

RESEARCH ARTICLE

Characterization of *ATM* Mutations in 41 Nordic Families With Ataxia Telangiectasia

K. Laake,¹ L. Jansen,¹ J.M. Hahnemann,² K. Brøndum-Nielsen,² T. Lönnqvist,³ H. Kääriäinen,⁴ R. Sankila,⁵ A. Lähdesmäki,⁶ L. Hammarström,⁶ J. Yuen,⁷ S. Tretli,⁸ A. Heiberg,⁹ J.H. Olsen,¹⁰ M. Tucker,¹¹ R. Kleinerman,¹¹ and A-L. Børresen-Dale^{1*}

¹Department of Genetics, Norwegian Radium Hospital, Oslo, Norway

²Department of Medical Genetics, J.F. Kennedy Institute, Glostrup, Denmark

³Children's Hospital, University of Helsinki, Helsinki, Finland

⁴Department of Medical Genetics, Väestöliitto, Family Federation of Finland, Helsinki, Finland

⁵Finnish Cancer Registry, Helsinki, Finland

⁶Division of Clinical Immunology, Karolinska Institute, Huddinge Hospital, Huddinge, Sweden

⁷Swedish University of Agricultural Science, Uppsala, Sweden

⁸Norwegian Cancer Registry, Oslo, Norway

⁹National Hospital of Norway, Oslo, Norway

¹⁰Institute of Cancer Epidemiology, Danish Cancer Society, Copenhagen, Denmark

¹¹National Cancer Institute, Division of Cancer Epidemiology and Genetics, National Institutes of Health, Bethesda, Maryland, USA

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The Ataxia Telangiectasia Mutation (*ATM*) gene is mutated in the rare recessive syndrome Ataxia Telangiectasia (AT), which is characterized by cerebellar degeneration, immunodeficiency, and cancer predisposition. In this study, 41 AT families from Denmark, Finland, Norway, and Sweden were screened for *ATM* mutations. The protein truncation test (PTT), fragment length and heteroduplex analyses of large (0.8–1.2 kb) cDNA fragments were used. In total, 67 of 82 (82%) of the disease-causing alleles were characterized. Thirty-seven unique mutations were detected of which 25 have not previously been reported. The mutations had five different consequences for the *ATM* transcript: mutations affecting splicing (43%); frameshift mutations (32%); nonsense mutations (16%); small in-frame deletions (5%); and one double substitution (3%). In 28 of the probands mutations were found in both alleles, in 11 of the probands only one mutated allele was detected, and no mutations were detected in two Finnish probands. One-third of the probands (13) were homozygous, whereas the majority of the probands (26) were compound heterozygote with at least one identified allele. Ten alleles were found more than once; one Norwegian founder mutation constituted 57% of the Norwegian alleles. Several sequence variants were identified, none of them likely to be disease-causing. Some of them even involved partial skipping of exons, leading to subsequent truncation of the *ATM* protein. *Hum Mutat* 16:232–246, 2000. © 2000 Wiley-Liss, Inc.

KEY WORDS: ataxia telangiectasia; *ATM*; ethnology; DNA mutational analysis; founder effect; nordic population

DATABASES:

ATM – OMIM:208900; GDB:593364; GenBank:U33841 (cDNA), U82828 (genomic); HGMD:*ATM*; <http://www.vmrsearch.org/atm.htm> (*ATM* Mutation Database)

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*Correspondence to: Anne-Lise Børresen-Dale, Dept. of Genetics, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway. E-mail: alb@radium.uio.no

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INTRODUCTION

Ataxia Telangiectasia (AT) is an autosomal recessive disorder that affects ~1:40,000–1:300,000 live births in various ethnic groups, hence about 0.35–1% heterozygotes in the general population (AT;MIM# 208900), [Swift et al., 1991; Sedgwick and Boder, 1991; Taylor et al., 1994; Olsen et al., submitted]. The disorder is characterized by defects in a number of distinct organ systems [for reviews see Boder, 1985; Regueiro et al., 2000]. Symptoms and findings include progressive cerebellar ataxia, telangiectasias in skin and conjunctiva of the eye, immunodeficiency, chromosomal instability, radiation sensitivity, and an increased incidence of malignancies, primarily of lymphoid origin.

The gene responsible for the disease, Ataxia Telangiectasia Mutated (*ATM*), was localized to chromosome 11q23.1 [Gatti et al., 1988], and subsequently cloned [Savitsky et al., 1995a, b]. It spans ~150 kb genomic DNA and contains 66 exons, including alternative splicing variants in the 5' untranslated region (5' UTR) [Savitsky et al., 1997]. The *ATM* gene gives rise to a ubiquitously expressed transcript of ~13 kb encoding a 350 kDa protein, mainly localized to the nucleus.

The 3' half of the gene contains two regions of homology with other proteins suggesting putative functions. At the carboxyl end of the *ATM* proteins there is a region (~350 amino acids) sharing homology with phosphatidylinositol-3 (PI3) kinase with highly conserved residues (2855–2875). This conserved C-terminal region is a common feature of the PI3 kinase gene family whose products are generally involved in DNA repair, recombination, and cell cycle checkpoints [Keith and Schreiber, 1995]. Protein kinase activity has been reported for some of these proteins, including *ATM* [Keegan et al., 1996]. A second region of the *ATM* gene, the Rad3 domain (~ residues 1,500–2,000), shares modest similarity with Rad-proteins [Savitsky et al., 1995b]. A leucine zipper is located at residues 1217–1238, probably involved in dimerization. A proline rich region, interacting with the SH3 domain of the non-receptor tyrosine kinase *c-Abl*, is located at residues 1373–1382. *c-Abl* has been identified as one of the downstream targets of the *ATM* protein [Shafman et al., 1997]. Whereas the complete diversity of functions fulfilled by the *ATM* protein is still not known, it is believed to sense DNA double strand breaks [Sikpi et al., 1998] and regulate physiological responses via TP53, CHK1, and CHK2 mediated pathways [Matsuoka et al., 1998; Canman et al., 1998; Banin et al., 1998; Kaneko et al., 1999].

The *ATM* protein has strong interaction with the TP53 protein at the N-terminus of the protein (~ residues 1–246) and weak interaction at the C-terminus or the PI3-kinase domain of the protein. *ATM* phosphorylates and dephosphorylates distinct serine residues in the TP53 protein (Ser-15 and Ser-376) leading to activation and stabilization of the protein [reviewed in Nakamura, 1998]. Recently, *ATM* was shown to phosphorylate the BRCA1 protein in response to γ -irradiation [Cortez et al., 1999]. The BRCA1 and BRCA2 proteins form a complex with RAD51, required for homologous recombinational repair of DNA double strand breaks [Chen et al., 1999]. It is also known that *ATM* and RPA (Replication A) co-localize along synapsed meiotic chromosomes and at sites for meiotic recombination [Plug et al., 1997].

