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Characterization of *ATM* gene mutations in 66 ataxia telangiectasia families

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Ataxia telangiectasia (AT) is an autosomal recessive disease characterized by neurological and immunological symptoms, radiosensitivity and cancer predisposition. The gene mutated in AT, designated the *ATM* gene, encodes a large protein kinase with a PI-3 kinase-related domain. In this study, we investigated the mutational spectrum of the *ATM* gene in a cohort of AT patients living in Germany. We amplified and sequenced all 66 exons and the flanking untranslated regions from genomic DNA of 66 unrelated AT patients. We identified 46 different *ATM* mutations and 26 sequence polymorphisms and variants scattered throughout the gene. A total of 34 mutations have not been described in other populations. Seven mutations occurred in more than one family, but none of these accounted for more than five alleles in our patient group. The majority of the mutations were truncating, confirming that the absence of full-length ATM protein is the most common molecular basis of AT. Transcript analyses demonstrated single exon skipping as the consequence of most splice site substitutions, but a more complex pattern was observed for two mutations. Immunoblot studies of cell lines carrying *ATM* missense substitutions or in-frame deletions detected residual ATM protein in four cases. One of these mutations, a valine deletion proximal to the kinase domain, resulted in ATM protein levels >20% of normal in an AT lymphoblastoid cell line. In summary, our results survey and characterize a plethora of variations in the *ATM* gene identified by exon scanning sequencing and indicate a high diversity of mutations giving rise to AT in a non-isolated population.

INTRODUCTION

Ataxia telangiectasia (AT) is an autosomal recessive disorder that affects ~1:40 000–1:300 000 children in various ethnic groups (1–4). AT is a pleiotropic disease characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia, thymus dysplasia, immunodeficiency and bronchopulmonary infections, gonadal dysgenesis, chromosomal instability, abnormal X-ray sensitivity and cancer predisposition (1,2). Cultured cells from AT patients are also hypersensitive to ionizing radiation and show defective activation of radiation-induced cell cycle checkpoints, including retarded p53 stabilization (3–8). Heterozygous carriers of an AT mutation are clinically unaffected, but there is reported evidence from epidemiological studies that AT heterozygosity predisposes to some epithelial cancers, in particular breast cancer (9–13). The gene mutated in AT, *ATM*, has been localized to chromosome 11q23 (14) and subsequently was isolated and characterized (15,16). It spans ~150 kb of genomic sequence and contains 66 exons (17,18). The *ATM* gene gives rise to a ubiquitously expressed transcript of ~13 kb which encodes a nuclear 350 kDa protein with homology to PI-3 kinases and related proteins involved in DNA damage responses and cell cycle regulation (16,19–22). While the precise function of the *ATM* gene is still unknown, the ATM gene product is yet believed to sense DNA double-strand breaks (7) and to regulate physiological responses via p53- or Chk1-mediated pathways (4,23). Furthermore, the non-receptor tyrosine kinase c-Abl has been identified as a downstream target of the ATM protein (4,24,25).

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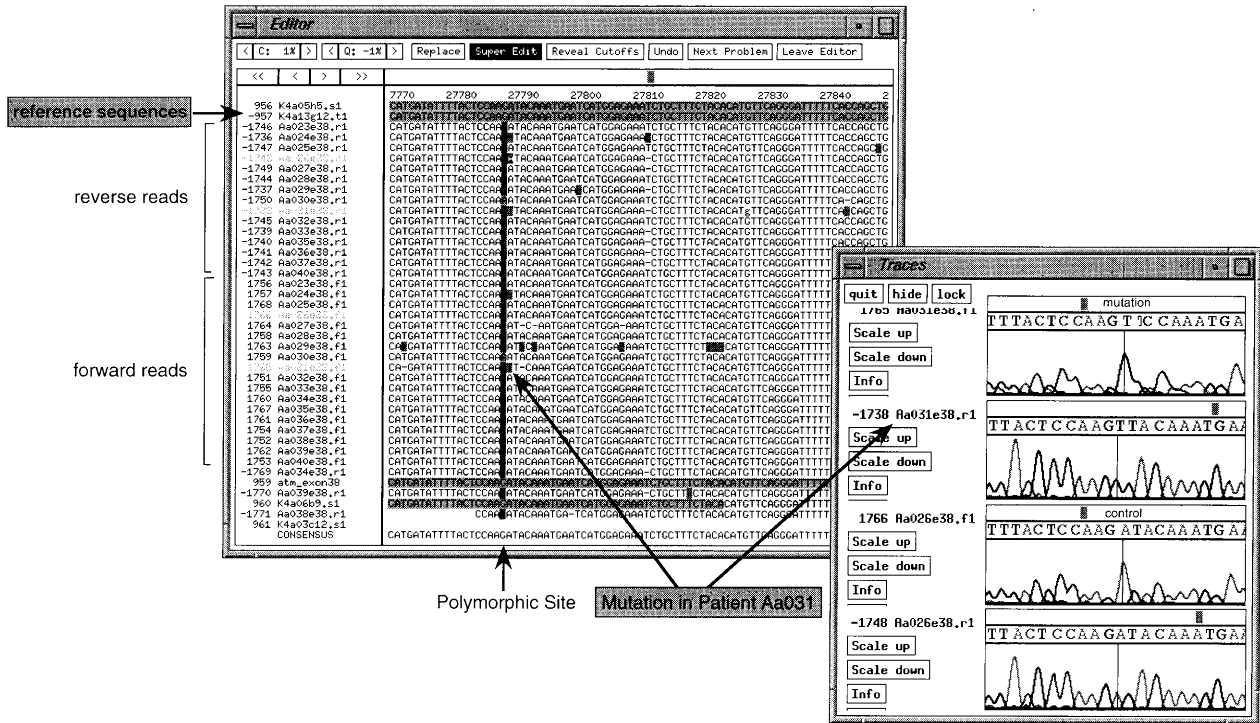

Figure 1. View of the XGAP editor and selected trace files of an AT sub-project with 18 patient samples.

