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Identical mutation in 55% of the *ATM* alleles in 11 Norwegian AT families: evidence for a founder effect

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The *ATM* gene is responsible for the autosomal recessive disorder Ataxia-Telangiectasia (AT). Many different mutations, located all across the gene, have been reported with a predominance of truncating mutations. By using PTT (protein truncation test) a mutation was found in one Norwegian AT family. Sequencing revealed that the mutation affected nucleotides 3245–3247, codon 1082, and changed the sequence from ATC to TGAT, inducing a stop codon downstream at codon 1095 and leading to early truncation of the *ATM* protein. Perpendicular DGGE (denaturing gradient gel electrophoresis) was used to screen 10 additional families for this mutation. The 3245 delATC instTGAT mutation was found in 12 of 22 proband alleles: five patients were homozygotes and two heterozygotes. Haplotype analyses were performed using eight microsatellite markers, within and flanking the *ATM* gene. All carriers of the mutation described were found to have a common haplotype of the five closest CA-repeat microsatellite markers. Genealogical investigations of the families identified a common ancestor for three of the families. The common ancestor was a woman born in 1684 in the area from which these families originate. The prevalence of this mutation in Norwegian patients now allows a major subset of AT heterozygotes to be identified, both in the general population and in breast cancer patients, so that their cancer risk can be evaluated

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Introduction

Ataxia-Telangiectasia (AT) is an autosomal recessive disorder characterised by unstable gait (ataxia), dilated blood vessels (telangiectasia), and immune deficiency in 70% of the cases.^{1,2} Ten to fifteen percent of patients develop lymphatic or leukaemic malignancies.³ Both heterozygotes and homozygotes show increased sensitivity to ionising radiation. This is seen by abnormal reaction in cell cultures after radiation exposure, where cells from heterozygotes react to an intermediate degree compared with cells from homozygotes and normals. Progressive degeneration of the cerebellum, abnormalities of coordinated eye movements (occulomotor apraxia), and increased levels of serum alpha-fetoprotein (90% of the cases) are other features characteristic of the syndrome.⁴

The world-wide frequency of the disorder is 1:40 000–1:300 000, whereas about 0.35–1% of the general population is heterozygous.^{5–7} It has been suggested that heterozygotes have a 4–10 fold increased risk of cancer, in particular breast cancer, compared to the normal population.^{6,8–10} Recently Athma and colleagues have haplotyped breast cancer patients among AT relatives and found that it was more frequent in the heterozygote carriers than in the non-carriers (odds ratio 3:8 (95% CI 1.7–8.4)).¹¹ Mutation analyses in a cohort of early onset breast cancers did not reveal higher mutation frequency than in controls,⁷ although the value of that study has been questioned.¹²

The *ATM* gene was cloned in 1995,^{13,14} following a seven-year positional cloning study by an international consortium.^{15–17} It spans approximately 150 kb genomic DNA and contains 66 exons including alternative splicing variants. The major *ATM* transcript is 12 kb and the protein is correspondingly large (approx. 350 kD).¹⁸ The protein is probably multifunctional. The carboxyl terminal end shows strong homology with a family of high molecular weight PI-3 kinases and a domain in the middle shows homology with *rad*-genes.¹⁹ Recent studies of *ATM* knockout mice^{20,21} confirm that the *ATM* protein is critical for cellular responses to ionizing radiation, for normal cell-cycle progression, and for presentation of cancer; 100% of the mice develop malignant thymic lymphoma by age 4 months.

Both missense and nonsense mutations within the *ATM* gene can cause the disorder, although nonsense mutations are the most common, including point mutations as well as small deletions or insertions.^{13,22–30} Most probands are compound heterozygous, whilst relatively few are found to be homozygous if the

parents are not consanguineous or do not live in isolated communities.^{23,26–28} A web-site (<http://www.vmmc.org/vmrc/ATM.htm>) lists the 130 mutations published to date.

The Norwegian population is known to be genetically homogeneous, and we know that some of the Norwegian AT families originated from Østerdalen, a valley in the south-east part of Norway. Therefore a founder mutation was anticipated. If a limited number of *ATM* mutations were to be found among the Norwegian AT probands, this would facilitate the assessment of cancer risk among heterozygotes. We now report just such a founder mutation in 12 out of 22 proband alleles, suggesting that this is an ideal population in which to assess the relationship between AT carrier status and the incidence of cancer or any other health effects.

