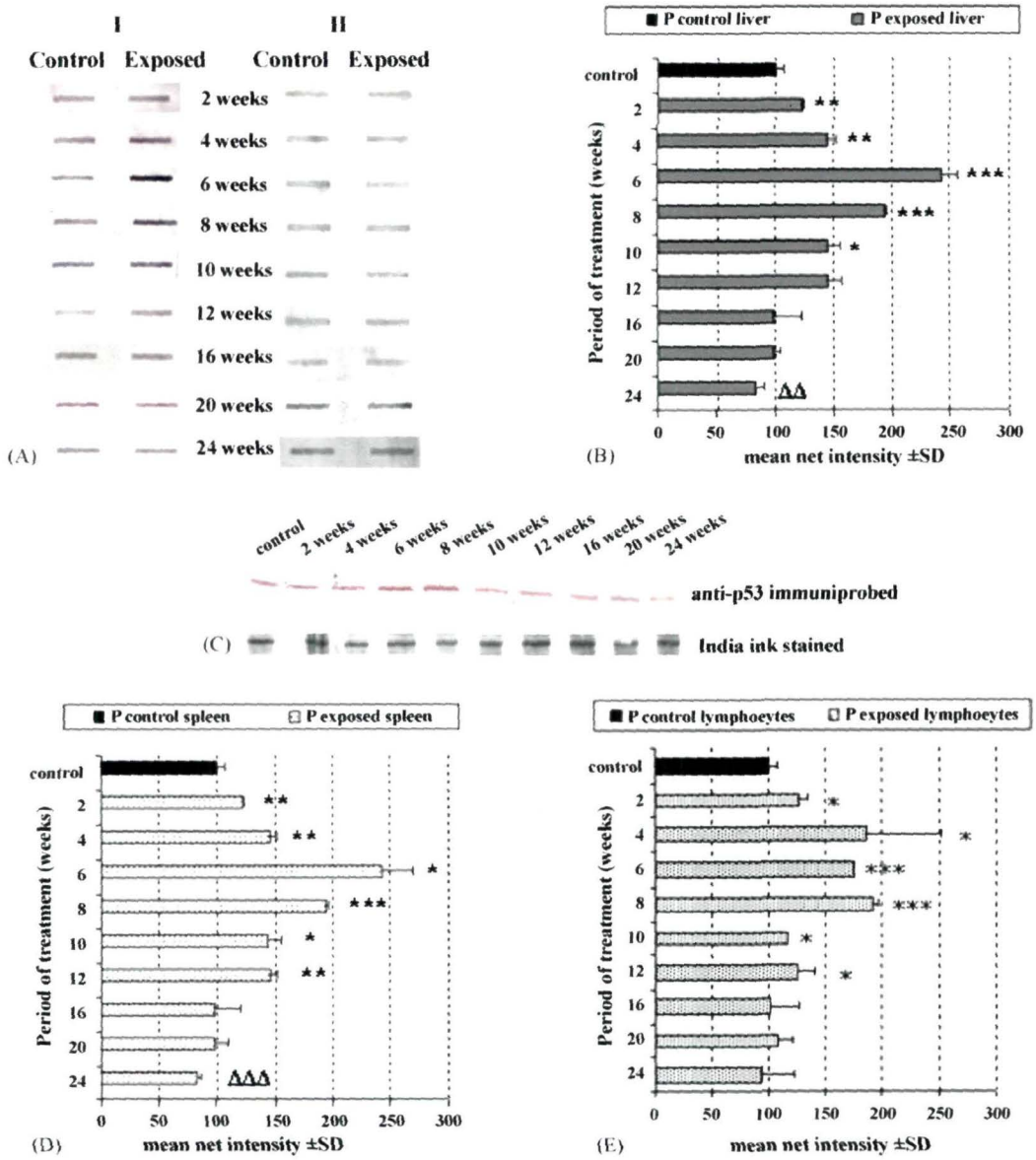


Fig. 3. p53 protein expression level in P generation Swiss Albino mice chronically exposed to AEBN drinking water (2 mg/ml) in comparison to age-matched controls. (A) Slot-blots of liver samples of AEBN exposed group and age-matched controls: panel I—liver samples immunoprobed with specific anti-p53 antibody; panel II—replica slot-blots stained with India ink for total protein. (B) Densitometric plot (% of age-matched controls; $x \pm$ S.D.) of the level of p53 protein expression in liver as obtained by densitometric analysis of the immunoprobed slot-blots (A) after normalization for equal protein loading. (C) Western blot of controls and AEBN exposed liver samples immunoprobed with specific anti-p53 antibody (I) and replicum blot stained with India ink for total protein (II). Densitometric plots (% of age-matched controls; $x \pm$ S.D.) of the level of p53 protein expression in spleen cell (D) and blood lymphocyte (E) samples as obtained by densitometric analysis of the immunoprobed slot-blots (not shown) after normalization for equal protein loading. * indicates significant increase at $P < 0.05$, ** indicates significant increase at $P < 0.01$, *** indicates significant increase at $P < 0.001$, Δ indicates significant decrease at $P < 0.05$, ΔΔ indicates significant decrease at $P < 0.01$ and ΔΔΔ indicates significant decrease at $P < 0.001$. Number of mice/point = 15 ± 1.

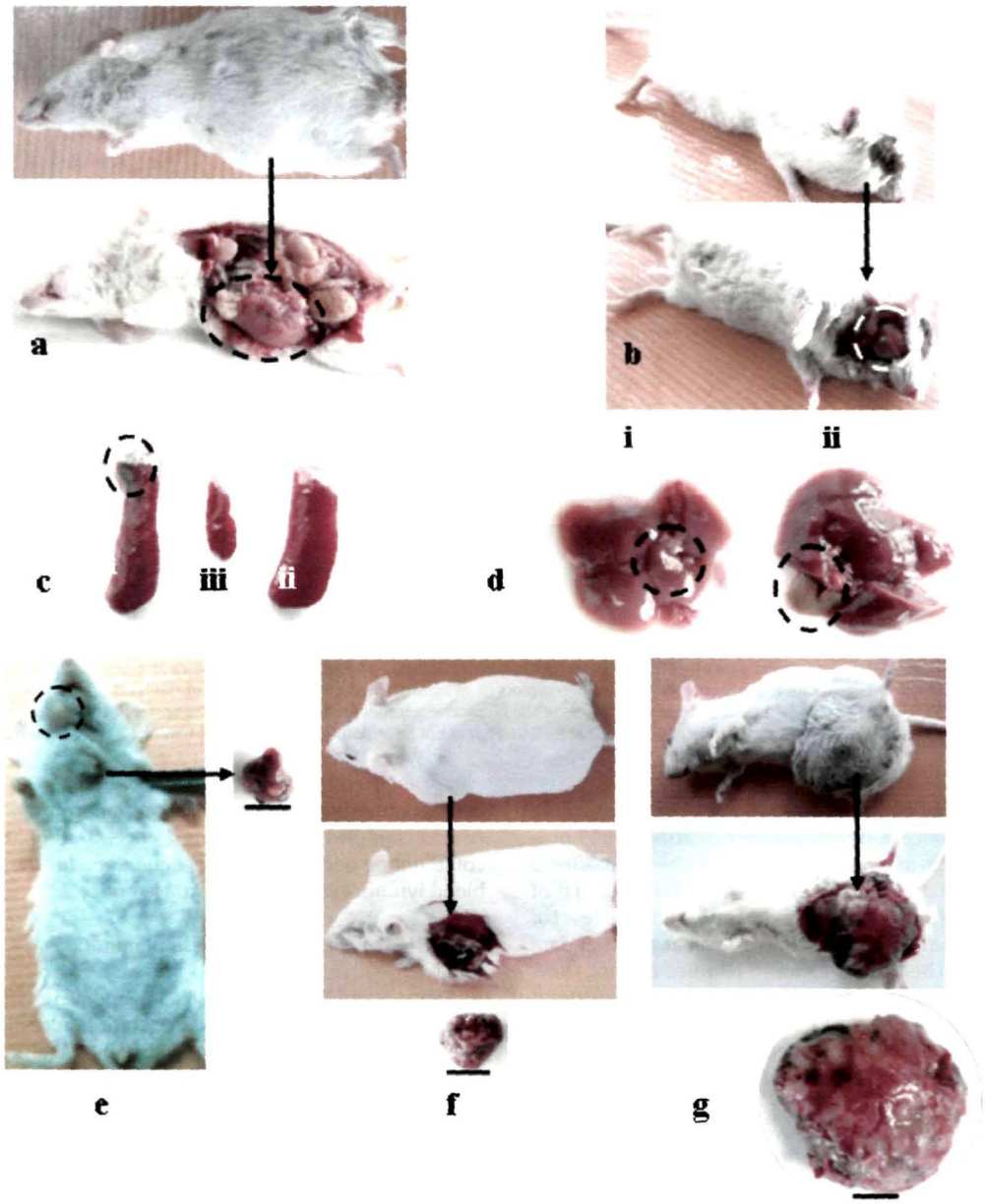


Fig. 4. Various anomalies/alterations observed in Swiss Albino mice transgenerationally exposed to AEBN drinking water (2 mg/ml). 24-week exposed F2 mouse showing pus-filled sac in gastrointestinal tract (circle) (a), enlarged neck node (circle) (b) or enlarged spleen (c) with protrusion (circle) (i) or without protrusion (ii); spleen of corresponding age-matched F2 generation control mouse is shown in (iii). (d) Necrotic areas (circle) in liver of F2 mice after 12 (i) or 24 (ii) week exposure. (e) A 24-week exposed F1 mouse with pus-filled sac on the mandible (circle) and solid tumor (7.5 mm in diameter) originating from epithelium of chest. (f) A 24-week exposed F3 mouse with solid tumor (9.3 mm in diameter) originating from skin epithelium of left forearm. (g) A 24-week exposed F3 mouse with solid tumor (30 mm in diameter) originating from epithelium of stomach (for (e), (f), and (g) scale bar = 7.5 mm).

normalization for equal loading of total protein the trend became clear in a bar diagram (Fig. 3B) showing progressive upregulation of the p53 protein beginning 2 weeks of exposure and recording a 2.5-fold increase at 6 weeks (Fig. 3). Downregulation of p53 began from 8 weeks of exposure reaching the control level after 16 weeks of exposure concomitant with the appearance of preneoplastic nodules in the liver (Fig. 2c). Subsequently, the level of p53 protein was maintained at control level in the livers of exposed mice up to 20 weeks of exposure, after which it was significantly below the control level (80%) after 24 weeks of exposure (Fig. 3A and B). The level of p53 protein was determined in preneoplastic nodules as well as the adjoining regions of the livers of mice exposed to AEBN for 16, 20 and 24 weeks and was found to be comparable. In order to determine if this effect of AEBN was specific to the liver, we also studied the changes in the level of p53 protein in the spleen cells (Fig. 3D) and blood lymphocytes (Fig. 3E). The levels of p53 protein in the spleen cells (Fig. 3D) and blood lymphocytes (Fig. 3E) of the exposed mice were largely found to mirror those in the liver (Fig. 3B). While the highest level of p53 protein was recorded at 6 weeks following AEBN exposure in liver (Fig. 3B) and spleen (Fig. 3D), it was slightly earlier (around 4 weeks) in blood lymphocytes (Fig. 3E). The level of p53 protein was essentially invariant during the entire period of treatment in the liver of exposed F1 mice (Fig. 5A and B), in comparison to age-matched controls, and was significantly lower (75%)

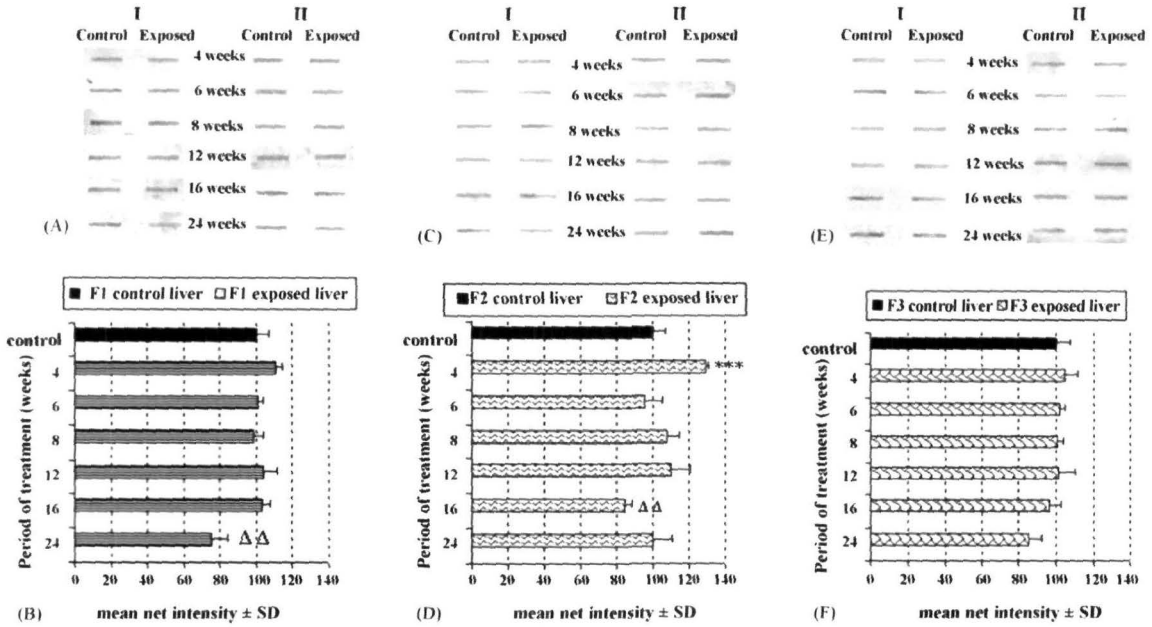


Fig. 5. p53 protein expression in liver of Swiss Albino mice transgenerationally exposed to AEBN drinking water (2 mg/ml) in comparison to age-matched controls. Slot-blots of AEBN exposed groups with age-matched controls: panel I—immunoprobed with specific anti-p53 antibody; panel II—replica slot-blots stained with India ink for total protein for the F1 (A), F2 (C) and F3 (E) generations, respectively. Densitometric plots (% of age-matched controls; $x \pm$ S.D.) of the level of p53 protein expression in liver samples of F1 (B), F2 (D) and F3 (F) mice as obtained by densitometric analysis of the respective immunoprobed slot-blots after normalization for equal protein loading. *** indicates significant increase at $P < 0.001$, ΔΔ indicates significant decrease at $P < 0.01$. Number of mice/point = 15 ± 1, except 24-week F2 and F3 exposure groups which had 17 and 20 mice, respectively.

than that of age-matched controls after 24 weeks of exposure. In the liver of exposed F2 mice, a significant upregulation of p53 protein (1.3-fold that of age-matched control) was observed after 2 weeks of exposure, and a significant downregulation (85% that of age-matched control) after 16 weeks of exposure, while the level of p53 protein was maintained near the control level at all other data points (Fig. 5C and D). The liver of exposed F3 mice did not show significant variation from that of age-matched controls throughout the period of treatment (Fig. 5E and F). The Western-blots immunoprobed with anti-p53 antibody confirmed that in contrast to the P generation, p53 protein was not upregulated in comparison to the control in the liver of F1 (Fig. 6A-i), F2 (Fig. 6B-i) and F3 (Fig. 6C-i) mice, and was maintained at or below control level. The India ink stained replica Western blots confirmed equal loading of the whole homogenate of liver (Fig. 6A-ii, B-ii and C-ii). Examination of the level of p53 protein in spleen cells and blood lymphocytes of F1 (Fig. 7A and B), F2 (Fig. 7C and D) and F3 (Fig. 7E and F) generations revealed that p53 protein was also essentially invariant in comparison to the age-matched controls, as in the liver, with exceptions at a few data points.

The slot-blots of whole homogenate of enlarged lymph nodes, pus-filled sacs and solid tumors obtained from transgenerationally exposed mice were also immunoprobed with anti-p53 antibody, and upon quantification, the level of p53 protein in terms of net-intensity was found to be low, and comparable to p53 level in the liver, spleen cells and blood lymphocytes of respective age-matched control mice (not shown). However, appropriate tissue-matched control samples were lacking. Hence, a comparative evaluation could not be performed as has been done for the liver, spleen and blood lymphocytes of AEBN exposed mice.

3.3. DNA sequencing and analysis of mutation

Exons 5 and 7 of the mouse *p53* gene were PCR amplified from liver nodules of P, F1, F2 and F3 generation mice along with respective controls, as well as from solid tumors obtained from F1 and F2 mice, followed by direct DNA sequencing. Both coding and non-coding strands of DNA were independently sequenced. Analysis of the DNA sequences revealed no mutations (see details under GenBank accession # EF570972 and EF634061).

4. Discussion

Mutations in the *p53* gene are observed in a variety of cancers in mice, but are rarely found in murine liver tumors, suggesting an alternate route of p53 inactivation in murine hepatocarcinogenesis (Jaworski et al., 2005). Exons 5 and 7 of the mouse *p53* gene were selected for the current investigation because both are constituents of its critical DNA binding domain. In addition, exon 7 is reported to be frequently mutated in hepatocellular carcinoma in humans (Staib et al., 2003). Amplification and sequence analysis of the DNA samples obtained from liver preneoplastic nodules of

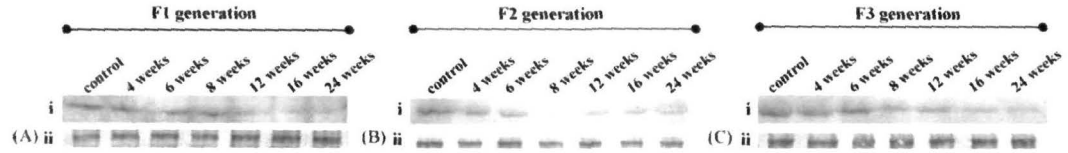


Fig. 6. Western blots of liver homogenates of controls and AEBN exposure groups of F1 (A), F2 (B) and F3 (C) mice immunoprobed with anti-p53 (i) and replica blot stained with India ink for total protein (ii). Number of mice/point = 15 ± 1, except 24-week F2 and F3 exposure groups which had 17 and 20 mice, respectively.

