

# Dominant Negative ATM Mutations in Breast Cancer Families

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**Background:** The ATM gene encoding a putative protein kinase is mutated in ataxia-telangiectasia (A-T), an autosomal recessive disorder with a predisposition for cancer. Studies of A-T families suggest that female heterozygotes have an increased risk of breast cancer compared with non-carriers. However, neither linkage analyses nor mutation studies have provided supporting evidence for a role of ATM in breast cancer predisposition. Nevertheless, two recurrent ATM mutations, T7271G and IVS10-6T→G, reportedly increase the risk of breast cancer. We examined these two ATM mutations in a population-based, case-control series of breast cancer families and multiple-case breast cancer families. **Methods:** Five hundred twenty-five or 262 case patients with breast cancer and 381 or 68 control subjects, respectively, were genotyped for the T7271G and IVS10-6T→G ATM mutations, as were index patients from 76 non-BRCA1/2 multiple-case breast cancer families. Linkage and penetrance were analyzed. ATM protein expression and kinase activity were analyzed in lymphoblastoid cell lines from mutation carriers. All statistical tests were two-sided. **Results:** In case and control subjects unselected for family history of breast cancer, one case patient had the T7271G mutation, and none had the IVS10-6T→G mutation. In three multiple-case families, one of these two mutations segregated with breast cancer. The estimated average penetrance of the mutations was 60% (95% confidence interval [CI] = 32% to 90%) to age 70 years, equivalent to a 15.7-fold (95% CI = 6.4-fold to 38.0-fold) increased relative risk compared with that of the general population. Expression and activity analyses of ATM in heterozygous cell lines indicated that both mutations are dominant negative. **Conclusion:** At least two ATM mutations are associated with a sufficiently high risk of breast cancer to be found in multiple-case breast cancer families. Full mutation analysis of the ATM gene in such families could help clarify the role of ATM in breast cancer susceptibility. [J Natl Cancer Inst 2002;94:205-15]

Mutations in two breast cancer susceptibility genes, BRCA1 and BRCA2, account for most multiple-case breast cancer families containing members with ovarian cancer or male breast cancer (1). However, mutations in BRCA1 and BRCA2 genes have been identified in only about 40% of families with four or five cases of female breast cancer but no cases of male breast cancer or ovarian cancer (1). It is, therefore, likely that mutations that confer a high risk of breast cancer exist in one or more other genes. Several groups are searching for these genes by use of whole genome scanning, but the success of this approach depends on the underlying heterogeneity of the remaining breast

cancer susceptibility loci. The failure to date to identify additional breast cancer susceptibility genes associated with a high risk of disease suggests that more than one may exist. A valid alternative approach is to examine candidate genes in families with multiple cases of breast cancer to determine whether such candidates carry mutations that might account for a proportion of these families with unknown genetic etiology.

One such candidate is the ATM gene, which is mutated in ataxia-telangiectasia (A-T). A-T is a rare autosomal recessive disorder characterized by progressive neuronal degeneration, immunologic deficiency, radiosensitivity, and an increased risk of cancer, particularly of lymphoid malignancies. At the cellular level, A-T is characterized by hypersensitivity to ionizing radiation and other agents that cause double-strand breaks in DNA, by defects in both the G<sub>1</sub>/S and G<sub>2</sub>/M checkpoint responses, by defective inhibition of DNA replication, and by defective production of p53 in response to ionizing radiation. An association between A-T and breast cancer was first suggested by Swift et al. (2), who reported more cases of breast cancer than expected in the relatives of A-T patients. This finding has since been confirmed in several studies of A-T families (3-6), where relative risks for breast cancer in the range of threefold to sevenfold have been reported. On the basis of early estimates of the A-T carrier frequency of 1% and an increased risk of about threefold, it was suggested that A-T heterozygosity may account for about 5% of all breast cancers (7), which is much greater than the 1%-2% attributable to mutations in BRCA1 and BRCA2. Although linkage analysis has not provided evidence of a major

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role for ATM in breast cancer predisposition (8,9), these analyses would have been hampered by lack of statistical power if the underlying susceptibility were heterogeneous.

The role of the ATM gene in breast cancer has been investigated in two other ways: by evaluating the risk of breast cancer in A-T carriers and by analyzing ATM mutations in patients with breast cancer. Four studies of A-T families (3,5,6,10) have reported an elevated frequency of breast cancer in female relatives, particularly in the obligate heterozygotes where risks range from threefold (95% confidence interval [CI] = twofold to sixfold) to sevenfold (95% CI = twofold to 17-fold). Stankovic et al. (11) found that a missense mutation in ATM (T7271G or Val2424Gly), which they identified in two A-T families, was associated with a 13-fold (95% CI = fourfold to 46-fold) increased risk of breast cancer in both heterozygotes and homozygotes. In a larger number of studies, the frequency of ATM mutations in sporadic or unselected case patients with breast cancer was compared with that in control subjects. These studies (12–18) have generally used methods that are biased toward detecting protein-truncating mutations and have been too small to have adequate statistical power. Most studies have not supported an increased prevalence of ATM mutations in patients with breast cancer. However, in a study of 82 Dutch patients with early-onset breast cancer, who had been exposed to low-dose ionizing radiation at an early age and who had survived for more than 5 years after their diagnosis of breast cancer (19), seven germline ATM mutations were uncovered including IVS10–6T→G, which was detected in three patients, and increased the risk of breast cancer risk by about ninefold. One German patient has been described with classical A-T with homozygous IVS10–6T→G mutations (20); this mutation is thus clearly pathogenic.

The ATM gene has 66 exons, is expressed in a wide range of tissues as an approximately 12-kilobase messenger RNA, and encodes a 350-kd protein with a protein kinase domain. ATM is activated by exposure to ionizing radiation. Activated ATM triggers a phosphorylation cascade that activates checkpoint and repair proteins (21). A number of well-characterized tumor suppressor genes (TP53, BRCA1, and CHK2) lie downstream of ATM that, after DNA damage is detected, trigger cell cycle arrest, DNA repair, or apoptosis. In addition, ATM and CHK2, which depends on ATM for activation, are both critical for the regulation of p53 stability after ionizing radiation. The tumor suppressor BRCA1 is also regulated by phosphorylation that is mediated by ATM and other kinases in response to DNA damage (22–24). Phosphorylation of BRCA1 in response to ionizing radiation is largely dependent on ATM, whereas phosphorylation in response to UV irradiation or hydroxyurea requires the ATR (i.e., A-T and rad3-related) protein (24,25). The physiologic relevance of ATM-dependent and ATR-dependent phosphorylation of BRCA1 is unclear at present, but these phosphorylation events probably affect the repair function of BRCA1.

