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ARTICLE

Rapid and efficient *ATM* mutation detection by fluorescent chemical cleavage of mismatch: identification of four novel mutations

Louise Izatt[1], Carina Vessey[2], Shirley V Hodgson[1] and Ellen Solomon[1]

[1]*Division of Medical and Molecular Genetics GKT, Guy's Hospital, London*
[2]*ICRF Molecular Oncology Laboratory, University of Oxford Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK*

Mutations in the Ataxia Telangiectasia Mutated (*ATM*) gene are responsible for the autosomal recessive disease Ataxia Telangiectasia (A-T). A wide variety of mutations scattered across the entire coding region (9168 bp) of *ATM* have been found, which presents a challenge in developing an efficient mutation screening strategy for detecting unknown mutations. Fluorescent chemical cleavage of mismatch (FCCM) is an ideal mutation screening method, offering a non-radioactive alternative to other techniques such as restriction endonuclease fingerprinting (REF). Using FCCM, we have developed an efficient, accurate and sensitive mutation detection method for screening RT-PCR products for *ATM* mutations. We have identified seven *ATM* mutations in five A-T families, four of which are previously unknown. We quantified ATM protein expression in four of the families and found variable ATM protein expression (0–6.4%), further evidence for mutant ATM protein expression in both classic and variant A-T patients. We conclude that FCCM offers a robust *ATM* mutation detection method and can be used to screen for *ATM* mutations in cancer-prone populations.

Keywords: ataxia telangiectasia; *ATM*; mutation detection; fluorescent chemical cleavage of mismatch; protein expression

Introduction

Ataxia telangiectasia (A-T) is an autosomal recessive disease affecting 1/40 000 to 1/300 000 individuals worldwide.[1,2] This complex disorder is characterised by progressive cerebellar degeneration, oculocutaneous telangiectasia, oculomotor apraxia, immunodeficiency, raised serum α-fetoprotein (AFP), chromosomal instability, radiosensitivity, and a 100-fold increase in cancer susceptibility, with lymphoreticular malignancies predominating.[3] Epidemiological studies have reported increased cancer susceptibility in A-T heterozygotes, particularly to breast cancer in women,[4] but debate continues over the role of A-T carrier status in breast cancer predisposition.[5]

The A-T gene, Ataxia Telangiectasia Mutated (*ATM*), was mapped to chromosome 11q22–23 in 1988,[6] and was isolated by positional cloning in 1995.[7] *ATM* is a large, complex gene, with 66 exons, spanning over 150 kb, with the start codon in exon 4.[8,9] This generates a 13 kb transcript, with an open reading frame of 9168 bp, predicting a protein of 3056 amino acids.[10] The ATM protein (about 350 kDa) is located primarily in the nucleus of normal cells[11] and has homology at its

Correspondence: Dr L Izatt, Division of Medical and Molecular Genetics GKT, 8th floor Guy's Tower, Guy's Hospital, London SE1 9RT, UK. Tel: +171 955 4648; Fax: +171 955 4644; E-mail: l.izatt@umds.ac.uk
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carboxy terminus to phosphatidylinositol (PI) 3-kinase. Related proteins that share PI 3-kinase domain homology are found in yeast (TEL1, MEC1, Rad3, TOR1, and TOR2), *Drosophila* (Mei-41) and mammals (DNA-PK), (Savitsky *et al*[10] for a review). PI 3-kinase family members are involved in a wide variety of regulatory events, including processing DNA damage, maintenance of genome stability and control of cell cycle progression.[10]

Over 250 *ATM* mutations have been reported to date, spanning the entire coding region.[12] No mutational hotspots have been found, although some founder mutations have been identified.[13,14] Most groups have developed scanning methods using cDNA to identify *ATM* mutations. Techniques include PTT,[15] SSCP,[16] RNAse cleavage,[17] and REF.[18] Others have used direct sequencing of cDNA.[19] The overall mutation detection rate has been high (about 70%), with base substitutions, small deletions and insertions predominating, resulting in truncation of the ATM protein.[12] An increasing number of in-frame deletions and missense mutations have recently been described.[13]

We chose solid-phase fluorescent chemical cleavage of mismatch (FCCM) to screen the entire *ATM* transcript, because it offers efficient and sensitive mutation detection, without bias towards any particular mutation type. Solid-phase FCCM has been used to detect mutations in other large, complex genes, eg Factor IX[20] and *BRCA1*,[21] with almost 100% accuracy,[20] locating any sequence change in long DNA segments (up to 1.8 kb). We describe its adaptation to detect *ATM* mutations using cDNA, with the aim of characterising mutations in A-T families for development of mutation-based prenatal diagnosis. This method is being extended to screen breast cancer patients for germline *ATM* mutations. In this study, we describe the identification of seven *ATM* mutations in five A-T families screened using FCCM, (four of which have not been previously identified), and examine expression of the mutant ATM protein.

Materials and Methods

Patients

Five non-consanguineous A-T families gave informed consent for this study. Their clinical features are listed in Table 1. Family 347 had two affected daughters; all other families had one affected child. The affected individuals, A-T 347, A-T 352, A-T 451, and A-T 537 have a classic A-T phenotype, whilst A-T 350 exhibits a variant A-T phenotype.[22]

Controls

DNA was isolated from 50 Caucasian males without a family history of A-T or cancer. BD2685 is a lymphoblastoid cell line (LCL) established from a breast cancer patient, negative for *ATM* mutations screened by FCCM. Molt 4 is a T cell LCL.

