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13. ABSTRACT ***(Maximum 200 Words)***

We are assessing whether a mutated allele of the Ataxia-telangiectasia gene, ATM, is a genetic risk factor for breast cancer, by screening for ATM mutations in patients with early-onset breast cancer. These patients were derived from a large population-based, case-control study of primary breast cancer in Western Washington. We are screening genomic DNAs by SSCP (single strand conformation polymorphism) analysis of the 62 coding exons amplified by multiplex PCR. In 142 breast cancer cases with early-onset or family history, we have found no truncation or deletion mutations in the ATM gene. However, possible ATM missense mutations were detected in 8.5% (12/142) of total cases, versus 1.25% (1/80) in controls. All 12 of the cases with possible mutations had some family history of breast cancer. In addition, 29 different rare variants were also identified. Some of these amino acid substitutions may also represent mutations, but their effects will require further functional studies. We have recently shifted our screening approach to utilize denaturing HPLC and have obtained increased sensitivity over SSCP for the detection of single nucleotide substitution in the ATM gene.

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Sharon Terada 9/16/99

PI - Signature Date

Table of Contents

SCREENING FOR ATAXIA-TELANGIECTASIA MUTATIONS IN A POPULATION-BASED SAMPLE OF WOMEN WITH EARLY-ONSET BREAST CANCER: FINAL REPORT, SEPT. 1999

Sharon N. Teraoka, Kathleen E. Malone, Elaine A. Ostrander, Suna Onengut, Aslihan Tolun, Janet R. Daling, and Patrick Concannon

Virginia Mason Research Center and Fred Hutchinson Cancer Research Center
Seattle, WA 98101, and Bogazici University, Bebek, Istanbul

Introduction

Recent studies have detected fewer mutations than expected in the major breast cancer susceptibility genes *BRCA1* and *BRCA2,* in families with high breast cancer incidence (1,2,3,4), in early-onset breast cancer cases (5,6) and in general populations (7,8). Therefore, other susceptibility genes should be considered. Heterozygosity in the gene for ataxia-telangiectasia (A-T), a rare, autosomal recessive disorder, has been suggested as a genetic risk factor for breast cancer (9). A-T homozygotes are characterized by progressive cerebellar ataxia and oculocutaneous telangiectases (dilated blood vessels), a 60-180 fold increased incidence of cancer, and hypersensitivity to ionizing radiation, among other multisystem effects (reviewed by Gatti (10)). The A-T gene, *ATM* (11,12), is a member of the phosphatidylinositol-3 kinase (PI-3 kinase) family and plays a role in detection of DNA damage and control of cell cycle progression. Although A-T is rare, A-T carriers are thought to be relatively common, estimated in epidemiological studies to be as much as 1.4% of the general population (13) and from a control population in a mutation screening study to be 1% of the general population (14). We have tested directly the hypothesis that a mutated A-T allele is a genetic risk factor for breast cancer, by screening for *ATM* mutations in patients with early-onset breast cancer or a family history of breast cancer. We screened the 62 coding exons of *ATM* in 142 cases and 80 controls derived from a large population-based study of primary breast cancer diagnosed by age 45 in Western Washington. A significant number of ATM missense mutations were detected among breast cancer cases and were associated with family history of breast cancer.

Materials and Methods

Breast Cancer Patient Population. The subjects for the current study were drawn from two previously completed population-based studies of breast cancer conducted the Fred Hutchinson Cancer Research Center. The cohort includes

cases of primary invasive breast cancer diagnosed before age 45 in a 3 county area of Washington. Controls were randomly ascertained and frequency age-matched. Methods for both studies, which together interviewed 1493 cases and 1571 controls, have been previously described (15,16). The subset of subjects for the current study includes two groups, those diagnosed before age 35, regardless of family history (99 cases) and those with a first-degree family history of breast cancer (43 cases). Laboratory investigators were blinded as to the case-control status and family history of the subjects at the time of mutation analysis.

Single Strand Conformation Polymorphism (SSCP) Analysis. *ATM* exons were amplified from 50 ng genomic DNA of each of the study subjects. Oligonucleotide primers, PCR, and SSCP conditions are described in the accompanying manuscript (Appendix IIIa). Conditions for amplifying 3 or 4 exons at once were empirically determined. PCR samples were denatured and separated on MDE (mutation detection enhancement) (FMC) gels with or without 10% or 5% glycerol.

Direct Sequencing of PCR Products. SSCP variant bands were isolated from gels, re-amplified and sequenced as described in the accompanying manuscript (Appendix IIIa).

Statistical Analysis. The Fisher exact test was used to assess whether proportions of women with and without an ATM missense mutation varied by age group, vital status, stage, and family history. Binomial exact 95% confidence intervals were calculated using Stata Statistical Software (Stat Corp., College Station, TX).

Results

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Genomic DNA samples from 142 breast cancer cases and 80 controls were each screened for mutations in all coding exons and flanking intronic sequences of the *ATM* gene. The results of the SSCP analysis are listed in Table 3, Appendix IIIa. No truncation mutations were detected. However, 13 missense mutations, of five different types, were identified. After the case-control status of the samples was decoded, 12 of the ATM missense mutations were assigned to breast cancer cases (8.5%, 95% CI 4.4%-14.3%) compared to 1 assigned to controls (1.3%, 95% CI 0.0-6.8%). The P-value for the difference = 0.04.

Because there are no reliable functional assays available for ATM, variants were scored as missense mutations based on several criteria, 1) whether they had been reported previously as mutations occurring in A-T

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patients, 2) whether they altered residues directly implicated in ATM function, 3) whether they altered residues conserved in ATM proteins of other species or in other members of the PI-3 kinase family, or 4) whether they would result in non-conservative amino acid substitutions. Mutation assignments were made prior to breaking the code on the samples.

Among the missense mutations detected, one mutation, S1691R, identified in two breast cancer cases in this study, had been previously reported as a mutation in an A-T patient (17) and also seen in a B-cell chronic lymphocytic leukemia (B-CLL) patient (18).

The mutation S707P, encoded in exon 15, occurred in six breast cancer patients. This non-conservative substitution is included within a mutation found in a Swedish A-T patient in which residues YSS (705-707) are replaced with FIP (19). The S707P variant occurred only once in 80 controls screened. The Annual Report for 1998 listed >80 controls screened for certain *ATM* variants. In the 1999 report, we list only 80 controls because that is the number of controls for which all *ATM* coding exons have been screened. Although *ATM* exon 15 was screened in 142 controls with only one S707P found, we could incorporate into the statistical analysis only the data from 80 controls for which all exons were screened.

Three breast cancer patients had single amino acid substitutions in regions of ATM of possible functional significance. In one patient, S1383L occurred immediately adjacent to a proline-rich motif recently identified as a c-abl binding site (20). ATM has been shown to interact with c-abl oncogene, and to kinase c-abl in fibroblasts that have been irradiated (20,21). The Serine at 1383 is conserved in ATM of mouse (22) and pufferfish, Spheroides nephelens (unpublished data). In a second patient, R2912G occurred in a highly conserved segment of the PI-3 kinase domain. In a third patient, M3011V, was located in the kinase domain at a less well conserved position, but one that is conserved in the yeast homologue of ATM, Tel1. None of these three variants has been seen in controls.

In addition, the putative splicing mutation IVS54+8A>G, created a potential new splice donor site 6 nucleotides after the canonical GT splice donor in a fourth patient. This variant was not detected in controls.

Relationships of *ATM* mutation frequency to different characteristics of the breast cancer cases were also assessed. All 12 *ATM* mutations occurred in the 91 breast cancer cases that had a family history (13%, 95% CI 7.0%-21.9%, P = 0.01). *ATM* mutation frequency was greater (14.3%, 95% CI 3.0%-36.3%) in cases with 4 or more affected family members as compared to those with 1-3 affected family members (7.4%, 95% CI 3.5%-13.7%). Mutation frequency increased with age of diagnosis, from 3.6% in those diagnosed before age 30 to 20.8% in those diagnosed from ages 40-44. Neither stage of disease nor vital status appeared to relate to *ATM* mutation status.

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We detected 29 additional rare variants in the *ATM* gene, 15 of which lead to amino acid substitutions (Table 6, Appendix IIIa). In light of recent studies suggesting that sequence variation in introns and exons distant from known splice consensus sequences can greatly influence correct splicing (23), and evidence of ATM missense mutations involved in T-cell prolymphocytic leukemia (T-PLL) (24,25), and B-CLL (18,26), the rare variants found in breast cancer patients cannot be ruled out as mutations.

Because of the large scale of the *ATM* screening project, we attempted to combine primers for several different exons in the same PCR amplification reaction. In practice, 4 exons at once was the upper limit. Table 2, Appendix IIIa lists many groups of *ATM* exons that are compatible for multiplex SSCP analysis. In the manuscript in Appendix IIIb, we optimized SSCP conditions for the detection of *ATM* mutations in A-T patients.

Discussion

We have accomplished technical objectives 1-4 of the Statement of Work of our research proposal by screening 142 breast cancer cases for germline *ATM* mutations. We have completed technical objective 5, task 1, screening for specific *ATM* variants in 80 controls. Studies of ATM protein function in this and other laboratories has been hampered by difficulty in reproducibly expressing the large and unstable protein in cell culture. Due to the lack of functional assays for ATM (task 2), we assigned mutation status to the DNA variants based on criteria listed in Results and in Appendix IIIa.

In the last year of the project, we assigned mutations, decoded case/control status, and subjected the data to statistical analyses. Because there were few cases/controls with prior radiation exposure, we replaced task 3 with the statistical analyses of *ATM* mutations in breast cancer cases with respect to family history, number of affected family members, age at onset, stage of disease, and vital status. Because of results indicating a role for single amino acid substitutions in ATM in breast cancer susceptibility, we have begun screening additional breast cancer cases utilizing a new technique with high sensitivity for detection of single nucleotide variants, temperature modulated heteroduplex analysis (TMHA), also referred to as denaturing HPLC. We found the Transgenomic™ TMHA platform to be more sensitive than SSCP for the detection of single nucleotide *ATM* mutations (Appendix IIIc).

Most published studies screening for *ATM* mutations in breast cancer cases reported no evidence of ATM truncation mutations in the breast cancer cases or found no significant increase in *ATM* mutations compared to controls (14,19,27,28,29). However, many of the studies, guided by early reports that ~90% of *ATM* mutations were chain terminating mutations (30), utilized the protein truncation assay (PTT). More recent reports of *ATM* mutations indicate frequency of truncation mutations is closer to 70% (13,31,32). Furthermore,

showed a high frequency of mostly missense ATM mutations clustered in the PI-3 kinase domain (24). ATM missense mutations were also found in British A-T patients who developed breast cancer, leukemia, or lymphoma (17) and in B-cell leukemia (B-CLL) patients (18,26). Our current study indicates ATM missense mutations may be involved in breast cancer cases with positive family history in a Western Washington population. Although the majority of A-T homozygotes have truncating ATM mutations, ATM missense mutations in heterozygotes may be of particular importance with regard to cancer susceptibility.

Conclusion

One of ATM's functions appears to be a response to cellular damage caused by ionizing radiation. Exposure to occupational and fluoroscopic diagnostic radiation and exposure to large doses of radiation such as in atomic bomb survivors and women with repeated fluoroscopy are risk factors for breast cancer (33,34,35). The radiosensitivity of A-T heterozygotes could put them at risk from standard diagnostic or occupational radiation exposures. The *ATM* gene possibly initiates the pathway through which cells deal with the environmental risk for breast cancer, ionizing radiation.

Recent evidence suggests that *BRCA1 and 2* participate in the cellular response to DNA damage via radiation as does *ATM* (36,37,38), giving us further reason to consider *ATM* as a third breast cancer susceptibility gene. *ATM* heterozygosity, at ~1% of the population, potentially accounts for a greater proportion of breast cancer cases than *BRCA1*, which accounts for an estimated 3 % of all U.S. breast cancer cases diagnosed by 80 years (10). In our study, 12 ATM missense mutations were detected in 142 breast cancer cases with early onset or positive family history diagnosed in western Washington, compared to 1 in 80 controls. Missense mutations can disrupt ATM functions and cause disease. Further analysis will be necessary to confirm whether a single ATM missense mutant confers susceptibility to breast cancer, but the results up to now are suggestive, based on position of the putative mutations within highly conserved regions, or previous sighting of the mutation in A-T patients. Because of the relatively high frequency of A-T carriers in the general population, it is important to identify them, as they may be predisposed to breast cancer and hypersensitive to standard therapeutic doses of radiation.

September 13, 1999

U.S. Army Medical Research and Materiel Command
ATTN: Judy Pawlus, Chief of Research Data Management
504 Scott Street
Fort Detrick, MD 21702-5012

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Sincerely,
Sharon Teraoka, Ph.D.

Appendix I Bibliography

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- Genomic DNA samples from 222 individuals were screened for mutations in each of the 62 coding exons of the ataxia-telangiectasia gene (*ATM*) using single-strand conformation polymorphism (SSCP) analysis; 142 of these samples were from breast cancer cases and 80 from matched controls.

- A total of 13 *ATM* mutations were identified, 12 among breast cancer cases (8.5%, 95% CI 4.4%-14.3%) and 1 among controls (1.3%, 95% CI 0.0-6.8%).

- All mutations detected were of the missense type; none were predicted to truncate the ATM protein.

- Among cases, mutations were found exclusively in patients with a family history of breast cancer (13.2%, 95% CI 7.0%-21.9%, P=0.01).

Appendix III

Reportable Outcomes Supported by DAMD17-96-1-6160

Papers

Teraoka, SN., Malone, KE., Doody, D., Ostrander, EA., Daling, JR., Concannon, P. (1999). Increased Frequency of *ATM* Mutations in Breast Cancer Patients with Early-Onset or Positive Family History. Submitted

Teraoka, SN., Telatar M., Becker-Catania S., Liang T., Onengut S., Tolun A., Chessa L., Sanal O, Bernatowska E., Gatti R., Concannon P. (1999) Splicing Defects in the Ataxia-Telangiectasia gene, *ATM:* Underlying Mutations and Consequences. Am. J. Hum. Genet. 64 (6):1617

Teraoka, S., Kuklin A, Concannon P (1999) Detection of Mutations in the ATM Gene: Comparison of Temperature Modulated Heteroduplex Analysis (THMA) with Single Strand Conformation Polymorphism (SSCP). Transgenomic Application Note 108.

Abstracts

Teraoka, SN., Malone, KE., Ostrander, EA., Daling, JR., Concannon, P. (1999). ATM Mutations in Breast Cancer Patients with Early-Onset or Family History. Proceedings of American Society of Human Genetics, Annual Meeting. In Press.