Materials and Methods

Patients

A total of 13 families with one or two AT children have been identified in Norway (Børresen *et al* 1990). This study includes samples from 11 of the families and 10 of the AT children all with a classical AT phenotype.

DNA Preparation

Peripheral blood samples (20 ml EDTA) were collected and kept frozen at –40°C until DNA could be extracted by phenol-chloroform extraction and ethanol precipitation using an ABI DNA extractor. DNA samples were stored at 4°C. Fibroblast samples were also collected from most families. They were grown in RPMI 1640 medium (GIBCO) with added 20% foetal calf serum, 2 mM L-glutamine, streptomycin/penicillin and 1 × NEAA mixture (Bio Whittaker, Verriers, Belgium). They were frozen by liquid nitrogen in this medium containing 10% DMSO. The fibroblasts were used as a source for RNA extraction.

PTT (Protein Truncation Test)

This was performed as described in detail elsewhere.²⁹ Essentially, mRNA was isolated from fibroblast cell lines from the proband and the parents in family NOAT 1. After cDNA synthesis seven PCR fragments were generated to perform the PTT analyses.

DGGE (Denaturing Gradient Gel Electrophoresis)

After locating the mutation by PTT, primers were designed to detect the mutation in genomic DNA using PCR-based DGGE. The forward PCR primer was 5'- CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GTG GGA AAA GAC TTT CCT G -3'; the reverse primer was 5'- CTA TTG ATT GAC TCT GCA GCC -3'. The PCR reaction contained 50–100 ng DNA as template, 50 pmol of each primer, 2 mM MgCl₂, 1 × PCR buffer II (Perkin Elmer) and 1.25 U AmpliTaq DNA polymerase (Perkin Elmer) in 50 µl. The reaction conditions were: preheating at 94°C for 5 min, 10 times cycling at 94°C for 15 s, 65°C for 30 s and 72°C for 30 s followed by 25 cycles at 94°C for 15 s, 50°C for 15 s and

72°C for 15 s; 1 min extension at 72°C, and heteroduplex formation at 65°C for 45 min.

Each PCR product was analysed on individual perpendicular DGGE gels^{33,34} containing 1 × TAE, 10% acrylamide/bis (37.5:1), 10–60% denaturant concentrations consisting of urea and formamide (100% denaturant corresponds to 7 M urea and 40% formamide). The PCR products were electrophoresed at 55°C, 130 V for 1.5 hours using a BioRad D-GENE electrophoresis system, stained in Sybr Green and photographed in UV-light.

Sequencing

All suspected mutants were sequenced on an ABI 373 Stretch Automated DNA Sequencer. PCR products of exon 24 were amplified in a mixture containing 50–100 ng genomic DNA, 35 pmol of each primer (forward: 5'-TAA CCA CAG TTC TTT TCC-3' and reverse: 5'-ATA TTT GAC CCA TTA CCT-3'), 2 mM MgCl₂, 1 × PCR buffer II (Perkin Elmer) and 1.25 U AmpliTaq DNA polymerase (Perkin Elmer) in 50 µl. The reaction mixture was preheated at 94°C for 5 min, cycled 35 times at 94°C for 15 s, 45°C for 15 s and 72°C for 15 s, followed by 1 min extension at 72°C. The PCR products were sequenced using an internal forward primer (5'-TAA TGT AAT GGG AAA AGA-3') and DNA sequencing kit, dye terminator cycle sequencing, ready reaction (Perkin Elmer).

The reactions were performed in accordance with the protocol recommended by the manufacturer.

Genotyping

The CA-repeat microsatellite markers D11S1325, D11S1347 and D11S927 were selected from the Genome Data Base, and the oligonucleotides purchased from Research Genetics. The microsatellite markers D11S1816, D11S1819, D11S2179, D11S1778 and D11S1818 were identified and physically mapped to 11q23 by Rotman *et al*³⁵ and Vanagaite *et al*³⁶. The location of the markers and the approximate distances between them are shown in Figure 1. Microsatellite genotyping was performed in microtitre plates by PCR in a 10 µl reaction volume containing 50–100 ng DNA, 200 µM dATP, dGTP and dTTP (of each); 2.5 µM dCTP; 0.7 µCi [α -³²P]-dCTP; 3 pmol of each primer and 0.4 U AmpliTaq polymerase (Perkin Elmer) in 1 × PCR buffer (1.5 mM MgCl₂ in the final solution). The PCR program started at 94°C for 5 min, continued with 27 cycles with three steps of 1:94°C for 30 s, 2:55°C for 75 s, 3: 72°C for 15 s, followed by 72°C for 2 min. PCR products were separated on 6% denaturing polyacrylamide gels (1:19 bis: acrylamide), containing 1 × TBE and 7.7 M urea, for 1.5–2 hours at 100 W constant in 0.5 × TBE buffer. After electrophoresis the gels were dried, and exposed to Fuji Medical X-ray films, RX