Molecular Methods

DNA and RNA were isolated from peripheral blood samples by standard methods.[23,24] Reverse transcription was performed on 0.5–1 μg of total RNA with 0.5 μg random hexamers primer.[25]

The *ATM* coding sequence was amplified from patient-derived cDNAs by nested PCR, in eight first round reactions, serving as templates for nine overlapping second round reactions, 0.9 to 1.7 kb in size (Figure 1).

Careful primer design allowed all primary and nested RT-PCR reactions to be amplified using one of two sets of conditions (primer details, Table 2). The PCR reaction mixture contained 200 μM dNTPs, 500 nM of each primer set (Table 2), 1.5 mM $MgCl_2$ Taq buffer (Promega, Southampton,

Table 1 Clinical and laboratory features of A-T patients in the study

Clinical feature	*Patient data*					
Family number	537	350	352	451	347[a]	347[a]
Age (years)	5	37	16	21	6	4
Sex	M	F	M	F	F	F
Age of onset of ataxia (years)	1	1	1	2	1	1
Telangiectasia	+	+	+	+	+	+
Raised AFP	+	+	+	+	+	+
Immunodeficiency	+	–	NT[b]	+	–[c]	+[c]
Malignancy	–	–	–	–	–	–
Radiosensitivity	Classical	Intermediate[d]	Classical	Classical	Classical	Classical
Chromosomal rearrangements	+	+	+	+	+	+
Clinical progression	Classical[e]	Delayed[f]	Classical[e]	Classical[e]	Classical[e]	Classical[e]

[a]These are two siblings from the same family; [b]NT=not tested; [c]Intrafamilial phenotypic variation in their measured immune function (IgM, IgA, and IgG subclasses) and frequency of infections; [d]Radiosensitivity measured in lymphocyte chromosomes exposed to 1 Gy X-rays at G_2 was intermediate between control patients and the high level observed in classical A-T patients, see Taylor et al;[45] [e]Classical clinical progression – for fuller description, see Bundey;[46] [f]Delayed clinical progression observed, eg moderate gait ataxia at age 37, see McConville *et al*.[29]

U.K.), 1 U *Taq* polymerase (Promega), and 1 U *Taq* extender (Stratagene, Cambridge, U.K.). 1 μl cDNA served as the template for the first round PCR; and 1 μl of first round PCR product, as the template in the second round PCR. Second round PCR products were internally labelled by incorporation of 1.5 μM R6G or R110, or 6 μM TAMRA fluorescent dUTPs ([F]dUTPs) (Perkin Elmer, Warrington, U.K.) (Figure 1). PCR conditions for first round PCR for all fragments, except 1AF/2BR (Figure 1), comprised 30 cycles of 94°C, 30 s, 55°C, 30 s, and 72°C, 2 min, followed by a 10 min extension at 72°C. Touchdown PCR[26] was used to amplify second round PCR reactions, fragment 1AF/2BR (Figure 1), the promoter fragment, and all probes. Conditions used were as follows: 30 cycles of 94°C, 30 s, 64°C, 30 s (for 5 cycles, then 60°C for 25 cycles), and 68°C, 90 s, and final extension at 68°C for 5 min. The promoter region was amplified from 100 ng of genomic DNA. Probe DNA was amplified using a modified second round primer set, (each primer biotinylated at the 5' end), internally labelled with [F]dUTPs, and gel-purified. cDNA clones (FB2-f, FB-2 and 7-9)[10] and a partial subclone of En-a (extending from –30 to 2028; Nucleotide numbering according to Savitsky *et al*[10]),[48] served as probe templates (Figure 1), except for fragment III, where a PCR product of known sequence was used.

Mutation Detection

Solid-phase fluorescent chemical cleavage of mismatch (FCCM) was used to identify *ATM* mutations[20] with modifications detailed below.

Three separate, non-overlapping multiplexes were formed by labelling nested PCR fragments as described (see legend, Figure 1). 1 μl of each biotinylated internally labelled probe, was combined with 4 μl internally labelled target per multiplex, favouring maximum heteroduplex formation between probe and patient DNA. Heteroduplex formation and chemical mismatch were as described,[20] using hydroxylamine and osmium tetroxide modification sequentially.

2 μl of supernatant was loaded on a 6% polyacrylamide/urea gel (34 lane, 12 cm well-to-read plates) on the ABI 377 DNA sequencer and separated by electrophoresis at 50 W for

