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Altered BRCA1 and BRCA2 responses and mutation of BRCA1 gene in mice exposed chronically and transgenerationally to aqueous extract of betel nut (AEBN)

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ABSTRACT

The *Brca1* and *Brca2* tumor suppressor genes are involved in the maintenance of genomic integrity as they facilitate error free DNA repair. This study was designed to understand the role of Brca1 and Brca2 in betel nut (BN) induced chronic and transgenerational carcinogenesis in mice. Young male and female Swiss Albino mice were chronically as well as transgenerationally exposed to aqueous extract of betel nut (AEBN) in drinking water (2 mg ml^{-1}) for up to 24 weeks. In chronically exposed mice, the levels of Brca1 and Brca2 proteins were elevated to approximately 1.4-fold over the age matched controls after 2 weeks of exposure to AEBN, followed by a decline below the controls. In transgenerationally exposed mice, both Brca1 and Brca2 proteins remained below the controls from the onset of AEBN exposure and rapidly declined further, indicating a loss of tumor suppressor protection. Nucleotide sequencing of exon 11 of *Brca1* and exon 27 of *Brca2* did not reveal mutation in liver nodules of chronically exposed mice, while a G → C mutation *Brca1* was observed in liver nodules as well as in solid tumors developing in transgenerationally exposed mice. Thus, the genomic instability arising due to the lowering in the levels of Brca1 and Brca2 proteins and mutation in exon 11 of *Brca1* gene contributed to the increased risk of cancer in mice exposed transgenerationally to AEBN.

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1. Introduction

Areca nut, also known as betel nut (BN), forms the basic ingredient of a variety of widely used social and habitual masticatory products consumed by over 600 million people across the globe (Sharan, 1996; Choudhury and Sharan, 2009; Sharan and Choudhury, in press). Today, there is sufficient evidence that BN as well as aqueous extract of betel nut (AEBN) are carcinogenic to humans as well as experimental animals (IARC, 1985; Sharan, 1996; IARC, 2004; Choudhury and Sharan, 2009). AEBN has also 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,b). The genotoxicity of BN or AEBN has also been widely reported. The frequency of sister chromatid exchange (SCE) was elevated in mouse bone marrow cells when mice were exposed to the AEBN and its tannin (Panigrahi and Rao, 1989). AEBN induced DNA strand breaks and enhanced cell proliferation in mouse kidney cells *in vitro* (Wary and Sharan, 1988) besides enhancing UDS (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 SCE probably involving *p53* dependent changes in cell proliferation (Kumpawat et al., 2003). We have previously characterized the alterations in tumor suppressor gene *p53* response associated with different stages of carcinogenesis induced in Swiss Albino mice by chronic exposure to AEBN and reported that transgenerational exposure of mice to AEBN resulted in a progressively increasing predisposition to cancer (Choudhury and Sharan, 2009). We also observed that chronic as well as transgenerational exposure to AEBN did not result in mutation of exons 5 and 7 of the mouse *p53* tumor suppressor gene and concluded that alterations in the level of p53 protein in AEBN exposed mice played a critical role in the development of cancer. These observations suggest that BN induced carcinogenesis is associated with genomic instability and may involve loss-of-function of genes involved in the maintenance of genomic integrity. In fact, global gene expression profiling in human gingival fibroblasts (HGF) exposed to arecoline, the major alkaloid of BN, revealed that four genes related to maintenance of genome stability and DNA repair, namely *FANCG*, *CHAF1* and *CHAF2*, and BRCA1, respectively, were repressed by arecoline in a dose-dependant manner (Chiang et al., 2007).

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The *BRCA1* and *BRCA2* genes are another class of tumor suppressor genes, which are associated with maintenance of genomic stability by maintaining error free DNA repair (Kinzler and Vogelstein, 1997; reviewed by Venkitaraman, 2002). For this reason these genes are also referred to as "caretaker" group of tumor suppressor genes. The BRCA proteins are proposed to be involved in the control of homologous recombination (HR) and double strand break (DSB) repair (Welsch and King, 2001). RAD51 is proposed to interact with BRCA1 at amino acid residues 758–1064 and mediate influence of BRCA1 in HR and DSB repair (Welsch and King, 2001). Besides, BRCA1 has also been implicated in cell cycle checkpoint regulation, transcription-coupled repair and apoptosis. BRCA2 also interacts with RAD51 and is primarily involved in DSB repair (Venkitaraman, 2002). Therefore, *BRCA1* and *BRCA2* genes also seem quite critical in carcinogenesis.

Thus, in addition to the reported response of the *p53* gene (Choudhury and Sharan, 2009), it was of interest to determine alterations in the responses of the *Brca1* and *Brca2* genes upon chronic and transgenerational exposure of mice to AEBN. Exon 11 of the *Brca1* gene was selected for PCR amplification and DNA sequencing studies because it codes for the vital RAD51 interaction domain of the Brca1 protein (Welsch et al., 2000). Deletion of exon 11 of murine *Brca1* has been reported to cause impaired DNA repair (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 tumor incidence in mice (McAllister et al., 2002).

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade and were used without further purification. Nitrocellulose membrane (NCM), anti-BRCA1 (rabbit polyclonal IgG, epitope corresponding to amino acids 1–100 mapping at the N-terminus of BRCA1 of human origin) and anti-BRCA2 (rabbit polyclonal IgG, epitope corresponding to amino acids 1–299 mapping the N-terminus of BRCA2 of human origin) were obtained from Santacruz Biotechnologies, USA. Anti-actin antibody (affinity isolated antibody raised in rabbit using C-terminal actin fragment C11 peptide attached to Multiple Antigen Peptide (MAP) backbone, as immunogen) and Histopaque-1083 were procured from Sigma Chemical Company, USA. Secondary antibody (alkaline-phosphatase labeled goat anti-rabbit IgG), Protein A-CL agarose bead suspension, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT), DNA Amplification Reagent Kit and Gel Extraction Kit were from purchased from Bangalore Genei Pvt. Ltd., India. PCR primers were supplied by Hysel India Pvt. Ltd., India.

2.2. Experimental animals

Six-week old inbred male and female Swiss Albino mice weighing 25 ± 1 g 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 groups. Standard mouse pellet and drinking water with or without AEBN were provided *ad libitum*. All experiments were conducted according to guidelines of the Institutional Ethics Committee for animal experimentation.

2.3. Carcinogen exposure protocol and experimental design

AEBN, prepared as described earlier, was administered to the mice in chronic and transgenerational exposure protocols at a dose of 2 mg ml^{-1} in drinking water *ad libitum* (Wary and Sharan, 1988; Choudhury and Sharan, 2009).

2.3.1. Chronic exposure regimen

AEBN was administered in drinking water for a period up to 24 weeks as detailed in Choudhury and Sharan (2009). The amount of drinking water consumed by each mouse per day was approximately 7 ml. Thus, the amount of AEBN consumed by each mouse was approximately 14 mg per day, which remained invariant throughout the exposure period. These mice have henceforth been 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 5 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. In the latter case, they were killed under chloroform anesthesia.