Figure 1 Schematic map of the ATM region in chromosome segment 11q23.1. The upper part shows the relative positions of the CA-repeat microsatellite markers used for haplotyping; below a map of the ATM ORF where the different exons are shown, including the position of the PTT (protein truncation test) fragment and the DGGE (denaturing gradient gel electrophoresis)-fragment used in this study. Numbering according to Savitsky *et al*¹⁴ and Uziel *et al*¹⁸

(normally between 30 min and 2 days). The autoradiograms were validated visually by two independent observers.

Results

The *ATM* open reading frame (ORF) was first analysed for mutations by the protein truncation test (PTT) in the proband and in the parents of one Norwegian AT family (NOAT 1). An aberrant migrating band in fragment **g**²⁹ was found, and the result of a PTT analyses of NOAT 1.3 (mother in family NOAT 1) is shown in Figure 2. After sequencing, an unusual delATC insTGAT mutation was detected which started at nucleotide 3245 and affected codon 1082, exon 24 (according to the numbering published by Savitsky *et al* and Uziel *et al*^{14,18}) (Figure 3). The mutation changes the amino acids from codon 1082 and downstream; a stop codon is induced in codon 1095 which leads to early truncation of the protein and deletion of the highly conserved PI3-kinase domain.

To be able to investigate the distribution and frequency of this mutation among other Norwegian AT patients and their relatives at the DNA level, a perpendicular denaturing gradient gel electrophoresis (DGGE) assay was designed. (Figure 4). Members from 11 Norwegian AT families were analysed, and the mutation was found in 12 of 22 proband alleles (55%).

Figure 2 A protein truncations test (PTT) of fragment **g** (see Figure 1 for localisation) of the *ATM* cDNA from three different samples. The sample from NOAT 1.3 (in the middle) displays one normal protein band and one truncated protein band

A: NOAT 1.2 (normal)

B: NOAT 1.1 (two mutated alleles)

C: NOAT 1.3 (one mutated allele)

Figure 3 Sequence analyses of exon 24 in three members of AT family NOAT 1 using an ABI Automated Sequencer. The sequence is normal in NOAT 1.2 (A), altered on both alleles from ATG to TGAT (nt 3245-3247) in NOAT 1.1 (B), and on one allele in NOAT 1.3 (C)

Five patients were homozygous and two were compound heterozygous for the mutation. The mutation was sequenced for verification in all probands.

Since seven out of 11 Norwegian AT patients carry this mutation, genotyping of the families was performed to investigate whether this mutation resided on the same haplotype. The families were genotyped for eight microsatellite CA-repeat markers within an area

Figure 4 Perpendicular DGGE (denaturing gradient gel electrophoresis) of a PCR product of genomic DNA from a compound heterozygous proband (NOAT 8.1) carrying the described mutation. Both the mutant and the wild-type homoduplexes as well as the hetero-duplexes formed by the mutation 3245 delATG insTGAT in exon 24 (nucleotide 3245-3247, codon 1082 and downstream) are seen.

of approximately 6.5 Mb upstream and 9 Mb downstream of the *ATM* gene, one marker residing inside the gene (Figure 1). The pedigrees of the families and the results of the haplotype analyses are presented in Figure 5. A common attendant haplotype was found in all individuals carrying the mutation, except one family. This haplotype was designated: F (D11S1819), E (D11S2179), M (D11S1778), G (D11S1818), D (D11S927). In family NOAT 5 the paternal haplotype has an E allele instead of G for marker D11S1818. Mutation in the CA-repeat markers is a possible explanation since these markers are known to have a relatively high mutation rate. However the chance of a double crossover may be in the same order of magnitude. Interestingly no other widespread common haplotypes were observed in these Norwegian AT patients (Figure 5).