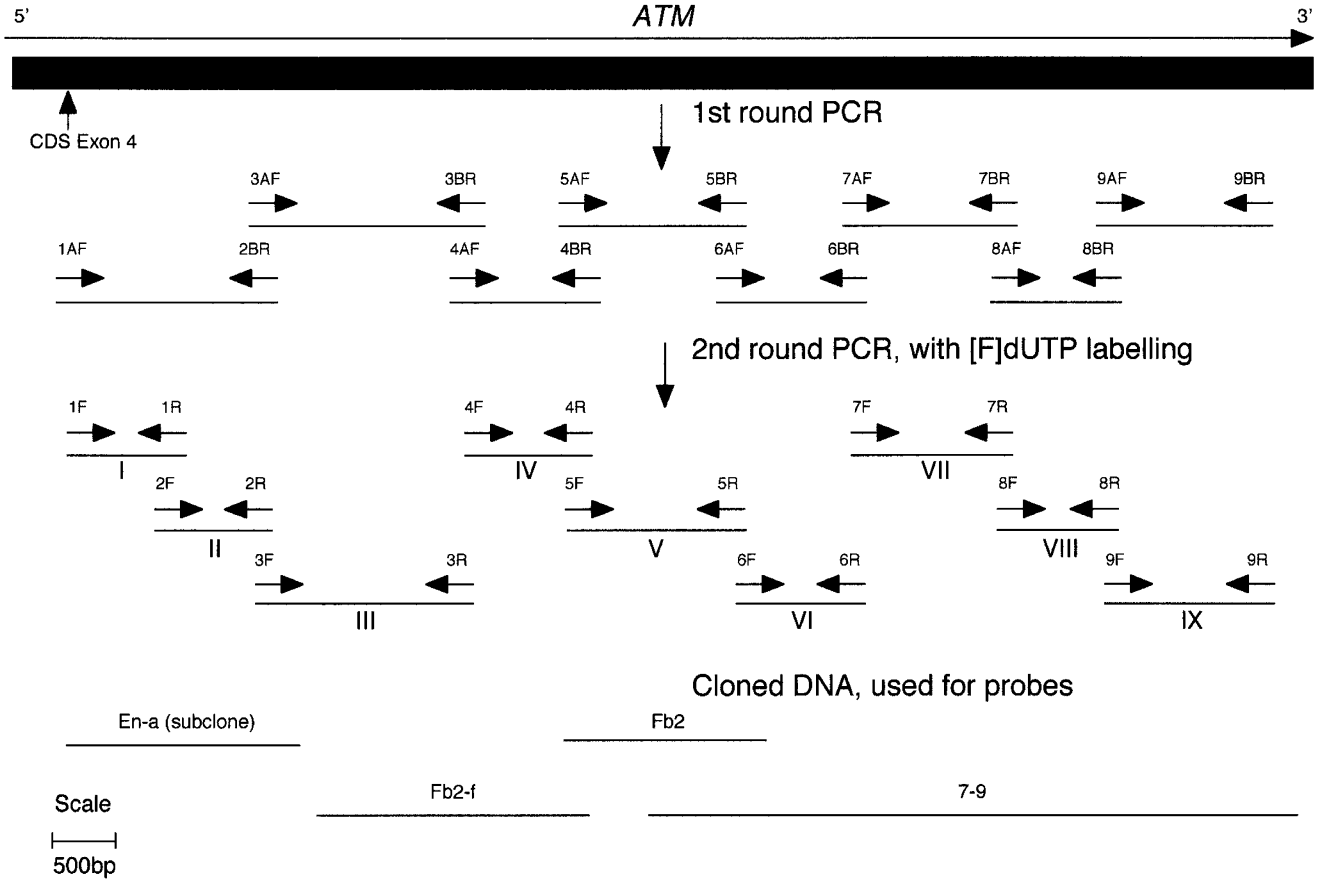

Figure 1 *Generation of PCR products for FCCM mutation analysis of* ATM. *Schematic representation of the* ATM *transcript showing RT-PCR amplified fragments and the position of cDNA clones,[10] amplified as probes for all fragments, except fragment III. The primer sequences and their positions are listed in Table 2. Fragments I and II are amplified as one primary fragment, but divided into two smaller second round products because of alternate splicing observed (results not shown). Nested second round PCR products are internally labelled by one of three fluorescent dyes ([F]dUTPs): R110, R6G, and TAMRA. PCR products V, VIII, and IX are labelled by R110; I, III, and IV by R6G; and II, VI, and VII by TAMRA. Three non-overlapping, differently labelled PCR products are combined and analysed concurrently. Multiplex A: products II, IV, VIII; Multiplex B: products III, VI, IX; Multiplex C: products I, V, VII.*

Table 2 Primers used in FCCM analysis and genomic sequencing

Primer	*Forward primer sequence 5'→3'*	*Primer*	*Reverse primer sequence 5'→3'*	*ATM position*	
		First round primers			
1AF	GTGAGGCATACATCACAATTTGG	2BR	GGTCTGCAGGCTGACCCAG	–120–1601[a]	
3AF	GCCTTACGGAAGTTGCATTG	3BR	CCCATTACATTAAGAATGGC	1373–3194[a]	
4AF	GATGTTCTTGAACTTCTG	4BR	TTGCTGGCTCATGTAACGTC	2893–4066[a]	
5AF	TCTATAGATCTTGTTATAAGG	5BR	AGGTAACAGCTGCTGATCGAA	3740–5206[a]	
6AF	GGCAATAAACCACACTGGTG	6BR	CCCACATTGCTTCGTGTTC	4971–6127[a]	
7AF	AGCCAGAGTACAACTATTTC	7BR	AGCCAGAGTGCAACTATTTC	5941–7277[a]	
8AF	AGAAAAGGCAGTAGAAGTTGC	8BR	CCTGCTAAGCGAAATTCTGC	7083–8081[a]	
9AF	ACTCAGTGGAAGACTCAGAG	9BR	CTACTTAAAGTATGTTGGCAG	7906–3'UTR[a]	
		Second round primers			*Overlap*[c]
1F	CAGTGATGTGTGTTCTGAAATTG	1R	GCACCTTTTTCTTGGGTTTTGGC	–28–905[a]	
2F	GCTATTCAGTGTGCGAGACAAG	2R	TGGCTCCAAGTAAGCCAAAG	646–1531[a]	259
3F	GAGGTCAAACCTAGAAAGCTCAC	3R	CCTCTCCTTGTTAGATGCC	1404–3096[a]	127
4F	CTAGGTCAAAGCAATATGGACTC	4R	CAATCTGTTTTCCCAATAAGTTTTC	3001–3997[a]	95
5F	CCACATCTGGTGATTAGAAGTCA	5R	GCATTATTATGATGGTCCACTGAAG	3769–5143[a]	228
6F	CCTATAGATTTCTCTACCATAGC	6R	GTTCGTAGTCTAGTAATGGGTTG	5038–6104[a]	105
7F	CTGGAATAAGTTTACAGGATCTTC	7R	GATGATTCATGTAGTTTTCAATTC	5990–7223[a]	114
8F	GATGGAGAAAGTAGTGATGAGC	8R	AGTCACCAGATTTCCATATTCTC	7114–8046[a]	109
9F	CCAGCAGACCAGCCAATTACTA	9R	AAGGCTGAATGAAAGGGTAATTC	7942–9206[a]	104
		Promoter primers			
PromF	CAAGCCGGGCTACGTCCGAGGGTAACAACAT	PromR	CTGCACTCGGAAGGTCAAAGTAGTATCAAC	10207–10882[b]	
		Sequencing primers			
96508F	CTTCTGGTTATTTTACCTTAGAG	96856R	GTTTTTGCCCAACATACTGTAC		
Exon 7F	GTTGCCATTCCAAGTGTCTTA	Exon 7R	CAAACAACAACCTTCAAAACA		
Exon 11AF	CAGGTTTTTAATGAAGATACCAG	Exon 11R	GAATGAGAAAATGGTAACACTT		
Exon 12FF	CAACCCAATTAATATCAAAG	Exon 12AR	AATAAATCTGACTTTTGTGAG		
Exon 23AF	CTTTTAGCTTGAATTTTTGG	Exon 23R	TGGTTAAATATGAAATAGAG		
Exon 31F	CCGAGTATCTAATTAAACAAG	Exon 31R	CAGGATAGAAAGACTGCTTAT		
Exon 65F	TTAAACTGTTCACCTCACTGA	4BR	CTACTTAAAGTATGTTGGCAG		

