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Altered p53 response and enhanced transgenerational transmission of carcinogenic risk upon exposure of mice to betel nut

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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

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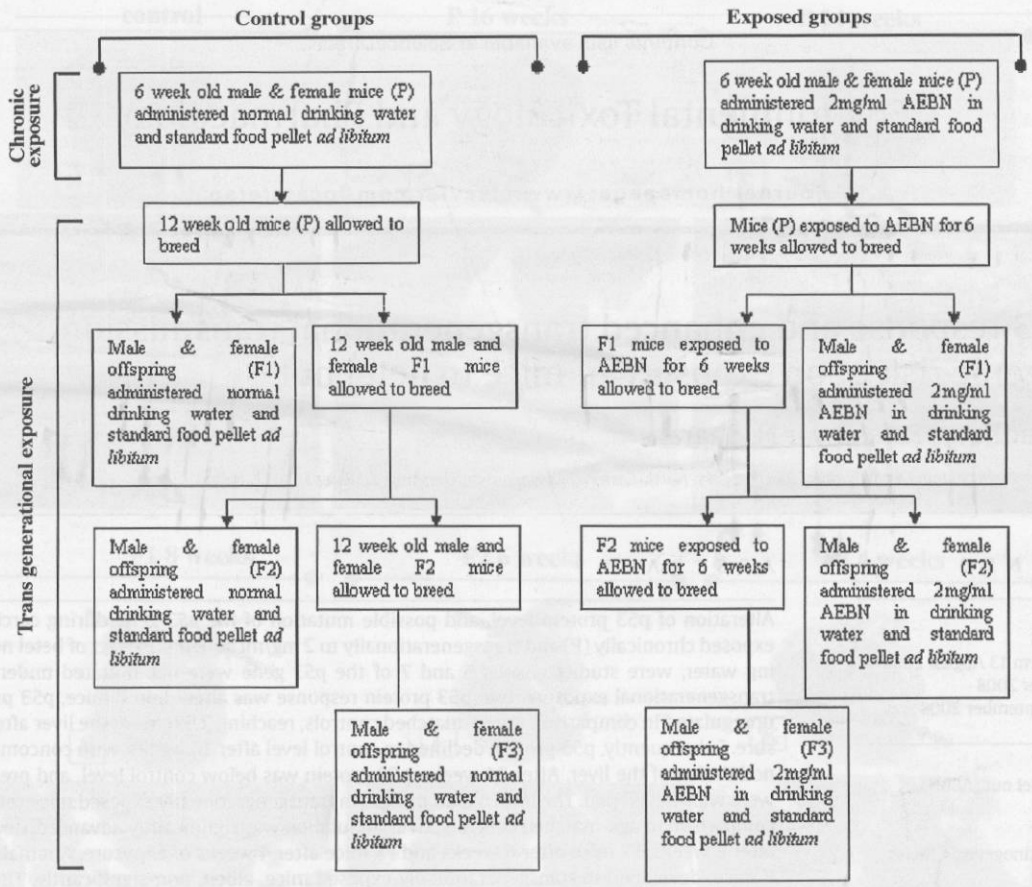

Fig. 1. Experimental designs of chronic and transgenerational exposure of Swiss Albino mice to AEBN drinking water (2 mg/ml).

through multiple mechanisms including sequence-specific transactivation 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,