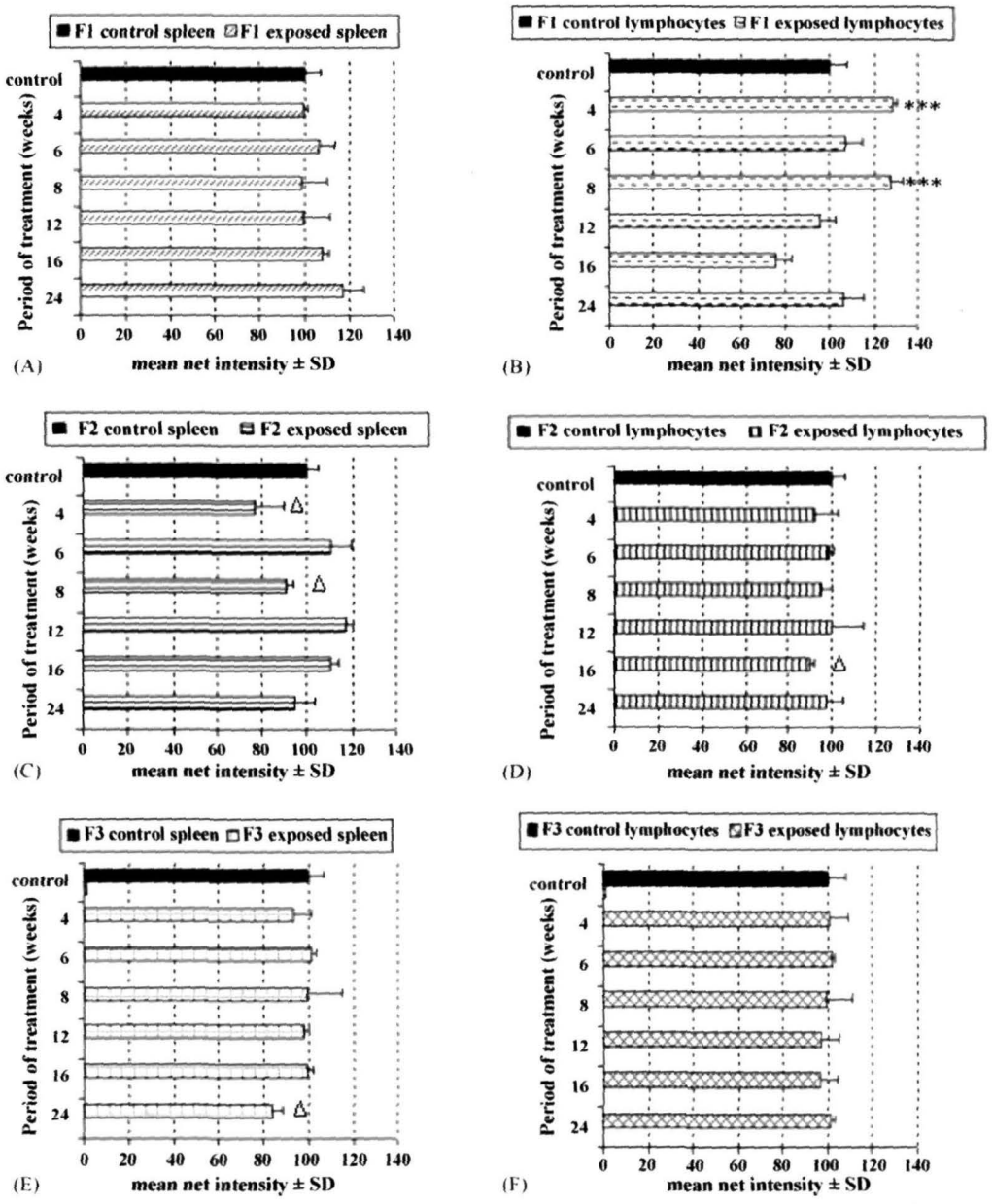


Fig. 7. Densitometric plots (% of age-matched controls; $x \pm$ S.D.) of the level of p53 protein expression in spleen cell samples of F1 (A), F2 (C) and F3 (E) and blood lymphocyte samples of F1 (B), F2 (D) and F3 (F). AEBN drinking water (2 mg/ml) exposed mice as obtained by densitometric analysis of the respective immunoprobed slot-blots (not shown) after normalization for equal protein loading. Number of mice/point = 15 ± 1, except 24-week F2 and F3 exposure groups which had 17 and 20 mice, respectively. *** indicates significant increase at $P < 0.001$ and Δ indicates significant decrease at $P < 0.05$.

chronically and transgenerationally exposed mice, as well as from solid tumors of transgenerationally exposed mice indicate that the selected exons 5 and 7 of the *p53* gene were not mutated under both chronic and transgenerational exposure regimes (see details under GenBank accession # EF570972 and EF634061). Thus, AEBN induced carcinogenesis in Swiss Albino mice does not involve mutations of exons 5 and 7 of the *p53* gene, though we cannot exclude the possibility of mutations occurring in other regions of the gene that have not been included in this study.

Rapid upregulation of p53 level by various types of stress prevents the proliferation of cells carrying damaged DNA with potentially oncogenic mutations (Moll and Petrenko, 2003). Thus, loss either of the ability to activate p53 or of p53 function is an important step in carcinogenesis (Evan and Vousden, 2001). Our results of the chronic exposure regimen show that exposure of Swiss albino mice to AEBN initially upregulated p53 protein in the liver up to 2.5 folds higher than age-matched controls, indicating an expected p53 response to the DNA damaging effect of AEBN (Fig. 3). This period of induction of p53 response is accompanied by a significant decrease in the relative liver weight of exposed mice (Table 2) indicating a possible suppression of cellular proliferation and perhaps death of cells that have incurred DNA damage (Moll and Petrenko, 2003). Continued chronic exposure to AEBN, however, led to a decline in cellular level of p53 to control level, concomitant with appearance of preneoplastic nodules in the liver (Fig. 2c and d). Thus, inability to maintain upregulated status of p53 in response to DNA damage may lead to carcinogenesis by disruption of the p53-mediated cell cycle arrest and/or apopto-

sis of damaged cells. The gradual, dose-dependent downregulation of p53 could be viewed as an adaptive mechanism by which cells evade the growth suppressive activities of p53 and continue to survive under conditions of continuing AEBN exposure. Further exposure to AEBN beyond 16 weeks did not upregulate p53, indicating a lack of p53 response. The preneoplastic nodules observed after 24 weeks of exposure were larger than those formed after 16 weeks of exposure, and the level of p53 in the liver was significantly lower than that after 16 weeks (Figs. 2e–f and 3A–C). We interpret our findings by suggesting that maintenance of p53 level at or below control level allows cells to undergo uncontrolled proliferation, leading to the development and subsequent enlargement of preneoplastic nodules as well as an overall increase in the relative weight of the liver (Table 2). Identical patterns were also observed in the spleen cells (Fig. 3D) and blood lymphocytes (Fig. 3E) of chronically exposed mice with noticeable increase in the relative weight of the spleen (Table 2), indicating uncontrolled proliferation of splenic cells upon AEBN exposure. The results obtained from studies with the spleen cells and blood lymphocytes support our contention that AEBN is a general, rather than tissue-specific carcinogen (Sharan, 1996; IARC, 1985; Pariat and Sharan, 1998). Based on the p53 response in Swiss Albino mice it appears that the initiation of AEBN-induced carcinogenesis involves a dose- or exposure period dependent upregulation of p53 protein, followed by its downregulation to control level during promotion. Final progression to cancer requires maintenance of p53 protein at or below control level (Fig. 3).

Swiss Albino mice exposed to AEBN in a transgenerational exposure regimen exhibited a strikingly different p53 response. The F1, F2 or F3 mice did not show upregulation of liver p53 protein level throughout the period of exposure, in comparison to age-matched controls (Figs. 5 and 6). A significant upregulation in liver p53 level of exposed F2 mice after 4 weeks of exposure was the only exception (Fig. 5C and D). In general, the liver p53 level was maintained at or below control level (Figs. 5 and 6). Thus, unlike the previously unexposed P generation mice, the prenatally exposed mice essentially failed to induce elevation of p53 protein level in the liver when challenged by the same carcinogen (that is, AEBN) and at the same dose (2 mg/ml in drinking water) in the postnatal environment. Such lack of p53 response predictably induced uncontrolled cell proliferation as in the P generation, but in the prenatally exposed mice significant increase in relative liver and spleen weight were observed earlier in comparison to the P generation (Table 2). Moreover, while preneoplastic nodules of the liver developed in P generation mice after 16 weeks of exposure, they developed in F1 mice after 8 weeks, F2 mice after 6 weeks and in F3 mice after 4 weeks of exposure (Fig. 2g–l). Thus, the development of preneoplastic nodules of the liver was significantly advanced in exposed F1 mice by 50%, F2 mice by 62.5% and F3 mice by 75% of the period of AEBN exposure required for initiation of nodulation in exposed P generation mice (Table 3). AEBN exposed F1, F2 and F3 mice also exhibited an increase in the frequency of nodulation—1–2 nodules per liver in the P generation, to 3–4 nodules per liver at the termination of exposure to AEBN in subsequent generations. This difference between P mice and transgenerationally exposed mice may be attributed to an early onset of uncontrolled cell proliferation in transgenerationally exposed mice, thereby enabling progression of carcinogenesis to a greater degree than in the P generation. Conforming with the role of AEBN as a general carcinogen, the lack of p53 response observed in liver of F1, F2 and F3 mice was mirrored by the spleen (Fig. 7A, C and E) and blood lymphocytes (Fig. 7B, D and F).

Previous studies have shown that following the exposure of germ cells to a mutagen or carcinogen, an initiating event could be inherited by subsequent generations and revealed after postnatal exposure to mutagens, carcinogens or non-genotoxic agents (Tomatis et al., 1992; Nomura, 1983). With respect to AEBN, the pattern of p53 response during AEBN-induced carcinogenesis as elucidated by our chronic exposure study is vital in this regard. Our study reveals that p53 protein is upregulated to elicit tumor-suppression during the initiation of AEBN-induced carcinogenesis, and the promotion stage involves downregulation of p53 protein level to control level. Since AEBN is a general carcinogen capable of affecting various tissues, it is likely that exposure of P generation parental mice causes an alteration in p53 protein level of their germ cells. The previously reported genotoxic potential of arecoline in mouse germ cells (Sinha and Rao, 1985a,b) supports this assumption. The P generation mice were exposed to AEBN for 6 weeks prior to mating. Thus, AEBN-induced carcinogenesis would have been initiated during the 6 weeks of exposure before mating (Wary and Sharan, 1988,1991), and, the initiating event could be inherited by F1 progeny through the germ cells. Subsequent exposure of the F1 progeny to AEBN postnatally would immediately induce promotion followed by progression, leading to the observed 50% advancement in the period of preneoplastic nodule appearance from the P to the F1 generation (Table 3). Similarly, the germ cells of the F1 and F2 mice would inherit a promoting event, hence, the progressive advancement of period of preneoplastic nodule development from F1 to F2 generations, and from F2 to F3 generations (Table 3). Keeping in view the reported transplacental effect of arecoline (Sinha and Rao, 1985b), another plausible explanation for our observations would be the transplacental exposure of the F1 fetus to AEBN, or, components derived from AEBN, leading to the initiation of AEBN-induced carcinogenesis at the fetal stage, and consequently an advancement in the period of preneoplastic nodule appearance in the successive generations of transgenerationally exposed mice.

In transgenerational carcinogenesis, it is assumed that heritable changes induced in germ cells will be present in all somatic cells of the offspring, thereby leading to an increase in frequency of multiple tumors or alterations in transgeneration cancer in comparison to sporadic cancer (Tomatis, 1994). Our results show that mice prenatally exposed to AEBN developed distinct anomalies at sites in addition to the liver (Fig. 4 and Table 4), upon subsequent postnatal exposure to AEBN. These anomalies possibly develop as a consequence of an inherited predisposition to cancer, but their development was not found to be significant. The progressive advancement in period of development of liver preneoplastic nodules was, however, significant in comparison to the P generation (Fig. 2 and Table 3), thereby indicating that transgenerational exposure to AEBN can lead to increased predisposition to cancer in the offspring. Unlike previous reports (Sharan, 1996; Sinha and Rao, 1985a), AEBN was not found to have teratogenic effect. This is possibly due to the nature of the extract or the dose of administration of AEBN.

Studies performed to monitor alterations in the *Brca1* and *Brca2* tumor suppressor genes under the same chronic and transgenerational exposure regimens (unpublished results), reveal a G → C transversion mutation in exon 11 of the *Brca1* gene, in solid tumors developing in mice transgenerationally exposed to AEBN. Thus, an absence of mutations in the selected regions of the *p53* gene upon AEBN exposure, are not indicative of the inability of AEBN to induce genome changes, as it is likely that AEBN induces carcinogenesis and influences transgenerational transmission of carcinogenic risk by attacking other targets which are required to prevent cellular transformation. The observed attenuation of p53 response upon AEBN exposure, manifested by maintenance of p53 protein at or below control level in the P and subsequent generations, is therefore critical because it removes a vital barrier required to protect against genomic instability, essentially by permitting cells that have incurred damage in other vital genes such as the *Brca1* gene, to evade cell-cycle arrest or apoptosis (Stewart and Pietenpol, 2001).

Table 4
Details of anomalies observed in mice transgenerationally exposed to 2 mg/ml AEBN in drinking water.

Type of anomaly	Generation	Anomaly present (+) or absent (−)	Afflicted site	AEBN exposure period (week)	Incidence (%)[a]
Solid tumor	F1[b]	+	Chest epithelium (tumor diameter = 7.5 mm; tumor load = 2.59)[c]	24	1/16 (6.25)
	F2	–	Skin epithelium (tumor diameter = 9.3 mm; tumor load = 3.40)[c]	–	0/17 (0)
	F3	+	Stomach epithelium (tumor diameter = 30 mm; tumor load = 50.64)[c]	24	2/20 (10)
Pus-filled sacs	F1[b]	+	Right mandible	24	1/16 (6.25)
	F2	+	Gastrointestinal tract		24
	F3[d]	+	Neck	24	1/20 (5)
Enlarged nodes	F1	–	–	–	0/16 (0)
	F2	+	Neck	24	1/17 (5.88)
	F3[d]	+		24	1/20 (5)
Necrosis	F1	–	–	–	0/16 (0)
	F2[e]	+	Liver	24	1/17 (5.88)
	F3	+		24	1/20 (5)
Protrusion	F1	–	–	–	0/16 (0)
	F2[e]	+	Spleen	24	1/17 (5.88)
	F3	–	–	–	0/20 (0)

Using 2×2 contingency χ^2-test, development of these anomalies was not significant in comparison to chronically exposed mice or between successive generations of transgenerationally exposed mice.

[a] Number of mice with anomaly/total number of mice exposed to AEBN for the same period of time.

[b] Anomalies developing in the same F1 mouse.

[c] Tumor weight in g/100 g body weight.

[d] Anomalies developing in the same F3 mouse.

[e] Anomalies developing in the same F2 mouse.

The MDM2 protein mediates cellular degradation of p53 (Levine, 1997), and over expression of MDM2 is therefore likely to cause lowering of p53 protein level below the optimum level required by a cell. Shwe et al. (2001) have reported that over expression of the MDM2 protein in tobacco and betel-chewing associated oral squamous cell carcinomas may constitute an alternative mechanism for p53 inactivation. Thus, the downregulation of p53 protein under our experimental regimens could be caused possibly by over expression of MDM2 protein, though this aspect remains to be investigated in future.

In conclusion, the present study suggests that chronic exposure of Swiss albino mice to AEBN induces carcinogenesis by inactivation of the tumor suppressor activities of the *p53* gene. This inactivation of the *p53* gene is most likely not through mutation of the *p53* gene, but involves a downregulation of the p53 protein expression after an initial elevation of p53 level in response to AEBN exposure. The mechanism of lowering of p53 protein expression in Swiss Albino mice upon AEBN exposure, however, remains to be elucidated. Further, prenatal exposure to AEBN can possibly lead to a transgeneration transmission of carcinogenic risk in Swiss Albino mice, which is manifested by significant early onset of cancer and non-significant development of multiple anomalies in a postnatal environment of exposure to AEBN.

Conflict of interest

None declared.

Acknowledgements

The authors gratefully acknowledge receipt of financial support for this work from UGC, India in form of doctoral fellowship to YC. Partial funds to RNS from INMAS (DRDO) and UPE (UGC) grant are also acknowledged.

References

Altschul, Stephen, F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., 1995. Short Protocols in Molecular Biology, 3rd edn. John Wiley and Sons Inc., USA, pp. 2–10.

Bradford, M.M., 1976. A rapid and sensitive method for quantification of microgram quantities of proteins using the principle of dye binding. Anal. Biochem. 72, 248–254.

Chang, B., Liao, M., Kuo, M.Y., Chen, C., 2001. Developmental toxicity of arecoline, the major alkaloid in betel nuts, in zebrafish embryos. Birth Defects Res. Part A 70, 28–36.

Corpet, F., 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. 16, 10881–10890.

Evan, G.I., Vousden, K.H., 2001. Proliferation, cell cycle and apoptosis in cancer. Nature 411, 342–348.

Hollstein, M., Sidransky, D., Vogelstein, B., Harris, C.C., 1991. p53 mutations in human cancers. Science 253, 49–53.

IARC, 1985. Tobacco chewing and some related nitrosamines. Monographs of Evaluation of Carcinogenic Risk of Chemicals to Humans, vol. 37. International Agency for Research on Cancer, Lyon, pp. 141–200.

IARC, 2004. Summaries & Evaluations: Betel-quid and Areca-nut Chewing, vol. 85. International Agency for Research on Cancer, Lyon, p. 39.

Jaworski, M., Hailfinger, S., Buchmann, A., Herhenhahn, M., Hollstein, M., Ittrich, C., Schwarz, M., 2005. Human p53 knock-in (hupki) mice do not differ in liver tumor response from their counterparts with murine p53. Carcinogenesis 26, 1829–1834.

Kma, L., Sharan, R.N., 2006. In vivo exposure of Swiss albino mice to chronic low dose of dimethylnitrosamine (DMN) lowers poly-ADP-ribosylation (PAR) of bone marrow cell and blood lymphocyte proteins. Mol. Cell. Biochem. 288, 143–149.

Levine, A.J., 1997. p53, the cellular gatekeeper for growth and division. Cell 88, 323–331.

Lohram, M.A.E., Vousden, K.H., 1999. Regulation and activation of p53 and its family members. Cell Death Differ. 6, 1162–1168.

Mohr, U., Dasenbrock, C., Tillmann, T., Kohler, M., Kamino, K., Hagemann, G., Morawietz, G., Campo, E., Cazorla, M., Fernandez, P., Hernandez, L., Cardesa, A., Tomatis, L., 1999. Possible carcinogenic effects of X-rays in a transgenerational study with CBA mice. Carcinogenesis 20, 325–332.

Moll, U.M., Petrenko, O., 2003. The MDM2-p53 interaction. Mol. Cancer Res. 1, 1001–1008.

Newbold, R.R., Hanson, R.B., Jefferson, W.N., Bullock, B.C., Haseman, J., McLachlan, J.A., 1998. Increased tumors but uncompromised fertility in the female descendants of mice exposed developmentally to diethylstilbestrol. Carcinogenesis 19, 1655–1663.

Nomura, T., 1983. X-ray induced germ line mutation leading to tumors. Its manifestation in mice given urethane post-natally. Mutat. Res. 121, 59–65.

Nomura, T., 2006. Transgenerational effects of radiation and chemicals in mice and humans. J. Radiat. Res. 47 (Suppl.), B83–B97.

Pariat, T., Sharan, R.N., 1998. Qualitative change in mice liver HMG proteins after low dose chronic administration of aqueous extract of betel nut and diethynitrosamine. Hepatol. Res. 12, 177–185.

Pitot, H.C., Beer, D., Hendrich, S., 1989. In: Inversen, O.H. (Ed.), Multistage Carcinogenesis. Hemisphere Publishing Company, Washington.