Numerous different mutations, spread all over the *ATM* gene, have been identified in AT patients [Concannon and Gatti, 1997]. A regularly updated *ATM* Mutation Database Web site is available [<http://www.vmrsearch.org/atm.htm>] containing 270 unique mutations in AT patients (updated 31 March 2000). There are no apparent hot spots for mutations in the gene. Mutations have been reported involving each of the coding exons (4–65), as well as the 5' and 3' UTR. From the published data, truncating mutations [Gilad et al., 1996a] and splice related mutations [Teraoka et al., 1999] seem to be predominant. Many single substitutions of unconserved residues of the protein and intronic variants have been reported, and it is difficult to predict whether these are disease-causing without access to functional studies. Recurrent mutations are reported in Norway, the Netherlands, Costa Rica, the English Midlands, Italy, Japan, and Poland; also among people of Irish English, Utah Mormon, African American, Israeli Jewish, and Amish/Mennonite descent [Gilad et al., 1996b; Sasaki et al., 1998; Ejima and Sasaki, 1998; Laake et al., 1998; Stankovic et al., 1998; Telatar et al., 1998]. For several of these mutations, the carriers share common haplotypes, indicating a founder effect.

Considerable phenotypic variations have been observed in AT patients. The variations are mainly in age of onset of the ataxia, level of immunodeficiency, level of cellular radiosensitivity, and presence or absence of tumors [Sedgwick and Boder, 1991; Taylor et al., 1996]. The heterozygotes are clinically unaffected, but there are several reports indicating that they are prone to develop cancer, diabetes, and cardiovascular diseases [Swift and

Chase, 1983; Morrell et al., 1986; Athma et al., 1996; Inskip et al., 1999; Janin et al., 1999; Olsen et al., submitted]. The phenotypic variation in both homozygotes and heterozygotes needs further elucidation.

The aim of the present study was to identify the ATM mutations present in Nordic AT families. The collected mutation data will be essential when exploring the differences in phenotype of both homozygous and heterozygous carriers of ATM mutations. In this study, 41 Nordic AT probands from Denmark, Finland, Norway, and Sweden have been screened for mutations in the ATM transcript using protein truncation test (PTT), fragment length and heteroduplex analyses.

MATERIALS AND METHODS

The study includes 41 AT families of Nordic origin; 10 Danish, seven Finnish, 15 Norwegian, and nine Swedish. Five of the families had two affected children (DAAT 1, NOAT 4, NOAT 9, SVAT 6, and SVAT 12). We anticipated two disease-causing alleles in each family, with a total number of 82. Three AT families in which both parents were born outside the Nordic countries were not included. Thirty-seven of these families were ascertained through an epidemiological study (Olsen et al., submitted). Clinical and biochemical examinations of the patients were undertaken by one of us, or the relevant information from the medical records was obtained and reviewed. Absolute criterion for inclusion was progressive cerebellar ataxia. Supporting criteria were telangiectasias, ocular apraxia, dysarthria, history of infections (minimum one episode of pneumonia), elevated alpha-fetoprotein, chromosome rearrangements involving chromosomes 7 and 14, and/or increased chromosome breakage, decreased IgA or IgG2. Information about consanguinity and birthplace of parents was recorded.

Blood samples, 20 ml EDTA whole blood, were obtained from the following family members: patient(s) when alive, mother, father, and siblings when available. Short-term lymphocyte cultures were grown from all blood samples. Lymphoblastoid cell lines were available from the Danish family members, and fibroblast cultures were available from some of the Norwegian family members. Short-term lymphocyte cultures were performed as follows: 6 ml EDTA blood was centrifuged at 125g for 20 min. The white blood cell layer with plasma was added 60 ml medium with 180 μ l Phythemagglutinin P (Bacto-Difco, Detroit, MI, US). The medium contained RPMI 1640 (Gibco-

BRL, Life Technologies, Paisley, Scotland), 20% fetal calf serum (Gibco-BRL, Life Technologies, Paisley, Scotland), 300 μ g/ml penicillin/streptomycin (Gibco-BRL, Life Technologies, Paisley, Scotland), and 10 I.E. heparin (Nycomed-Pharma, Asker, Norway). The culture was divided into six tubes of 10 ml each and incubated at 37°C. 10 ml fresh medium was added after two to three days. The cells were harvested after three to five days by centrifugation at 500g for 20 min. They were subsequently washed four times in 1 X PBS. Dry cell pellets containing approximately 10^7 cells were stored at -70°C. The remaining blood was frozen and subject to DNA extraction using a standard phenol/chloroform method followed by ethanol precipitation (340A Nucleic Extractor, PE Biosystems, Foster City, CA, US).

mRNA was extracted from short term lymphocyte cultured cells, lymphoblastoid cell lines or fibroblast cultures using the Quick Prep Micro mRNA Purification Kit (Pharmacia-Amersham Biotech Inc., Piscataway, NJ, US) according to the protocol provided by the supplier. When lymphoblastoid cell lines or fibroblast cultures were available, they were preferred. The mRNA was subject to random hexamer cDNA synthesis using the Gene Amp RNA PCR KIT (PE Biosystems, Foster City, CA, US). The cDNA was further PCR amplified in nine different overlapping fragments of sizes 1-1,5 kb [Telatar et al., 1998]. Analyses to detect fragment length alterations or heteroduplexes were performed as follows: The PCR products were denatured at 95°C for 1 min and allowed to form heteroduplexes at 65°C for 30 min. The PCR fragments were analyzed using 7.5% PAGE, stained in ethidium bromide and photographed under UV-light. The PTT analyses were performed by *in vitro* translating the PCR products using the TNT T7 Reticulocyte System (Promega, Madison, WI, US) followed by SDS-PAGE electrophoresis and autoradiography. All was performed according to the protocol provided by the supplier.

Samples containing fragments with altered mobility were subsequently investigated both on the cDNA level and on the genomic level. Overlapping PCR fragments of different sizes (0.1-1.5 kb) were analyzed for heteroduplex formation, change in fragment size, and loss or gain of restriction enzyme sites. PCR primer sequences and conditions are available upon request. Finally, the fragments of interest were cycle-sequenced using an 373 DNA sequencer Stretch and Big Dye (PE Biosystems, Foster City, CA, US) according to the protocol provided by the supplier. The sequences

were confirmed on both strands, on the cDNA and on the genomic DNA level, and visually read by two independent observers.

