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## ***ATM* germline mutations in women with familial breast cancer and a relative with haematological malignancy**

Laura La Paglia · Anthony Laugé · Jérémie Weber · Jérôme Champ · Eve Cavaciuti · Antonio Russo · Jean-Louis Viovy · Dominique Stoppa-Lyonnet

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**Abstract** Biallelic inactivation of the *ATM* gene causes ataxia–telangiectasia (A–T), a complex neurological disease associated with a high risk of leukaemias and lymphomas. Mothers of A–T children, obligate *ATM* heterozygote mutation carriers, have a breast cancer (BC) relative risk of about 3. The frequency of *ATM* carriers in BC women with a BC family history has been estimated to be 2.70%. To further our clinical understanding of familial BC and examine whether haematological malignancies are predictive of *ATM* germline mutation, we estimated the frequency of heterozygote mutation carriers in a series of 122 BC women with a family history of both BC and haematological malignancy and without *BRCA1/2* mutation. The gene screening was performed with a new high throughput method, EMMA (enhanced mismatch mutation analysis).

Amongst 28 different *ATM* variants, eight mutations have been identified in eight patients: two mutations leading to a putative truncated protein and six being likely deleterious mutations. One of the truncating mutations was initially interpreted as a missense mutation, p.Asp2597Tyr, but is actually a splice mutation (c.7789G>T/p.Asp2597\_Lys2643>LysfsX3). The estimated frequency of *ATM* heterozygote mutation carriers in our series is 6.56% (95% CI: 2.16–10.95), a significantly higher figure than that observed in the general population, estimated to be between 0.3 and 0.6%. Although a trend towards an increased frequency of *ATM* carriers was observed, it was not different from that observed in a population of familial BC women not selected for haematological malignancy as the frequency of *ATM* carriers was 2.70%, a value situated in the confidence interval of our study.

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**Keywords** Ataxia–telangiectasia · Breast cancer · *BRCA1* · *BRCA2* · *ATM* · EMMA

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## Introduction

Breast cancer (BC) is the most common cancer in western countries. Most cases of BC are sporadic, but almost 13% of women have at least one first-degree relative with BC [1]. The *BRCA1* and *BRCA2* genes are the two genes whose germline monoallelic inactivating mutations are identified in 15–20% of women with BC and a strong family history of BC [2], suggesting the presence of other BC predisposing genes explaining at least a portion of the remaining non-mutated familial cases. Three other genes are associated with an increased BC risk: *TP53*, *STK11*, and *PTEN*. As mutations of these genes are associated with rare autosomal dominant syndromes, these genes must be considered in a specific familial context. They also have a very small contribution to familial aggregations (<1% of familial cases) [2]. The *ATM* gene (ataxia telangiectasia mutated) encodes a lipid kinase phosphatidylinositol 3-kinase (PI3K) expressed in a wide range of tissues. It is the apex of a signalling cascade that detects and repairs DNA double-strand breaks, a cascade in which the *BRCA1* and *BRCA2* proteins are involved [3, 4]. Biallelic inactivation of the *ATM* gene causes ataxia–telangiectasia (A–T), a neurodegenerative disorder that develops in early childhood. Ataxia–telangiectasia children present a severe clinical phenotype characterised by progressive cerebellar ataxia and dysarthria, associated with ocular telangiectasia, immunodeficiency, sensitivity to ionising radiation, and an increased risk of cancers, especially leukaemias and lymphomas [5]. A large number of *ATM* mutations have been identified, extending over a very long 10 kb coding sequence scattered in 62 exons. Point or small size mutations and large gene rearrangements have been identified and some deleterious missense mutations located in the PI3K domain have been reported (*ATM* mutation database: <http://chromium.liacs.nl/LOVD2/home.php>) [6, 7].