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Appendix IIIa

Increased Frequency of *ATM* Mutations in Breast Cancer Patients with Early-onset Disease or Positive Family History

Increased frequency of ATM mutations in breast cancer patients with early-onset disease or positive family history

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ABSTRACT

BACKGROUND: Studies of relatives of individuals affected with the rare recessive inherited disorder, ataxia-telangiectasia (A-T), suggest that heterozygosity for mutation in the ataxia-telangiectasia mutated (ATM) gene is associated with an increased risk for breast cancer. An increased frequency of ATM mutations in breast cancer cases compared to controls has not been reported, to date, but prior studies have focused largely on truncating mutations. METHODS: Genomic DNA samples from 222 individuals were screened for mutations of all types in each of the 62 coding exons of the ATM gene using single-strand conformation polymorphism (SSCP) analysis; 142 of these samples were from "high risk" breast cancer cases due to family history or early age at diagnosis and 80 were from matched controls. RESULTS: A total of 13 ATM mutations were identified, 12 among breast cancer cases (8.5%, 95% CI 4.4%-14.3%) and 1 among controls (1.3%, 95% CI 0.0-6.8%). All mutations detected were of the missense type; none were predicted to truncate the ATM protein. Among cases, mutations were found exclusively in patients with a family history of breast cancer (13.2%, 95% CI 7.0%-21.9%, P=0.01). CONCLUSIONS: Among women tested in this study, missense mutations within the ATM gene are observed significantly more frequently in breast cancer cases than controls. We propose that the increased cancer incidence observed with A-T heterozygosity may reflect the effects of specific ATM missense mutations that predispose to cancer, possibly via a dominant negative mechanism.

An increased incidence of breast cancer has been reported among relatives of patients with the autosomal recessive disorder ataxia-telangiectasia (A-T) (1-3). A-T is characterized by progressive cerebellar ataxia, oculocutaneous telangiectases (dilated blood vessels), increased incidence of cancer, hypersensitivity to ionizing radiation, chromosomal instability, cellular and humoral immunodeficiencies, and developmental defects in various organ systems (reviewed in 4). Upon exposure to ionizing radiation, cultured fibroblasts from affected individuals exhibit a characteristic phenotype that includes reduced colony forming capacity and an inability to limit DNA synthesis (reviewed in 5). The gene that is mutated in A-T, ATM, encodes a member of the phosphatidylinositol-3 kinase (PI-3 kinase) family of proteins defined by sequence similarity in the carboxy-terminal tenth of the protein (~350 amino acids) to the catalytic domain of PI-3 kinase (6,7). Based on the phenotype of A-T patients and their cells in culture, ATM appears to play a role in monitoring genomic integrity and triggering appropriate cell cycle checkpoints or apoptotic pathways in response to DNA damage, specifically double strand breaks.

Although the disease A-T is rare (seen only in homozygotes), A-T carriers (heterozygotes) are thought to be relatively common, with an estimated frequency in the U. S. population of 1-3% (8-10). Several lines of evidence implicate ATM heterozygosity in cancer susceptibility. Studies of American A-T families have revealed an increased incidence of cancer in general, and breast cancer, in particular, among A-T blood relatives as compared to spouse controls (1,2,11). Haplotyping for markers near the ATM gene within these A-T families, allowing reliable identification of A-T carriers, has confirmed these observations (3). Studies of British (12-14) and

Norwegian (15) A-T families have also reported an excess of breast cancer among A-T heterozygotes. In addition, 2 types of leukemia, T-prolymphocytic leukemia (T-PLL) and B-cell chronic lymphocytic leukemia (B-CLL), have been associated with heterozygosity for ATM mutations (16-21). In each case, predominantly missense mutations are observed.

Given the high estimates of A-T heterozygosity in the population and the increased breast cancer incidence among A-T heterozygotes reported in A-T families, ATM mutations could potentially be an important contributor to breast cancer incidence in the general population. Several studies have attempted to assess the public health impact of A-T heterozygosity by screening breast cancer patients for A-T mutations. These studies have reached disparate conclusions (9, 22-25) but, in most cases, have had only limited power to test the underlying hypothesis (26). Moreover, the recent reports of increases specifically in missense types of ATM mutations in patients with other cancers such as T-PLL and B-CLL (16-21) suggest that those prior studies, which relied exclusively on protein truncation testing, may have overlooked relevant missense mutations in the ATM gene.

In this current study, a series of women diagnosed with breast cancer before age 45 was screened for mutations in the ATM gene by SSCP (single strand conformation polymorphism) analysis. In order to insure that as broad a spectrum of mutations as possible was detected in the screening process, multiple sets of conditions were used. These conditions had been optimized in prior mutation screens of the ATM gene in A-T patients (27,28).

MATERIALS AND METHODS

Study Population

The subjects for the current study were drawn from two previously completed population-based case-control studies of breast cancer conducted at the Fred Hutchinson Cancer Research Center. Cases diagnosed with breast cancer before age 45 in a three county area in Western Washington State were ascertained via a population-based cancer registry; controls (women of similar age with no history of breast cancer) were ascertained via random digit dialing. Methods for both studies, which altogether interviewed 1493 cases and 1571 controls, have been previously described (29,30). Updated pedigrees and additional blood samples were obtained as part of a subsequent genetic-epidemiology investigation. Written informed consent was obtained from all subjects prior to their enrollment. Samples for the current analyses were accessed from a subset of study subjects originally targeted for BRCA1 and BRCA2 mutation screening (31, Malone KE, Daling JR, Neal C, Suter NM, O'Brien C, Cushing K, et al. manuscript submitted). This subset included two groups of cases: those with a first-degree family history of breast cancer (i.e. an affected mother and/or sister) and those diagnosed before age 35 (regardless of family history), for a total of 386 cases along with an additional 251 controls. The specific subjects enrolled in the current study represent the first 142 consecutive case samples and 80 control samples available for analysis from these 386 cases and 251 controls. The 142 cases were comprised of 76 with diagnosis before age 35, 43 with a first degree family history of breast cancer, and 23 that fulfilled both criteria.

Single Strand Conformation Polymorphism (SSCP) Analysis

ATM exons and intron/exon boundaries were amplified from 50 ng samples of genomic DNA. Oligonucleotide primers are listed in Table 1. PCR reactions contained 200 μM dCTP, dGTP, dTTP, 20 μM dATP, 3 μM of each primer, .05 μC ^{33}P-dATP (Amersham), 1.25 units Hot Tub polymerase (Amersham), Amplitaq (Perkin-Elmer) or Taq DNA polymerase (Boehringer Mannheim), and 1X of the corresponding manufacturer's reaction buffer in a total volume of 50 μl. After denaturing for 5 min. at 94°C, amplification was carried out for 35 cycles of 94°C, 30 s, 54°C, 30 s, 72° C, 1 min. Samples were denatured and separated on MDE (FMC) gels with or without 5% or 10% glycerol.

In order to increase the speed of screening, conditions were established such that 3 to 4 exons could be amplified simultaneously and screened in a single reaction. In these multiplex PCR reactions, primer concentration was reduced by as much as 50% per primer and ^{33}P-dATP increased to 0.1 μC. All other reagents and volumes were kept identical to those in the single exon reactions. Using the pools of primers indicated in Table 2, 24 PCR reactions are sufficient to screen all 62 coding exons of ATM for mutations.

Direct Sequencing of PCR Products

PCR products were purified using QIAquik Spin PCR Purification kits (Qiagen) or High Pure PCR Product Purification kits (Boehringer Mannheim). 5-10 μl of PCR product served as the template in reactions using the Taq DyeDeoxy terminator cycle sequencing kit (ABI). Unincorporated nucleotides were removed using Centri-sep

columns (Princeton Separations). Sequencing reactions were analyzed on an ABI 373A sequencer.

Classification of Mutations and Variants

Observed nucleotide substitutions were categorized as either mutations or rare variants based on 4 criteria, (1) whether they had been reported previously as mutations occurring in A-T patients or controls, (2) whether they altered residues directly implicated in ATM function (e.g. such as specific, functionally important residues in the kinase domain), (3) whether they altered residues conserved in more distantly related members of the PI-3K family of kinases, or (4) whether they altered residues conserved in the ATM proteins of most other species where sequence data are available, specifically mouse (32), pufferfish (unpublished data), and yeast (33). In all instances, assignment of a specific change to either the "mutation" or "rare variant" category was made prior to breaking the code on the samples. Only after screening and mutation classification was completed, was information regarding the samples (i.e. case or control, family history, etc.) decoded.

Statistical Analyses

The Fisher exact test was used to assess whether proportions of women with and without an ATM missense mutation varied by age group, vital status, stage, and family history. Binomial exact 95% confidence intervals were calculated for the proportions with mutation. P-values and confidence intervals were calculated using Stata Statistical Software (Stata Corp., College Station, TX).

RESULTS

Genomic DNA samples from 142 breast cancer cases and 80 controls were screened for mutations in all coding exons and flanking intronic sequences of the ATM gene. No coding region mutations predicted to frameshift or truncate the ATM protein were detected. However, more than 40 unique nucleotide alterations, many predicted to change the amino acid sequence of the ATM protein, were identified. Based on the criteria described above, 13 changes were scored as missense mutations (Table 3). After the case-control status of samples was decoded, 12 of the 142 breast cancer cases screened, (8.5%, 95% CI 4.4%-14.3%) had a germline missense mutation, compared to 1 (1.3%, 95% CI 0.0-6.8%) of the 80 control samples tested (P-value for difference = 0.04).

The specific nucleotide changes scored as missense mutations met 1 or more of the criteria outlined in the Materials and Methods section. One mutation, S1691R, identified in two breast cancer patients in this study, had been previously reported as a mutation in an A-T patient (13). This mutation had also been reported in a B-CLL patient whose leukemic cells failed to produce ATM protein and displayed loss of heterozygosity (LOH) for the 11q23 chromosomal region, where the ATM gene is located (20).

The most common mutation detected was S707P, in exon 15, which was observed in 6 breast cancer cases and one control sample. While this specific non-conservative substitution has not been previously described in an A-T patient, it is

included within a previously reported A-T mutation in which 3 residues, YSS (705-707) are substituted with FIP (705-707) (34).

One mutation, IVS54+8G>T occurs in the intron between exons 54 and 55. This change creates a new potential splice donor site, 6 nucleotides after the end of exon 54. The predicted outcome of the use of this site would be an insertion of 2 amino acids, maintaining the open reading frame of the ATM protein.

Three of the substitutions assigned as mutations occurred in conserved regions of the ATM gene with likely functional significance. The substitution in exon 30, S1383L, is immediately adjacent to the reported c-abl binding site in ATM and is conserved in ATM homologs in other species. The substitution in exon 62, R2912G, alters a residue that is conserved in all ATM homologues including the Tel1 protein of *Saccharomyces cerevisiae*. It is also conserved, along with the 6 amino acids that precede it, in most other members of the PI-3 kinase family of proteins including distantly related molecules such as the catalytic subunit of the DNA-dependent protein kinase, DNA-PKcs. The substitution in exon 65, M3011V, occurs in the kinase domain at a position that is conserved among ATM homologues, but not among other PI-3 kinase family members.

In addition to case-control status, mutation frequency was also assessed for relationship to proband characteristics and family history. Mutation frequency increased with age of diagnosis, from 3.6% in those diagnosed before age 30 to 20.8% in those diagnosed ages 40-44 (Table 4). Neither stage of disease nor vital status appeared to relate to ATM mutation status.

ATM mutation frequency varied by family history (Table 5). There were no mutations observed in the 43 breast cancer cases that had no family history of breast cancer, whereas 12 (13.2%, 95% CI 7.0%-21.9%) mutations were found among the 91 breast cancer cases that had any family history of breast cancer (P = 0.01). Mutations were slightly more frequent among the 66 women with first-degree family history (13.6%, 95% CI 6.4%-24.3%) than in those with only a second-degree affected relative (8.3%, 95% CI 1.0%-27.0%).

The frequency of mutations also varied by the number of family members with breast cancer. Mutation frequency was greater (14.3%, 95% CI 3.0%-36.3%) in those with 4 or more affected family members as compared to those with 1-3 affected family members (7.4%, 95% CI 3.5%-13.7%, p=0.39). There was no variation in mutation frequency in cases whose affected first-degree relative(s) had only been diagnosed at age 45 or later (12.9%) when compared with cases with at least one affected first-degree relative diagnosed before age 45 (12.5%). Similar consideration of the age of diagnosis of all affected relatives (including both first- and second-degree relatives) found that mutations were more common in cases whose affected relatives had all been diagnosed at age 45 or later (16.3%, 95% CI 6.8-30.7) compared to cases who had at least one relative diagnosed before age 45 (9.3%, 95% CI 2.6%-22.1%); however, this difference was not statistically significant (P=0.52).

All subjects in this study have been screened for mutations in the BRCA1 and BRCA2 genes. Among the cases in this study with BRCA1 mutations, 2 also had mutations in ATM. While the BRCA1 mutations in these cases, 185delAG and

3875del4, were distinct, the ATM mutations were the same, 2119T>C. There were no cases with both BRCA2 and ATM mutations.

A total of 29 additional alterations in the ATM gene were detected that were classified as rare variants (Table 6). Fifteen of these rare variants occurred within coding regions, and were predicted to result in amino acid substitutions. There was no significant difference in the overall frequencies of rare variants in the case and control populations. However, one silent substitution, 4578C>T, was substantially more frequent among cases compared to controls. Although listed as rare variants, the changes in Table 6 cannot be ruled out as mutations given reports that alterations within exons or at some distance from consensus splice sites can affect splicing (35,36), and that the functional consequences of amino acid substitutions in ATM cannot be readily tested (37).

DISCUSSION

Prior studies have documented an excess of breast cancers occurring in blood relatives of A-T patients (1,2,12,15). Haplotyping with highly informative genetic markers flanking the ATM gene (3) and mutation screening of ATM (13) have further confirmed that increased risk for breast cancer is associated with heterozygosity for A-T mutations. These findings raise an important public health question--do mutations in the ATM gene make a significant contribution to the incidence of breast cancer in the general population? In order to address this question, it is necessary to obtain

accurate estimates of the A-T carrier frequency both in the general population and among individuals with breast cancer.

Initial surveys of breast cancer cases for mutations in the ATM gene were guided by reports suggesting that approximately 90% of A-T alleles carried truncating or otherwise null mutations (38). Thus, the protein truncation test (PTT) appeared to be the method of choice for mutation detection. Studies such as that reported by FitzGerald et al. 1997 (9), in which 401 breast cancer patients and 202 controls were screened by PTT, revealed only modest numbers of truncating ATM mutations, 2 each in cases and controls. In the current study, no truncating or null mutations were detected among either 142 cases or 80 controls.

How can the seemingly disparate results obtained from studies of A-T families as opposed to population screening of breast cancer cases be reconciled? Bishop and Hopper (26) have suggested that the confidence intervals for estimates of the heterozygote frequency and risk associated with A-T heterozygosity are quite wide and overlap over a substantial portion of their range. Thus, the results from these different types of studies may well be consistent.

We have recently proposed an additional mechanistic explanation (Gatti RA, Tward A, Concannon P, submitted) based on 2 hypotheses; (1) that different ATM mutations may vary in their penetrance for cancer susceptibility in the heterozygous state and (2) that homozygosity for some ATM missense mutations may not result in the neurologic features associated with a diagnosis of A-T. Recent mutation screening studies in A-T patients indicate that the frequency of truncating mutations is less than originally thought, closer to 70% rather than 90% (13,27,39). Truncating mutations in

ATM are predominantly null alleles that make no detectable protein of any length (13). Missense mutations, which constitute most of the remaining 30% of mutations in A-T patients, are much more frequently associated with production of some level of ATM protein, albeit a mutant form (13). The ATM protein is known to exist in cells as part of a multiprotein complex (40). Expression of a mutant form of the ATM protein, as would occur from certain missense alleles, could disrupt the function of this complex via a "dominant negative" mechanism. Thus, the expression of a mutant form of the ATM protein might have phenotypic effects beyond what would be observed if overall ATM protein levels were reduced by the presence of null mutations.