The current controversy regarding the role of ATM in breast cancer susceptibility could have arisen from two misconceptions. First, only certain ATM mutations may predispose to breast cancer (12,26–28), perhaps because of the effect they have on phosphorylation of BRCA1 and/or p53. This hypothesis could be tested by analyzing genotype–phenotype associations within A-T families. Second, analysis of patients with breast cancer who are unselected for a family history of breast cancer will be an inefficient way to detect ATM mutations if they do

confer high increased risks of cancer, and analysis of multiple-case breast cancer families may be more rewarding. Although this idea seems obvious, it has not been a popular approach, perhaps because the average increased risk for breast cancer found in A-T families, which is only threefold to sevenfold, has been considered too low to generate multiple-case families. However, because these are estimates of average relative risk with very wide CIs, we hypothesized that some ATM mutations might confer risks for breast cancer high enough to identify multiple-case breast cancer families (11,29). To test this hypothesis and to determine whether we could identify such families, we have screened a panel of non-BRCA1/2 multiple-case breast cancer families and a large number of unselected patients with breast cancer and control subjects for two ATM mutations that appear to confer high relative risks for breast cancer.

## SUBJECTS AND METHODS

### Ascertainment

Case patients and control subjects for genotyping the T7271G (Val2424Gly) and IVS10–6T→G ATM mutations were identified by the Australian Breast Cancer Family Study/Cancer Family Registry for Breast Cancer Studies (ABCFS/CFRCBS), a population-based, case–control–family study conducted by use of the Victorian and New South Wales Cancer Registries and the electoral rolls from 1992 through 1999 (30,31). We genotyped the T7271G mutation in 525 incident case patients with breast cancer and in 381 control subjects and the IVS10–6T→G mutation in 262 case patients and in 68 control subjects (all ascertained irrespective of family history). The T7271G and IVS10–6T→G ATM mutations were also genotyped in index patients from 76 families with four or more members with breast cancer ( $n = 50$ ) in the same or adjacent generations or only two individuals with breast cancer if one had high-risk features such as bilateral disease, or breast plus ovarian cancer, or was diagnosed before the age of 40 years ( $n = 26$ ). These families were ascertained by the Kathleen Cuninghame Foundation Consortium for Research into Familial Breast Cancer (kConFab), an Australasian consortium of familial breast cancer researchers (32) (<http://www.kconfab.org>). None of the kConFab families contained members with male breast cancer or carried a mutation in BRCA1 or BRCA2 as determined by various testing strategies. Thus, these families are defined as non-BRCA1/2 families. The index patient was the youngest affected member in the family. For each family, extensive cancer histories had been taken through interviews with multiple relatives, and attempts were made to verify reported cancers through medical records. All of the subjects gave written informed consent for this study, and the study was approved by the ethics committees of the Queensland Institute of Medical Research, the Peter MacCallum Cancer Institute, The University of Melbourne, The Anti-Cancer Council of Victoria, and the New South Wales Cancer Council.

### Mutation Detection

DNA was obtained from blood samples (33) or, for the two deceased individuals with gastric cancer in family A, from paraffin blocks of uninvolved tissue (34). The tumor sections used for loss of heterozygosity (LOH) analysis were obtained from paraffin blocks that contained at least 80% tumor tissue. Polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) assays were used for low-throughput screen-

ing. For the ATM T7271G variant, the primers used were 5'-TGAAAAGAGCCAAAaAGGAGG-3' (forward) and 5'-TAACTGGTGAACATAAAATTGTAC-3' (reverse). The lowercase "a" in the forward primer is a mismatch (the nucleotide is G in the published sequence) that was specifically introduced to destroy an *MnII* site adjacent to and overlapping with the *MnII* site created by the G substitution at nucleotide 7271, thus allowing the 7271 nucleotide substitution to be detected by *MnII* digestion. The PCR was carried out in a 10- $\mu$ L reaction mixture containing 15 ng of genomic DNA, all four deoxyribonucleoside triphosphates (each at 200 nM), 1 $\times$  PerkinElmer/Cetus PCR buffer II (PerkinElmer/Cetus, Norwalk, CT), 1 U of *Taq* polymerase, and 1.5 mM MgCl<sub>2</sub>. Cycling conditions were 94°C for 5 minutes, followed by 35 cycles of 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 20 seconds, with a final extension at 72°C for 7 minutes. Next, 2  $\mu$ L of the 101-base-pair (bp) amplified product was digested with 2.5 U of *MnII* restriction enzyme (New England Biolabs, Beverly, MA) in a 20- $\mu$ L reaction with 1 $\times$  New England Biolabs buffer 2 and bovine serum albumin (0.1 mg/mL) at 37°C for 2–3 hours. The digested products were separated by electrophoresis on a 4.5% NuSieve agarose gel (BioWhittaker Molecular Applications, Rockland, ME), and the genotype was determined by the banding pattern observed. The G allele was identified by the presence of a 13-bp fragment (not resolved by this method) and an 88-bp fragment. The T allele, which lacks an *MnII* digestion site, was identified by a single 101-bp fragment. Alleles from T/G heterozygotes identified by the restriction endonuclease assay were amplified and sequenced by use of the primers described above and the ABI Prism BigDye Terminator Cycle Sequencing Kit (PerkinElmer/Cetus) for automated sequencing. For the PCR-RFLP assay used to detect the IVS10-6T→G variant, a 193-bp PCR product was amplified with 8 pmol of each primer (forward primer = 5'-ACAGCGAAACTCTGGCTCAA-3'; reverse primer = 5'-TGATCTTTTATTACTTCCCAGCCTAGT-3') in a 10- $\mu$ L reaction as described above. Cycling conditions were 94°C for 4 minutes, followed by 35 cycles at 94°C for 20 seconds, 53°C for 20 seconds, and 72°C for 20 seconds, with a final extension at 72°C for 7 minutes. Next, 3  $\mu$ L of the 193-bp product was digested with 4 U of *RsaI* restriction enzyme (Promega Corp., Madison, WI) in a 15- $\mu$ L reaction with 1 $\times$  Promega buffer C and bovine serum albumin (0.1 mg/mL) at 37°C for 2–3 hours. The digested products were separated by electrophoresis on a 4.5% NuSieve agarose gel, and the genotype was determined by the banding pattern observed. The G allele was identified by the presence of 58-bp and 135-bp fragments, and the T allele, which lacks a *RsaI* restriction site, was identified by a single 193-bp product. Heterozygotes identified by the PCR-RFLP assays were amplified and sequenced by use of the primers described above and the ABI Prism BigDye Terminator Cycle Sequencing Kit (PerkinElmer/Cetus) for automated sequencing.

For high-throughput screening of population-based case patients with breast cancer and control subjects, the ATM T/G polymorphism at position 7271 was detected by use of the Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) and the standard protocol for allelic discrimination assays, as described previously (35). A 123-bp PCR product was amplified with the primers 5'-AAACAAGCAAGCTCTCCTGAAA-3' and 5'-AACCTAGTTACCTGTTTGTCTGAATTTT-3', and fluorescently labeled probes 5'-6-carboxy-fluorescein-

TCCCTAAGGAGACCTaCTTCCTCTTTGGCT-TAMRA-3' (100 nM = final concentration; where TAMRA is 6-carboxy-tetramethylrhodamine) and VIC<sup>TM</sup>-TCCCTAAGGAGACCTcCTTCCTCTTTGG-TAMRA-3' (200 nM = final concentration) were used to detect the T and G alleles, respectively. For high-throughput screening of population-based case patients with breast cancer and control subjects, the ATM IVS10-6T→G mutation was detected by denaturing high-performance liquid chromatography with the automated Helix system (Varian Inc., Palo Alto, CA) (36). PCR products were denatured for 3 minutes at 95°C and then gradually cooled to 65°C over a 30-minute period. The heteroduplex observed with the IVS10-6T→G mutation, which was used in all of the analyses as a positive control, was successfully resolved when eluted from the column at 55°C.

