Atm Heterozygosity Cooperates with Loss of Brca1 to Increase the Severity of Mammary Gland Cancer and Reduce Ductal Branching

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Abstract

The role of homozygous ataxia telangiectasia mutated (ATM) mutations in familial and sporadic forms of cancer is well established, but the contribution of ATM heterozygosity to mammary gland and other cancers has been controversial. To test the effect of Atm heterozygosity on mammary gland cancer, mice with complete loss of exon 11 of Brca1 specifically in mammary epithelium (Brca1-MG-Δex11) were studied in either Atm heterozygous or Atm wild-type backgrounds. Targeted deletion of Brca1 in mammary epithelium resulted in carcinomas and adenocarcinomas of varying histology with long (>9 months) latency. Latency to tumorigenesis was found to be unchanged in the Brca1-MG-Δex11;Atm heterozygous mice compared with Brca1-MG-Δex11;Atm wild-type mice. However, the mice displayed variable tumor severity and differences in mammary tissue development. Mammary tumors from Brca1-MG-Δex11;Atm heterozygous mice were anaplastic and undifferentiated in all 20 tumors tested, whereas tumors from mice that were Brca1-MG-Δex11 but wild-type for Atm displayed variable histologic profiles, with some anaplastic tumors and other differentiated and less invasive tumor types. Previously reported developmental defects for Brca1-deficient mice were also observed in our model with and without Atm heterozygosity, but Brca1-MG-Δex11;Atm heterozygous mice displayed decreased ductal branching during puberty, a phenotype that was not observed in Brca1-MG-Δex11;Atm wild-type mice. Our results provide evidence that Atm heterozygosity influences severity of mammary gland tumors in the Brca1-MG-Δex11 tumor-prone mouse and suggest that this mutation leads to a newly characterized developmental defect during glandular maturation. (Cancer Res 2005; 65(19): 8736-46)

Introduction

Breast cancer is the most common nondermatologic cancer diagnosed in humans and the second leading cause of cancer death among women. Tumorigenesis can result from the dysfunction of any of several important oncogenes or tumor suppressors. For example, the tumor suppressor BRCA1 was one of the first genes to be associated with familial breast cancer susceptibility in humans. Carriers of mutations in BRCA1 display a much higher (50-85%) lifetime risk of developing breast cancer than noncarriers (1). The pathologic phenotype of tumors associated with BRCA1-deficient breast cancer varies widely from nonmalignant adenomas to malignant anaplastic carcinomas that are undifferentiated, aggressive, and highly proliferative.

Mutations in other tumor suppressor genes lead to an increased susceptibility to breast cancer as well. One such gene is ataxia telangiectasia mutated (ATM). Homozygous mutation of the ATM gene causes the human disease ataxia telangiectasia, a disease characterized by progressive cerebellar ataxia, ocultaneous telangiectasias, immunodeficiency, retarded somatic growth, gonadal dysgenesis, radiosensitivity, and lymphoreticular malignancies (2, 3). Many of these phenotypes are a result of the inability to properly control the cell cycle or respond to DNA damage in the absence of ATM. ATM is a critical kinase with many target proteins that are responsible for the maintenance of cell cycle, including known tumor suppressors, such as p53. ATM has also been shown to directly phosphorylate BRCA1 in activation of the cell cycle checkpoints at G2-M and S-G2 (4, 5). The loss of BRCA1 results in cell cycle checkpoint dysfunction, improper DNA damage repair, and genomic instability in the form of translocations, deletions, and duplications (6-8).

Heterozygous carriers of mutations in ATM display no overt phenotypes associated with clinical, epidemiologic, and molecular studies of obligate ataxia telangiectasia homozygotes, but some show an increase in susceptibility to cancers, including an increased risk of breast cancer (9-18). This relative risk varies widely among studies from 1.3- to 12.7-fold increase in susceptibility compared with control groups. However, no increased risk levels in the general population have been reported, perhaps because the exact mutations of random carriers are not all known. Most of the studies done have used assays for truncation mutations, and these studies have generally revealed no increase in the susceptibility of female ATM carriers relative to noncarriers in populations of women with breast cancer (14, 19-22). By contrast, other studies suggest that there may be a higher prevalence of certain types of ATM mutations in breast cancer arising in familial breast cancers but not in nonfamilial sporadic mammary gland cancers (23). For example, a recent study analyzing several different ATM mutations, including five that caused a protein truncation and two causing missense mutations, suggested that ATM heterozygosity may cause an 8.5% increased...
risk in families with hereditary breast and ovarian cancers (24). These same mutations were analyzed in the general population and no increased risk for breast cancer was found, suggesting that the effects may be limited to familial cases (25). It has also been shown that a low concentration of ATM transcripts is highly correlated with breast carcinomas, moderate expression with benign lesions, and expression at highest levels in normal mammary tissue (26, 27).

Finally, a mouse model of an Atm heterozygous missense mutant (ΔSR1) that recapitulates the increased susceptibility to cancers, including mammary tumors, has also been generated (28). The data all suggest that ATM loss may play some role in the initiation, progression, and/or maintenance of breast tumors when a familial breast cancer gene, such as BRCA1, is also absent, although this has not been specifically addressed in human studies.