There is accumulating data suggesting an association between ATM missense mutations and malignancy. Missense mutations represent the majority of ATM mutations documented in sporadic cases of cancers such as T-PLL and B-CLL. In a comprehensive study of A-T patients in the British Isles, Stankovic et al. (13) reported that A-T patients developing leukemia or lymphoma were much more likely than other A-T patients to carry at least one A-T allele with either a missense mutation or short in-frame deletion that still allowed for some production of ATM protein. They also described 2 families segregating a missense mutation, 7271T>G, that was associated with increased risk of breast cancer in the heterozygous state and with an extremely mild neurological phenotype, not typical of A-T, in the homozygous state. This mutation allowed for the production of mutant ATM protein. The 7271 T>G mutation has been detected independently in a B-CLL patient in conjunction with loss of heterozygosity for the normal ATM allele (20). Therefore, in other cancers, specifically leukemias and lymphomas, the spectrum of ATM mutations is weighted towards missense mutations,

both in sporadic cases occurring among A-T heterozygotes, and in cases occurring among compound heterozygote A-T patients.

In the current study, SSCP was employed as a screening method to insure that all types of alterations in the ATM gene, whether truncating mutations, missense mutations or rare polymorphic variants could potentially be detected. The screening conditions were optimized in the course of identifying ATM mutations in 2 studies of A-T patients in which 154 mutations were identified (27,28). All samples in the current study were screened using at least 2 different gel conditions to enhance sensitivity. As a result, a larger number of variants and mutations were identified than in previous ATM mutation screens in breast cancer and a majority of these were novel.

Unambiguous assignment of alterations in the ATM gene as either deleterious mutations or rare variants with minor phenotypic consequences remains a significant challenge. The ATM gene is unstable in a wide range of cloning vectors and hosts. Disregulated expression, as occurs when the gene is introduced exogenously into cells under the control of a strong promoter, typically is toxic (37). Therefore, although complementation of A-T cell lines by transfection with expression vectors containing the ATM gene has been achieved (41,42), routine success with such approaches has not.

Lacking reliable functional approaches to evaluate the consequences of variation in ATM, the current study relies on a series of criteria based on consideration of functional domains and evolutionary conservation in assigning observed changes as mutations or as rare polymorphic variants. Application of these criteria still requires some subjective decision-making on the status of individual variants. In order to limit the possibility of bias in this decision process, investigators who carried out the

screening and evaluation of all of the samples were blinded as to whether they were derived from patients or controls. Samples were selected for study and coded as to their identity by investigators at one institution and laboratory screening and assignment as mutation or rare variant was then carried out at a separate institution.

The specific increase observed in the frequency of ATM missense mutations in breast cancer cases in this study is consistent with the explanation proposed above, that is, that different ATM mutations may differ in their penetrance for cancer susceptibility and that ATM missense alleles may be of particular importance in this regard. However, the benefits of studying cases drawn from a population-based study, as reported here, need to be balanced with the acknowledgment that the subgroup of 142 breast cancer cases studied may not be representative of all women from the original study. Since this set of subjects was specifically drawn from the subgroup targeted for BRCA1 and BRCA2 analyses, the cases are rather unique having been selected for early age of diagnosis and/or positive family history. Generalizability may also be influenced by the loss of otherwise eligible subjects who died before samples were obtained, as previously described (31).

The results reported here suggest several directions for possible follow-up studies. First, in order to evaluate fully the effects of ATM missense mutations, it will be necessary to carry out larger, population-based screens of breast cancer cases for ATM mutations utilizing techniques that are highly efficient for the identification of single nucleotide substitutions. Second, the observed pattern of increasing mutation frequency with age at diagnosis suggests that future studies should also explore the role of ATM heterozygosity in breast cancer cases with later ages of diagnosis. A

similar conclusion was reached by Athma et al. (3) in their study of breast cancer among A-T heterozygotes identified by marker haplotyping within A-T families. They reported an increased risk ratio for breast cancer among older patients, specifically those with age at diagnosis greater than 60 years. Finally, all of the breast cancer cases in the current study that had ATM missense mutations also had a family history of breast cancer. While it could be argued that the history of breast cancer in these families might result largely from the segregation of predisposing ATM alleles, it might also be worth considering whether mutant ATM alleles interact with predisposing alleles at other loci in such families. For example, 2 cases among those studied here had mutations in both the ATM and BRCA1 genes.

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NOTES

S. N. Teraoka and K. E. Malone contributed equally to this work.

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Table 1. ATM primers used to amplify genomic DNA

Exon	Primer sequence 5' to 3'	Product size in bp
Exon 4 F	CCTCTTTCTCTCTATATATGC	160
Exon 4R	AATAATGGGTTACTAATCACA	
Exon 5.1F	GATTAGTAACCCATTATTATTC	210
Exon 5R	CAACAGAAATAAATATGAAAGAG	
Exon 6.1F	GATGGCATGAACAGCTTTTG	280
Exon 6R	CTCACGCGACAGTAATCTG	
Exon 7F	TAGTTGCCATTCCAAGTGTC	288
Exon 7R	TGAAGTTTCATTTCATGAGG	
Exon 8F	TTTTTCTGTATGGGATTATGGA	327
Exon 8R	CATGGTCTTGCAAGATC	
Exon 9F	CCCCCTGTTATACCCAGTT	318
Exon 9R	TGAAGAAGCAAATTCAAAACAG	
Exon 10F	TTTGTGGGGAGCTAGCAGTG	262
Exon 10.1R	TCTAAATGTGACATGACCTAC	
Exon 11.1F	GGCTCAAAAAAAAAAAAAAG	265
Exon 11R	ACAAGAGATTAAAATGACACT	
Exon 12F	GTTTGTTAATGTGATGGAATA	467
Exon 12R	GTGTGTTTATCTGTAAGTCAG	
Exon 13F	ATAAAGTCTTTGCCCCTCCA	320
Exon 13R	AAATAAGTGGAGAGAGCCTG	
Exon 14F	GGCTTTTGGTCTTCTAAGTG	192
Exon 14R	ATCTTTGTAATTAAAGCTATAGC	
Exon 15F	GTAGTCTTTGAATGATGTAGA	377
Exon 15R	CTATTTCTCCTTCCTAACAGT	
Exon 16.2F	GTTCTTACAAAAGATAGAGTATAC	329
Exon 16.2R	TTCACAGGAATACATTTCATTC	
Exon 17.2F	GTCCAAGATCAAAGTACACTG	314
Exon 17.2R	GTGACAGAGAAAGATCCTATC	
Exon 18F	ATATTGGCCCTAATAGTAAAC	292
Exon 18.2R	CCTTATTTACAAAGATATTTCAAC	
Exon 19F	AATTGCTGAGATTACAGATGT	352
Exon 19.1R	GCCTCTTATACTGCCAAATCA	
Exon 20.2F	TATATATGGCTGTTGTGCCC	314

Exon 20R	TACATTTAGTCAGCAACATCA	
Exon 21F	CCGGCCTATGTTTATATACTT	225
Exon 21R	TTAACAGAACACATCAGTTAT	
Exon 22.2F	AACTGATGTGTTCTGTTAAGC	274
Exon 22R	CTTGCATTCGTATCCACAGAT	
Exon 23F	TTAGCACAGAAAGACATATTG	259
Exon 23R	AATTACTCATTAACAAACAAA	
Exon 24F	GCAGTCTTTGTTTGTTAATGA	274
Exon 24R	CTATGTAAGACATTCTACTGC	
Exon 25F	GTTTGTTTGCTTGCTTGTTT	203
Exon 25R	ATTTATGGGATATTCATAGC	
Exon 26F	TGGAGTTCAGTTGGGATTTTA	304
Exon 26R	TTCACAGTGACCTAAGGAAGC	
Exon 27F	GTTGTTTCTAGGTCCTACTCT	333
Exon 27R	GACTTGCTAAGTATTGTTAAC	
Exon 28F	TGATACTTTAATGCTGATGGT	409
Exon 28R	GGTTATATCTCATATCATTCA	
Exon 29F	TCCTCTTAGTCTACAGGTTG	257
Exon 29R	GACATTGAAGGTGTCAACCA	
Exon 30F	TGGAAGTTCACTGGTCTATG	283
Exon 30R	TACTTTTCCTCTTTAAGATGTAT	
Exon 31F	TTTATTGTAGCCGAGTATCTAA	318
Exon 31R	AAACAGGAAGAACAGGATAGA	
Exon 32F	TGCTGAACCAAAGGACTTCT	334
Exon 32R	CACTCAAATCCTTCTAACAATA	
Exon 33F	CAGTAAGTTTTGTTGGCTTAC	315
Exon 33R	CTGCTAGAGCATTACAGATTT	
Exon 34F	TGTCTATAAATGGCACTTAACT	311
Exon 34R	CCAAGAGCAAGACTTTGCAAA	
Exon 35F	TAGAAGTTTTCTAGTCAGATAAT	255
Exon 35R	AATCTGTCCTATATGTGATCC	
Exon 36F	CTTGAAGTACAGAAAAACAGC	336
Exon 36R	GTATCATTCTCCATGAATGTC	
Exon 37F	TGGAGGTTAACATTCATCAAG	287
Exon 37R	ATTTAACAGTCATGACCCACA	
Exon 38F	GGAAAGGTACAATGATTTCCA	312
Exon 38R	ATGTGCAGTATCACAGCACT	

Exon 39F	GTATGTTGAGTTTATGGCAGA	376
Exon 39R	ATCCATCTTTCTCTAGAACTG	
Exon 40F	ACCAGAACCTTATAGCATAGT	247
Exon 40R	TTCAGCCGATAGTTAACAAGT	
Exon 41F	TAAGCAGTCACTACCATTGTA	314
Exon 41R	ATACCCTTATTGAGACAATGC	
Exon 42F	GTATATGTATTCAGGAGCTTC	238
Exon 42R	ATGGCATCTGTACAGTGTCT	
Exon 43F	CAGAACTGTATTTCAGAATCAT	387
Exon 43R	ACATAACTCCTTCATAAACAGT	
Exon 44F	CCAAAGCTATTTTCACAATCTT	262
Exon 44R	TACTGAAATAACCTCAGCACT	
Exon 45F	CTCTGGTTTTCTGTTGATATC	236
Exon 45R	CCCCATGAAGAATCAAGTC	
Exon 46F	TTTATACATGTATATCTTAGGGTTCTG	220
Exon 46R	TTCAGAAAAGAAGCCATGACA	
Exon 47F	TATTTCCCTGAAAACCTCTTC	233
Exon 47R	CACTATTGGTAACAGAAAAGC	
Exon 48F	TCATTTCTCTTGCTTACATGAA	314
Exon 48R	AAAGGAAAGTCAAGAGGTAAG	
Exon 49F	ATGGTAGTTGCTGCTTTCATT	365
Exon 49R	TTACTAATTTCAAGGCTCTAATA	
Exon 50F	AGTTGGGTACAGTCATGGTA	230
Exon 50R	GAAAAGATGAAGCATATTCATG	
Exon 51F	TTTGAGTGATTCTTTAGATGTAT	352
Exon 51R	AACAACTCACTCAGTTAACTG	
Exon 52F	TGTGTGATTTTGTAGTTCTGTT	340
Exon 52R	ACATCAAGGGGCTTATGTCT	
Exon 53F	ACTTACTTGCTTAGATGTGAG	282
Exon 53R	CCATTTCTTAGAGGGAATGG	
Exon 54F	CACTGCAGTATCTAGACAGT	322
Exon 54R	CTAGGAAAGACTGAATATCAC	
Exon 55F	AATGTTGGGTAGTTCCTTATG	308
Exon 55R	GCTTTTGGATTACGTTTGTGA	
Exon 56F	TGACTATTCCTGCTTGACCT	253
Exon 56R	TTTCACCAATTTTGACCTACAT	
Exon 57F	TAACCACTATCACATCGTCAT	385

Exon 57R	CTTCCTCATTTGTAAGTATTCA	
Exon 58F	CCTTTGCTATTCTCAGATGACTCTGT	290
Exon 58R	GCATTATGAATATGGGCATGA	
Exon 59F	GATCATCAAATGCTCTTTAATG	286
Exon 59R	TATCTGACAGCTGTCAGCTT	
Exon 60F	GTGTATATTAGTTTAATTGAACAC	279
Exon 60R	AACCTGCCAAACAACAAAGTG	
Exon 61F	TAGAAAGAGATGGAATCAGTG	317
Exon 61R	ATCTTGGTAGGCAAACAACAT	
Exon 62F	AAAGTTCACATTCTAACTGGAA	272
Exon 62R	ATTACAGGTGCAAAGAACCAT	
Exon 63F	GATAAAGATACGTTGTTGACAACATTGG	199
Exon 63R	GTGACTTCCTGATGAGATACACAG	
Exon 64F	CTGGTTCTACTGTTTCTAAGT	298
Exon 64R	GTTTCAGTGAGGTGAACAGT	
Exon 65F	TCCTGTTGTCAGTTTTTCAGA	354
Exon 65R	ACTTAAAGTATGTTGGCAGGT	

Table 2. ATM exon groupings for multiplex SSCP analysis.

Pool	Exons
A	5, 10, 38, 65
B	8, 29, 61, 44
C	19,26, 11, 6
D	51, 31, 55
E	36, 18, 42
F	33, 17, 56, 50
G	49, 48, 30, 47
H	32, 35, 40, 4
I	14, 45, 52
J	24, 27, 53, 63
K	12, 15, 13
L	28, 59, 58, 25
M	57, 54, 21
N	41, 22, 46
O	7, 9, 64

Table 3. ATM Mutations in Breast Cancer Cases and Controls

Exon	Nucleotide change[1,2]	Codon change[3]	Number in breast cancer cases (n=142)	Number in controls (n=80)
15	2119T>C	707S>P	6	1
30	4148C>T	1383S>L	1	0
36	5071A>C	1691S>R	2	0
54	IVS54+8G>T	--	1	0
62	8734A>G	2912R>G	1	0
65	9031A>G	3011M>V	1	0
Totals			12[4]	1

[1]Nucleotide numbering is based on the sequence GenBank accession number U33841. The first nucleotide of the start codon is taken as position 1

[2]IVS refers to introns and nucleotides therein are numbered such that the splice acceptor AG is numbered -2,-1 and the splice donor GT is numbered +1,+2.

[3]The first methionine in the open reading frame is at position 1.

[4]P-value for difference with controls, 0.04.

Table 4. Distribution of ATM missense mutations according to disease characteristics in women diagnosed with breast cancer before age 45 in Western Washington.

Characteristic	Number tested n	Missense Mutations n	%	95% C.I.[1]	P-Value[2]
All tested	**142**	**12**	**8.5**	**(4.4-14.3)**	
Age of reference					
21-29	28	1	3.6	(0.1-18.3)	
30-34	71	4	5.6	(1.6-13.8)	
35-39	19	2	10.5	(1.3-33.1)	
40-44	24	5	20.8	(7.1-42.2)	.09
Stage of disease					
in situ	17	0	--	(0-19.5) [3]	
local	84	8	9.5	(4.2-17.9)	
regional/distant	41	4	9.8	(2.7-23.1)	.51
Vital status					
alive	123	10	8.1	(4.0-14.4)	
deceased	19	2	10.5	(1.3-33.1)	.66

[1] Confidence interval for the proportion with missense mutations.
[2] P-value for the differences in mutation frequency between groups, Fisher's Exact
[3] One-sided, 97.5% confidence interval

Table 5. Distribution of ATM missense mutations according to features of family history in women diagnosed with breast cancer before age 45 in Western Washington.

