Human Molecular Genetics, 1996, Vol. 5, No. 4 **433–439**

ARTICLE

Predominance of null mutations in ataxia-telangiectasia

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Received December 5, 1995; Revised and Accepted January 16, 1996

GenBank accession no. U33841

Ataxia-telangiectasia (A-T) is an autosomal recessive disorder involving cerebellar degeneration, immunodeficiency, chromosomal instability, radiosensitivity and cancer predisposition. The responsible gene, ATM, was recently identified by positional cloning and found to encode a putative 350 kDa protein with a PI 3-kinase-like domain, presumably involved in mediating cell cycle arrest in response to radiation-induced DNA damage. The nature and location of A-T mutations should provide insight into the function of the ATM protein and the molecular basis of this pleiotropic disease. Of 44 A-T mutations identified by us to date, 39 (89%) are expected to inactivate the ATM protein by truncating it, by abolishing correct initiation or termination of translation, or by deleting large segments. Additional mutations are four smaller in-frame deletions and insertions, and one substitution of a highly conserved amino acid at the PI 3-kinase domain. The emerging profile of mutations causing A-T is thus dominated by those expected to completely inactivate the ATM protein. ATM mutations with milder effects may result in phenotypes related, but not identical, to A-T.

INTRODUCTION

Ataxia-telangiectasia (A-T) is a highly pleiotropic disorder inherited in an autosomal recessive manner, with an average worldwide frequency of 1:40 000–1:100 000 live births (see refs 1–3 for recent reviews). Cerebellar ataxia resulting from gradual loss of the Purkinje cells in the cerebellum is usually the first clinical sign, and leads to progressive neuromotor deterioration. Its co-occurrence with telangiectases (dilated blood vessels) in the conjunctivae and occasionally on the facial skin—the second early hallmark of the disease—usually establishes the differential diagnosis of A-T from other cerebellar ataxias. Additional characteristics of this disorder are cellular and humoral immunodeficiency, thymic and ovarian degeneration, growth retardation, occasional endocrine abnorma-

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lities, profound predisposition to lymphoreticular malignancies, and acute sensitivity to ionizing radiation. A-T heterozygotes are moderately cancer prone, and cultured cells from these individuals are moderately radiosensitive.

The cellular phenotype of A-T includes reduced life span of cultured cells, chromosomal instability, hypersensitivity to ionizing radiation and radiomimetic chemicals, and defective checkpoints at the G1, S and G2 phases of the cell cycle (2–5). The G1 and G2 checkpoint defects are evident as reduced delay in cell cycle progression following treatment with ionizing radiation or radiomimetic chemicals (5), while the rise in the p53 protein level usually associated in normal cells with radiation-induced G1 arrest is delayed in A-T cells (6,7). The defective checkpoint at the S phase is readily observed as radioresistant DNA synthesis (RDS) (4). Cellular sensitivity to these DNA damaging agents and RDS are usually considered an integral part of the A-T phenotype.

Although these clinical and cellular features are considered common to all 'classical' A-T patients, variations have been noted. Milder forms of the disease with later onset, slower clinical progression, reduced radiosensitivity and occasional absence of RDS have been described in several ethnic groups (8–11). Additional phenotypic variability possibly related to A-T is suggested by several disorders that show 'partial A-T phenotype' with varying combinations of ataxia, immunodeficiency and chromosomal instability without telangiectases (12–16). Still, other disorders display the A-T phenotype and additional features; most notable is the Nijmegen breakage syndrome that combines A-T features with microcephaly, sometimes with mental retardation, but without telangiectases (17).