The mothers of A–T children, obligate *ATM* heterozygote mutation carriers, have been shown to present an increased BC risk [8]. Additional studies conducted in families of A–T children have shown a relative risk (RR) of about three attributable to *ATM* heterozygosity in women [9–12]. Various BC population studies have tried to estimate the risk of developing BC amongst *ATM* mutation carriers, sometimes with controversial results, as reviewed in Ahmed and Rahman [13]. In a more recent study, Renwick et al. analysed 443 cases of familial BC and 521 controls, and found 12 women carrying an *ATM* mutation in the case group (2.70%) and two in the control group (0.38%) [14]. They estimated that *ATM* mutation heterozygosity was associated with a BC RR of 2.37 (95% CI: 1.51–3.78) [14]. As the rate of *BRCA1/2* mutation detection in BC women with a family history of BC is 15–20%, the

rate of *ATM* mutations is five to seven times lower than the rate of *BRCA1/2* mutations.

*ATM* mutations are involved in the development of lymphoid malignancies as A–T patients are at high risk of T-cell acute lymphocytic leukaemia (T-ALL) and especially prolymphocytic leukaemia (T-PLL) [15]. Somatic inactivation of the *ATM* gene has been reported in T-PLL and B-cell chronic lymphocytic leukaemia (B-CLL) occurring in patients outside an A–T context [16–18]. Mutations observed in these reports are essentially missense mutations and mostly occur in the PI3 K domain. Monoallelic *ATM* germline mutations have also been reported in some patients with B-CLL, mantle cell lymphoma (MCL), and T-cell ALL [19–22]. In Stankovic's series, 18% of patients were carriers of a monoallelic *ATM* germline mutation [19]. In a short series of T-ALL affected children, a monoallelic *ATM* germline mutation was identified in 20% of cases [23]. In a Nordic study of relatives of A–T children, an increased standardised incidence ratio (SIR) of 37 (95% CI: 13–80) was observed for leukaemias and non-Hodgkin lymphomas [11]. A trend towards an increased RR of leukaemias and lymphomas was also observed in a French study of the relatives of A–T children [24].

To further our clinical understanding of familial BC without *BRCA1/2* mutation and to limit the volume of *ATM* gene screening, we estimated the frequency of *ATM* mutation carriers in BC women with a family history of both BC and haematological malignancy. In other words, this study was designed to examine whether the presence of leukaemia and/or lymphoma amongst relatives in BC families was a predictive factor for *ATM* mutation. A second objective was to report a new high throughput method, called enhanced mismatch mutation analysis (EMMA), that we have adapted for *ATM* gene mutation screening. We report the results of EMMA *ATM* gene analysis in a series of 122 unrelated *BRCA1* and *BRCA2* mutation negative women with at least one-first or second degree relative with leukaemia or lymphoma.

## Patients and methods

### Patients

Between January 1995 and December 2005, 2,009 unrelated patients with BC (index case) were tested for *BRCA1* and *BRCA2* mutations at the Institut Curie. They had a family history of breast and/or ovarian cancer and/or developed BC before the age of 36. A family history was defined as either two first-degree relatives with cancer: (1) at least one with invasive BC before the age of 41, or (2) one with ovarian cancer or breast cancer at any age, or

three first-degree or second degree relatives from the same lineage with invasive BC or ovarian cancer at any age, where the index case constituted one of the cases. Amongst the 1,629 patients with negative *BRCA1* and *BRCA2* screening, 122 had at least one first or second degree relative with leukaemia or lymphoma, and this relative belonged to the branch of the family in which breast cancers were segregating. Informed consent for breast cancer genetic studies was obtained from each woman at the family cancer clinic.

#### DNA preparation

Two DNA extraction methods from whole blood were used: (1) a modified perchlorate/chloroform procedure [25], or (2) a rapid method using the NucleoSpin Blood extraction kit (Macherey–Nagel; ref. <http://www.mn-net.com>). DNA concentrations and the quality of each sample were determined spectrophotometrically at 260/280 nm (NanoDrop<sup>®</sup> ND-1000, NanoDrop technologies).