The mouse provides an alternative way to address the relationship of Atm and Brca1 in mammary gland cancer. Both genes have been knocked out in mice and allow the controlled manipulation of these genes to determine if there is a synergistic effect when both are disrupted. Brca1-deficient mice display an early embryonic lethal phenotype (29–35). Because of this, conditional knockout mice have been generated that spatial and temporal deletion of exon 11 of Brca1 (35). When Cre-recombinase is expressed from either the mouse mammary tumor virus long terminal repeat promoter (MMTV-LTR) or the whey acidic protein promoter, Brca1 was deleted specifically in mammary epithelium (35). These mice displayed variable types of mammary carcinomas, commonly ductal and lobular carcinomas as well as more rare cases of squamous carcinomas, medullary carcinomas, and anaplastic carcinomas, all of which are found in human familial breast cancers (36, 37). Mouse models of ataxia telangiectasia have also been generated and they recapitulate the lymphoma tumor phenotype seen in humans with the disease (38, 39). Atm-deficient mice develop lymphomas between 2 and 4 months of age (38, 39). These tumors display the characteristic genomic aberrations and cell cycle defects that are found in the human disease and these defects are very similar to those also seen in Brca1-deficient mammary gland tumors (34, 40, 41). Cancers from both Atm and Brca1 knockouts display several obvious similarities, including diminished DNA damage response, cell cycle defects, and extensive genomic rearrangements.

These studies together suggest that there may be a link between Atm haploinsufficiency and loss of Brca1 that can be effectively investigated using the mouse as a model. We hypothesized that Atm heterozygosity might exacerbate the cancer phenotype associated with Brca1 loss in mammary. Therefore, we produced mice with complete loss of exon 11 of Brca1 in mammary epithelium that were either wild-type or heterozygous for an Atm-null allele. Latency to tumorigenesis and tumor types as well as mammary gland development were studied. We found that Atm haploinsufficiency resulted in absence of differentiated tumor types and increased invasiveness as well as reduced ductal branching in virgin mice, supporting a role for Atm in mammary gland tumorigenesis and development.

Materials and Methods

Mice, genotyping, and tumor latency. Brca1 conditional knockout mice (35), with the MMTV-Cre A1 line (35, 42, 43), were mated with Atm heterozygous mice (38) and back-crossed to obtain Atm+/−; Brca1-MG-Δex11, Atm+/−; Brca1-MG-Δex11, and Atm+/−; Brca1-Δex11 mice as a control for loss of Brca1 in the mammary gland. The Atm, Brca1, and MMTV-Cre status of the mice, mammary tumors, and cell lines were determined by PCR using Atm, Brca1, and MMTV-Cre probes as described previously (35, 38, 40, 41). Animals in all groups were checked weekly for tumors. At the onset of tumors, the mice were noted and tumors were harvested at no more than 1.5 cm in diameter. Complete autopsies were done on all mice that developed tumors. Survival time of mice was used to generate Kaplan-Meier survival curves that were compared using log-rank (Mantel-Cox) test.

Western blot and quantitative real-time PCR analysis. Frozen tumor tissue was homogenized and lysed in Triple Lysis Buffer (50 mmol/L Tris, 1 mmol/L EDTA, 0.5% Tween) with protease inhibitors added. A Bradford assay was used to quantify protein. For Western blot analysis, protein (100 μg) was subjected to PAGE in a 3% to 8% Tris-acetate gel and transferred to Immobilon membranes. Western analysis was done using standard procedures. Antibodies to c-Myc, estrogen receptor (ER)-α, erbB2, cyclin D1, cyclin A, cdc2, and p27 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and BRCA1 was purchased from Oncogene Research Products (La Jolla, CA).

RNA was prepared from 16 Atm+/−; Brca1-MG-Δex11 and 16 Atm+/−; Brca1-Δex11 tumors and 2 Atm+/− mouse embryonic fibroblast cell lines. Superscript (Invitrogen, Carlsbad, CA) enzyme was used for reverse transcription and random hexamers were used as primers. Quantitative real-time PCR assays were done at the Center for AIDS Research Genomics Core (Veteran’s Medical Research Foundation, La Jolla, CA). Forward and reverse primers were created for Atm quantitative PCR (forward primer CCAGCTTTGGATCGATACCA and reverse primer CCCAGCCTACGTCATTGTTTCT). Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used for normalization (GAPDH-forward CCACGCCCTATGGACATACCA and reverse primer CCACCTGATGCATTATCC). Amplification and detection of cDNA in quantitative real-time PCR.

Immunohistochemistry, histology, and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays. Samples were fixed in 4% paraformaldehyde and stored at 4°C. Sections (5 μm) were cut for tumor and mammary tissue and mounted on slides. Tumor and mammary sections, mammary tissue whole mounts, and H&E staining were done by University of California-San Diego (UCSD) Cancer Center Histology and Immunohistochemistry Shared Resource (La Jolla, CA) using standard procedures. For immunohistochemistry, samples were also prepared using standard protocol. Antibodies against smooth muscle actin, cytokeratin 5, and vimentin were used for tumor typing (Santa Cruz Biotechnology). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was carried out on 5-μm mammary gland cross-sections using standard protocol.