### Microsatellite Genotyping

Microsatellite markers D11S2179, D11S1294, D11S2180, and D11S2178 were genotyped as described previously (37).

### Analysis of ATM Expression and Kinase Activity

ATM expression and kinase activity were analyzed as described previously (24,38). For *in vitro* kinase assays, cells were lysed in TGN buffer (i.e., 50 mM Tris-HCl [pH 7.5], 50 mM  $\beta$ -glycerophosphate, 150 mM NaCl, 10% glycerol, 1% Tween 20, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, pepstatin [2  $\mu$ g/mL], aprotinin [10  $\mu$ g/mL], leupeptin [5  $\mu$ g/mL], and 1 mM dithiothreitol) as described previously (39). The immunoprecipitates were washed twice with TGN buffer, once with 100 mM Tris-HCl (pH 7.5) and 0.5 M LiCl, and twice with kinase buffer (10 mM HEPES [pH 7.5], 50 mM  $\beta$ -glycerophosphate, 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, 5  $\mu$ M adenosine triphosphate [ATP], and 1 mM dithiothreitol). Kinase reaction mixtures were prepared by resuspending washed beads in 30  $\mu$ L of kinase buffer containing 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP and 1  $\mu$ g of glutathione *S*-transferase (GST)-p53 fusion protein. Immune complex reactions were incubated at 30°C for 30 minutes and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography. For *in vivo* kinase assays, cellular extracts were prepared by resuspending the cells in lysis buffer (i.e., 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EGTA [i.e., ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N,N'*-tetraacetic acid], 2 mM EDTA, 25 mM NaF, 25 mM  $\beta$ -glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, leupeptin [5  $\mu$ g/mL], aprotinin [1  $\mu$ g/mL], 0.2% Triton X-100, and 0.3% Nonidet P-40) and incubating the mixture on ice for 30 minutes. Supernatants were collected after centrifugation at 14000g for 15 minutes at 4°C. Protein (40  $\mu$ g per sample) was then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with the appropriate antibodies.

### Linkage and Penetrance Analysis and Statistical Methods

Parametric logarithm of odds (LOD) scores were calculated for the relevant ATM mutations by FASTLINK (40) by assuming autosomal dominant inheritance of risk and a disease allele frequency of 0.0033 as in Easton et al. (41). LOD scores were calculated for breast cancer status only, and individuals with other cancers were classified as unaffected for the purposes of these analyses. The average cumulative risk to a given age (penetrance) was estimated by a modified segregation analysis, fitted

under maximum likelihood theory with the software MENDEL (42), by assuming that the hazard ratio (age-specific incidence in mutation carriers as a multiple of age-specific incidence in the population) was a constant. This approach uses information on all family members, not just on those who have been tested, and allows appropriate adjustment for ascertainment as follows: For family A (the pedigree of the T7271G mutation carrier detected from testing the unselected population-based case patients), the likelihood was conditioned on the proband (0013, identified by screening population-based case patients and control subjects) being a mutation carrier with a given age at diagnosis. A similar design has been used previously to estimate the penetrance of mutations in BRCA1 and BRCA2 (43). For the pedigrees in which the IVS10-6G→T mutations were segregating, the likelihood was conditioned on the affected members of the family and the mutation status of the index person/proband, who was the first identified with the mutation, by the approach used to estimate penetrance of BRCA1 and BRCA2 mutations in the Breast Cancer Linkage Consortium families (1). We assumed a phenocopy rate of 0.083 and penetrance of 0.60 and used the S-PLUS function for the rare-allele case, as described previously by Petersen et al. (44), to compute the Bayes factor and posterior probability of a disease-causing effect.

All statistical tests and *P* values were two-tailed, and statistical significance was taken as a nominal *P* value of less than .05.