Rosenberg, I.M., 1996. Getting started with protein purification. In: Protein Analysis and Purification, Bench Top Techniques. Birkhäuser, Boston, pp. 99–109.
Saikia, J.R., Schneeweiss, F.H.A., Sharan, R.N., 1999. Arecoline-induced changes of poly-ADP-ribosylation of cellular proteins and its influence on chromation organization. Cancer Lett. 139, 59–65.
Sharan, R.N., 1996. Association of betel nut with carcinogenesis. Cancer J. 9, 13–19.
Sharan, R.N., Devi, B.J., Humtsoe, J.O., Kma, L., 2005. Detection and quantification of poly-ADP-ribosylated cellular proteins of spleen and liver tissues of mice in vivo by slot and Western blot immunoprobing using polyclonal antibody against mouse ADP-ribose polymer. Mol. Cell. Biochem. 278, 213–221.
Sharan, R.N., Wary, K.K., 1992. Study of unscheduled DNA synthesis following exposure of human cells to arecoline and extracts of betel nut in vitro. Mutat. Res. 278, 271–276.
Shwe, M., Chiguchi, G., Yamada, S., Nakajima, T., Maung, K.K., Takagi, M., Amagasa, T., Tsuchida, N., 2001. P53 and MDM2 co-expression in tobacco and betel-chewing associated oral squamous cell carcinomas. J. Med. Dent. Sci. 48, 113–119.
Sinha, A., Rao, A.R., 1985a. Induction of shape abnormality and unscheduled DNA synthesis by arecoline in the germ cells of mice. Mutat. Res. 158, 189–192.
Sinha, A., Rao, A.R., 1985b. Transplacental micronucleus inducing ability of arecoline, a betel nut alkaloid in mice. Mutat. Res. 158, 193–194.
Staib, F., Hussain, S.P., Hofseth, L.J., Wang, X.W., Harris, C.C., 2003. TP53 and liver carcinogenesis. Human Mutat. 21, 201–216.
Stewart, Z.A., Pietenpol, J.A., 2001. p53 signaling and cell cycle checkpoints. Chem. Res. Toxicol. 14, 243–261.
Tomatis, L., Narod, S., Yamasaki, H., 1992. Transgeneration transmission of carcinogenic risk. Carcinogenesis 13, 145–151.
Tomatis, L., 1994. Transgeneration carcinogenesis: a review of the experimental and epidemiological evidence. Jpn. J. Cancer Res. 85, 443–454.
Wary, K.K., Sharan, R.N., 1988. Aqueous extract of betel-nut of North-East India induces DNA strand breaks and enhances rate of cell proliferation in vitro. J. Cancer Res. Clin. Oncol. 114, 579–582.
Wary, K.K., Sharan, R.N., 1991. Cytotoxic and cytostatic effects of arecoline and sodium nitrite on human cells *in vitro*. Int. J. Cancer 47, 396–400.

FIU

FLORIDA INTERNATIONAL UNIVERSITY
Miami's public research university

09/17/08

To: Dr. Rajesh Sharan

RE: Ms. "Betel Nut and Susceptibility to Cancer by Yashmin Choudhury and R. N. Sharan"

Dear Rajesh,

I am happy to inform you that your invited contribution for a chapter on the subject of "Betel Nut and Susceptibility to Cancer by Yashmin Choudhury and R. N. Sharan" has been accepted for publication in a Springer Science + Business Media and Humana Press new monograph titled "The Environment and Cancer: Gene-Environment Interactions and Individual Susceptibility".

Best,

Deodutta Roy, PhD

Editor of the Monograph "The Environment and Cancer,

Department of Environmental & Occupational Health
Robert Stempel School of Public Health
11200 S.W. 8th Street, HLS-2 595 • Miami, FL 33199 • Tel: (305) 348-4903 • Fax: (305) 348-4901

"The Environment and Cancer: Gene-Environment Interactions and Individual Susceptibility". Ed Roy, D. and Warren, R., Springer Science - Humana Press, USA (In press)

Betel Nut and Susceptibility to Cancer

Yashmin Choudhury and R. N. Sharan*
Radiation & Molecular Biology Unit
Department of Biochemistry
North-Eastern Hill University
Shillong 793 022
India

* Corresponding author, E-mail rnsharan@nehu ac in, FAX (0364) 255 0108, Telephone (0364) 272 2121

ABSTRACT Betel nut is a widely masticated natural product, which is consumed by over 600 million people across the globe The ancient habit of betel nut chewing, either as dry or raw/wet nut, in association with betel leaf and a host of region specific additives, including chewing tobacco, is believed to be an important etiological factor for human cancer Alkaloids and their betel nut specific nitrosamine derivatives produced upon metabolic activation interact with DNA and other cellular targets to produce highly variable mutagenic, genotoxic, cytostatic, immunostatic and teratogenic effects At molecular level the betel nut or its constituents strongly influence gene expression patterns, especially that of tumor suppressor genes Structural damage to nucleus and mitochondria, etc are also induced The review dwells upon these aspects of betel nut induced carcinogenesis to show that genetic susceptibility to cancer through generations progressively increased due to exposure to betel nut.

INTRODUCTION

Areca nut is the seed of fruit of a tropical palm, *Areca catechu* L (Fig 1A) It forms the most basic ingredient of a variety of widely used social and habitual masticatory products, which are often wrapped in the leaf of another tropical creeper, *Piper betle* L , commonly known as the betel leaf Hence, the *Areca* nut is more commonly known as betel nut (BN) (Warnakulasuriya 2002) The earliest use of BN as a masticatory by humans has been mentioned by Theophrastus in scripts dating around 430 BCE (Before Common Era), which described use of *Areca* nut as a component of the betel morsel Chinese texts of 150 BCE, also mention BN as '*pinlang*' In Persia (modern Iran), it is believed that around 30000 shops sold BN in the capital town during the reign of Khosrau II, the King of Persia during 590 to 628 AD There is also mention of use of BN in one or the other form in different parts of the world including South and South-East Asia, several Pacific islands, many regions of the former Soviet Union, parts of North America and Europe (Sharan 1996) The use of BN is deeply ingrained in highly variable socio-cultural and religious practices across the globe (Warnakulasuriya 2002) BN is believed to be used by both men and women across all age groups and social classes though in some societies the latter predominate (Warnakulasuriya 2002) In old Indian scripts such as *Vagbhata* (4th century), and *Bhavamisra* (13th century), BN has also been described as a 'therapeutic agent' BN users report increased well-being and stamina, a soothing effect on the digestion, protection of the mouth and gums, and some euphoria Its use was recommended in wide ranging human diseases and other disorders, which included vitiligo or leucoderma, leprosy, anemia, digestive disorders and infections, urinary and dental infections, and obesity It has been suggested that BN chewing may confer protection against dental caries and other infections *In vitro* evidence indicates that *Areca* tannins may have anti-microbial activity, which may contribute to the cariostatic properties of BN Furthermore, betel stain, which coats the teeth of chewers, may act as a protective varnish (Trivedy, Craig and Warnakulasuriya 2002) BN is also reported to have aphrodisiac property and has been recommended as a general stimulant In China, it has been used as a vermifuge since the 6th century (Sharan 1996) The BN is predominantly consumed in its dry form, which is usually a very hard nut (Fig 1D) To make it easy to masticate or chew, the BN is cut into small to very small pieces (Fig 1E) In contrast, people in several parts of the world, including the whole of the north-eastern region of India, masticate the raw and wet form, which is relatively soft (Fig 1G) Hence, larger pieces of the nut are masticated (Fig 1I) Aged people may masticate even powdered form of raw/wet or dry variety of BN

Areca nut is normally harvested as unripe (green) or ripe (orange/red) fruit from the *Areca* palm (Fig 1B-C) The *Areca* fruits may be sun dried for several weeks, fibrous shells removed and the hard, dry nuts are ready for use (Fig 1D) Alternatively, the ripe *Areca* fruits are boiled for several hours in an aqueous solution containing the bark of the plant *Eugenia jambolana*, jaggery or brown sugar, and various edible oils, to 'cure' it. The cured fruits are sun dried for several weeks, fibrous shell removed and very hard, brown nuts are ready for use (Fig 1D) In contrast, ripe, partly ripe or unripe *Areca* fruits are freshly picked (Fig 1B-C), fibrous shells removed and the relatively soft nuts are ready for masticated (Fig 1G) Occasionally, the fruits can be cured by burying them into moist pits for one to two weeks for fermentation (maturation) before deshelling and use Such raw and wet variety of BN in the north-eastern part of India is locally called '*kwai*'or '*tambul*' (Fig 1G-I)

The BN is either consumed alone or with a wide variety of region and socio-culture specific additives as betel quid (BQ) In latter case, dry variety of BN is usually wrapped along with slaked lime (calcium oxide and calcium hydroxide or slacked lime) and catechu (*Acacia catechu*) without or with a host of additives, which may also include a variety of tobacco

products, perfumes, stimulants, etc , in a piece of betel leaf (Fig 1E-F) The raw/wet variety of BN is usually masticated with slaked lime wrapped in a betel leaf (Fig 1E, J) and occasionally supplemented with chewing tobacco (IARC 1985, Sharan 1996, Warnakulasuriya 2002) In India, most habitual chewers of BQ add tobacco, while in some countries, such as Papua New Guinea and China, tobacco is not added Betel leaf is perishable and the preparation of BQ is somewhat complex (Fig 1E) Hence, over the past three decades, commercial BQ substitutes, flavored and sweetened dry mixture of *Areca* nut, catechu and slaked lime with tobacco (*gutkha*) or without tobacco (*pan masala*), have become increasingly popular among habitual BN chewers (Nair, Bartsch and Nair 2004)

1. CONSTITUENTS OF BETEL NUT AND ITS ACTIVE PRINCIPLES

The constituents of BN include carbohydrates, crude fiber, fats, polyphenols, alkaloids, tannins, proteins, ash and water Trace amounts of fluorine, sapogenein, and free amino acids have also been reported in some forms The relative amounts of these constituents are highly variable in dry or raw/wet variety of BN Geographical and climatic conditions of growth of the *Areca* palm tree and the methods of curing BN also contribute to the observed variation in the constituents (Sharan 1996) Table - I shows the approximate content of different constituents of dry and raw/wet variety of BN The raw and wet variety of BN is relatively rich in all constituents as compared to the dry variety Notwithstanding these variations, the active components of both forms of BN, which produce betel nut associated effects, are primarily the alkaloids, polyphenols, and tannins

(a) **Alkaloids** Alkaloids are reduced pyridines BN contains primarily two alkaloids that are biologically highly relevant. Arecoline (1,2,4,5-tetrahydro-1-methyl-pyridinecarboxylic acid, molecular weight 155 19 Da) is the most abundant alkaloid of BN followed by arecaidine (1,2,5,6-tetrahydro-1-methyl-3-pyridinecarboxylic acid, molecular weight 141 17 Da) Other alkaloids such as, guvacine (methyl ester of arecaidine), guvacoline (methyl ester of guvacine) and arecolinidine are also present in small to very small or trace amounts (Table I) (Sharan 1996)

(b) **Polyphenols and tannins** The main polyphenols of BN are catechin, flavanoids, flavan-3 4-diols, leucocyanidins and hexahydroxyflavans When oxidized in the presence of lime, these give the characteristic red color to saliva, teeth and lips of BQ masticator The predominant tannin of BN is gallotannic acid In addition, minor amounts of gallic acid, D-catechol and phlobatannin are also present (Sharan 1996)

(c) **Betel nut specific nitrosamines (BSNA)** Numerous and highly complex nitrosamine derivatives are produced from different alkaloids of BN essentially by nitrosation of the alkaloid in the mouth and stomach, especially in acidic milieu, and in the presence of nitric oxide generated by bacterial action (Wary and Sharan 1991, Boucher and Mannan 2002) Figure 2 shows a typical and representative metabolic pathway of arecoline nitrosation and production of different derivatives The major biologically relevant nitrosamines of arecoline, appropriately grouped as betel nut specific nitrosamines (BSNA), are N-(methylnitrosamino) propionaldehyde (NMPA), N-(methylnitrosamino) propionitrile (NMPN) and N-nitosoguvacoline Of these, MNPA was reported to be the most potent BSNA on a molar basis effecting both survival and thiol content of cultured human buccal epithelial cells and causing significant formation of DNA single strand breaks (Sundqvist et al 1989) It is proposed that NMPA may further generate N-(methylnitrosamino) 3-hydroxypropionaldehyde and N-(methanoylnitrosamino) propionaldehyde derivatives, each of which can potentially produce several diazohydroxide derivatives (see Fig 2) Presence of most of these derivatives has been demonstrated in the saliva of BQ chewers (IARC 1985, Nair et al 1985)

(d) **Reactive oxygen species (ROS)** - Aqueous extracts of *Areca* nut and catechu were found to be capable of generating superoxide anion radicals (O_2) and hydrogen peroxide (H_2O_2) at pH greater than 9 5 (Nair et al 1987) While saliva was found to inhibit both O_2 and H_2O_2 formation from BQ ingredients, ROS are formed in the alkaline chewing mixture within the saliva of a chewer due to the addition of slaked lime (Stich and Anders 1989)

2. GENERAL EFFECTS OF BETEL NUT CONSUMPTION

BN is masticated or chewed for its psycho-stimulating effects (Norton 1998) When BN is masticated, it usually produces mild psychoactive and cholinergic effects Due to this, it is estimated that over 600 million individuals are habitual consumers of BN in one form or the other world-wide (Sharan 1996) Only three other 'addictive' substances - nicotine, ethanol and caffeine, are reported to be more widely used by human beings (Norton 1998) In north-east India, a raw/wet variety of BN called *kwai* or *tambul*, consumed with betel leaf and slaked lime, causes an immediate thermogenic physiological response lasting 2-3 min with significant perspiration on the forehead and reddening of ear pinnae (Sharan 1996) There is copious production of blood-red saliva that stains oral structures After years of chewing, the teeth may become red-brown to nearly black (Sharan 1996, Boucher and Mannan 2002) *In vitro* studies have demonstrated that *Areca* extracts containing arecoline inhibit growth and attachment of and protein synthesis in human cultured periodontal fibroblasts These findings suggest that *Areca* may be cytotoxic to periodontal fibroblasts and may exacerbate preexisting periodontal disease as well as impair periodontal reattachment (Trivedy, Craig and Warnakulasuriya 2002) The use of BQ was also found to be associated with the appearance of lichenoid lesions on the buccal mucosa and tongue, and betel chewer's mucosa, characterized by a brownish-red discoloration of the oral mucosa, often accompanied by encrustation of the affected mucosa with quid particles which are not easily removed, and with a tendency for desquamation and peeling (Trivedy, Craig and Warnakulasuriya 2002)

Acute ill effects are also reported at high rates of usage of BN and include cardiac arrhythmia, exacerbation of asthma, acute psychosis and acute gut upset (Boucher and Mannan 2002) Significant hyperglycemia was observed in male mice administered with BSNA, NMPN In fact, a population study revealed increase in waist size and weight, taken as markers for hyperglycemia, in direct relation to *paan*, a type of Indian BQ, usage among Asians These studies, thus, suggest that BN may be diabetogenic (Boucher and Mannan 2002) BN chewing was found to be independently associated with increased urinary albumin excretion and albuminuria in Taiwanese male patients of type - 2 diabetes (Tseng 2006) BN alkaloids, especially arecoline, have anti-muscarinic effects on the smooth muscle They are proposed to bind to GABA receptors in the brain, contributing to their psychoactive effects BN chewing is thought to reduce the severity of symptoms in schizophrenia with reduction in both positive and negative symptoms Withdrawal symptoms such as mood swings, anxiety, irritability, reduced concentration, sleep disturbance and craving were found to be associated with trying to quit the habit of BN chewing These findings are regarded to be consistent with the existence of a dependence syndrome among regular users In rare cases, *Areca* nut psychosis has been reported to occur in heavy users following abrupt cessation of the habit

(IARC 2004) One study of cases between 1988 and 1998 also reports toxicity of BN manifested in different individuals by tachycardia/palpitations, tachypnea/dyspnea, hypotension, sweating, vomiting, dizziness, chest discomfort, abdominal colic, nausea, numbness, coma, and acute myocardial infarction with its related manifestations (Deng et al 2001)

3. LINK BETWEEN BETEL NUT AND CARCINOGENESIS

Today, there is sufficient evidence that *Areca* nut or BN as well as BQ without or with tobacco is carcinogenic to humans (Sharan 1996, IARC 1985, 2004) BQ without tobacco causes oral cancer, while BQ with tobacco causes cancers of the oral cavity, pharynx and oesophagus (IARC 2004) A causal association between tobacco and BQ chewing habits and oral mucosal diseases such as leukoplakia, oral submucous fibrosis (OSF) and oral cancer has been established, and heavy users have a significantly increased mortality rate Oral cancer is the fifth most common cancer worldwide (Nair, Bartsch and Nair 2004) Of the 390000 oral and oro-pharyngeal cancers estimated to occur annually worldwide, 58% occur in south and south east Asia In India, there is reported addition of 75000 to 80000 new cases of oral cancer each year and the incidence rates of cancers of the oral cavity in both males and females in all urban cancer registries are among the highest in the world Time-trend analysis of cancers at all sites for the period 1990-1996 showed a decrease in cancers of the oral cavity in Indian population based registries, but an increase in the incidence of mouth cancer was reported among those aged < 50 years between 1983-1987 and 1995, consistent with the hypothesis of an increase in oral cancer among the young due to increased consumption of the alternative chewing products such as, *gutkha* and *pan masala* (Nair, Bartsch and Nair 2004) In Taiwan, data on oral cavity cancer from the period between 1986 and 1997 indicated that those who chew BN belong to a high-risk group (Lin et al 2005)

3.1. Induction Of Pre-Cancerous Lesions By Betel Nut

As an early sign of damage to the oral mucosa, chewers of BN or BQ with or without tobacco often develop clinically visible whitish (leukoplakia) or reddish (erythroplakia) lesions, which may or may not be accompanied by stiffening of the oral mucosa and OSF These manifestations are well established precancerous lesions and are taken as early and important indicators of oral cancer risk to an individual Some 2 – 12 % of these lesions have been reported to turn malignant over several years OSF, which is predominantly caused by the use of *Areca* nut, is a seriously debilitating and progressive disease marked by stiffening of the oral mucosa, development of fibrous bands and loss of elasticity of the mucosa, resulting in a progressive restriction of mouth opening Flavonoids, catechins and tannins of BN cause collagen fibers to crosslink making them less susceptible to collagenase This can cause increased fibrosis due to increased collagen production and decreased collagen breakdown OSF is irreversible and persists even after cessation of the chewing habit, suggesting that components of the *Areca* nut initiate OSF and then affect gene expression in the fibroblasts, which then produce greater amounts of normal collagen (Nair, Bartsch and Nair 2004) Considerable amounts of copper have been found in BN products Copper salts significantly increase the production of collagen by oral fibroblasts *in vitro* supposedly by upregulation of activity of a copper-dependent enzyme, lysyl oxidase, which catalyses the cross linking of collagens and elastin and is implicated in the pathogenesis of OSF (Nair, Bartsch and Nair 2004) In recent years, studies in India, China, south east Asia and South Africa, and on Asian migrants in the UK have shown a clear link between *Areca* nut chewing and OSF (Nair, Bartsch and Nair 2004)