2.3.2. Transgenerational exposure regimen

The transgenerational exposure protocol has also been described in details earlier (Choudhury and Sharan, 2009). Briefly, male and female mice of the P generation, which has been exposed to AEBN in drinking water for 6 weeks, were allowed to breed while continuing on AEBN drinking water *ad libitum* through pregnancy and postnatal weaning period. The offspring of the exposed P generation mice constituted the F1 generation exposed mice. Post-weaning, the F1 mice were separated from their parents at 6 weeks, male and female mice being maintained separately, and maintained on AEBN drinking water for a period to 24 weeks. The F2 and F3 generations were similarly raised from AEBN exposed F1 and F2 mice, respectively. Similarly, age matched unexposed control mice of the P generation were bred in parallel up to F3 generation to serve as respective controls. 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 2, 6, 8, 12, 16 and 24 weeks.

2.4. Preparation of whole homogenates of liver, spleen cells, enlarged lymph nodes, pus-filled sacs and solid tumors

A 10% (w/v) whole homogenate of liver, spleen cells, enlarged lymph nodes, pus-filled sacs, and solid tumors was prepared using a cell extraction buffer (100 mM Tris–HCl pH 7.5, 250 mM sucrose, 100 mM NaCl, 3 mM EDTA, 10 mM 2-mercaptoethanol and 1 mM PMSF) as described earlier (Choudhury and Sharan, 2009). The total protein content of the homogenates was determined by the method of Bradford (Bradford, 1976) using BSA protein as a standard.

2.5. Preparation of peripheral blood lymphocytes

Peripheral blood lymphocytes (PBL) were isolated as previously described (Kma and Sharan, 2006) using a Histopaque density gradient, and were lysed by freezing and thawing in a cell lysis buffer (20 mM Tris–Cl, pH 8, 10 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 3 mM $MgCl_2$ and 10 mM PMSF), as described earlier (Choudhury and Sharan, 2009). The protein content of the PBL lysate was determined by the method of Bradford (1976).

2.6. Immunoprecipitation of Brca1 and Brca2 proteins

The Brca1 and Brca2 proteins were immunoprecipitated from the whole homogenates of liver and spleen and PBL lysate (150 mg of total protein) following the method described by Rosenberg (1996) with some modifications. Briefly, 7 μl of supplied anti-BRCA1 and anti-BRCA2 antibodies containing 3.5 μg protein each and protease inhibitor PMSF (final strength of 10 mM) were added to the whole homogenate/lysate. The mixture was incubated at 4 °C overnight. The following day, 40 μl of Protein A-CL agarose bead suspension was added to the homogenate/lysate, mixed and incubated at 4 °C for 2 h. The Protein A-CL agarose beads were recovered by centrifugation (18,000 × *g*) for 3 min at 4 °C. The beads were washed thrice with cold immunoprecipitation buffer (50 mM Tris–Cl, pH 8, 500 mM NaCl, 5 mM EDTA, 0.02% NaN_3, 0.5% Triton X-100 and 0.1% SDS) by centrifugation (18,000 × *g*) for 3 min at 4 °C. The washed beads were resuspended in 40 μl of 2 X Laemmli buffer (0.5 M Tris–Cl buffer, pH 6.8, 10% glycerol, 10% (w/v) SDS, 0.7% 2-mercaptoethanol and 0.05% (w/v) bromophenol blue), incubated in a boiling water bath for 5 min and centrifuged (18,000 × *g*) for 3 min. The supernatant was used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.7. Slot blot and Western blot immunoprobing

Equal quantities of protein (400 ng for slot, and all immunoprecipitated Brca1 and Brca2 proteins and 150 μg actin protein for Western blotting) were slot or Western blotted onto 0.45 μm NCM using Bio-Dot SF Microfiltration Apparatus or Mini-Protean II Electrophoretic Cell & Trans-Blot Electrophoretic Transfer Cell (Bio-Rad), respectively. The NCM was immunoprobed as well as stained with India ink as previously described (Sharan et al., 2005). Briefly, for immunoprobing slot blots, the NCM was incubated with either anti-actin antibody (dilution: 1:250), anti-BRCA1 or anti-BRCA2 antibodies (dilution: 1:2000) overnight at 37 °C. In case of immunoprobing the Western blots, the dilutions were 1:150 for anti-actin or 1:1000 for anti-BRCA1 and anti-BRCA2 antibodies. The secondary antibody (dilution 1:10,000) incubation was for 2 h at 37 °C. Color development was done using BCIP/NBT at 37 °C for approximately 10 min. Each experimental set comprised 4–5 replicate slot blots of control and exposed samples. A replica blot of each slot or Western blot was also stained with India ink for total protein.

2.8. DNA extraction, PCR amplification, direct DNA sequencing and nucleotide sequence analysis

DNA was extracted from the preneoplastic nodules of livers of chronically and transgenerationally exposed mice after 24 week exposure to AEBN. The corresponding regions of livers of age matched controls and from the solid tumors

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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. For the purpose of amplification of exon 11 of the *Brca1* gene, which is large in size (1386 bp), the exon was divided into five overlapping regions, including segments of the flanking introns, in order to ensure accurate sequencing of smaller sized amplicons. The scheme has been shown in Fig. 1. Amplification of various regions of the *Brca2* gene did not yield satisfactory products, with the exception of one segment of exon 27, 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 1. PCR amplifications were performed in a 25 μl reaction mixture containing 0.5 μg genomic DNA, 1 × Taq DNA polymerase buffer, 1.5 mM $MgCl_2$, 15 mM of dNTPs, 1 U Taq polymerase and 100 pmol of each primer. Amplification was carried out using Thermal Cycler 2720 (Applied Biosystems) for 30 cycles under optimized conditions (see details in Table 2). Amplification of the targeted region of exon 27 of the *Brca2* gene yielded multiple amplification products, including the expected 365 bp amplicon. The 365 bp band was, therefore, eluted out from the gel using gel extraction kit (Bangalore Genei Pvt. Ltd.). The amplified products were

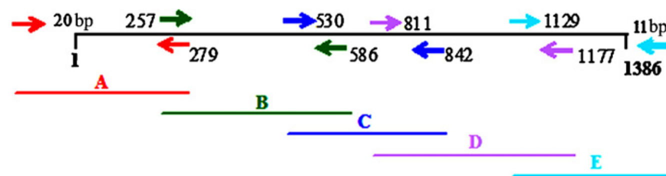

Fig. 1. Experimental design for amplification of exon 11 of the mouse *Brca1* gene to yield 5 overlapping amplicons (A through E). The arrows indicate forward primer (→) and reverse primer (←) for each amplicon, respectively.

lyophilized (Heto Lyolab 3000) and sequenced by direct nucleotide sequencing using ABI's AmpliTaq FS dye terminator cycle sequencing chemistry (Bangalore Genei Pvt. Ltd.). The nucleotide sequences were analyzed with BLASTN (Altschul et al., 1997) and Multalin (Corpet, 1988).

Table 1
Sequences of primers used for amplifying selected regions of the *Brca1* and *Brca2* genes.

Serial no.	Gene/target region	Primer (site of annealing)	Annealing temperature (°C)	Amplicon size (bp)
1	*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
2	*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
3	*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
4	*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
5	*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
6	*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

Table 2
Conditions used for amplifying selected regions of the *Brca1* and *Brca2* genes.