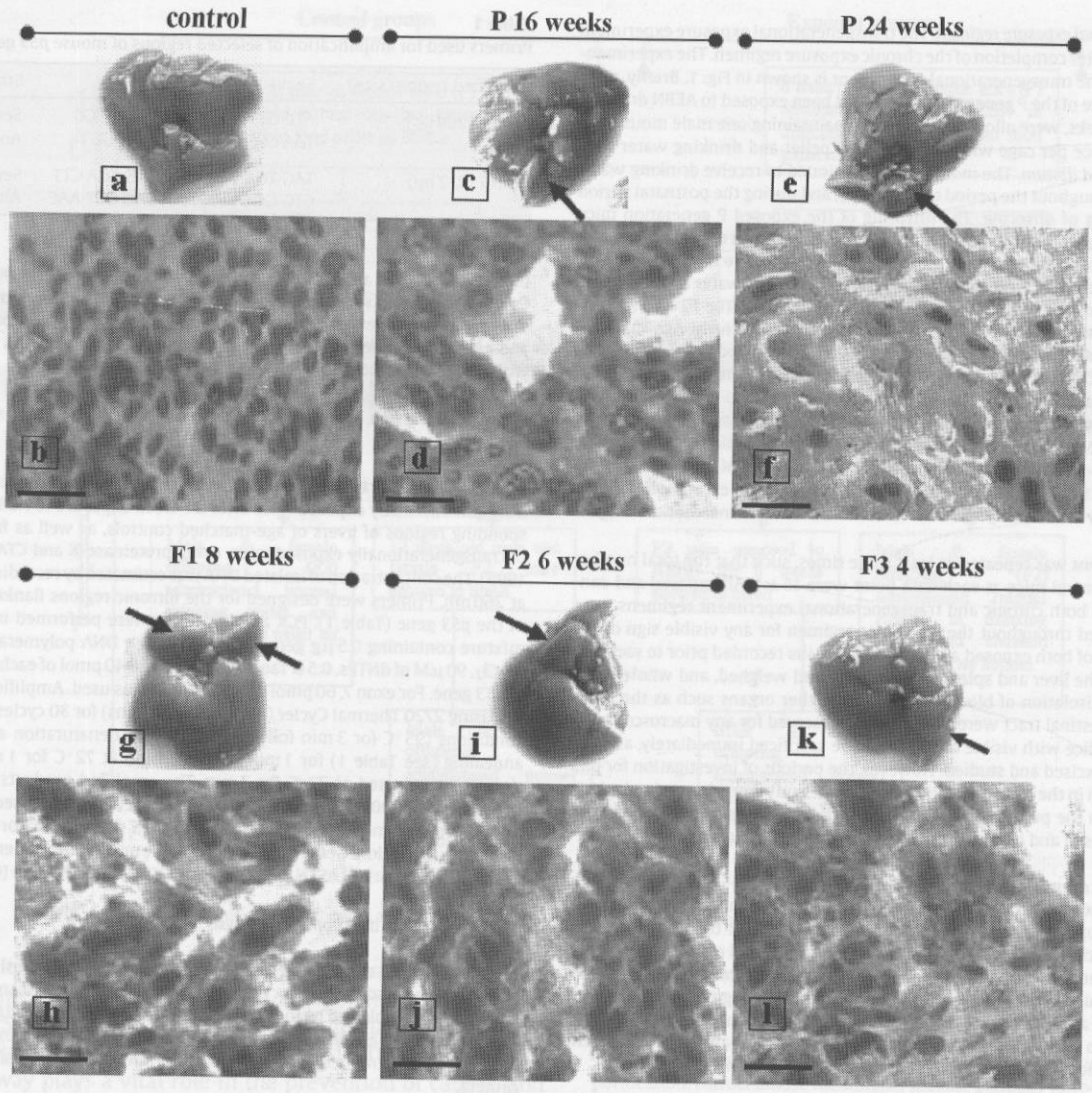

Fig. 2. Preneoplastic nodule formation in the liver of Swiss Albino mice exposed to AEBN drinking water (2 mg/ml) and their respective hematoxylin–eosin stained histological sections at magnification 400× (bar = 20 μm). Normal, control liver (a) and histological section of normal liver (b); nodule formation (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).

for increased susceptibility to cancer. Three generations of progeny were examined in this manner.

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.