The A-T gene was mapped to chromosome 11q22–23 (18), and recently cloned in our laboratory using positional cloning and designated ATM (19,20). This gene spans about 150 kb of genomic DNA and produces a 13 kb transcript. The predicted 350 kDa protein has a carboxy terminal region of 350 amino acids which shows strong similarity to the catalytic domain of the p110 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) (19,20). This protein is similar to several large proteins in various species that contain PI 3-kinase-like domains at their carboxy termini, and are involved in DNA damage processing and cell cycle control. Among these proteins are TEL1p and MEC1p in budding yeast, rad3p in fission yeast, the TOR proteins in yeast and their mammalian counterpart, FRAP (RAFT1), mei-41 in *Drosophila melanogaster*, and the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{CS}) in mammals. Mutations in the genes encoding these proteins result in a variety of phenotypes that share features with A-T, such as radiosensitivity, chromosomal instability, telomere shortening, and defective cell cycle checkpoints (reviewed in refs 20,21). A possible working model for the ATM protein's function is DNA-PK, a serine/threonine protein kinase that is activated *in vitro* by DNA double-strand breaks and responds by phosphorylating several regulatory proteins (22). The ATM protein may be responsible for conveying a signal evoked by a specific DNA damage to various checkpoint systems, possibly via lipid or protein phosphorylation. Elucidating the nature of the mutations responsible for A-T should give insight into the molecular and physiological bases of the disease. In our search for ATM mutations in A-T patients from a variety of ethnic populations, we report here the unique profile of these mutations.

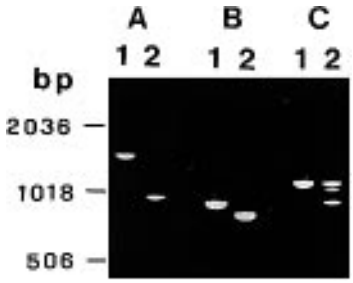

Figure 1. Detection of large deletions in the ATM transcript in three A-T patients (Table 1). RT-PCR products obtained from control (1) and patient (2) RNAs using primers flanking the deletions were separated on a 1.2% agarose gel. (**A**) Patient AT12RM showing homozygous deletion of 403 nt; (**B**) AT21RM, a patient homozygous for a 90 nt deletion; (**C**) AT13BER, a patient heterozygous for a 159 nt deletion. The minor band of intermediate size represents a heteroduplex of normal and mutant strands typically observed in such heterozygotes.

RESULTS

The ATM transcript was scanned for mutations in fibroblast and lymphoblast cell lines derived from an extended series of A-T patients from 13 countries, all of whom were characterized by the classical A-T phenotype. The analysis was based on RT-PCR followed by restriction endonuclease fingerprinting (REF). REF is a modification of the single-strand conformation polymorphism (SSCP) method, and enables efficient detection of sequence alterations in DNA fragments up to 2 kb in length (23). Briefly, after PCR amplification of the target region, multiple restriction endonuclease digestions are performed prior to SSCP analysis, in order to increase the sensitivity of the method and enable precise localization of a sequence alteration within the analyzed fragment. The coding sequence of the ATM transcript, which spans 9168 nucleotides (20), was thus divided into eight partly overlapping portions of 1.0–1.6 kb, and each one was analyzed separately. Sequence alterations causing abnormal REF patterns were located and disclosed by direct sequencing. Mutations identified in this way were reconfirmed by repeating the RT-PCR and sequencing, or by testing the presence of the same mutations in genomic DNA. In compound heterozygotes, the two alleles were separated by subcloning and individually sequenced. In some cases, agarose gel electrophoresis showed large deletions in the ATM transcript manifested as RT-PCR products of reduced sizes (Fig. 1). The breakpoints of such deletions were delineated by direct sequencing of these products.

The 44 mutations identified to date in our patient cohort (Table 1 and Fig. 2) include 34 new ones and 10 previously published ones (19). These mutations were found amongst 55 A-T families: many are unique to a single family, while others are shared by several families, most notably the 4 nt deletion, 7517del4, which is common to six Italian A-T families (Table 1). These families come from six towns and villages surrounding Naples. According to this sample, there is a considerable heterogeneity of mutations in A-T, and most of them are 'private'. The proportion of homozygotes in this sample is relatively high due to a high degree of consanguinity in the populations studied. It should be noted, however, that apparently homozygous patients from non-consanguineous families may in fact be compound heterozygotes with one allele not expressed or producing an unstable message.