Serial no.	Gene/target region	Step/temperature/time	No. of cycles
1	*Brca1*/exon 11 region A	Initial denaturation/95 °C/3 min	1
		Denaturation/94 °C/1 min	35
		Annealing/55.5 °C/1 min	
		Extension/72 °C/1 min	1
		Final extension/72 °C/7 min	
2	*Brca1*/exon 11 region B	Initial denaturation/95 °C/3 min	1
		Denaturation/94 °C/1 min	30
		Annealing/55.5 °C/1 min	
		Extension/72 °C/1 min	
		Final extension/72 °C/7 min	1
3	*Brca1*/exon 11 region C	Initial denaturation/95 °C/3 min	1
		Denaturation/94 °C/1 min	30
		Annealing/55.5 °C/1 min	
		Extension/72 °C/1 min	
		Final extension/72 °C/7 min	1
4	*Brca1*/exon 11 region D	Initial denaturation/95 °C/3 min	1
		Denaturation/94 °C/1 min	30
		Annealing/58.5 °C/1 min	
		Extension/72 °C/1 min	
		Final extension/72 °C/7 min	1
5	*Brca1*/exon 11 region E	Initial denaturation/95 °C/2 min	1
		Denaturation/95 °C/1 min	30
		Annealing/52 °C/1 min	
		Extension/72 °C/1 min	
		Final extension/72 °C/7 min	1
6	*Brca2*/segment of exon 27 (Touchdown PCR)	Initial denaturation/95 °C/2 min	1
		Denaturation/95 °C/30 s	15
		Annealing/59.7 °C/30 s decrement in temperature by 0.5 °C per cycle	
		Extension/72 °C/40 s	
		Denaturation/95 °C/30 s	20
		Annealing/52.7 °C/30 s	
		Extension/72 °C/40 s	
		Final extension/72 °C/5 min	1

2.9. Imaging and densitometric analysis

Hematoxylin-eosin (HE) stained sections were examined and photographed using Olympus B × 60 bright field microscope at 400× magnification. Immunoprobed and India ink stained slot- or Western blotted NCM images were digitized (HP Scanjet 7400C) for densitometric analysis using KDS-1D software (Kodak). Agarose gels of PCR products were photographed on a BioRad mini transilluminator using a Kodak digital camera.

2.10. 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. The amino acid sequences so obtained were used to simulate and generate 3-D models of the corresponding region of the Brca1 protein in order to predict the effect of the observed mutation on the protein conformation. The molecular models were constructed and analyzed using Swiss-Pdb Viewer version 4.0.

2.11. Statistical analyses

All data presented are the mean ± SD of 3 independent experiments each with 4–5 replicates. The significance of differences in the levels of Brca1 and Brca2 proteins were analyzed by Student's *t*-test. Significance of the progressive advancement in the period of appearance of preneoplastic nodules in the liver of AEBN-exposed mice and the increase in multiplicity of nodules were determined using χ^2-test with Yate's correction. The significance of the development of anomalies in transgenerationally exposed mice, in comparison to chronically exposed P mice or between F1, F2 and F3 mice, was determined using 2 × 2 contingency χ^2-test.

3. Results

3.1. General observations

None of the mice showed any signs of illness throughout the treatment period in P, F1, F2 and F3 generations. Similarly, no congenital malformations were observed in F1, F2 or F3 progeny of parents exposed to AEBN.

3.2. Cellular levels of Brca1 and Brca2 proteins

The levels of Brca1 and Brca2 proteins in control and AEBN exposed mice were monitored by quantitative slot blotting and confirmed by Western blotting. The India ink stained and the actin immunoprobed slot blots, which served as loading controls, did not show significant differences between the net intensities of control and AEBN exposed samples upon densitometric analysis, thus, confirming the loading of equal amounts of protein (Figs. 2A and C, 3A, C and E, 4A, C and E, and 6(I) and (II)). Immunoprobing with anti-BRCA1 and anti-BRCA2 antibodies (Fig. 2A and C, respectively) revealed significant changes in the levels of both Brca1 and Brca2 proteins in the liver of AEBN exposed mice of P generation in comparison to controls. Upon quantification of slot blots and normalization for equal loading of total protein, it was observed that both Brca1 and Brca2 proteins were significantly upregulated to approximately 1.4-fold over the controls at 2 weeks of exposure to AEBN (Fig. 2B and D, respectively). Subsequently, the levels of both proteins were downregulated with Brca1 recording a significant decline to 66% of the control (Fig. 2B) and Brca2 declining to 60% of the control after 16 weeks of exposure (Fig. 2D). This coincides with the reported appearance of preneoplastic nodules in the liver (Choudhury and Sharan, 2009). After 24 weeks of exposure to AEBN, the cellular levels of Brca1 and Brca2 proteins were approximately 50% of the respective controls (Fig. 2B and D). The levels of Brca1 and Brca2 proteins in the preneoplastic nodules were comparable to the levels in the adjoining regions of the liver of mice exposed to AEBN for 16, 20 and 24 weeks (results not shown).

In contrast, the Brca1 and Brca2 proteins did not record any increase throughout the period of exposure in the liver of transgenerationally AEBN exposed F1, F2 and F3 mice (Figs. 3A, C and E, and 4A, C and E, respectively). Instead, the levels of Brca1 and Brca2 proteins in the liver of F1, F2 and F3 mice were found to decline rapidly below the controls from 2 weeks onwards (Figs. 3B, D and F, and 4B, D and F, respectively). A striking difference was, thus, observed in the response of Brca1 and Brca2 proteins in the livers of chronically and transgenerationally AEBN exposed mice (Fig. 5A and B, respectively).

The Western blots immunoprobed with anti-BRCA1 and anti-BRCA2 antibodies confirmed that in contrast to the chronically exposed P generation, the Brca1 and Brca2 proteins were not upregulated above the control level in the liver of transgenerationally exposed F1, F2 and F3 mice at any point during the entire exposure period, and instead, declined rapidly below control level (Fig. 6(I) and (II), respectively). The replica Western blots immunoprobed for actin protein and stained with India ink confirmed equal loading of samples from AEBN exposed mice and controls (Fig. 6(I) and (II), respectively).

3.3. DNA sequencing and analysis of mutation

Exon 11 of the mouse *Brca1* gene and a segment of exon 27 of the *Brca2* 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. The NT sequences of the amplicons were determined. Both strands of DNA were independently sequenced. Analysis of the NT sequences revealed no mutations in exon 11 of the *Brca1* gene in the liver nodules of P generation mice (GenBank Accession # FJ497232). However, a point mutation (G → C) was observed in the liver as well as solid tumors of F1, F2 and F3 mice exposed transgenerationally to AEBN (Fig. 7A(I) and (II); GenBank Accession # FJ589202), in the region corresponding to the amplified region B of exon 11 of the *Brca1* gene (Fig. 1). The occurrence of this transversion point mutation was confirmed by a complementary (C → G) change in the corresponding position when the samples were sequenced with the reverse primer (result not shown). No mutation was observed in the amplified segments of exon 27 of the *Brca2* gene in the P, F1, F2 and F3 mice as in their respective controls (GenBank Accession # FJ825143).