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

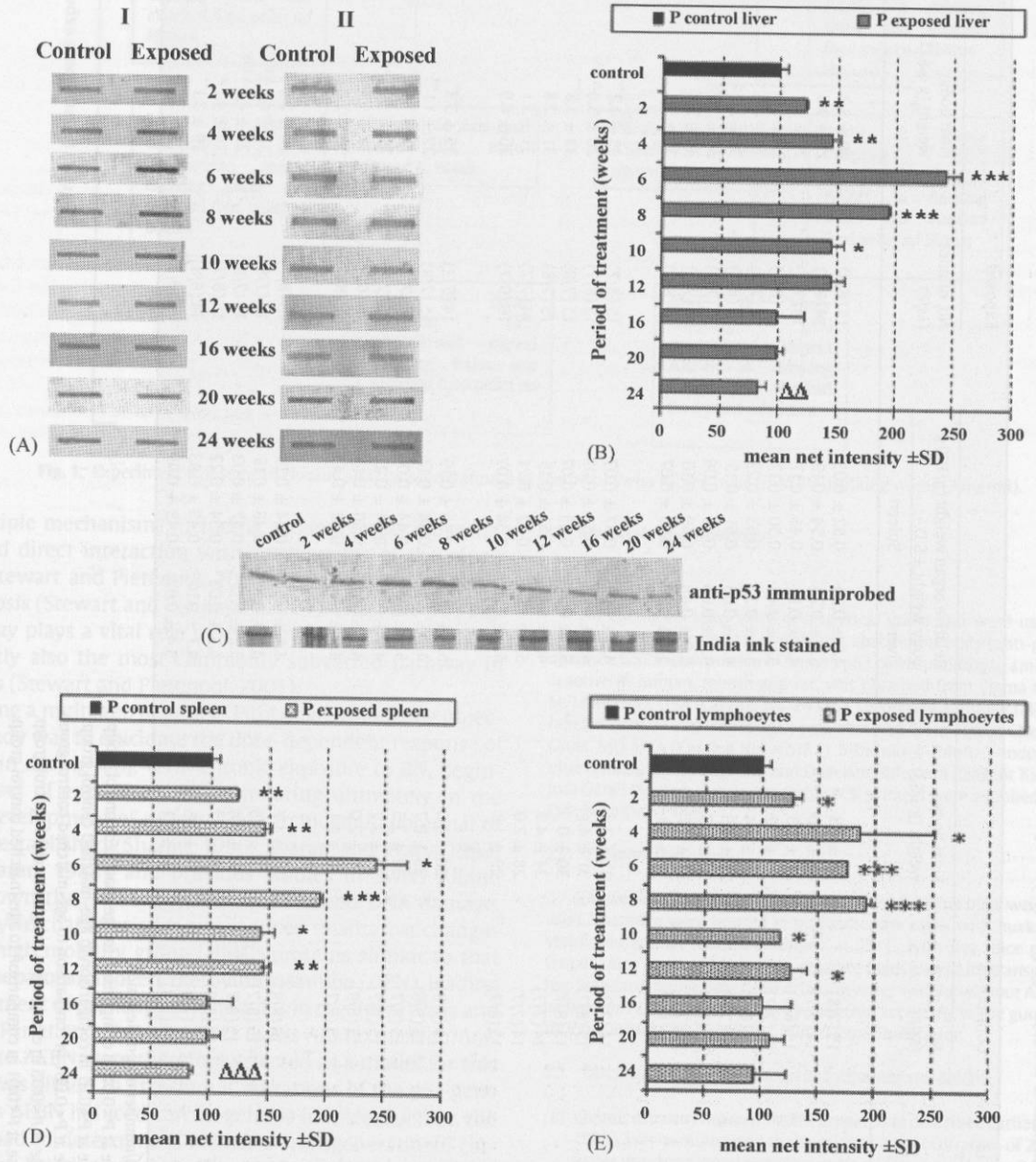

Fig. 3. p53 protein expression level in P generation Swiss Albino mice chronically exposed to AEBN drinking water (2 mg/ml) in comparison to age-matched controls. (A) Slot-blots of liver samples of AEBN exposed group and age-matched controls: panel I—liver samples immunoprobed with specific anti-p53 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.