[a]The first nucleotide of the open reading frame was designated at +1, GDB[47]: U33841 Savitsky *et al*;[10] [b]nucleotide numbers refer to the genomic sequence with the first nucleotide designated +1, GDB:[47] U82828 Platzer *et al*;[9] [c]the overlap, in base pairs, corresponds to the overlap between that fragment and the 5' neighbouring fragment.

5–7 h using GS12A-2400. Data was collected and analysed using Genescan 2.02 software (ABI Prism, Warrington, U.K.).

DNA Sequencing and Restriction Assays

RT-PCR was repeated without labelling, and products sequenced in both directions.[27] Sequence variants were confirmed by PCR and sequencing genomic DNA, using primers listed (Table 2).

For the L1465P mutation, a naturally occurring restriction site was created in exon 31 by the base substitution, allowing *Xho*I digestion in mutant alleles.

Cell Lines/Western Blotting

Control BD2685, and four A-T patient LCLs were cultured in RPMI 1640 (Gibco BRL, Paisley, U.K.) with 10% foetal calf serum. Total cellular lysate preparation was as described.[14] 50 μg protein was loaded per lane, separated electrophoretically through 7.5% SDS-Polyacrylamide gels, and blotted on to nitrocellulose membranes (ImmobilonP-Millipore, Bedford, MA, USA) by semi-dry transfer. ATM protein was detected with a polyclonal antiserum (CN-12) raised against a recombinant polypeptide corresponding to 992–1144 of ATM protein. A β-tubulin monoclonal antibody (Amersham, Little Chalfont, Bucks, U.K.) was used to quantitate protein loading. Western blotting was as described[28] and blots were developed by enhanced chemiluminescence (Amersham). Protein levels were measured using the BioImage Whole Band Analyser system (Millipore), normalised against β-tubulin readings, using BD2685 as a standard.

Results

FCCM on Patient Samples

The optimised multiplexed FCCM technique was used to screen A-T patient samples, allowing analysis of the entire *ATM* coding region of up to ten patients per gel. Cleavage bands detected by Genescan analysis were used to predict the mismatched base site within the fragment (Figures 2A and 2B). Accurate sizing of the two separate cleavage products on the electropherogram enables location of the mismatched base to within 10–15 bp of one of two sites in the fragment screened (Figures 2C and 2D).

Seven different *ATM* mutations were detected in five A-T families analysed (Table 3). Four of these, 480del5, 1158delG, IVS22-1G→A, and 4394T→C, are novel.

Frameshift Mutations

The 480del5 mutation in *ATM* exon 7 detected in A-T 347 was seen in both affected siblings in this family and