3.4. Molecular modeling of the observed mutation in exon 11 of the Brca1 gene

The consensus NT sequences obtained for control and transgenerationally exposed samples were translated into the corresponding amino acid sequences. The mutation TGT → TCT (Fig. 7A(I) and (II)) was found to change the amino acid residue cysteine (Cys) → serine (Ser) (substitution) at position 933 of the Brca1 protein (GenBank Accession # FJ589202). The effect of the observed Cys → Ser mutation on the structure of Brca1 protein was studied by molecular modeling. The –SH (thiol) group of Cys in the wild-type protein, and the –OH (hydroxy) group of Ser in the mutant protein were found to be involved in hydrogen bonding with the backbone oxygen atom(s) of the neighboring amino acid residues. Three different modes of hydrogen bonding corresponding to three different rotamers, were identified. In the first rotamer, the –SH group of Cys and the –OH group of Ser were not involved in hydrogen bonding with the neighboring amino acid residues (not shown). In the second rotamer, the –SH group and the –OH group formed hydrogen bonds with the backbone oxygen of the neighboring Asp 38 residue (Fig. 7B(I) and (II), respectively). 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. 7B(I) and (II), respectively) and the bond angle was altered from 66.07° in the wild-type protein to 70.70° in the mutant protein (Fig. 7C(I) and (II), respectively). In the third rotamer, the –SH group of Cys in the

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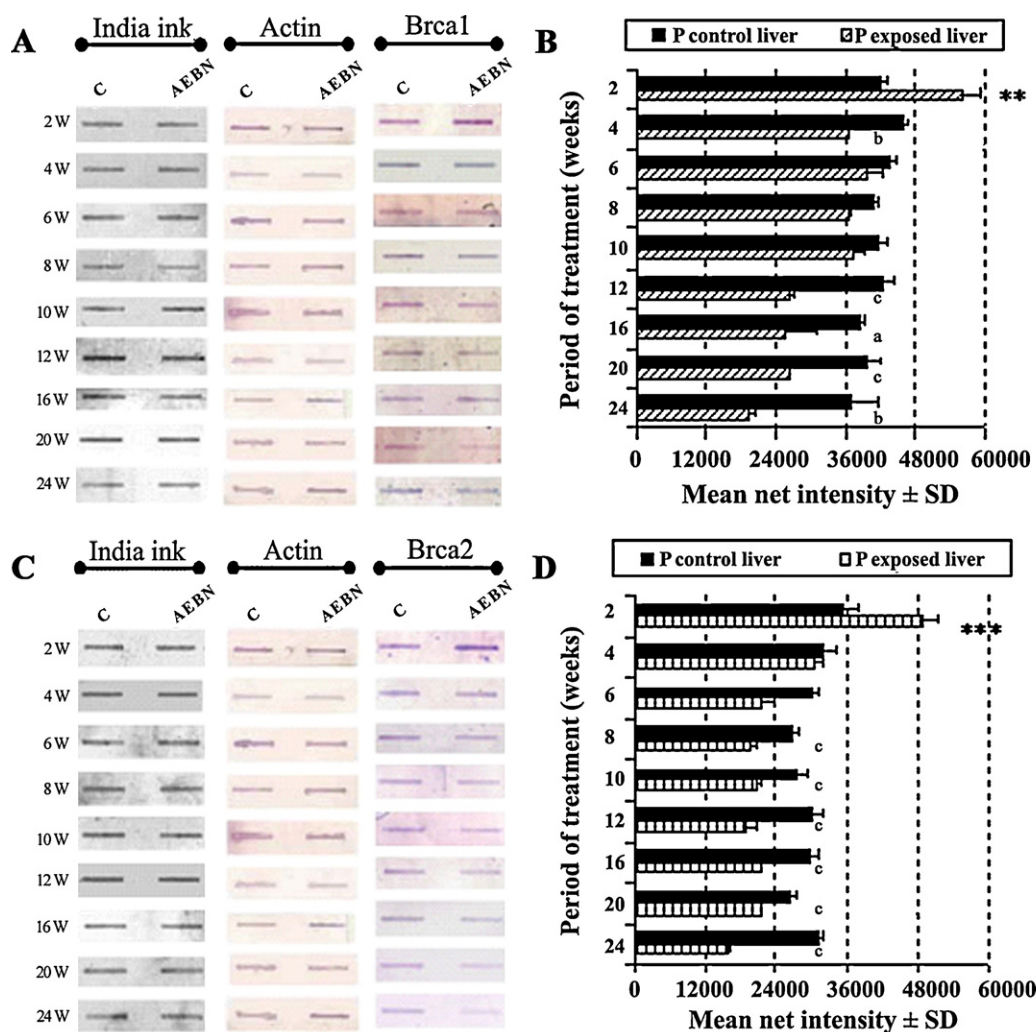

Fig. 2. Slot blot analysis for alterations in levels of Brca1 and Brca2 proteins in liver of P generation AEBN-exposed mice. (A) Slot blots immunoprobed with anti-BRCA1 antibody for Brca1 protein, slot blots stained with India ink for total protein, serving as loading control, and slot blots immunoprobed with anti-actin antibody for actin protein serving as loading control. (B) Densitometric plot (% of age matched controls; mean ± SD) of the level of Brca1 protein expression in liver as obtained by densitometric analysis of the immunoprobed slot blots (A – Brca1) after normalization for equal protein loading (A – India ink and A – actin). (C) Slot blots immunoprobed with anti-BRCA2 antibody for Brca2 protein, slot blots stained with India ink for total protein, serving as loading control, and slot blots immunoprobed with anti-actin antibody for actin protein, serving as loading control. (D) Densitometric plot (% of age matched controls; mean ± SD) of the level of Brca2 protein expression in liver as obtained by densitometric analysis of the immunoprobed slot blots (C-Brca2) after normalization for equal protein loading (C – India ink and C – actin). ** indicates significant increase at $P<0.01$, *** indicates significant increase at $P<0.001$, a indicates significant decrease at $P<0.05$, b indicates significant decrease at $P<0.01$ and c indicates significant decrease at $P<0.001$. Number of mice/point = 15 ± 1.

wild-type protein was not found to be involved in hydrogen bonding with the backbone oxygen of the neighboring Cys 37 (Fig. 7D(I)), while the –OH group of Ser in the mutant protein formed a hydrogen bond with the backbone oxygen of the Cys 37 residue (Fig. 7D(II)).

4. Discussion

Biochemical, genetic and physiological studies have revealed multiple functions for BRCA1 and BRCA2 (Welsch and King, 2001). We have previously reported that chronic exposure of Swiss Albino mice to AEBN elicited an upregulation of p53 protein above control level, after 2–8 weeks of exposure, thereby leading to p53-mediated cell-cycle arrest and inhibition of cellular proliferation (Choudhury and Sharan, 2009). In the present study, chronic exposure to AEBN was also found to induce an immediate cellular response of the Brca1 and Brca2 tumor suppressor proteins, by an immediate elevation of both proteins to approximately 1.4-fold the age matched control levels in the liver, after 2 weeks of exposure to AEBN (Figs. 2 and 5A and B, 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 (Fig. 5C and D, respectively) and approximately 1.2-fold the age matched control levels in the PBL (Fig. 5E and F, 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. In keeping with the known functions of Brca1 and Brca2, such a rise in their levels would result in the arrest of cells which have incurred AEBN-induced damage, accompanied by the facilitation of error-free DNA repair processes (Welsch et al., 2000; Scully and Livingston, 2000; Venkitaraman, 2002), and the likely induction of apoptosis of cells which have incurred irreparable damage (Venkitaraman, 2002).