Characteristics	Number tested	Missense mutations		95% C.I.[1]	P-Value[2]
	n	n	(%)		
Family history of breast cancer[3]					
none	43	0	--	(0-8.2) [5]	
mother and/or sister	66	9	13.6	(6.4-24.3)	
aunt/grandmother only	24	2	8.3	(1.0-27.0)	
other relative only	1	1	100.0	(2.5-100) [5]	
Insufficient information	8	0	--	(0-36.9)	.01
none	43	0	--	(0-8.2) [5]	
any	91	12	13.2	(7.0-21.9)	.01
Age of breast cancer diagnosis in relatives					
mother/sister < age 45	32	4	12.5	(3.5-29.0)	
mother/sister ≥ age 45	31	4	12.9	(3.6-29.8)	0.99
any relatives < age 45	43	4	9.3	(2.6-22.1)	
all relatives ≥ age 45	43	7	16.3	(6.8-30.7)	.52
Number of family members with breast cancer[4]					
1-3	121	9	7.4	(3.5-13.7)	
4 or more	21	3	14.3	(3.0-36.3)	.39

[1] Confidence interval for the proportion with missense mutations
[2] P-value for differences in mutation frequency between groups, Fisher's Exact
[3] One Proband was adopted and did not provide family history
[4] Includes proband's cancer
[5] One-sided, 97.5% confidence interval

Table 6. ATM Rare Variants in Breast Cancer Cases and Controls

Nucleotide change[1,2]	Codon change[3]	Number in breast cancer cases (n=142)	Number in controls (n=80)
370A>G	124I>V	1	0
378T>A	126D>G	0	1
IVS7+28insA	--	0	1
IVS7+18T>C	--	1	0
609C>A	203D>E	1	0
998C>T	--	0	1
1541G>A	514G>E	1	1
IVS16+22A>C,	--	2	0
IVS16+34A>C	--	2	0
2572T>C	858F>L	3	6
IVS19-17G>T	--	1	0
2805G>C	--	1	0
IVS25+32delCAT	--	1	0
4258C>T	1420L>F	0	2
4578C>T	--	23	1
IVS33-20A>G	--	0	1
IVS35-66T>G	--	0	1
IVS38-112G>A	--	1	1
IVS38-66T>G	--	0	1
IVS38-62A>G	--	1	0
IVS38-15G>C	--	1	2
IVS38-8T>C	--	5	5
5558A>T	1853D>V	2	2
5793T>C	--	1	1
6088A>G	2030I>V	1	1
6919C>T	2307L>F	1	0
6988C>G	2330L>V	1	0
8000T>C	2667M>T	1	0
IVS62+8A>C	--	4	2

[1]Nucleotide numbering is based on the sequence GenBank accession number U33841. The first nucleotide of the start codon is taken as position 1

[2]IVS refers to introns and nucleotides therein are numbered such that the splice acceptor AG is numbered -2,-1 and the splice donor GT is numbered +1,+2.

[3]The first methionine in the open reading frame is at position 1.

Appendix IIIb

Splicing Defects in the Ataxia-Telangiectasia gene, *ATM:* Underlying Mutations and Consequences

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Splicing Defects in the Ataxia-Telangiectasia Gene, *ATM*: Underlying Mutations and Consequences

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Summary

Mutations resulting in defective splicing constitute a significant proportion (30/62 [48%]) of a new series of mutations in the *ATM* gene in patients with ataxia-telangiectasia (AT) that were detected by the protein-truncation assay followed by sequence analysis of genomic DNA. Fewer than half of the splicing mutations involved the canonical AG splice-acceptor site or GT splice-donor site. A higher percentage of mutations occurred at less stringently conserved sites, including silent mutations of the last nucleotide of exons, mutations in nucleotides other than the conserved AG and GT in the consensus splice sites, and creation of splice-acceptor or splice-donor sites in either introns or exons. These splicing mutations led to a variety of consequences, including exon skipping and, to a lesser degree, intron retention, activation of cryptic splice sites, or creation of new splice sites. In addition, 5 of 12 nonsense mutations and 1 missense mutation were associated with deletion in the cDNA of the exons in which the mutations occurred. No ATM protein was detected by western blotting in any AT cell line in which splicing mutations were identified. Several cases of exon skipping in both normal controls and patients for whom no underlying defect could be found in genomic DNA were also observed, suggesting caution in the interpretation of exon deletions observed in *ATM* cDNA when there is no accompanying identification of genomic mutations.

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Introduction

Ataxia-telangiectasia (AT [MIM 208900]) is an autosomal recessive disorder with a diverse phenotype that includes progressive cerebellar ataxia, oculocutaneous telangiectasias, radiation hypersensitivity, increased cancer incidence, immunodeficiency, chromosome instability, and elevated levels of serum alpha-fetoprotein (reviewed in Gatti 1991, 1998). Genetic studies of families with AT indicate that heterozygous carriers for AT, although generally asymptomatic, have an increased risk for the development of breast cancer (Swift et al. 1991; Athma et al. 1996). The gene mutated in AT, *ATM*, was identified in 1995 (Savitsky et al. 1995*a*, 1995*b*; Uziel et al. 1996). In light of both the public-health implications of the reported cancer risk in known AT carriers and estimates that place their population frequency at 0.5%–1%, there is substantial interest in detection and characterization of mutations in *ATM* (Swift et al. 1991; Easton 1994; FitzGerald et al. 1996; Vorechovsky et al. 1996).

The majority of mutations detected thus far in the *ATM* gene in patients with AT are predicted to result in protein truncation (Gilad et al. 1996; Concannon and Gatti 1997). Western blot analyses of cell lines of patients with AT, with antibodies directed against ATM, indicate that most of these truncating mutations are associated with an absence of detectable ATM protein (Lakin et al. 1996; Stankovic et al. 1998). Some studies have reported that patients with AT whose cells are capable of producing even modest amounts of ATM may have distinct phenotypic features when compared with those whose cells produce no detectable protein. One class of mutations that has the potential to be "leaky" and to allow for the production of at least some ATM protein are those that affect the fidelity of splicing. Indeed, two such mutations with effects on splicing of *ATM* have been reported as being associated with either a milder phenotype (McConville et al. 1996) or a variant

Table 1

ATM Primers

Primers Used	Nucleotides	Designation
For amplification of cDNA:		
CAGAAGAGCACCTAGGCTAA	327–346	14F
CTGCCGTTATAATGCTTTAGG	1023–1003	14R
CGAGTGTGTGAATTAGGAGAT	748–778	AF3
CATTAAGCCTATGAAGAG	817–800	AR2
TGTTCTCTGTTTACTTCAG	500–685	ex8.2F
AGAATGATTTTGATCTTGTGC	1231–1211	11AR
GCTTACTTGGAGCCATAATTC	1517–1537	16F
GTGAACACCGGACAAGAGTT	2158–2139	16R
CGCTGTCTTCTGGGATTATC	2124–2093	17F
GTAGGTTCTAGCGTGCTAGA	2816–2797	17R
GTGTAACTACTGCTCAGACC	2720–2739	18F
GTTTCAGGGTTCTCAGCACT	3428–3409	18R
TGCATACTTGAAAGCTCAGGA	3366–3386	19F
TCAGTGCTCTGACTGGCACT	4091–4072	19R
AGACAGCCGTGACTTACTGTA	4508–4528	3F
CAGGATTATGAAGGTCCACTG	5145–5115	3R
TTGTCTTCGAAGATCC	4860–4845	R5
CAGTGGAGGCACAAAATGTGA	5445–5466	5F
CTGGCTTCCTTCTTCAAATGC	5948–5926	5R
CTCTATGCAGAAATCTATGCAG	5866–5887	6F
CTGGTTCCTTCTACTTCTTTGC	6368–6347	6R
CACTGCATATTCCTCCATGCTGC	6316–6304	R309
CAACCTGATTGTGTGGGATAG	6528–6507	B2
T7–ATGCAGTGGGACCATTGC	6319–6335	7F
GAGACTCCACAGCTAACTGAA	6866–6846	7R
GAAGTAGGTCTCCTTAGGGAA	7267–7287	9F
TTCTGACCATCTGAGGTCTCC	7855–7834	9R
TTGATGAGGATCGAACAGAGG	7780–7801	10F
CATTCAAGAACACCACTTCGC	8306–8285	10R
CACGGAAACTAGGAAGAGGAA	8226–8246	11F
CCCTGGTTTTCTCACAGCAT	8806–8787	11R
ATGAGCAGTCAGCAGAACTTG	8636–8656	12F
CTAAAGGCTGAATGAAAGGGTAAT	9209–9185	12R
	Exon	Product Size (bp)
For amplification of genomic DNA:		
CCCCCTGTTATACCCAGTT	9F	318
TGAAGAAGCAAATTCAAAACAG	9R	
TTTGTGGGGAGCTAGCAGTG	10F	262
TCTAAATGTGACATGACCTAC	10R	
GTTTGTTAATGTGATGGAATA	12F	467
GTGTGTTTATCTGTAAGTCAG	12R	
GTTCTTACAAAAGATAGAGTATAC	16.2F	329
TTCACAGGAATACATTTCATTC	16.2R	
GTCCAAGATCAAAGTACACTG	17.2F	314
GTGACAGAGAAAGATCCTATC	17.2R	
ATATTGGCCCTAATAGTAAAC	18F	292
CCTTATTTACAAAGATATTTCAAC	18.2R	
CCGGCCTATGTTTATATACTT	21F	225
TTAACAGAACACATCAGTTAT	21R	
TGGAGTTCAGTTGGGATTTTA	26F	304
TTCACAGTGACCTAAGGAAGC	26R	
TGCTGAACCAAAGGACTTCT	32F	334
CACTCAAATCCTTCTAACAATA	32R	
GTATGTTGAGTTTATGGCAGA	39F	376
ATCCATCTTTCTCTAGAACTG	39R	
ACCAGAACCTTATAGCATAGT	40F	247

(*continued*)

Table 1 (continued)

Primers Used	Nucleotides	Designation
	Exon	Product Size (bp)
TTCAGCCGATAGTTAACAAGT	40R	
TAAGCAGTCACTACCATTGTA	41F	314
ATACCCTTATTGAGACAATGC	41R	
GTATATGTATTCAGGAGCTTC	42F	238
ATGGCATCTGTACAGTGTCT	42R	
CAGAACTGTATTTCAGAATCAT	43F	387
ACATAACTCCTTCATAAACAGT	43R	
CCAAAGCTATTTTCACAATCTT	44F	262
TACTGAAATAACCTCAGCACT	44R	
CTCTGGTTTTCTGTTGATATC	45F	236
CCCCATGAAGAATCAAGTC	45R	
TTTATACATGTATATCTTAGGGTTCTG	46F	220
TTCAGAAAAGAAGCCATGACA	46R	
CACTGCAGTATCTAGACAGT	54F	322
CTAGGAAAGACTGAATATCAC	54R	
AATGTTGGGTAGTTCCTTATG	55F	308
GCTTTTGGATTACGTTTGTGA	55R	
CCTTTGCTATTCTCAGATGACTCTGT	58F	290
GCATTATGAATATGGGCATGA	58R	
TAGAAAGAGATGGAATCAGTG	61F	317
ATCTTGGTAGGCAAACAACAT	61R	
AAAGTTCACATTCTAACTGGAA	62F	272
ATTACAGGTGCAAAGAACCAT	62R	
TCCTGTTGTCAGTTTTTCAGA	65F	354
ACTTAAAGTATGTTGGCAGGT	65R	

[a] Sequence is from Vorechovsky et al. (1996).

clinical presentation (Gilad et al. 1998), even in compound heterozygotes.

There is suggestive evidence that splicing-related mutations may be unusually frequent in AT. A recent survey of published reports of *ATM* mutations included 115 alterations detected predominantly through the examination of *ATM* cDNA from patients with AT (Concannon and Gatti 1997; for updates, see the Ataxia-Telangiectasia Mutations Database Website). A significant fraction (45/115 [39.1%]) of these changes corresponded to the loss of either exons or portions of exons, presumably reflecting the presence of underlying splice-site mutations. Unfortunately, for the majority (39/45) of these *ATM* splicing alterations, only the observed effects in cDNA were reported, not the identity of specific causative mutations. If it is assumed that such mutations can be identified in the majority of cases, then the frequency of splicing mutations in the *ATM* gene would be substantially higher than that reported in surveys of other human genetic disorders, in which ~15% of point mutations are found to affect mRNA splicing (Krawczak et al. 1992; Ruttledge et al. 1996).

In the present study, we have sought confirmation of the implied high frequency of splicing mutations in *ATM*, through the study of an independent population of patients with AT. Initial mutation screening was done at the cDNA level, to identify any cell lines from patients with altered splicing patterns. In all such cases, the underlying mutation was then identified by sequencing of genomic DNA. To evaluate the possible implications that the different splicing mutations have for ATM expression, all cell lines of patients were tested for the production of ATM, by western blotting. Where appropriate, clinical features of specific patients were reexamined in light of the mutation data.

Patients, Material, and Methods

Patients

Genomic DNA and mRNA were isolated, and cDNA was synthesized, from B-lymphoblastoid cell lines previously established from patients with AT. Patients were ascertained in Turkey, Poland, Italy, and the United States. The clinical features for all patients were reviewed by one of us (R.A.G.) and were found to be consistent with the standard diagnostic criteria for AT. All clinical diagnoses were confirmed by radiation-sensitivity testing of cell lines by the colony-survival assay (Huo et al. 1994). Cell lines AT25RM, AT39RM, AT117LA, and

Table 2

Frequency of Mutations Associated with Splicing Defects in Patients with AT

AT Cell Line	Genomic Mutation[a]	Effect on cDNA	Intron/Exon	Codon Change	Codon Number[b]	Status	Consequence	Second Mutant Allele
AT68LA	380delA	380delA	7	Frameshift	128	Heterozygote	Truncation	Unknown
AT113LA	748C→T	663del239	9	R→X	221	Heterozygote	Exon 9 skipped, truncation	Unknown
AT51LA	802C→T	663del239	9	Q→X	268	Heterozygote	Exon 9 skipped, truncation	Unknown
AT134LA	IVS9−1G→T	902del164	10	Frameshift	301	Heterozygote	Exon 10 skipped, truncation	3802delG
AT139LA	1024delAAAG	1024delAAAG	10	Frameshift	343	Heterozygote	Truncation	Unknown
AT17LA	1180delGG	1180delGG	11	W→X	393	Heterozygote	Truncation	Unknown
AT81LA	1563delAG	1563delAG	12	Frameshift	522	Heterozygote	Truncation	6100C→T
AT115LA	1563delAG	1563delAG	12	Frameshift	522	Homozygote	Truncation	...
AT39LA	1563delAG	1563delAG	12	Frameshift	522	Heterozygote	Truncation	3382C→T
AT39RM	IVS12+1G→T	IVS12ins764	12	Frameshift	535	Homozygote	Intron 12 retained, truncation	...
AT54LA	2250G→A	2125del126	16	Silent	709	Heterozygote	Exon 16 skipped	8266A→T
AT111LA	IVS16−10T→G	IVS16−1ins9	17	Frameshift	750	Heterozygote	Splice acceptor created, truncation	2809insCTAG
AT114LA	IVS18+1G→A	2237del90	18	del30	792	Heterozygote	Exon 18 skipped	Unknown
AT25RM	2503insA	2503insA	19	Frameshift	835	Heterozygote	Truncation	Unknown
TAT50	2503insA	2503insA	19	Frameshift	835	Homozygote	Truncation	...
TAT49	IVS21+3insT	2839del83	21	Frameshift	946	Homozygote	Exon 21 skipped, truncation	...
AT84LA	3372C→G	3372C→G	25	Y→X	1124	Heterozygote	Truncation	Unknown
AT142LA	3576G→A	3403del174	26	Silent	1135	Heterozygote	Exon 26 skipped	Unknown
TAT48	3576G→A	3403del174	26	Silent	1135	Homozygote	Exon 26 skipped	...
AT117LA	3576G→A	3403del 174	26	Silent	1135	Heterozygote	Exon 26 skipped	Unknown
AT136LA	3625delTT	3625delTT	27	Frameshift	1209	Heterozygote	Truncation	Unknown
AT36LA	3663G→A	3663G→A	27	W→X	1221	Heterozygote	Truncation	1110delC
AT80LA	3802delG	3802delG	28	Frameshift	1268	Heterozygote	Truncation	Unknown
AT18RM	3894insT	3894insT	28	Frameshift	1299	Homozygote	Truncation	Unknown
AT137LA	4052delT	4052delT	29	Frameshift	1351	Heterozygote	Truncation	Unknown
AT140LA	4373delG	4373delG	31	Frameshift	1458	Heterozygote	Truncation	Unknown
AT11LA	IVS32−12A→G	IVS32−1ins11	32	Frameshift	1479	Homozygote	Splice acceptor created, truncation	...

