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DHPLC screening of *ATM* gene in Italian patients affected by ataxia-telangiectasia: Fourteen novel *ATM* mutations

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Abstract. The gene for ataxia-telangiectasia (A-T:MIM:#208900), *ATM*, spans about 150 kb of genomic DNA and is composed of 62 coding exons. *ATM* mutations are found along the entire coding sequence of the gene, without evidence of mutational hot spots. Using DNA as the starting material, we used denaturing high performance liquid chromatography (DHPLC) technique to search for *ATM* gene mutations. Initially, DHPLC was validated in a retrospective study of 16 positive control samples that included 19 known mutations; 100% of mutations were detected. Subsequently, DHPLC was used to screen for mutations a cohort of 22 patients with the classical form of A-T. A total of 27 different mutations were identified on 38 of the 44 alleles, corresponding to a 86% detection rate. Fourteen of the mutations were novel. In addition, 15 different variants and polymorphisms of unknown functional significance were found. The high incidence of new and individual A-T mutations in our cohort of patients demonstrates marked mutational heterogeneity of A-T in Italy and corroborate the efficiency of DHPLC as a method for the mutation screening of A-T patients.

Keywords: Ataxia-Telangiectasia, *ATM*, mutations, DHPLC, polymorphisms

1. Introduction

Ataxia-Telangiectasia (A-T; MIM# 208900) is an autosomal recessive disorder characterized by cerebellar ataxia, telangiectases, immune defects and predisposition to malignancy. A-T is caused by mutations in the *ATM* (Ataxia-Telangiectasia Mutated) gene. The gene extends over 150 kb of genomic DNA, includes 66 exons, and has an open reading frame of 9168 nt. The *ATM* gene product contains 3,056 amino acids and is a member of the phosphatidylinositol (PI) 3-kinase family of proteins, with the kinase domain in its C-terminal region [1]. The *ATM* gene plays a key role in several pathways involved in cell-cycle control, oxidative stress, and DNA repair [2,3].

As of December 2005, the Human Gene Mutation Database (HGMD, Cardiff, http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html) listed more than 415 different *ATM* mutations. These include deletions, insertions, and point mutations. Most of these changes are predicted to give rise to a truncated protein that is highly unstable, effectively producing a null phenotype (∼85%), but a significant number of missense mutations have also been recorded (∼10%) [4–10]. It has been hypothesized that *ATM* missense mutations are implicated in breast cancer, and there is some ev-

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idence to support this [11–13]. Mutations are spread all over the *ATM* gene without evidence of mutational hot spots. Nonetheless, recurrent mutations have been reported in Norway, the Netherlands, Costa Rica, the English Midlands, Italy, Japan, and Poland, and among people of Irish, English, Utah Mormon, African American, Israeli Jewish, and Amish/Mennonite descent [5, 7,14–17]. For several of these mutations, the carriers share common haplotypes, suggestive of a founder effect [4,7,15,16,18–24]. To better assess the spectrum and distribution of mutations leading to A-T in the Italian population, we performed a systematic mutation analysis of the *ATM* gene in 22 unrelated patients with A-T, 21 coming from Italy. For this purpose, a new DHPLC-based mutation screening method was developed. In this survey, we identified 38 mutant alleles in 22 families, which consisted of 27 different mutations including 14 novel ones.

2. Materials and methods

2.1. Patients

Lymphoblastoid cell lines of 21 unrelated patients from the Italian Ataxia Telangiectasia Registry (RIAT) [25], and of one Japanese patient (ATJ3), all presenting with the classical form of A-T, were analysed for the presence of mutations within the *ATM* gene. Inclusion criteria were the presence of progressive cerebellar ataxia, oculocutaneous telangiectasias, increased alpha-fetoprotein levels, chromosomal instability and cellular radiosensitivity. Included in these 22 cases there are three A-T patients (AT42RM, AT50RM and AT57RM) in whom one of the mutations was previously detected [5,26,27].