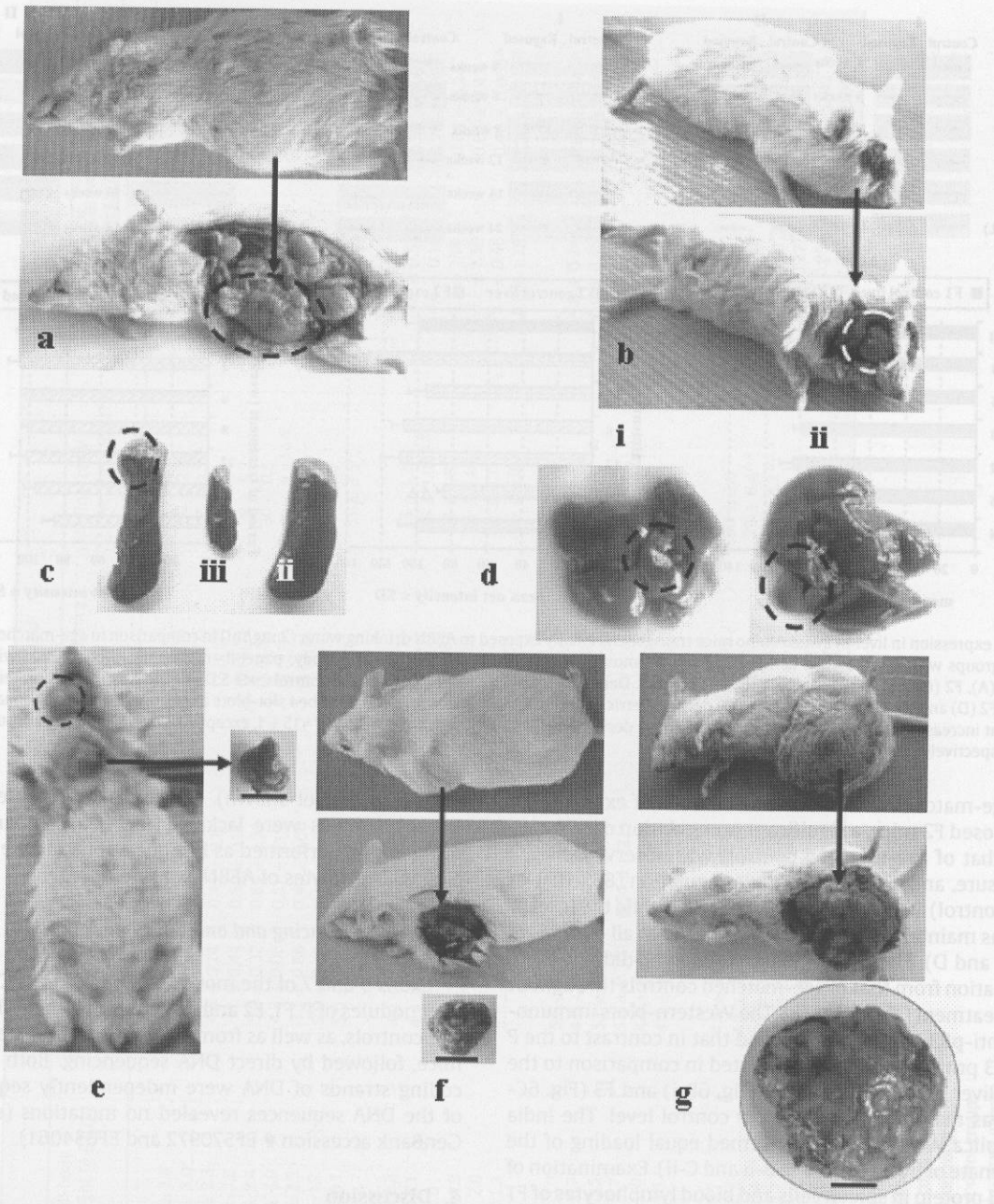

Fig. 4. Various anomalies/alterations observed in Swiss Albino mice transgenerationally exposed to AEBN drinking water (2 mg/ml). 24-week exposed F2 mouse showing pus-filled sac in gastrointestinal tract (circle) (a), enlarged neck node (circle) (b) or enlarged spleen (c) with protrusion (circle) (i) 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%)