3.2. Betel Nut And Betel Nut Extracts In Carcinogenesis

An increased incidence of local tumors was observed in mice after subcutaneous injection of aqueous extracts of BQ without tobacco Local tumors were produced in mice and local mesenchymal tumors in rats following subcutaneous injection of aqueous extracts of betel nut (AEBN) In hamsters, administration of *Areca* nut and application of its aqueous or dimethyl sulphoxide extracts to the cheek-pouch mucosa resulted in squamous cell carcinomas (SCC) of the cheek pouch and carcinomas of the fore-stomach (IARC 1985) While BQ has various components (IARC 1985, Sharan 1996, Warnakulasuriya 2002), a study on Syrian hamsters revealed that BN fiber and cold aqueous extract are the major components of BQ that may promote carcinogenesis in the hamster buccal pouch, leading to tumor formation AEBN has been shown to induce conformational changes in mouse liver high mobility group (HMG) proteins similar to that induced by a hepatocarcinogen, diethylnitrosamine (DEN), leading to the development of preneoplastic nodules in the liver (Pariat and Sharan 1998a, 1998b) The post-translational modification of proteins such as, poly-ADP-ribosylation of HMG (Pariat, Balachandran and Sharan 1999, Pariat and Sharan 2002) and histone (Saikia, Schneeweiss and Sharan 1998, 1999a, 1999b) proteins was also strongly affected by exposure to BN resulting in alterations in chromatin organization

a) Cytotoxicity - *Areca* nut extract was found to decrease cell survival, vital dye accumulation and membrane integrity of cultured human buccal epithelial cells in a dose-dependent manner BN also caused formation of both DNA single strand breaks and DNA protein cross links (Wary and Sharan 1988, Sundqvist et al 1989, Wary and Sharan 1991) Different extracts of BN such as, AEBN, acetic acid extract (AAEBN), HCl extract (HEBN) and ethanol extract (EEBN) as well as arecoline showed different extents of cytostatic and cytotoxic effects on Hep2 cells *in vitro*, with arecoline, HEBN and EEBN being the most potent (Sharan and Wary 1992) Cultured normal human oral keratinocytes (NHOK) exposed to ripe BN extract also showed significant decrease in population doubling, increase in senescence, cell cycle arrest at G_1/S phase and decrease in cell proliferation (Lu et al 2006) Hamsters fed with powdered diet containing BN or BQ showed significant decrease in the survival rate, body weight, and hyperkeratosis and acanthosis of cheek pouch indicating that BN and BQ components may induce alterations in proliferation and differentiation of oral epithelial cells (Chiang et al 2004)

b) Genotoxicity - BQ and its components were found to be genotoxic Interestingly, they also stimulated cell proliferation making the observed biological effects very complex For instance, while the extracts of BN and inflorescence of *Piper betle* (IPB) induced DNA strand break, the extracts of BN, IPB, the BN polyphenol (+)-catechin and arecoline decreased cell survival and proliferation On the other hand, another component of BQ, the aqueous extract of lime, was found to increase cell proliferation (Jeng et al 1994) AEBN was found to reduce GSH levels, induce chromosomal aberrations (CA) and delay cell kinetics in mouse bone marrow cells with the induction of sister chromatid exchange (SCE) probably involving p53 dependant changes in cell proliferation (Kumpawat et al 2003) Ethyl acetate and *n*-butanol extracts of BN as well as betel leaf are reported to induce CA in human lymphocytes and Chinese hamster ovary (CHO) cells (IARC 1985) All components of BQ have been shown to individually enhance chromatid breaks and exchanges in the range of 12 to 37 % in human cells *in vitro* Frequency of SCE was elevated in mouse bone marrow cells when mice were exposed to the AEBN and its tannin (Panigrahi and Rao 1989) AEBN also

induced DNA strand breaks and enhanced cell proliferation in mouse kidney cells *in vitro* (Wary and Sharan 1988) A study revealed that OSF was largely associated with BN and the exfoliated oral mucosal cells of such patients had significantly higher numbers of micronucleated cells The patients also exhibited increased SCE in circulating lymphocytes indicating that the carcinogenic agents in BN produce damage not only in target tissue but also in other host cells such as circulating lymphocytes (Desai et al 1996)

c) **Immunotoxicity** - Aqueous extracts of raw *Areca* nut without husk as well as with husk were found to inhibit the phagocytic activity of human neutrophils in a dose dependent manner (Hung et al 2005) BQ also influenced cytokine production of peripheral blood mononuclear cell The mononuclear cells of persons suffering from SCC, with a long history of BQ chewing, produced lower levels of TGF-β, TNF-α and IFN-γ in comparison to normal persons (Hsu et al 2001) This was indicative of compromised immune system under the influence of BQ or BN chewing

d) **Mutagenicity** - Aqueous extracts of BQ without tobacco induced mutations in *Salmonella typhimurium* but not in Chinese hamster V79 cells BQ also did not induce any significant micronuclei in Swiss albino mice (IARC 1985) AEBN, on the other hand, induced mutations in *Salmonella typhimurium* and in Chinese hamster V79 cells besides inducing gene conversion in *Saccharomyces cerevisiae* as well as CA in CHO cells It also induced micronuclei in bone marrow cells of Swiss albino mice while BN tannin fraction induced gene conversion in *Saccharomyces cerevisiae* (IARC 1985) Arecoline, AEBN, AAEBN, HEBN and EEBN induced variable levels of dose dependent unscheduled DNA synthesis (UDS) in Hep2 cells *in vitro* (Sharan and Wary 1992, Sharan 1996) Ames test using *Salmonella typhimurium* strain TA 1535 revealed that arecoline, AEBN and HEBN were weak mutagens while AAEBN and EEBN were strong mutagens suggesting that the mutagenic potential of alkaloids (arecoline) could be significantly enhanced by other constituents of BN (Sharan 1994, Balachandran and Sharan 1995, Sharan 1996) Exposure to BN extracts was found to induce mutations at the *hypoxanthine phosphoribosyltransferase* (*HPRT*) locus in human keratinocytes (HaCaT cells), which also increased frequency of appearance of micronuclei, intracellular levels of reactive oxygen species and 8-hydroxyguanosine in the cells suggesting that stress caused by long-term BN extract exposure enhanced oxidative stress and genetic damage in HaKaT cells (Lai and Lee 2006) When aqueous extracts of different brands of *pan masala* and scented BN or *supari* were tested for mutagenicity by the *Salmonella typhimurium* assay using tester strains TA98 and TA100 their mutagenic effects were found to be similar to that produced by BN extracts (Polasa , Babu and Shenolikar 1993) A study involving patients of head and neck cancer suggested that BQ chewing may increase mitochondrial DNA (mtDNA) mutation in human oral tissues and that accumulation of mtDNA deletions and subsequent cytoplasmic segregation of these mutations during cell division could be important contributors to the early phase of oral carcinogenesis (Lee et al 2001)

e) **Teratogenesis:** - Aqueous extracts of dry as well as raw/wet varieties of BN were reported to be fetotoxic in Swiss albino mice leading to death, enhanced resorption and reduced weight of fetuses Other abnormalities such as hematomas, curved tails, abnormal ribs and delay in skeletal maturity have also been reported (Sinha and Rao 1985a)

3.3. Betel Nut Alkaloids In Carcinogenesis

Alkaloids of BN are suspected to be its main carcinogenic constituent (IARC 1985, 2004, Sharan 1996, Norton 1998, Jeng, Chang and Hahn 2001, Trivedy, Craig and Warnakulasuriya 2002) Early studies found that the application of arecaidine to the oral mucosa of experimental animals failed to have any carcinogenic effects unless it was supplemented with a known promoter such as, croton oil (Trivedy, Craig and Warnakulasuriya 2002) Arecoline given by gavage produced lung adenocarcinomas, stomach SCC and liver haemangiomas in male mice (IARC 2004) Cheek-pouch application of arecoline following application of slaked lime produced an esophageal papilloma in female hamsters, while local application of arecaidine to the cheek pouch did not produce tumors in male hamsters (IARC 2004) To explain the variable observation, it is proposed that the alkaloids first required metabolic activation via nitrosation to develop its carcinogenicity (Wary and Sharan 1991) In rats, the major metabolic pathway of arecoline activation is via de-esterification and production via conjugated mercapturic acid *In vitro* data suggest that arecoline is metabolized by carboxylesterase (EC 3 1 1 1) in mouse liver and kidney Male Swiss albino mice fed *Areca* nut powder or arecoline showed enhanced levels of the hepatic cytochrome P450 and b_5 and decreased levels of hepatic glutathione (GSH) (IARC 2004) Exposure of Swiss albino mice to arecoline was found to lower poly-ADP-ribosylation of most cellular and histone proteins and induce relaxation of chromatin, thereby allowing the N-nitrosamines of arecoline easy access to genomic DNA for interaction, while the absence of PADPR induced repair may favor the accumulation of DNA damage (Saikia, Schneeweiss and Sharan 1999b)

a) **Cytotoxicity** - Arecoline was found to inhibit cell attachment, cell spreading and cell migration in a dose dependent manner in cultured human gingival fibroblasts (HGF) (Jeng et al 1996) In Hep2 cells *in vitro*, arecoline inhibited both DNA and protein syntheses in a dose dependent manner, which ultimately resulted in cytostatic effect on cell division (Wary and Sharan 1991) Crude alkaloid extracts of green *Areca* fruit consumed in Taiwan and arecoline were found to be mutagenic in *Salmonella typhimurium* TA100, and N-Nitrosoguvacoline (NG) was weakly mutagenic in TA98 and TA100, with the formation of NG being favored at neutral pH (Wang and Peng 1996) Arecoline alone was also only weakly mutagenic (Balachandran and Sharan 1995) *In vitro* studies have shown that arecoline and arecaidine may stimulate cultured fibroblasts to proliferate and synthesize collagen, an important step in development of OSF However, subsequent *in vitro* studies have failed to show similar effects of arecoline on cultured OSF fibroblast It has also been shown that arecoline inhibited collagen synthesis and fibroblast proliferation *in vitro*, indicating the cytotoxic properties of arecoline The disparity of results from *in vitro* studies might be indicative of other agents, in addition to arecoline, being important in the pathogenesis of OSF (Trivedy, Craig and Warnakulasuriya 2002) The cytotoxicity of arecoline on the oral mucosal fibroblast (OMF) or on Hep2 cells was found to be associated with cellular GSH levels and esterase activities on one hand (Jeng et al 1999), and the agents that facilitate metabolic activation and nitrosation of alkaloids, on the other (Wary and Sharan 1991) In fact, GSH depletion and reduction of glutathione *S*-transferase activity have been demonstrated in cultured human oral keratinocytes and in fibroblasts treated with arecoline (IARC 2004) Arecoline was also reported to be cytotoxic to human buccal fibroblasts in a dose dependent manner wherein the cellular glutathione-S-transferase (GST) activity was downregulated in a dose dependent manner without increase in lipid peroxidation Addition of extracellular nicotine acted synergistically on the arecoline-induced cytotoxicity, indicating that arecoline may render human OMF more vulnerable to other reactive agents in cigarettes via GST reduction These observations could explain why patients who practice the combined habit of BQ chewing

and cigarette smoking are at greater risk of contracting oral cancer (Chang et al 2001a)

Global gene expression profiling in HGF exposed to arecoline revealed that four genes related to maintenance of genome stability and DNA repair were repressed by arecoline (Chiang et al 2007) They are *FANCG*, also known as *XRCC9* (tumor suppressor capable of correcting CA), *CHAF1* and *CHAF2* (encoding chromatin assembly factor I, CAF1), and *BRCA1* (breast cancer susceptibility gene implicated in DNA damage response and DNA repair) Among them, at least the *BRCA1* response was dose dependent. *COX-2/ PTGS2*, which are involved in cancer initiation and progression, were over expressed in HGF cells *HSP4A1* and *DNAAJA1*, which belong to the *HSP70* family of stress induced proteins, and GDF15/ MIC-1 were also upregulated by arecoline in a dose dependent manner (Chiang et al 2007)

b) **Genotoxicity** - Arecoline was found to induce mutations in *Salmonella typhimurium* and Chinese hamster V79 cells, and CA in CHO cells It also induced micronuclei, CA and SCE in bone marrow cells of Swiss albino mice (IARC 1985, Deb and Chatterjee 1998) However, upon withdrawal of arecoline exposure regime from Hep2 cells *in vitro* the inhibited DNA synthetic index fully recovered (Wary and Sharan 1991) suggesting existence of weak interaction between BN genotixin and DNA Arecaidine induced mutations in *Salmonella typhimurium* and Chinese hamster V79 cells It also induced SCE but not micronuclei in bone marrow cells of Swiss albino mice (IARC 1985) This arecoline induced DNA damage was found to be influenced by endogenous GSH levels with the frequency of CA and SCE increasing when arecoline was given to mice treated with buthionine sulfoximine (BSO), a GSH depleting agent (Lu et al 2006)

c) **Immunotoxicity** - Arecoline was found to cause inhibition of both humoral and cell-mediated immune responses in mice (IARC 2004) It is reported to interfere with the immune system by targeting the muscarinic acetylcholine receptors of the non-neuronal cholinergic system (Wen et al 2006) Arecoline was also found to inhibit the phagocytic activity of human neutrophils (Hung et al 2005)

d) **Cell-cycle alterations** - Arecoline inhibited growth of human KB epithelial cells in dose- and time dependent manners by causing cell cycle arrest in late-S and G2/M phases due to induction of cyclin B1, Wee 1, and phosphorylated cdc2 proteins and inhibition of p21 protein expression in KB cancer cells In primary human gingival keratinocyes (HGK) arecoline effect was mediated differently In this case, arecoline induced p21 but inhibited cdc2 and cyclin B1 proteins This clearly suggests that differential regulation of S and/or G2/M cell cycle related proteins in the HGK and KB cells play crucial roles in different stages of BQ mediated carcinogenesis (Lee et al 2006) Arecoline, which was cytotoxic to HGF cells due to depletion of intracellular thiols and inhibition of mitochondrial activity, induced cell cycle arrest in HGF cells at G2/M phase in a dose dependent manner (Chang et al 2001b)

e) **Teratogenicity:** - Arecoline has been reported to induce abnormality in the shape of sperm heads and unscheduled DNA synthesis (UDS) in the early spermatid stages of Swiss albino mice (Sinha and Rao 1985b) It also induced micronuclei formation in fetal mouse blood after transplacental exposure to BN (Sinha and Rao 1985c) Arecoline caused general developmental retardation of zebra fish embryos predominantly due to a general cytotoxic effect induced by depletion of intracellular thiols (Chang et al 2001c) Arecoline hydrobromide has been reported to have teratogenic effects on developing chick embryos leading to embryo mortality, retarded development of fetuses and other abnormalities The abnormalities included reduced body size, scanty feathering, general edema with light body color, shortened lower beak, clubfoot, missing or unossified rib and shortening of long bones (Paul et al 1999).

4. BETEL NUT AND TUMOR SUPPRESSOR GENES *P53, BRCA1* AND *BRCA2*

Tumor suppressor genes are critical in carcinogenesis because loss of their function(s) results in promotion of malignancy (Kinzler and Vogelstein 1997) Prominent among them is *p53* gene encoding a 393-amino acid residue long p53 protein, which is maintained at low cellular level in normal cells due to MDM2 mediated rapid turnover (Lane 1992, Levine 1997) Cells exposed to carcinogen or other stresses rapidly accumulate p53 due to its stabilization and/or mutation Mutated or stabilized p53 induces cell cycle arrest at G1/S or G2 checkpoints The quiescent cells are now in a position to repair the damage caused by the carcinogen or other stress factors and come out of it Thus, p53 functions as a 'gatekeeper' tumor suppressor The breast cancer susceptibility genes *Brca1* and *Brca2* are other two tumor suppressor genes relevant to human carcinogenesis Both Brca1 and Brca2 proteins are functionally grouped as 'caretakers' as they are involved with repair of DNA breaks, especially the critical double stranded breaks (DBS), via homologous recombination (HR) repair pathway in association with RAD family and other proteins (Welsch, Owen and King 2000, Yilun and West 2002)

Consistent with projected functions of *p53*, *Brca1* and *Brca2* tumor suppressor genes, mutation or alteration in expression or both is expected in these tumor suppressor genes/proteins during carcinogenesis Indeed, *p53* gene, one of the most extensively studied tumor suppressor genes, is known to be mutated in a variety of human and experimental animal cancers Similarly, change in cellular level of p53 protein is also known to occur Accumulation of p53 protein or its stabilization is an important indicator of the presence of mutant p53 protein (Hollstein et al 1991, Harris and Hollstein 1993) However, reports pertaining to *p53* mutation status of cancers associated with BN chewing have been widely contradicting A study of Sri Lankan subjects with histologically confirmed oral squamous cell carcinoma (OSCC) and the habit of BN chewing with tobacco revealed low expression of p53 protein (Ranasinghe, Warnakulasuriya and Johnson 1993) A similar study in BN and tobacco associated OSCC from Southern India showed nuclear p53 staining and p53 expression indicating that carcinogens derived from tobacco and BN chewing may induce p53 mutations (Kuttan et al 1995) BQ chewers in Taiwan exhibited significantly higher incidence of *p53* gene mutations than non-chewers in esophageal squamous cell carcinoma (ESCC) The A T → G C transition and G C → T A transversion were the prevalent spectra of *p53* gene mutations and alcohol consumption could enhance this peculiar spectrum of *p53* mutation in ESCC suggesting that *p53* might be an important molecular target of BQ carcinogens in the development of ESCC in Taiwanese (Goan et al 2005) Another study on patients of OSCC in Taiwan revealed that G C → A T transitions were the predominant mutations in the *p53* gene associated with BQ and tobacco use (Hsieh et al 2001) Mutations in the *p53* gene were also frequent in OSCC specimens from Sri Lanka obtained from BQ chewers They exhibited point, small deletion and addition type of mutations mainly clustered in exon 5 of the *p53* gene These results indicate that exon 5 of the *p53* gene could be one of the specific targets for some BQ ingredients, and BQ chewing may be a critical environmental factor in the development of OSCC (Chiba et al 1998) A study of potentially malignant oral lesions (leukoplakia) and OSCC associated with BQ

consumption in northern India revealed a good correlation between p53 missense mutations, p53 antibodies and p53 protein accumulation in matched potentially malignant and malignant oral lesions (Ralhan et al 2001) Alternatively, incidence of *p53* mutations was reported to be infrequent or absent in oral premalignant lesions and OSCC in subjects chewing BQ with tobacco (Kannan, Munirajan and Krishnamurthy 1999) and without tobacco (Thomas, Brennan and Martel 1994, IARC 2004) Mutations in both BRCA genes are known to be prevalent in familial as well as sporadic breast cancers (Rajan et al 1996, Nadeau et al 2000) However, not much is known about the status of these two important tumor suppressor proteins in BN associated carcinogenesis in mice or men

We have made a systematic effort to study the effect of long term and transgenerational exposure of Swiss albino mice to AEBN on expression of p53, Brca1 and Brca2 proteins as well induction of mutation in exons 5 and 7 of the *p53* gene and exon 11 of the *Brca1* gene Chronic exposure to AEBN in drinking water led to an upregulation of p53 protein in liver, spleen and peripheral blood lymphocytes (PBL) of exposed parental (P) generation mice from 2 weeks onwards reaching a maximum (2 5 folds of the age-matched control) after 6 weeks of exposure in the liver and spleen and 4 weeks of exposure in PBL (Fig 3, panel A) Subsequently, the level of p53 protein declined gradually reaching control level after 16 weeks of exposure concomitant with the appearance of pre-neoplastic nodules in the liver (Fig 3, panel A) After 24 weeks of exposure p53 protein was below control level, and the pre-neoplastic nodules were well developed The expression of Brca1 (Fig 3, panel B) and Brca2 (Fig 3, panel C) proteins showed immediate elevation in liver, spleen and PBL after 2 weeks of exposure followed by a decline to 60 % of that of age-matched control after 16 weeks of exposure and 50 % after 24 weeks of exposure No mutation in exons 5 and 7 of the *p53* gene (GenBank accession # EF570972 and EF634061) and exon 11 of the *Brca1* gene (Yashmin and Sharan 2008, unpublished) were detected Transmission electron microscope (TEM) study of the liver pre-neoplastic nodules after 24 weeks of exposure revealed a large number of binucleated cells with enlarged and abnormally shaped nuclei (Fig 4B) as compared to the controls (Fig 4A) Disruption of nuclear membrane as well as chromatin condensation and marginalization were also observed in a significant number of nuclei (Fig 4C-D) Damage to mitochondria was most noticeable The size of normal mitochondria (Fig 4E) was significantly reduced (Fig 4F) in all cases showing shrinkage This was also accompanied with membrane disruptions (Fig 4C, F, arrow head) The rough endoplasmic reticulum membrane organization (Fig 4H) was also severely damaged (Fig 4I, arrow head)