Spectral karyotyping and comparative genome hybridization. Tumor metaphases were fixed from passage 0 or 1 tumor cells cultured in DMEM/F-12 supplemented with 5% fetal bovine serum, epidermal growth factor, and insulin. Metaphases were generated from four cell lines representing two Atm+/−; Brca1-MG-Δex11 and two Atm+/−; Brca1-MG-Δex11 anaplastic tumors. Spectral karyotyping and comparative genome hybridization (CGH) were done as described previously (41). Clones were labeled by nick translation and hybridized to tumor metaphase chromosomes according to standard procedures. Images were acquired using a DMRXA microscope (Leica, Wetzlar, Germany) connected to a Sensys charge-coupled device camera (Roper Scientific, Tucson, AZ) using Q-FISH software (Leica Microsystems, Cambridge, United Kingdom).

Expression analysis. Total RNA was isolated from six anaplastic carcinomas using Trizol reagent and standard protocol (three Atm+/−; Brca1-MG-Δex11 tumors and three Atm+/−; Brca1-Δex11 tumors). Total RNA was also isolated from two less aggressive tumors (one ductal carcinoma and one papillary carcinoma) from two mice from the Atm+/−; Brca1-Δex11; MMTV-Cre−/− cohort. These were the only tumors that developed from this cohort. The RNA was checked for integrity and hybridized to Affymetrix U74Av2 probe arrays according to the manufacturer’s protocol by the UCSD Cancer Center Microarray Shared Resource (VA Gene Chip Core).

Analysis was done using both dChip and Robust Multichip Average (RMA) analyses for hierarchical clustering and scatter plot analysis using
the Biocductor: open source software for bioinformatics.® RMA pre-
processing consisted of three steps: background adjustment, normalization, and summarization. The RMA background noise adjustment corrected for processing effects and cross-hybridization. We implemented normalization to reduce the nonbiological variation across the chips in an experiment, including technical variability, which is related to the experimental procedure of the assay. RMA used the quantile normalization technique to adjust the gene expression intensities such that the distributions of the probe intensities are the same across all chips. The last step, summarization, calculated a single gene expression value by mathematically combining the probe intensities from each probe set. RMA employs the robust multichip linear model fitted on a log scale. Note that the RMA-
transformed gene expression intensity calculations were based only on perfect match values from each probe set, because the inclusion of the mismatch probes is believed to contaminate true expression. Aside from measuring nonspecific hybridization, mismatch probes also measure true expression, so valuable signal may be lost when incorporating mismatch probes in expression calculations. By contrast, dChip relied on "invariant set nonlinear normalization" to reduce the variability for low expression estimates and account for both nonlinear and nonbiological effects. We again analyzed perfect match probes only without correction for mismatch hybridization. Note that background adjustment was not conducted here.

The second stage of analysis employed unsupervised clustering to determine if the two classes of tumors could be recovered from the data. Clustering was separately done after preprocessing by RMA and dChip. Dendrograms of expression patterns and samples were computed via hierarchical clustering using average linkage as the between-cluster distance criteria and Euclidean distance as the similarity measure.

Results

Generation of Atm heterozygous, Brca1 conditional knock-
out mice. To analyze the possible effects that Atm heterozygosity may have during tumorigenesis in mammary tissue that lacks intact Brca1, mice carrying mutations for either Brca1 or Atm were mated to generate a compound genotype. Mice bearing two conditional Brca1 alleles (called Brca1Co/C0) as well as a Cre-recombinase transgene under control of the promoter of the MMTV-LTR gene (MMTV-LTR A1) as described previously were used in the study (called MMTV-Cre-; refs. 42, 43). MMTV-Cre deletes exon 11 of the Brca1 gene in mammary epithelium causing a Brca1 mutation (35, 40, 41). These mice (called Brca1-MG-Δex11) were crossed with mice heterozygous for mutant Atm (called Atm+/−; ref. 38). After crossing mice to generate either Atm+/−:Brca1-MG-Δex11 or Atm+/−:Brca1Co/C0:MMTV-Cre− genotypes, all were in a mixed background. Littermate controls were generated for the study having one of three genotypes. Atm+/−:Brca1-MG-Δex11 and Atm+/−:Brca1-MG-Δex11 mice were the experimental groups and would express Brca1 exon 11 mutant alleles in the mammary epithelium. Atm+/−:Brca1Co/C0:MMTV-Cre− mice were used as a control group.

Atm haploinsufficiency does not alter latency to tumori-
genesis in Brca1 mutant mice. Females of all three groups were continuously mated to maintain high levels of MMTV-Cre and monitored for mammary gland tumor formation. No mammary tumors were detected between 0 and 9 months of age in any cohort. However, between 9 and 26 months, 43% of Atm+/−:Brca1-MG-Δex11 (20 of 46) and 56% of Atm+/−:Brca1-MG-Δex11 (28 of 50) mice developed mammary tumors. This was slightly higher than previous reports of 25% to 30% penetrance (35, 40). Kaplan-Meier analysis of survival indicated a significant difference in overall survival between the control Atm+/−:Brca1Co/C0:MMTV-Cre− group and the two Brca1-MG-Δex11 groups (Fig. 1A). The mice that did not express the MMTV-Cre transgene retained both alleles of Brca1 and would display normal Brca1 expression. However, there was no difference in the latency to tumorigenesis between Atm+/−:Brca1-MG-Δex11 and Atm+/−:Brca1-MG-Δex11 mice (P > 0.56; Fig. 1B). Two control mice that were Atm+/−:Brca1Co/C0:MMTV-Cre− (2 of 40) developed mammary tumors at 18 months of age.