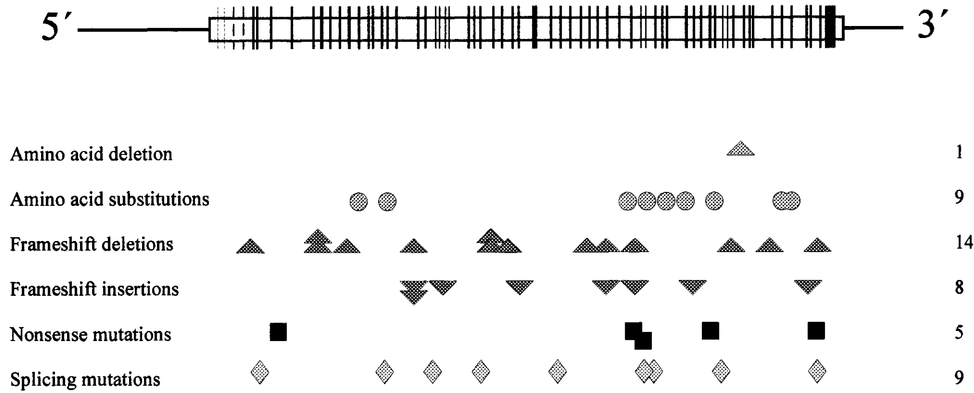

Figure 2. Distribution of mutations throughout the *ATM* gene in a group of 66 AT patients. In-frame or frameshift deletions, frameshift insertions, nonsense, missense and splicing mutations are indicated using different symbols.

Numerous different mutations in the *ATM* gene have been identified in classical AT and in some patients with variant forms of AT (26). The majority of the published mutations in the *ATM* gene are truncating, although missense substitutions and in-frame-deletions have also been found (15,27–36). Until now most studies have used cDNA-based screening techniques for mutation analysis and, thus, a number of the reported exon-skipping mutations have remained uncharacterized at the genomic level (26). Now that the exon–intron structure of the *ATM* gene has been fully elucidated (17) and complete sequence information is available for the entire *ATM* gene (18), mutation scanning methods using genomic DNA have become feasible. In the present study, we established primers and PCR conditions to amplify all exons and exon-flanking intron regions of the *ATM* gene. We applied automated sequencing of the PCR products to mutation scanning. As a result, we report the spectrum of *ATM* gene mutations identified in 66 AT families diagnosed in Germany.

RESULTS

We designed a set of 68 PCR primer pairs based on the published genomic sequence information to amplify all 66 exons and the adjacent intron regions of the *ATM* gene. The primers were arranged in a fashion to allow for amplification of most exons under the same conditions. Due to its large size of 3.8 kb, the last exon 66 was amplified in nine overlapping fragments. In a few other instances, two exons were amplified jointly together with a small intervening sequence. All primers and PCR conditions are compiled in Materials and Methods.

Table 1. Ataxia telangiectasia mutations

Mutation	Nucleotide change	Location	Predicted effect	Patient ID (status)
26delT	Deletion of T at 26–27	Exon 4	Truncation	Aa053 (hom)
72+2T→C	T→C at 72+2	Intron 4	Aberrant splicing	Aa005 (het)
Y171X	T→G at 513	Exon 8	Truncation	Aa050 (hom)
788delT	Deletion of T at 788–790	Exon 9	Truncation	Ae005 (het)
822delT	Deletion of T at 822	Exon 9	Truncation	Ae009 (hom)
				Aa037 (het)
1561delAG	Deletion of 2 bp at 1561–1565	Exon 12	Truncation	Aa029 (het)
F570S	T→C at 1709	Exon 13	Phe→Ser at 570	Aa003 (het)
2250G→A	G→A at 2250	Exon 16	Aberrant splicing	Aa033 (het)
				Ac048 (het)
				Ac053 (het)
				Ae006 (het)
R785C	C→T at 2353	Exon 17	Arg→Cys at 785	Ac046 (het)
2502insA	Insertion of A after 2502	Exon 19	Truncation	Aa037 (het)
2565delGins21	Deletion of G and insertion of 21 bp[a] at 2565	Exon 19	Truncation	Ac045 (het)
2921+3A→T	A→T at 2921+3	Intron 21	Aberrant splicing	Ae014 (hom)
3085insA	Insertion of A after 3085	Exon 23	Truncation	Ae007 (het)
3576G→A	G→A at 3576	Exon 26	Aberrant splicing	Aa025 (hom)
				Aa026 (het)
				Aa040 (hom)
3801delG	Deletion of G at 3801–3802	Exon 28	Truncation	Aa036 (het)
				Aa039 (hom)
3849delA	Deletion of A at 3849–3850	Exon 28	Truncation	Aa010 (hom)
4051delT	Deletion of T at 4051–4052	Exon 29	Truncation	Aa032 (het)
4143insT	Insertion of T after 4143	Exon 30	Truncation	Aa032 (het)
4630del4	Deletion of 4 bp at 4630–4635	Exon 33	Truncation	Aa030 (hom)
4909+1G→A	G→A at 4909+1	Intron 34	Aberrant splicing	Aa031 (hom)
				Ac044 (hom)
5309delC	Deletion of C at 5309	Exon 37	Truncation	Aa011 (het)
5441insT	Insertion of T after 5441	Exon 38	Truncation	Aa006 (het)
5546delT	Deletion of T at 5546–5549	Exon 39	Truncation	Ae015 (het)
V1913G	T→G at 5738	Exon 40	Val→Gly at 1913	Aa021 (het)
5876delA	Deletion of A at 5876–5878	Exon 41	Truncation	Ae017 (het)
E1978X	G→T at 5932	Exon 42	Truncation	Aa035 (het)
5977insA	Insertion of A after 5977	Exon 42	Truncation	Ae010 (het)
E2014X	G→T at 6040	Exon 43	Truncation	Ae004 (hom)
D2016G	A→G at 6047	Exon 43	Asp→Gly at 2016	Aa017 (hom)
R2032K	G→A at 6095	Exon 43	Aberrant splicing	Aa006 (het)
6096–9del5	Deletion of TTCTT at 6096–9	Intron 43	Aberrant splicing	Aa014 (het)
A2067D	C→A at 6200	Exon 45	Ala→Asp at 2067	Ae003 (het)
R2227C	C→T at 6679	Exon 48	Arg→Cys at 2227	Aa055 (het)
6867insT	Insertion of T after 6867	Exon 49	Truncation	Aa001 (hom)
R2443X	C→T at 7327	Exon 52	Truncation	Ae006 (het)
				Ae015 (het)
Y2470D	T→G at 7408	Exon 52	Tyr→Asp at 2470	Aa038 (het)
7630–2A→C	A→C at 7630–2	Intron 53	Aberrant splicing	Aa002 (het)
				Aa009 (het)
				Aa051 (het)
				Ac049 (hom)
7668del4	Deletion of 4 bp at 7668–7674	Exon 54	Truncation	Aa028 (hom)
V2662del	Deletion of 3 bp at 7983–7991	Exon 56	Deletion of Val at 2662	Ac052 (hom)
8385del10	Deletion of 10 bp at 8385–8404	Exon 59	Truncation	Aa005 (het)
R2849P	G→C at 8546	Exon 60	Arg→Pro at 2849	Aa034 (het)
G2867R	G→C at 8599	Exon 61	Gly→Arg at 2867	Aa027 (het)
8720C→TT	C→TT at 8720	Exon 62	Truncation	Ae017 (het)
8787–1G→T	G→T at 8787–1	Intron 62	Aberrant splicing	Aa013 (het)
C2931X	T→A at 8793	Exon 63	Truncation	Aa002 (het)
8814del11	Deletion of 11 bp at 8814–8824	Exon 63	Truncation	Aa020 (het)