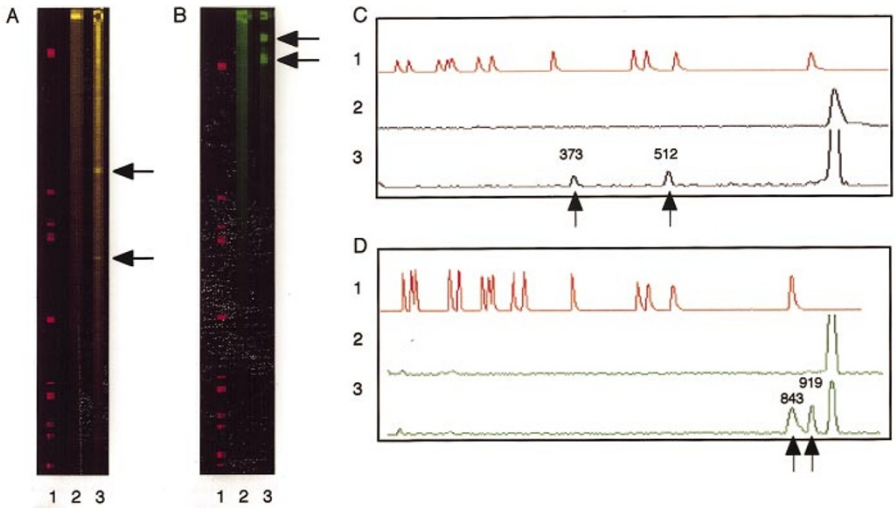

Figure 2 *Detection of* ATM *gene mutations by FCCM. Gel images and electropherograms of mismatch cleavage products detected in A-T 537.* **A** *and* **C***: FCCM of ATM fragment II, internally labelled with TAMRA. Heteroduplexes were formed between probe DNA (clone En-a) and RT-PCR products from A-T 537, subjected to hydroxylamine treatment and separated by electrophoresis on a 6% polyacrylamide gel. Lane 1: Genescan Rox-2500 marker (Perkin Elmer). Lane 2: Probe DNA only. Lane 3: 537/probe DNA heteroduplex. A mismatched base is detected, generating two shorter products (373/512 bp). These add up to the full-length of fragment II, 885 bp (Lane 3), shown best in the electropherogram image in* **C***. The mutation lies at one of these two sites, but cannot be assigned to sense or antisense strands because both strands are internally labelled. Sequencing, shown in Figure 3A, revealed a 1 bp deletion, 1158delG in exon 11.* **B** *and* **D***: FCCM of* ATM *fragment IV, internally labelled with R6G. Heteroduplexes were formed between probe DNA (clone FB2-f) and RT-PCR products from A-T 537, subjected to hydroxylamine treatment and separated by electrophoresis on a 6% polyacrylamide gel. Lane 1: Genescan Rox-2500 marker (Perkin Elmer). Lane 2: Probe DNA only. Lane 3: 537/probe DNA heteroduplex. Two shorter fragments, below the full-length band of 996 bp can be seen in Lane 3, corresponding to the boundaries of exon 23, which is 76 bp in length. The mismatch is sized more accurately (919/843 bp) from the electropherogram image shown in* **D***. Sequencing of genomic DNA, shown in Figure 3C, revealed the mutation IVS22-1G→A. Arrows indicate mismatch bands due to mutations and numbers correspond to the size of each mismatch band detected.*

Table 3 *ATM* mutations in A-T families identified by FCCM

A-T patient	*Ethnic origin*[a]	*Genomic mutation*[b]	*Transcript mutation*[c]	*Predicted protein alteration*	*Exon/intron*[c]	*Heterozygosity*[d]
347	Irish[p]	5-bp deletion in exon 7	480del5	Frameshift at codon 160, leading to a stop codon after 23 amino acids	7	Cpd het[e]
347	Greek-Cypriot[m]	?				Cpd het
350	English[p]	IVS40-1050A→G	5762^5763ins137[f]	Frameshift at codon 1921, leading to a stop codon after 9 amino acids	IVS40	Cpd het
350	English[m]	Base substitition in exon 65	9139C→T	Arg→ter at codon 3047	65	Cpd het
352	English	?				NK
352	English	?				NK
451	English[m]	1-bp deletion in exon 12	1355delC	Frameshift at codon 452, leading to a stop codon after 20 amino acids	12	Cpd het
451	English[p]	Base substitution in exon 31	4394T→C	Leucine to proline at codon 1465	31	Cpd het
537	Irish[m]	1-bp deletion in exon 11	1158delG	Frameshift at codon 386, leading to a stop codon after 3 amino acids	11	Cpd het
537	Irish[p]	IVS22-1G→A	3078-1G→A (exon 23 deleted)	Deletion of 26 amino acids at codon 1026, leading to a stop codon after 2 amino acids in exon 24	IVS22	Cpd het

[a]=allele, [p]=paternal allele, [m]=maternal allele; [b]=exon numbers are according to Uziel *et al*;[8] [c]=mutations are as proposed by the Nomenclature Working Group.[48] The first nucleotide of the open reading frame was designated as +1, GDB[47]: U33841; [d]=Cpd het: compound heterozygote; NK: not known; [e]=second allele expressed at the RT-PCR level, detected on sequencing RT-PCR product; [f]=137bp of intron 40 aberrantly spliced into the A-T transcript between exon 40 and 41; mutations were confirmed by amplification and sequence analysis of genomic PCR products using primers listed in Table 2.

their father (results not shown) (Table 3). Sequencing of the RT-PCR product in A-T 347 showed bi-allelic expression (results not shown). This 5 bp deletion (underlined) is associated with a direct repeat (italicised) in the DNA sequence TA*TCTC*AGCAACA. The 1158delG mutation in *ATM* exon 11 was detected in A-T 537 and his mother (results not shown) (Table 3; Figure 3A). This deletion (underlined) occurs in a run of homonucleotides (italicised) CAAAA*GG*AAGA. These two deletions may occur as a result of DNA slippage during replication. The 1355delC in *ATM* exon 12, found in A-T 451, (Table 3) was described previously.[13]

Splicing Mutations

Two close mismatch bands were detected in fragment IV from A-T 537 (Figures 2B and 2D). Sequencing cDNA across this region identified exon 23 skipping in A-T 537 (Figure 3B), deleting 76 bp, resulting in a frameshift in exon 24 (Table 3). Sequence analysis of genomic DNA from A-T 537 revealed IVS22-1G→A at the intron 22-exon 23 splicing junction (Figure 3C; Table 3). This mutation alters the invariant splicing acceptor site from AG to AA, explaining the observed exon 23 skipping (Figure 3B). This splicing variant is unlikely to occur in the absence of the splice site mutation, because it was not observed in FCCM analysis of 250 chromosomes from A-T and breast cancer families, but was found in the father of A-T 537 (results not shown). IVS40-1050A→G, found in A-T 350, was described previously,[29] (Table 3).