To determine whether there was loss of heterozygosity (LOH) of Atm in Atm+/− tumors, both standard PCR (done with DNA from both tails and tumors of corresponding animals) and quantitative real-time PCR (done with total RNA isolated from frozen tumor tissue) were done. All Atm+/− tumors retained both Atm mutant and wild-type alleles, whereas all Atm wild-type tumors retained wild-type alleles (Fig. 1C and D). All tumors were analyzed for loss of BRCA1 protein via Western blot, which revealed normal levels of expression in Atm+/− and Atm+/−/Brca1-MG-Δex11 or Atm+/−: Brca1-MG-Δex11 mice. Quantitative real-time PCR indicated that the amount of Atm RNA from Atm+/−:Brca1-MG-Δex11 tumors was approximately half the level of RNA from Atm+/−:Brca1-MG-Δex11 animals (Fig. 1F). These findings show that the Atm alleles remain intact in tumors from the Atm+/−:Brca1-MG-Δex11 mice and do not undergo LOH and that any phenotypic differences may be the result of Atm haploinsufficiency.

Atm haploinsufficiency alters tumor severity in Brca1 mutant mammary tissue. Histologic analysis revealed that the tumors from Atm+/−:Brca1-MG-Δex11 mice were variable in type as reported previously for mouse mammary tumors of Brca1-deficient tissues (40). These tumors varied in type from well differentiated to undifferentiated (Fig. 2A-E) and included adenocarcinomas with some squamous carcinomas, fibroadenomas, fibrillary carcinomas, ductal carcinomas, papillary carcinomas, and spindle cell and anaplastic carcinomas. These tumor histologic types observed are representative of the human breast cancers seen in familial cancer and cancers associated with BRCA1 mutations (36, 37). Interestingly, when the heterozygous Atm+/−:Brca1-MG-Δex11 tumors were analyzed histologically, we found that all 20 tumors were anaplastic, invasive carcinomas, with a completely undifferentiated appearance (Fig. 2F), whereas none showed the less invasive, encapsulated, ductal, or squamous features that are associated with more differentiated tumor types, such as many of those seen in the Atm+/−:Brca1-MG-Δex11 (Fig. 2A-D). The anaplastic tumors of both genotypes were consistent in appearance and rate of growth.

Tumor cell lines were established from several anaplastic tumors of both Atm+/− and Atm+/− genotypes and analyzed with immuno-

histochemical markers for cell lineage, proliferation, and common mammary gland tumor markers. We compared the expression levels of some typical markers for Brca1-deficient tumors from mice expressing the MMTV-polyomavirus middle T antigen used previously as a positive control for some typically expressed histochemical markers for cell lineage, proliferation, and common mammary gland tumor markers. We compared the expression levels of some typical markers for Brca1-deficient tumors from mice expressing the MMTV-polyomavirus middle T antigen used previously as a positive control for some typically expressed markers in the Brca1-deficient tumors (44). Both Atm+/−:Brca1-MG-Δex11 and Atm+/−:Brca1-MG-Δex11 tumor cells expressed the markers cytokeratin 5, cytokeratin 8, and vimentin, indicating that they were of ductal origin as expected (data not shown).

It has been shown that the overexpression of cell cycle and oncogenic proteins, such as erbB2, c-Myc, and cyclin D1, occur in tumors from mice with loss of exon 11 of Brca1 (41, 45, 46). To determine if the Atm+/−:Brca1-MG-Δex11 and Atm+/−:Brca1-MG-Δex11 mice were similar or different in the regulation of these
proteins, we did Western blot analysis on several tumors (15 Atm\textsuperscript{+/−};Brca1-MG-Δex11 and 15 Atm\textsuperscript{+/+};Brca1-MG-Δex11 mice). We found that cyclin D1, cdc2, c-Myc, and erbB2 were all overexpressed in Brca1 exon 11–deficient mammary tumors of either Atm genotype compared with normal mammary tissues (Fig. 3). Previous studies suggest that cyclin A was not up-regulated in asynchronous tumor cells grown in culture (40, 45). No tumors displayed expression of cyclin A, whereas normal mammary tissues did, supporting previous finding (40). Protein levels of p27 were slightly up-regulated in these tumors compared with control samples, supporting aberrant cell cycle regulation. No obvious differences were observed between differentiated and undifferentiated tumors from Atm\textsuperscript{+/−} or undifferentiated tumors from Atm\textsuperscript{+/+} mice. This suggests that Atm heterozygosity does not affect protein marker expression levels seen in other Brca1-deficient mammary tumor models. Tumors were evaluated for ER expression because it was found that tumors resulting from Brca1 deficiency fall into a basal subtype and are typically ER negative (47). We found that all Atm\textsuperscript{+/−};Brca1-MG-Δex11 mice and 13 of 15 Atm\textsuperscript{+/+};Brca1-MG-Δex11 mice were ER negative (Fig. 3).