Mutations have been named according to the recommended nomenclature of Beaudet and Tsui (58). Nucleotide numbering follows the cDNA sequence reported by Savitsky *et al*. (16) (GenBank accession no. U33841), with nucleotide position 1 assigned to the first nucleotide of the ATG initiation codon in exon 4. Exon–intron boundaries and numbers are as defined by Uziel *et al*. (17). Homozygous and heterozygous samples are indicated as ‘hom’ and ‘het’, respectively.
[a]The inserted sequence at 2565 in sample Ac045 reads <TACTGATCTAAATAGATCAGT>.

Table 2. Polymorphisms and rare sequence variants of the *ATM* gene

Nucleotide change	Location	Allele frequency	Amino acid change	Restriction site
72+36insAA	Intron 4	0.57	None	
146C/G	Exon 5	0.01 (G)	S49C	*Hin*fI (+)
496+221T/C	Intron 7	0.39 (C)	None	*Msp*I (+)
901+25T/G	Intron 9	0.01 (G)	None	
901+99C/A	Intron 9	0.02 (A)	None	*Mnl*I (–)
1176C/G	Exon 11	0.01 (G)	None	
1488T/C	Exon 12	0.01 (C)	None	*Alu*I (+)
2148C/G	Exon 16	0.01 (G)	None	*Msp*I (–)
2377–56G/A	Intron 17	0.36 (A)	None	
2572T/C	Exon 19	0.02 (C)	F858L	
3078–77T/C	Intron 22	0.37 (C)	None	*Mse*I (–)
3161C/G	Exon 24	0.07 (G)	P1054R	*Alw*I (–)
3285–9delT	Intron 24	0.18	None	
3403–15delA	Intron 25	0.37	None	
4777–20A/G	Intron 33	0.01 (G)	None	
5320–8T/C	Intron 38	0.09 (C)	None	
5557G/A	Exon 39	0.18 (A)	D1853N	
5558A/T	Exon 39	0.03 (T)	D1853V	*Mae*III (+)
6199–61C/G	Intron 44	0.09 (G)	None	
7515+45delTT	Intron 52	0.01	None	*Dde*I (–)
7630–123insCAGG	Intron 53	0.01	None	
8787–56T/C	Intron 62	0.39 (C)	None	*Mae*II (+)
8850+60G/A	Intron 63	0.37 (A)	None	
10775C/T	3′-UTR	0.32 (T)	None	
12564T/G	3′-UTR	0.37 (G)	None	

Sequence alterations and their allele frequencies are listed here as polymorphisms or neutral variants if the variations were found in similar frequencies on control chromosomes or if there was no obvious evidence for a potential disease-causing effect from the nature and location of a rare variation. Some of the variants have previously been described by Vorechovsky *et al*. (31,62). Restriction enzymes to distinguish between variant alleles are as indicated with (+) designating the creation and (–) the abolition of a recognition site.

A cohort of 66 German AT patients were investigated for their mutations in the *ATM* gene. From genomic DNA samples of all patients, PCR products were obtained for all exons and exon-flanking intron regions and were sequenced on both strands. We obtained ~25 kb of edited sequence information from each patient (Fig. 1; see also Materials and Methods). A total of 46 different mutations were identified on 75 of the 132 alleles, corresponding to a 57% detection rate. In addition, 26 variants and polymorphisms of unknown biological significance were found. The sequence alterations suggesting pathological consequences (mutations) are compiled in Table 1 and the sequence variants that are likely to represent polymorphisms or neutral variations are compiled in Table 2. The distribution of mutations throughout the *ATM* gene is illustrated in Figure 2. Each of the mutations was confirmed by an independent screening method in the parental DNA samples whenever available.

A major fraction of 27 different mutations (57%) were nonsense mutations or small frameshift insertions/deletions leading to a premature translation termination codon. This subset of the mutations included 14 frameshift deletions and seven insertions comprising between 1 and 11 nt, as well as one more complex frameshift mutation that had emerged from the deletion of a single guanine and the insertion of a 21 nt quasi-palindromic sequence (Table 1). The truncating mutations were scattered throughout the entire *ATM* gene without apparent clustering. Our data did not allow us to determine whether differences in the AT phenotype in our patients may result from different locations of the premature termination codon within the *ATM* gene. At any rate, three termination mutations were located downstream of the deduced kinase domain, indicating that even premature stop codons in the very final part of the protein impair ATM expression, stability or function to a disease-causing degree.