Nonsense/Missense Mutations

The 4394T→C base substitution found in *ATM* exon 31 in A-T 451 results in a leucine-to-proline amino acid substitution at codon 1465 (Table 3). This base change creates an X*ho*I cutting site in the exon 31 PCR product of 294 bp. Upon digestion, the mutant allele gives rise to two fragments of 206 bp and 88 bp, while the normal allele is resistant to X*ho*I digestion, giving an undigested fragment of 294 bp (Figure 4). 4394T→C, is unlikely to be a polymorphism, since this base change was not detected after PCR and X*ho*I digestion of 100 control chromosomes (results not shown), and because leucine is conserved at this position in mouse *Atm*.[30]

The 9139C→T single base substitution in *ATM* exon 65 found in A-T 350 creates a stop codon at 3047, with loss of ten amino acids from the C-terminus of the protein. This mutation has been described previously.[29]

Two other coding sequence variations were detected in A-T 537 and A-T 451, both patients being heterozygous for polymorphism 5557G/A (exon 39). No sequence variations were detected in A-T 352.

Western Blotting

ATM protein expression was investigated in four of the A-T families where LCLs were available, to examine the consequences of different *ATM* mutations on protein expression and clinical phenotype (Figure 5). The highest amount of full-length ATM protein observed (6.4%) was in A-T 350 (Figure 5). The presence of full-length ATM protein occurs as a result of some normal splicing at IVS40-1050A→G,[29] and expression of some stable minimally truncated ATM protein from 9139C→T.[31] This patient exhibits a variant phenotype (Table 1). A-T 451 and A-T 347 have classic phenotypes (Table 1) and significantly reduced full-length ATM expression (Figure 5). Each has one truncated A-T allele upstream of the antibody-binding site (Table 3), so that any ATM protein detected must arise from the second allele. In A-T 451, the low level of ATM expression shown (1%) (Figure 5) indicates that the L1465P missense mutation (Table 3) may lead to instability of the protein. The trace of full-length ATM protein expression in A-T 347 (Figure 5), may indicate that the unidentified mutation is an in-frame deletion/insertion, or a missense mutation. ATM protein was not detected in A-T 352 (Figure 5).

Mutant Allele Origins

Three of the five families studied were native to the British Isles. Family 537, and the father of A-T 347 were of Celtic/Irish origin, whilst the mother of A-T 347 was of Greek-Cypriot origin (Table 3). Only one mutation, IVS40–1050A→G, has previously been shown to be a founder mutation in the British Isles.[29] Although 9139C→T has been described in an Italian,[31] Japanese,[17] and UK families, no common haplotype was reported.[13] We identified all three alleles of Celtic/Irish origin, each mutation being novel to this study (Table 3).

Discussion

We have shown that FCCM is an effective mutation detection method in scanning the entire *ATM* coding region for mutations. Our study identified seven out of a possible ten *ATM* mutations in five A-T families, giving a sensitivity of 70%, comparable with other *ATM* mutation detection methods used, eg PTT[15] and REF.[13]

FCCM offers several advantages in comparison with other methods used, screening up to ten A-T patients per gel by multiplexing samples, and accurately locating the variant base within the segment analysed. By labelling both the patient and control DNA samples, an almost twofold redundancy in the ability to detect base