Similar genomic abnormalities between Atm\textsuperscript{+/−} and Atm wild-type mammary tumors in a Brca1 exon 11–deficient background. Previous studies showed that loss of Brca1 exon 11 in the mammary gland resulted in tumors with genomic instability, including aneuploidy, chromosomal translocations, and loss and gain of chromosomal regions (40, 41). To address whether genomic differences were occurring between the aggressive tumors of each genotype, spectral karyotyping and CGH were used to analyze anaplastic carcinomas of both Atm\textsuperscript{+/−};Brca1-MG-Δex11 and Atm\textsuperscript{+/+};Brca1-MG-Δex11 genotypes. Primary cell lines were established from four anaplastic tumors, two from each genotype. Attempts to culture cell lines from the less aggressive tumors were not successful. Spectral karyotyping analysis was done on four different cell lines that were derived from primary tumors at passage 0 or 1. All four tumors revealed numerous chromosomal aberrations, including complex rearrangements.
duplications, and deletions (Fig. 4A). Complex rearrangements were observed involving chromosomes 1, 10, and 17. Duplications, deletions, and translocations were commonly observed on most chromosomes. However, quantification of the number of aberrations per chromosome revealed no difference between Atm<sup>+/−</sup>;Brca1-MG-Δex11 and Atm<sup>+/−</sup>;Brca1-MG-Δex11 genotypes (Fig. 4B). CGH was used to analyze the loss or gain of chromosomal regions in these four tumor lines and revealed gains and losses representing regions on nearly all chromosomes (Fig. 4C). Amplification of the distal end of chromosome 11, and the amplification of the proximal end of chromosome 15, including band 15D, which contains the c-Myc oncogene, was observed. Only two major distinctions were noted between the two genotypes of tumors via CGH. The distal region of chromosome 4 showed loss in both Atm<sup>+/−</sup>;Brca1-MG-Δex11 tumors, whereas there was no loss in the Atm<sup>+/−</sup>;Brca1-MG-Δex11 tumors, indicating the loss of the INK4A/ARF locus (Fig. 4C). In addition, there was a gain of the proximal portion of the X chromosome in all four tumors as well as the distal end of the X chromosome for the Atm wild-type tumors, whereas the Atm<sup>+/−</sup> tumors showed no amplification or deletion of the distal region (Fig. 4C).

**Expression analysis reveals tumor similarity between genotypes.** To determine if there were expression differences based on genotype or tumor type, microarray analysis was done on six anaplastic carcinomas (three tumors from each genotype) and two more highly differentiated tumors from Atm<sup>+/−</sup>;Brca1-MG-Δex11 mice. RNA was isolated from frozen tissue and hybridized to Affymetrix oligonucleotide arrays containing ~12,000 genes. The resulting expression profiles for each tumor were normalized and compared with both dChip and RMA analyses. Hierarchical clustering resulted in the two noninvasive carcinomas being grouped separately, whereas all of the anaplastic carcinomas clustered more closely. The separate clustering of the noninvasive and invasive carcinomas was expected based on their differences in histologic degree of differentiation. The anaplastic carcinomas could not be differentiated based on Atm genotype, though, suggesting that these tumors were very similar in expression and that genotype differences have no obvious effect on global gene expression (Fig. 5). Of the 12,000 genes that were analyzed in this

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**Figure 2.** Histologic analysis of tumors from both Atm<sup>+/−</sup>;Brca1-MG-Δex11 and Atm<sup>+/−</sup>;Brca1-MG-Δex11 tumors reveals differences. Atm<sup>+/−</sup>;Brca1-MG-Δex11 tumors (A-E) were variable in type and level of differentiation. Examples of types of tumors occurring were secretory carcinomas (A), squamous carcinomas (B), papillary carcinomas (C), ductal carcinomas (D), and anaplastic carcinomas (E), all of varying degrees of differentiation. Atm<sup>+/−</sup>;Brca1-MG-Δex11 tumors were all undifferentiated, invasive, anaplastic carcinomas (F). The anaplastic carcinomas from the two different genotypes were indistinguishable histologically (E and F).
expression panel, only 16 were consistently expressed at a significantly different level in the Atm<sup>+/−</sup>:Brca1-MG-Δex11 anaplastic carcinomas compared with the Atm<sup>+/−</sup>:Brca1-MG-Δex11 carcinomas with the RMA analysis method. Thirteen genes were differentially expressed using the dChip method for the same group of genes. Of these genes, only 5 were considered significant by both methods of analysis. These genes include two Riken clones, DEAD box polypeptide 6, T-box 15, and protein kinase, lysine deficient 1 (Supplementary Table S1). The low number of genes identified by both methods again suggests that the anaplastic tumors were very similar independent of Atm genotype.

**Atm haploinsufficiency leads to decreased ductal branching during early mammary development.** Brca1 conditional knockout mice display defects in mammary gland development (35), including incomplete penetrance of the mammary fat pad and increased levels of apoptosis. These observations as well as the more anaplastic tumor phenotype in Atm<sup>+/−</sup>:Brca1-MG-Δex11 mice lead us to analyze mammary development to ascertain if there are any differences between genotypes. Glandular development was analyzed using whole mounts of mammary glands from mice of each genotype at several stages of development, 7-week-old virgins, pregnancy days 8.5 and 16.5, lactation days 1 and 10, and involution. We observed the expected developmental defects in Brca1 conditional knockout mice during pregnancy and lactation, such as the incomplete penetrance of the mammary fat pad and increased levels of apoptosis (data not shown). However, when analyzing 7-week-old virgin glands for ductal branching, we found that Atm<sup>+/−</sup>:Brca1-MG-Δex11 glands (Fig. 6C) displayed significantly fewer bifurcations than either control (Fig. 6A) or Atm<sup>+/+</sup>:Brca1-MG-Δex11 glands (P = 0.0004; Fig. 6B, quantitated in Fig. 6D). Glands analyzed at early pregnancy showed no differences, whereas those analyzed at late pregnancy displayed slightly decreased density in glandular structure but were not significantly different (P = 0.158; data not shown). There were no differences between the two experimental groups during lactation or involution stages. Differences in infiltration into the adipose tissue were observed between the experimental groups and the wild-type Brca1 control groups as reported previously (35). These defects include failure to completely infiltrate the fat tissue during pregnancy and some underdeveloped areas during lactation (data not shown).