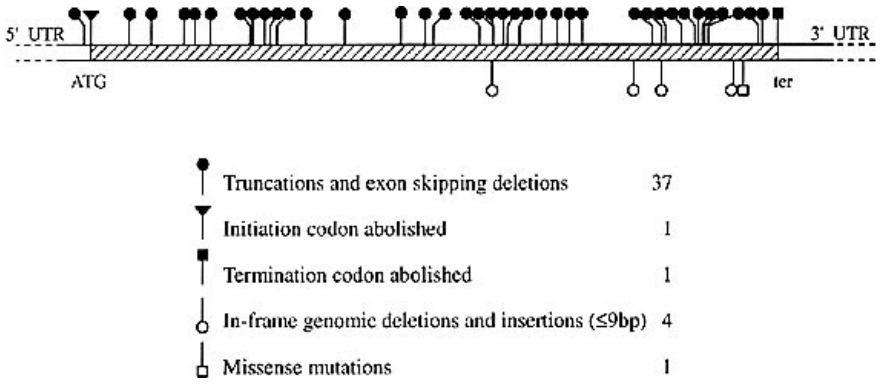

Figure 2. Distribution of 44 A-T mutations along the open reading frame (stippled box) of the ATM transcript. For large deletions, the symbols mark the locations of the 5′ breakpoints. Above the line: mutations expected to inactivate the ATM protein. Below the line: mutations which *a priori* may have a milder nature. By inference, the latter probably also exert the same effect on the protein.

Table 1. Mutations in the ATM gene in patients with classical A-T

mRNA sequence change[1]	Predicted protein alteration	Codon[8]	Patient	Ethnic/ geographical origin	Genotype[11]
Truncations and exon skipping deletions:					
9001delAG	Truncation	3001	91RD90[9]	Turkish	Hmz
8946insA	Truncation	2983	AT103LO	American	Hmz
8307G→A	Trp→ter; truncation	2769	AT2SF	American	Compd Htz
8283delTC	Truncation	2762	AT28RM	Italian	Compd Htz
8269del403[2]	Truncation	2758	AT12RM	Italian	Hmz
8269del150[3]	Del, 50 aa	2758	F-2086	Turkish	Compd Htz
			GM9587	American	Compd Htz
8140C→T	Gln→ter; truncation	2714	IARC12/AT3	French	Hmz
7883del5	Truncation	2628	ATF104	Japanese	Hmz
			JCRB316	Japanese	Compd Htz
7630del298/7789del139[4,5]	Truncation	2544	AT4LA	Carribean Black	Comp Htz
7630del159[3]	Del, 53 aa	2544	F-2086	Turkish	Compd Htz
			AT13BER	German	Compd Htz
7517del4	Truncation	2506	AT43RM[10]	Italian	Hmz
			AT59RM[10]	Italian	Hmz
			AT22RM[10]	Italian	Hmz
			AT57RM[10]	Italian	Compd Htz
			AT7RM[10]	Italian	Compd Htz
			AT8RM[10]	Italian	Compd Htz
6573del5	Truncation	2192	AT12ABR	Australian	Compd Htz
6348del105[3]	Del, 35 aa	2116	IARC15/AT4	French	Hmz
6199del149[3]	Truncation	2067	WG1101	Canadian	Hmz
5979del5	Truncation	1994	AT5RM	Italian	Compd Htz
5712insA	Truncation	1905	AT15LA	Philippino	Compd Htz
5554insC	Truncation	1852	F-2079[9]	Turkish	Hmz
5539del11	Truncation	1847	AT2RO[9]	Arab	Hmz
5320del355[6]	Truncation	1774	AT7RM	Italian	Compd Htz
5320del7	Truncation	1774	AT2SF	American	Compd Htz
5178del142[3]	Truncation	1727	AT50RM	Italian	Compd Htz
4612del165[3]	Del, 55 aa	1538	ATL105	Japanese	Hmz
4437del175[3]	Truncation	1480	AT29RM	Italian	Hmz
4110del127[3]	Truncation	1371	AT2TAN[9]	Turkish	Hmz
3403del174[3]	Del, 58 aa	1135	F-2095	Turkish	Compd Htz
2839del83[3]	Truncation	947	F-2080[9]	Turkish	Hmz
			AT10TAN[9]	Turkish	Hmz
2467del372[3,5]	Del, 124 aa	823	AT6LA	English/Irish	Hmz
2377del90[3]	Del, 30 aa	793	AT21RM[9]	Italian	Hmz
2284delCT	Truncation	762	F-169[9]	Palestinian Arab	Hmz