Extensive damage of the mitochondrial membrane is a pro-apoptotic signal and extensive disruption of the ER could lead to calcium release from the ER lumen, which can potentially trigger ER-stress induced apoptosis Thus, chronic exposure to AEBN caused serious molecular and metabolic damage to cells characterized by enlarged nuclei, high frequency of abnormally shaped nuclei, chromatin condensation and marginalization, and damaged membrane (Fig 4) along with downregulated p53, Brca1 and Brca2 proteins (Fig 3) After 16 weeks of chronic exposure to AEBN, an inability to upregulate p53 beyond control level, combined with compromised DNA repair due to downregulation of Brca1 and Brca2, are sufficient to allow progression of hepatocarcinogenesis

The effect of prenatal and transgenerational chronic exposure to AEBN has been followed up to F3 generation by breeding In striking contrast to the P generation, the liver, spleen and PBL of AEBN exposed F1, F2 and F3 generation mice exhibited invariant expression of p53 protein in comparison to age matched controls throughout the period of exposure (Fig 3A) Similarly, the expression of Brca1 (Fig 3B) and Brca2 (Fig 3C) proteins progressively declined to approximately 80 % that of age matched controls only after 2 weeks of exposure in all the three tissues examined Thus, while the P generation mice exhibited an induction of the tumor suppressive functions of the p53, Brca1 and Brca2 proteins during the initial periods of AEBN exposure, the transgenerationally exposed mice failed to induce these tumor suppressors (Fig 3) Concomitantly, a significant advancement in the appearance of liver pre-neoplastic nodules was observed with each subsequent generation suggesting progressive enhancement of transmission of carcinogenic risk due to exposure to BN constituents (Table II) Abnormalities, which were not observed in P generation mice, also developed in the transgenerationally exposed mice Though statistically insignificant up to F3 generation, these abnormalities included enlargement of lymph nodes of the neck, development of protuberant pus-filled sacs, necrosis of the liver and development of solid tumors No mutations in exons 5 and 7 of the *p53* gene were observed in the liver nodules as well as solid tumors of even the transgenerationally exposed mice Thus, while inactivation of the p53 gene apparently plays a crucial role in BN associated cancer in mice, the inactivation is not ubiquitously through *p53* mutation and other routes of inactivation require to be investigated One possible alternative mechanism for p53 inactivation in BN carcinogenesis may be over-expression of MDM2 protein as has been shown in OSCC (Shwe et al 2001) In contrast BN induced solid tumors in Swiss albino mice carried a G → C (codon 156, –TGT– → –TCT–) transversion mutation in exon 11 of the *Brca1* gene (Fig 5) Exon 11 of the *Brca1* gene encodes the two nuclear localization motifs and a region of the gene believed to be essential for binding of RAD51 (Cressman at al 1999) Mutation in exon 11 would, therefore, disrupt normal functioning of the *Brca1* gene leading to DNA repair defects, which could be pivotal for the development of the solid tumors Thus, AEBN can possibly lead to transgenerational transmission of carcinogenic risk in Swiss Albino mice by compromising the functions of the tumor suppressor genes *p53*, *Brca1* and *Brca2* via different mechanisms

5. BETEL NUT POLYPHENOL AND TANNINS IN CARCINOGENESIS

Toxicity studies relating to BN specific polyphenols and tannins are not conclusive with both carcinogenic and anti-carcinogenic effects being reported It is reported that ROS produced during auto-oxidation of BN polyphenols in the BQ chewer's saliva are crucial in the initiation and promotion of oral cancer (Jeng, Chang and Hahn 2001) Incidences of certain cancers, such as esophageal cancer, have been reported to be related to consumption of tannins-rich foods such as BN suggesting that tannins might be carcinogenic However, other reports indicated that the carcinogenic activity of tannins might be related to components associated with tannins rather than tannins themselves (Chung et al 1998)

6. BETEL NUT AND HUMAN GENETIC SUSCEPTIBILITY TO ORAL CANCER

Exposure to BN carcinogens, particularly the alkaloids, enhances the risk of cancer in BN or BQ chewers in general However, correlation between prevalence of cancer in human populations in different parts of the world and habit of BN/BQ mastication is not absolute This suggests that the genetic makeup of the masticator has its own influence on the ultimate manifestation of BN induced cancer It is becoming obvious that the interplay between the genetic constitution and the environmental factor(s), determine the final risk of human oral cancer following exposure to BN or BQ alone or in combination with additives, including tobacco Mere exposure

to BN or BQ does not commit the chewer to cancer For any given level of exposure to BN carcinogen, only a proportion of exposed individuals will develop cancer, indicating the prevalence of inter-individual differences in susceptibility (Spitz and Bondy 1993) Individual susceptibility to cancer may result from several factors including (a) differences in metabolism, (b) status of DNA repair pathways and related genes, (c) patterns of expression of proto-oncogenes and tumor suppressor genes, and (d) nutritional status of the masticator, etc Variations in an individual's metabolic phenotype, i e , phenotypic polymorphism, have also been detected in a variety of enzymes involved in activation and detoxification of chemical carcinogens It is becoming clearer now that different phenotypic and/or metabolic variations stem from genetic polymorphisms prevalent in different population groups (Bartsch and Hietanen 1996) A number of genetic polymorphisms have been identified, which seem to be associated with risk of BN induced oral cancer in human sub-populations Table III depicts the up to date list of polymorphisms observed in BN exposed human sub-populations with manifestation of oral cancer

7. POSSIBLE MECHANISM OF BETEL NUT INDUCED CARCINOGENESIS

BN is a natural plant product characterized by a very complex and highly variable mixture of different biochemical and nutraceutical constituents (Table I) Some of these are recognized as potent carcinogens (e g , alkaloids, polyphenols, tannins, etc) However, many others, especially those present in small to trace amounts, have largely unknown biological functions As with nutraceuticals, it is anticipated that in a complex cellular environment some of these may function as mediators, some as modulators, affecters, promoters, and/or inhibitors, *etc eliciting a variety of biological effects and* responses The highly variable constituents of BN should also chemically and otherwise interact differently with different biomolecules The situation is further complicated by the fact that a host of region, culture and society specific additives, notably different types of chewing tobaccos, are invariably added to BN preparation by a traditional masticator (Fig 1) Therefore, it is only expected that the mechanism of BN induced carcinogenesis would also be highly variable and complex Nonetheless, certain conclusions can be drawn from the wealth of knowledge available to us (see Fig 6) The overall perception is that alkaloids are the main carcinogenic constituents of BN Polyphenols and tannins may also contribute positively to carcinogenic potency of the alkaloids It is *now accepted that alkaloids, the primary suspected* carcinogen of BN, should first undergo metabolic activation and nitrosation to produce the ultimate carcinogenic derivatives together called BSNA (Fig 2) This may be achieved by de-esterification using carboxylesterase and may also involve cytochrome P450, b_5 and GSH besides other metabolites The activated or ultimate carcinogen(s) acquires capability of interaction with target biomolecules The interaction of BSNA or their activated derivatives with cellular targets forming adducts of different kinds may be the beginning of carcinogenesis Using different physic-chemical methods evidence of existence of BSNA adducts has been amply demonstrated (IARC 1985) Recently, formation of DNA adducts by AEBN and its consequences has been directly shown on a plasmid DNA construct, pMTa4 (Bhattacharjee and Sharan 2008) Using the plasmid model *in vitro* and *in vivo* it has been shown that under chronic exposure condition up to one BN specific adduct could be formed every 3 nucleotides In other words, up to 3 adducts can potentially be formed per helical turn of DNA double helix These adducts were essentially unstable and dissociated from DNA in about 24 hours in line with known weak, non-covalent and reversible nature of interaction of BSNA or their metabolites with DNA (Wary and Sharan 1991, Sharan 1996) However, we have also discovered that the BN adducts became stable in the presence of trace amounts of monovalent cations, Na^+ and K^+ (Bhattacharjee and Sharan 2008) Since physiological cellular concentrations of these monovalent ions are more than the concentration required to confer stability to BSNA adducts on DNA, it explains, at least in part, why habitual BN chewers are at high risk of stable adduct formation on their genetic material and consequent risk of mutagenesis/carcinogenesis It has been shown that the risk of carcinogenesis progressively increased under continuing environment of BN exposure (Table II) Possible biological consequence of adduct formation on DNA as well as damage inflicted upon the genetic material due to presence of such adducts could be many At first, adducts on DNA may induce strand break, induce CA, SCE, UDS, etc Secondly, damage to the genetic materials may cause alteration in pattern of gene expression In particular, the changes in tumor suppressor genes *p53*, *Brca1* and *Brca2* either by way of p53 stabilization or mutation in critical domains are likely to diminish their tumor suppressor properties and favor carcinogenesis (Fig 3) Thirdly, BN and their constituents, especially arecoline, has been shown to differentially dysregulate cell cycle control, mitochondrial membrane potential, GSH level and intracellular H_2O_2 production in the pathogenesis of OSF and oral cancer (Chang et al 2001d) Reduction of GSH content by arecoline and BN extract and enhanced cytochrome

P450 activity, which were observed in the liver of mice treated with BN, could cause increased oxidative metabolism of carcinogens and reduced detoxification GSH depletion leads to increased oxidative stress that can cause DNA damage and trigger several response signals implicated in the carcinogenic process (Nair, Bartsch and Nair 2004) Thus, BN and its constituents potentially interfere with cell signaling pathways Little is understood about these aspects and more research is needed to unravel the influence of BN exposure on the complex cell signaling pathways In spite of this, it is known that BQ chewing contributes to the pathogenesis of cancer and OSF also by impairing T cell activation and by induction of PGE2, TNF-α and IL-6 production, which favors oral mucosal inflammation and growth of OMF and oral epithelial cells (Jeng et al 2003) Similar end may also be achieved by activation of the MEK1/ERK/c-Fos pathway, which promotes keratinocyte inflammation, cell survival, and affects cell cycle progression (Chang et al 2004) Alternatively, MMP-2, an enzyme belonging to matrix metalloproteinases (MMP) group of *proteins that degrade extracellular matrix proteins* and contribute to the tumor invasion and metastasis, was found to be elevated in most oral tumor patients with long term BQ usage while short term BQ usage increased the secretion of MMP-2 by oral epithelial cells and fibroblasts This is suggestive of BQ consumption promoting oral tumor progression through the induction of MMP-2 secretion (Kato et al 2005, Liu et al 2005a) Elevation of MMP-9 was also observed following BQ chewing showing its role in the pathogenesis of oral mucosal lesions (Liu et al 2005b) Due to this, levels of both MMP-2 and MMP-9 have been suggested as possible markers of human oral cancer (Patel et al 2007) In all this, it has to be kept in mind that mere exposure to BN does not commit a cell or an organism to carcinogenesis There are metabolic escape routes available to the exposed cell or organism by way of complete repair of damage and attainment of normalcy or necrotic or apoptotic programmed cell death (Fig 6) Metabolic, cellular and other genetic factors, in complex and largely unclear ways, influence the path of carcinogenesis triggered by exposure to BN

ACKNOWLEDGEMENTS

RNS acknowledges contributions of all research associates and scholars whose work has been cited in this review Grants of UGC, CSIR, DST and ICMR, which supported different aspects of research embodied in this review, are also acknowledged Part of the ongoing research is being carried out under a research scheme partly funded by the UPE-NEHU grant YC was supported by a fellowship (NET-JRF/SRF) from the UGC and is currently working as a Lecturer, Department of Biotechnology, St. Anthony's College, Shillong

8. REFERENCES

Balachandran, B, and Sharan, R. N 1995 Induction of mutations by different extracts of betel nut and radiation Their implication in carcinogenesis In Radiation Research 1895-1995, eds U Hagen, H Jung, and C Streffer, pp 165, vol 1, Würzburg Universitatsdruckerei H Stürtz AG

Bartsch, H and Hietanen, E 1996 The role of individual susceptibility in cancer burden related to environmental exposure Environ Health Perspect 104 569-577

Bhattacharjee, C, and Sharan, R. N 2008 Aqueous extract of betel nut (AEBN) induced adducts on pMTa4 DNA acquires stability in the presence of Na^+ and K^+ ions Mol Med Rep 1 435-441

Boucher, B J, and Mannan, N 2002 Metabolic effects of the consumption of *Areca catechu* Addict Biol 7 103-110

Chang, B, Liao, M, Kuo, M Y, and Chen, C 2001c Developmental toxicity of arecoline, the major alkaloid in betel nuts, in zebrafish embryos Birth Defects Res 70 28-36

Chang, K W, Lee, T C, Yeh, W I, Chung, M Y, Liu, C J, Chi, L Y, and Lin, S C 2004 Polymorphism in heme oxygenase-1 (HO-1) promoter is related to the risk of oral squamous cell carcinoma occurring on male areca chewers Br J Cancer 18 1551-1555

Chang, M C, Ho, Y S, Lee, P H, Chan, C P, Lee, J J, Hahn, L J, Wang, Y J, and Jeng, J H 2001d Areca nut extract and arecoline induced the cell cycle arrest but not apoptosis of cultured oral KB epithelial cells association of glutathione, reactive oxygen species and mitochondrial membrane potential Carcinogenesis 22 1527-1535

Chang, M C, Wu, H L, Lee, J J, Lee, P H, Chang, H H, Hahn, L J, Lin, B R, Chen, Y J, and Jeng, J H 2004 The induction of prostaglandin E2 production, interleukin-6 production, cell cycle arrest, and cytotoxicity in primary oral keratinocytes and KB cancer cells by areca nut ingredients is differentially regulated by MEK/ERK activation J Biol Chem 279 50676-50683

Chang, Y C, Hu, C C, Liu, C K, Tai, K W, Yang, S H, and Chou, M Y 2001b Cytotoxicity and arecoline mechanisms in human gingival fibroblasts *in vitro* Clin Oral Investig 5 51-56

Chang, Y C, Hu, C C, Tseng, T H, Tai, K W, Liu, C K, and Chou, M Y 2001a Synergistic effects of nicotine on arecoline-induced cytotoxicity in human buccal mucosal fibroblasts J Oral Pathol Med 30 458-464

Chen, C L, Chi, C W, Chang, K W, and Liu, T Y 1999 Safrole-like DNA adducts in oral tissue from oral cancer patients with a betel quid chewing history Carcinogenesis 20 2331-2334

Chiang, C, Chang, M, Lee, J, Chang, J Y, Lee, P, Hahn, L, and Jeng, J 2004 Hamsters chewing betel quid or areca nut directly show a decrease in body weight and survival rates with concomitant epithelial hyperplasia of cheek pouch Oral Oncol 40 720-727

Chiang, S, Jiang, S, Wang, Y, Chiang, H, Chen, P, Tu, H, Ho, K, Tsai, Y, Chang, I, and Ko, Y 2007 Characterization of arecoline-induced effects on cytotoxicity in normal human gingival fibroblasts by global gene expression profiling Toxicol Sci 100 66-74

Chiba, I, Muthumala, M, Yamazaki, Y, Zaman, A U, Iizuka, T, Amemiya, A, Shibata, T, Kashiwazaki, H, Sugiura, C, and Fukuda, H 1998 Characteristics of mutations in the *p53* gene of oral squamous-cell carcinomas associated with betel-quid chewing in Sri Lanka Int J Cancer 77 839-842

Chiu, C J, Chang, C P, Hahn, L J, Hsieh, L L, and Chen, C J 2002 Interaction of collagen-related genes and susceptibility to betel quid-induced oral submucous fibrosis Cancer Epidemiol Biomarkers Prev 11 646-653

Chiu, C J, Chiang, C P, Chang, M L, Chen, H M, Hahn, L J, Hsieh, L L, Kuo, Y S, and Chen, C J 2001 Association between genetic polymorphism of tumor necrosis factor-alpha and risk of oral submucous fibrosis, a pre-cancerous condition of oral cancer J Dent Res 80 2055-2059

Chung, K T, Wong, T Y, Wei, C I, Huang, Y W, and Lin, Y 1998 Tannins and human health a review Crit Rev Food Sci Nutr 38 421-64

Cressman, V L, Backlund, D C, Avrutskaya, A, Leadon, S A, Godfrey, V, and Koller, B H 1999 Growth retardation, DNA repair defects, and lack of spermatogenesis in BRCA1-deficient mice Mol Cell Biol 19 7061–7075

Deb, S, and Chatterjee, A 1998 Influence of buthionine sulfoximine and reduced glutathione on arecoline-induced chromosomal damage and sister chromatid exchange in mouse bone marrow cells *in vivo* Mutagenesis 13 243-248

Deng, J F, Ger, J, Tsai, W J, Kao, W F, and Yang, C C 2001 Acute toxicities of betel nut rare but probably overlooked events J Toxicol Clin Toxicol 39 355-360

Desai, S S, Ghaisas S D, Jakhi, S D, and Bhide, S V 1996 Cytogenetic damage in exfoliated oral mucosal cells and circulating lymphocytes of patients suffering from precancerous oral lesions Cancer Lett 109 9-14

Goan, Y G, Chang, H C, Hsu, H K, Chou, Y P, and Cheng, J T 2005 Risk of *p53* gene mutation in esophageal squamous cell carcinoma and habit of betel quid chewing in Taiwanese Cancer Sci 96 758-765

Harris, C C, and Hollstein, M 1993 Clinical implications of the p53 tumor-suppressor gene N Engl J Med 329 1318-1327

Hollstein, M, Sidransky, D, Vogelstein, B, and Harris, C C 1991 P53 mutation in human cancer Science 253 49-53

Hsieh, L L, Wang, P F, Chen, I H, Liao, C T, Wang, H M, Chen, M C, Chang, J T, and Cheng, A J 2001 Characterisics of mutations of the *p53* gene in oral squamous cell carcinoma associated with betel quid chewing and cigarette smoking in Taiwanese Carcinogenesis 22 1497-1503

Hsu, H J, Chang, K L, Yang, Y H, and Shieh, T Y 2001 The effects of arecoline on the release of cytokines using cultured peripheral blood mononuclear cells from patients with oral mucous diseases Kaohsiung J Med Sci 17 175-182

Hung, S, Cheng, Y, Peng, J, Chang, L, Liu, T and Chen, Y 2005 Inhibitory effects of areca nut extracts on phagocytosis of *Actinobacillus actinomycetemcomitans* ATCC 33384 by neutrophils J Periodontol 76 373-379

IARC 1985 International Agency for Research on Cancer (IARC) - Tobacco habits other than smoking Betel quid and Areca-nut chewing and some related nitrosamines In Monographs on the Evaluation of Carcinogenic Risks to Humans, 37 263, Lyon IARC