AT46LA	5290delC	5290delC	37	Frameshift	1764	Heterozygote	Truncation	Unknown
AT32LA	IVS38−2A→C	5497del61	39	Frameshift	1832	Heterozygote	Cryptic splice acceptor, truncation	Unknown
TAT51	5623C→T	5623C→T	39	R→X	1875	Homozygote	Truncation	...
AT56LA	IVS40+1126A→G	IVS40ins137	40/41	Frameshift	1921	Heterozygote	Cryptic splice sites, truncation	Unknown
CAT13	IVS40+1126A→G	IVS40ins137	40/41	Frameshift	1921	Heterozygote	Cryptic splice sites, truncation	Unknown
AT130LA	5932G→T	5919del88, 5932G→T	42	R→X	1973	Homozygote	Exon 42 skipped, truncation	...
AT63LA	5932G→T	5919del88, 5932G→T	42	R→X	1973	Heterozygote	Exon 42 skipped, truncation	432insA
AT31LA	5971G→T	5919del88, 5971G→T	42	E→X	1991	Heterozygote	Exon 42 skipped, truncation	7636del9
AT65LA	6095G→A	6007del89	43	Frameshift	2003	Heterozygote	Exon 43 skipped, truncation	214G→T
AT138LA	6154G→A	6096del103	44	Frameshift	2032	Homozygote	Exon 44 skipped, truncation	...
AT127LA	IVS45+1G→A	6007del326, 6347insAG, 6347ins78nt	45	Frameshift	2002	Homozygote	Exon 43, 43–45 skipped, truncation	...
AT30LA	IVS46+1G→A	6348del105	46	del35	2176	Heterozygote	Exon 46 skipped	6372insG
GM01525C	6404insTT	6404insTT	46	Frameshift	2136	Heterozygote	Truncation	Unknown
AT72LA	IVS53−2A→C	7630del159	54	del53	2544	Heterozygote	Exon 54 skipped	3085insA
AATV26	7705delGA	7705del GA	54	D→X	2569	Heterozygote	Truncation	Unknown
AT119LA	7865C→T	7864del64	55	Frameshift	2621	Homozygote	Splice donor created, truncation	...
AT121LA	8264delATAAG	8152del117	58	del39	2718	Heterozygote	Exon 58 skipped	3485T→G
TAT45	IVS60−14del27	8585del87	61	del29	2886	Homozygote	Exon 61 skipped	...
AT147LA	IVS62+1G→A	8672del115	62	Frameshift	2891	Heterozygote	Exon 62 skipped, truncation	6998insA
AT122LA	IVS62+1G→A	8672del115	62	Frameshift	2891	Heterozygote	Exon 62 skipped, truncation	Unknown
AT73LA	8769insT	8769insT	62	Frameshift	2924	Heterozygote	Truncation	Unknown
AT34LA	IVS64−1G→C	8988del13	65	Frameshift	3045	Heterozygote	Cryptic splice acceptor, truncation	Unknown

NOTE.—Mutations associated with splicing defects are enclosed in boxes.

[a] Numbering of nucleotides is based on the sequence reported by Savitsky et al. (1995*b*), which designates the first nucleotide of the initiating ATG codon as "1"; mutations are designated according to the convention of Beaudet and Tsui (1993), as modified by Antonarakis (Recommendations for a Nomenclature System for Human Gene Mutations). Exon/intron boundaries and numbering are as defined by Uziel et al. (1996), with introns numbered such that intron X follows exon X, −2 and −1 are the positions of the splice acceptor AG, and +1 and +2 are the positions of the splice donor GT.

[b] In the case of a frameshift mutation, the codon given is that at which the predicted protein sequence diverges from the normal ATM protein sequence.

AT18RM were provided by one of us (L.C.). SV40-transformed fibroblast cell lines LM217 and GM00637, from two individuals with normal radiosensitivity, were obtained from Dr. L. Kapp (University of California, San Francisco). A B-lymphoblastoid cell line was established from unaffected individual NAT2.

Primers

Primers used for the protein-truncation test (PTT) assay have been described by Telatar et al. (1998). Primers used to amplify portions of the cDNA for SSCP and sequence analyses, as well as primers used to amplify exons from genomic DNA, are listed intable 1.

RNA Isolation, cDNA Synthesis, and the PTT

Specific methods for RNA isolation, cDNA synthesis, and application of the PTT to *ATM* have been described elsewhere (Telatar et al. 1996). Protein products from the coupled in vitro transcription-translation reaction were separated on 10%–20% gradient SDS-PAGE gels for 5 h at 250 V.

SSCP

SSCP analyses (Orita et al. 1989) were done on both cDNA and genomic DNA. PCR amplifications were done in 50-μl reactions containing 200 mM each of dCTP, dGTP, and dTTP (Boehringer Mannheim), 10 mM dATP (Boehringer Mannheim), 0.1–0.2 mCi of [^{33}P]-dATP (Amersham), 1 ng of each primer, 1.25 units of *Taq* DNA polymerase (either Ampli*Taq* from Perkin-Elmer/ABI or *Taq* polymerase from Boehringer Mannheim), and 50 ng of either cDNA or genomic DNA. The amplification profile was 35 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by a 7-min extension at 72°C. A 4-μl aliquot of the PCR product was mixed with 10 μl of dye buffer (98% formamide, 10 mM EDTA pH 8, 0.05% bromophenol blue, and 0.05% xylene cyanol) and was heated for 5 min at 95°C, quick-cooled to 4°C, and held on ice. Four microliters of each sample was loaded on both a standard 0.5 × MDE (mutation-detection enhancement [FMC]) gel and a 0.5 × MDE gel supplemented with 5% glycerol. After electrophoresis for 15 h at 7 W, gels were dried and exposed to x-ray film for 24–48 h. Variant or wild-type bands were cut from the gel, rehydrated in 20 μl of distilled water, reamplified by PCR, and sequenced.

Sequencing

PCR reactions for direct sequencing of 50 ng of genomic DNA were done in 50-μl volumes containing 200 mM each of dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim), 0.3 mM of each primer, and 1.25 units of *Taq* DNA polymerase (Perkin-Elmer/ABI or Boehringer Mannheim) in PCR buffer. Amplification of cDNA for sequencing was done as described for genomic DNA, except that 1 ng of each primer was used. High Pure™ columns (Boehringer Mannheim) were used to purify the PCR products to be sequenced. Purified PCR products (1–10 μl) were sequenced with an FS sequencing kit (Perkin-Elmer/ABI), were purified with Centri-sep™ columns (Princeton Separations), and were electrophoresed on an ABI 373 automated sequencer.

Western Blotting

Epstein-Barr virus–transformed lymphoblastoid cells were grown in RPMI 1640 supplemented with 15% fetal bovine serum, 100 μg of penicillin/ml, and 100 μg of streptomycin/ml. Cell pellets of ~1 × 10^6 cells were washed twice with PBS (80 mM Na_2HPO_4, 15 mM KH_2PO_4, 1.4 M NaCl, and 27 mM KCl) pH 7.4 and then were lysed by sonication in 100 μl of cell lysis solution (Analytical Chemiluminescent Laboratories) containing 2 μg of aprotinin/ml and 550 μM phenylmethylsulfonylfluoride. During the procedure, the samples were kept on ice whenever possible. After sonication, the samples were centrifuged at 15,300*g* for 7 min at 4°C. The supernatants were recovered, and the protein concentration was measured, by a modified Bradford method (Bio-Rad). A total cell lysate of 25 μg was electrophoresed on 6% SDS-polyacrylamide gels and was blotted onto PVDF membranes (Bio-Rad) overnight at 4°C. The efficiency of the transfer was verified by silver-staining of the gel. Membranes were blocked with 10% nonfat milk for 2 h. ATM protein was detected by a monoclonal antibody generated against peptide 980–1512 in the leucine zipper and proline-rich region of the ATM protein (Chen and Lee 1996). The primary antibody was applied for 1 h. The membrane was then washed six times with PBS-Tween 0.05%. Peroxidase-linked anti-mouse Ig (Amersham) was applied for 30 min, and the membranes were washed six times with PBS-T. Detection was done by the ECL system (Amersham). Blotting of serial dilutions of lysates from normal control cell line NAT2 was used to establish conditions under which 10% of the normal level of ATM could be reliably detected.

Results

Alterations in *ATM* cDNA were detected by either SSCP or PTT in 49 cell lines derived from patients with AT. Within these cell lines, a total of 62 mutations, corresponding to 75/98 (76%) of the disease chromosomes, were identified in genomic DNA by both SSCP analysis and DNA sequencing of exons (table 2). Some of the cell lines are representative of founder-effect mutations in specific ethnic groups, as described elsewhere (Telatar et al. 1998). However, 32/62 (52%) of the mutations reported here are novel. Of the 62 mutations, 30 (48%)

affected the accuracy of splicing of the *ATM* transcript. In all cell lines in which mutations were identified, western blotting was used to determine whether any detectable normal-size ATM protein was produced.

Mutations in Canonical Splice Sites

Only 10 of the 30 *ATM* splicing mutations detected in the present study altered either a conserved GT or AG dinucleotide. Of the five mutations in the GT dinucleotide splice donor, three predictably resulted in skipping of the adjacent exon, whereas the remaining two mutations had unexpected effects (table 2).

In AT39RM, homozygous for the splice-donor substitution GT→TT in intron 12, two cDNA products were visualized by SSCP, as shown in figure 1*B* and as diagrammed in figure 2*B*. The upper band in lane AT39RM in figure 1*B* corresponds to an aberrantly spliced cDNA from which intron 12 has not been removed. A small amount of the correctly spliced cDNA is also seen (and confirmed by direct sequencing of the cDNA), despite the absence of an intact splice-donor sequence. Of the 30 splicing mutations detected in the present study, only AT39RM exhibited intron retention. Retention of intron 12 is predicted to lead to a frameshift and truncation of the protein. No ATM protein was detected by western blotting in AT39RM, indicating that, although some correctly spliced *ATM* transcript was produced in this cell line, it did not lead to the accumulation of detectable amounts of ATM protein (fig. 3).

In AT127LA, a homozygous mutation, GT→AT, of the splice-donor sequence at the end of exon 45 (IVS45+1G→A) resulted in the production of multiple incorrectly spliced transcripts identified in cDNA (see the numbered bands in fig. 1C, lane AT127LA). These transcripts corresponded to an insertion of 72 nucleotides from intron 45, an insertion of 80 nucleotides, an insertion of 2 nucleotides between exons 45 and 46, and a fourth species (data not shown) that had a deletion of exons 43–45. No normally spliced transcripts (shown in band 3a in fig. 1C, lanes N1 and N2) were detected in AT127LA, and, as expected, no ATM protein could be detected in cell lysates by western blotting (fig. 3).

The effects of mutations at the conserved AG dinucleotide of the splice acceptor were similarly diverse. Two mutations, IVS9-1G→T (in AT134LA) and IVS53-2A→C (in AT72LA), resulted in skipping of exons 10 and 54, respectively (table 2). However, two other similar mutations, IVS64-1G→C (in AT34LA) and IVS38-2A→C (in AT32LA), each resulted in the activation of cryptic splice sites (table 2).

Mutations at Positions Flanking Canonical Splice–Site Sequences

Three mutations were identified that altered nucleotides at the less-conserved positions (Padgett et al. 1986;

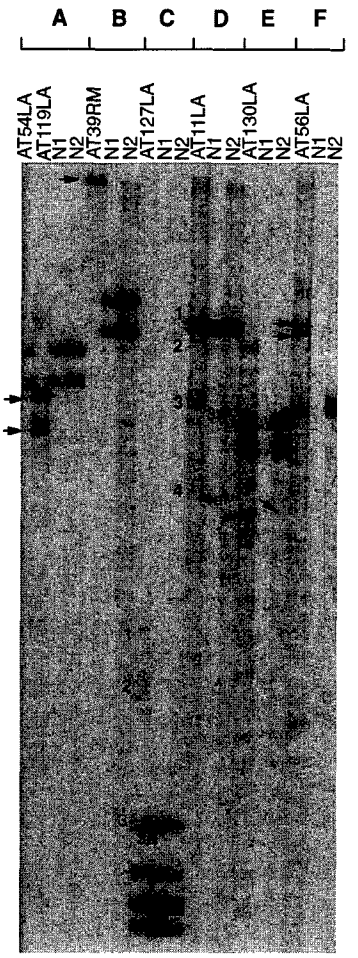

Figure 1 SSCP of *ATM* subfragments from patients with AT and from controls. In each of the panels *A–F*, cDNAs from AT cell lines and from controls GM637 (lane N1) and LM217 (lane N2) are compared. *A*, *ATM*7781-8305, including portions of exons 55–58. The arrows in lane AT119LA indicate the two strands of the mutant transcript deleting 64 nucleotides at the end of exon 55. *B*, *ATM*1517-2159, including portions of exons 12–16. The arrow in lane AT39RM indicates the mutant transcript in which intron 12 was not removed. *C*, *ATM*6319-6529, including portions of exons 45–47. In lane AT127LA, band 1 represents a 72-nucleotide insertion from intron 45, band 2 represents an 80-nucleotide insertion from intron 45, and bands in the region below band 3 all have AG inserted between exon 45 and exon 46. Bands in region 3a of the control lanes show the slightly shifted positions of the wild-type transcripts. *D*, *ATM*4508-5145, including portions of exons 32–36. In lane AT11LA, band 1 refers to the doublet representing the two strands of the mutant transcript in which 11 nucleotides of intron 32 were inserted. Bands near 3 and 4 represent transcripts that contained the 11 nucleotides of intron 32 and skipped either exon 34 or exons 34 and 35, respectively. Corresponding bands 3a and 4a of the control (lane N2) did not contain the intron 32 insertion but skipped exon 34 or exons 34 and 35, respectively. Band 2 of lane AT11LA contained the intron 32 insertion and had deletion of the first 25 nucleotides of exon 34. *E*, *ATM*5866-6368, including portions of exons 41–45. The arrow in lane AT130LA indicates a transcript lacking exon 42. The arrow in the control (lane N2) indicates a transcript that had deletion of the first 49 nucleotides of exon 45. *F*, *ATM*5445-5948, including exons 39–41. The arrows indicate the two strands of the mutant transcript in which 137 nucleotides from the middle of intron 41 were inserted.