The mutation nomenclature used is according to Beaudet and Tsui [1993] updated by Antonarakis [1998]. cDNA nucleotide numbering was according to Savitsky et al. [1995b] (GeneBank number U33841), and genomic DNA sequence was compared to the sequence published by Platzer et al. [1997] (GeneBank number U82828). We have noted the following discrepancy between those sequences: In Savitsky et al. [1995b] cDNA base 2250 is a cytosine whereas in Platzer et al. [1997] it was a guanine. In our analysis, the Platzer sequence seemed to be the correct wild type sequence. Shapiro and Senapathy [1987] have described a scoring system for calculation of the likelihood that a particular splice-donor-acceptor sequence, as a whole, would be functional. This system gives a score between 0 and 100. Subsequently, the ratio between the score of the splice mutations and the corresponding wild type sequence was computed to evaluate the difference between them.

RESULTS

The Spectrum of Mutations

Mutations that are most likely to be disease-causing were found in 67 of 82 alleles, corresponding to an 82% detection rate. The detection rate varied between the populations. For Finnish probands the detection rate was only 50% (seven of 14 alleles), whereas it was 95% for Denmark (19 of 20 alleles), 87% for Norway (26 of 30 alleles), and 83% for Sweden (15 of 18 alleles). Thirty-seven different unique mutations were detected, of which 25 have not been reported previously. The mutated alleles detected had five different consequences for the transcript. Sixteen (43%) affected splicing, 12 (32%) were frameshift mutations, six (16%) were nonsense mutations, two (5%) were in-frame deletions, and one (3%) a double substitution (Table 1). Mutations in both alleles were found in 28 of the probands, only one mutated allele was detected in

11 of the probands, whereas no mutations were detected two Finnish probands (FIAT 1 and FIAT 6). One-third of the probands (13), with at least one identified allele, were homozygous, whereas the majority of the probands (26) were compound heterozygous (Table 2).

The ATM mutations identified in the Nordic probands are spread across the entire gene (Fig. 1). They are described in detail in Table 3. Exon 24 might appear as a hot spot, but is not, since one of the mutations found in this exon was the Norwegian founder mutation, present on 17 alleles. Most of the mutations lead to a significant truncation of the ATM protein. However, one double substitution in exon 55, DA 2625-2627 EP, was seen upstream of the kinase domain of the ATM protein. These amino acids are in a stretch of 12 amino acids conserved between mouse and man. The amino acid changes lead to insertion of a proline residue, which introduces a bend in the protein chain. The two small in-frame deletions, two or five residues, both affecting the PI3-kinase domain of the protein, are most likely deleterious for the protein function. In three alleles, a mutation in the last exon, resulting in loss of only 10 amino acids downstream of the catalytic domain, was seen. A complex mutation was found in FIAT7P. One amino acid substitution (E2257A) was found to segregate on the paternal mutated allele nine base pairs upstream of a two base pairs deletion (del TA) in exon 48.

Interestingly, two of the mutations were combined deletions and insertions: ATC>TGAT (exon 24) and GAAA>AT (exon 64). Three other mutations had deletions in mono nucleotide repeats: 4C>3C (exon 21), 4A>3A (exon 48) and 7A>6A (exon 60). This could be incidental, due to environmental exposure (for instance radiation) or reflect an underlying mechanism (for instance poor DNA repair capacity).

Splice Mutations

As many as 16 (43%) of the unique disease-causing mutations affected correct splicing. Mu-

TABLE 1. Distribution of Mutations by Consequence for the Protein

Consequence for the protein	Number of unique mutations	Percent of total
Splice mutations	16	43%
Frameshift	12	32%
Nonsense	6	16%
Deletion in frame (2-5 amino acids)	2	5%
Substitution (double)	1	3%
Total	37	100%

TABLE 2. Distribution of Homozygote and Compound Heterozygote AT Probands

Category	Number of probands
Homozygote	13
Compound heterozygote, two mutations detected	15
Compound heterozygote, one mutation detected	11
No mutations detected	2
Total	41

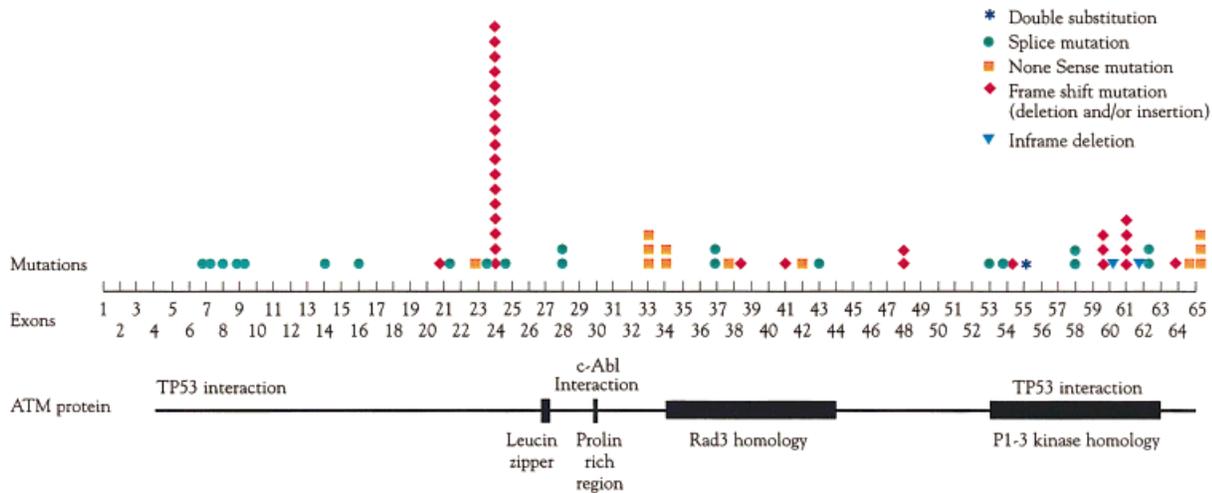


FIGURE 1. Spectrum of mutations in the Nordic AT patients. Each mutation is represented by a symbol showing its consequence to the transcript (key to the symbols is shown in figure). Mutations in exonic or flanking intronic sequences are located in the nearest exon in the *ATM* transcript. Mutations found more than once are placed on top of each other, whereas the unique mutations are placed beside each other. Note that in exon 24 there is a Norwegian founder mutation constituting 17 alleles.

tations in the 3' splice junction all involved the canonical AG splice-acceptor site. Mutations affecting the 5' splice junction were more diverse. Six of 17 alleles were mutated in the canonical GT splice-donor site, whereas nine alleles were mutated in non-canonical sites (six of these were in the last base of the exon). Computation of splice site strength showed reduction in splice site efficiency in the range 13 to 50% for these mutations [Shapiro and Senapathy, 1987]. A strong new cryptic splice site was created in intron 37 in two Finnish alleles. The cryptic splice site was 24% more efficient as 5' splice-donor than the wild type sequence.

