Atm-haploinsufficiency enhances susceptibility to carcinogen-induced mammary tumors

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Ataxia-telangiectasia (A-T), which is due to mutations in the ATM gene, is a rare autosomal recessive genomic instability syndrome characterized by radiosensitivity and predisposition to cancer. Epidemiological studies have suggested that relatives of A-T patients (A-T carriers) have increased risks of developing breast cancer. We propose that increased breast cancer risks in A-T carriers may be due to exposure to various environmental carcinogens and/or dietary consumption. To test this hypothesis, we treated a congenic strain of Atm<sup>−/−</sup> mice with DMBA (7,12-dimethylbenz(α)anthracene), a mammary carcinogen, and observed mammary tumor incidence. It was found that Atm<sup>−/−</sup> mice have a 2-fold increase, as well as early onset, in mammary tumor incidence relative to wild-type mice (P < 0.005). The increased mammary tumor development is correlated with a 3-fold increase in the development of mammary dysplasia in Atm<sup>−/−</sup> compared with wild-type mice (P < 0.05). We also found that Ras signaling pathway was not activated in DMBA-induced mammary tumors irrespective of the Atm status. At the cellular level, Atm-haploinsufficiency confers increased cellular stress stress manifested by an increased p53 expression and a slightly enhanced survival of mammary epithelial cells in response to radiation. Our results demonstrate that Atm heterozygotes are predisposed to mammary tumor development and support the hypothesis that exposure to environmental carcinogens contributes to the increased rate of breast cancer development in A-T carriers. Given that 1% of the general population are ATM heterozygotes (A-T carriers), this study has great implications in breast cancer development in this population.

Introduction

ATM, which encodes a serine/threonine protein kinase of the PIKK family, is known to ensure genomic integrity by controlling cell cycle checkpoint in response to DNA damage signals. ATM is required for the phosphorylation and activation of downstream genes such as p53, hChk2/hCds1, MDM2 and BRCA1. Functional mutations or amplifications of these genes have frequently been associated with the development of breast cancer (1–3). The importance of ATM in suppressing breast cancer development is supported by recent studies demonstrating that ATM is epigenetically silenced in advanced breast cancer (4) and that ATM mutations contribute to the development of familial breast and ovarian cancer (5).

Consistent with these observations, a number of epidemiological studies have suggested that blood relatives of ataxia-telangiectasia (A-T) patients have increased risks of developing breast cancer (6–12). Indeed, haploinsufficiency of Atm has been shown to result in increased sensitivity to radiation-induced aging phenotypes, such as hair graying and cataracts, in mouse models (13,14). About 80% of the mutations in A-T patients are due to truncating mutations that lead to the loss of ATM protein (15,16). Atm truncating/knockout mouse models have faithfully recapitulated most of the A-T-associated phenotypes (17–20). However, none of the truncated Atm knockout mouse models develop mammary tumors when housed in a controlled environment. On the other hand, ~10% of the mothers of A-T patients (obligate A-T carriers) are more likely to develop breast cancers at an early age (8,10–12). These observations suggest that additional environmental factors may influence the breast cancer development in A-T carriers.

In light of the varied environmental exposures encountered by the A-T carriers, it is suggested that A-T carriers are more sensitive to environmental carcinogens, and hence have higher incidence of breast cancer than the general population. In this study, we tested this hypothesis and analyzed the incidence of carcinogen-induced mammary tumors in an A-T carrier mouse model system.