Matched cross-sections from both 7-week-old virgin and midpregnancy mammary glands were analyzed for apoptosis and proliferation, TUNEL assays were done on sections from each of the two experimental groups and a control group to analyze the level of apoptosis in virgin female mammary glands. Similar numbers of apoptotic cells were found in all three groups (n = 10; P = 0.463; data not shown). The proliferation marker Ki-67 was done on serial sections matching those used in the TUNEL assay to determine the level of proliferation, and similar levels of proliferation were found in both control and experimental groups (n = 10; P = 0.671; data not shown). Five-week-old virgin mammary glands from Atm<sup>+/−</sup>:Brca1-MG-Δex11 mice were also sectioned and compared with age-matched wild-type glands to analyze terminal end bud (TEB) morphology and marker expression. Atm<sup>+/−</sup>:Brca1-MG-Δex11 TEBs appeared normal in morphology with no signs of defective organization in the cap cell layer or prelumenal cells (Fig. 6E-G) compared with wild-type controls (Fig. 6H-J). Expression of E-cadherin, which adheres prelumenal and luminal epithelial cells, appears normal as does the expression of P-cadherin, which binds myoepithelial layers (data not shown).

**Discussion**

We have analyzed the role that Atm heterozygosity plays in tumorigenesis and development in Brca1-null murine mammary tissue. Previous human studies revealed contradictory evidence for the role that Atm heterozygosity may play in tumorigenesis. Early studies based on obligate carrier status in families with affected ataxia telangiectasia individuals suggested that the loss of one allele of Atm might result in an increased susceptibility to cancer (10, 12, 48–50). Later studies using molecular genotyping methods revealed no association of ATM mutations and early onset of mammary gland cancer (14, 19, 21, 22). However, recent evidence analyzing seven different mutations suggests that ATM mutations in individuals with hereditary mammary gland and ovarian cancers may increase the risk of breast cancer (24). There is, however, no evidence reported previously that suggests that a null mutation in Atm may confer any developmental or tumorigenic phenotype.

Our study strongly suggests that the heterozygous loss of Atm in the context of Brca1-deficient mammary tissue affects the tumor type and invasiveness. In 20 Atm<sup>+/−</sup>:Brca1-MG-Δex11 mice that developed tumors, all developed invasive, anaplastic carcinomas that displayed atypical mammary architecture, invaded blood vessels, and completely displaced the mammary fat pad after infiltration. In the 24 Atm<sup>+/−</sup>:Brca1-MG-Δex11 mice, 13 resulting tumors were of the same anaplastic, invasive type as the Atm<sup>+/−</sup>:Brca1-MG-Δex11 mice. The other 11 tumors, however, were variable in histologic appearance a phenotype reported previously for mammary tumors in Brca1-null mice (40). None of the nonanaplastic tumors were found to invade the bloodstream. Although several of the Atm<sup>+/−</sup>:Brca1-MG-Δex11 tumors were found to penetrate blood vessels, no metastases were discovered. This may be because animals were sacrificed at a tumor burden of ~1 cm, which was reached only a few days after initial detection by palpation. This may not allow sufficient time for a distant tumor to develop to the point of detectability. Of interest, in the control
group (Atm\textsuperscript{+/−}:Brca1\textsuperscript{+/−};MTV-Cre−), two tumors formed, both of these tumors were adenocarcinomas, suggesting that our mixed strain background may be predisposed to cancer due to Atm haploinsufficiency. The low number of tumors and the design of our study do not allow us to make a definitive conclusion.

Of all 49, none were found to have metastasized to secondary tissues. This was surprising considering that cells from anaplastic tumors often invaded blood vessels. A previous study reported metastasis in only 3 of 56 Brca1\textsuperscript{+/−};MTV-Cre+p53\textsuperscript{−/−} mice, suggesting low probability of metastasis due to Brca1 mutations in the mouse model (40). It is possible that cells were able to establish in secondary tissues but not able to proliferate in those microenvironments. This phenomenon has been observed in other studies using human metastatic mammary gland cancer cells that were inoculated into mouse mammary glands. The cells were found in secondary tissues and able to grow again in culture but not able to proliferate in the secondary tissues (51). Finally, as noted above, mice were sacrificed when tumor burden reached 1 to 2 cm in diameter, so it is also possible that the mice were sacrificed before there was sufficient time for metastasis to occur.