In most cases, the mRNA alteration seen could be predicted from its underlying genomic mutation. However, two unexpected consequences for the mRNA were seen. Variant splicing appeared when sequencing cDNA of the Danish mother (DAAT 21) carrying the genomic IVS53-2A>C mutation. The mutant allele resulted in both skipping of exon 53, and the use of a cryptic splice site. The mRNA was from leukocytes grown in short-term lymphocyte culture. Such variant splicing has been reported previously in the *ATM* gene [Fukao et al., 1998; Teraoka et al., 1999]. The second unexpected observation was a nonsense mutation 10 base pairs into exon 7 that lead to skipping of exon 7.

Recurring Mutations

Ten different mutations were recurring in the Nordic AT probands, whereas an additional six previously had been published in AT probands living outside the Nordic countries (Table 4). In only three

out of 13 families with homozygous probands the parents knew they were consanguineous. The Norwegian founder mutations, 3245-3247 delATC insTGAT in exon 24, constituted 17 of 30 (57%) of all Norwegian AT alleles investigated. It was present in seven homozygous and three compound heterozygous probands. Six of the families could be traced back to a common ancestor born in 1495 [Laake et al., 1998, unpublished results]. These families also have a common haplotype [Laake et al., 1998]. Birthplaces of all parents are shown in Figure 2.

The father in family NOAT 7 and the mother in family NOAT 8 carried the same mutation and haplotype [Laake et al., 1998]. They were born in the same community. The mother in family NOAT 7 and the parents in family NOAT 13 exhibited the same deletion in exon 60. In all carriers of the deletion in exon 60 one silent mutation in exon 9 segregated with the disease suggesting a common haplotype. The parents in family NOAT 13 probably have common ancestors. The maternal and the paternal grandfathers carry the same mutation (unpublished results).

Three mutations were recurrent in Denmark, but the descents of the mutations were more complex. The mothers in the families DAAT 1 and DAAT 9 carried a splice mutation in intron 28. Surprisingly, one of them was a Canadian immigrant whereas the other was born in Denmark. The father in family DAAT 6 and the mother in family DAAT 19 both had a splice mutation in intron 62 and were born in two closely situated communities in Denmark. In the family DAAT 19, the ma-

TABLE 3. Sequence Alterations in the ATM Gene, Most Likely Disease-Causing

Family	Descent	Sequence alteration genomic DNA ^a	Exon/intron ^b	First codon(s) affected	mRNA/protein alteration
DAAT 5	P	IVS6-1 G>A	IVS6	111	Skipping of exon 7 (165 bp, 55 aa)
SVAT 8	—	487 C>T	7	Q163X/111 ^c	Nonsense mutation at codon 163 leading to skipping of exon 7 (165 bp, 55 aa) ^c
SVAT 13	—	662 G>T	8	166	Skipping of exon 8 (166 bp). Frame shift, stop after 8 aa subst.
SVAT 9	P	IVS9+2 T>A	IVS9	QEK222-224CLX	Skipping of exon 9 (239 bp). Frame shift, stop after 3 aa substi.
SVAT 3	—	IVS9+3 A>T	IVS9	QEK222-224CLX	Skipping of exon 9 (239 bp). Frame shift, stop after 3 aa substi.
FIAT 4	M	IVS14+3-4 delAT	IVS14	Del 602-633	Skipping of exon 14 (96 bp, 32 aa)
DAAT 5	M	2250 G>A	16	Del 709-750	Skipping of exon 16 (126 bp, 42 aa)
NOAT 3	P	2880 delC	21	960	Frame shift, stop after 9 aa substitutions
SVAT 9	M	IVS21+3 insT	IVS21	947	Skipping of exon 21 (83bp). Frame shift, stop after 8 aa substi.
DAAT 7	—	3102 T>A	23	Y1034X	Nonsense mutation
DAAT 19	P	IVS23-2 A>G	IVS23	1052	Cryptic 5' splice site -> ins 14 bp. Frame shift, stop after 16 aa
NOAT 1	M+P	3245-3247 delATC insTGAT	24	1082	Frame shift, stop after 12 aa subst.
NOAT 2	M+P	3245-3247 delATC insTGAT	24	1082	Frame shift, stop after 12 aa substit.
NOAT 5	P	3245-3247 delATC insTGAT	24	1082	Frame shift, stop after 12 aa substit.
NOAT 6	M+P	3245-3247 delATC insTGAT	24	1082	Frame shift, stop after 12 aa substit.
NOAT 8	P	3245-3247 delATC insTGAT	24	1082	Frame shift, stop after 12 aa substit.
NOAT 10	M+P	3245-3247 delATC insTGAT	24	1082	Frame shift, stop after 12 aa substit.
NOAT 11	M+P	3245-3247 delATC insTGAT	24	1082	Frame shift, stop after 12 aa substit.
NOAT 14	M+P	3245-3247 delATC insTGAT	24	1082	Frame shift, stop after 12 aa substit.
NOAT 16	M+P	3245-3247 delATC insTGAT	24	1082	Frame shift, stop after 12 aa substit.
NOAT 17	M	3245-3247 delATC insTGAT	24	1082	Frame shift, stop after 12 aa substit.
SVAT 8	—	3284 G>C	24	1052	Skipping of exon 24 (131 bp). Frame shift, stop after 25 aa substitutions
DAAT 1	M	IVS28+1 G>A	IVS28	Del 1292-1331	Cryptic 3' splice site. Deletion of 120 bp, 40 aa
DAAT 9	M	IVS28+1 G>A	IVS28	Del 1292-1331	Cryptic 3' splice site. Deletion of 120 bp, 40 aa
DAAT 16	M+P	4632-4535 delCTTA	33	Y1544X	Nonsense mutation
NOAT 9	M	4632-4635 delCTTA	33	Y1544X	Nonsense mutation
SVAT 4	M+P	4858 C>T	34	Q6120X	Nonsense mutation
FIAT 3	M	IVS37+9 A>G	IVS37	1773-1774 insVSX	Creation of new 5' cryptic splice site in intron 37. Ins 9 bp
FIAT 5	P	IVS37+9 A>G	IVS37	1773-1774 insVSX	Creation of new 5' cryptic splice site in intron 37. Ins 9 bp
DAAT 21	P	5326 G>T	38	E1776X	Nonsense mutation
DAAT 8	M	5405-5406 insA	38	HD1802QX	Frame shift, stop after 1 aa substitution
DAAT 7	—	5809-5813 delAATTA	41	1937	Frame shift, stop after 25 aa substitution
DAAT 15	M	5932 G>T	42	E1978X	Nonsense mutation
SVAT 1	P	6095 G>A	43	DLLLE2003-2007TTNIX	Skipping of exon 43 (89 bp). Frame shift, stop after 4 aa substitution
DAAT 15	P	6710 delA	48	2237	Frame shift, stop after 19 aa substitution
FIAT 7	P	6779-6780 delTA ^d	48	2260	Frame shift, stop after 11 aa substitution ^d

(continued)

TABLE 3. (Continued).