Materials and methods

Mice

Male Atm<sup>−/−</sup> mice in a mixed genetic background [129SvEv x Black Swiss, (20)] were backcrossed with female FVB/N mice to generate FVB.129S6-Atm F1 mice. The F1 mice were then backcrossed again with female FVB/N mice for a total of 13 generations to obtain FVB.129S6(BlkSw)-Atm<sup>tm1Led</sup> mice. PCR assay was used to identify Atm heterozygotes with the following primers: M1: 5’-tgtagagctcagcattggat-3’; M10: 5’-gtcaataaagttgctgct-3’; Neo1: 5’-ctctattactgaggctctata-3’. PCR amplification was performed in 1x GPCR buffer (16.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 67 mM Tris–HCl (pH 8.3), 6.7 mM MgCl<sub>2</sub>, 5 mM [ME, 1% DMSO, 80 μg/ml BSA, 1 mM dNTP, 150 nM PCR primers and 2 U Taq polymerase]. PCR amplification was initially denatured for 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 45 s at 56°C and 60 s at 72°C. PCR product was resolved onto 2% agarose gel and then stained with ethidium bromide.

Abbreviations: A-T, ataxia-telangiectasia; DMBA, 7,12-dimethylbenz(α)anthracene; LOH, loss of heterozygosity; MAPK, mitogen-activated protein kinase.

These authors contributed equally to this work.
concentration of 5 mg/ml and administered to mice ~6 weeks of age by oral gavage at a dose of 1 mg once a week for five consecutive weeks. Mammary tumor development was followed by weekly palpation of DMBA-treated mice.

**Whole mount and histological analysis of mammary gland**

Whole mount analysis was performed as described (22). For histological analysis of the whole mount mammary gland, the tissue was removed from the slide and placed in xylene for 30 min. The whole mount was then paraaffin-embedded and sectioned with a Zeissmicrotome. Sections were rehydrated in a series of descending concentrations of alcohol followed by counterstaining in fast green (Sigma). Histological analysis of mammary tumors was performed as described (23).

**Protein expression analysis**

Frozen mammary glands or mammary tumors were thawed on ice followed by homogenization in NP 40 buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8.0 and 1% IGEPA CA630) containing a cocktail of proteinase inhibitors (Roche). Cleared lysate were resolved on SDS–PAGE gel and transferred to PVDF membrane. Protein expression was detected by enhanced chemiluminescence following incubations of various antibodies. The following antibodies were used: anti-ATM (MAT3), 1:1000 (kindly provided by Y.Shiloh); anti-Mcm2 (Santa Cruz, N-19, sc-9639), 1:200; anti-Smc1 (Novus, NB-100-204), 1:500; Ahl (Santa Cruz, H-211, sc-5579), 1:500; anti-H-Ras (Santa Cruz, F235, sc-29), 1:500; anti-Raf (Pharmingen, Cat#13981A), 1 µg/ml; anti-MAPKpT202/ Y204 (Cell Signaling, #9101), 1:1000; anti-MAPK (ERK2, Santa Cruz, C-14, sc-154), 1:400; anti-p53-S15 (Cell Signaling, #9284), 1:1000. For quantitative analysis, the scanned gel images were analyzed by ImagePro 5.0 software, and relative levels were normalized against the expression levels of β-actin.

**Tumor genomic DNA isolation and Ras mutation assay**

Following NP40 extraction of tumor sample as described above, insoluble pellet was briefly rinsed in PBS, and 200 µl of lysis buffer (100 mM NaCl, 10 mM EDTA, 20 mM Tris–HCl, pH 7.5 and 0.5% SDS) containing 100 µg/ml proteinase K (Roche) was added followed by overnight incubation at 50°C. Saturated NaCl (100 µl) was then added to the samples and this was followed by vigorous shaking for 5 min. Tumor samples were spun at 6000 g for 10 min after sitting on ice for 10 min. Supernatant was transferred to a new tube and tumor genomic DNA was precipitated by addition of 100% ethanol. Tumor DNA was dissolved in TE and was used for PCR assay directly without further treatment.

Detection of H-Ras mutation at codon 61 was done essentially as described, with modification (24). In brief, following amplification of exon 2 of Hras, DNA sample was digested with XbaI for 2 h at 37°C. Digested samples were resolved onto 5% polyacrylamide gel in 1x TBE buffer to distinguish the parent DNA (207 bp) and the mutated parental DNA (204 bp) from each other.