Nine different mutations were identified within splice sites of the *ATM* gene. Mutations within the conserved AG and GU dinucleotides of the acceptor and donor splice sites were regarded as pathogenic, since this mutation type results in aberrant splicing by exon skipping or activation of cryptic splice sites (42). Both effects were observed to occur together in lymphoblasts from patients with the common mutation 7630–2A→C (Table 3). Another mutation, 2921+3A→T, only affected the poorly conserved position +3 in the donor splice site of intron 21, but nevertheless caused a complex aberrant splicing pattern in *ATM* mRNA, in which exon 21 alone or both exons 20 and 21 were deleted (Table 3). Three other mutations, 2250G→A, 3576G→A and R2032K substituted the final nucleotide of exons 16, 26 and 43, respectively. All of them were confirmed to represent true splicing mutations since skipping of the affected exon was observed in transcripts from patient lymphoblastoid cell lines or fibroblasts (Table 3). No alternative splicing of these exons was detected in lymphocytes from control persons (43; M. Nicke, personal communication). The families of the three patients with the splicing mutation 3576G→A were of Italian, Turkish and Georgian descent, respectively, suggesting that this splicing mutation may be more common in south or south east Europe than

in Germany itself. Finally, a deletion of 5 bp, 6096–9del5, within the polypyrimidine tract of intron 43 was found to be associated with skipping of the downstream exon in fibroblasts of the heteroallelic patient. Since this deletion retains only four pyrimidines immediately preceding exon 44, it apparently impedes splice site recognition and thereby acts as a splicing mutation similar to previously reported polypyrimidine sequence alterations in *in vitro* reactions and in other genetic diseases (44,45).

Missense substitutions constituted ~20% of the identified mutations. Two missense mutations, R2849P and G2867R, reside within the kinase domain and affect residues that are highly conserved among ATM-homologous proteins from different species (16). No mutations were found within the putative leucine zipper region encoded by exon 27 (16) or within the proposed c-Abl binding site encoded by exon 30 (25). Most missense mutations resulted in the substitution of a conserved amino acid identical in the human and murine ATM proteins, the exception being R785C (46). We screened ~100 control chromosomes from anonymous donors for the presence of missense substitutions identified in the AT patients, in order to exclude the possibility that such amino acid mutations represent common polymorphisms. Five missense substitutions (S49C, F858L, P1054R, D1853V and D1853N) were classified as probable polymorphisms after this analysis, since they were also found in our analysis of control chromosomes (P1054R, D1853V and D1853N) or have been previously reported in DNA samples from random blood donors [e.g. by Vorechovsky *et al.* (31,62)]. Although P1054R was significantly more frequent in our AT patient group (10/132 chromosomes) than in random donors (4/100 chromosomes), this difference appeared to be due to the association of the relatively common AT splicing mutation 3576G→A with the P1054R substitution on the same allele in our AT patients and, thus, to a linkage disequilibrium. With regard to the remaining missense substitutions listed in Table 1, it must cautiously be noted that the absence of an amino acid substitution in a limited number of controls does not fully prove its causative role in the pathogenesis of AT disease. In order to further characterize the effect of non-truncating mutations, we analysed cell lines carrying *ATM* missense substitutions or in-frame deletions by immunoblot analysis for their levels of ATM protein expression (Fig. 3 and Table 4). A minority of these mutant cell lines revealed detectable but very low amounts of ATM protein. Only the cell line homozygous for the V2662del mutation exhibited higher ATM levels, which varied from culture to culture: This mutant exhibits levels which range between apparently normal and 20% of the normal level. A typical example is shown in Figure 3 (designated L106), which in this case showed a level of ~41%. This protein is probably not very stable and therefore fluctuates according to physiological conditions. It may be possible, but could not be determined, that it is very unstable in certain tissues. The underlying mutation which deletes one of three consecutive valines in exon 56 was identified in a single homozygous German AT patient. The 7-year-old girl developed ataxia by the age of 3 years. She has remained free of recurrent infections so far, despite laboratory evidence of absent IgA and lowered IgG3 levels. Chromosomal instability was shown by increased bleomycin-induced chromosome breakage rates. Hence, clinical and cytogenetic examinations of this patient reveal features of classic AT and further functional analysis of the mutant ATM protein is in progress to yield insight into the molecular pathogenesis of the disease.

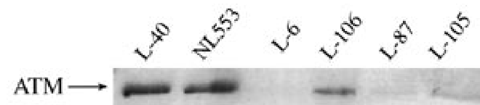

Figure 3. Immunoblot analysis of ATM protein levels in lymphoblastoid cell lines. Equal amounts of total protein were loaded in all lanes. L-40 and NL553, normal cell lines; L-6, an AT cell line homozygous for a stop mutation at position 35 of the ATM protein (49); L-106, L-87 and L-105, AT cell lines from patients included in this study (L-106 is homozygous for the V2662del mutation and corresponds to sample Ac052 in Table 1, L-87 is heterozygous for mutation 2250G→A and corresponds to sample Ac053 in Table 1 and L-105 is from a patient whose mutations remained unknown after sequencing all ATM exons and adjacent intron regions).

Table 3. Effects of splicing mutations on *ATM* mRNA transcripts

Mutation	Location	Cell type	Observed effects
2250G→A	Exon 16	B-LCL	Skipping of exon 16
2921+3A→T	Intron 21	B-LCL	Skipping of exon 21 (90%) and joint skipping of exons 20+21 (10%)
3576G→A	Exon 26	B-LCL	Skipping of exon 26
		Fibroblasts	Skipping of exon 26
R2032K	Exon 43	Lymphocytes	Skipping of exon 43
6096–9del5	Intron 43	Fibroblasts	Skipping of exon 44
7630–2A→C	Intron 53	B-LCL	Skipping of exon 54 (60%) and skipping of the first 11 nt of exon 54 (40%)

The effect of splicing mutations was characterized by RT–PCR experiments on total RNA isolated from the listed cell types using the primers given in Table 7. Exon skipping has been observed for all investigated mutations. In addition, one cell line exhibited joint skipping of two neighbouring exons and one exhibited an additional alternatively spliced cDNA species due to the use of a cryptic acceptor splice site.