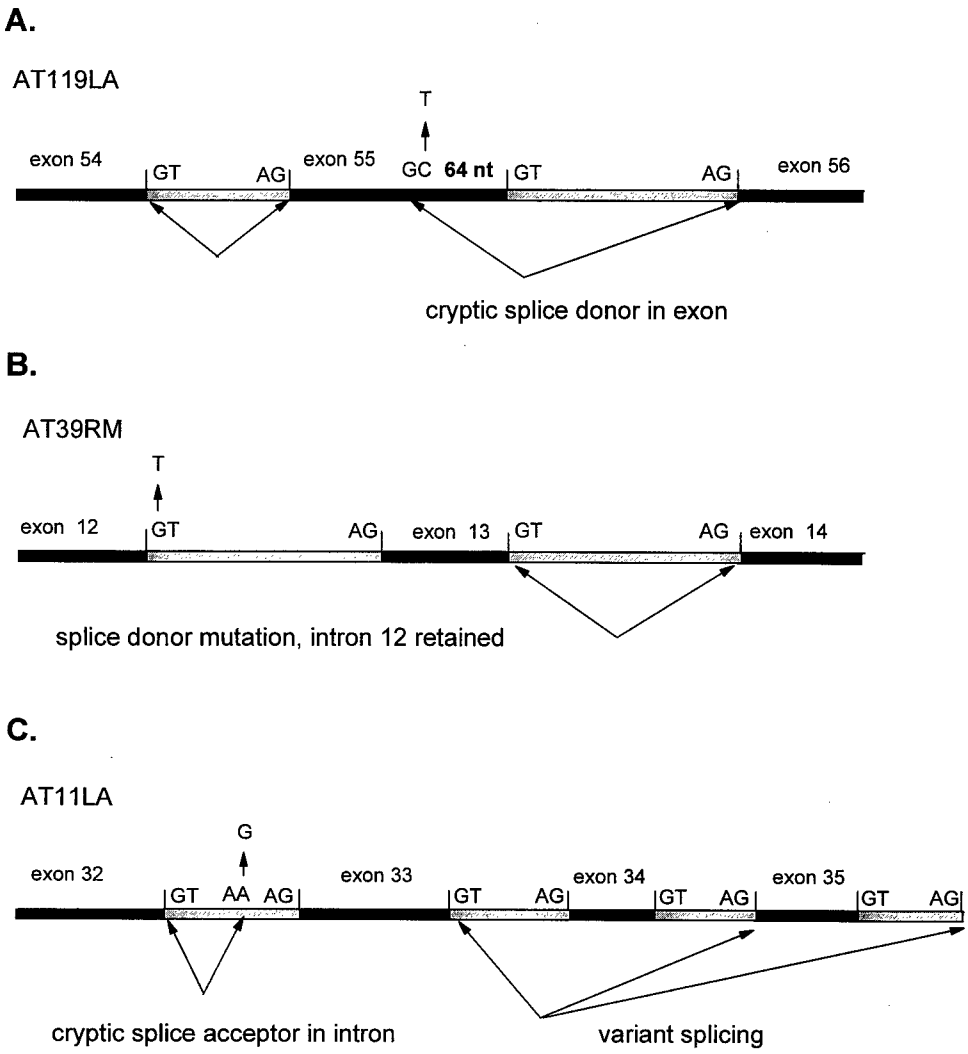

Figure 2 Effects of *ATM* splice mutations. *A*, Mutation 7865C→T, which creates a splice donor (GT) within exon 55, resulting in the deletion of 64 nucleotides at the end of the exon. Arrows indicate sites joined by splicing on this mutant allele. *B*, Mutation IVS12+1G→T of the splice donor in intron 12, which abolishes normal splicing of intron 12. Intron 13 is correctly spliced, as indicated by arrows. *C*, Mutation IVS 32-12A→G, which creates a splice acceptor (AG) in intron 32, resulting in the inclusion of 11 nucleotides of intron 32 in the transcript. No mutations were detected in the splice sites of exon 34 or exon 35, but either one or both of these exons were deleted in some of the transcripts from AT11LA, as indicated by arrows, suggesting that variant splicing occurred.

Krawczak et al. 1992) flanking the invariant GT dinucleotide of the splice donor. Two patients (TAT48 and AT117LA) had a 3576G→A mutation altering the last nucleotide of exon 26, and another patient (AT54LA) had an analogous mutation affecting the last nucleotide of exon 16 (2250G→A) (table 2). In all three cases, the nucleotide substitution itself is silent with respect to the amino acid sequence. Patient TAT48 is homozygous for the 3576G→A mutation and thus is particularly illustrative with regard to the effect that such a substitution has at the last nucleotide of this particular exon. The only transcript detected in this cell line had exon 26 deleted (fig. 4), as shown by SSCP and as confirmed by direct sequencing, indicating that the G→A substitution was sufficient to completely disrupt normal splicing. Deletion of exon 26 should result in a transcript that, if translated, would leave the reading frame of the ATM protein intact but that would eliminate 58 amino acids. Nevertheless, no ATM protein of normal or altered molecular weight was detected in patient TAT48 by western blotting (fig. 3).

The mutation IVS21+3insT in patient TAT49 highlights the significance of two other, less well-conserved positions in the consensus splice site. Patient TAT49 is homozygous for the insertion of T at the +3 position, leading to the altered splice-donor sequence TC | GTTAAGAA (position +3 is underlined, and the vertical bar denotes the intron/exon boundary), compared with

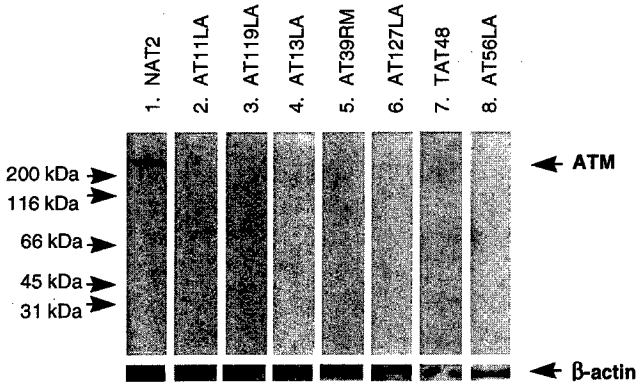

Figure 3 Western blots of proteins from cell lysates for seven patients with AT and for a control. ATM protein was detected with a monoclonal antibody generated against peptide 980-1512 in the leucine zipper and proline-rich region of the ATM protein (Chen and Lee 1996). Control NAT2 is represented in lane 1. β-Actin antibody provides an internal standard for the amount of protein per lane. Lanes 1–3, 5, and 6 were taken from one blot, whereas lanes 4, 7, and 8 represent separate experiments. In all cases, ATM was readily detected in the control.

the wild-type sequence TC | GTAAGAAA. The mutation in patient TAT49 disrupts the consensus sequence, placing the rarely used residue T (2%) at the +3 position and the rarely used residue A (7%) at the +5 position.

Shapiro and Senapathy (1987) have described a scoring system for calculation of the likelihood that a particular splice-donor or -acceptor sequence as a whole would be functional. In their rating of 5′ splice-donor sequences, no functional donor sequences scored <60 on this scale. The mutation identified in patient TAT49 reduces the score of this splice-donor sequence from 72.8 to 49.3, suggesting that it is unlikely to be functional. The consequence of this mutation was that only *ATM* transcripts lacking exon 21 were detected by either SSCP or sequencing of cDNA from this cell line (fig. 4). Western blotting of lymphoblastoid-cell lysates from patient TAT49 failed to detect any ATM protein (data not shown).

Mutations Creating Novel Splice Sites

Three mutations were identified that created novel splice sites: IVS32-12A→G in AT11LA, 7865C→T in AT119LA, and IVS16-10T→G in AT111LA (table 2). In two of these cases, AT11LA and AT119LA, the families were consanguineous, and the mutations were homozygous, simplifying the interpretation of their effects. The IVS32-12A→G mutation created a new splice-acceptor dinucleotide site, 11 nucleotides 5′ of the normally used splice acceptor for exon 33 (diagrammed in fig. 2C). This mutated sequence scored high (91.6) when its potential as a splice acceptor was calculated (Shapiro and Senapathy 1987). SSCP analysis of cDNA from this region revealed several different transcripts (fig. 1*D*, bands 1–4), all of which contain an insertion of the last 11 nucleotides of intron 32. This insertion results in a frameshift predicted to cause premature truncation of the protein. The new splice-acceptor site created by the mutation was used exclusively, despite the continued presence of the normal splice acceptor downstream. The proximity of the normal and mutant splice sites suggests that preference for the mutant site may arise from a suppressing effect on the normal site. In creating a novel splice acceptor, the mutation significantly reduces the splice site–potential score for the wild-type acceptor. No ATM protein was detected in AT11LA by western blotting, consistent with the exclusive production of mutant transcripts observed by SSCP (fig. 3).

Although two of the smaller *ATM* transcripts detected in AT11LA represent the skipping of either exon 34 or exons 34 and 35 (fig. 1*D*, bands 3 and 4, respectively), control cell line LM217 (fig. 1*D*, lane N2) also produced minor transcripts with deletion of either exon 34 (fig. 1*D*, lane N2, band 3a) or exons 34 and 35 (fig. 1*D*, lane N2, band 4a), albeit without the inclusion of 11 extra nucleotides from intron 32. On the SSCP gel, an

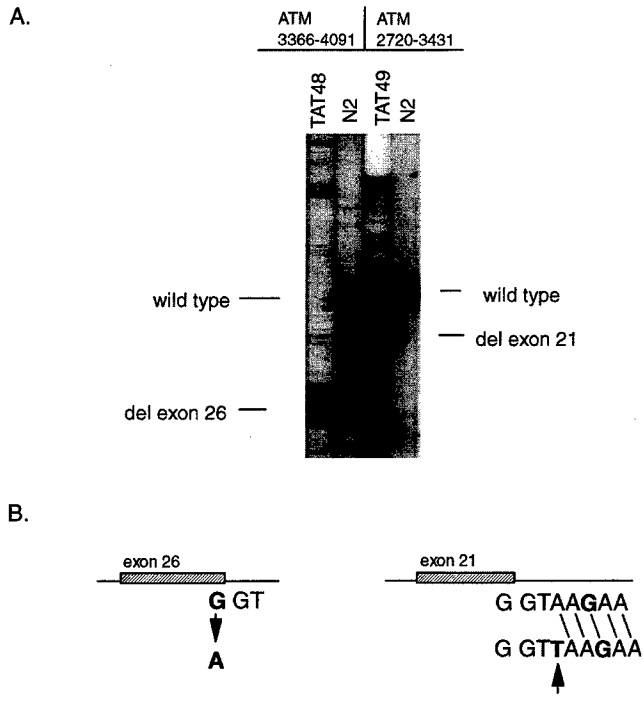

Figure 4 *A*, SSCP of *ATM* cDNA subfragments demonstrating absence of wild- type transcript in homozygous ATM patients TAT48 and TAT49. Control LM217 is represented in lane N2. Nucleotide positions of the subfragments are indicated at the top of the figure. *B*, Diagrams of the splicing consensus site defects in the genomic DNA of the patients with AT that led to the cDNA SSCP pattern in 3*A*.

additional faint band present in both the sample from AT11LA and that from control LM217 (fig. 1*D*, lane AT11LA, band 2) represents a transcript with the first 25 nucleotides of exon 34 deleted. Because no corresponding genomic mutations were detected for any of these three deleted products, and because they were found in controls, they are most likely rare, aberrantly spliced transcripts that are normally present and not the consequence of a mutation.

Patient AT119LA is homozygous for a C→T mutation at nucleotide 7865, creating a new splice-donor site within exon 55 (diagrammed in fig. 2*A*). Despite the continued presence of the normal splice-donor site, the new site was used exclusively, resulting in the deletion of 64 nucleotides at the end of the exon. Unlike the mutation in AT11LA, the novel splice site created by the mutation in AT119LA does not have an improved score for its potential as a splice site. In figure 1*A*, the SSCP pattern for this region in AT119LA displayed only the aberrantly spliced transcript. Sequencing revealed that the two major SSCP bands had 64 nucleotides deleted in exon 55. No ATM protein was detected in this cell line by western blotting (fig. 3).

Nonsense and Missense Mutations with Indirect Effects on Splicing

Two distinct nonsense mutations in exon 9 were detected in patients AT113LA (748C→T) and AT51LA (802C→T), both of whom are compound heterozygotes. Despite the fact that neither mutation altered an existing splice site or created a novel site, these cell lines produced comparable amounts of both properly spliced and exon 9–deleted transcripts (fig. 5). No additional mutations were detected in any splice-consensus sites within exon 9, including purine-rich or AC-rich splicing-enhancer sequences (reviewed by Cooper and Mattox 1997) or the ~50 bp of flanking sequences to either side. The deletion of exon 9 was also noted in two other cDNAs—that of AT140LA and that of control LM217 (fig. 5)—although in very small amounts; neither had mutations in either exon 9 or flanking introns.

Patient AT130LA is homozygous for a nonsense mutation in exon 42, resulting from the mutation 5932G→T. As shown in figure 1*E*, AT130LA produced a properly spliced transcript comparable in amount to that produced in control LM217 (fig. 1*E*, lane N2); however, AT130LA also produced a unique transcript lacking exon 42 (indicated by the arrow in fig. 1*E*, lane N2). A faint band with comparable migration to the exon 42–deleted band in AT130LA was also detected in the control cell line (indicated by the diagonal arrow in fig. 1*E*, lane N2). This band was isolated, sequenced, and found to represent a transcript lacking the first 49 nucleotides of exon 45, apparently a minor, alternatively spliced species in normal cells.

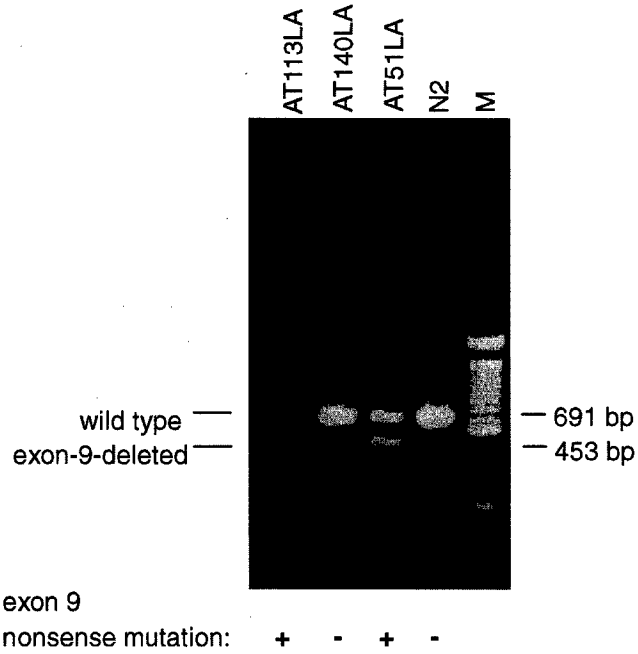

Figure 5 Missplicing of exon 9, in the absence of splice-site mutations. Shown are nested-PCR products of *ATM* nucleotide positions 500–1191 from cDNAs of AT113LA, AT140LA, and AT51LA and from control LM217 (lane N2). Patients AT113LA and AT51LA are heterozygous for the exon 9 nonsense mutations 748C→T and 802C→T, respectively. AT140LA and control LM217 have no mutation in either exon 9 or the flanking splice consensus sites. All cell lines produce varying amounts of transcripts with exon 9 deleted. The 123-bp ladder marker is shown in lane M.