**Results**

In an effort to better understand the function of Atm in mammary tumor development, we generated a congenic strain of Atm knockout mice by backcrossing 129SvEv x Black Swiss Atm<sup>tm1Led</sup> mice (20) to FVB/N congenic background for 13 generations. Reciprocal mammary gland transplantation can be performed with this new strain, which we referred to as FVB.129S6(BKLSW)-Atm<sup>tm1Led</sup> (Lu and Wang, unpublished data). To test the hypothesis that A-T heterozygotes are predisposed to mammary tumorigenesis and to identify the environmental factors that may influence the development of breast cancer of A-T heterozygotes in the general population, we performed the following experiments with mice and cells derived from FVB.129S6(BKLSW)-Atm<sup>tm1Led</sup> (Atm<sup>+/−</sup> hereafter).

Wild-type and Atm<sup>+/−</sup> nulliparous female mice were treated with DMBA at 6 weeks of age, and mammary tumor development was monitored weekly by palpation. Once tumor mass was identified, the mice were killed and the tumors dissected for histopathological analysis (Table I). Nearly twice as many Atm<sup>−/−</sup> heterozygotes developed mammary tumors (64.7%) as the wild-type mice (37.5%). The relative risk factor for DMBA-induced mammary tumors is 1.7 for Atm heterozygotes. Histopathological analysis of tumor samples indicates a wide variety of mammary tumor histological types in both wild-type and Atm heterozygotes (Table I and data not shown). Most importantly, Atm heterozygotes developed mammary tumors earlier with an average onset of 189 days compared with 229 days for wild-type mice (Figure 1A, P < 0.005 by log-rank test). In contrast, control untreated wild-type (n = 6) and Atm<sup>−/−</sup> (n = 11) mice did not develop mammary tumors over a 1-year period (data not shown). Interestingly, following DMBA treatment, ovarian tumors were observed in both wild-type and Atm<sup>−/−</sup> mice, whereas uterine tumors were observed in some of the Atm<sup>−/−</sup> mice but not in wild-type mice (Table I).

Since tissue dysplasia is an early event during tumorigenesis, the extent of DMBA-induced dysplasia was determined in the mammary glands of the treated mice. In these experiments, tumor-free mammary glands were harvested from tumor-bearing mice and processed for whole mount analysis. Poisson regression analysis was performed to determine whether mammary dysplasia might be affected by mammary tumor development. Analysis suggests that mammary tumor development had no effect on mammary dysplasia of adjacent glands (P = 0.169, Chi-Square test). Over 56% (n = 53) of the
Table I. DMBA-induced tumors in wild-type and Atm¹/² mice

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Tumor pathology</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>112 Lymphoma</td>
</tr>
<tr>
<td>2</td>
<td>142 Mammary adenocarcinoma</td>
</tr>
<tr>
<td>3</td>
<td>168 Mammary adenocarcinoma</td>
</tr>
<tr>
<td>4</td>
<td>169 Mammary adenocarcinoma, uterine adenocarcinoma</td>
</tr>
<tr>
<td>5</td>
<td>171 Mammary adenocarcinoma</td>
</tr>
<tr>
<td>6</td>
<td>183 Mammary adenoma</td>
</tr>
<tr>
<td>7</td>
<td>185 Mammary papillary tumor</td>
</tr>
<tr>
<td>8</td>
<td>202 Mammary cribriform tumor</td>
</tr>
<tr>
<td>9</td>
<td>204 Mammary solid tumor, uterine adenocarcinoma</td>
</tr>
<tr>
<td>10</td>
<td>205 Mammary adenocarcinoma</td>
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<tr>
<td>11</td>
<td>206 Mammary adenocarcinoma</td>
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<tr>
<td>12</td>
<td>208 Uterine squamous cell carcinoma</td>
</tr>
<tr>
<td>13</td>
<td>212 Ovarian granulosa cell tumor</td>
</tr>
<tr>
<td>14</td>
<td>215 Ovarian granulosa cell tumor</td>
</tr>
<tr>
<td>15</td>
<td>253 Lung adenocarcinoma, gastric adenocarcinoma</td>
</tr>
<tr>
<td>16</td>
<td>254 Mammary squamous carcinoma and mammary solid tumor</td>
</tr>
<tr>
<td>17</td>
<td>263 Uterine adenocarcinoma, lung adenocarcinoma</td>
</tr>
</tbody>
</table>

Six wild-type tumors and 22 Atm¹/² tumors were analyzed. Three of the eight wild-type mice developed mammary tumors compared with 11 of the 17 Atm¹/² mice.