Continued exposure of P generation mice to AEBN was, however, found to result in a decline in the levels of Brca1 and Brca2 proteins. Both Brca1 and Brca2 proteins declined below age matched control levels after 4 weeks onwards of exposure, declining to approximately 50% that of age matched control level in the liver (Figs. 2 and 5A and B), SC (Fig. 5C and D, respectively) and PBL

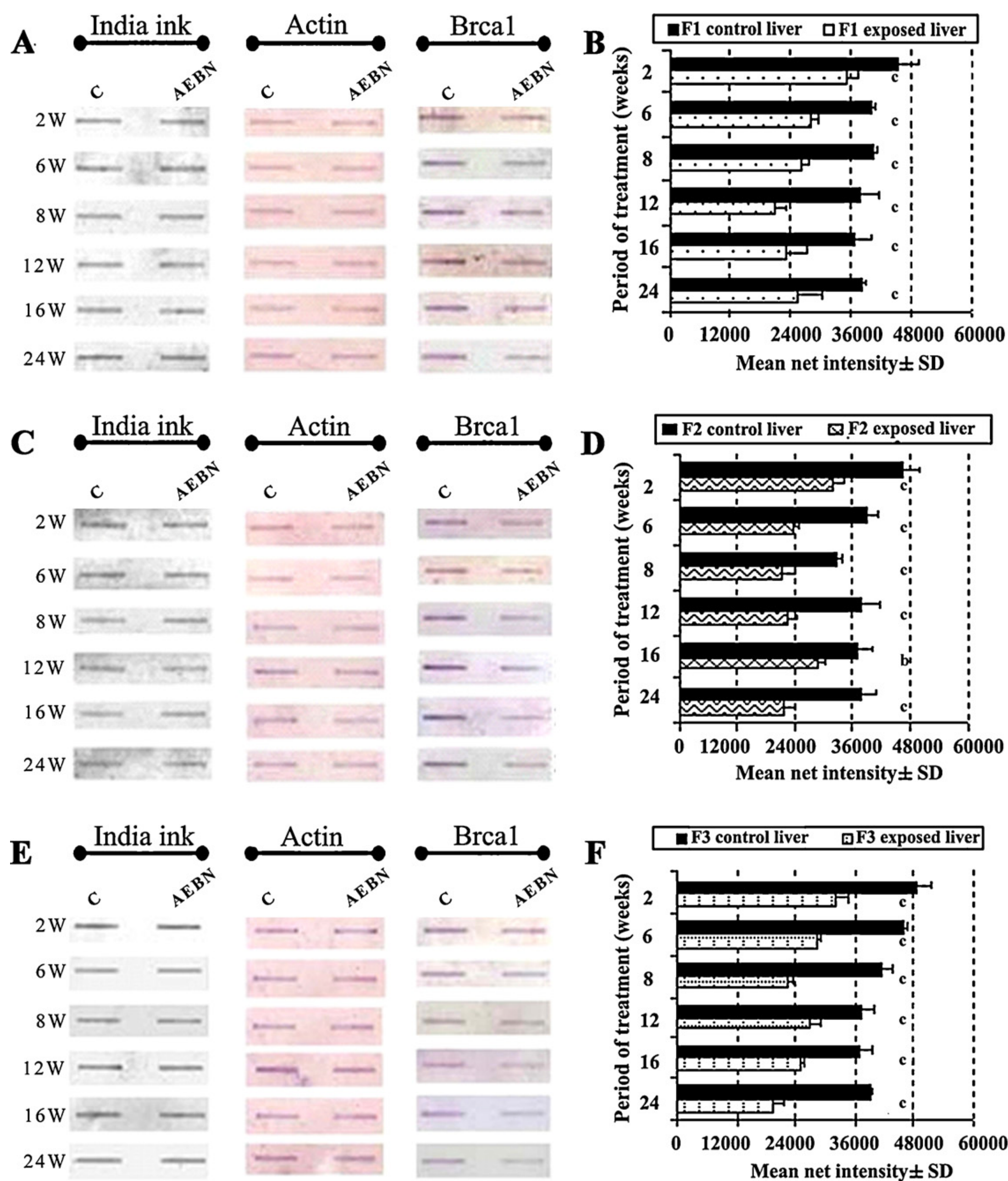

Fig. 3. Slot blot analysis for alterations in levels of Brca1 protein in liver of mice exposed transgenerationally to AEBN. (A, C and E) slot blots immunoprobed with anti-BRCA1 antibody for Brca1 protein in liver of F1, F2 and F3 mice, respectively, slot blots stained with India ink for total protein in liver of F1, F2 and F3 mice, respectively, serving as loading control, and slot blots immunoprobed with anti-actin antibody for actin protein in liver of F1, F2 and F3 mice respectively as loading control. (B, D and F) Densitometric plot (% of age matched controls; mean ± SD) of the level of Brca1 protein expression in liver of F1, F2 and F3 mice respectively as obtained by densitometric analysis of the immunoprobed slot blots (A – Brca1, C – Brca1 and E – Brca1) after normalization for equal protein loading (A, C and E – India ink, and A, C and E – actin). b indicates significant decrease at $P < 0.01$ and c indicates significant decrease at $P < 0.001$. Number of mice/point = 15 ± 1, except 24-week F2 and F3 exposure groups which had 17 and 20 mice, respectively.

(Fig. 5E and F, respectively) at 24 weeks. Thus, chronic exposure to AEBN resulted in immediate elevation, followed by decline in the levels of both Brca1 and Brca2 proteins well below the basal level maintained in age matched control mice. The patterns of alteration of Brca1 and Brca2 proteins were 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 of these proteins following DNA damage would, therefore, compromise the efficiency of DNA repair and probably result in erroneous repair of damaged DNA (Huber et al., 2001; Venkitaraman, 2002; Lu et al., 2005). Thus, the ability of AEBN to induce a decline in the cellular levels of the Brca1 and Brca2 proteins, could, at least partly, explain the previously reported induction of CA and SCE upon exposure to AEBN (Kumpawat et al., 2003). The resultant genomic instability might also contribute to the appearance and development of preneoplastic nodules of the liver, as reported earlier (Wary and Sharan, 1988; Sharan and Wary, 1992; Pariat and Sharan, 1998a,b; Choudhury and Sharan, 2009).

A previous study reported that the treatment of cultured cells with the DNA damaging agents adriamycin and mitomycin C