We analyzed chromosome rearrangements, amplifications, and deletions in the Atm\textsuperscript{+/−}:Brca1-MG-\textsuperscript{Δ}\textsubscript{x11} tumors to determine if haploinsufficiency of Atm exacerbated the genomic instability phenotype seen in Brca1 tumors. Spectral karyotyping and CGH analysis revealed that there were high levels of genomic aberrations in both Atm\textsuperscript{+/−}:Brca1-MG-\textsuperscript{Δ}\textsubscript{x11} and Atm\textsuperscript{+/−}:Brca1-MG-\textsuperscript{Δ}\textsubscript{x11} tumors (Fig. 4). There were no significant differences in the average number of chromosome breaks per chromosome between the two genotypes of tumor (Fig. 4B). Global analysis of copy number changes suggests gross dysregulation of chromosomal stability (Fig. 4C). The degree of chromosomal instability appears to be similar regardless of genotype, aside from the two regions of chromosome 4 and the X chromosome. It is possible that any differences in chromosomal integrity resulting from Atm heterozygosity may be masked by the high levels of genomic instability that occur in Brca1 mutant cells. This may make it impossible to detect the subtle differences between the tumor types. The data obtained correlate with findings reported previously in Brca1-MG-\textsuperscript{Δ}\textsubscript{x11} mice (41) with few differences. In the four tumors analyzed in the current study, we could not detect loss of the proximal region of chromosome 11 where TRP53 maps, in addition to the deletion of distal chromosome 14 containing the Rb1 locus. Intriguingly, the amplification on chromosome 11 maps to bands E1 to E2 also in the current study, suggesting that the amplicon might be the result of a high copy number amplification of a novel oncogene (Spet9) that we found as being amplified in a variety of mouse models for breast cancer, including BRCA1-associated tumors (52).

Microarray analysis showed that, as expected, noninvasive and invasive tumors displayed different patterns of gene expression, reflecting their obvious histologic differences and state of differentiation. By contrast, Atm haploinsufficiency did not detectably affect global gene expression once tumorigenesis has been initiated. Two different methods of analysis were used. We found that only 16 genes were differentially expressed by RMA and 13 by dChip in anaplastic tumors of Atm wild-type and heterozygotes. Importantly, only 5 were considered significant by both types of analysis. These findings suggest that the major variable in gene expression is the tumor type, not the tumor genotype. We thought that differences in expression might be found in regions where there were differences in copy number by CGH (Fig. 4C). Interestingly, though, none of the differentially expressed genes

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**Figure 4.** Spectral karyotyping and CGH analysis of tumor chromosomal spreads. A, spectral karyotyping revealed high levels of chromosomal aberrations, including complex rearrangements (chromosomes 1, 10, 17), translocations (chromosomes 6, 8, 9(2), 11(2), 12, 16), deletions (chromosomes 1, 2, 15), and duplications (chromosomes 2, 3, 5, 6, 8, 9, 11, 12, 15, 17, 18, 19). There were no recurrent rearrangements seen in any of the tumors analyzed. B, numbers of chromosome breakpoints were compared between samples. A total of 890 chromosomes were analyzed in two samples from the Atm\textsuperscript{+/−}:Brca1-MG-\textsuperscript{Δ}\textsubscript{x11} genotype (gray) and 988 chromosomes were analyzed for two Atm\textsuperscript{+/−}:Brca1-MG-\textsuperscript{Δ}\textsubscript{x11} samples (black). The total numbers of breaks detected per chromosome are represented. C, map of genomic imbalances derived by CGH. All chromosomes were affected in at least one sample, except chromosomes 9, 12, and 18. A loss of the distal region of chromosome 4 occurred for both Atm\textsuperscript{+/−}:Brca1-MG-\textsuperscript{Δ}\textsubscript{x11} samples (purple 81.1, blue 161.3) but neither of the Atm\textsuperscript{+/−}:Brca1-MG-\textsuperscript{Δ}\textsubscript{x11} samples (yellow 81.2, green 85.1). In addition, amplification of the distal end of the X chromosome occurred in the Atm\textsuperscript{+/−}:Brca1-MG-\textsuperscript{Δ}\textsubscript{x11} samples but not in the Atm\textsuperscript{+/−}:Brca1-MG-\textsuperscript{Δ}\textsubscript{x11} tumors (arrowheads).
found in our array analysis correspond to regions on the X chromosome or chromosome 4 where copy number was altered. Although we recognize that our sample size was small, we employed several practical methods to alleviate the statistical issues related to our sample size. First, we obtained gene expression intensities based on two different methods, dChip and RMA, and repeated the analyses using both preprocessing techniques. The purpose of conducting the analyses using two different preprocessing and normalization methods was that genes detected as being differentially expressed between the two types of tumors for both types of analytic methods would have a greater likelihood of being of practical interest. We detected a few genotype differences in anaplastic tumors by two separate methods, supporting a similarity of gene expression based on histologic type and not by genotype. In addition, the clustering dendograms provided a measure for how similarly dChip and RMA classify the data, and in both methods, tumors clustered by histologic type and not by genotype. Thus, our findings indicate that we could differentiate tumors only by histologic type and not by genotype among the anaplastic tumors that are clustered differently between the two methods.

Whole mount analysis revealed that Brca1-deficient developing mammary tissue displayed delayed growth and a failure to extend completely into mammary fat tissue during development. Some defects in pregnancy that have been reported previously for Brca1-null mammary glands were also noted (35). The Atm+/−; Brca1-MG-Δex11 and Atm+/++; Brca1-MG-Δex11 mice recapitulate these defects, but the Atm+/−; Brca1-MG-Δex11 mice displayed additional subtle developmental abnormalities. The ductal branching during mammary development in virgin females was significantly less than in wild-type and Brca1-deficient tissues (Fig. 6D). There were no abnormalities of organization of the TEBs, nor were there differences observed in proliferation or apoptosis in these tissues.