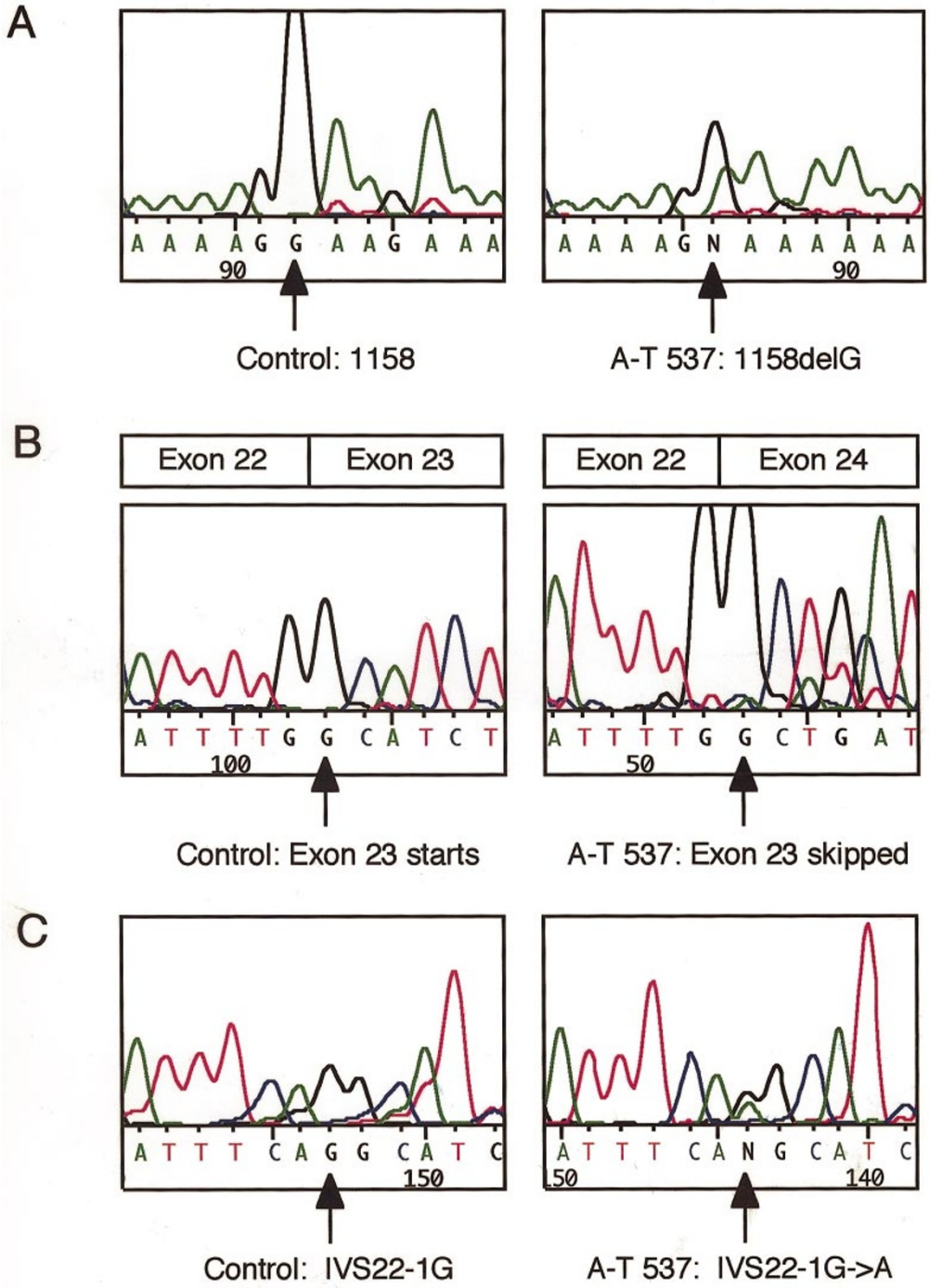

Figure 3 *Sequence analysis of the mismatch bands observed in Figure 2.* **A***: Identification of 1158delG in* ATM *exon 11 (1) control (Molt 4) and (2) A-T 537.* **B***: Sequence of* ATM *cDNA across the exon 22/23 boundary in (1) control (FB2-f) and (2) A-T 537 RT-PCR products from fragment IV, showing exon 23 skipping.* **C***: Identification of genomic mutation underlying skipping of exon 23 observed in A-T 537. Sequencing the acceptor splice site,* ATM *intron 22/exon 23 revealed IVS22-1G→A in A-T 537. (1) control (Molt 4) and (2) A-T 537. An arrow indicates the mutant base in each instance.*

changes is seen, increasing sensitivity and detection.[20] If differential mRNA expression of each mutant allele occurs, the sensitivity of FCCM may enable detection of as little as 10% of the mutant transcript.[32] Finally, because analysis of FCCM gels is automated, comparisons between samples are easier.

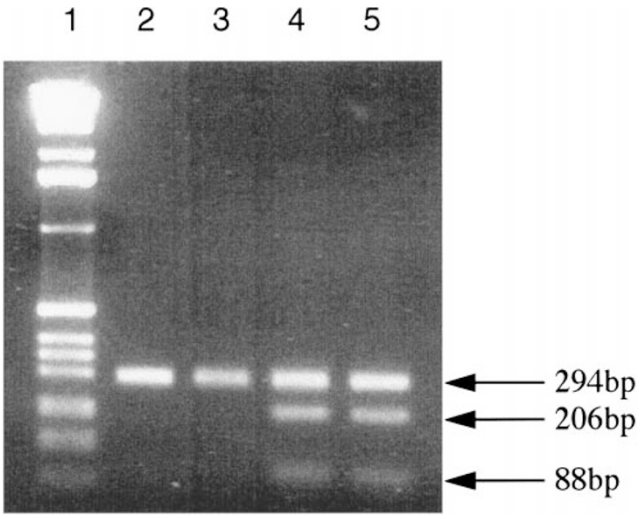

Figure 4 *XhoI digest of* ATM *exon 31 products in A-T 451. 2% agarose gel showing detection of the 4394T→C (L1465P) missense mutation in exon 31 of the* ATM *gene by PCR (primers listed in Table 2) and XhoI digestion. 4394T→C creates a XhoI cutting site, giving digested smaller fragments of 206 bp and 88 bp from the full length PCR product of 294 bp. Lane 1: 1 kb ladder (Gibco BRL). Lane 2: Undigested PCR product. Lanes 3–5 XhoI digested PCR products. Lane 3: Mother of A-T 451; Lane 4: Father of A-T 451; Lane 5: A-T 451. Arrows indicate the full length and digested products*

The seven *ATM* mutations detected in this study reflect the spectrum of *ATM* mutations already characterised worldwide, with small deletions and exon-skipping mutations predominating. Although *ATM* mutations are found to span the entire open reading frame, our small study showed three deletions occurring in exons 7–12. Exon-skipping mutations have been reported to involve at least 34 of the 66 exons in *ATM*, but only in about 20% of instances has the underlying genomic mutation been reported.[33] We found exon 23 skipping in A-T 537, as a consequence of IVS22-IG→A. Exon skipping is the commonest outcome following a mutation in the acceptor splice site; however, cryptic splice site activation, intron retention, and new splice site creation can occur.[34] There has been one previous description of exon 23 skipping, in tandem with exon 24, but the underlying genomic mutation was not reported.[16]

We detected one new missense mutation (L1465P) in *ATM*, adding to the 24 already reported in A-T patients.[33] This mutation leads to a proline substitution in the middle of a predicted α-helix from amino acids 1460 to 1476 (consensus predictions generated by Jpred,[35] a protein prediction algorithm service). This is predicted to change the secondary structure of the