By researching old Norwegian parish records and regional history books, a common ancestor was identified for families NOAT 1, NOAT 6 and NOAT 11, a woman who lived from 1684 to 1755 (Figure 6). She married twice, founding two different branches of the family, and gave birth to eight children in total. Family NOAT 6 belongs to one branch of the family and NOAT 1 and NOAT 11 to the other. NOAT 1 and NOAT 11 are related through their common great-

great grandparents and great-great-great grandparents respectively (Figure 6). At this writing none of the other families carrying the mutation (NOAT 2, NOAT 5, NOAT 8 and NOAT 10) can be traced in this extended pedigree.

From the haplotype analyses, one can anticipate that probands NOAT 3.1, NOAT 4.1 and NOAT 7.1 are compound heterozygotes. Noteworthy is the fact that NOAT 7.1 and NOAT 8.1 have one haplotype in common (FEDD) which may indicate that they carry the same mutation. The parents of probands NOAT 9.1 and 9.2 might possibly share one haplotype, accounting for their two affected children. The proband in family NOAT 5 is also a compound heterozygote with the 3245 delATC insTGAT mutation segregating from the father. The maternal mutation is likely to be a *de novo* mutation since the brother of the proband NOAT 5.2 has exactly the same genotype as his affected sister, and he is healthy and non symptomatic at age 25. This is one of the six non-linked families in the 176 AT families used positionally to clone the *ATM* gene.^{16,17}

Discussion

The mutation found in 12 of 22 Norwegian AT proband alleles leads to early truncation of the protein. Nonsense mutations and deletions seem to be the dominating type of aberration in AT patients.²⁴ One exception is a report from McConville *et al*²⁷ describing 14 British AT families, some with a milder proband phenotype. They found that ten of those patients were compound heterozygous for a leaky splice mutation and two were compound heterozygous for two other missense mutations outside the conserved PI3-kinase domain. All other mutations published are nonsense point mutations, deletions or insertions.^{22-24,28-30}

The mutation found in the Norwegian families leads to truncation 13 aminoacids downstream of codon 1082 and thereby loss of the PI3-kinase domain which is the most conserved part of the *ATM* protein. The *ATM* mutations seem to be spread over the entire gene with no 'hot spots'. One possible trend may be that deletions of the PI3-kinase domain are over-represented,³⁰ including a 7638 del9 (codon 2546-2548) mutation reported both in British, Australo-British, Celtic-Irish and American AT families.^{23,24,30} This 9 bp deletion was also found in a Swedish family with a history of breast cancer but not AT.³¹ Recently we haplotyped five of these AT patients with the 7638 del9 (codon 2546)

Figure 5 Pedigrees of the Norwegian AT families included in the study (NOAT 1–11). The results of the haplotyping using eight CA-repeat microsatellite PCR markers are shown for each individual (for location see Figure 1). The different alleles are given the symbols A–Z which refer to a given size of the PCR product of that specific microsatellite marker. The size range is different for each marker. When the letter is accompanied by an asterisk this denotes that it is deduced from information from the rest of the family members. An unknown genotype is denoted with a question mark, and possible overcrossing is shown by a horizontal line in the haplotype. For family NOAT 9 the haplotypes for the offspring are inferred; the tested genotypes are shown. A common haplotype F (D11S1819), E (D11S2179), M (D11S1778), G (D11S1818), D (D11S927), can be seen in individuals carrying the 3245 delATC *insTGAT* mutation

Figure 5 Continued

Figure 6 Pedigree of the relationship between families NOAT 1, NOAT 6 and NOAT 11. The common ancestor was a woman who lived from 1684 to 1755. NOAT 1 and NOAT 11 are related through common great-grand grandparents and great-great-great grandparents, respectively.

mutation; four share a common haplotype.²⁹ Thus, this potential 'hot spot' is also questionable.

The diversity of ATM mutations appears to be low in the Norwegian population as compared with other populations studied. Further screening in these families will reveal how many different ATM mutations exist in the Norwegian AT population and the relationship between the families. Simple tests can be designed for each mutation with the purpose of screening AT family members and cancer populations to ascertain the cancer susceptibilities that have been estimated from epidemiological studies of AT families.^{6,8,9} Such studies are already in hand for the 3245 delATC insTGAT mutation in Norwegian breast cancer patients.

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