Family	Descent	Sequence alteration genomic DNA ^a	Exon/intron ^b	First codon(s) affected	mRNA/protein alteration
DAAT 6	M	7629-7629+4 delTgtaa	53 & IVS53	Del 2506-2543	Skipping of exon 53 (114 bp, 38 aa)
DAAT 21	M	IVS53-2 A>C	IVS53	Del 2544-2596 and 2544	Skipping of exon 54 (159 bp, 53 aa) and cryptic 3' splice site. Frame shift, stop after 22 aa substitution ^e
SVAT 12	M	7671-7668 delTTTG	54	2557	Frame shift, stop after 12 aa substitution
NOAT 4	M	7875-7876 TG>GC	55	DA2625-2627EP	Double substitution just before the PI-3 kinase domain
NOAT 7	P	8264-8268 delATAAG	58	Del 2718-2756	Skipping of exon 58 (117 bp, 39 aa)
NOAT 8	M	8264-8268 delATAAG	58	Del 2718-2756	Skipping of exon 58 (117 bp, 39 aa)
NOAT 7	M	8432 delA	60	2811	Frame shift, stop after 45 aa substitution
NOAT 13	M+P	8432 delA	60	2811	Frame shift, stop after 45 aa substitution
DAAT 9	P	8504-8518 del 15	60	Del 2835-2839	Deletion of 5 aa
SVAT 6	M+P	8656 insT	61	2886	Frame shift, stop after 9 aa substitutions
SVAT 11	M+P	8656 insT	61	2886	Frame shift, stop after 9 aa substitutions
FIAT 3	P	8710-8715 delGAGACA	62	2904-2905 delET	Deletion of 2 aa in the PI-3 kinase domain
DAAT 6	P	IVS62+1 G>A	IVS62	2891	Skipping of exon 62 (115 bp). Frame shift, stop after 8 aa substitutions
DAAT 19	M	IVS62+1 G>A	IVS62	2891	Skipping of exon 62 (115 bp). Frame shift, stop after 8 aa substitutions
NOAT 4	P	8978-8981 delGAAA insAT	64	RNLS2993-2996HSQX	Frame shift, stop after 2 aa substitutions
SVAT 1	M	9026 T>G	65	L3010X	Nonsense mutation, loss of only 46 aa
DAAT 1	P	9139 C>T	65	R3947X	Nonsense mutation, loss of only 10 aa
FIAT 2	M+P	9139 C>T	65	R3947X	Nonsense mutation, loss of only 10 aa

^aMutations are designated according to recommended nomenclature [Antonarakis, 1998], the nucleotide sequence numbering is according to the cDNA sequence [Savitsky et al., 1995b], while the genomic sequence used is published by Platzer et al., 1997.

^bExon numbers are according to Uziel et al., 1996.

^cGenomic alteration was a nonsense mutation (10bp upstream of 3' splice site), while the cDNA sequence show deletion of the whole exon 7.

^dA nine base pair upstream substitution 6770A>C co-segregated with the deletion leading to an amino acid substitution E2557A upstream of the frameshift.

^eBoth skipping of exon 54 and cryptic splice site was present on cDNA sequence.

DAAT, Danish AT family; FIAT, Finnish AT family ; NOAT, Norwegian AT family, SVAT, Swedish AT family; M, maternal; P, paternal.

TABLE 4. Recurring Mutations

Sequence alteration	Exon/ intron	Number of alleles detected in this study				Previously published AT alleles and origin (number of alleles)	Total occurrence of alleles	Reference ^a
		Danish	Finnish	Norwegian	Swedish			
2250 G>A	16	1	—	—	—	Denmark (1) ^b , Great Britain (3), Germany (2), Latvia (1) ^b , US (1)	9	1-4
IVS21+3 insT	IVS21	—	—	—	1	Turkey (1) ^b	2	4
3245-3247 delATC insTGAT	24	—	—	17 ^c	—	US (1) ^d	18	5,6
IVS28+1 G>A	IVS28	2	—	—	—		2	
4632-4535 delCTTA	33	2	—	1	—		3	
4858 C>T	34	—	—	—	2 ^e		2	7
IVS37+9 A>G	IVS37	—	2 ^e	—	—		2	8
5932 G>T	42	1	—	—	—	Poland (3), Germany (1), Caucasian (1) ^f , US (2)	8	3-5, 90
6095 G>A	43	—	—	—	1	Poland (2), Germany (2), US (1)	6	3-5
IVS53-2 A>C	IVS53	1	—	—	—	Germany/East Europe (5) ^b , Scandinavia (1) ^b	7	3
7875-7876 TG>GC	55	—	—	1	—	The Netherlands (3)	4	10
8264-8268 delATAAG	58	—	—	2	—	Great Britain (1), US (1)	4	2,4
8432 delA	60	—	—	3	—		3	
8656 ins T	61	—	—	—	4		4	
IVS62+1 G>A	IVS62	2	—	—	—	Great Britain (4)	6	2
9139 C>T	65	1	2	—	—	Italy (2), Great Britain (2), Japan (1)	8	2,11-13

^a1Byrd et al., 1996; ²Stankovic et al., 1998; ³Sandoval et al., 1999; ⁴Teraoka et al., 1999; ⁵Telatar et al., 1998; ⁶Laake et al., 1998; ⁷Vorechovsky et al., 1996; ⁸Gilad et al., 1996b; ⁹Hacia et al., 1998; ¹⁰van Belzen et al., 1998; ¹¹Lakin et al., 1996; ¹²Toyoshima et al., 1998; ¹³Gilad et al., 1998.

^bAT patients living in Germany, but of the given origin (T. Dörk, personal communication).

^cTwelve of the alleles previously published.

^dPersonal communication RA Gatti.

^eOne of the alleles previously published (the same patent were studied).

^fNationality unknown.