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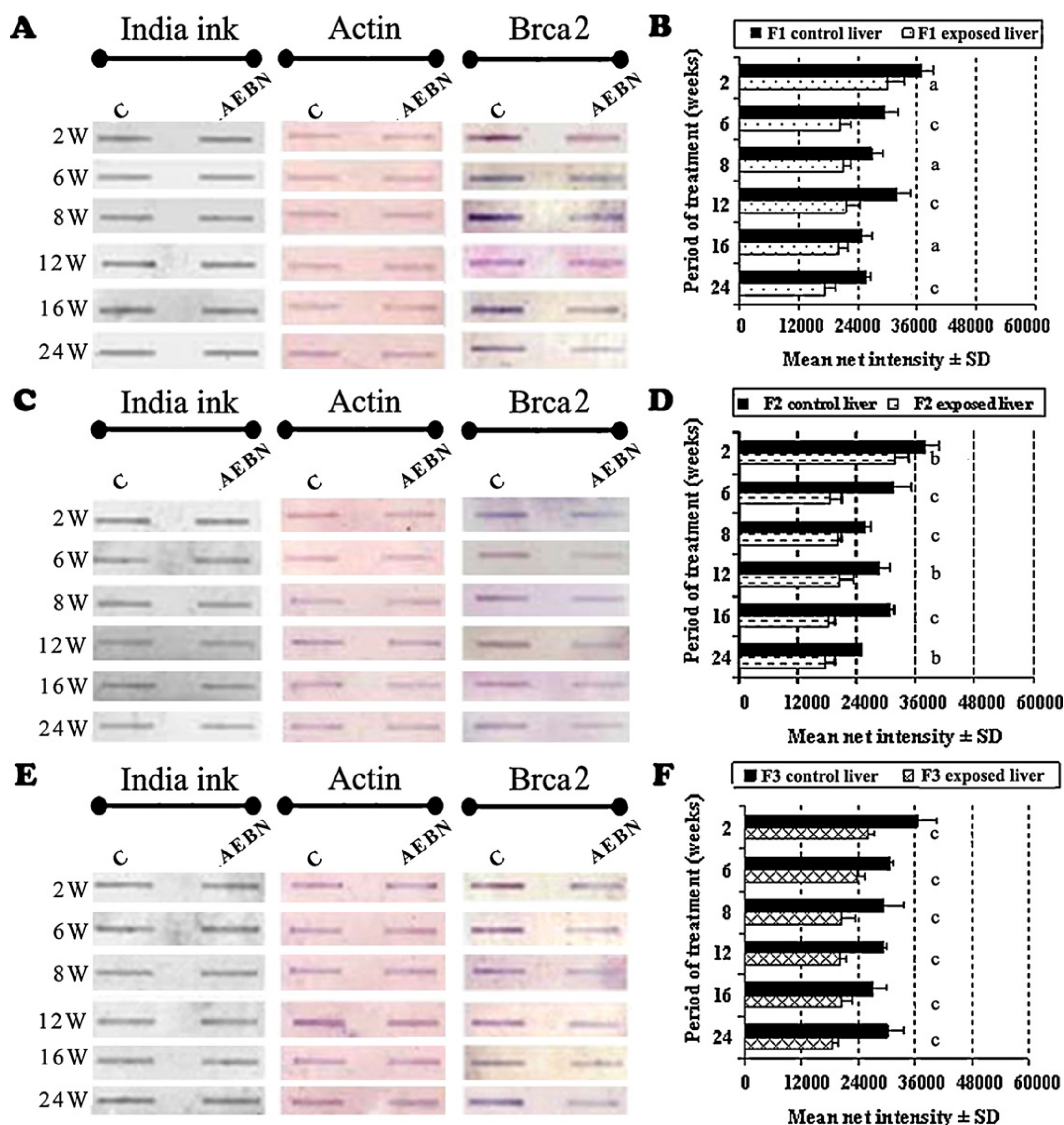

Fig. 4. Slot blot analysis for alterations in levels of Brca2 protein in liver of mice exposed transgenerationally to AEBN. (A, C and E) Slot blots immunoprobed with anti-BRCA2 antibody for Brca2 protein in liver of F1, F2 and F3 mice, respectively, slot blots stained with India ink for total protein in liver of F1, F2 and F3 mice, respectively, serving as loading control, and slot blots immunoprobed with anti-actin antibody for actin protein in liver of F1, F2 and F3 mice respectively as loading control. (B, D and F) Densitometric plot (% of age matched controls; mean ± SD) of the level of Brca2 protein expression in liver of F1, F2 and F3 mice respectively as obtained by densitometric analysis of the immunoprobed slot blots (A – Brca2, C – Brca2 and E – Brca2) after normalization for equal protein loading (A, C and E – India ink, and A, C and E – actin). a indicates significant decrease at $P < 0.05$, b indicates significant decrease at $P < 0.01$ and c indicates significant decrease at $P < 0.001$. Number of mice/point = 15 ± 1, except 24-week F2 and F3 exposure groups which had 17 and 20 mice, respectively.

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 dose- and time-dependent manners 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). We have earlier reported that exons 5 and 7 of the *p53* gene were not mutated upon chronic exposure to AEBN (Choudhury and Sharan, 2009). It, thus, appears likely that upon chronic exposure to AEBN, wild-type p53 mediates the decline in the levels of Brca1 and Brca2 proteins by a mechanism similar to those reported earlier (MacLachlan et al., 2000; Wu et al., 2003). This hypothesis however, needs to be firmed up as it has not been ascertained whether or not other regions of the *p53* gene besides exons 5 and 7 were mutated following chronic exposure to AEBN.

The responses of the Brca1 and Brca2 tumor suppressors upon transgenerational exposure to AEBN were found to be in striking contrast to those elicited by chronic exposure. The cellular levels of both Brca1 and Brca2 proteins were not elevated during the entire period of transgenerational exposure to AEBN. Instead, Brca1 and Brca2 declined rapidly below age matched controls from the second week of postnatal exposure to AEBN in the liver of F1, F2 and F3 mice (Figs. 3, 4, and 5A and B, respectively). Response was similar in the spleen cells and PBL of the AEBN exposed mice (Fig. 5C and F). Thus, the protective response elicited by an upregulation in the levels of