IARC 2004 International Agency for Research on Cancer (IARC) - Summaries & Evaluations Betel-quid and areca-nut chewing, 85:39, Lyon IARC

Jeng, J H, Tsai, C L, Hahn, L J, Yang, P J, Kuo, Y S, and Kuo, M Y 1999 Arecoline cytotoxicity on human oral mucosal fibroblasts related to cellular thiol and esterase activities Food Chem Toxicol 37 751-756

Jeng, J H, Chang, M C, and Hahn, L J 2001 Role of areca nut in betel quid-associated chemical carcinogenesis current awareness and future perspectives Oral Oncol 37 477-492

Jeng, J H, Kuo, M L, Hahn, L J, and Kuo, M Y 1994 Genotoxic and non-genotoxic effects of betel quid ingredients on oral mucosal fibroblasts *in vitro* J Dental Res 73 1043-1049

Jeng, J H, Lan, W H, Hahn, L J, Hsieh, C C, and Kuo, M Y 1996 Inhibition of the migration, attachment, spreading, growth and collagen synthesis of human gingival fibroblasts by arecoline, a major areca alkaloid, *in vitro* J Oral Pathol Med 25 371-375

Jeng, J H, Wang, Y J, Chiang, B L, Lee, P H, Chan, C P, Ho, Y S, Wang, T M, Lee, J J, Hahn, L J, and Chang, M C 2003 Roles of keratinocyte inflammation in oral cancer regulating the prostaglandin E2, interleukin-6 and TNF-α production of oral epithelial cells by areca nut extract and arecoline Carcinogenesis 24 1301-1315

Kannan, K, Munirajan, A K, and Krishnamurthy, J 1999 Low incidence of p53 mutation in betel quid and tobacco chewing-associated oral squamous carcinoma from India Int J Oncol 15 1133-1136

Kao, S Y, Wu, H C, Lin, S C, Yap, S K, Chang, C S, Wong, Y K., Chi, L Y, and Liu, T Y 2002 Genetic polymorphism of cytochrome P4501A1 and susceptibility to oral squamous cell carcinoma and oral precancer lesions associated with smoking/betel use J Oral Pathol Med 31 505-511

Kato, K, Hara, A, Kuno, T, Kitaori, N, Huilan, Z, Mori, H, Toida, M, and Shibata, T 2005. Matrix metalloproteinases 2 and 9 in oral squamous cell carcinomas manifestation and localization of their activity J Cancer Res Clin Oncol 131 340-346

Kietthubthew, S, Sriplung, H and Au, W W 2001 Genetic and environmental interactions on oral cancer in Southern Thailand Environ Mol Mutagen 37 111-116

Kinzler, K. W and Vogelstein, B 1997 Gatekeeprs and caretakers Nature 386 761-763

Kumpawat, K, Deb, S, Ray, S, and Chatterjee, A 2003 Genotoxic effect of raw betel-nut extract in relation to endogenous glutathione levels and its mechanism of action in mammalian cells Mutat Res 538 1-12

Kuttan, N A A, Rosin, M P, Ambika, K, Priddy, R W, Bhakthan, N M G, and Zhang, L 1995 High prevalence of expression of p53 oncoprotein in oral carcinomas from India associated with betel and tobacco chewing Eur J Cancer 31 169-173

Lai, K, and Lee, T 2006 Genetic damage in cultured human keratinocytes stressed by long-term exposure to areca nut extracts *Mutat Res Fundam Mol Mech Mutag* 599 66-75

Lane, D P 1992 Cancer p53 guardian of the genome Nature 358 15-16

Lee, H, Yin, P, Yu, T, Chang, Y, Hsu, W, Kao, S, Chi, C, Liu, T, and Wei, Y 2001 Accumulation of mitochondrial DNA deletions in human oral tissues — effects of betel quid chewing and oral cancer Mutat. Res Genetic Toxicol Environ Mutagen 493 67-74

Lee, P, Chang, M, Chang, W, Wang, T, Wang, Y, Hahn, L, Ho, Y, Lin, C, and Jeng, J 2006 Prolonged exposure to arecoline arrested human KB epithelial cell growth Regulatory mechanisms of cell cycle and apoptosis Toxicol. 220 81-89

Levine, A J 1997 P53, the cellular gatekeeper for growth and division Cell 88 323-331

Lin, S C, Liu, C J, Yeh, W I, Lui, M T, Chang, K. W, and Chang, C S 2006 Functional polymorphism in NFKB1 promoter is related to risks of oral squamous cell carcinoma occurring on older male areca (betel) chewers Cancer Lett. 243 47-54

Lin, Y, Jen, Y, Wang, B, Lee, J, and Kang, B 2005 Epidemiology of oral cavity cancer in Taiwan with emphasis on the role of betel nut chewing ORL J Otorhinolaryngol Relat Spec 67 230-236

Liu, S Y, Lin, M H, Yang, S C, Huang, G C, Chang, L, Chang, S, Yen, C Y, Chiang, W F, Kuo, Y Y, Chen, L L, Lee, C H, and Liu, Y C 2005a Increased expression of matrix metalloproteinase-2 in oral cells after short-term stimulation and long-term usage of areca quid J Formos Med Assoc 104 390-397

Liu, S Y, Lin, M H, Yang, S C, Huang, G C, Chang, L, Chang, S, Yen, C Y, Chiang, W F, Lee, C H, Kuo, Y Y, and Liu, Y C 2005b Areca quid chewing enhances the expression of salivary matrix metalloproteinase-9 J Formos Med Assoc 104 113-119

Liu, T Y, and Chi, C W 1999 Areca nut extracts stimulate DNA strand breaks by generation of nitric oxide Toxicol Lett 95 112

Liu, T Y, Chung, Y T, Wang, P F, Chi, C W, and Hsieh L L 2004 Safrole-DNA adducts in human peripheral blood—an association with areca quid chewing and CYP2E1 polymorphisms Mutat. Res Genetic Toxicol Environ Mutagen 559 59-66

Liu, T , Chen, C , and Chi, C 1996 Oxidative damage to DNA induced by areca nut extract Mutat Res Genetic Toxicol 367 5-31

Lu, S , Chang, K , Liu, C , Tseng, Y , Lu, H , Lee, S , and Lin, S 2006 Ripe areca nut extract induces G_1 phase arrests and senescence-associated phenotypes in normal human oral keratinocyte Carcinogenesis 27 1273-1284

Nadeau, G , Boufaied, N , Moisan, A , Lemieux, K M , Cayanan, C , Monteiro, A N A , and Gaudreau, L 2000 BRCA1 can stimulate gene transcription by a unique mechanism EMBO Rep 3 260-265

Nair, J , Ohshima, H , Friesen, M , Croisy, A , Bhide, S V , and Bartsch, H 1985 Tobacco-specific and betel nut-specific N-nitroso compounds occurrence in saliva and urine of betel quid chewers and formation *in vitro* by nitrosation of betel quid Carcinogenesis 6 295-303

Nair, U J , Nair, J , Mathew, B , and Bartsch, H 1999 Glutathione S-transferase M1 and T1 null genotypes as risk factors for oral leukoplakia in ethnic Indian betel quid/ tobacco chewers Carcinogenesis 20 743-748

Nair, U , Bartsch, H , and Nair, J 2004 Alert for an epidemic of oral cancer due to use of the betel quid substitutes gutkha and pan masala a review of agents and causative mechanisms Mutagenesis 19 251-262

Nair, U , Floyd, R. A , Nair, J , Bussachini, V , Friesen, M , and Bartsch, H 1987 Formation of reactive oxygen species and of 8-hydroxydeoxyguanosine in DNA in vitro with betel quid ingredients Chem Biol Interact 63 157-169

Norton, S A 1998 Betel consumption and consequences J Am Acad Dermatol 38 81-88

Panigrahi, G B , and Rao, A R. 1989 Study of the genotoxicity of the total aqueous extract of betel nut and its tannin Carcinogenesis 7 37-39

Pariat, T , and Sharan, R. N 1998a Betel nut and diethylnitrosamine induced conformational changes in the liver HMG proteins *in vivo* In Trends in Radiation and Cancer Biology, ed R. N Sharan, pp 162-165, vol 29, International co-operation bilateral seminars series, Juelich Forschungszentrum Juelich GmbH.

Pariat, T , and Sharan, R. N 1998b Qualitative change in mice liver HMG proteins after low dose chronic administration of aqueous extract of betel nut and diethynitrosamine Hepatol Res 12 177-185

Pariat, T , and Sharan, R. N 2002 Role of mouse spleen cell HMG proteins and its poly-ADP-ribosylation in betel nut induced carcinogenesis Indian J Biochem Biophys 39 130-132

Pariat, T , Balachandran, B , and Sharan, R. N Effects of carcinogen exposure on poly-ADP-ribosylation of HMG Proteins and on chromatin organization 1999 In Recent Aspects of Cellular and Applied Radiobiology, eds F H A Schneeweiss and R. N Sharan, pp 158-161, vol 30, International co-operation bilateral seminars series, Juelich Forschungszentrum Juelich GmbH

Patel, B P , Shah, S V , Shukla, S N , Shah, P M , and Patel, P S 2007 Clinical significance of MMP-2 and MMP-9 in patients with oral cancer Head Neck 29 564-572

Paul, K , Moitra, P K , Mukherjee, I , Maity, C , and Ghosal, S K. 1999 Teratogenicity of arecoline hydrobromide on developing chick embryos A preliminary report. Bull Environ Contam Toxicol 62 356-362

Polasa, K , Babu, S , and Shenolikar, I S 1993 Dose-dependent genotoxic effect of pan masala and areca nut in *Salmonella typhimurium* assay Food Chem Toxicol 31 439-442

Rajan, J V , Wang, M , Marquis, S T , and Chodosh, L A 1996 Brca2 is coordinately regulated with Brca1 during proliferation and differentiation in mammary epithelial cells Proc Natl Acad Sci (USA) 93 13078-13083

Ralhan, R , Agarwal, S , Nath, N , Mathur, M , Wasylyk, B , and Srivastava, A 2001 Correlation between p53 gene mutations and circulating antibodies in betel- and tobacco-consuming North Indian population Oral Oncol 37 243-250

Ramachandran, S , Ramadas, K , Hariharan, R., Rejnish, K R , and Radhakrishna, P M 2006 Single nucleotide polymorphisms of DNA repair genes XRCC1 and XPD and its molecular mapping in Indian oral cancer Oral Oncol 42 350-362

Ranasinghe, A W , Warnakulasuriya, K. A A S , and Johnson, N W 1993 Low prevalence of expression of p53 oncoprotein in oral carcinomas from Sri Lanka associated with betel and tobacco chewing Eur J Cancer 29 147-150

Saikia, J R , Schneeweiss, F H A , and Sharan, R. N 1998 Effects of chronic low-dose arecoline administration on the macromolecular components of bone marrow and spleen cells of mice Cancer J , 11 94-98

Saikia, J R , Schneeweiss, F H A , and Sharan, R. N 1999b Arecoline-induced changes of poly-ADP-ribosylation of cellular proteins and its influence on chromatin organization Cancer Lett 139 59-65

Saikia, J R , Schneeweiss, F H A , and Sharan, R N 1999a Chronic low dose arecoline exposure affects DNA, RNA and protein contents and cellular poly-ADP-ribosylation in mice *in vivo* In Recent Aspects of Cellular and Applied Radiobiology, eds F H A Schneeweiss, and R. N Sharan, pp 149-152, vol 30, International co-operation bilateral seminars series, Juelich Forschungszentrum Juelich GmbH

Sharan, R. N 1994 Biochemical investigation of carcinogenic potency of betel nut (*Kwai*) of north-east India In, Oral Oncology, pp 190-193, vol III, New Delhi Mac Millan India Ltd

Sharan, R N 1996 Association of betel nut with carcinogenesis – A review Cancer J 9 13-19

Sharan, R. N , and Wary, K K 1992 Study of unscheduled DNA synthesis following exposure of human cells to arecoline and extracts of betel nut *in vitro* Mutat Res 278 271-276

Shwe, M , Chiguchi, G , Yamada, S , Nakajima, T , Maung, K. K., Takagi, M , Amagasa, T , and Tsuchida, N 2001 P53 and MDM2 co-expression in tobacco and betel chewing-associated oral squamous cell carcinomas J Med Dent Sci 48 113-119

Sinha, A , and Rao, A R 1985b Induction of shape abnormality and unscheduled DNA synthesis by arecoline in the germ cells of mice Mutat Res 158 189-192

Sinha, A , and Rao, A R 1985c Transplacental micronucleus inducing ability of arecoline, a betel nut alkaloid in mice Mutat Res 158 193-194

Sinha, A , and Rao, A R , 1985a Embryotoxicity of betel nuts in mice Toxicol 37 315-26

Spitz, M R , and Bondy, M L 1993 Genetic susceptibility to cancer Cancer 72 991-995

Stich, H F , and Anders, F 1989 The involvement of reactive oxygen species in oral cancers of betel quid/tobacco chewers Mutat Res 214 47-61

Sundqvist, K , Liu, Y , Nair, J , Bartsch, H , Arvidson, K , and Grafstrom, R C 1989 Cytotoxic and genotoxic effects of areca nut-related compounds in cultured human buccal epithelial cells Cancer Res 49 5294-5298

Thomas, S , Brennan, J , and Martel, G 1994 Mutations in the conserved regions of p53 are infrequent in betel-associated oral cancers from Papua New Guinea Cancer Res 54 3588-3593

Topcu, Z , Chiba, I , Fujieda, M , Shibata, T , Ariyoshi, N , Yamazaki, H , Sevgican, F , Muthumala, M , Kobayahi, H , and Kamataki, T 2002 CYP2A6 gene deletion reduces oral cancer risk in betel quid chewers in Sri Lanka Carcinogenesis 23 595-598

Wary, K K , and Sharan, R N 1991 Cytotoxic and cytostatic effects of arecoline and sodium nitrite on human cells *in vitro* Int J Cancer 47 396-400

Wen, X , Zhang, Y , Liu, X , Guo, S , and Wang, H 2006 Immune responses in mice to arecoline mediated by lymphocyte muscarinic acetylcholine receptor Cell Biol Int 30 1048-1053

Trivedy, C R , Craig, G , and Warnakulasuriya, S 2002 The oral health consequences of chewing areca nut Addict Biol 7 115- 125

Tseng, C 2006 Betel nut chewing is independently associated with urinary albumin excretion rate in type 2 diabetic patients Diabetes Care 29 462-463

Tu, H F , Liu, C J , Chang, C S , Lui, M T , Kao, S Y , Chang, C P , and Liu, T Y 2006 The functional (-1171 5A-->6A) polymorphisms of matrix metalloproteinase 3 gene as a risk factor for oral submucous fibrosis among male areca users J Oral Pathol Med 35 99-103

Tu, H F , Wu, C H , Kao, S Y , Liu, C J , Liu, T Y , and Lui, M T 2007 Functional -1562 C-to-T polymorphism in matrix metalloproteinase-9 (MMP-9) promoter is associated with the risk for oral squamous cell carcinoma in younger male areca users J Oral Pathol Med 36 409-414

Wang, C K , and Peng, C H 1996 The mutagenicities of alkaloids and *N*-nitrosoguvacoline from betel quid Mutat Res Environ Mutagen Relat Subj. 360 165-171

Warnakulasuriya, S 2002 Areca nut use an independent risk factor for oral cancer Br Med J 324 799–800

Wary, K K , and Sharan, R N 1988 Aqueous extract of betel-nut of North-East India induces DNA strand breaks and enhances rate of cell proliferation *in vitro* J Cancer Res Clin Oncol 114:579-582

Table I – Constituent of betel nut (BN): Approximate average percent constituent of dry and raw/wet varieties of BN (IARC 1985; 2004; Sharan 1996).

Constituent		Dry variety (%)	Raw/wet variety (%)
Alkaloids	Combined	0.25	0.35 – 0.49
	Arecoline	0.15	0.18 - 0.24
	Arecaidine	0.10	0.10 – 0.20
	Others	Trace	0.14
Polyphenols		15	23
Tannins		18	22
Carbohydrates		25	30
Proteins		7.5	12
Fats		1.2	2.5
Fiber		15	18
Water		Low	High
Ash		Low	High

Table II – Estimated transgenerational cancer transmission risk in mice from BN (Yashmin and Sharan 2008; unpublished).

Chronic AEBN administration in drinking water to mice	Appearance of pre-neoplastic nodules on liver
P generation	16 weeks
F1 generation	8 weeks
F2 generation	6 weeks
F3 generation	4 weeks

Table III: Genetic polymorphism and susceptibility to oral cancer in humans

#	Gene/region	Polymorphism	Effect	Population group	Reference
1	Matrix metallo-proteinase-9 (MMP9) promoter	1562 C-to-T polymorphism	MMP-9-1562 C>T polymorphism - enhanced OSCC risk in young male BN chewers	Taiwanese	Tu et al 2007
2	Matrix metallo-proteinase-3 (MMP3) promoter	Insertion/deletion (-1171 5A-->6A) polymorphisms	5A genotype polymorphism - enhanced risk of OSF but not OSCC among male BN users	Asian	Tu et al 2006
3	NFKB1 promoter	Insertion (ins)/deletion (del) polymorphism (-94 ins/del ATTG) in NFKB1 promoter	NFKB1 ins and HO-1 L allelotypes – significantly enhanced risks for different subsets of OSCC in male BN chewers	Asian	Lin et al 2006
4	DNA repair genes *XRCC1* and *XPD*	Polymorphisms Arg194Trp, Arg280His, and Arg399Gln of the XRCC1 gene and Lys751Gln of the XPD gene	Variant allele of XRCC1 399 codon and XPD – enhanced risk of oral cancer among BQ chewers and smokers	South Indian	Ramachandran et al 2006
5	Heme oxygenase-1 (*HO-1*)	Polymorphisms in a (GT)n microsatellite repeat in HO-1 promoter in short (S), medium (M) and long (L) alleles	Longer (GT)n repeat allele L – higher risk of BN related OSCC, (GT)n repeat allele S - may be protective for OSCC	Asian	Chang et al 2004
6	Cytochrome gene *CYP2A6*	*CYP2A6*4C* mutation-gene deletion type of polymorphism	Deficient *CYP2A6* activity due to deletion – reduced risk of oral cancer risk in BQ chewers	Sri Lankan	Topcu et al 2002
7	Cytochrome gene *CYP1A1*	*CYP1A1 A/G* genotype (Ile/Val) and *G/G* genotype (Val/Val) in exon 7	*CYP1A1* exon 7 containing *G* allele - enhanced risk for OSCC and oral precancerous lesion (OPL) in BN chewer and smoker	Chinese	Kao et al 2002
8	Collagen related genes Collagen 1A1 and 1A2 (COL1A1 and COL1A2), Collagenase-1 (COLase), transforming growth factor β1 (TGF-β1), Lysyl oxidase (LYOXase), and Cystatin C (CST3)	Polymorphisms of six collagen related genes, COL1A1, COL1A2, COLase, TGF-β1, LYOXase, and CST3	Multigenic mechanisms involving the collagen related genes enhance susceptibility to OSF among BQ chewers	Taiwanese	Chiu et al 2002
9	Tumor necrosis factor-α (TNF-α)	Bi-allelic promoter region (-308) polymorphism on the TNFα gene	The high production allele, TNF2 - significantly lower among individuals with OSF	Taiwanese	Chiu et al 2001
10	Glutathione-S-transferase genes GSTM1 and GSTT1	GSTM1 and GSTT1 null genotypes (GSTM1*2 and GSTT1*2)	Null genotypes of either or both GSTM1 and GSTT1 - enhanced risk of development of leukoplakia following exposure to tobacco with or without BQ	South Indian	Nair et al 1999
11		Genetic polymorphism of GSTM1 and GSTT1	Homozygous deletion of GSTM1 gene – enhanced risk for oral cancer, which is further compounded by exposure to cigarette smoke, alcohol, and BQ	Thai	Kietthubthew, Sriplung and Au 2001