#### EMMA

The enhanced mismatch mutation analysis, EMMA (Fluigent, France), is a method of mutation screening based on heteroduplex analysis (HDA). Heteroduplexes and homoduplexes are separated by their different electrophoretic mobility due to their conformational differences. The resolution of EMMA is considerably better than that of standard HDA due to a combination of high resolution block-copolymer sieving matrix, and nucleosides as additives in the electrophoretic medium [26, 27]. The medium was loaded on the ABI3130XL sequencing system (Applied Biosystems 2006). EMMA also allows simultaneous analysis of numerous amplicons by multiplex analysis. This method allows the simultaneous detection of both small mutations and large gene rearrangements (LGR) by applying semi-quantitative PCR conditions and an external control amplicon [28]. As a result of multiplex analysis and LGR detection, EMMA is a high throughput screening mutation method. Data analysis was performed with Emmalys<sup>®</sup> software (Fluigent, France) for both point and small mutations and Excel Visual Basic macro for LGR analysis.

#### Amplicon and multiplex design

Primers were designed using the web-based Primer3 software ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) to cover the 62 coding exons of the *ATM* gene and the splice site junctions. A series of 36 multiplexes of 2–4 amplicons, including the external gene control, was prepared. Amplicon sizes ranged between 217 and 567 bp (basepair). In a given multiplex, the length difference

between two adjacent amplicons was at least 50 bp. Primer sequences and multiplex amplicon PCR conditions are reported in Table S1 (supplementary data).

#### EMMA PCR conditions

PCR conditions were identical for all multiplexes. About 100 ng of DNA were amplified using Taq ABgene 0.06 U/μl (ABgene UK, ref: [www.abgene.com](http://www.abgene.com)), 5 mM MgCl<sub>2</sub>, 4 × 0.2 mM dNTPs, and 0.08–0.3 μM of each primer in a final reaction volume of 20 μl. Cycling conditions were as follows: 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by two steps of 72°C for 7 min and 98°C for 10 min. Heteroduplex formation consisted of 73 cycles of 98°C for 30 s, with temperature decreasing by 1° at each cycle, and a final step at 25°C for 10 min.

#### DNAs for EMMA validation study

As small insertions or deletions have been shown to be easily detected by EMMA, we focused our validation study on nucleotide substitutions previously detected by direct sequence analysis [27]. DNAs from A–T children or their *ATM* heterozygote carrier relatives, previously referred to our laboratory, were selected for nucleotide substitutions. DNAs carrying a substitution were available for 49 amplicons. The substitutions studied are reported in Table S2 (supplementary data).