2.2. DHPLC

Sixty-one out of the 62 coding exons (from exon 4 to 15 and from exon 17 to 65) of *ATM*, along with exon-intron junctions, were PCR-amplified according to Bernstein and Colleagues [28]. *ATM* exon 16 primers (exon 16 Forward 5'-ATCCAGGATATGCCACCTTT-3' and exon 16 Reverse 5'-CATTTGTTTGGAAGACA TTAATTTT-3') were newly designed to obtain improved PCR amplification. Web Primer software available on-line (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used for primer design. PCR reactions were carried out on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) in

a final volume of 50 μl containing 100 ng of genomic DNA, 1 μM primers, 200 μM dNTPs, 1,75U of Optimase^{TM} Polymerase (Transgenomic, Crewe, UK), 5μl of 10X Optimase^{TM} Polymerase Reaction Buffer and 1.5 mM MgSO$_4$. Initial denaturation at 94°C for 3 min was followed by 38 cycles of denaturation at 94°C for 30 sec, specific annealing temperature for 30 sec and extension at 72°C for 45 sec. A final extension step was performed at 72°C for 5 min. Denaturing high-performance liquid chromatography was carried out using the WAVE DNA Fragment Analysis System (Transgenomic, Crewe, UK) equipped with a DNASep column (Transgenomic, Crewe, UK). After denaturation at 95°C for 5 min and then gradual re-annealing by decreasing temperature from 95°C to 25°C over 60 min, PCR products were injected into the column and separated (at a flow rate of 1.5 ml/min) through a 5% linear acetonitrile gradient, using commercially available Wave Optimised^{TM} buffers A, B and D and syringe solution (Transgenomic, Crewe, UK). Analysis lasted 2.5 min per sample. All peaks falling below 2 mV was considered not reliable and was re-analysed. Buffer B start concentrations and oven temperature for optimal heteroduplex separation were determined using the WAVEmaker software version 4.1.40 (Transgenomic, Crewe, UK). Where distinct melting domains were predicted by the software, DHPLC analysis was performed at two different temperatures. For 17 exons one temperature was adequate, while two temperatures were necessary to analyze 45 exons. Regarding the DHPLC detection of *ATM* homozygous mutations, in order to have detectable heteroduplex peaks, all exons were re-screened after a preceding step of mixing patients' PCR products with normal reference wild type amplicons. A full list of annealing temperatures for PCR amplification, resolution temperatures and buffer B start concentrations for DHPLC analysis is reported in Table 1.

2.3. Direct sequencing

Any amplification product showing an abnormal elution profile was re-amplified and sequenced in the forward and reverse direction using the BigDye Terminator chemistry and an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA). The pathogenic role of novel missense and intronic changes was evaluated by screening 384 control chromosomes from 192 unrelated healthy individuals.

Table 1
PCR and DHPLC analysis conditions for the *ATM* amplicons

Exonic fragment	PCR annealing temp. °C	DHPLC oven temp. °C	Start % buffer B	Exonic fragment	PCR annealing temp. °C	DHPLC oven temp. °C	Start % buffer B
4	53	52–57	57–55	35	52	53.5–57.5	57–56
5	54	53–56	59–58	36	54	52.9–55.9	60–59
6	54	54.3–57.3	60–56	37	54	55.5–57.1	59–58
7	54	55	59	38	54	53.9–56.1	60–59
8	54	51.2–55.2	61–58	39	54	56	56
9	54	55.3–57.3	60–57	40	54	58	56
10	54	54.4–56	59–58	41	54	53.5–55.2	59–55
11	54	56.5	56	42	54	54.7–56.7	55–54
12	54	56.2–58.2	62–61	43	54	57.2	50
13	54	56.3	60	44	54	56.6–60	55–53
14	54	54.9–56.9	56–63	45	54	57	55
15	54	54–57	60–57	46	54	54–56	59–58
16	58	52.3–56.3	61–55	47	54	56–60	58–54
17	54	56	57	48	54	57.3	60
18	54	56.5	54	49	54	56–59.5	59–57
19	54	56.8–58.8	59–59	50	53	56–58.5	57–57
20	54	57	60	51	54	51–55.8	62–60
21	54	54–56	57–56	52	54	52.4–57.4	61–59
22	54	57	56	53	54	54–57	57–55
23	54	55	57	54	54	54–56.5	61–57
24	54	56.9–58	56–54	55	55	57	57
25	54	55.7–58.7	55–53	56	53	52–55	59–55
26	54	55–57	58–57	57	54	53–56	62–58
27	54	52.2–55	60–59	58	54	54–58.2	58–55
28	54	54.5–56.5	60–60	59	55	58	57
29	54	55.1–58	57–56	60	54	56.5	58
30	54	54.5–56.5	58–57	61	54	54.5–56.5	57–54
31	54	54	60	62	54	56.8–58.5	58–56
32	54	54.9–57	60–59	63	54	56–57	55–53
33	54	53.6–55.6	59–57	64	54	56.5–58	58–56
34	54	55–57.6	56–55	65	54	57.5–59	59–58