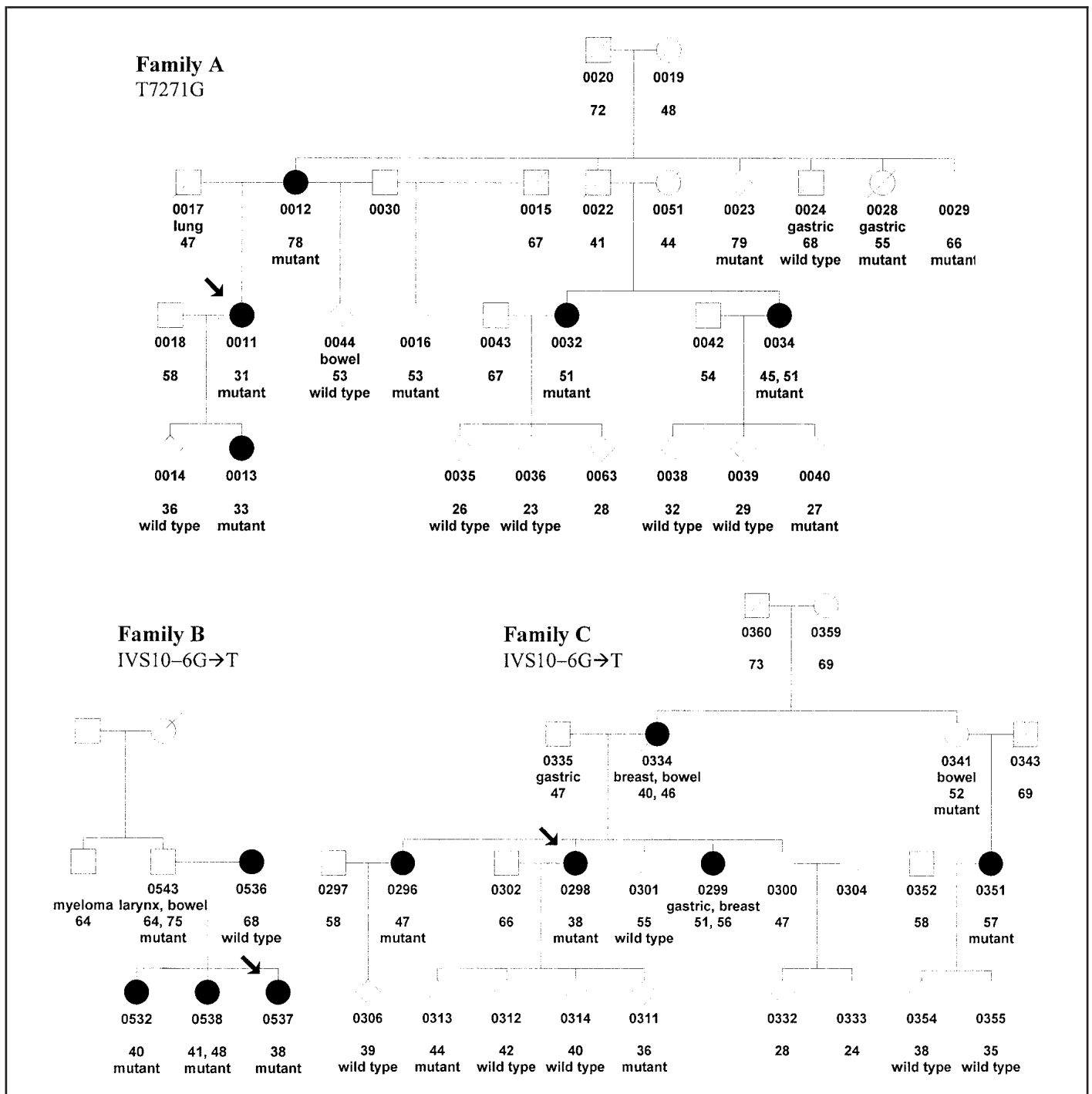
## RESULTS

To estimate the frequency of the ATM T7271G missense mutation in the Australian population, we first screened 525 case patients and 381 control subjects younger than 40 years of age, all unselected for a family history of breast cancer, who were obtained from the ABCFS/CFRCBS, a population-based, case-control family study (30,31). We identified only one heterozygote, a woman (0013 in family A) who developed breast cancer at age 33 years and had a family history of breast cancer. We then genotyped the T7271G mutation in index patients from 76 multiple-case Australian families identified by kConFaB (32). Analysis of these index patients revealed one heterozygote, who turned out to be the mother of the woman identified previously in the population-based study. Further genotyping revealed that, in family A, all of the case patients with breast cancer were carriers of the T7271G mutation, as was the woman with gastric cancer (Fig. 1). Five unaffected carriers were also identified in the family. Although the exact ages and sex of these unaffected carriers cannot be disclosed without loss of confidentiality, we have included these data in estimating penetrance. Three breast tumors (case patients 0012, 0013, and 0032) and one gastric tumor (case patient 0028) from carriers were examined for LOH by analysis of the T7271G ATM variant and the flanking microsatellite marker D11S1294. We found no evidence of LOH for either the wild-type or mutant allele (data not shown). Four microsatellite markers were used for haplotype analysis of family A and of the A-T T7271G homozygote mutant reported previously in a Scottish family (11). This analysis showed that the ATM mutation in family A occurred on the same haplotype as the Scottish T7271G mutations, consistent with the possibility that the two families share an ancestral haplotype, although we were unable to find any genealogic connection. Penetrance of the T7271G mutation was estimated to be 55% (95% CI = 26% to 88%) to age 70 years, equivalent to an increased risk of 13.7-fold (95% CI = 5.1-fold to 36.6-fold).

We genotyped 262 case patients and 68 control subjects, all unselected for family history of breast cancer, obtained from the ABCFS/CFRCBS, to determine the frequency of the IVS10-6T→G ATM mutation in the Australian population and identified no carriers. Index patients from the 76 multiple-case breast cancer families were then genotyped, and two IVS10-6 heterozygotes were identified (Fig. 1). Family B contained three sisters, all of whom developed bilateral or unilateral breast cancer and all of whom were heterozygous carriers of the mutation. Their mother, who had no family history of breast cancer except for the breast cancers in her daughters, was diagnosed with breast cancer at age 69 years but was not a carrier of the mutation. Instead, the mutation was inherited from their father, who was diagnosed with squamous cell carcinoma of the larynx at age 65 years, after which he had a very severe reaction to radiotherapy, and colon cancer at age 75 years. Only limited information on family history of cancer was available from this man. One of these carriers has a *de novo* t(21;22) Robertsonian translocation and a child with trisomy 21, who surprisingly has a Robertsonian t(21;21), and miscarried a child with trisomy 21 with an unbalanced t(21;21). Family C contained five patients with breast cancer, of whom four were shown or inferred to be carriers. The fifth patient with breast cancer, who also developed gastric cancer, was not available for testing. One tumor, from carrier 0538, was examined for LOH by RFLP-PCR analysis of the IVS10-6 ATM variant. We found no evidence of LOH for the wild-type or mutant allele (data not shown).

In total, we therefore identified three multiple-case families with ATM T7271G or IVS10-6T→G mutations. Penetrance of the IVS10-6T→G mutation was estimated to be 78% (95% CI = 36% to 99%) to age 70 years, equivalent to an increased risk of 26.0-fold (95% CI = 7.5-fold to 90.0-fold). When the T7271G and IVS10-6T→G mutations were combined, the overall average penetrance was estimated to be 60% (95% CI = 32% to 90%), equivalent to an increased risk of 15.7-fold (95% CI = 6.4-fold to 38.0-fold). The ATM mutations segregated with breast cancer in the three families, giving LOD scores for families A, B, and C of 0.40, 0.14, and 0.64, respectively, at zero recombination and a total LOD score of 1.18 (*P* = .01). Bayesian analysis of genetic linkage in these families confirmed this increased risk of breast cancer, producing Bayes factors of 50.99, 0.75, and 7.14 and posterior probabilities of .98, .43, and .88, for families A, B, and C, respectively. These values resulted in a combined Bayes factor of 273.19, indicating that test results from the three families are 273 times more likely under the hypothesis of causality than under the hypothesis of no causality. Moreover, if prior odds for causality are even across families, the posterior probability of causality is .996.

To understand the biologic effects of these mutations, we characterized lymphoblastoid cell lines (LCLs) derived from individuals who were either homozygous or heterozygous for these mutations. Immunoblot analysis using ATM-specific antibodies showed that LCLs derived from individuals from the Scottish family who were homozygous for the T7271G mutation (11) (109II-5 and 109II-6) (Fig. 2, A) contained full-length ATM protein in amounts comparable to those found in LCLs established from control subjects who were homozygous for wild-type ATM (C3ABR) and heterozygous for the T7271G mutation (Fig. 2, A: 109II-3 and 109III-1 from Scotland; and Fig. 2, E: 0011, 0029, and 0012 from family A). Immediately after exposure to ionizing radiation, ATM contributes to the