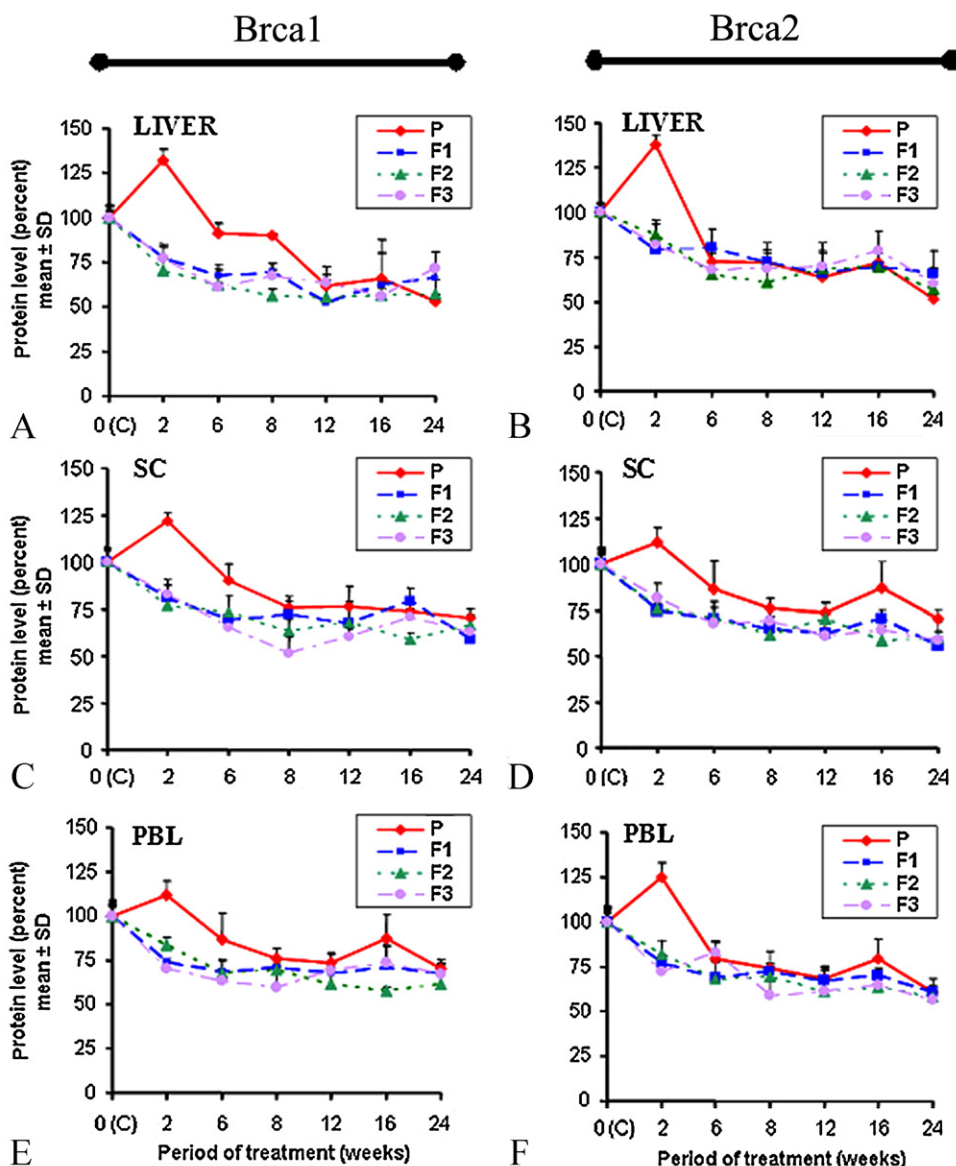

Fig. 5. Graphical representation of expression of Brca1 and Brca2 proteins in P, F1, F2, and F3 generation mice after exposure to AEBN (% age matched control; mean ± SD) as determined by slot blot analysis. (A) Samples from liver immunoprobed for Brca1, (C) samples from spleen cells (SC), immunoprobed for Brca1 and (E) samples from peripheral blood lymphocytes (PBL) immunoprobed for Brca1. (B) Samples from liver immunoprobed for Brca2, (D) samples from spleen cells (SC), immunoprobed for Brca2 and (F) samples from peripheral blood lymphocytes (PBL) immunoprobed for Brca2. Number of mice/point = 15 ± 1, except 24-week F2 and F3 exposure groups which had 17 and 20 mice, respectively.

the Brca1 and Brca2 proteins, during the early period of the chronic exposure regimen, was lost during transgenerational exposure to AEBN. This pattern is identical to the pattern of the p53 protein response in transgenerationally exposed mice as reported earlier (Choudhury and Sharan, 2009).

The rapid decline of the Brca1 and Brca2 proteins in the F1, F2 and F3 mice below basal level, from the very onset of postnatal exposure to AEBN, would indicate severely compromised repair of AEBN induced DNA damage in the exposed mice. Such a shortcoming could lead to increasing genomic instability (Venkitaraman, 2002) 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. In such a cellular milieu, the complete abrogation of the p53 response to AEBN, and maintenance of p53 protein at control level, as reported earlier (Choudhury and Sharan, 2009), would, at best induce a basal level of p53 mediated protection. This would be insufficient to deal with the increasing gamut of damaged DNA. This could result in uncontrolled proliferation of cells as well as inefficient induction of cell death. The alteration in levels of Brca1 and Brca2 proteins observed in the spleen cells and PBL of chronically and transgenerationaly exposed mice, were found to largely mirror those observed in the liver (Fig. 5C and F). Thus, these results support our contention that AEBN is a general, rather than a tissue-specific carcinogen (IARC, 1985; Sharan, 1996; Pariat and Sharan, 1998a,b; Choudhury and Sharan, 2009).

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 nongenotoxic agents (Nomura, 1983; Tomatis et al., 1992). With respect to AEBN, the chronic exposure study reveals that the Brca1 and Brca2 proteins were transiently elevated above control level as an immediate response after 2 weeks of exposure to AEBN (Fig. 2), followed by subsequent decline below age matched controls level which was

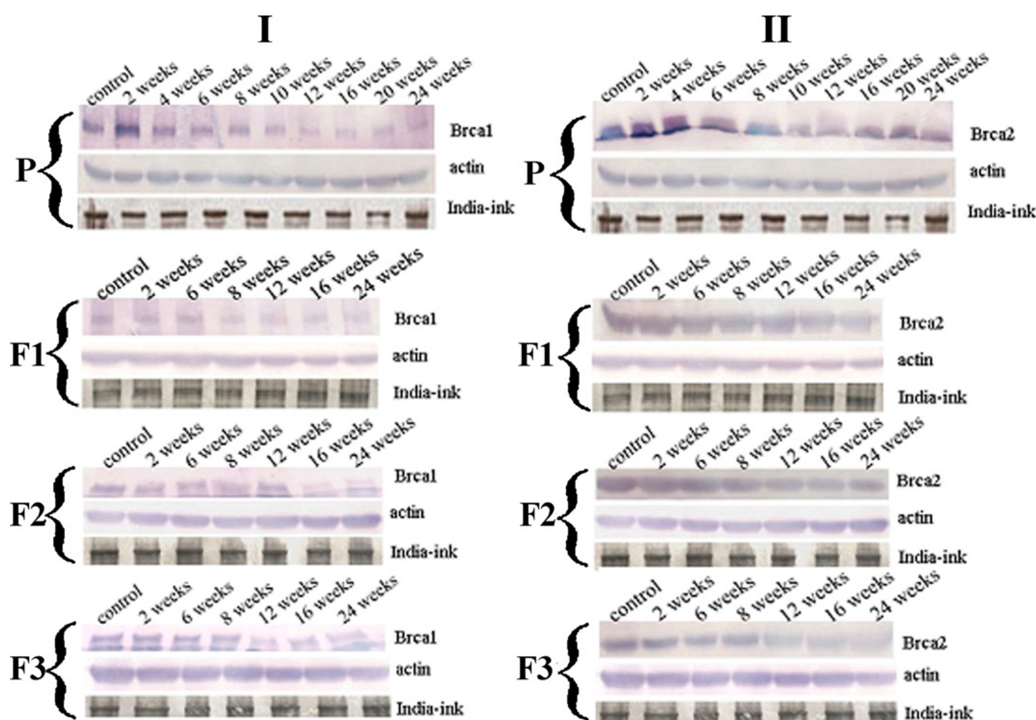

Fig. 6. Western blot analysis for alterations in levels of (I) Brca1 and (II) Brca2 proteins in the liver of P, F1, F2 and F3 mice after exposure to AEBN. Each panel shows Western blot immunoprobed with anti-BRCA1/anti-BRCA2 antibodies for Brca1/Brca2 proteins, Western blot immunoprobed with anti-actin antibody for actin protein serving as loading control, and Western blot stained with India ink for total protein, serving as loading control.

maintained through these generations that followed. Since AEBN is a general carcinogen capable of affecting various tissues, it is likely that exposure of P generation parental mice caused alterations in the Brca1 and Brca2 proteins of their germ cells. The previously reported genotoxic potential of arecoline in mouse germ cells (Sinha and Rao, 1985a,b) supports this contention. Thus, AEBN induced decline in Brca1 and Brca2 protiens would have been initiated in P generation during the 6 weeks of exposure prior to mating (Wary and Sharan, 1988; Choudhury and Sharan, 2009). The initiating event could be inherited to F1 progeny through the germ cells. Subsequent exposure of the F1 progeny to AEBN postnatally would immediately induce promotion followed by progression of AEBN induced carcinogenesis, leading to the observed rapid decline in Brca1 and Brca2 levels, and progressive advancement in the period of preneoplastic nodule appearance through transgenerationally exposed mice (Choudhury and Sharan, 2009). Keeping in view the reported transplacental effect of arecoline (Sinha and Rao, 1985b) another plausible explanation for the observed results would be the transplacental exposure of the F1 fetus to AEBN, or to the 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. Whether the effect of parental exposure to AEBN is inherited through the germ cells, or transmitted transplacentally to the offspring at the fetal stage, it leads to a rapid decline in the Brca1 and Brca2 proteins from the onset of postnatal exposure of F1, F2 and F3 mice (Figs. 3–5, respectively). The F1, F2 and F3 mice were, thus, deprived of the early protective response provided by the Brca1 and Brca2 proteins to the P generation mice, and, therefore, became predisposed to cancer.