Table 4. Effects of ATM missense and in-frame splicing mutations on ATM protein levels

Mutation	Genotype	Location	Cell type (line[a])	Protein level
2250G→A	2250G→A/unknown	Exon 16	B-LCL (L87[a])	Trace (6%)
3576G→A	Homozygous	Exon 26	B-LCL	None detected
			Fibroblasts	None detected
D2016G	Homozygous	Exon 43	B-LCL	Trace (4%)
Y2470D	Y2470D/unknown	Exon 52	Fibroblasts	None detected
7630–2A→C	Homozygous	Intron 53	B-LCL	None detected
V2662del	Homozygous	Exon 56	B-LCL (L106[a])	Variable, ranging from 20% to apparently normal (see text)
R2849P	R2849P/unknown	Exon 60	B-LCL	None detected
G2867R	G2867R/unknown	Exon 61	B-LCL	Trace (2%)

Cell lines with at least one missense mutation or in-frame deletion of the ATM cDNA were analysed for ATM protein levels by immunoblotting.
[a]The designation of lymphoblastoid cell lines refers to the designation of samples shown in Figure 3.

When we examined the clinical features of other patients carrying missense mutations, some differences in their disease phenotypes became apparent. One patient homozygous for the missense mutation D2016G was found to produce trace amounts of ATM protein and presented with a protracted disease course. He does not manifest telangiectasia or immunodeficiency at his current age of 7 years. Two siblings who were heterozygotes for the missense mutation F570S, combined with an unidentified second mutation, also showed mild manifestations of AT. Both died in their mid-thirties, which is considered unusually long survival. Unfortunately, no cells were available to study ATM protein levels in these cases. In contrast, two patients heteroallelic for the missense mutations R2849P and G2867R, each in combination with a non-identified second mutation, both expired at age 17 after the classical AT disease course. They showed no (R2849P) or low (G2867R) detectable amounts of ATM protein, suggesting that carrying one missense allele affecting the kinase region may not be sufficient to lessen the disease manifestations. Another two patients homozygous for the in-frame splicing mutations 3576G→A and 7630–2A→C, respectively, likewise showed severe AT manifestations and had no detectable ATM protein.

Several intragenic dimorphisms were identified as a by-product in the sequence analysis during the course of this study (Table 2), in addition to the known intragenic microsatellite at locus D11S2179 present within the exon 62 PCR product. The allele frequencies of the new dimorphisms are compiled in Table 2. Of these dimorphisms, nine surpassed the frequency of 0.3 for both alleles and could be used to construct combined haplotypes over the gene. For example, the dimorphisms in introns 7, 17, 62 and 63 and in the 3′-UTR were in strong linkage disequilibrium and defined two major haplotypes, suggesting that intragenic recombination within the *ATM* gene is a rare event. No efforts were made in this study to analyse the allele frequencies of these dimorphisms in the general population, since a previous analysis of another linked marker in intron 48 had not revealed any significant differences between the alleles of healthy, breast cancer or AT individuals in the German population (48). Some of the dimorphisms described in our study can be assayed as natural RFLPs (Table 2), which may be useful for indirect genetic diagnosis within AT families or to study loss of heterozygosity in malignancies.

At the end of our sequencing efforts, we were able to identify 46 different ATM mutations on 75 of 132 AT alleles. None of the detected mutations accounted for more than five alleles, and only seven mutations, namely 822delT, 2250G→A, 3576G→A, 3801delG, 4909+1G→A, R2443X and 7630–2A→C, occurred in more than one apparently unrelated AT family. Some of the identified mutations are also present in other populations. There are 12 mutations in our panel whose independent detection has been reported by other investigators: 822delT, 1561delAG, 2250G→A, 2502insA, 3085insA, 3576G→A, 3801delG, E1978X, R2032K, R2443X, 7630–2A→C and 8814del11 (29,35,36). In contrast, the high incidence of new and individual AT mutations in our cohort of patients demonstrates marked mutational heterogeneity of AT in Germany. Finally, it is notable that the complete AT genotype could only be determined in 24 of our patients (17 homozygotes and seven compound heterozygotes), whereas one of both mutations was uncovered in 26 patients and no *ATM* mutation was found in 16 patients. Thus, further molecular analyses apart from our automated sequencing approach are required to determine the molecular basis of AT disease in the patient group where the complete *ATM* genotype could not yet be defined.

DISCUSSION

Although numerous mutations have been previously identified in AT families, the present study is the first which systematically examines the whole coding region and all flanking intron portions of the *ATM* gene by high throughput automated sequencing of PCR products from genomic DNA in a large cohort of AT patients. This approach provides insight into the mutational heterogeneity of AT in Germany and into the polymorphic variability of the *ATM* gene. Some previous studies reported either a high frequency of distinct AT mutations or uncovered singular mutations that were common in isolated populations due to founder effects (15,27–36,49,59,60). In our cohort, direct sequencing identified 46 different mutations of the *ATM* gene in 50 of 66 AT patients. These mutations were well distributed over the whole gene, which appears in contrast to hitherto published studies, where mutations were more frequent in the 3′-region of the gene (26). However, the over-representation of C-terminal *ATM* gene mutations may be explained by more intense screening for mutations in the initially published 3′-part of the gene (26). None of the mutations in our cohort occurred in more than four families and only 12 of the 46 mutations have been independently published for other populations (29,32,34–36). Hence, common mutations exist in AT, but they seem to be present at only low frequencies in Caucasians. It will be interesting to determine by haplotype comparison whether such mutations have a common origin or whether they represent recurrent mutational events. Those mutations which in our study were present in more than

one family shared the same intragenic marker haplotype, as exemplified by the association of the 3576G→A splicing mutation with the missense variant P1054R on the same allele in three families with different population backgrounds. This mutation has also been identified by other investigators in Italian AT patients (35). Furthermore, heterozygosity for the P1054R allele has recently been implicated in the aetiogenesis of breast cancer (61), suggesting that either the amino acid substitution P1054R could have a modifying effect on ATM function or that the associated 3576G→A mutation may predispose to malignancy. The mutations 2250G→A and 3801delG have previously been reported in AT families from the British Isles, suggesting an origin in common with the German AT patients in our study (36). Similarly, the mutations 3085insA, R2032K and 7630–2A→C have recently been identified in Polish patients and may share the same origin with the German families reported here (35). In contrast, the previously reported occurrence of mutation R2443X in African-Americans (35) may reflect an independent mutational event as the underlying nucleotide substitution affects a CpG dinucleotide, known hot spots of mutations in general (50).