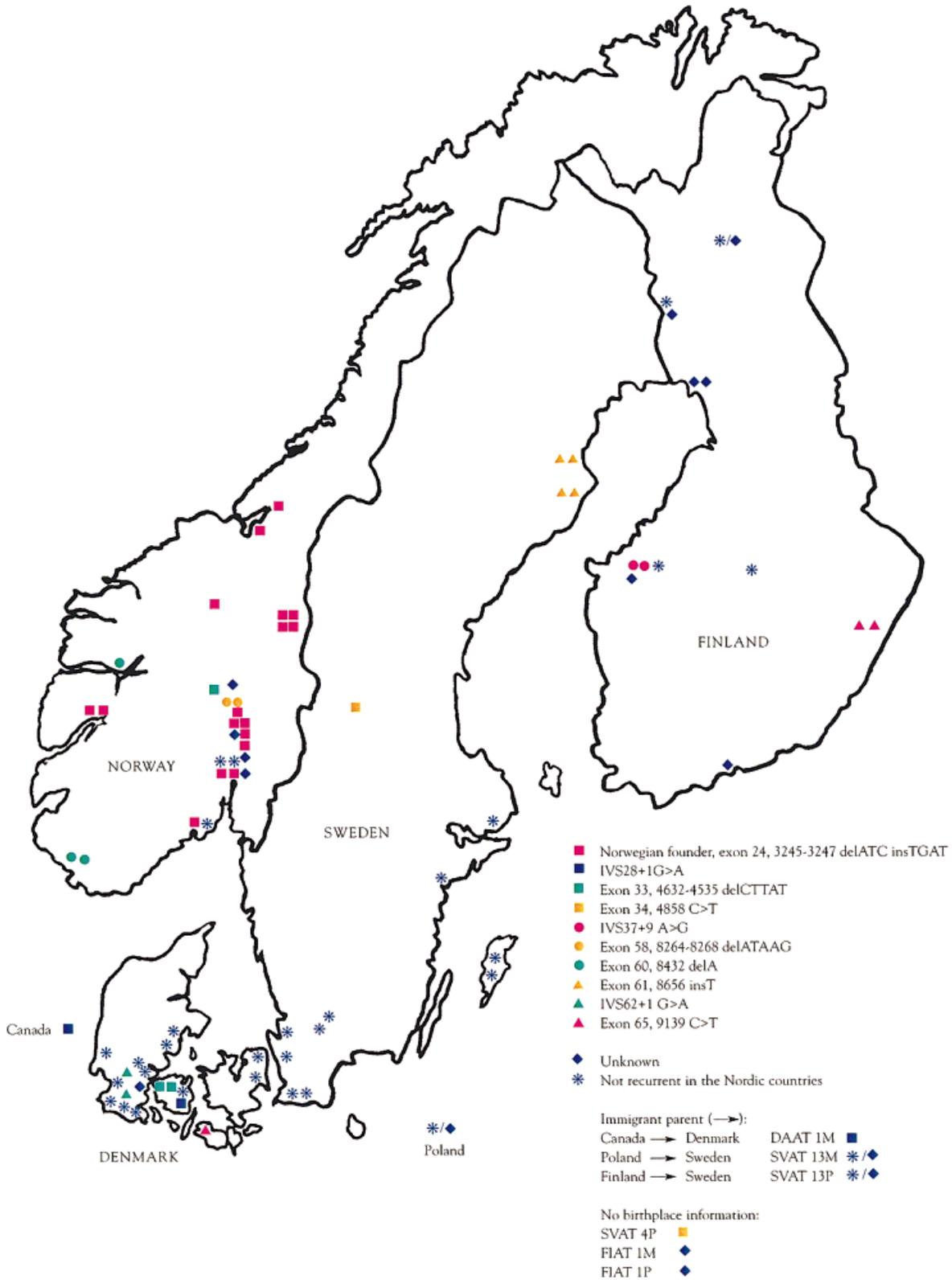


FIGURE 2. Birthplace of the Nordic AT parents. Each individual parent has a symbol, except those with unknown birthplace (see legend).

ternal and paternal grandmothers were sisters, but surprisingly the AT child was compound heterozygous. The parents in family DAAT 16 were born in the same community, and both carried a four base pair deletion in exon 33. A Norwegian AT mother (NOAT 9) also carried this mutation.

Two recurrent mutations were found in Sweden. A frameshift mutation in exon 61 was found in two families (SVAT6 and SVAT 11) both with probands homozygous for the mutations. These families were from two relatively close communities in the north of Sweden (Fig. 2). The proband in family SVAT 4 was homozygous for a nonsense mutation in exon 34. Their mother was born close to the Norwegian border, whereas the birthplace of their father was unknown.

In Finland, one would expect founder effects for historic reasons [Peltonen et al., 1999], but only two mutations were recurrent, present on two alleles each. One splice mutation in intron 37, leading to creation of a cryptic splice site, was carried by the mother in family FIAT 3 and the father in family FIAT 5. It is very likely that the families are related since they originate from the same rural community, some ancestors have the same family name, and they have a common haplotype (unpublished results). However, a common ancestor has not been proven. The other recurrent Finnish mutation was present on both alleles in the proband in family FIAT2, in which the paternal grandmother and the maternal grandfather were siblings. The mutation was a nonsense mutation in the very last exon, leading to loss of 10 amino acids only, also present in the father of family DAAT1 and in other countries (Table 4 and discussion).

De Novo Mutation

In a previous linkage study, the proband in family NOAT 5 and her healthy brother (born in the early 1970s) were shown to carry the same haplotypes both from their father and mother [Laake et al., 1998]. The mutation from the father was the Norwegian founder mutation. We therefore concluded that the allele of maternal origin had to be a de novo mutation. In search for this mutation, a deletion of the 22 first base pairs of exon 8 was found in cDNA. The alteration lead to a frameshift and early truncation of the protein at codon 169, but the sequencing signal of the mutant allele was weak compared to the wild type. When genomic DNA was examined, an intronic deletion was discovered, IVS7-18 delT in a row of thymine bases (Table 5). However, the exon 8 variant was present in the mother, and the healthy brother. The variant was

also observed in a Finnish immigrant AT proband of Arabian origin, and by Teraoka et al. [1999] in both patients and controls. Thus, this is most likely not a disease-causing alteration, and the de novo mutation has still to be found.

Sequence Variants of Uncertain Function

In addition to the low abundant splicing variant in exon 8 described above, several sequence variants were detected. The different alterations leading to missense substitutions, splicing variants, and silent mutations in exon or intron coding sequence are described in Table 5.