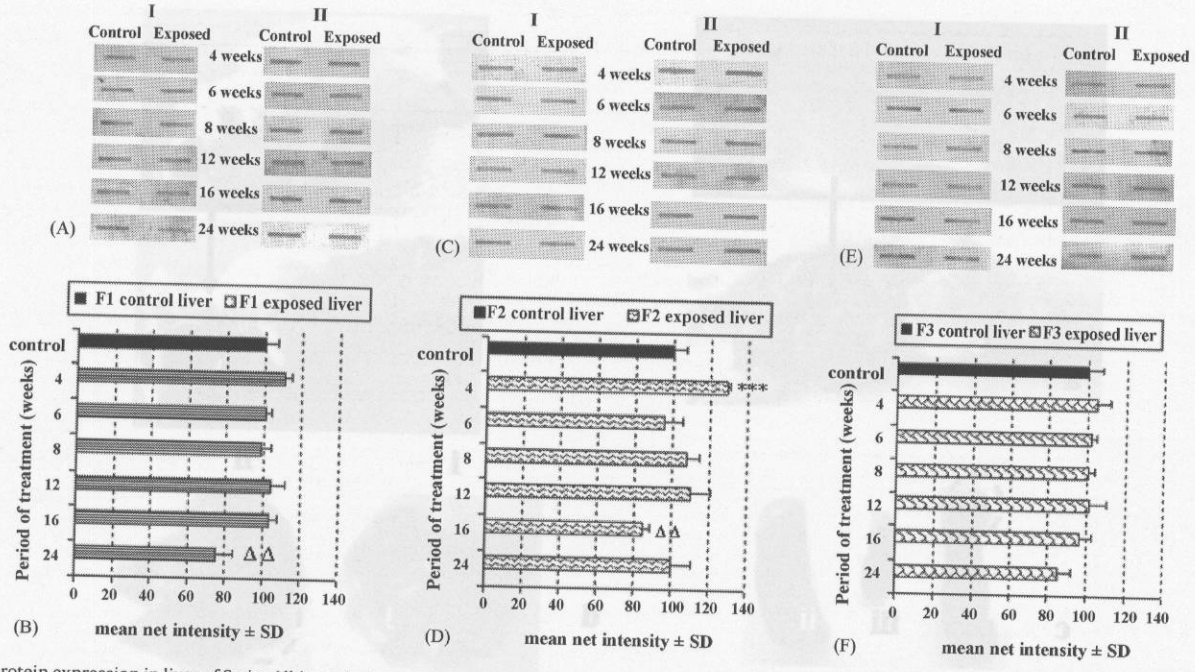

Fig. 5. p53 protein expression in liver of Swiss Albino mice transgenerationally exposed to AEBN drinking water (2 mg/ml) in comparison to age-matched controls. Slot-blots of AEBN exposed groups with age-matched controls: panel I—immunoprobed with specific anti-p53 antibody; panel II—replica slot-blots 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

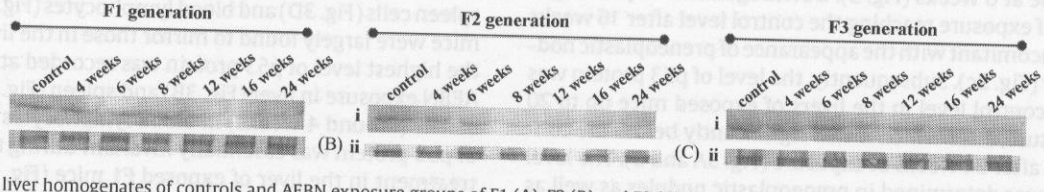

Fig. 6. Western blots of liver homogenates of controls and AEBN exposure groups of F1 (A), F2 (B) and F3 (C) mice immunoprobed with anti-p53 (i) and replica blot stained with India ink for total protein (ii). Number of mice/point = 15 ± 1, except 24-week F2 and F3 exposure groups which had 17 and 20 mice, respectively.