Table 1. Continued

2125del126[3]	Truncation	709	F-2078[9]	Turkish	Hmz
2113delT	Truncation	705	AT5RM	Italian	Compd Htz
1563delAG[5]	Truncation	522	AT8LA[9]	Swiss/German	Hmz
1339C→T	Arg→ter; truncation	447	F-2005[9]	Druze	Hmz
1240C→T	Gln→ter; truncation	414	AT26RM	Italian	Compd Htz
755delGT	Truncation	252	AT24RM	Italian	Hmz
497del7514[7]	Truncation	166	F-596[9]	Palestinian-Arab	Hmz
30del215	Incorrect initiation	5′ UTR	F-303	Bedouine	Hmz
In-frame genomic deletions and insertions:					
8578del3	Del, 1 aa	2860	AT3NG	Dutch	Compd Htz
7636del9	Del, 3 aa	2547	AT2BR	Celtic/Irish	Hmz
			AT1ABR	Australian (Irish)	Hmz
			AT1SF	American	Compd Htz
7278del6[5]	Del, 2 aa	2427	AT5BI	Indian/English	Compd Htz
			GM5823	English	Compd Htz
5319ins9	Ins, 3 aa	1774	251075–008T	Finnish	Compd Htz
Other base substitutions:					
9170G→C	ter→Ser Extension of protein by 29 amino acids	ter	F-2089[9]	Turkish	Hmz
8711A→G	Glu2904Gly	2904	AT41RM	Italian	Hmz
2T→C	Met→Thr Initiation codon abolished	1	AT8BI	British	Compd Htz

[1]Presented according to the nomenclature proposed by Beaudet & Tsui (41). Nucleotide numbers refer to their positions in the sequence of the ATM transcript (ref. 20; accession number U33841). The first nucleotide of the open reading frame was designated +1.
[2]Three adjacent exons skipped.
[3]One exon skipped.
[4]This allele produces two transcripts, with one or two ajacent exons skipped.
[5]The same mutation was found in two affected siblings.
[6]Two exons skipped.
[7]This transcript is produced by an allele containing a large genomic deletion spanning approximately 85 kb within the ATM gene in Family ISAT 9 (19).
[8]For deletions, the number of the first codon on the amino terminus side is indicated. Codon numbers are according to the ATM protein sequence published by Savitsky *et al.* (20). In each section of the table, the mutations are ordered according to the codon numbers in this column, beginning with the one closest to the carboxyl terminus.
[9]Consanguineous family.
[10]All patients are from the same region.
[11]Genotypic combinations in which the mutation was found. Hmz: homozygote; Compd Htz: compound heterozygote. Each patient represents one family.

This series of 44 A-T mutations is dominated by deletions and insertions. The smaller ones, of less than 12 nt, reflect identical sequence alterations in genomic DNA. Deletions spanning larger segments of the ATM transcript were all found to reflect exon skipping. This phenomenon usually results from sequence alterations at splice junctions or within introns, or mutations within the skipped exons, mainly of the nonsense type (24–28). One large deletion spans about 7.5 kb of the transcript and represents a genomic deletion of about 85 kb within the ATM gene (ref. 19 and unpublished data). Of these deletions and insertions, 25 are expected to result in frameshifts. Together with the four nonsense mutations, truncation mutations account for 66% of the total number of mutations in this sample. Seven in-frame deletions span long segments (30–124 aa) of the protein, and similarly to the truncation mutations, are expected to have a severe effect on the protein's structure. It should be noted that two base substitutions abolish the translation initiation and termination codons. The first methionine residue downstream to the initiator codon is at position 94. The mutation eliminating the termination codon is expected to result in an extension of the ATM protein by an additional 29 amino acids. This mutation may affect the conformation of the nearby PI 3-kinase-like domain.