More than half of the identified AT mutations were nonsense mutations or small frameshift deletions or insertions. Including splicing mutations, four out of five AT mutations are predicted to severely truncate the ATM protein. This finding is an interesting contrast with the mutational spectrum of ATM in T-PLL patients (51,52), but it confirms the predominance of null mutations in AT, consistent with what has been reported in other studies (26,29). Although there are examples of polymorphic stop codons in other genes, e.g. in BRCA2 (53), truncating mutations would usually be expected to result in the loss of ATM function. This view is corroborated by the observation of significantly reduced amounts or the complete absence of truncated ATM proteins with this type of mutation (20,54,55). The sample of ATM missense mutations in our cohort of AT patients is more difficult to address, because only a few of these affect the highly conserved ATM kinase domain and information about other functional domains of the ATM protein is still limited. Most of the identified amino acid substitutions were not present in a series of control chromosomes, however, their absence in a restricted sample from the general population does not yet prove their physiological significance in causing AT disease. Five amino acid substitutions were classified as probably benign on the basis of their occurrence in the general population, but large case–control studies are required to finally exclude the possibility that these are missense variants predisposing towards disease symptoms. Interestingly, our immunoblot analysis of selected missense mutations and in-frame deletions uncovered one amino acid deletion to be associated with variable ATM protein levels in an AT cell line. In the absence of detailed knowledge about ATM structure and substrates, the identification of such naturally ocurring missense substitutions and deletions can be helpful in elucidating residues of physiological importance and may guide *in vitro* studies to analyse the processing and function of the ATM protein.

In view of the marked allelic heterogeneity of AT in our patients, no general recommendations concerning screening strategies can presently be deduced from our study. The diversity of many distinct mutations scattered throughout a large gene challenges mutation analysis in AT families and hampers the molecular assessment of AT heterozygosity in other clinical entities, such as breast cancer or leukaemias. In addition, the number of mutations presented here accounts for little more than half of all investigated AT alleles. This detection rate is unexpectedly low given the fact that all exons and adjacent intron regions of the *ATM* gene were sequenced and carefully evaluated on both strands. Direct sequencing is commonly thought to provide a sensitivity close to 100% in the analysed regions and although some problems with heterozygote detection in automated sequencing are known to impair the identification rate of point mutations, this alone is unlikely to account for missing >40% of the *ATM* mutations. Another reason would be the ascertainment of patients who have a genetically distinct disease, but genetic heterogeneity also seems unlikely in view of the many heterozygotes in whom *ATM* gene mutations were identified on one of both alleles. Furthermore, some cell lines from patients with unidentified mutations displayed reduced or absent ATM protein in western blot analyses (as exemplified by sample L105 in Fig. 3). A more plausible explanation thus would be the assumption of mutations outside the analysed region, e.g. promotor mutations or intronic splicing mutations of the type that have already been observed in variant AT (33). It is also possible that gross genomic deletions or large inversions account for a substantial portion of AT alleles and have escaped our PCR-based mutation scanning. Similar observations have been made in other inherited diseases, for example familial hypercholesterolemia and haemophilia A (55,56). Further studies at the mRNA level and a search for gross deletions and genomic rearrangements must therefore follow to characterize the unidentified AT alleles. Such studies will complement our automated exon scanning sequencing approach and finally result in an optimized strategy for *ATM* gene mutation analysis at the genomic DNA level in AT families and for heterozygote detection in conditions where *ATM* involvement is considered.

MATERIALS AND METHODS

Patients

AT patients and their family members were ascertained at one of the participating centres in Wuerzburg, Hannover or Berlin. The diagnosis was established on the basis of typical clinical features, such as ataxia, telangiectasia, elevated α-fetoprotein levels and altered immunoglobulin profiles, and was confirmed by laboratory evidence for X-ray or bleomycin sensitivity, such as cytogenetic analysis of chromosome breakage rates or radio-resistant DNA synthesis (37) or by flow cytometric analysis of cell cycle abnormalities after specific induction (38). More detailed information on the clinical and laboratory features of patients with particular ATM genotypes can be obtained from the authors upon request. Only apparently unrelated AT patients were included into this study. Four AT patients in our study have been investigated in parallel using cDNA-based mutation screening and the results have been published elsewhere (27). The genotypes of these patients (designated Aa009, Aa025, Aa027 and Aa028 in Table 1) were resolved at the genomic level in the present study and are consistent with the results obtained by Baumer *et al.* (27). Little more than half of our 66 AT patients were of German descent, whereas the others originated from Turkey, Italy, Denmark, Switzerland, the Czech Republic, Romania, Georgia or Lithuania. After informed consent had been obtained, blood samples were collected from the AT patients and, wherever available, their parents. Genomic DNA was extracted from white blood cells according to standard procedures. In some cases, established lymphoblastoid or fibroblast cell lines were used as the source for the extraction of genomic DNA.