2.4. Genotyping of microsatellite markers

We genotyped five microsatellite markers (D11S18 19-NS22-D11S2179-D11S1778-D11S1294) closely linked with *ATM* in homozygous patients. Primer sequences and PCR conditions in detail are available on request. The forward primers were labelled with 6-Fam fluorochromes, and the fluorescent PCR products were resolved on an ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA) and analysed with Genotyper software (Applied Biosystems, Foster City, CA, USA).

3. Results

The sensitivity of DHPLC was evaluated in a retrospective study of a cohort of 16 positive samples that included 19 known germline mutations previously identified using other mutation detection methods including SSCP, DNA heteroduplex or RFLP (Table 2). All 26 sequence variants were identified, under one or more run conditions, as unique elution profiles (data not shown). All 62 coding exons of the *ATM* gene were screened by DHPLC analysis of a panel of 22 unrelated A-T patients. A total of 27 different mutations -including 3 *ATM* mutations previously published by us [5,26,27] -were identified on 38 of the 44 alleles, corresponding to a 86% detection rate. Fourteen of the mutations were found to be novel according to "The Human Gene Mutation Database (HGMD), http://www.hgmd.cf.ac.uk/hgmd0.html". DHPLC revealed two putative mutations in 17 A-T patients, and one mutation in six patients. In addition, 15 different variants and polymorphisms of unknown functional significance were found. The sequence alterations suggesting pathological consequences are reported in Table 3; the sequence variations that are likely to represent polymorphisms or neutral variations are compiled in Table 4. Among the 38 mutations identified, we observed 6 (16%) nonsense alleles and 26 (68%) frameshift alleles, including 16 deletions, 1 insertion, 7 out-of-frame splicing events and 1 homozygous insertions. Thus, as many as 32 (84%) alleles caused,

Table 2
Known mutations in the *ATM* gene detected by DHPLC

Nucleotide substitution	Exon	Patient	Genotype	Method of identification of the mutation
755delGT	9	AT24RM	Homozygote	DNA heteroduplex
1240C>T	12	AT26RM	Heterozygote	SSCP
IVS12+1G>T	12	AT44RM	Homozygote	RFLP
2250G>A	16	AT73RM	Heterozygote	SSCP
2192insA	16	AT13RM	Heterozygote	DNA heteroduplex
IVS18+1delG	18	AT21RM	Homozygote	DNA heteroduplex
2413C>T	18	AT73RM	Heterozygote	SSCP
2718delGGCC	20	AT79RM	Heterozygote	DNA heteroduplex
3109delG	23	AT14RM	Heterozygote	SSCP
3625delTT	27	AT40RM	Homozygote	DNA heteroduplex
3802delG	28	AT4RM	Heterozygote	DNA heteroduplex
3894insT	28	AT18RM	Homozygote	SSCP
5229A>T	37	AT81RM	Heterozygote	SSCP
IVS47-9G>A	48	AT53RM	Heterozygote	SSCP
7146delA	51	AT14RM	Heterozygote	SSCP
8283delTC	59	AT28RM	Heterozygote	DNA heteroduplex
8365delA	59	AT52RM	Heterozygote	SSCP
8545C>T	60	AT13RM	Heterozygote	SSCP
9139C>T	65	AT9RM	Homozygote	RFLP