While the effect of the four small (1–3 aa) in-frame deletions and insertions on the ATM protein remains to be studied, it should be noted that one such deletion (8578del3) leads to a loss of a serine residue at position 2860. This amino acid is part of a conserved motif within the PI 3-kinase-like domain typical of the protein family to which ATM is related, and is present in seven of nine members of this family (Fig. 3). The single missense mutation identified in this study, which leads to a Glu2904Gly substitution, results in a nonconservative alteration of another extremely conserved residue within this domain, which is shared by all of these proteins (Fig. 3). The patient homozygous for this mutation, AT41RM, shows the typical clinical A-T phenotype. Measurement of radioresistant DNA synthesis in the patient's cell line revealed a typical A-T response (not shown), demonstrating that this patient has the classical A-T cellular phenotype.

DISCUSSION

The recently discovered ATM gene is probably involved in a novel signal transduction system that links DNA damage surveillance to cell cycle control. A-T mutations affect a variety of tissues and lead to cancer predisposition. This striking

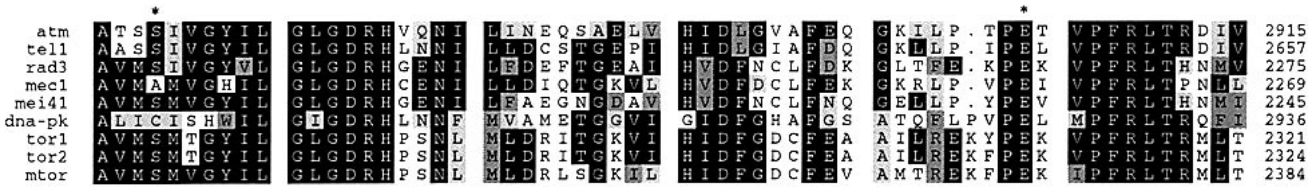

Figure 3. Alignment of amino acid sequences within the PI 3-kinase-like domains of the ATM protein and related proteins in various species (see refs 20,21 for details). One asterisk denotes the serine residue at position 2860 which is deleted in the 8578del3 allele, and two asterisks denote the glutamic acid residue substituted in the Glu2904Gly allele. tel1: TEL1p of *S. cerevisiae*; rad3: rad3p of *S. pombe*; mec1: MEC1p of *S. cerevisiae*; mei41: mei-41 protein of *D. melanogaster.* dna-pk: the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{CS}) in humans; tor1, tor2 and mtor: products of the TOR1 and TOR2 genes in *S. cerevisiae* and mTOR (RAFT) in rat.

phenotype together with the existence of 'partial A-T phenotypes' endow the study of ATM mutations with special significance.

The large size of the ATM transcript renders its systematic screening for mutations laborious. The recently developed REF method (23) appears to offer a particularly suitable tool for this purpose. This technique was used in our laboratory to identify ATM as the gene mutated in A-T (19). The power of this method to efficiently detect sequence variations in large DNA segments led us to streamline the procedure for a mutation search throughout the ATM transcript in a larger series of A-T patients.