Atm¹/² mammary glands exhibited severe dysplasia, compared with 21% (n = 28) of the wild-type mammary glands that showed only mild dysplasia (Figure 1B and Figure 2, P = 0.0248 by Poisson regression analysis). The dysplastic mammary structures in the wild-type mice were more localized by whole mount analysis and exhibited a single epithelial layer by histological analysis (Figure 2A–D). For example, after DMBA treatment, 58% of the Atm heterozygotes with dysplastic mammary glands appeared to be diffusive, whereas only 11% of the wild-type with dysplastic mammary glands had such feature (Figure 2E–L, data not shown). Histopathological analysis revealed the presence of various stages of mammary tumorigenesis including hyperplastic alveolar nodule (HAN) (Figure 2M–P) and mammary intraductal neoplasia (MIN) (Figure 2G–H) in Atm¹/² mammary glands.

Although increased mammary dysplasia in Atm¹/² mice could contribute to enhanced mammary tumorigenesis in those mice, it is not clear whether mammary tumors from Atm¹/² mice exhibit loss of heterozygosity (LOH) at the Atm locus. To further characterize the enhanced carcinogen-induced mammary tumor development in Atm¹/² mice, genomic DNA from three wild-type and five Atm¹/² mammary tumors was harvested from fresh frozen tissues. PCR analysis was performed to determine whether LOH occurred in tumors from Atm¹/² mice. Our results demonstrated that the wild-type Atm allele was retained in all five mammary tumors analyzed (Figure 3A). It is possible that Atm LOH might have occurred in the tumors and that wild-type PCR products detected may be derived from normal cells intermingling with tumor cells.

Therefore, we compared PCR products derived from wild-type and Atm¹/² mammary tissues with that derived from tumors and found that the intensity of the wild-type bands were comparable in tissues or tumors derived from Atm¹/² mice (Figure 3B). Moreover, Atm expression was clearly detectable in all five mammary tumor samples and three ovarian tumor samples derived from Atm¹/² mice (Figure 3C). Therefore, increased mammary tumor development in Atm¹/² mice is not due to loss of Atm expression from the wild-type allele. Collectively, these results suggest that Atm haploinsufficiency confers increased sensitivity to chemical carcinogenesis. Interestingly, Mini-Chromosome Maintenance protein 2 (Mcm2), which was recently described as a target of ATM/ATR (27,28), was strongly expressed in three of the five Atm¹/² mammary tumors but none in wild-type tumors. Furthermore, Mcm2 expression was only weakly detected in one of the three Atm¹/² ovarian tumors (Figure 3B). In addition, the expression of Structural Maintenance Chromosome 1 (Snc1), another downstream target of Atm (29,30), appears to be positively correlated with the levels of Atm protein except in one wild-type tumor (#2) expressing high amount of
The levels of Atm protein in these tumors were not due to differential protein loading since Aryl hydrocarbon Receptor (AhR) and Raf were all expressed similarly in these tumors (Figures 3B and 4B). It has been demonstrated recently that ATM is epigenetically silenced in advanced breast cancers (4). It is possible that variable expression of Atm in the mammary tumors may also be under epigenetic regulation.