AT138LA produced only *ATM* transcripts with exon 44 deleted. No other evidence of a mutation elsewhere in the gene was detected by PTT. Sequencing of exons 43–46 and flanking intronic sequences revealed only a single mutation, 6154G→A, within exon 44, resulting in a nonconservative amino acid substitution, 2032E→K. Deletion of exon 44 results in a frameshift. Western blotting of lysates from this cell line failed to reveal any ATM protein (data not shown).

Variant Splicing Not Associated with Mutations

During the course of screening *ATM* cDNA subfragments for mutations, several regions were encountered where transcripts had exon(s) or portions of exons deleted but for which no underlying genomic mutations within either the exon or the flanking splice sites could be found; for example, PTT assays revealed aberrantly migrating bands in the region encompassing portions of exons 10–12 in patients AT115LA, AT81LA, and AT139LA. The cDNA from these patients contained substantial amounts of products lacking exon 11, as shown in the SSCP assay in figure 6 and as confirmed by isolation and sequencing of the shortened products. However, no splice-site mutations were detected flanking

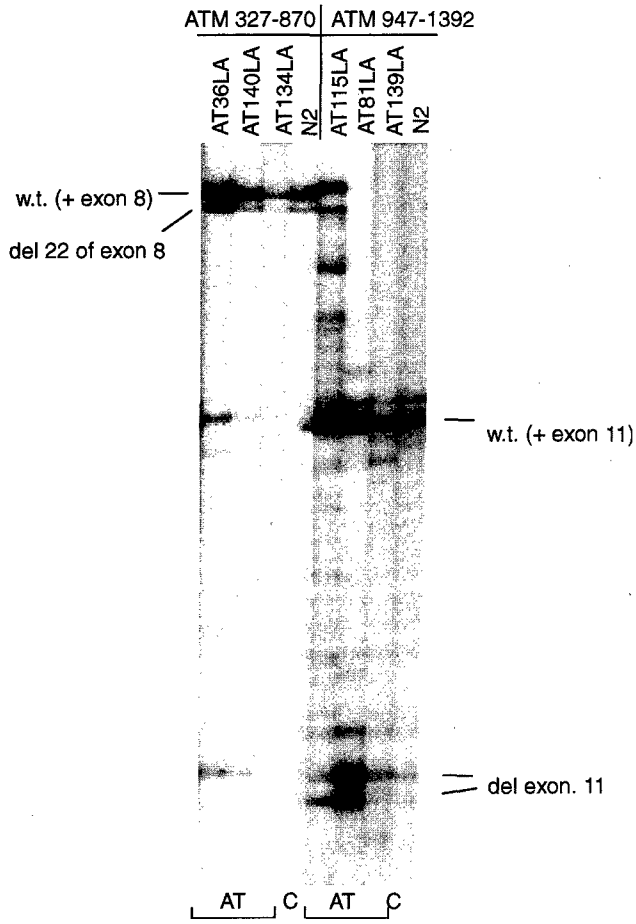

Figure 6 Variant splicing of *ATM* transcripts, in the absence of mutations. Nested PCR of *ATM* nucleotides 327–870 from three patients with AT and from control LM217 (lane N2) show an alternatively spliced transcript missing the first 22 nucleotides of exon 8. Nested PCR of *ATM* nucleotides 947–1392 from three patients and the control LM217 (N2) show an alternatively spliced transcript with exon 11 deleted. Patient AT139LA had bands below the wild-type and exon 11–deleted bands, representing its mutation in exon 10, as 1024delAAAG. Patients AT115LA and AT81LA are homozygous and heterozygous, respectively, for an exon 12 mutation (1563delAG) not contained in the amplified fragment.

exon 11 in the genomic DNA of these individuals. Examination of surrounding exons did reveal truncating mutations in three of these patients: AT115LA and AT81LA had a deletion of two nucleotides in exon 12, and AT139LA had a deletion of four nucleotides in exon 10 (table 2). Transcripts lacking exon 11 were also readily detected in control LM217 (fig. 6), again without any underlying mutation being detected.

Amplified cDNAs from cell lines of patients with AT that had truncated fragments in the PTT assay for the region containing exons 7–10 also displayed a wide variety of bands on SSCP gels (fig. 6). The cDNA from patients AT45LA, AT51LA, AT84LA, AT113LA, AT134LA, and AT140LA contained a product lacking the first 22 nucleotides of exon 8. The presence of an AG dinucleotide at the end of the 22 deleted nucleotides is consistent with the use of a cryptic splice-acceptor sequence. However, there was no mutation of the original splice acceptor in the genomic DNA of any of the individuals. Moreover, cDNA from control LM217 also contained the 22 nucleotide–deleted product (fig. 6).

Other examples of cryptic-splice-site utilization were seen in minor transcripts from a number of patients with AT and from controls. These include the deletion of the first 41 nucleotides of exon 15 (in AT117LA), the deletion of the first 86 nucleotides of exon 14 (in AT63LA), and either the skipping of exons 11, 34, and/or 35 or deletion of the first 19 nucleotides of exon 17 (in control LM217). In all these cases, no splice-site mutations were detected in the genomic DNA; however, the sequences at the end of the cDNA deletions indicated that cryptic splice sites had been used.

Discussion

Most studies of *ATM* mutations suggest that a high percentage are inactivating mutations resulting in protein truncation and the production of little or no ATM protein. In the *ATM* gene, with its large complement of exons, splice sites represent a sizable target for mutations that would have severe effects on the protein product. In a previous review of AT mutations (Concannon and Gatti 1997), we noted that a high percentage (39%) of alterations detected in *ATM* cDNA from patients with AT reflected the loss of one or more exons, a result consistent with this hypothesis. However, because the majority of published studies have examined only cDNA transcripts of *ATM*, there is a potential for ascertainment bias in the reported number of splicing mutations in the *ATM* gene. The loss of an entire exon is easily apparent by visual examination of enzymatically amplified cDNA products, regardless of what mutation-screening technique is being used. Therefore, exon skipping should be among the defects easiest to identify in cDNA. Furthermore, in cases in which an underlying genomic mutation has not been identified, exon skipping may simply reflect low-level missplicing that occurs normally but is more apparent in AT cell lines in which overall *ATM* transcript levels may be reduced.

In the set of 62 *ATM* mutations reported in the present study, a significant fraction (30/62 [48%]) affected splicing of the ATM transcript. These results are consistent with our earlier suggestion that splicing-related mutations are unusually frequent in AT. The canonical GT and AG dinucleotides that flank most mammalian exons are frequent targets of mutations that affect splicing (59% of mutations compiled by Krawczak et al. [1992] and 71% of mutations compiled by Schwarze et al. [1997]). In contrast, the majority of mutations effecting splicing that were detected in the present study did not

directly alter the canonical AG or GT exon-flanking dinucleotides but, instead, either targeted less-conserved sequences surrounding splice junctions or created novel splice sites. Several other instances of exon skipping that were observed in ATM were not associated with any detectable underlying mutation, suggesting that some degree of caution is necessary in the interpretation of such alterations when they are observed only in cDNA.

Two different mutations detected in the present study, 3576G→A and IVS40+1126A→G, have previously been described (by Gilad et al. [1998] and McConville et al. [1996], respectively) in patients reported to exhibit an atypical clinical course of AT. The mutation 3576G→A in patient TAT48 resulted in exclusive production of transcripts with exon 26 deleted. Deletion of exon 26 does not alter the translational reading frame for the ATM protein. No normal-length transcripts were detected, despite the sensitivity of PCR as an assay. If an exon 26–deleted transcript were to be translated into a stable protein, it would be only 58 amino acids (~2% of the total length) shorter than normal. On an SDS-PAGE gel, which is commonly used for protein assays, such a protein would be indistinguishable from the normal one. Gilad et al. (1998) have described several cell lines that are homozygous for this mutation and in which they could detect a low level of ATM protein (~5% of the level in controls). In the present study, no ATM protein was detected in patient TAT48. The discrepancy between our results and those of Gilad et al. may be due to differences in experimental conditions. However, in light of the absence, in patient TAT48, of any transcripts containing exon 26, only ATM protein with an internal deletion of 58 amino acids (encoded by exon 26) could be produced. If such a shortened protein is produced in these cells, it is unclear whether it would be functional or have any significant impact on the clinical phenotype.

An additional patient in the present study, AT117LA, was heterozygous for the 3576G→A mutation and had clinical features consistent with the AT variant disorder AT_{Fresno}. AT_{Fresno} encompasses all of the features of AT but includes microcephaly and mental retardation in the phenotype (Curry et al. 1989). AT117LA is only the second patient with AT_{Fresno} who has been screened for *ATM* mutations. The other previously characterized AT_{Fresno} patient, AT25LA, is homozygous for a splice-site mutation in intron 33 (Gilad et al. 1998). The 3576G→A mutation in AT117LA has been observed in several patients with AT who have classic phenotypes, including two patients (AT142LA and TAT48) in the present study. The IVS33+2T→C mutation in AT25LA is unique, but there are several patients with AT who have a very similar IVS33+2T→A mutation, one of whom is homozygous. Neither AT117LA nor AT25LA produces detectable levels of ATM protein. Thus, the variant phenotype observed in these patients with AT_{Fresno} seems unlikely to result from the nature of their *ATM* mutations alone, given (1) the occurrence of these same mutations in typical patients with AT and (2) the fact that absence of detectable ATM protein is also frequent among classic patients with AT. The AT_{Fresno} phenotype may result instead from epistatic effects of other, as yet unidentified genes.

The IVS40+1126A→G mutation, reported to be associated with a milder clinical course of AT (McConville et al. 1996), was detected in two patients (AT56LA and CAT13; fig. 1*F*) in the present study and in one patient (AT13LA) from a previously published study (Wright et al. 1996), all of whom are compound heterozygotes. In AT56LA, the two *ATM* alleles could be distinguished because of a nearby common polymorphism (5557G/A in exon 39; Dork et al. 1997). The allele containing the IVS40+1126A→G mutation produced both wild-type and mutated (insertion of 137 nucleotides) transcripts in comparable, albeit low, amounts. However, no *ATM* protein was detected in any of these cell lines (see fig. 3, lanes AT13LA and AT56LA), raising concerns as to whether the production of this small amount of normal transcript could significantly affect the phenotype of these patients.

All 12 patients whom McConville et al. (1996) described as having a milder clinical phenotype were compound heterozygotes for the IVS40+1126A→G mutation, as were patients AT56LA, CAT13, and AT13LA. However, in 8 (67%) of these 12 patients, the ages at onset of ataxia were 1–3 years, which is quite characteristic of classic AT. The age at onset of ataxia in the three affected siblings in the family of patient AT56LA were also characteristic of AT: 8 mo, 18 mo, and 3 years. In the family of patient AT13LA, the ages at onset were 5 years and 6 years for the two affected siblings. McConville et al. (1996) also suggest a decreased frequency of cancer in IVS40+1126A→G patients. This is corroborated in the families of patients AT56LA and AT13LA; in the latter, the affected siblings lived to ages 40 and 44 years without cancer. Thus, some aspects of the milder phenotype reported in conjunction with the IVS40+1126A→G mutation appear to recur in separate patient groups. However, identification and clinical characterization of additional patients with this mutation will be necessary to clarify the exact relationship between phenotype and genotype.

Twelve (~20%) of the patients in the present study had mutations that resulted in nonsense premature-termination codons (PTCs), and in five of these patients the PTCs were associated with some degree of deletion of the exon containing them. All prokaryotic and eukaryotic cells that have been examined appear to have mechanisms to degrade RNAs that harbor PTCs (Maquat 1995, 1996). However, in cell lines from patients with AT who are compound heterozygotes for a PTC

mutation and a second unrelated mutation, equimolar amounts of mRNA from PTC-containing alleles and from the other allele are detected by reverse transcriptase–PCR. The observed skipping of the *ATM* exon containing the PTC appears to be a specific event, one that is not associated with general degradation of transcripts containing the PTC. PTC mutations that lead to specific deletion of the exons containing them have also been reported for *BRCA1* (Mazoyer et al. 1998), the gene for fibrillin (Dietz et al. 1993), *CFTR* (Hull et al. 1994), the gene for mouse immunoglobulin κ (Aoufouchi et al. 1996), and the gene for mouse urinary protein (Belgrader and Maquat 1994), among others. Reports that even missense mutations can cause the deletion of exons containing them (Belgrader and Maquat 1994; Liu et al. 1997) suggest that additional mechanisms, not dependent on the presence of PTCs, may lead to effects on splicing.

In the present study, one patient (AT138LA) had only *ATM* transcripts with exon 44 deleted. Examination of the genomic sequence in and around exon 44 revealed only a single homozygous change, a G→A substitution at position 6154, a position that is conserved in the mouse *ATM* sequence. The resulting nonconservative amino acid substitution, E→K, may alter protein structure or function, but it would not necessarily be expected to terminate protein translation. Despite the observed missplicing of exon 44, this mutation does not affect any known splicing-control motif (including exonic splicing-enhancer sequences; see review by Cooper and Mattox [1997]). Hoffmeyer et al. (1998) have suggested that particular PTCs, while not altering any established splice-consensus sites, may cause defective splicing by inducing a significant alteration in the secondary structure of the transcript, disrupting the formation of the spliceosome. In a study of mutations in the *HPRT* gene, Steingrimmsdottir et al. (1992) noted that all of seven missense mutations that were associated with splicing defects disrupted base pairing in stem-loop structures in the predicted secondary structure of the corresponding RNA. In light of the observations, in AT138LA, of both a deletion of exon 44 and the 6154G→A missense mutation, it seems reasonable to postulate that this missense mutation may also cause defective splicing, by a similar mechanism. When the RNA secondary structure of exon 44 was predicted by the program mfold, position 6154 was projected to lie at the base of a stem-loop structure. The G→A mutation in AT138LA disrupts the GC base pair at this site, resulting in a predicted destabilization of a portion of the stem structure, a result consistent with the model of Steingrimmsdottir et al. (1992)

For some regions of *ATM*, exon-deleted transcripts have been detected even in controls. Although multiple, differentially spliced transcripts have been described for *ATM*, all were due to alternative splicing in the untranslated regions, not in the coding exons (Savitsky et al. 1997). Because the *ATM* mRNA is large, and because mutation screening involves amplification of only portions of the mRNA at a time, we cannot rule out the possibility that alternatively spliced products described in the present study are derived from either nonfunctional, truncated transcripts or transcripts in the process of being degraded. However, regardless of whether they play a functional role, the fact that these exon-deleted products are easily detected in significant amounts in standard mutation-screening assays indicates that caution should be applied in the interpretation of observed cases of exon skipping in *ATM* cDNA, until a credible underlying mutation in genomic DNA can be identified.