PCR amplification and analysis of the DNA sequences of exon 27 of the *Brca2* gene revealed that it was not mutated upon chronic as well as transgenerational exposure to AEBN (GenBank Accession # FJ825143). While exon 11 of the *Brca1* gene was also not mutated in chronically exposed mice (Fig. 7A(I); GenBank Accession # FJ497232), it was found to be mutated in the solid tumors as well as liver nodules developing in mice exposed transgenerationally to AEBN (Fig. 7A(II); GenBank Accession # FJ589202). 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 observed Cys → Ser amino acid substitution in transgenerationally exposed mice occurs at position 933 of the Brca1 protein, which falls within its RAD51 interaction domain (Welsch and King, 2001). Molecular modeling was used to study the bonding/s of the thiol (–SH) group of Cys in the wild-type protein and the hydroxy (–OH) group of Ser in the mutant protein with the neighboring amino acids in the Brca1 protein. These studies revealed altered hydrogen bonding specificities of the substituted amino acid Ser in the mutant protein (Fig. 7B and D). The observed alterations suggest a conformational change in the RAD51 interaction domain of the mutant Brca1 protein, which could influence its interaction with RAD51, and consequently, 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 concluded that the abrogated tumor suppressor responses of the Brca1 and Brca2 proteins as well as mutation of exon 11 of the *Brca1* gene are responsible for the increased predisposition to cancer of Swiss Albino mice exposed transgenerationally to AEBN, when they were postnatally challenged with the same dose of AEBN. This predisposition is manifested by significant advancement in the period of appearance of preneoplastic nodules and non-significant increase in the multiplicity of these nodules in the liver of the P generation and transgenerationally exposed mice as well as the development of various anomalies, including solid tumors, in transgenerationally exposed mice.

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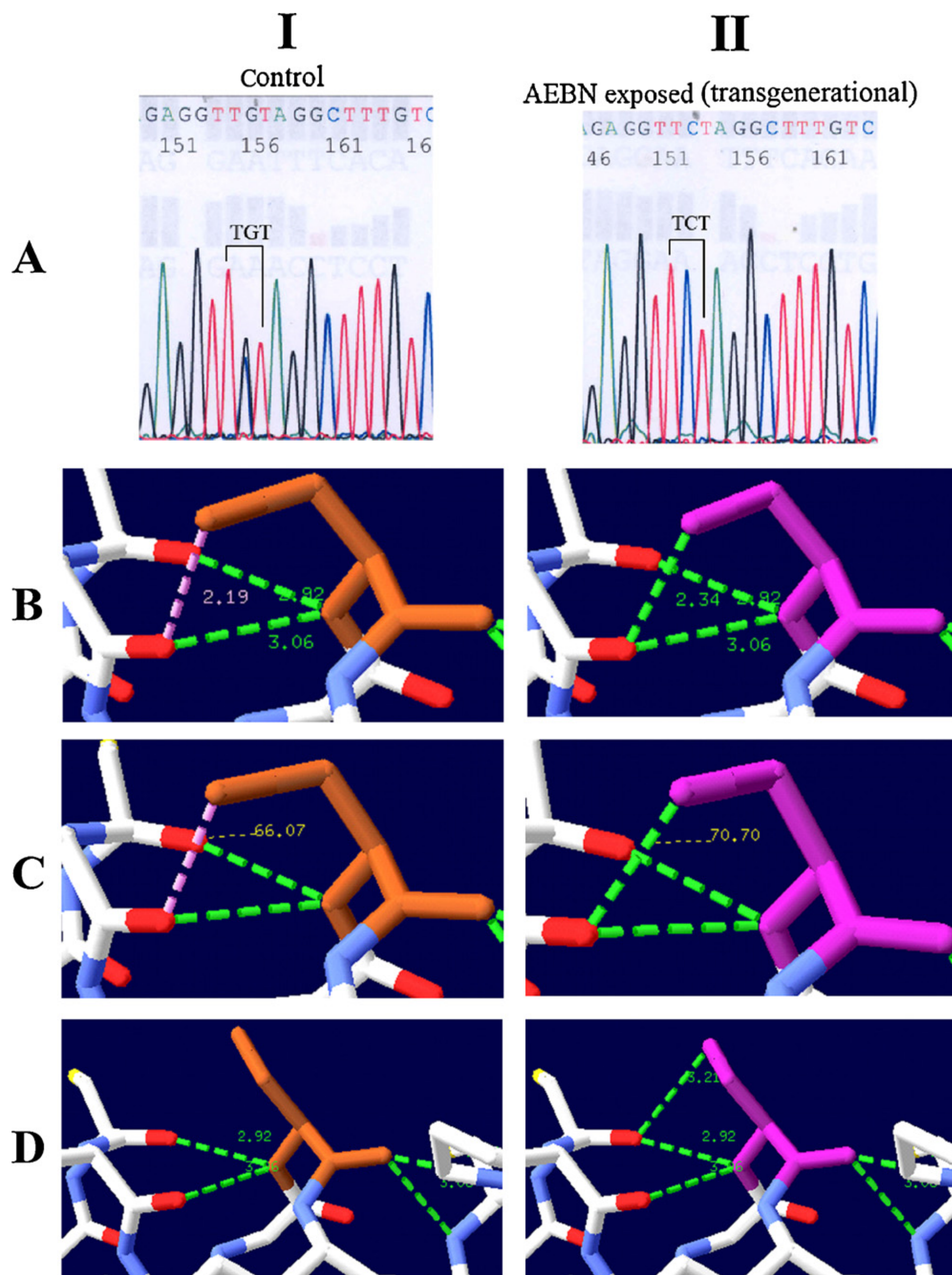

Fig. 7. G → C transversion mutation observed in mice exposed transgenerationally to AEBN. (A) Sequencing electropherograms of control (I) and AEBN exposed samples (II), showing the TGT → TCT mutation in exon 11 of the *Brca1* gene of mice exposed transgenerationally to AEBN. The corresponding amino acid substitution is Cys → Ser. (B) Difference in length of H-bond formed between –SH group of Cys 41 in wild-type (I, 2.19 Å) and –OH group of Ser 41 in mutant (II, 2.34 Å) with the backbone oxygen of Asp 38 in rotamer 2. (C) Difference in angle of H-bond formed between –SH group of Cys 41 in wild-type (I, 66.07°) and –OH group of Ser 41 in mutant (II, 70.70°) with the backbone oxygen of Asp 38 in rotamer 2. (D) Difference H-bonding between –SH group of Cys 41 in wild-type (I, no bond) and –OH group of Ser 41 in mutant (II, bond formed) with the backbone oxygen of Cys 37 in rotamer 3.

The current finding, in association with our previous report (Choudhury and Sharan, 2009), indicates that AEBN induces carcinogenesis in Swiss Albino mice by attenuating their tumor suppressor response through concerted decline of the levels of the Brca1, Brca2 and p53 tumor suppressor proteins below the basal level. In addition, exon 11 of the *Brca1* gene is also mutated in mice exposed transgenerationally to AEBN. Thus, AEBN not only leads to a loss of genomic integrity by abrogating the Brca1 and Brca2 “caretaker” response, but also removes the control over cellular proliferation and apoptosis by abrogating the p53 “gatekeeper” response (Kinzler and Vogelstein, 1997). These synergistic events triggered by AEBN exposure induced genomic instability which is strengthened through continued transgenerational exposure.

Conflict of interest statement

Nothing declared.

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