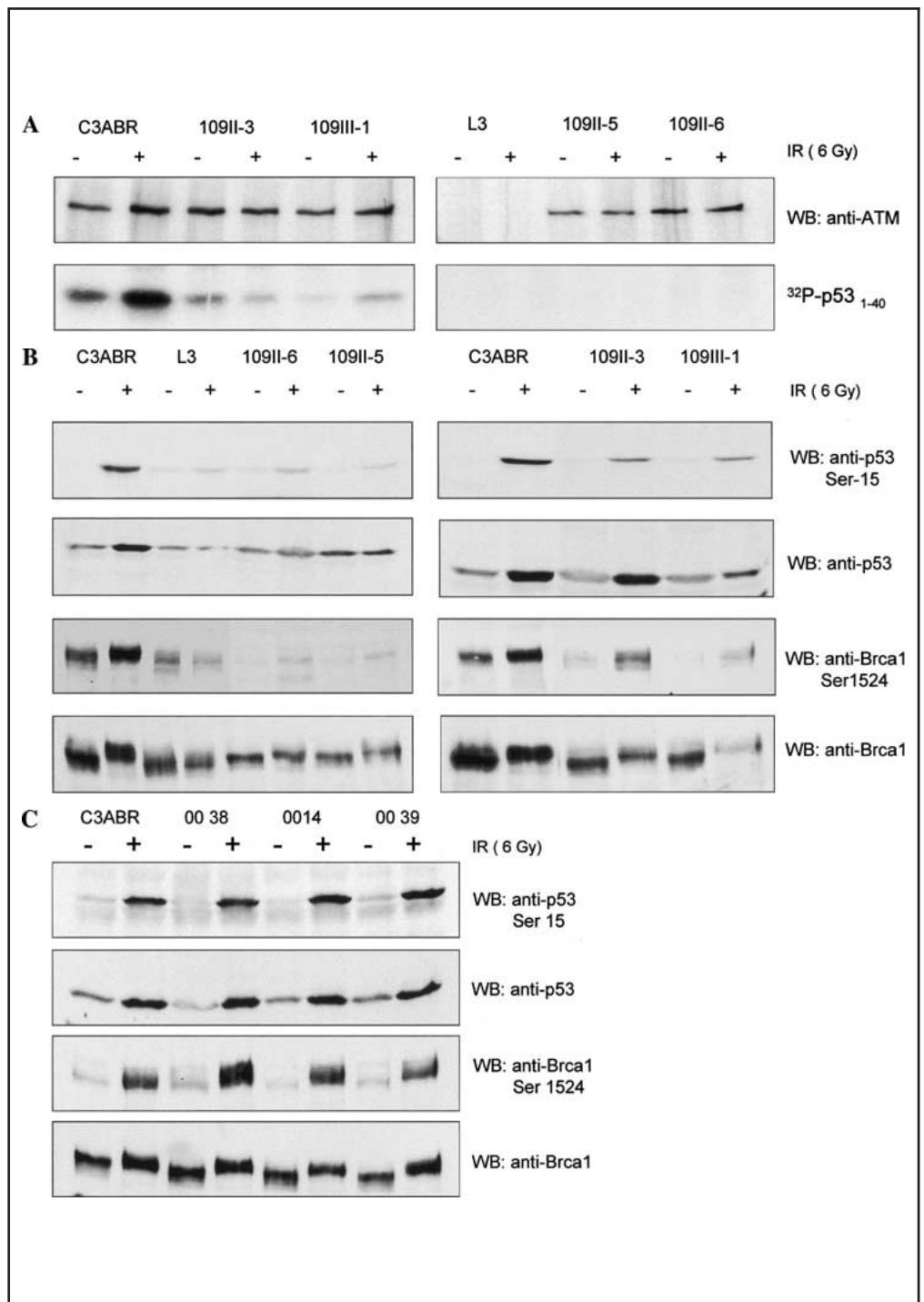


**Fig. 1.** Pedigrees of breast cancer families with mutations in ATM. **Solid symbols** = women with breast cancer; **open symbols** = unaffected individuals. The diagnoses of non-breast cancers are **below the symbols** as is the age (in years) at onset of all the cancers or the age (in years) at interview for unaffected individuals. Bilateral breast cancer is indicated by two ages at onset. **Arrows** indicate the index patient. These pedigrees have been slightly altered to protect confidentiality, including the use of the **diamond symbol**, indicating unspecified sex, but they essentially reveal the correct relationships and mutation status.

stabilization of p53 by phosphorylating p53 on Ser-15 (38,39,45). When ATM kinase activity *in vitro* was assayed with p53 as the substrate, the kinase activity in LCLs homozygous for the T7271G mutation (109II-6 and 109II-5) and in an ATM-nonexpressing cell line (L3) was completely abolished (Fig. 2, A); however, kinase activity in LCLs heterozygous for the T7271G mutation (109II-3, 109III-1, 0011, 0029, and 0012) was detected but at less than 10% of the activity detected in control cells (Fig. 2, A and D). Exposure to ionizing radiation caused a

rapid, ATM-dependent, DNA damage-induced shift in the electrophoretic mobility of BRCA1, as a result of the phosphorylation of BRCA1 at multiple sites by ATM (Ser-1387, Ser-1423, Ser-1457, and Ser-1524) (23,24). We used specific antibodies to detect sites phosphorylated by ATM in p53 (phospho-Ser-15) and BRCA1 (phospho-Ser-1524) (24) to assay for the *in vivo* activation of ATM kinase after ionizing radiation. Immunoblotting of cell extracts with antibodies that detect either the phosphorylated form or the total pool of p53 in cells showed that p53

**Fig. 2.** ATM expression and kinase activity in lymphoblastoid cell lines (LCLs). Growing cultures of LCLs from individuals either homozygous (109II-5 and 109II-6) or heterozygous (109II-3, 109III-1, 0011, 0029, and 0012) for the T7271G mutation were exposed to ionizing radiation (IR; 6 Gy) (+) to activate the ATM kinase and harvested 30 minutes after treatment. Control cells were not irradiated (-). C3ABR, 0038, 0014, and 0039 cells = positive controls; L3 cells = negative control for ATM expression and activity. **A)** ATM expression was analyzed by western blotting with an anti-ATM antibody (WB:anti-ATM), and *in vitro* ATM kinase activity was assayed by use of a glutathione *S*-transferase (GST)-p53 fusion protein (<sup>32</sup>P-p53<sub>1-40</sub>) and autoradiography for <sup>32</sup>P incorporation in cell lines from the Scottish family homozygous (109II-5 and 109II-6) and heterozygous (109II-3 and 109III-1) for the T7271G mutation. **B)** *In vivo* analysis of ATM kinase activity with p53 and BRCA1 as substrates is shown by western blotting (WB) with anti-phospho-specific antibodies against ATM phosphorylation sites in p53 (anti-p53 Ser-15) or BRCA1 (anti-BRCA1 Ser-1524) and with anti-p53 or anti-BRCA1 antibodies that detect the total pools, both phosphorylated and unphosphorylated, of these proteins. **C)** *In vivo* analysis of ATM kinase activity in LCLs from three individuals with wild-type alleles (0038, 0014, and 0039) from family A as described in **panel B** is shown. **D)** Analysis of ATM kinase activity from three mutation carriers (0011, 0029, and 0012) from family A shows ATM kinase activity against p53 *in vitro* by use of GST-p53 fusion protein as the substrate (<sup>32</sup>P-p53<sub>1-40</sub>) and the *in vivo* assessment of ATM kinase activity, as described in **panel B**. **E)** Analysis of three mutation carriers (0011, 0029, and 0012) and an individual with wild-type alleles (0014) for ATM kinase activity against BRCA1 is shown. BRCA1 expression and phosphorylation were analyzed by western blotting as described in **panel B**. ATM and DNA-dependent protein kinase (DNAPK) expression was analyzed by western blotting with anti-ATM and anti-DNAPK antibodies, as a loading control. ATM monomers and dimers are shown.