Figure 5. RMA and dChip scatter plot and hierarchical clustering of eight tumor-derived RNA profiles. A, hierarchical clustering using RMA indicated that the nonanaplastic tumors (gray boxes) clustered the furthest away, whereas the anaplastic tumors seemed to be more similar regardless of genotype (white boxes, Atm+/−; Brca1-MG-Δex11; black boxes, Atm+/++; Brca1-MG-Δex11). B, dChip analysis indicated again that the two nonanaplastic tumors were the most different, whereas the anaplastic tumors clustered more closely without respect to genotype (same designations). C, RMA scatter plot indicated very few genes differentially expressed between three Atm+/−; Brca1-MG-Δex11 and three Atm+/++; Brca1-MG-Δex11 tumors. D, dChip analysis revealed mostly similar expression of genes as well, with a few genes appearing to be differentially expressed.
suggesting that these differences may be resulting from developmental mechanisms affecting branching but not affecting the rate of proliferation and/or death. The relevance of this phenotype to the increase in anaplastic phenotype is not known, but it may be that certain cells affected by this dysregulation, such as those that are undifferentiated (TEB cap stem cells, for example), could be important during early tumorigenesis. We hypothesize that the branching defects may be related to the developmental defects by influencing the type of cell that becomes tumorigenic, affecting earlier and less differentiated cells in the Atm/Brcal double mutant than in the Brcal mutant alone.

In summary, our results support the clinical data suggesting that ATM heterozygosity may play a role in breast tumorigenesis. Atm heterozygosity does not alter the time-dependent development of mammary gland cancers resulting from loss of Brcal, but Atm haploinsufficiency is able to promote the development of invasive, anaplastic carcinomas in all of the tumors we analyzed. Data from previous clinical studies have identified obligate ATM mutant carriers as having an increased risk of certain types of cancer, including hereditary breast cancers (9–18). This implied risk in developing hereditary breast cancer would require primary mutations in other tumor suppressors known to be associated with hereditary breast cancer, such as the BRCAl gene. Because ATM directly activates BRCAl following ionizing radiation, and both BRCAl and ATM have also been shown to act in response to DNA damage, deficiency of both ATM and BRCAl may result in increased dysregulation of cell cycle control leading to genomic instability and cancers. Although no differences in chromosomal aberrations and copy number of RNA expression were observed in Atm+/−:Brcal-MG-Δex11 versus Atm−/-:Brcal-MG-Δex11 tumors, the severe phenotype of Brcal tumors may obscure any uncovered changes due to haploinsufficiency of Atm. These observations indicate that the heterozygous loss of ATM in addition to the homozygous mutation of BRCAl may be of clinical importance.

Atm haploinsufficiency also had an effect on developing mammary glands in virgin female mice. A defect in ductal branching during extension of the mammary ducts into the adipose tissue compared with wild-type and Brcal mutant controls was observed. Levels of apoptosis and proliferation were similar in

![Figure 6](Image)

**Figure 6.** Analysis of abnormalities associated with mammary gland development. A, analysis of ductal branching in 7-week-old virgin females from five Atm+/−:Brcal-MG-Δex11 (A), five Atm−/-:Brcal−/−:MMTV-Cre−/− (B), and five Atm−/-:Brcal-MG-Δex11 mice (C) revealed that Atm−/-:Brcal-MG-Δex11 have a decreased amount of ductal bifurcations compared with both controls (D). Analysis of ductal morphology revealed that Atm−/-:Brcal-MG-Δex11 ducts had normally developed ducts on examination of cross-sections (E), longitudinal sections (F), and TEB morphologies (G) compared with Atm−/-:Brcal−/−:MMTV-Cre−/− controls (H-J).
Atm+/−;Brca1-MG-Δex11 versus Atm+/−;Brca1-MG-Δex11, and the morphology of the TEBs appeared normal as well. We offer two potential nonexclusive explanations for our findings. First, it is possible that Atm haploinsufficiency affects development (as well as tumorigenesis) by further reducing DNA repair, cell cycle control, and possibly transcriptional regulation than are present in the Brca1 mutant alone. Because of the similarities in function and because ATM activates BRCA1, it is possible that the additional reduction in ATM protein levels may exacerbate phenotypes associated with mutations in Brca1 under certain situations, such as hyperproliferation (as in mammary duct elongation) or tumorigenesis. It is tempting to speculate that in the presence of Atm haploinsufficiency ATP fails to activate alternative pathways that otherwise compensate for the lack of Brca1. The phenotypes associated with absence of functional Brca1, such as mammary developmental defects and tumorigenesis, could then be exacerbated when Atm, which has similar and somewhat redundant functions with Brca1, is reduced in its absence.

Second, Atm haploinsufficiency may impair an important damage response mechanism early during the onset of tumorigenesis. Recent reports suggest that tumorigenic events occurring early in cancer progression activate the ATM-regulated checkpoint through deregulation of DNA replication and DNA damage, creating a barrier against tumor progression (53). Further progression of these tumors may rely on the disruption of the DNA damage response components, such as ATM. Atm haploinsufficiency may partially repress this response, leading to the occurrence of more invasive tumors.

Although we do not know the precise mechanism of action, our study provides clear evidence that the disruption of Atm affects both mammary development and tumorigenesis in a Brca1 mutant background, suggesting a synergistic interaction in tumorigenesis when both proteins are depleted. In addition, this work corroborates other recent findings suggesting that the disruption of DNA repair machinery early during tumorigenesis may be critical in the progression to cancer (53).

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Atm Heterozygosity Cooperates with Loss of Brca1 to Increase the Severity of Mammary Gland Cancer and Reduce Ductal Branching

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