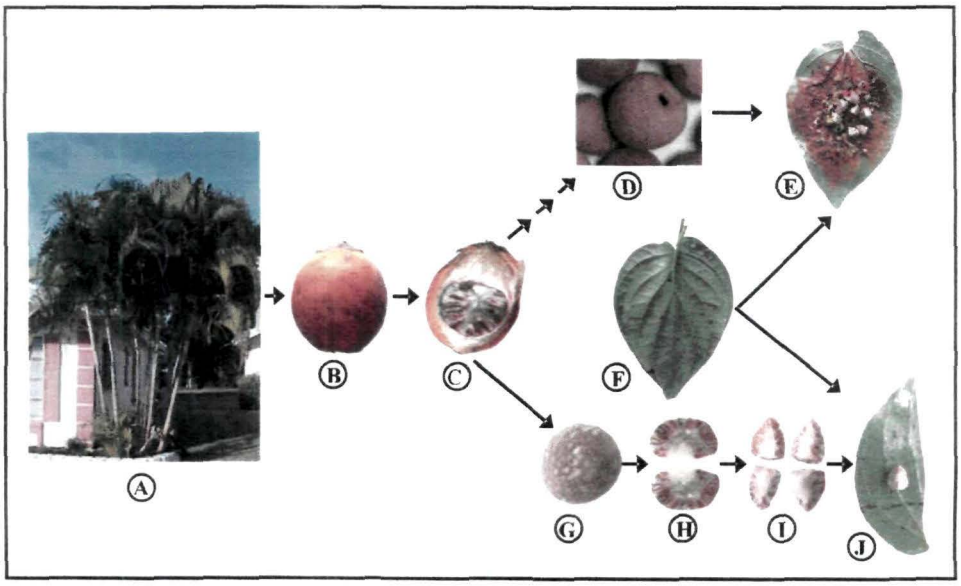


Figure 1. Patterns of betel nut usage: *Areca catechu* L. palm trees in its natural habitat (A); a ripe betel fruit (B) and its cross section showing the betel nut (BN) encased within its fibrous shell (C). After appropriate curing, sun drying and removal of the shell, the dry and very hard variety of BN (D) is prepared, which is usually cut into small to very small pieces for mastication along with *Piper betle* leaf (F) as a betel quid (BQ) supplemented with a large variety of additives (E) (see text for details). BN is also masticated in its raw and wet form (G), which is usually cut into 4 pieces (H, I) and consumed as a simple BQ (J) comprising betel leaf (F), slacked lime and a piece of wet/raw variety of nut.

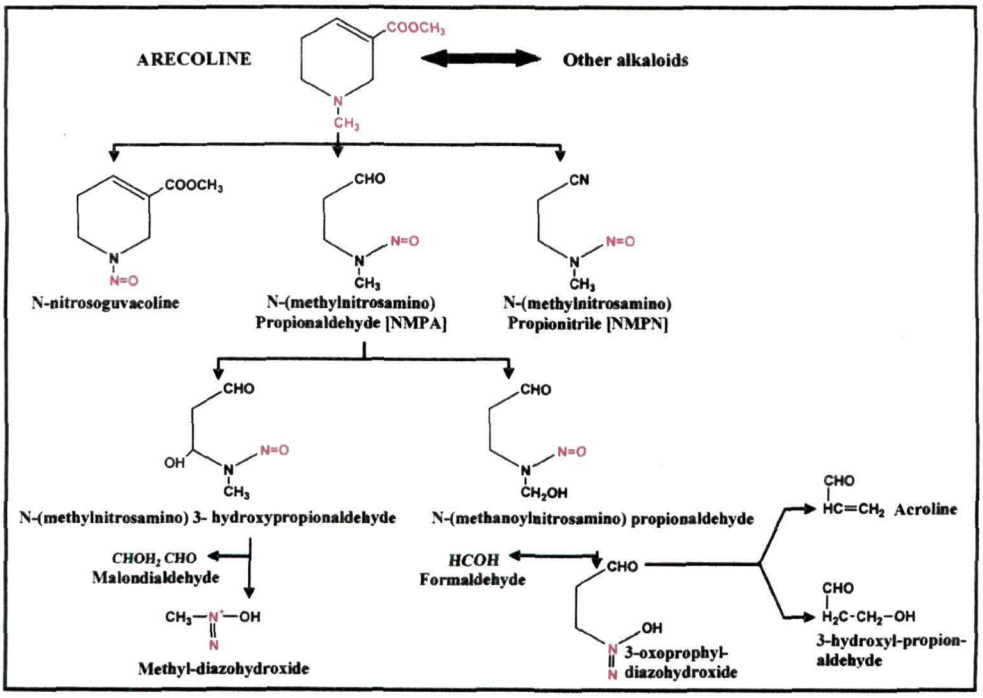


Figure 2. Representative chemical pathway of metabolic activation of arecoline, the major carcinogenic alkaloid of BN. Different nitrosamine and their derivative are produced from the alkaloids, which have been called as betel nut specific nitrosamines (BSNA)

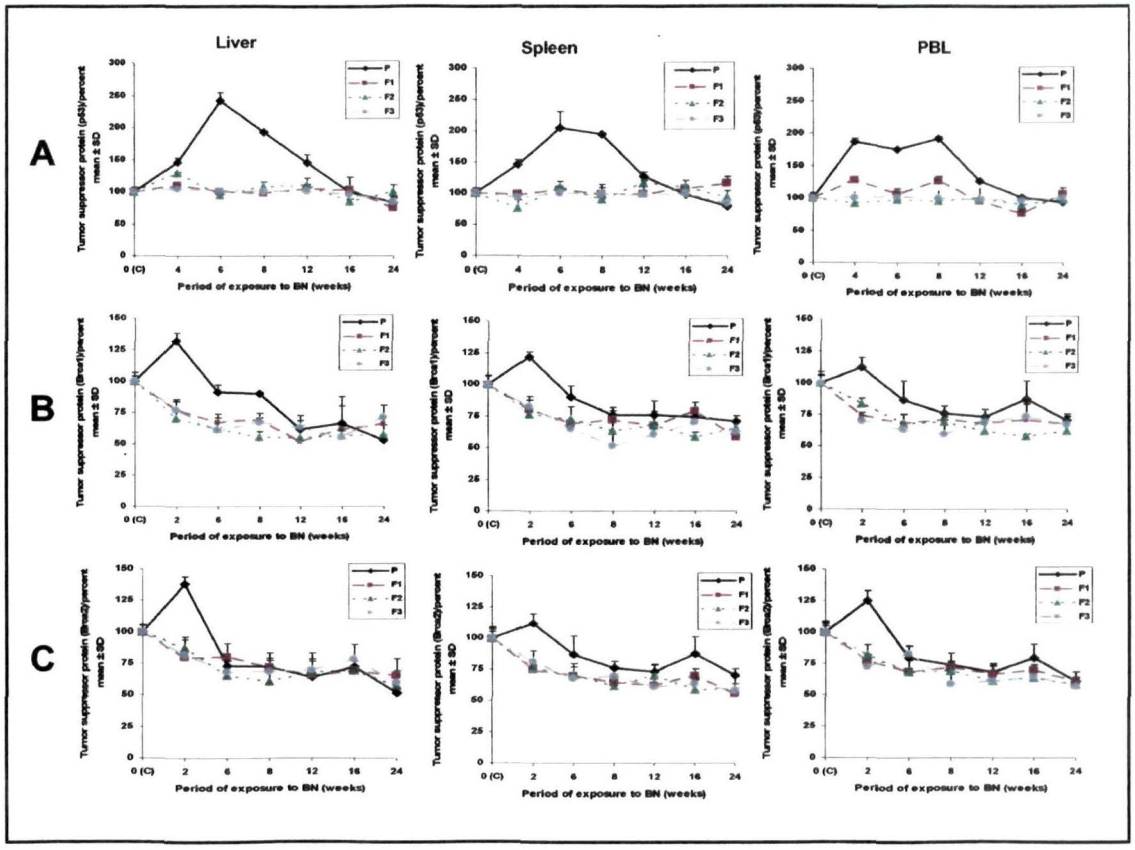


Figure 3. Graphs showing cellular levels of three tumor suppressor proteins, p53 (A), Brca1 (B) and Brca2 (C), in liver, spleen and peripheral blood lymphocytes (PBL) of mice chronically and transgenerationally exposed to aqueous extract of betel nut (AEBN) in drinking water from parental (P) generation to F1 through F3 generations of mice.

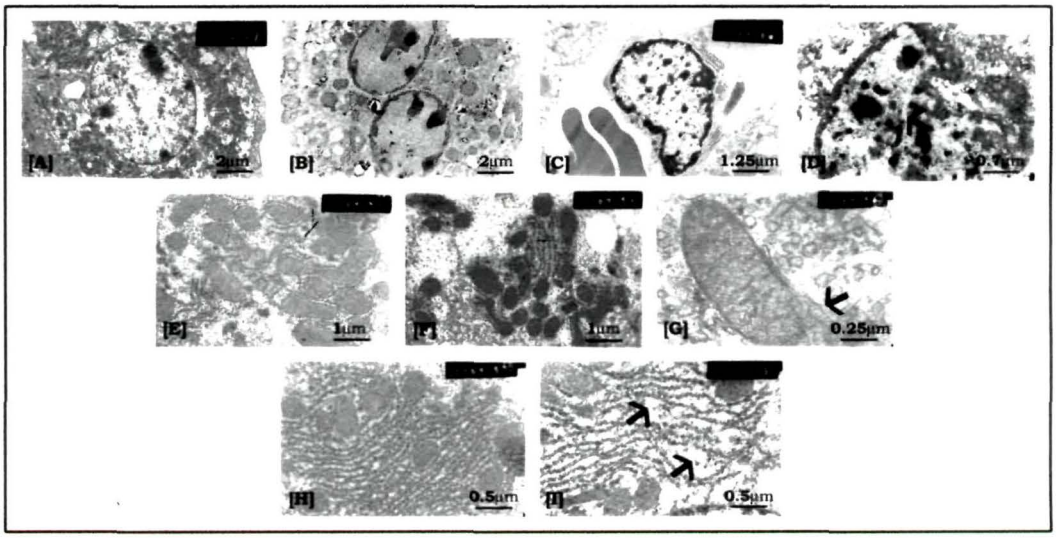


Figure 4. Transmission electron micrographs of normal and transformed liver sections of mice exposed to aqueous extract of betel nut (AEBN) in drinking water. A normal liver cell with regular nucleus (A), which upon exposure to AEBN often showed binucleated cells (B), deformed nucleus (C) and/or nucleus with condensed and marginalized chromatin (D). The regular mitochondria of a normal cell (E), exhibited shrinkage and reduction in size upon exposure to AEBN (F) often accompanied with disrupted mitochondrial membrane (G). The normal arrangement of membrane in the endoplasmic reticulum (ER) (H) also exhibited pronounced disruptions (I).

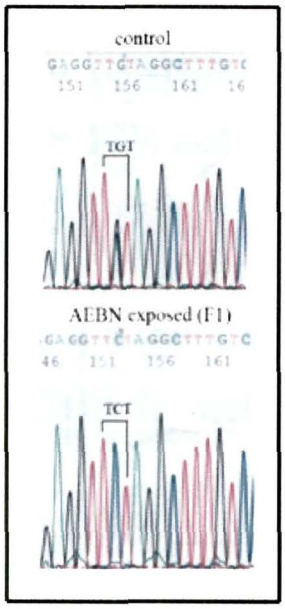


Figure 5. Part of the nucleotide sequence chromatograms of PCR amplicons representing exon 11 of *Brca1* gene of control (top) and F1 generation of AEBN exposed (bottom) mice liver. It shows induction of a G → C transversion type point mutation.

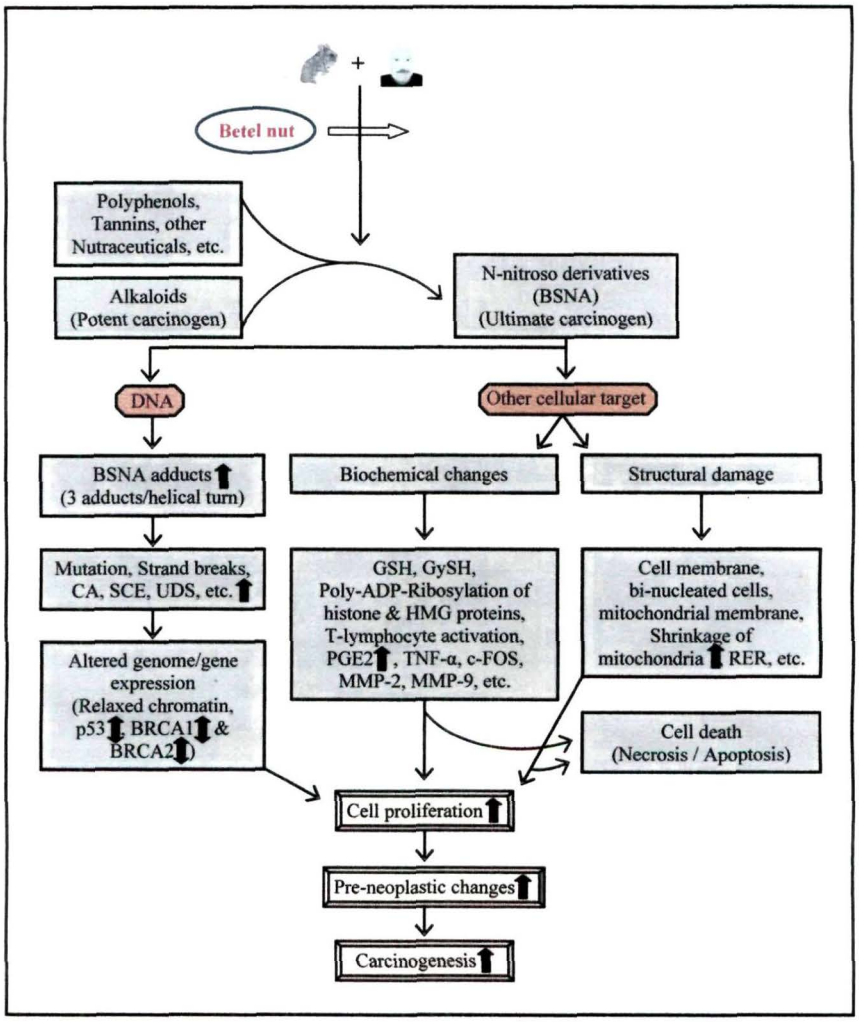


Figure 6: Schematic diagram of major metabolic events and milestones in the pathway of betel nut induced carcinogenesis (see text for details).

APPENDICES

APPENDIX I

Mus musculus p53 gene, exon 5 and partial cds

```
LOCUS       EF570972                214 bp    DNA     linear   ROD 28-NOV-
2008
DEFINITION  Mus musculus p53 gene, exon 5 and partial cds.
ACCESSION   EF570972
VERSION     EF570972.1  GI:146761198
KEYWORDS    .
SOURCE      Mus musculus (house mouse)
  ORGANISM  Mus musculus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia;
            Sciurognathi; Muroidea; Muridae; Murinae; Mus.
REFERENCE   1  (bases 1 to 214)
  AUTHORS   Choudhury,Y. and Sharan,R.N.
  TITLE     Altered p53 response and enhanced transgenerational transmission
            of carcinogenic risk upon exposure of mice to betel nut
  JOURNAL   Environ. Toxicol. Pharmacol. (2008) In press
  REMARK    Publication Status: Available-Online prior to print
REFERENCE   2  (bases 1 to 214)
  AUTHORS   Choudhury,Y. and Sharan,R.N.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-APR-2007) Biochemistry, North Eastern Hill
            University, Umshing, Shillong, Meghalaya 793022, India
FEATURES             Location/Qualifiers
     source          1..214
                     /organism="Mus musculus"
                     /mol_type="genomic DNA"
                     /strain="Swiss Albino"
                     /db_xref="taxon:10090"
                     /chromosome="11"
                     /map="39.0 cM"
                     /PCR_primers="fwd_seq: atcgttactcggcttgtccc, rev_seq:
                     taaccccacaggcggtgtt"
     mRNA            <32..>208
                     /product="p53"
     CDS             <32..>208
                     /codon_start=1
                     /product="p53"
                     /protein_id="ABQ44583.1"
                     /db_xref="GI:146761199"

/translation="YSPPLNKLFCQLAKTCPVQLWVSATPPAGSRVRAMAIYKKSQHM
                     TEVVRRCPHHERCSD"
     exon            32..208
                     /number=5
ORIGIN
        1 cccgacctcc gttctctctc ctctcttcca gtactctcct cccctcaata agctattctg
       61 ccagctggcg aagacgtgcc ctgtgcagtt gtgggtcagc gccacacctc cagctgggag
      121 ccgtgtccgc gccatggcca tctacaagaa gtcacagcac atgacggagg tcgtgagacg
      181 ctgcccccac catgagcgct gctccgatgt gatg
//
```

APPENDIX II

Mus musculus p53 gene, exon 7 and partial cds

```
LOCUS       EF634061                176 bp    DNA     linear   ROD 28-NOV-
2008
DEFINITION  Mus musculus p53 gene, exon 7 and partial cds.
ACCESSION   EF634061
VERSION     EF634061.1  GI:149784072
KEYWORDS    .
SOURCE      Mus musculus (house mouse)
  ORGANISM  Mus musculus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia;
            Sciurognathi; Muroidea; Muridae; Murinae; Mus.
REFERENCE   1  (bases 1 to 176)
  AUTHORS   Choudhury,Y. and Sharan,R.N.
  TITLE     Altered p53 response and enhanced transgenerational transmission
            of carcinogenic risk upon exposure of mice to betel nut
  JOURNAL   Environ. Toxicol. Pharmacol. (2008) In press
  REMARK    Publication Status: Available-Online prior to print
REFERENCE   2  (bases 1 to 176)
  AUTHORS   Choudhury,Y. and Sharan,R.N.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-MAY-2007) Biochemistry, North-Eastern Hill
            University, Umshing, Shillong, Meghalaya 793022, India
FEATURES             Location/Qualifiers
     source          1..176
                     /organism="Mus musculus"
                     /mol_type="genomic DNA"
                     /strain="Swiss Albino"
                     /db_xref="taxon:10090"
                     /chromosome="11"
                     /map="39.0 cM"
                     /PCR_primers="fwd_seq: tagtgaggtagggagcgactt, rev_seq:
                     ctggggaagaaacaggctaac"
     mRNA            <22..>131
                     /product="p53"
     CDS             <22..>131
                     /codon_start=1
                     /product="p53"
                     /protein_id="ABR29561.1"
                     /db_xref="GI:149784073"
                     /translation="AGSEYTTIHYKYMCNSSCMGGMNRRPILTIITLEDS"
     exon            22..131
                     /number=7
ORIGIN
        1 ggatcctgtg tcttccccca ggccggctct gagtatacca ccatccacta caagtacatg
       61 tgtaatagct cctgcatggg gggcatgaac cgccgaccta tccttaccat catcacactg
      121 gaagactcca ggtaggaagg cgcgtggtag gttaggttag cctgtttctt ccccag
//
```