RFLP – Restriction Fragment Length Polymorphism, SSCP – Single Strand Conformation Polymorphism.

directly or indirectly, a premature termination codon (PTC). Two alleles (5%) caused in-frame splicing mutations. In addition, 4 (11%) missense mutations were observed. None of the novel missense and splice site mutations was detected in 192 anonymous control subjects (384 chromosomes). Sixteen (73%) of the investigated patients were compound heterozygous, and six (27%) were homozygous for an *ATM* mutation. To evaluate if homozygosis was due to consanguinity, 4 of the 6 patients with homozygous mutations were analysed by microsatellite markers flanking the *ATM* gene. Three patients AT97RM, AT105RM and AT71RM showed homozygous haplotypes, whereas one patient (AT19RM) carried different haplotypes.

4. Discussion

Identifying mutations in the *ATM* gene is difficult due to its large size and high number of exons, as well as the fact that most exons are small and scattered with large introns. In this study we used a DHPLC-based method to analyse 21 unrelated Italian patients and 1 Japanese patient affected by A-T. DHPLC analysis disclosed 27 different mutations in 38 of the 44 alleles, corresponding to 86% detection rate. This is the highest recorded mutation detection rate using a single technique for the *ATM* gene. Given that the current estimate for large heterozygous genomic rearrangements (duplications, inversions and full gene,

single and multi-exon deletions) is approximately 10% (The Human Gene Mutation Database (HGMD), http://www.hgmd.cf.ac.uk/hgmd0.html) DHPLC technique may therefore pick up almost all the remaining classes of mutations and potentially replaces all existing technologies, used either alone or together, with advantages of increased specificity and sensitivity associated with decreased cost and considerably reduced analysis times. One of the operational limitations of DHPLC is its inability to differentiate homozygous normal individuals from homozygous mutant individuals when run as singletons and thus requires the mixing of the patient sample with wild-type sequence in order to create heteroduplexes for recessive diseases [29]. Thus, the cost for DHPLC analysis of an A-T patient includes i) the PCR amplification of 62 exons of the *ATM* gene in the patient and in the wild-type control; ii) 107 DHPLC analyses (patient) plus 107 DHPLC analyses (mix between the patient and the wild type control); iii) an average of 4 bi-directional sequence reactions (2 germline mutations and an average of 2 polymorphisms per patient). However, when 96 well plates are used, an approximate cost for DHPLC analysis of the entire *ATM* coding region for one patient is around 400 euros, which is significantly less than the price for the corresponding full sequence analysis of the *ATM* gene. Furthermore, the frequency of the mutated alleles detected in our study (86%) was similar to that observed in a previous analysis in which comprehensive sequencing was performed (85%) [30],