It was shown previously that DMBA-induced skin carcinogenesis was due to mutation of the \textit{Hras} gene at codon 61, which could be detected in over 90% of the DMBA-induced skin tumor samples (31,24). In addition, mammary tumors induced by DMBA appear to be correlated with ras mutations (32–34). To determine whether mammary tumor induced by DMBA harbours \textit{Hras} mutation at codon 61 in our experimental model, an allele-specific \textit{Hras} mutation assay was performed (24). This assay takes advantage of the fact that DMBA-induced \textit{Hras} mutation creates a novel \textit{XbaI} restriction site, which could be conveniently employed to distinguish the wild-type allele from mutated \textit{Hras} allele after PCR amplification of sequence surrounding codon 61. Our data clearly indicate that \textit{Hras} mutation at codon 61 was not involved in either DMBA-induced mammary or ovarian tumor development since all tumors from wild-type and \textit{Atm}\textsuperscript{+/---} mice display only wild-type \textit{Hras} allele following \textit{XbaI} digestion of the PCR products (Figure 4A). Although we were unable to detect \textit{Hras} mutation at codon 61, the presence of other activating mutations in \textit{Ras} gene cannot be excluded. Since \textit{Ras} mutations would activate mitogen-activated protein kinase (MAPK) signaling pathway, we therefore determined the expression of activated MAPK in these mammary tumors. As shown in Figure 4B, phosphorylation of MAPK at Thr202 and Tyr204 was variably observed in mammary and ovarian tumors derived from both wild-type and \textit{Atm}\textsuperscript{+/---} mice. In a limited comparison of Ras signaling between mammary gland and mammary tumors from the same wild-type or \textit{Atm}\textsuperscript{+/---} mice, we also could not find correlative evidence of Ras activation and mammary tumor development.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig2.png}
\caption{Whole mount and histopathological analysis of mammary dysplasia. (A–D) Wild-type mammary gland. (E–P) \textit{Atm}\textsuperscript{+/---} mammary gland. (A, B, E, F, I, J, M and N) Whole mount analysis of mammary gland. (C, D, G, H, K, L, O and P) Histopathological analysis of the corresponding whole mount mammary gland. (G and H) Representative images showing MIN. (K and L) Representative images of ductal epitheliums hyperplasia were presented. (M–P) Representative images indicating the presence of HAN. Magnifications, A, E, I, M = 7.5x; B, F, J, N = 20x; C, G, K, O = 100x; D, H, L, P = 200x.}
\end{figure}
since phosphorylated MAPK was expressed at a lower level in the Atm$^{+/-}$ mammary tumors than that in the mammary glands (Figure 4C). Although our data did not provide evidence for a maintenance role of Ras activation in DMBA-induced mammary tumor development.

It was shown previously that ionizing radiation induces mammary dysplasia in about 10% of the Atm$^{+/-}$ mice although the mechanism of dysplasia induction was not determined (35). To further characterize the molecular mechanism of mammary tumor development in an Atm$^{+/-}$ background, we generated mammary epithelial cell lines from Atm$^{+/-}$ (AHME1 and AHME2) and wild-type control (WTME1 and WTME2) litters. Both wild-type and Atm$^{+/-}$ cell lines display epithelial morphology and express pan-cytokeratin marker, confirming the nature of mammary epithelial cells (data not shown). To determine whether Atm-haploinsufficiency affects DNA damage signaling, we examined the induction of p53 in both wild-type and Atm$^{+/-}$ mammary cells in response to DNA damage. Interestingly, ~2-fold increased p53 phosphorylation at serine 18 (equivalent to human Ser15) was detected in AHME1 (2.07 ± 0.58, n = 3) and AHME2 (3.1-fold) Atm$^{+/-}$ mammary cell lines without DNA damage (Figure 5), suggesting that Atm-haploinsufficiency elevated endogenous DNA damage in unstressed cells. However, upon DNA damage, Ser18 phosphorylation was detected to a similar extent in both wild-type and Atm$^{+/-}$ mammary epithelial cells in a range of doses and time points examined (Figure 5). The expression of Atm was verified in these cells and it was confirmed that Atm$^{+/-}$ cells expressed half the amount of Atm protein found in wild-type cells (Figure 6A). Interestingly, a slightly decreased expression of Atm was observed in wild-type mammary epithelial cells in response to irradiation, while a slightly increased expression of Atm was seen in Atm$^{+/-}$ mammary epithelial cells after normalization to the expression levels of $\beta$-actin (Figure 6A).