was activated rapidly in extracts from the C3ABR cell line, expressing wild-type ATM, as judged by its stabilization and enhanced phosphorylation on Ser-15 within 1 hour after exposure to ionizing radiation (Fig. 2, B: C3ABR; and Fig. 2, C: 0038, 0014, and 0039). In contrast, stabilization and phosphorylation of p53 were barely detectable in cell extracts from individuals homozygous for the T7271G mutation (109II-6 and 109II-5) and in the ATM-nonexpressing L3 cell line (Fig. 2, B). DNA damage-induced phosphorylation and BRCA1 mobility shift were also dramatically lower in cell lines established from T7271G mutant homozygotes and in the L3 control (Fig. 2, B) than in cells expressing wild-type ATM (Fig. 2, B: C3ABR; and Fig. 2, C: 0038, 0014, and 0039). Cell lines established from

heterozygotes (109II-3, 109III-1, 0011, 0029, and 0012) showed only 15%–25% of the ATM kinase activity detected in positive control lines, as assayed by phosphorylation of p53 and BRCA1 (Fig. 2, B, D, and E), even though comparable amounts of ATM were detected. It is interesting that an immunoreactive band of the size predicted for an ATM dimer was observed in extracts from control cells but not from heterozygous mutant cells. The appearance of this band was associated with the presence of wild-type ATM (Fig. 2, E). The level of expression of BRCA1 in heterozygous carriers (0011, 0012, and 0029) was also lower than in wild-type cells (C3ABR and 0014) (Fig. 2, E), suggesting that mutant ATM protein might interfere with the expression of the BRCA1 gene. This result was not an artifact due to dif-

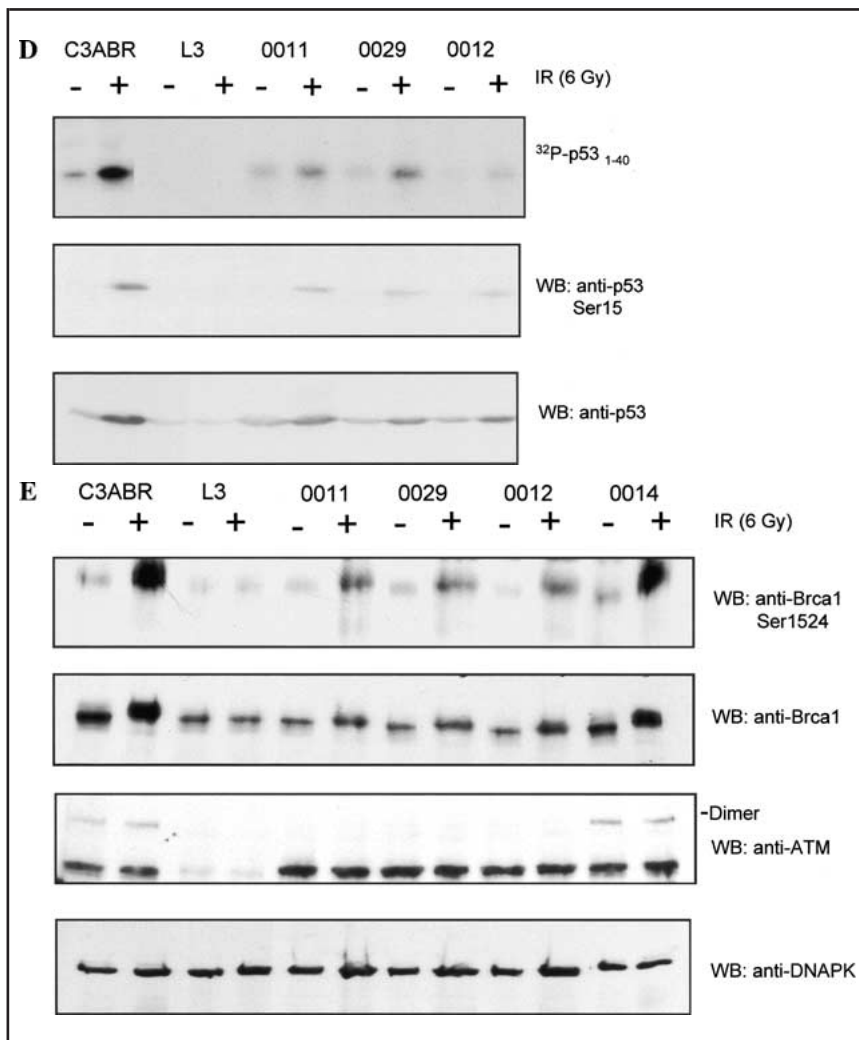


Fig. 2. (Continued).

ferences in protein loading, because similar levels of ATM and DNA-dependent protein kinase were detected in the mutant and wild-type cell lines (Fig. 2, E).

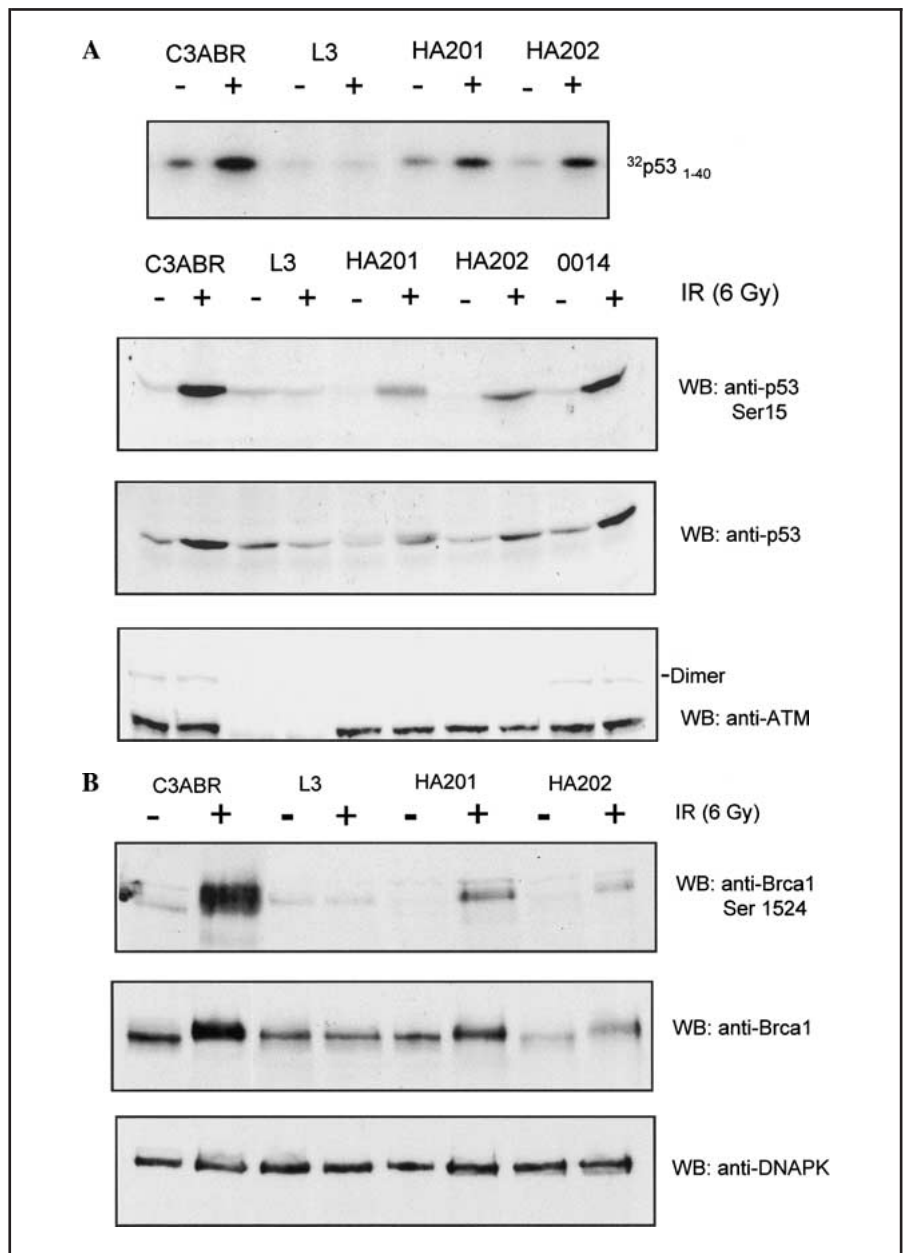
A similar functional analysis was performed for the IVS10-6T→G variant in cell lines established from German heterozygotes (HA201 and HA202) and heterozygotes from families B and C (0532, 0296, 0298, and 0351). The expression of ATM protein in the IVS10-6T→G German homozygote was less than 5% of levels detected in control subjects, consistent with the observation that less than 10% of the mutant exon is spliced correctly (data not shown). The truncated protein was not detectable by western blotting with an anti-ATM polyclonal antibody raised against the amino terminus of protein (residues 250–522), indicating that the truncated protein may be unstable or not recognized by the polyclonal antibody. When the cell lines from individuals heterozygous for this mutation were tested for activation of ATM kinase *in vitro* with p53 as the substrate, the specific activity (ratio of level of ATM to phosphorylated p53) was similar to that in wild-type control lines (Fig. 3, A). This result might be explained if the product of the mutant allele were unstable and thus unavailable to suppress the activity of wild-type ATM under these assay conditions. In contrast, the heterozygous cell lines (HA201, HA202, 0296, 0298, 0351, and 0532) had 25%–40% of the *in vivo* ATM kinase activity detected in positive control lines, as assayed by phosphorylation of p53 on