The ATM gene leaves a great deal of room for mutations: it encodes a large transcript and contains 65 exons (29). The variety of mutations identified in this study indeed indicates a rich mutation repertoire. Despite this wealth of mutations, their structural characteristics point to a definite bias towards those that inactivate or eliminate the ATM protein. The nature or distribution of the genomic deletions among these mutations do not suggest a special preponderance of the ATM gene for such mutations, such as that of the dystrophin (30) or steroid sulfatase (31) genes which are particularly prone to such deletions. Thus, one would have expected also a strong representation of missense mutations, which usually constitute a significant portion of the molecular lesions in many disease genes (24,25). However, only one such mutation was identified in the present study. Other point mutations reflected in this series are the nonsense mutations and those that probably underlie the exon skipping deletions observed in many patients, again, exerting a severe structural effect on the ATM protein.

A technical explanation for this bias towards deletions and insertions could be a greater ability of the REF method to detect such lesions versus its ability to detect base substitution. Liu and Sommer (23) have shown, however, that the detection rate of this method in a sample of 42 point mutations in the factor IX gene ranged between 88% and 100%, depending on the electrophoresis conditions. The seven base substitutions detected directly by the REF method in the present study (Table 1), indicate that such sequence alterations are detected in our hands as well.

Since the expected result of most of these mutations is complete inactivation of the protein, this skewed mutation profile might represent a functional bias related to the studied phenotype, rather than a structural feature of the ATM gene that lends itself to a particular mutation mechanism. The classical A-T phenotype appears to be caused by homozygosity or compound heterozygosity for null alleles, and hence is probably the most severe expression of defects in the ATM gene. The plethora of missense mutations expected in the large coding region of this gene is probably rarely represented in patients with classical A-T, unless such a mutation results in complete functional inactivation of the protein. By inference, the only missense mutation identified in this study, Glu2940Gly, which substitutes a conserved amino acid at the PI 3-kinase domain and clearly gives rise to a classical A-T phenotype, points to the importance of this domain for the biological activity of the ATM protein. The only deletion of a single amino acid found in this study, 8578del3, is also located in this region. Mutations in this domain abolish the telomere-preserving function of the TEL1 protein in *Saccharomyces cerevisiae* (32), a protein which shows a particularly high sequence similarity to ATM (20,21). Another member of the family of PI 3-kinase-related proteins that includes ATM is the mammalian FRAP. Mutations in the PI 3-kinase domain abolish its autophosphorylation ability and biological activity (33). These observations, together with the mutation shown here, suggest that this domain in ATM is also likely to include the catalytic site, which may function as a protein kinase.

Genotype-phenotype relationships associated with the ATM gene may therefore extend beyond classical A-T. There are several examples of genes in which different mutations lead to related but clinically different phenotypes. For example, different combinations of defective alleles of the *ERCC2* gene may result in xeroderma pigmentosum (group D), Cockayne's syndrome or trichothiodystrophy—three diseases with different clinical features involving UV sensitivity (34,35). Different mutations in the CFTR gene may lead to full-fledged cystic fibrosis, or only to congenital bilateral absence of the vas deferens which is one feature of this disease (36,37). A particularly interesting example is the X-linked *WASP* gene responsible for Wiskott Aldrich syndrome (WAS), characterized by immunodeficiency, eczema and thrombocytopenia. Most of the mutations responsible for this phenotype cause protein truncations; however, certain missense mutations may result in X-linked thrombocytopenia, which represents a partial WAS phenotype, while compound heterozygosity for a severe and mild mutation results in females in an intermediate phenotype (38,39). In a similar manner, genotypic combinations of mutations with different severities create a continuous spectrum of phenotypic variation in many metabolic diseases.

Which phenotypes are most likely to be associated with milder ATM mutations? Since cerebellar damage is the early and severe manifestation of A-T, it is reasonable to assume that the cerebellum might also be affected to some extent in phenotypes associated with milder ATM mutations. Such phenotypes may include cerebellar ataxia, either isolated (40) or coupled with various degrees of immunodeficiency. The latter combination has

indeed been described, sometimes with chromosomal instability, and is often designated 'ataxia without telangiectasia' (12–16). Friedman and Weitberg (16) recently suggested a new clinical category of 'ataxia with immune deficiency' that would include A-T as well as other cases of cerebellar degeneration with immune deficits. Routine immunological evaluation of patients with cerebellar disorders may reveal a higher frequency of such cases than previously estimated. However, in view of the pleiotropic nature of the ATM gene, the range of phenotypes associated with various ATM genotypes may be even broader, and include mild progressive conditions not always defined as clear clinical entities. Screening for mutations in this gene in such cases may reveal wider boundaries for the molecular pathology associated with the ATM gene.