One of the major features of A-T patients is increased sensitivity to radiation (3). At the cellular level, the increased radiation sensitivity can be determined by clonogenic survival assay. To determine whether Atm-haploinsufficiency could confer radiosensitivity in mammary epithelial cells, we performed clonogenic survival assay in response to various doses of ionizing radiation in both wild-type and Atm$^{+/-}$ mammary cell lines. Surprisingly, we found that both Atm$^{+/-}$ mammary epithelial cell lines (AHME1 and AHME2) were less sensitive than wild-type mammary epithelial cell lines (WTME1 and WTME2) to radiation in this clonogenic survival assay (Figure 6B). Hence, Atm-haploinsufficiency...
It was shown previously that 7271T heterozygotic or homozygotic state in the general population. An experiment was performed in triplicate and results were presented as mean + SEM.

**Discussion**

It was estimated that 1% of the general population are A-T carriers who are at an increased risk of breast cancer development (6). This hypothesis is supported by the observations that obligate A-T carriers are more likely to develop breast cancer at an early age (8,10–12). The majority of the mutations in A-T patients are truncating mutations, whereas the remaining are missense mutations (1,15). However, the relative contribution of the truncating and missense mutations of ATM to breast cancer development in A-T family is not clear. It is inferred that blood relatives of A-T patients where full-length ATM protein can be seen confers enhanced survival in response to radiation in mammary epithelial cells.

Fig. 6. Atm heterozygotes displayed enhanced survival to radiation. (A) Atm expression in wild-type (WTME2) or Atm<sup>−/−</sup> (AHME1) mammary epithelial cell line in response to different doses of irradiation. The expression of Atm was determined 1 h post-radiation. The relative expression levels were quantitated using ImagePro 5.0 software after normalization against β-actin, and fold differences compared with the level of Atm in non-irradiated wild-type cells were shown at bottom of the gel. (B) Mammary epithelial cell lines (WTME1, WTME2, AHME1 and AHME2) were irradiated at given doses and survival clones were scored after 2 weeks in culture. Survival fractions were expressed as percentage of irradiated survival clones normalized to non-irradiated survival clones. The experiment was performed in triplicate and results were presented as mean ± SEM.

A mouse model with missense mutation (ATM-ΔSRI) in which three amino acids of ATM at positions 2556–2558 have been deleted has been developed recently (42). This model is representative of missense mutations observed in some A-T patients where full-length ATM protein can be detected. However, ATM protein kinase activity is lost in this mutant protein (42). Interestingly, ~9% of the heterozygotes of ATM-ΔSRI mice developed tumors, a 3-fold increase over that of the wild-type mice (43). However, only 2% of the ATM-ΔSRI heterozygotes developed mammary tumors (43), suggesting that additional factors are required for mammary tumor development in this model. It will be interesting to determine whether ATM-ΔSRI mice are also sensitive to chemical carcinogen-induced mammary tumorigenesis.

In Atm<sup>−/−</sup> mice treated with ethynitrosourea (ENU), a direct alkylating agent that is thought to produce DNA lesions without DNA double-strand breaks, no increased mammary tumors were observed within 100 days of treatment (44). The inability to detect increased tumors induced by ENU in Atm<sup>−/−</sup> mice may be due to a short incubation time or that Atm<sup>−/−</sup> mammary epithelial cells are not sensitive to ENU treatment. Umesaka et al. (45) have recently reported that Atm-haploinsufficiency promotes mammary tumor development in a p53 heterozygotic background and that the Atm-haploinsufficiency effect was further enhanced by ionizing radiation. However, it is not clear whether mammary dysplasia occurred in this cohort. The importance of ATM in suppressing breast cancer development is also supported by a recent study demonstrating that ATM is epigenetically silenced in advanced breast cancer (4).