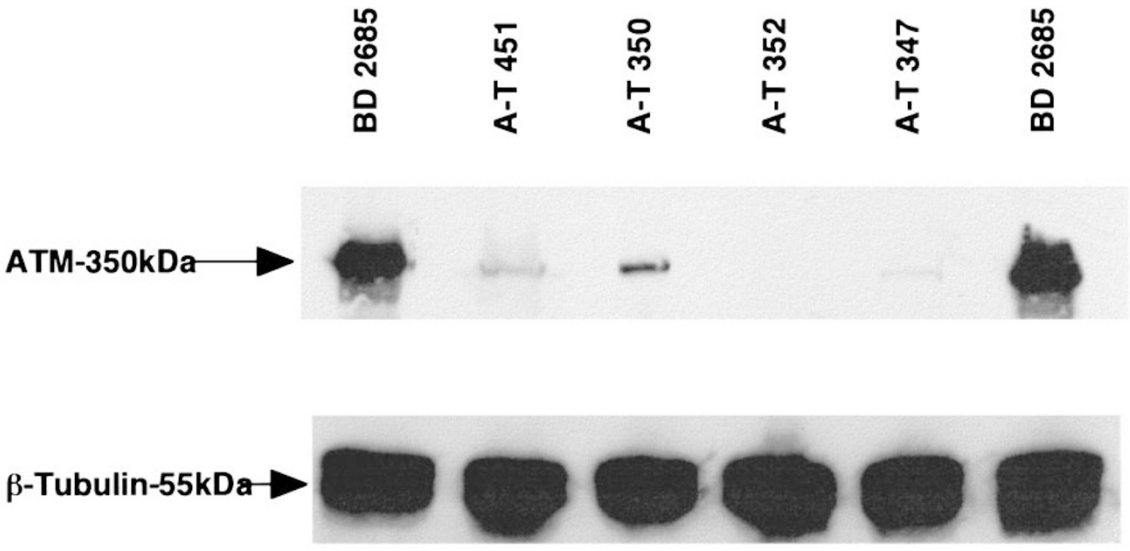

Figure 5 *Western analysis of ATM protein extracted from lymphoblastoid cell lines. Whole cell lysates, containing 50 μg protein, were fractionated on a 7.5% SDS-PAGE gel and blotted with polyclonal ATM antibody CN-12, and monoclonal β-tubulin antibody (Amersham). Lane 1: BD2685 (Control); Lane 2: A-T 451; Lane 3: A-T 350; Lane 4: A-T 352; Lane 5: A-T 347; Lane 6: BD2685 (Control)*

protein, destabilising the protein.[36] Examples of leucine-to-proline substitutions resulting in protein instability are reasonably common, eg L12P in λ repressor.[37] We observed reduced ATM protein expression from this allele in A-T 451, in keeping with this prediction.

By demonstrating reduced or absent ATM protein expression in four cases, we were able to confirm the diagnosis of A-T. Residual ATM protein expression is often associated with alleles harbouring 'leaky' splicing mutations,[31] as seen in A-T 350, or in-frame deletions, or missense mutations, as seen in A-T 451. Correlation of expressed ATM protein levels with clinical and cellular phenotypic features is difficult, because 'variant' A-T patients have been shown to express 1–100% of the normal level of ATM protein,[13,31] and this overlaps with the protein levels seen in classic A-T patients.[13]

Several founder mutations have been described in *ATM*, including eleven recurrent *ATM* mutations in the British Isles.[13] Two of these, 2639del200nt (codon 880), and 7639del9nt (codon 2546–2548), were found in families of Irish/Celtic descent.[38] Three of our A-T alleles were of Celtic/Irish origin, but did not share these founder mutations.

Three *ATM* alleles were not detected in this study, despite analysing the *ATM/E14* bi-directional promoter region for mutations. *ATM* promoter mutations have not been found in A-T patients,[39] but bi-allelic deletion of 5' exons of *ATM*, has been observed in T-prolymphocytic leukaemia tumour DNA.[40] Possible explanations for non-detection of these three mutations include technical difficulties in FCCM, absent mRNA expression and other types of mutations, not detectable by RT-PCR analysis, eg gross deletions/insertions of the gene, RNA editing, position effect variegation,[41] or gene modifiers altering expression or function of *ATM*.[42] The sensitivity of FCCM had previously been tested against a panel of eight known and seven 'blind' *ATM* mutations and all were detected (details not shown), making technical limitation of FCCM unlikely. Evidence for both mutant mRNA alleles being expressed was seen in all patients, except A-T 352, where no coding sequence variations were found. *ATM* has a relative paucity of coding sequence variants (5557G/A found in 0.82/0.18,[43] is the most common), and therefore verifying that both mRNA alleles are expressed at adequate levels may prove impossible. One of the undetected *ATM* mutations in A-T 352 may be a regulatory mutation in remote promoter elements, as reported for other genes, eg *BRCA1*,[27] or may destabilise the mRNA so that only a single copy of the transcript is amplified.[44]

In summary, we have shown FCCM to be a fast, accurate and sensitive method for detecting mutations in *ATM* and have examined the resultant protein expression patterns in four A-T families. Three of the four novel mutations described are from Celtic/Irish families, which extends the known mutation repertoire in this population. The detection of both *ATM* mutations in three families, allows us to offer direct mutation-based prenatal tests for A-T in future pregnancies, which is preferable to and more accurate than the radiosensitive chromosomal breakage assay or linkage analysis. Finally, having confirmed the efficacy of FCCM, we can now use FCCM in further studies, testing the hypothesis that A-T heterozygotes may have increased susceptibility to breast cancer.

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