MATERIALS AND METHODS

RT-PCR

Total RNA was extracted from cultured fibroblast or lymphoblast cells using the Tri-Reagent system (Molecular Research Center, Cincinnati, OH). Reverse transcription was performed on 2.5 µg of total RNA, using an oligo(dT) primer and the Superscript II Reverse Transcriptase (Gibco BRL, Gaithersburg, MD), in 10 µl reactions containing the buffer recommended by the supplier, 125 U/ml of RNAsin (Promega) and 1 mM dNTPs (Pharmacia). The reaction products were used as templates for PCR performed with specific primers. These reactions were carried out in 50 µl containing 2 U *Taq* DNA Polymerase (Boehringer Mannheim, Mannheim, Germany), 200 µM dNTPs, 0.5 µM of each primer, and one tenth of the RT-PCR products. The products were purified using the QIA-quick spin system (Qiagen, Hilden, Germany).

Restriction endonuclease fingerprinting

The protocol of Liu and Sommer (23) was followed with slight modifications. RT-PCR was performed as described above, using primers defining PCR products of 1.0–1.6 kb. One hundred ng of amplified DNA was digested separately with five or six restriction endonucleases in the presence of 0.2 U shrimp alkaline phosphatase (United States Biochemicals, Cleveland, OH). Following heat inactivation at 65°C for 10 min, the digestion products corresponding to the same PCR product were pooled, denatured at 96°C for 5 min and immediately chilled on ice. Ten ng of this fragment mixture was labeled in the presence of 6 µCi of [^{33}P]ATP and 1 U of T4 polynucleotide kinase (New England Biolabs, Beverly, MA) at 37°C for 45 min. Twenty µl of stop solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol, and 10 mM NaOH were added, and the samples were boiled for 3 min and quick-chilled on ice. Electrophoresis was performed in 5.6% polyacrylamide gels in 50 mM Tris-borate, pH 8.3, 1 mM EDTA at constant power of 12 W for 3 h at room temperature, with a fan directed to the glass plates, keeping them at 22–24°C. The gels were dried and subjected to autoradiography. A detailed protocol including primer sequences and restriction enzymes used is available on request.

Direct sequencing of PCR products

Five hundred ng of PCR products was dried under vacuum, resuspended in reaction buffer containing the sequencing primer, and the mixture was boiled and snap-frozen in liquid nitrogen. The Sequenase II system (Unites States Biochemicals) was used to carry out the sequencing reaction in the presence of 0.5 µg of single-strand binding protein (T4 gene 32 protein, United States Biochemicals). The reaction products were treated with 0.1 µg of proteinase K at 65°C for 15 min, separated on a 6% polyacrylamide gel, and visualized by autoradiography.

Sequence accession number

The nucleotide sequence of the ATM gene transcript was submitted to the GenBank database under accession no. U33841.

ACKNOWLEDGEMENTS

We are indebted to Drs Steve Sommer and Qiang Liu for technical information regarding the REF method and to Drs Francis Collins and Dan Tagle for valuable discussions. We are grateful to Drs Auli Nuutila and Helena Kaariainen for the cell line 251075–008T. This study was supported by research grants from the A-T Children's Project, the A-T Medical Research Foundation, the Thomas Appeal (A-T Medical Research Trust), The United States-Israel Binational Science Foundation, Consiglio Nazionale Ricerche Progetto Finalizzato Applicazioni Cliniche Ricerca Oncologica, and the National Institute of Neurological Disorders and Stroke (NS31763). This work was carried out in partial fulfillment of the requirements for the Ph.D. degree to S.G.

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