In this study, we found that truncating Atm<sup>−/−</sup> mice had a 2-fold increased mammary tumor incidence in the DMBA-induced mammary tumorigenesis model. In addition, we found that there was about a 3-fold increase in mammary dysplasia induced by DMBA treatment in Atm<sup>−/−</sup> mice.
compared with wild-type mice. Importantly, there was more severe mammary dysplasia in Atm heterozygotes than in wild-type mice (see Figure 2E–L), which is consistent with the notion that DMBA may interfere with the hormone response pathway (46,47). Mammary dysplasia appears to be specifically associated in mice treated with either DMBA (this study) or ionizing radiation (35) since untreated Atm+/− mice do not develop this phenotype (data not shown, also see ref. 35). The severe mammary dysplasia observed in Atm heterozygotes implies that Atm+/− mammary epithelial cells are more sensitive to hormone-induced proliferation than wild-type cells. Therefore, increased tumor incidence and dysplasia in Atm heterozygotes may be partly explained by the combined actions of increased mutation accumulations (deficient DNA repair system) and increased sensitivity to hormone-induced proliferation of mammary epithelial cells. DMBA causes bulky DNA–protein adducts formation, which could be repaired by base/nucleotide excision repair. Activation of Ras pathway has been implicated in DMBA-induced carcinogenesis in general (32–34,48,49). The possibility of DMBA-induced Ras mutation in the initiation of mammary tumorigenesis was evaluated in this study. Our results suggest that Ras mutation at codon 61 was not involved in DMBA-induced mammary tumors irrespective of the status of Atm. However, it is likely that DMBA may also induce other Ras mutations. If true, then Ras mutations would activate downstream signaling, and such events should be detected. Surprisingly, examination of Raf and MAPK levels did not provide such evidence. Therefore, our results suggest that DMBA induces distinct signaling pathways in tumor progression dependent on cell types. However, we could not exclude the possibility that activation of Ras signaling pathway is involved in the initiation of mammary carcinogenesis but is not required for the maintenance once mammary tumor mass formed. Alternatively, DMBA-induced DNA-adducts may interfere with replication fork movement and therefore may cause replication fork collapse. Restart of the replication fork is thought to invoke a DNA recombination step induced by double-strand breaks. It is possible that in Atm heterozygotes the restart of the replication fork progression is defective, thereby leading to the accumulation of mutations and chromosome instability. Consistent with this interpretation, DMBA and other bulky-adduct-forming agents, such as PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine), have been shown to induce aneuploidy in Chinese hamster cells and colon cancer cells, respectively (50,51).

Mammary epithelial cells from Atm heterozygotes might be deficient in the correction of environmental mutagenic insults and hence predisposed to breast cancer. Consistent with this hypothesis, we found that Atm+/− mammary epithelial cells exhibit elevated basal levels of p53 phosphorylation compared with that in wild-type cells. Constitutive induction of p53 phosphorylation in Atm+/− mammary cells indicates that these cells are constantly under low levels of stresses compared with wild-type cells. Therefore, Atm-haploinsufficiency confers elevated cellular stresses in mammary epithelial cells.

Atm-deficient cells exhibit increased radiation sensitivity. The cause of radiation sensitivity in Atm-deficient cells is not well understood although both cell cycle checkpoints and DNA repair defects could contribute to such effect (26,52). It was shown previously that ATM heterozygotic lymphocytes showed intermediate radiation sensitivity using a short-term survival assay (43,53), whereas radiation sensitivity was not observed in mouse Atm+/− ES cells using a clonogenic survival assay (18). In this study, we were therefore surprised to found that Atm-haploinsufficiency confers slightly enhanced survival in mammary epithelial cells in response to DNA damage. The variations in radiation sensitivity in these different cell types are not clear at present but could be dependent on cell context and/or due to the history of cellular stresses. Atm-haploinsufficiency may predispose mammary epithelial cells to genome instability and therefore leads to enhanced tumorigenesis in response to chemical carcinogens. In summary, our results suggest that gene–environmental interactions may dictate the breast cancer development in ATM heterozygotes.

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References


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