Table 3
Mutations detected in the *ATM* gene

Exon	Patient ID (status)	Nucleotide substitution	Amino acid substitution	Type of mutation/ Predicted effect	References
5	AT16RM (het)	128T>C	L43P*	Missense/Amino acid change	This report
7	AT57RM (het)	450delTTCT		Deletion/Truncated protein	This report
7	AT106RM (het)§	692A>G	H231R	Missense/Amino acid change	This report
9	AT97RM (hom)	717delCCTC		Deletion/Truncated protein	This report
12	AT101RM (het)§	1463G>A	W488X	Nonsense/Truncated protein	This report
12	AT95RM (het)	IVS12 + 1G>T		Splicing/Truncated protein	[7]
16	AT58RM (het)	2250G>A	K750K	Splicing/Truncated protein	[31]
18	AT6RM (het)	2385C>A	P795T	Missense/Amino acid change	This report
19	AT25RM (hom)	2501insA		Insertion/Truncated protein	[7]
23	AT96RM (het)§	3111delT		Deletion/Truncated protein	This report
26	AT3RM (het)	3576G>A	K1192K	Splicing/Truncated protein	[26]
26	AT95RM (het)	3576G>A	K1192K	Splicing/Truncated protein	[26]
26	AT85RM (het)§	3576G>A	K1192K	Splicing/Truncated protein	[26]
26	AT42RM (het)*	3576G>A	K1192K	Splicing/Truncated protein	[26]
28	AT42RM (het)	3802delG		Deletion/Truncated protein	[17]
28	AT105RM (hom)	3802delG		Deletion/Truncated protein	[17]
34	AT3RM (het)	4842insCT		Insertion/Truncated protein	This report
34	AT16RM (het)	4842delA		Deletion/Truncated protein	This report
37	AT50RM (het)*	IVS37 + 2T>C		Splicing/Truncated protein	[17]
40	AT50RM (het)	5692C>T	R1898X	Nonsense/Truncated protein	This report
42	AT6RM (het)	5932G>T	E1978X	Nonsense/Truncated protein	[7]
42	AT61RM (het)	5979delTAAAG		Deletion/Truncated protein	[5]
46	AT90RM (het)	6442delA		Deletion/Truncated protein	This report
49	AT61RM (het)	6913C>T	Q2305X	Nonsense/Truncated protein	[36]
53	AT57RM (het)*	7517delGAGA		Deletion/Truncated protein	[5]
53	AT76RM (hom)	7517delGAGA		Deletion/Truncated protein	[5]
53	ATJ3 (het)§	7519delGA		Deletion/Truncated protein	This report
55	AT71RM (hom)	IVS54-3T>G		Splicing/Skipping of exon 55	This report
57	AT58RM (het)	8122G>A	D2708N	Missense/Amino acid change	This report
59	AT90RM (het)	8283delTC		Deletion/Truncated protein	[5]
63	AT45RM (het)§	8833delCT		Deletion/Truncated protein	[4]
64	AT19RM (hom)	8977C>T	R2993X	Nonsense/Truncated protein	[30]

Nucleotide numbering is based on cDNA sequence (GenBank accession no. U82828.1). The first base (position +1) of the initiator methionine is taken as the start of the cDNA. *These samples and related mutations have been previously reported [5,26,27]. § These samples had only one mutation identified. Hom – homozygote, het – heterozygote.

and greater than the frequency obtained in another such study (57%) [31]. Nonetheless, 6 of 44 alleles could not be identified in our study. Previous DHPLC analysis performed on the *ATM* gene described a similar proportion of undetected mutations [28]. These results might have different possible explanations: first, we did not screen the regulatory regions (promoter, 5' and 3' UTRs) of the gene, known to contain a minority of A-T mutations [32]; second, our DHPLC-based technique cannot detect large rearrangements or single and multi-exon deletions, that have been reported in previous studies [5,7,20,27]; third, the DHPLC protocol used here might have failed to detect some mutations, although all the 19 known sequence alterations in the *ATM* gene were precisely identified in our retrospective analysis, showing that DHPLC approaches ∼100% sensitivity in detecting *ATM* alleles. We plan to set up a multiplex ligation dependent probe amplification

assay (MLPA) to look for whole exon and whole gene deletions in the patients in whom we have not found pathogenic mutations by DHPLC analysis [33].

Among the mutations identified, 84% of the alleles caused, directly or indirectly, a premature termination codon (PTC). These proportions are similar from those in a previously published study, which suggested that as many as 89% of mutations might inactivate the *ATM* protein by truncation [5]. Two alleles (5%) caused in-frame exon skipping. In addition, 4 (11%) missense mutations were also observed. Among these, 8122G>A (D2708N) in exon 57 resides within the kinase domain and affects amino acids conserved in Mus musculus (NP_031525.1; GI:6680740), Xenopus laevis (AAT72929.1; GI:51599115), Drosophila melanogaster (NP_650435.1; GI:24647031) and Saccaromices cerevisiae (CAA56016.1; GI:496689). Mutation 2385C>A (P795T) in exon 18 is conserved