Three different sequence alterations leading to missense substitutions were seen. These substitutions either did not segregate with the disease or two truncating mutations were seen in at least one of the probands carrying them. Two of them were seen in more than one family, D1853N in exon 39 and S2518N in exon 54. The substitution in exon 39 frequently has been reported [Appleby et al., 1997; Sasaki et al., 1998; Maillet et al., 1999; Li et al., 1999; Castellvi-Bel et al., 1999]. Consequently, none of these amino acid substitutions were likely to be disease-causing in AT patients.

Several different families showed heterozygous deletion of exon 11 when sequencing cDNA. The deleted allele was present in a very small amount compared to the wild type allele when sequencing. The ratio between the wild type allele and the aberrant allele varied between different mRNA populations from the same individual. Three different genomic changes in intron 10 were seen in those probands. All were in stretches of adenine or thymine bases: IVS10-18 delT, IVS10-29 delA, and IVS10-44 delA. The cDNA deletion was, for instance, identified in the family NOAT 13, which was homozygous for a truncating mutation in exon 60. All family members tested showed partial deletion of exon 11.

Deletion of exon 9 was also frequently seen in this set of samples. Two Swedish parents had two different 5' splice mutations, most likely disease-causing (Table 3). In one Norwegian (NOAT 13) and two Finnish (FIAT 1P, FIAT 7M) families, deletion of exon 9 coincided with an apparently silent mutation, 735C>T, 70 bases into the exon. No mutations were found in exon 9 or more than 50 base pairs upstream and downstream of the exon in those individuals. Long range PCR including introns 8 and 9 gave full-length products, ruling out a genomic deletion between exon 8 and 9 as a cause of the exon skipping. The Norwegian family was homozygous for a truncating mutation in exon

TABLE 5. Sequence Variants in the *ATM* Gene and Transcript of Uncertain Function

Sequence alteration genomic DNA ^a	Exon/ intron ^b	First codon(s) affected	mRNA/protein alteration	Classification ^c	Reference ^d
Missense substitutions:					
5557 G>A	39	D1853N	Substitution	B,C	1,3
7742 G>A	54	S2581N	Substitution	B,C	
8414 T>C	59	M2805T	Substitution	B	
Splicing variants:					
IVS7-18 delT ^e	IVS7	ELFS166-169GSIX	Partial skipping of first 22bp of exon 8, use of 3' cryptic splice	B,C	2
735 C>T ^f	9	QEK222-224CLX	Frame shift, stop after 3 aa substitutions	A,B	
			Skipping of ex 9 (239bp). Frame shift, stop after 2 aa substitutions		
IVS10-18 delT	IVS10	356	Partial skipping of ex 11 (170bp). Frame shift, stop after 16 aa substitutions	A	2
IVS10-29 delA	IVS10	356	Partial skipping of ex 11 (170bp). Frame shift, stop after 16 aa substitutions	B	2
IVS10-44 delA	IVS10	356	Partial skipping of ex 11 (170bp). Frame shift, stop after 16 aa substitutions	B	2
Silent mutations:					
1356 A>C	12	T452T	Silent		
6114 C>T	43	H2038H	Silent		
Silent intronic variants:					
IVS4+(37) insAA	IVS4	—	Silent. No cDNA change		3
IVS8-154 delT	IVS8	—	Silent. No cDNA change		
IVS20+(29) insA	IVS20	—	Silent. No cDNA change		
IVS21-22 delT	IVS21	—	Silent. No cDNA change		
IVS26-24 delT	IVS26	—	Silent. No cDNA change		
IVS38-8 T>C	IVS38	—	Silent. No cDNA change		3
IVS62+8 A>C	IVS62	—	Silent. No cDNA change		

^aMutations are designated according to recommended nomenclature [Antonarakis, 1998], the nucleotide sequence numbering is according to the cDNA sequence published by Savitsky et al. [1995b], while the genomic sequence used was published by Platzer et al. [1997].

^bExon numbers are according to Uziel et al. [1996].

^cA, Only one truncating mutation identified in the patient; B, Two truncating mutations identified in at least one patient; C, Do not segregate with the disease.

^eFound in proband NOAT 5, her healthy brother, which is carrier of the Norwegian founder mutation, and in an Arabian immigrant in Finland with AT.

^{d1}Appleby et al. [1997]; Sasaki et al. [1998]; Castellvi-Bel et al. [1999]; Li et al. [1999]; Maillat et al. [1999]; ²Teraoka et al. [1999]. No underlying genomic alteration were reported; ³Hacia et al. [1998].

^fThis silent mutation coincides with deletion of exon 9. Wild type sequence 50 bp upstream and 70 bp downstream in addition to exon 9 itself. Longrange PCR including introns 8 and 9 gave full length product.

60. No other disease-causing alleles were detected in the family FIAT 1 nor FIAT 7M.

DISCUSSION

Recurring Mutations and Descent

Mutations that have been published previously in AT probands from Italy, Germany, Great Britain, Japan, The Netherlands, Poland, and/or US were found in the Nordic AT probands (references in Table 4). A significant proportion of the Nordic AT alleles were either found in immigrants (from Canada or Poland) or previously published in AT probands of American, European, or Japanese origin. This was the case for seven of the 20 alleles in Denmark (35%), three of 18 alleles in Sweden (17%), two of 14 alleles in Finland (14%), and three of 30 alleles in Norway (10%) (Table 4 and Fig. 2).

The proportion of foreign related alleles was highest in Denmark, which historically had closer relations to the European continent than Finland, Norway, or Sweden. Whether this recurrence was due to immigration or emigration was unknown. The Norwegian founder mutation has been found in the U.S. in an AT proband of Norwegian ancestry [Telatar et al., 1998; and R.A. Gatti, personal communication]. A common ancestor, born in 1495, has been identified [unpublished results], and emigration to the U.S. from that kindred has been ascertained.

Ten of the mutations identified in the Nordic AT probands were also observed in AT probands outside the Nordic countries (Table 4). Mutations in a total of 10 AT alleles in probands in Great Britain, nine AT alleles in Germany, eight alleles in AT probands from the U.S., and two Polish founder mutations were recurring in the studied Nordic AT probands. A Norwegian mother (NOAT 4), born in Oslo, carried a mutation recurring in the Netherlands. A mutation seen in a Swedish mother (SVAT 9) born in Stockholm, was also reported in a German AT proband of Turkish descent [Sandoval et al., 1999; and T Dörk, personal communication]. Unexpectedly, one of the mutations has been seen previously in AT probands in Japan, showing truncation of 10 amino acids in the last exon (discussed below).