Table 5. Primers used for genomic PCR amplification

Exon	Forward primer	Reverse primer	Annealing (°C)	Size (bp)
1a/1b	GGTAGCTGCGTGGCTAACGGAG	CCACATACATTATCATCCACTGTAG	59	658
1b/2	AGATAATCTTGACCTGTGGTGAG	TCACTAATCTCTTCAAACCTCAG	59	557
3	TTGATTGGCACCAGTTAGTTCAG	AGAAGATGCTCATTCACTGATAG	59	303
4+5	TGACCAGAATGTGCCTCTAATTG	CACACAAAAGTAATATCACAACAG	59	521
6	ATGTAGTAATCTAAGCAAGGTGG	ACAAACTTATGCAACAGTTAAGTC	59	376
7	AAATAGTTGCCATTCCAAGTGTC	GATGGAGATGGTATTTTTCAACTG	59	491
8	CAGCAGTTTATAGTTATTCCTGTC	TAGTTCTGTTATGATGGATCAATG	59	502
9	CTTTCAGCATACCACTTCATAAC	CATAAGTAGCTCCTAGAGGGAAC	54	601
10	TTCTAACGCTGATGCAGCTTGAC	CTGATAGTTTGAAAGGAATAGTTG	54	411
11	TGAGTTCCCTGTATCTTCATTTC	GTTGAGATGAAAGGATTCCACTG	54	674
12	CTATGGAAATGATGGTGATTCTC	GCATCTGAAATAGAATTTGACATC	59	621
13	CTGTCCTGATAGATAAAGTCTTTG	TATAAAATAAAGCCATCTGGCATC	59	357
14	ATGGTTGTCCTCCTTAAATTGTC	AAACAACCTCTTCCCTGGCTAAC	54	429
15	TAAGGCAAAGCATTAGGTACTTG	TTTCTCCTTCCTAACAGTTTACC	54	351
16	GCCACCTTTAACTCAGTTTAACTG	CCTGCATAAAATTTAATGTCTTCC	54	354
17	TGAGTTTTGCTTATACTGTATGAC	ACTCAGATTCCTATTGCTTATTTG	54	979
18	ATCTACATTCCATTCAAGATAGAG	ATGTTAAGAGCTATATGTTGTGAG	59	296
19	GTACTGTAGTTTTCATACTTACAC	GGAACAATCCTAAAAGGCTATAC	59	735
20	CAGGTAGTTGCTTTTCTTGATAG	ATTACATTTAGTCAGCAACATCAG	59	632
21+22	GTAAATGATTTGTGGATAAACCTG	GAAGAAATCACTGATGTGGATAC	59	480
23+24	AGCACAGAAAGACATATTGGAAG	ACTATGTAAGACATTCTACTGCC	59	495
25	GTATGTTATTATGTCTCACAGAGTG	CTCTCTTATACCAAACTTGGTGAAG	59	378
26	CTGAAATGTGTATAGCTTGTC	CACAGTGACCTAAGGAAGCTTC	59	331
27	AATGTTGTGTCTAGGTCCTACTC	TGCTTCTCATACTATGTAGGCTC	59	406
28	AGTATGATACTTTAATGCTGATGG	AGGTTATATCTCATATCATTCAGG	59	414
29	CCTTTTGAGCTGTCTTGACGTTC	GAAATAGACATTGAAGGTGTCAAC	59	271
30	TCTGGACTGTGATATGTCATTTG	CTAAAGTGTCACAAGATTCTGTTC	59	513
31	GTGTATTTATTGTAGCCGAGTATC	GAAGAACAGGATAGAAAGACTGC	59	315
32	CTGAACAAAAGGACTTCTGAATG	ACACTCAAATCCTTCTAACAATAC	54	334
33	CTGGGTATCTTAGACGTAATTAG	GCTAGAGCATTACAGATTTTTGAA	59	379
34	GTAGTAATAGAGACATGAGTCAG	GACAATGAAACCAAGAGCAAGAC	59	344
35	GGAAGTTCAGATCATTCCCTAC	CTATACTGAATACTACAGGCAAC	54	380
36	TATGTATGATCTCTTACCTATGAC	CTGAGAATATCTATTTGTGTGAAG	59	302
37	AGTTGCAGTGATTAGTAATTCAAG	CAGAACTGTTTTAGATATGCTGG	59	400
38	TTGCCATACCACTCTGCCTCTTG	GAAGATGATGTGCAGTATCACAG	59	447
39	ATTCTGTTTCATTATGGTAATGGC	TTCCATCTTAAATCCATCTTTCTC	59	529
40	TATCATATTGTGTTGTAATGTCCC	ATCATGTTAAAATTCAGCCGATAG	59	524
41	GTCACTACCATTGTATTCTATATC	TATACCCTTATTGAGACAATGCC	59	310
42	TGTATTCAGGAGCTTCCAAATAG	GCTTAGTCCAGTAAGTAAATTCAG	54	269
43+44	TAGAGTTGGGAGTTACATATTGG	TACTGAAATAACCTCAGCACTAC	59	456
45	GGATTTTAAATGATATTGTGAAC	CAACTCCTGTTATATTCATAGAAG	54	365
46	AGAGCATATTTAGAACCAGGCAG	CTCTTCATCAATGCAAATCCTTAC	59	439
47	GTAGCAAAGCCTATGATGAGAAC	TATTGGTAACAGAAAAGCTGCAC	54	279
48	ACAAGTTCTAGTCTTGTCACTAC	TAATGACATTTCTTTTTCCCTCAG	59	466
49	GTTAAGTCCTCAATGAATGGTAG	CATGATGGACTGATAGAATTGTG	59	479
50	TTGTATGGCAAAAGCAGATGAGG	TGAAGCATATTCATGCTAAGTAAC	59	466
51	GAAAATGTACGAATTTGTGTTGGG	AGAACCAAGTCACTCTTTCTATG	59	513
52	TTGTAGTTCTGTTAAAGTTCATGG	CAAGTGCTAGGAATACAAAGAGG	54	369
53+54	ACTTACTTGCTTAGATGTGAG	GGAAAGACTGAATATCACACTTC	59	718
55	GACTGTTTTGTTTGTATCTGAGG	TCTACAGAGAGTAACACAGCAAG	59	341
56	CTGCTGACTATTCCTGCTTGACC	GCCAATATTTAACCAATTTTGACC	59	253
57	TTCTTAACCACTATCACATCGTC	GCATTTCTACTCTACAAATCTTCC	59	405
58	TGAGTGCCCTTTGCTATTCTCAG	AGACTCCTGGTCCAAATAATGGC	59	597
59	ATGCTTTGCACTGACTCTGATAG	CTTAGTATCTTTGACAATTACCTG	54	367
60	GTAGGTAATGTATCCTGTTCATC	AGAATGTAGAAAAAGTGCTGAATC	54	377
61	TATTAGAAAGAGATGGAATCAGTG	GGCAAACAACATTCCATGATGAC	59	210
62	AAAGTTCACATTCTAACTGGAAAG	GCACTGGAATACGATTCTAGCAC	59	467
63	TATTAAGCATAGGCTCAGCATAC	TGCTCTTCACATCAGTGACTTCC	59	257
64	GGTTCTACTGTTTCTAAGTATGTG	CCCTACTTAAAGTATGTTGGCAG	54	587
65.1	ATGAATTACCCTTTCATTCAGCC	GTAACTACACTAACTGTGTCTTTG	54	630
65.2	ATTTTTCCTAGTAAGATCACTCAG	TGGATGAACAGCCTATCAAATAC	59	464
65.3	ACTTGATAGACACTGTAATAGTTC	ATCTATATGGGGAGCAAAGAACC	59	454
65.4	CTTCCATGTGTCCCACCTTTATG	AACAGAACACAGAGAAATGCTGG	59	600
65.5	CCTTTCTTGTAAGTTCTGCTATG	CTTCAAGGTCCAAATACGAGGAG	59	311
65.6	CCTTAGGAAAATGTTCATCCCAG	TGCAACAACTAGCAAGAATTAGC	59	479
65.7	TGAAATCTTCATGGGTGAAATTAG	ATTGGGATAGTATCACTAATCTTG	54	547
65.8	GTTGTATGCTAAGTCACTGACCC	TACTTGAAATTGGGACACATCTC	59	571
65.9	TAAAATGTATGCACTTAGGAATG	ACTAGGATATTTAGACTGAGATG	59	497