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The authors wish to thank Patsy Byers, for oligonucleotide synthesis, and Tim Williams, Amanda Cisler, and Lindsey Johnson, for sequencing. We would also like to thank the many patients with AT, as well as their families, who contributed to these studies. This work was supported by National Institutes of Health grants CA57569 (to P.C.) and NS35322 (to R.A.G.), by the support of the AT Medical Research Foundation and Department of Energy grant 87ER60548 (to R.A.G.), by North Atlantic Treaty Organization grant 900264 (to A.T.), and by an AT Children's Project grant (to S.Ö.).

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Ataxia-Telangiectasia Mutations Database, http://www.vmmc.org/vmrc/atm.htm
mfold, http://www.ibc.wustl.edu/~zuker/rna/form1.cgi
Online Mendelian inheritance in man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for AT [MIM 208900])
Recommendations for a Nomenclature System for Human Gene Mutations, http://ariel.ucs.unimelb.edu.au:80/~cotton/antonara.htm

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Appendix IIIc

Transgenomic™ Application Note 108

Detection of Mutations in the *ATM* Gene: Comparison of Temperature Modulated Heteroduplex Analysis (TMHA) with Single Strand Conformation Polymorphism (SSCP)

by Sharon Teraoka[1], Alexander Kuklin[2] and Pat Concannon[1]

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Temperature Modulated Heteroduplex Analysis (TMHA) with the WAVE system was successfully used to detect mutations in exon 42 of the ATM gene. Using various protocols of SSCP did not reveal these mutations.

Introduction

Ataxia-Telangiectasia (A-T) is an autosomal recessive disorder characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia, cellular and humoral immune deficiency, chromosomal instability, sensitivity to ionizing radiation, and increased incidence of cancer.[1] In A-T families, an increased incidence of breast cancer has been reported among A-T heterozygotes.[2] To facilitate the identification of A-T heterozygotes among sporadic breast cancer cases, we are screening for unknown mutations in the gene responsible for A-T, *ATM*, in an automated fashion using Temperature Modulated Heteroduplex Analysis (TMHA), also known also as DHPLC. [3,4]

The *ATM* gene is large, containing 66 exons encoding a 13-kb transcript.[5,6] Most mutations are unique, and they are distributed uniformly along the length of the gene, with no obvious "hot spots".[7,8] An automated approach for mutation detection is desirable because all exons must be screened. Before embarking on large- scale screening of breast cancer patients, we evaluated the performance of TMHA for detecting known single nucleotide mutations of ATM in A-T patients. Here we compare results obtained with single strand conformation polymorphism (SSCP)[9] and the WAVE DNA Fragment Analysis System (Transgenomic Inc., San Jose, CA) and demonstrate the accuracy of TMHA.

Results and Discussion

SSCP has long been a standard, effective approach for detecting mutations in DNA due to its ease of application and reasonable (50-90%) sensitivity of detection.[10,11] However, some mutations in *ATM* were not detected even when a variety of SSCP conditions were applied. Among these were two single nucleotide mutations that lead to nonsense codons in exon 42; 5932 G→T in AT63LA (Mutation 1) and 5971 G→T in AT31LA (Mutation 2).[8]

Addition of glycerol and/or HEPES buffer has been reported to increase the sensitivity of SSCP.[12] However, as shown

in Figure 1, none of these modifications improved the detection of the exon 42 mutations, even though mutations in other exons were detected by at least one of the four conditions. In contrast, analysis of exon 42 PCR products by TMHA on the WAVE platform yielded three different chromatogram patterns for the wild-type (control), mutation 1, and mutation 2 (Figure 2).

Conclusions

The WAVE DNA Fragment Analysis System has proved to be useful for detection of *ATM* mutations that are recalcitrant to detection by a variety of modifications of SSCP analysis. Sensitivity and accuracy of detection combined with automation, ease of use (Table 1), and low cost per sample make the WAVE DNA analytical platform a useful tool for the large scale mutation screening required in assessing whether *ATM* heterozygosity plays a significant role in breast cancer populations.

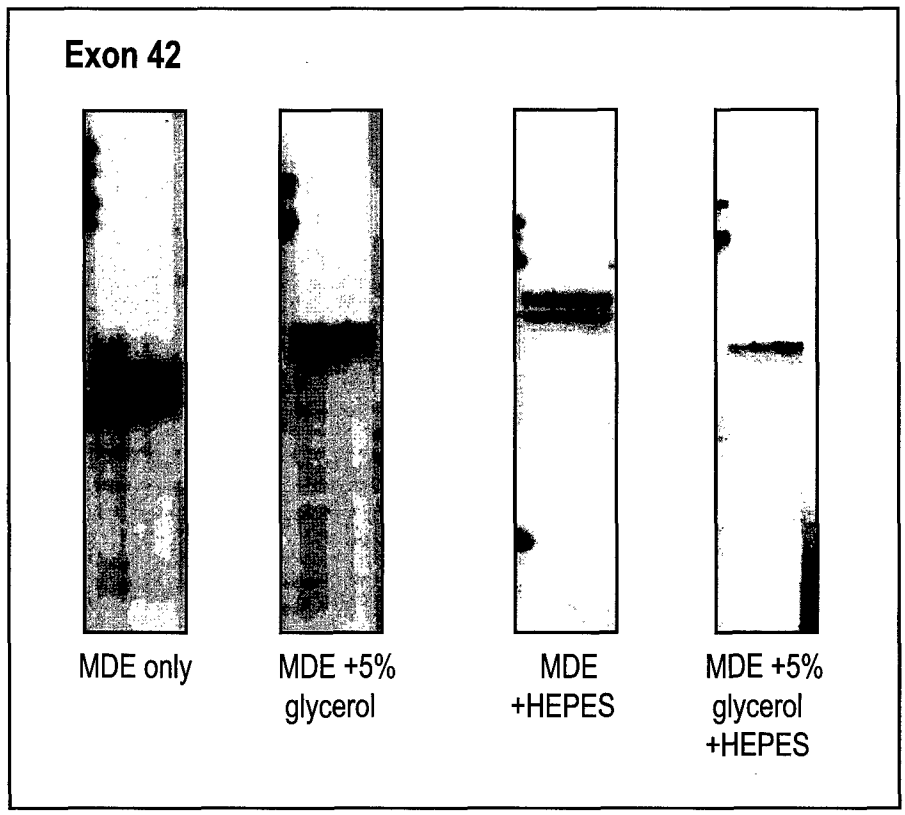

Figure 1. No mutations were detected in exon 42 under various SSCP conditions. SSCP was performed on ATM exons amplified by PCR from genomic DNA as described by Teraoka et al.[8] Samples (4-µL each) were loaded on standard mutation detection enhancement (MDE, FMC BioProducts, Rockland, ME) gels with or without supplements. For each gel, the lane on the right is the wild-type control, and the left and middle lanes are samples with known AT mutations. Addition of 20 mM HEPES buffer or of both glycerol (5%) and HEPES (20 mM) buffer, did not facilitate detection of mutations in ATM exon 42 by SSCP.

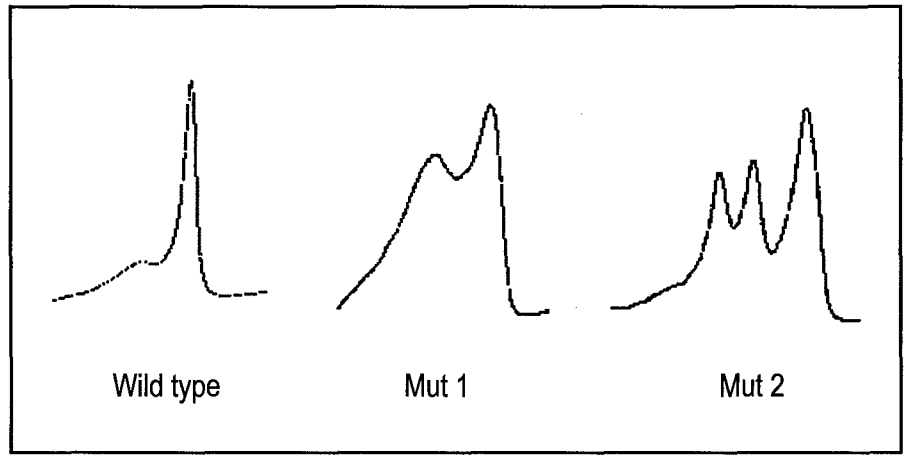

Figure 2. Samples of ATM Exon 42 were evaluated by Temperature Modulated Heteroduplex Analysis (TMHA) with the WAVE system instrument platform. The control (wild-type DNA sample) is characterized by one peak. Different mutations were distinguished in samples 1 and 2 as indicated by variant chromatograms, which were also different from the wild-type. Differences in chromatograms were due to resolution of the homoduplexes from the heteroduplexes (formed between wild-type and variant DNA strands in the compound heterozygote samples). Analysis of each sample required less than 8 minutes without pooling.

SSCP	WAVE System
1. Amplify *ATM* exons by PCR with radiolabeled nucleotide.	1. Amplify *ATM* exons by PCR and form heteroduplexes with the thermocycler in a 96 well plate.
2. Prepare two large format standard mutation detection enhancement gel (FMC). Wait 2 hours for gel polymerization.	2. Load PCR plate into the autosampler.
3. Denature PCR products and place on ice.	3. Use software to optimize analytical conditions.
4. Load 4 µL of each sample per gel lane with a micropipette.	4. Run samples (<7.4 min per sample). Automated injection and electronic data storage.
5. Electrophorese for 15 hours at 7 W.	
6. Dry gels for 2 hours.	
7. Expose dried gels to X-ray film for 24 - 48 hours.	

Table 1. Outline of SSCP and WAVE system protocols. SSCP was performed on ATM exons amplified by PCR from genomic DNA as described by Teraoka et al.[8]

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Appendix IIId

ATM Mutations in Breast Cancer Patients with Early-onset or Family History.
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We are screening for mutations in the ATM gene among individuals drawn from a population-based study of breast cancer in a 3 county area of Western Washington. Breast cancer cases have been selected based on meeting one of 2 criteria, early-onset (diagnosed by age 35), or having a first degree relative with breast cancer. To date, we have screened 142 cases and 80 matched controls. The 62 ATM coding exons were amplified by multiplex PCR and screened by SSCP. No truncation or deletion mutations were detected. However, possible ATM missense mutations were detected in 7.7% (11/142) of total cases, versus 1.25% (1/80) in controls. All 11 of the cases with possible mutations had some family history of breast cancer. Of 64 cases with a first degree relative with breast cancer, 8 (12.5%) had ATM missense mutations. Among the 99 cases diagnosed at age 35 or less, 4 (4%) had ATM missense mutations. An additional 27 individuals with rare variants that resulted in amino acid substitutions were also identified (17 in cases, 10 in controls). Some of these rare variants may also represent mutations, but their effects will require further functional studies. We have recently shifted our screening approach to utilize denaturing HPLC and have obtained increased sensitivity over SSCP for the detection of single nucleotide substitution in the ATM gene.

Teraoka, SN., Malone, KE., Ostrander, EA., Daling, JR., Concannon, P. (1999). ATM Mutations in Breast Cancer Patients with Early-Onset or Family History. Proceedings of American Society of Human Genetics, Annual Meeting. In Press

Appendix IIIe

ATM Mutations in Breast Cancer Patients with Early-onset or Family History

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Appendix IIIf

A High Frequency of ATM Splicing Mutations With Diverse Consequences in Ataxia-Telangiectasia.

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Mutations resulting in defective splicing comprise a significant proportion (30/62, 48%) of a series of mutations in the ATM gene in Ataxia-Telangiectasia patients that were detected by the Protein Truncation Assay (PTT) followed by sequence analysis of cDNA and genomic DNA. The remainder of mutations were small deletions or nonsense mutations. Less than half of the splicing mutations involved the canonical AG splice acceptor site or GT splice donor site. There was an increased percentage of mutations at less stringently conserved sites, including silent mutations of the last coding nucleotide of exons, mutations in other nucleotides in the consensus splice sites, and creation of splice acceptor or donor sites in either introns or exons. These splicing mutations found in the genomic DNA led to a variety of consequences at the cDNA level, including exon skipping, or to a lesser degree, intron retention, activation of cryptic splice sites, or use of new splice sites. In addition, 7 of 12 nonsense mutations in the genomic DNA led to deletion in the cDNA of the exons in which the stop codons occurred. These data are consistent with evidence from others that some premature termination codons cause defective splicing, presumably by altering secondary structure. We also observed many cases of exon deletions at the cDNA level in normal controls for which no underlying defect could be found in genomic DNA. This indicates the existence of alternatively spliced transcripts in normal individuals and suggests caution in the interpretation of prior reports of exon deletions in ATM when not accompanied by identification of genomic mutations. Determining the exact nature of splicing mutations has potential prognostic implications for AT patients and may aid in studies of genomic mutations in AT heterozygotes at risk for cancer.

Teraoka, S., Telatar M., Becker-Catania S., Liang T., Onengut S., Tolun A., Chessa L., Sanal O, Bernatowska E., Gatti R., Concannon P. (1998) A High Frequency of Splicing Mutations with Diverse Consequences in Ataxia-telangiectasia. Proceedings of American Society of Human Genetics Annual Meeting. 63(4) supplement:A195.

Appendix IIIg

Screening for Ataxia-Telangiectasia Mutations in a Population-based Sample of Women with Early-onset Breast Cancer.

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Although mutations in the genes BRCA1 and BRCA2 are thought to account for much of the genetic susceptibility of individuals diagnosed with breast cancer before age 30, heterozygosity in the gene for ataxia-telangiectasia (AT), a rare, autosomal recessive disorder, has also been suggested as a risk factor. Prior epidemiologic studies have implied that AT carriers are at increased risk for breast cancer, with a relative risk of 3.8. We are assessing whether a mutated allele of the AT gene, ATM, is a genetic risk factor for breast cancer, by screening for ATM mutations in patients with early-onset breast cancer. These patients were derived from a large population-based, case-control study of primary breast cancer in Western Washington. We are screening genomic DNAs by SSCP (single strand conformation polymorphism) analysis of the 62 coding exons amplified by multiplex PCR. Variants detected by SSCP were confirmed by sequencing and checked against age-matched controls. In 87 early-onset breast cancer cases, we have found no truncation or deletion mutations in the ATM gene. We have detected three cases of possible missense mutations. Two of these occur in regions of the ATM gene of potential functional significance; a putative SH3 binding site, and a highly conserved region of the phosphatidyl inositol-3-kinase domain. At present, our data do not support a role for ATM heterozygosity in early-onset breast cancer. However, the numbers screened to date are small, and some observed variants may, in fact, be mutations. We plan to increase screening efficiency and expand significantly the number of cases to be screened. Assessment of the prevalence of ATM mutations in breast cancer patients and controls has implications for diagnosis and treatment, given the estimated frequency of AT carriers of 1.4\% and their possible hypersensitivity to standard therapeutic doses of radiation.

Teraoka, SN., Malone, KE., Ostrander, EA., Onengut, S., Tolun, A., Daling, JR., Concannon, P. (1997). Screening for Ataxia-Telangiectasia Mutations in a Population-based Sample of Women with Early-Onset Breast Cancer. (1997) Proceedings of American Society of Human Genetics Meeting. 61(4) supplement:463

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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