In the Finnish AT families this study had a remarkable low detection rate (50%) compared to the other countries (83–95%). There are no obvious technical reasons for this low detection rate. However, one of the Finnish probands (FIAT1) had a more severe clinical picture with symptoms from early infancy. Haplotype analyses of the ATM locus

revealed that three of the AT parents carried a common haplotype (FIAT6M, FIAT6P, and FIAT7M) (unpublished results). Recently, Stewart et al. [1999] showed that mutations in the double-strand break repair gene *hMRE11* were present in patients with AT-like disorder (ATLD). It would be of interest to screen the *hMRE11* gene for mutations in the families with no common ATM haplotype.

Function of Mutations

A high proportion (43%) of the mutations affected correct splicing. They led to a variety of consequences, including exon skipping, and to a lesser degree activation of cryptic splice sites, or creation of new splice sites. In addition, a nonsense mutation was associated with deletion of exon 7 in cDNA. There are similar observations in the ATM gene [Ejima and Sasaki, 1998; Teraoka et al., 1999]. Both silent mutations and truncating mutations have been reported to result in skipping of exons in different genes [for instance Liu et al., 1997; Dietz et al., 1993]. Valentine [1998] notes that almost all exonic mutations leading to exon skipping occur in splice enhancers.

Splicing is thought to be dependent on mRNA configuration, and the efficiency of the splice-donor and -acceptor sites. The efficiency of a mutated splice site can be mildly or severely reduced compared to the wild type sequence. Consequently, the splice apparatus will fully or partly choose another donor or acceptor sequence. It has been reported previously that some of the mutated ATM splice sites have the ability to produce low levels of correctly spliced mRNA and wild type protein [McConville et al., 1996; Gilad et al., 1998]. The probands carrying these alterations exhibited mild phenotypes, and small amounts of wild type transcript and protein were seen. The splice site scores of these leaky mutations were in the same range as the splice mutations in the present study. On the other hand, none of the classical AT patients carrying a wide range of splice mutations reported by Teraoka et al. [1999], possessed detectable amounts of normally spliced transcript or protein. It is not conclusive to what extent splicing mutations are leaky, and whether this leads to a milder phenotype of the patients.

The truncation of the last 10 amino acids of the ATM protein, downstream of the catalytic PI3-kinase domain, was seen in one Danish and one Finnish AT proband in the present study. The probands were homozygotes and compound heterozygotes, respectively. To date this is the smallest truncation noted among ATM mutant alleles; and it has previ-

ously been reported in two British, one Italian, and one Japanese AT proband [Stankovic et al., 1998; Gilad et al., 1998; Toyoshima et al., 1998]. The Finnish proband (FIAT 2) and the Italian proband (AT9RM) were both homozygous for this alteration. Both probands lacked recurrent infections and malignancies. The Italian proband exhibited 17% ATM protein level compared to the wild type protein level, which is among the highest protein levels noted among AT patients. Her truncated protein was less stable than wild type protein, and the catalytic activity was presumed reduced [Gilad et al., 1998]. It is noteworthy that this mutation has been found in Northern and Southern Europe in addition to Japan. In comparison, a polymorphic stop codon has been found in the *BRCA2* gene leading to truncation of the last 93 amino acids [Mazoyer et al., 1996]. Whether this alteration is a polymorphism that by incidence was present on the disease-causing allele has to be verified by functional analyses and further ATM mutation screening.

It is not investigated whether the mutated alleles in this study give rise to stable ATM protein. Detectable protein levels are usually reported in AT patients exhibiting milder clinical phenotypes [Lakin et al., 1996; McConville et al., 1996; Gilad et al., 1998]. In classical AT patients it is rarely reported. Investigation of genetic diseases such as cystic fibrosis, alpha-1-antitrypsin deficiency, phenylketonuria, and mitochondrial acylCoA dehydrogenase deficiency has shown that enhanced proteolytic degradation of mutant protein is a common mechanism [Bross et al., 1999]. Detailed studies of the fate of mutated proteins have revealed that impaired or aberrant folding typically resulted in prolonged interaction with chaperones and degradation by proteases before functional conformation was acquired. This appeared to be the case also for many missense mutations and short in-frame deletions or insertions as well as nonsense mutations [Bross et al., 1999].

Exon Skipping as Natural Variants

Variant or alternative splicing leading to loss of parts of the cDNA sequence and premature truncation of protein were observed for exons 8, 9, and 11. They were seen in a lower amount than the wild type transcript for exon 8 and 11, and the accompanying genomic alterations did not affect the conserved splice site sequence. The variants in exon 8 and 11 were also observed by Teraoka et al. [1999] without any underlying genomic alterations. Broeks et al. [2000] reported DNA alteration, IVS10-6 T>G, associated with skipping of exon 11 in breast

cancer patients, which they interpret as a truncating splice mutation. This genomic alteration is situated in a row of redundant pyrimidines, and the computed splice site score is not changed between wild type and mutant splice site sequence. There is a possibility that skipping of exon 11 is a natural sequence variation. In conclusion, the partial skipping of exon 8 and 11 doesn't seem to cause the AT disease, at least not alone. The skipping of exon 9 was more pronounced and needs to be further investigated even if it is present in a family with two truncating mutations.

The phenomenon of partial skipping leading to transcripts with premature stop codons has been reported for several genes expressed in brain tissue [Stamm et al., 1994; Leeb and Brenig, 1998]. For instance, variant mRNA constituted about 30% of the total ryanodine receptor 3 (RYR3) transcripts, measured densitometrically from reverse transcribed PCR amplified mRNA. Lately, it has become clear that a conserved mRNA degradation system exists whose function is to degrade aberrant mRNAs [Hilleren and Parker, 1999]. This process plays an important role in monitoring that mRNAs are properly synthesized and functional. The system increases the fidelity of gene expression by degrading aberrant mRNAs that, if translated, would produce truncated proteins. A critical issue is how normal and aberrant mRNAs are distinguished and how that distinction leads to differences in mRNA stability.

CONCLUSIONS

In the present study, protein truncating mutations or mutations displaying heteroduplex patterns, all sequence verified, were detected in 82% of the investigated AT alleles. The remaining AT alleles are most likely substitutions of conserved residues, large genomic deletions, mutations affecting transcript initiations, or alterations in other genes giving a similar phenotype like the *hMRE11* gene. The present information gives us the unique possibility to study phenotype-genotype correlations in detail, in addition to modifier genes, and/or environmental exposure that may affect phenotype.

Detecting mutations causing AT in a family can be very useful clinically since the clinical diagnosis of AT is difficult, especially in the early stages of the disease. Confirming the diagnosis at the gene level is important. Some families may also want to consider prenatal diagnosis. This study, however, demonstrates that mutation detection in AT families is laborious because of the high number of dif-

ferent mutations in the large gene. In addition, there are splice variants of unknown function in the gene complicating the interpretation.

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