Table 6. Internal sequencing primers

Exon	Designation	Sequence
1b	s1f2	TCACTCCATCTTTCCTG
1b	s1r2	AGGAGGAGGTTATTGGC
2	s1cf3	AGATAATCTTGACCTGTGGTGAG
6	s6r2	CTCACGCGACAGTAATCTG
8	s8f4	TTCTCTGTGTACTTCAGG
11	s11f2	AATGAAGATACCAGATCC
11	s11r2	GGTAGACAAATGACTTAGTTCTG
12	s12f2	TTTTAGGCTACAGATTGC
12	s12r2	GTAAGTCAGACATAATGC
17	s17f4	TCCAAGATCAAAGTACAC
17	s17r1	ATGAGTTGTGACAATCCC
19	s19f1	GATTAGGTAAATTTTGACTACAG
20	s20f2	GCTGTTGTGCCCTTCTCTTAGTG
21	s21r1	CTTAACAGAACACATCAG
22	s22f2	ACCACAGCAATGTGTGTTC
26	s26f5	TTAAGTCCCATAGTGCTG
26	s26r5	TGAGGTTCTAATCCATTC
39	s39f1	GAAATTTAATATGTCAACGGG
45	s45r2	CAAGTCAAATTTCTTACCTG
47	s47r4	GCTGCACTTTAGGATAAC
48	s48f2	TCTTGCTTACATGAACTC
49	s49f4	CAGTAGTAAAAGTATTTATTCCC
53	s53f2	AGGCATACACGCTCTAC
53	s53r2	ATGCCTGCATGTGTGAG
58	s58r1	ACTTCACCCAACCAAATG
62	s62r2	CTCCTTTACTTCATATCAC
65	s64r1	CATATACTGAAGATCACAC

Sequence analysis

PCR conditions were established to separately amplify all exons and exon-flanking intron sequences of the *ATM* gene for every patient sample (Table 5). PCR amplifications were performed in 50 µl reaction volumes containing 100 ng of genomic DNA, 0.2 mM each dNTP and 2 U Taq DNA polymerase in the reaction buffer supplied by the manufacturer (Boehringer Mannheim). Primer concentrations were 0.5 µM each. As the standard procedure, 35 PCR cycles were performed with 1 min denaturation at 94°C, 1 min annealing at 54 or 59°C, depending on the primer pair, and 1 min elongation at 72°C. The PCR products were purified by PEG precipitation according to a previously published protocol (39) and sequenced on both strands using dye-terminator chemistry (Perkin Elmer). Whenever possible, the PCR primers served as sequencing primers. Because of some background in the sequence trace files or due to poly(A) or poly(T) stretches, 34 sequencing primers had to be chosen anew. These sequencing primers are compiled in Table 6. Sequence information for all exons was generated and filed using ABI 377 automated sequencers. This data was then assembled using the XGAP program (40). To facilitate editing, four XGAP sub-projects were generated, each of which consisted of a backbone of reference sequences derived from genomic clones and the data from 18 patients (Fig. 1). A second round of editing served as an independent confirmation of identified sequence changes.

Transcript analysis

Total RNA was extracted from white blood cells, fibroblasts or lymphoblastoid cell lines by lysis of the cells with guanidinium thiocyanate and extraction of the RNA with acid phenol/chloroform according to the protocol of Chomczynski and Sacchi (41). An aliquot of 1 µg of total RNA was reverse transcribed using a First Strand cDNA Synthesis Kit (Pharmacia) with random hexamers as primers. One-fifth of the cDNA was used as the template in the subsequent PCR amplification of a cDNA region spanning several exons and containing the mutation of interest. The primers used for RT–PCR are compiled in Table 7. Splicing products were analysed after 32 cycles of PCR by electrophoresis in NuSieve agarose, excised and eluted from the gel and sequenced. For quantitative determination of the relative frequencies of the splicing products, the PCR products were radioactively labelled and separated on a 6% denaturing polyacrylamide gel. The incorporated radioactivity was then quantitated by phosphorimager analysis (Fuji).

Western blotting

Protein extraction and immunoblotting analysis were performed as described by Ziv *et al*. (55) using a monoclonal antibody, ATM 132, raised against positions 819–844 (L. Moyal *et al*., in preparation).

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Table 7. Primers used for cDNA analysis

Exons	Forward primer	Reverse primer
15–19	CAGACAACTTTTGACAAGATGGAC	GGTACTTTGGCTCTCTCCAGGTTC
19–22	AGGTGGAGGATCAGTCATCCATG	CATCCCTTGTGTTCTCAGAGTCC
22–28	GACTCTGAGAACACAAGGGATGCTC	GCATATCATAGACCTTGGTAGCAG
42–45	GCATTTGAAGAAGGAAGCCAG	CTTCTAGTTCAGGACACCAGTC
52–55	CGCTTCTATGTAAAGCAGTTG	GTCTTCCACTGAGTGGCATC

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