Ser-15 (Fig. 3, A and C) and of BRCA1 on Ser-1524 (Fig. 3, B and C), although the total amount of ATM protein in these lines was almost comparable (heterozygous cell lines had 80%–90% of the normal level of expression) (Fig. 3, A and C). These functional analyses suggest that both ATM mutations tested act in a dominant negative manner.

## DISCUSSION

We have analyzed two ATM mutations, T7271G and IVS10-6T→G, in a large number of population-based case patients and control subjects and in index patients from 76 non-BRCA1/2 multiple-case breast cancer families. A total of 1812 and 660 chromosomes were genotyped from the case-control study for T7271G missense and IVS10-6T→G protein-truncating mutations, respectively. We found no IVS10-6G→T mutations and only one T7271G mutation in a case patient with a family history of breast cancer. Among the 76 non-BRCA1/2 families, we found one family who carried the missense mutation and two families who carried the protein-truncating mutation. The T7271G (Val2424Gly) mutation was the only ATM mutation found in the A-T family reported by Stankovic et al. (11) and is, therefore, likely to be pathogenic, although weakly so, because the three A-T case patients in this family had a mild clinical phenotype. The IVS10-6T→G ATM mutation has been identi-

**Fig. 3.** ATM expression and kinase activity in lymphoblastoid cell lines from individuals heterozygous for the IVS10-6T→G mutation. Cells were left untreated (-) or exposed to ionizing radiation (6 Gy) (+) and harvested 30 minutes after exposure. C3ABR and 0014 cells served as positive controls, and L3 cells served as a negative control for ATM expression and activity. Cell lines HA201 and HA202 were established from individuals from the German family who were heterozygous for IVS10-6T→G. Cell lines 0296, 0298, 0351, and 0532 were derived from individuals heterozygous for the IVS10-6T→G mutation from families B and C. **A)** ATM kinase activity was analyzed *in vitro* with a glutathione *S*-transferase (GST)-p53 fusion as a substrate (<sup>32</sup>P-p53<sub>1-40</sub>) and *in vivo* with p53 by western blotting (WB) of a cell extract with anti-p53 Ser-15 and anti-p53 antibodies. ATM expression was analyzed by blotting with anti-ATM antibody. ATM monomers and dimers are shown. **B)** ATM kinase activity was analyzed with BRCA1 as the substrate. BRCA1 expression and phosphorylation were analyzed by western blotting with anti-BRCA1 and anti-BRCA1 Ser-1524 as described in Fig. 2, B. Anti-DNA-dependent protein kinase (DNAPK) antibodies were used as control for protein loading. **C)** *In vivo* analysis of ATM kinase activity with p53 and BRCA1 in four mutation carriers (0296, 0298, 0351, and 0532) from families B and C as described in Fig. 2, B.



fied in the homozygous state in one German patient with full-blown A-T (26,29). This mutation leads to incorrect splicing of exon 11 and exon skipping, resulting in a frame shift starting at codon 355 and subsequent truncation of the protein at amino acid 419. All of the three Australian families with ATM mutations have been tested (protein truncation tests, single-stranded conformation polymorphism analysis, and DNA sequencing) for mutations in BRCA1 and BRCA2 and also haplotyped for markers flanking these loci. No mutations were detected in either gene, and in none of the families did all of the affected case patients share the same haplotype at either the BRCA1 or BRCA2 gene.

The ATM mutations that we identified segregated with breast cancer in the three families, giving a total LOD score of 1.18 ( $P = .01$ ). The penetrance of the IVS10-6T→G mutation was 78% (95% CI = 36% to 99%) to age 70 years, and the penetrance of the T7271G mutation was 55% (95% CI = 26% to 88%), giving an overall average penetrance of 60% (95% CI =

32% to 90%). These penetrance estimates equate to an overall 15.7-fold (95% CI = 6.4-fold to 38.0-fold) increased risk for development of breast cancer averaged up to age 70 years, which is not statistically significantly different from estimates derived from analysis of obligate carriers in A-T families (3-6,46) or carriers of pathogenic mutations in BRCA1 and BRCA2. Apart from female breast cancer, the only other cancer that appears to be elevated in frequency in heterozygous A-T carriers is gastric cancer (5), although this effect may be limited to female carriers (4). We observed four case patients with gastric cancer among women from two of the kConFaB families reported herein, but the association of these cancers with the ATM mutations was not clear (Fig. 1).

*In vivo* analyses of cell lines established from individuals heterozygous for the T7271G and IVS10-6T→G mutations suggest that both of these ATM mutations act in a dominant negative manner so that the wild-type enzyme is unable to function normally in the presence of the mutant protein. Similarly, *in*