APPENDIX III

Mus musculus breast cancer associated 1 (Brca1) gene, exon 11 and partial cds

```
LOCUS       FJ497232               1385 bp    DNA     linear   ROD 24-DEC-
2008
DEFINITION  Mus musculus breast cancer associated 1 (Brca1) gene, exon 11 and
partial cds.
ACCESSION   FJ497232
VERSION     FJ497232.1  GI:218533673
KEYWORDS    .
SOURCE      Mus musculus (house mouse)
  ORGANISM  Mus musculus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia;
            Sciurognathi; Muroidea; Muridae; Murinae; Mus.
REFERENCE   1  (bases 1 to 1385)
  AUTHORS   Choudhury,Y. and Sharan,R.N.
  TITLE     Exon 11 of mouse Brca1 gene
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1385)
  AUTHORS   Choudhury,Y. and Sharan,R.N.
  TITLE     Direct Submission
  JOURNAL   Submitted (25-NOV-2008) Biochemistry, North Eastern Hill
            University, Umshing, Shillong, Meghalaya 793022, India
FEATURES             Location/Qualifiers
     source          1..1385
                     /organism="Mus musculus"
                     /mol_type="genomic DNA"
                     /strain="Swiss Albino"
                     /db_xref="taxon:10090"
                     /chromosome="11"
                     /map="11D (60.5 cM)"
                     /PCR_primers="fwd_seq: agtcctggaacgctcaca, rev_seq:
                     cagttcctttgagggcaca"
     gene            <1..>1385
                     /gene="Brca1"
     mRNA            <1..>1385
                     /gene="Brca1"
                     /product="breast cancer associated 1"
     CDS             <1..>1385
                     /gene="Brca1"
                     /codon_start=1
                     /product="breast cancer associated 1"
                     /protein_id="ACK87008.1"
                     /db_xref="GI:218533674"
                    /translation="GTAQCMTQSTPRENPKELLNRSNNAGSGTEGLKPPLRHALNLSQ
                    EKVEMEDSELDTQYLQNTFQVSKRQSFALFSKPRSPQKDCAHSVPSKELSPKVTAKGK
                    QKERQGQEEFEISHVQAVAATVGLPVPCQEGKLAADTMCDRGCRLCPSSHYRSGENGL
                    SATGKSGISQNSHFKQSVSPIRSSIKTDNRKPLTEGRFERHTSSTEMAGGNENILQST
                    VHTVSLNNRGNACQEAGSGSIHEVCSTGDSFPGQLGRNRGPKVNTVPPLDSMQPGVCQ
                    QSVPVSDKYLEIKKQEGEAVCADFSPCLFSDHLEQSMSGKVFQVCSETPDDLLDDVEI
                    QGHTSFGEGDIMERSAVFNGSILRRESSRSPSPVTHASKSQSLHRASRKLESSEESDS
                    TDDEDLPCFQHLLSRISNTPELTRCSSAVTQRMPEKAEGTQAPWKGSSSDCNNEVIMI
                                          EASQEHQFSEDP"
     exon            <1..>1385
                     /gene="Brca1"
                     /number=11
ORIGIN
        1 ggaaccgctc agtgtatgac tcagagcaca ccaagagaaa accccaagga actcctcaat
       61 cgctctaaca atgctgggag tggcacagag ggtctcaagc ccccttgag acacgcgctt
      121 aacctcagtc aggagaaagt agaaatggaa gacagtgaac ttgatactca gtatttgcag
      181 aatacatttc aagtttcaaa gcgtcagtca tttgctttat tttcaaaacc tagaagtccc
      241 caaaaggact gtgctcactc tgtgccctca aaggaactga gtccaaaggt gacagctaaa
      301 ggtaaacaaa aagaacgtca gggacaggaa gaatttgaaa tcagtcacgt acaagcagtt
      361 gcggccacag tgggcttacc tgtgccctgt caagaaggta agctagctgc tgatacaatg
      421 tgtgatagag gttgtaggct ttgtccatca tctcattaca gaagcgggga gaatggactc
```

```
      481 agcgccacag gtaaatcagg aatttcacaa aactcacatt ttaaacaatc agtttctccc
      541 atcaggtcat ctataaaaac tgacaatagg aaacctctga cagagggacg atttgagaga
      601 catacatcat caactgagat ggcgggggga aatgagaaca ttcttcagag tacagtgcac
      661 acagttagcc tgaataacag aggaaatgct tgtcaagaag ccggctcggg cagtattcat
      721 gaagtatgtt ccactggtga ctccttccca ggacaactag gtagaaacag agggcctaag
      781 gtgaacactg tgcctccatt agatagtatg cagcctggtg tctgtcagca aagtgttcct
      841 gtaagtgata agtatcttga aataaaaaag caggagggtg aggctgtctg tgcagacttc
      901 tctccatgtc tattctcaga ccatcttgag caatctatga gtggtaaggt ttttcaggtt
      961 tgctctgaga cacctgatga cctgctggat gatgttgaaa tacagggaca tactagcttt
     1021 ggtgaaggtg acataatgga gagatctgct gtctttaacg gaagcatcct gagaagggag
     1081 tccagtagga gccctagtcc tgtaacccat gcatcgaagt ctcagagtct ccacagagcg
     1141 tctaggaaat tagaatcgtc agaagagagc gactccactg acgatgaaga tcttccctgc
     1201 ttccaacact tactgagcag aataagcaac acacctgagc ttaccagatg cagcagtgct
     1261 gtgacacagc gtatgccaga gaaagcggag gggacccaag caccatggaa gggtagcagc
     1321 agtgactgca ataatgaggt gatcatgata gaggcatctc aggagcatca gtttagtgag
     1381 gatcc
//
```

APPENDIX IV

Mus musculus mutant breast cancer associated 1 (Brca1) gene, exon 11 and partial cds

```
LOCUS       FJ589202               1386 bp    DNA     linear   ROD 01-FEB-
2009
DEFINITION  Mus musculus mutant breast cancer associated 1 (Brca1) gene, exon
11 and partial cds.
ACCESSION   FJ589202
VERSION     FJ589202.1  GI:221706358
KEYWORDS    .
SOURCE      Mus musculus (house mouse)
  ORGANISM  Mus musculus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia;
            Sciurognathi; Muroidea; Muridae; Murinae; Mus.
REFERENCE   1  (bases 1 to 1386)
  AUTHORS   Choudhury,Y. and Sharan,R.N.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-DEC-2008) Biochemistry, North Eastern Hill
            University, Umshing, Shillong, Meghalaya 793022, India
FEATURES             Location/Qualifiers
     source          1..1386
                     /organism="Mus musculus"
                     /mol_type="genomic DNA"
                     /strain="Swiss Albino"
                     /db_xref="taxon:10090"
                     /chromosome="11"
                     /map="11D"
                     /note="transgenerationally exposed to aqueous extract of
                     betel nut (AEBN); exhibit greater susceptibitlity to
                     cancer"
     gene            <1..>1386
                     /gene="Brca1"
     mRNA            <1..>1386
                     /gene="Brca1"
                     /product="mutant breast cancer associated 1"
     CDS             <1..>1386
                     /gene="Brca1"
                     /codon_start=1
                     /product="mutant breast cancer associated 1"
                     /protein_id="ACM24793.1"
                     /db_xref="GI:221706359"
                     /translation="GTAQCMTQSTPRENPKELLNRSNNAGSGTEGLKPPLRHALNLSQ
                     EKVEMEDSELDTQYLQNTFQVSKRQSFALFSKPRSPQKDCAHSVPSKELSPKVTAKGK
                     QKERQGQEEFEISHVQAVAATVGLPVPCQEGKLAADTMCDRGSRLCPSSHYRSGENGL
                     SATGKSGISQNSHFKQSVSPIRSSIKTDNRKPLTEGRFERHTSSTEMAGGNENILQST
                     VHTVSLNNRGNACQEAGSGSIHEVCSTGDSFPGQLGRNRGPKVNTVPPLDSMQPGVCQ
                     QSVPVSDKYLEIKKQEGEAVCADFSPCLFSDHLEQSMSGKVFQVCSETPDDLLDDVEI
                     QGHTSFGEGDIMERSAVFNGSILRRESSRSPSPVTHASKSQSLHRASRKLESSEESDS
                     TDDEDLPCFQHLLSRISNTPELTRCSSAVTQRMPEKAEGTQAPWKGSSSDCNNEVIMI
                                          EASQEHQFSEDP"
     exon            <1..>1386
                     /gene="Brca1"
                     /number=11
     variation       434
                     /gene="Brca1"
                     /note="S145C"
                     /compare=FJ497232.1
                     /replace="g"
ORIGIN
        1 ggaaccgctc agtgtatgac tcagagcaca ccaagagaaa accccaagga actcctcaat
       61 cgctctaaca atgctgggag tggcacagag ggtctcaagc ccccccttgag acacgcgctt
      121 aacctcagtc aggagaaagt agaaatggaa gacagtgaac ttgatactca gtatttgcag
      181 aatacatttc aagtttcaaa gcgtcagtca tttgctttat tttcaaaacc tagaagtccc
      241 caaaaggact gtgctcactc tgtgccctca aaggaactga gtccaaaggt gacagctaaa
      301 ggtaaacaaa aagaacgtca gggacaggaa gaatttgaaa tcagtcacgt acaagcagtt
```

```
 361 gcggccacag tgggcttacc tgtgccctgt caagaaggta agctagctgc tgatacaatg
 421 tgtgatagag gttctaggct ttgtccatca tctcattaca gaagcgggga gaatggactc
 481 agcgccacag gtaaatcagg aatttcacaa aactcacatt ttaaacaatc agtttctccc
 541 atcaggtcat ctataaaaac tgacaatagg aaacctctga cagagggacg atttgagaga
 601 catacatcat caactgagat ggcgggggga aatgagaaca ttcttcagag tacagtgcac
 661 acagttagcc tgaataacag aggaaatgct tgtcaagaag ccggctcggg cagtattcat
 721 gaagtatgtt ccactggtga ctccttccca ggacaactag gtagaaacag agggcctaag
 781 gtgaacactg tgcctccatt agatagtatg cagcctggtg tctgtcagca aagtgttcct
 841 gtaagtgata agtatcttga aataaaaaag caggagggtg aggctgtctg tgcagacttc
 901 tctccatgtc tattctcaga ccatcttgag caatctatga gtggtaaggt ttttcaggtt
 961 tgctctgaga cacctgatga cctgctggat gatgttgaaa tacagggaca tactagcttt
1021 ggtgaaggtg acataatgga gagatctgct gtctttaacg gaagcatcct gagaagggag
1081 tccagtagga gccctagtcc tgtaacccat gcatcgaagt ctcagagtct ccacagagcg
1141 tctaggaaat tagaatcgtc agaagagagc gactccactg acgatgaaga tcttccctgc
1201 ttccaacact tactgagcag aataagcaac acacctgagc ttaccagatg cagcagtgct
1261 gtgacacagc gtatgccaga gaaagcggag gggacccaag caccatggaa gggtagcagc
1321 agtgactgca ataatgaggt gatcatgata gaggcatctc aggagcatca gtttagtgag
1381 gatccc
//
```

CURRICULUM VITAE

1. **Name:** Yashmin Choudhury
2. **Date of birth:** 28th October, 1978
3. **Permanent residential address:**

   Goraline, Revenue Block,
   Lumshngain, Plot No-13,
   Shillong-793006

4. **Telephone number:** 0364-2231729
   +91-9612159389
5. **Email:** yashminchoudhury@yahoo.co.in

6. **EDUCATIONAL QUALIFICATIONS:**

• Master of Science, **M. Sc** (Biochemistry), 2002	North-Eastern Hill University, Shillong **(First class, First rank with 76.61 percent)**
• Bachelor of Science, **B. Sc** (honours in Biochemistry), 2002	North-Eastern Hill University, Shillong **(First class, Second rank with 76.25 percent)**
• Higher Secondary School Leaving Certificate, **HSSLC**, 1997	Meghalaya Board of School Education, Shillong **(First division with 84.11 percent)**
• Secondary School Leaving Certificate, **SSLC**, 1995	Meghalaya Board of School Education, Shillong **(First division, fourth rank with 78.55 percent)**

7. **ADDITIONAL QUALIFICATIONS:**
   - Graduate Aptitude Test in Engineering **(GATE)** in Life Sciences with percentile score of 86.89., March, 2003.
   - National Eligibility Test conducted by Council of Scientific Research and University Grants Commission, India **(CSIR-UGC NET)** in Life Sciences, December 2002.

Advanced Computing, Pune and Computer Centre, North-Eastern Hill University, from 3rd to 6th March, 2009.

b. Training course on **"Applications of Bioinformatics"** conducted by the Bioinformatics Centre, North-Eastern Hill University, at North-Eastern Hill University, from 13th to 15th February, 2007.

c. CME programme on **"Cancer challenges in India with particular reference to North-East"** organized by Department of Radiation Oncology, NEIGHRIHMS, Shillong, at Shillong on 13th and 14th July, 2006.

d. Workshop on **"Statistical data analysis using SPSS"** organized by Computer and Statistical Service Centre, Indian Statistical Institute, Kolkata and St. Anthony's College, Shillong, at St. Anthony's College, Shillong, from 23rd to 27th March, 2004.

e. Training course on **"Basic techniques in Animal Cell and Tissue Culture"**, conducted by Department of Zoology, North-Eastern Hill University, and National Centre for Cell Science, Pune, at North-Eastern Hill University, from 15th to 20th September, 2003.

11. PUBLICATIONS:

a. Refereed Journals

- Choudhury, Y. and Sharan, R.N. (2009) **Altered p53 response and enhanced transgeneration transmission of carcinogenic risk upon exposure of mice to betel nut**, *Environmental Toxicology and Pharmacology*, **27**, 127-138.

b. Book Chapter

- Choudhury, Y. and Sharan, R.N. **Betel nut and susceptibility to cancer**. *In: The Environment and Cancer: Gene-Environment Interactions and Individual Susceptibility*. Ed. Roy, D. Springer Science & Business Media and Humana press. (In press)

c. Seminar/ Conference proceedings (abstracts)

1. Choudhury, Y. and Sharan, R.N. (2009) **Intrinsic involvement of the *p53*, *Brca1* and *Brca2* tumor suppressor genes in betel nut induced carcinogenesis in mice.** *In: Proceedings of the 96th Indian Science Congress, New Biology Section.*

2. Choudhury, Y. and Sharan, R.N. (2008) **Genomic instability increases risk of cancer in mice exposed transgenerationally to aqueous extract of betel nut.** In: *Indian Journal of Radiation Research, 5, p 81.*

3. Choudhury, Y. and Sharan, R.N. (2008) **Tumor suppressor genes p53, BRCA1 and BRCA2 as biomarkers of human cancer.** *In: Proceedings of National Seminar on Advances in Medical and Microbial Biochemistry.*

4. Sharan, R.N. and Choudhury, Y. (2008) **Insight into interrelationships of p53, BRCA1 and BRCA2 proteins in peripheral blood lymphocytes of patients in advanced stages of cancers vis-à-vis betel nut induced carcinogenesis.** *In: Proceedings of 5th World Assembly on Tobacco Counters Health, page 81, 2007.*

5. Choudhury, Y. and Sharan, R.N. (2007) **Chronic exposure of mice to aqueous extract of betel nut enhanced genomic instability by disrupting organelle functions and inhibiting expressions of BRCA1 and BRCA2 proteins in mouse liver.** *In: Proceedings of National Seminar on Adaptation Biochemistry, held at Department of Biochemistry.*

6. Choudhury, Y. and Sharan, R.N. (2006) **Effect of long term and transgenerational exposure to chronic low dose of aqueous extract of betel nut on the tumor suppressor p53 in mice.** *In: Proceedings of Seminar on Trends in Biochemical Research.*

7. Choudhury, Y. and Sharan, R.N. (2005) **Long term and transgenerational effect of betel nut exposure to mice and**

advanced human cancers on p53 protein: A preliminary comparative study. *In: Proceedings of Seminar on Advances in Biochemical Education and Research,* page 100, 2005.

8. Choudhury, Y. and Sharan, R.N. (2005) **Upregulated p53 plateaus with pre-cancerous nodulation in liver of mice chronically exposed to low-dose aqueous extract of betel nut (AEBN) in drinking water.** *In: Proceedings of at Fourth JSH Single Topic Conference on Hepatocellular Carcinoma: International Consensus and Controversies, No. 101: page 113, 2005.*

12. AWARDS/ DISTINCTIONS:

a. **ISCA Best Poster Presentation Award** of the Indian Science Congress Association, Kolkata, in the section of New Biology during the 96th Indian Science Congress, 2009.

b. **Young Scientist Award** for oral presentation at the **International Conference on Radiation Biology and Translational research in Radiation Oncology**, Jaipur, 2008.

c. **University Gold Medal, NEHU, Shillong**, for securing 1st rank in M.Sc Biochemistry Examination, 2002.

d. **Award of Excellence by St. Edmund's College, Shillong**, for securing 2nd rank in University Examination (Biochemistry Honours), 2000.

e. **Awarded by Meghalaya Board of School Education**, Tura, for securing 4th position in the SSLC examination, 1995.

13. RESEARCH EXPERIENCE:

- Ph. D scholar in Radiation and Molecular Biology Unit, Department of Biochemistry, NEHU, since March 2003.

14. EXPERTISE FOR SCIENTIFIC RESEARCH:

- **Skills developed/ techniques known:** Maintenance and ethical handling of laboratory animals, preparation of histological slides, light microscopy, standard molecular biology procedures such as isolation of protein and DNA,

slot-blotting, SDS-Polyacrylamide Gel Electrophoresis, Western Blotting, design of primers for PCR, amplification of DNA using PCR, RT-PCR, Agarose Gel Electrophoresis etc.

- **Computer skills:** MS Office (Word, Excel, PowerPoint), Kaleidagraph, Digital Image Processing, Documentation and Analysis (Kodak Digital Science, Photoshop) and Bio-informatics tools and packages such as Genamics expression, Genefisher, BLAST, Multalin, Molecular modeling tools such as Swiss PDB Viewer etc.

15. TEACHING EXPERIENCE:

1. Lecturer, Department of Biochemistry, St. Edmund's College, Shillong, August 2002 to July 2003

   Instructing undergraduate students of Biochemistry in
   - Theoretical courses of Metabolic pathways, Nutritional & Medical Biochemistry, and Molecular Biology etc.
   - Practical courses in spectrophotometry and molecular biology techniques

2. Lecturer, Department of Biotechnology, St. Anthony's College, Shillong, May 2006 till date

   Instructing undergraduate students of Biotechnology and Biochemistry in
   - Theoretical courses on Cell Biology, Molecular Biology and Genomics etc.
   - Practical courses in spectrophotmetry, cell biology and molecular biology techniques etc.

   Instructing postgraduate students of Biotechnology and Biocinformatics in
   - Theoretical courses on Molecular Biology, Genomics, Proteomics, Human Molecular Genetics etc.
   - Practical courses in Molecular Biology