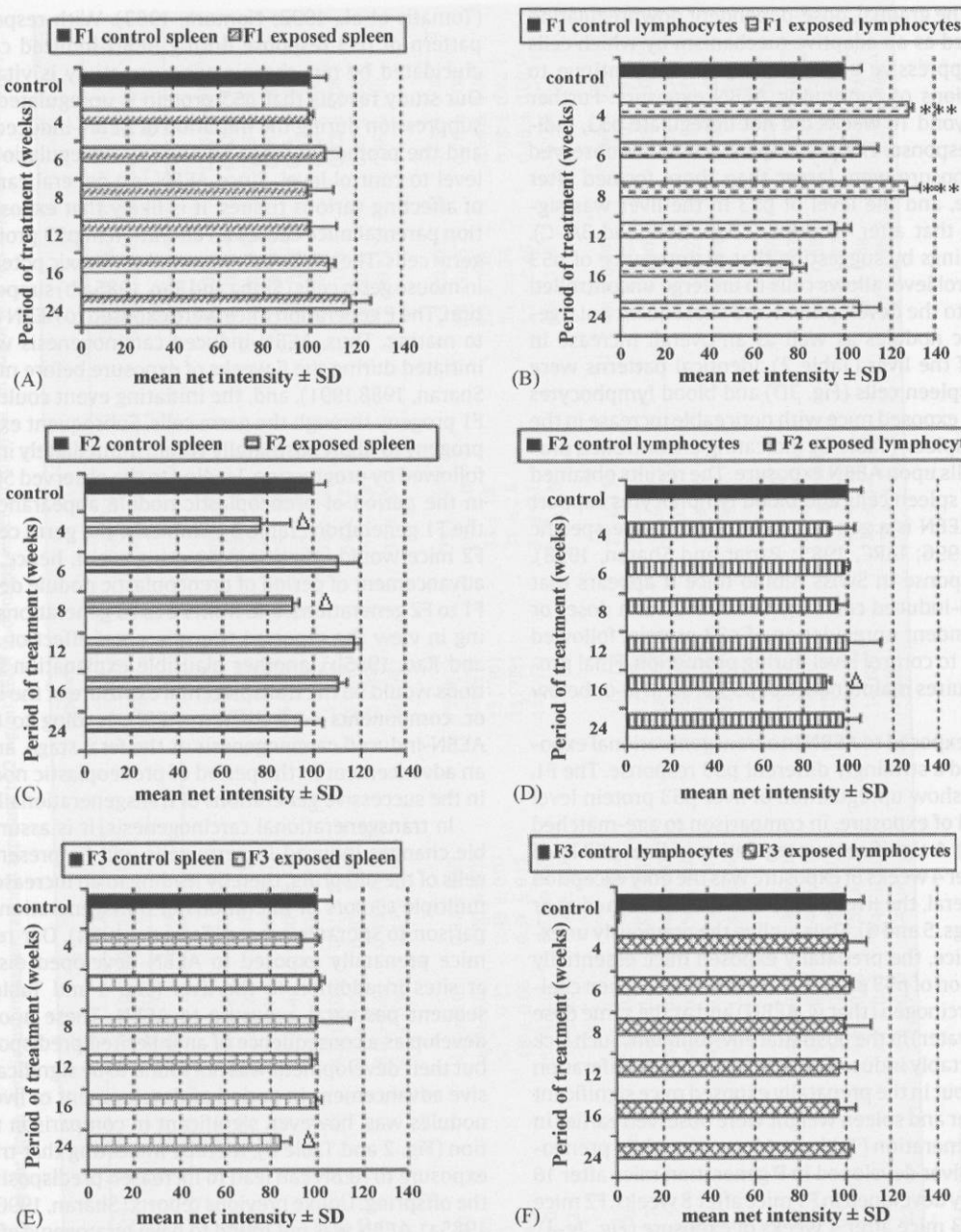

Fig. 7. Densitometric plots (% of age-matched controls; $x \pm$ S.D.) of the level of p53 protein expression in spleen cell samples of F1 (A), F2 (C) and F3 (E) and blood lymphocyte samples of F1 (B), F2 (D) and F3 (F). AEBN drinking water (2 mg/ml) exposed mice as obtained by densitometric analysis 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	–	–		
	F2	+	Neck	–	0/16 (0)
	F3[d]	+		24	1/17 (5.88)
Necrosis	F1	–	–	24	1/20 (5)
	F2[e]	+	Liver	–	0/16 (0)
	F3	+		24	1/17 (5.88)
Protrusion	F1	–	–	24	1/20 (5)
	F2[e]	+	Spleen	–	0/16 (0)
	F3	–	–	24	1/17 (5.88)
				–	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.

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