Table 4
Summary of the polymorphisms detected in the *ATM* gene

Exon	Nucleotide substitution	Amminoacidic substitution	Type of change	Reference
5	146C/G	S49C	Missense	[37]
7	378T/A	D126E	Missense	[38]
11	1176C/G	G392G	Silent	[31]
13	1744T/C	F592L	Missense	[38]
14	IVS14-55T/G	—	Intronic	[8]
16	IVS15-68T/C	—	Intronic	[38]
19	2572T/C	F858L	Missense	[39]
24	3161C/G	P1054R	Missense	[39]
26	IVS25-12insA	—	Intronic	[38]
32	4578C/T	P1526P	Silent	[31]
36	IVS35-68T/G	—	Intronic	[38]
37	5185G/C	V1729L	Missense	[38]
39	5557G/A	D1853N	Missense	[31]
39	5558A/T	D1853V	Missense	[31]
62	IVS62+8A/C	—	Intronic	[8]

in Mus musculus and Xenopus laevis, but not in Drosophila melanogaster and Saccaromices cerevisiae. Mutation 692A>G (H231R) in exon 7 was localized within the N-terminus of the *ATM* protein (~residues 1–246), a domain that strongly interacts with the p53 protein and affects amminoacid conserved in Mus musculus, but not in Xenopus laevis, Drosophila melanogaster and Saccaromices cerevisiae. None of the novel missense and splice site mutations was detected in 192 anonymous controls. Nevertheless, with regard to the novel missense substitutions, it must be cautiously noted that the absence of an aminoacid substitution in a limited number of controls does not fully prove its causative role in the pathogenesis of A-T. Three mutations [3576G>A (K1192K), 3802delG and 7517delGAGA] were recurrently detected; particularly, 3576G>A (K1192 K) was the most frequent (4/38; 11%), consistent with reported data [7,31,34]. Two mutations, 2250G>A and 3576G>A substitute the final nucleotide of exon 16 and 26, respectively. Both mutations were previously confirmed to represent true splicing mutations since skipping of the affected exon was observed from transcript of patient lymphoblastoid cell lines and fibroblasts [31]. However, the splicing mutation 3576G>A (K1192 K) seems to be a leaky mutation that leaves a residual amount of normally spliced *ATM* transcript [27]. The 3576G>A mutation has been previously described in other families of Italian, Georgian and Turkish descent [31], suggesting that this splicing mutation may be very common in south or southeast Europe. The same mutation as been found in other Turkish A-T individuals (AT17RO, AT22RO, and AT23RO), who share the same haplotype, pointing to a founder effect [34]. However,

most of the remaining mutations were detected in a single patient, i.e. "private mutation". What is more, mutations were scattered across the whole coding sequence of the *ATM* gene. Fourteen (37%) of the mutations were novel, demonstrating a high incidence of new and individual A-T mutations in our cohort of patients and a marked mutational heterogeneity of A-T in Italy. Among the 22 investigated patients, 16 (73%) were compound heterozygous, and 6 (27%) were homozygous for an *ATM* mutation (Table 2). Four of the six patients with homozygous mutations were analysed by microsatellite markers flanking the *ATM* gene. Patients AT97RM, AT105RM and AT71RM had homozygous haplotypes, suggesting that both alleles had a common origin, whereas patient AT19RM carried a different haplotype, suggesting that the mutation occurred independently on two different chromosomes. Thus, the relatively high proportion of homozygotes in this sample (6/22, 27%) seems to be due to a relatively high degree of consanguinity in the population studied. It should be noted, however, that apparently homozygous patients from non-consanguineous families might in fact be compound heterozygotes with a deletion encompassing the entire *ATM* gene.

The present study adds to the large number of reports showing the usefulness of DHPLC for mutation screening in disorders where disease-causing mutations are heterogeneous [28,29,35]. In these conditions, DHPLC has marked advantages over classical approaches in term of time, cost and flexibility. The mutations identified in the current study increase the fraction of characterized *ATM* genotypes in the RIAT. The identification of new mutations in Italy is important for genetic counselling, prenatal testing, carrier detection, and for

designing rapid assays for specific founder effect mutations.

Anknoledgements

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