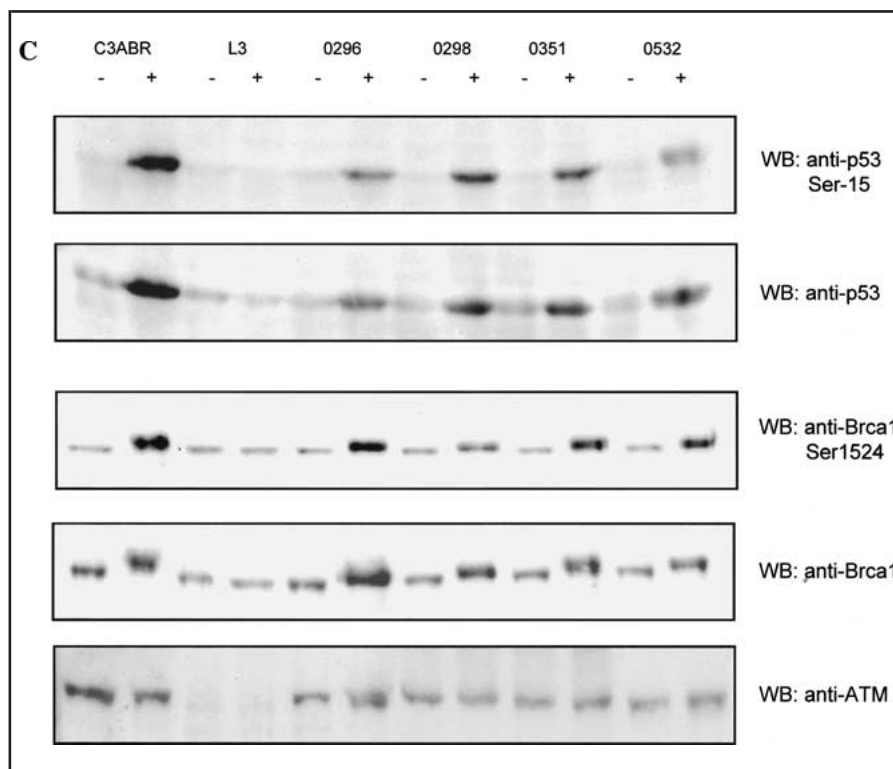


Fig. 3. (Continued).

*vitro* kinase assays suggest that the T7271G mutation acts as a dominant negative, although this activity was not evident for the IVS10-6T→G mutation, perhaps because of the instability of the truncated peptide in this assay. Our results show that the mutated ATM protein from the T7271G heterozygotes, in which Val-2424 is replaced by Gly, is stable but intrinsically defective as a kinase. It appears that, in cells with these mutant ATM alleles, p53 phosphorylation and stabilization are reduced, which presumably decreases the effectiveness of the G<sub>1</sub>/S checkpoint. These cells are also defective in DNA damage-induced phosphorylation of BRCA1. The mutant ATM allele may exert a dominant negative effect on the wild-type allele for a variety of reasons, including direct competition with wild-type protein for binding to key substrates and regulators, such as the p53 and BRCA1 proteins (23,38).

These *in vivo* assays of the heterozygote cell lines support the concept that the truncated ATM protein containing only the amino terminus, resulting from the IVS10-6T→G mutation, exerts a dominant negative effect on wild-type ATM activity, consistent with an earlier report (47) in which expression of the amino-terminal ATM fragment caused wild-type cells to become checkpoint deficient. However, the effect of the amino-terminal ATM fragment on endogenous ATM kinase activity was not addressed in that study. The dominant negative effect of mutant alleles is consistent with the absence of LOH observed in tumors arising in carriers of the mutations. This observation is reminiscent of a previous report of two ATM mutations (7636del9 and 3246insG) in two patients with breast cancer and with family histories of breast, gastric, and other cancers (48), in whom no LOH was detected in the breast tumors. Few family members could be genotyped in these families, but the authors concluded that the ATM mutations were unlikely to be related to breast cancer risk because no LOH was observed in index patients. The proposed dominant negative effect of the T7271G

and IVS10-6T→G ATM mutations with respect to BRCA1 and p53 phosphorylation may explain their high penetrance in these families. However, it is important to recognize that segregation of breast cancer in family A cannot solely be explained by the reduction in ATM kinase activity because both affected and unaffected carriers in the family showed markedly reduced ATM kinase activity compared with control subjects. The incomplete penetrance of mutations, which is also observed in BRCA1 and BRCA2 families, may be attributable to the influence of modifying genes and/or environmental factors.

Cultured cells from ATM heterozygotes are apparently moderately sensitive to radiation (49), which raises concerns about mammography and radiotherapy in ATM heterozygotes. The T7271G ATM homozygote mutant affected with breast cancer reported by Stankovic et al. (11) had a severe reaction to radiotherapy. However, we have found that T7271G heterozygous LCLs were not radiosensitive *in vitro* (data not shown). In contrast, the IVS10-6T→G heterozygous LCLs were moderately radiosensitive (data not shown), and one of the carriers (0536) in family B with this mutation had an extremely severe reaction to radiotherapy. It has been suggested previously that exposure to low-dose radiation at a young age may be an important component of breast cancer risk in the presence of an ATM mutation and that germline ATM mutations may be common in long-term survivors of breast cancer (29). However, none of the carriers studied herein had been exposed to x-rays in childhood or adolescence. Furthermore, four of the women with breast cancer in these ATM families, including one in family B, were known to have had radiotherapy, but there have been no reports of adverse reactions. However, it is notable that, of the 12 ATM carriers with breast cancer reported in these families, which was bilateral in two women, 11 are alive 2-14 years after diagnosis and the remaining case patient developed bowel cancer and died 2 years later. Given the dominant negative nature of these mutations, it

would be worthwhile to examine the carriers clinically for mild symptoms of A-T. It is interesting that one of the carriers of the IVS10-6T→G mutation carries a Robertsonian translocation and has had two trisomic conceptions, each carrying a different Robertsonian translocation. Whether these aberrations are related to her ATM carrier status is not clear, but it is interesting, given the chromosomal instability observed in A-T homozygotes.

Hereditary breast cancer is a heterogeneous disorder. The current inability to find convincing evidence for additional susceptibility genes from genome-wide scans of multiple-case families in which mutations in, or linkages to, BRCA1 and BRCA2 have not been found suggests that breast cancer in these families may be due to mutations in several other genes. The ATM gene has long been hypothesized to be a breast cancer susceptibility gene, but the evidence has been contradictory. Most mutation analyses of ATM in patients with breast cancer and in control subjects have not found the increased frequency of mutations in case patients that would be expected if these mutations did predispose to breast cancer (10,12,13,27). However, these studies have been limited by a lack of statistical power (14) and by the choice of method for mutation detection, most of which exclusively or preferentially detect protein-truncating mutations (50). Furthermore, these studies have focused largely on patients with sporadic breast cancer (i.e., those without a known family history of breast cancer), and few have tested for ATM mutations in multiple-case breast cancer families in which segregation between mutation and cancer can be examined (48). Our analysis of just two ATM mutations (IVS10-6T→G and T7271G) identified mutations in three (4%) of 76 multiple-case BRCA1/2-negative breast cancer families that segregated with disease with a total LOD score of 1.18 ( $P = .01$ ). This LOD score could have occurred by chance alone, but surprisingly the penetrance estimates were similar to those reported for BRCA1 and BRCA2 mutations in breast cancer families (1). This study, therefore, provides convincing evidence that at least two specific mutations in ATM can contribute to the development of breast cancer, and, furthermore, the strength of risk associated with these mutations is sufficient for them to cause multiple-case families. Our data suggest that full mutation analysis of the ATM gene would be worthwhile in other multiple-case breast cancer families, population-based and clinic-based, to clarify further the role of this gene in breast cancer susceptibility.

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## NOTES

*Note added in proof:* Since submission of this article, 68 additional index cases from non-BRCA1/2 multiple-case families have been screened for these mutations and two more have been found to carry the IVS10-6T→G ATM mutation.

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