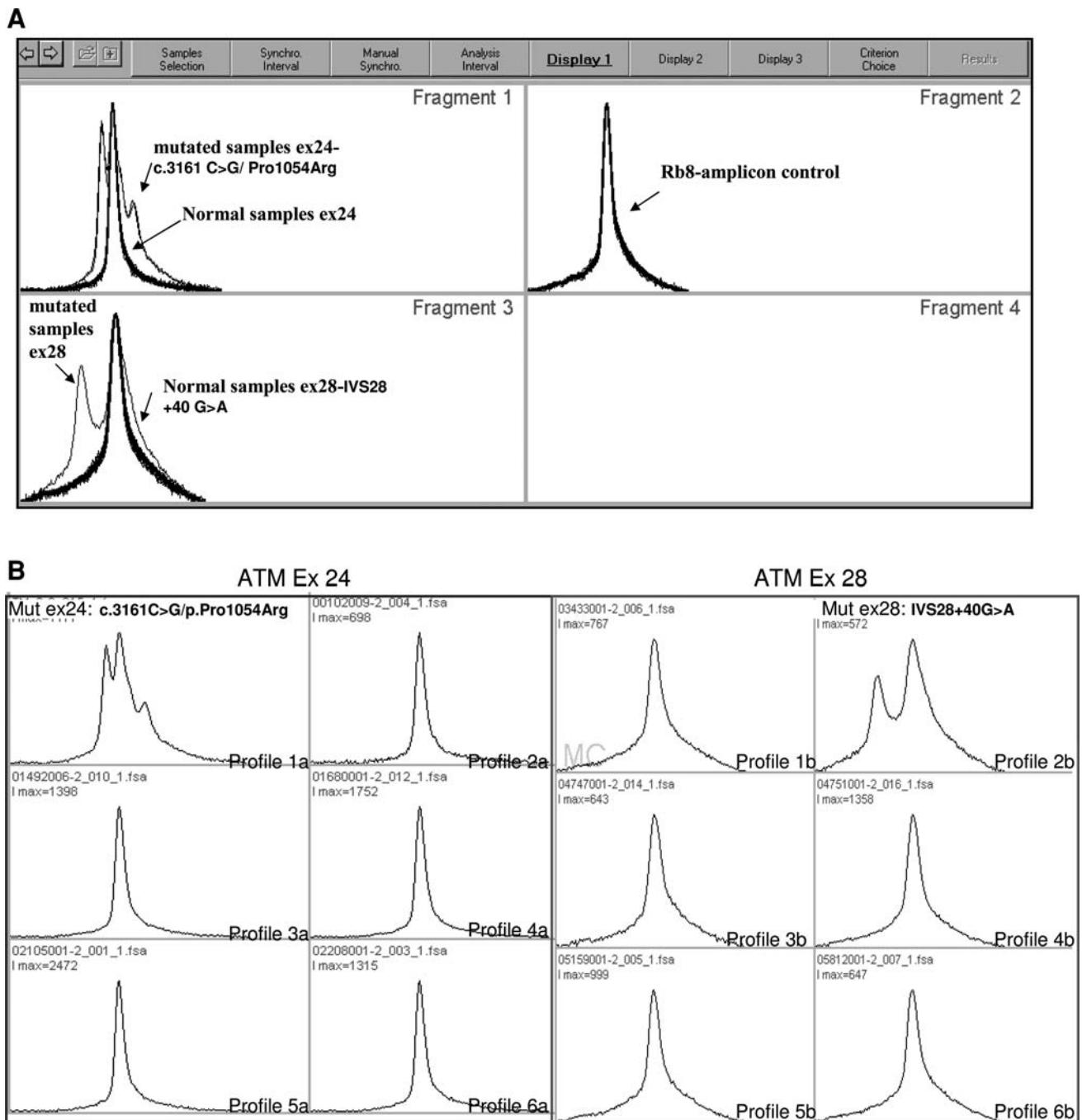
A heterozygote *ATM* deletion from exons 26–65 was also used to validate LGR detection. An amplicon containing exon 8 or 11 of the *RB1* gene was used as external control [29]. DNAs from these A–T patients were used as experimental controls in the study of the 122 BC patients.

#### Sequencing of EMMA variant profiles

Each DNA corresponding to a different EMMA profile as compared with the majority of profiles was sequenced.

100 ng of DNAs were amplified using Taq ABgene 0.025 U/μl (ABgene UK, ref: [www.abgene.com](http://www.abgene.com)), 4 × 0.2 mM dNTPs, 1.5 mM MgCl<sub>2</sub>, and 0.3 μM of each primer in a final reaction volume of 50 μl. Amplification was performed with a touchdown PCR protocol consisting of an initial denaturation step at 94°C for 5 min followed by 35 PCR cycles: denaturation at 94°C for 30 s, annealing for 30 s with temperature gradually decreasing from 60°C to 52°C for 35 cycles, and elongation at 72°C for 30 s.

PCR products were separated by agarose gel electrophoresis, purified (Macherey Nagel), and sequenced, using one of the PCR primers (usually the forward primer, except in the case of poor sequence quality), Big Dye Cycle Sequencing Reactions and an ABI3130XL automated



**Fig. 1** Emma analysis of the multiplex amplified *ATM* exons 24, 28, and *RBI* exon 8. **a** The profiles of 93 EMMA analysed DNAs were superimposed with Emmalys<sup>®</sup> software. Each peak corresponds to a single amplified exon or amplicon. *Fragment 1* is *ATM* exon 24. Two profiles were identified: (1) a single peak profile detected in 90 DNAs, and (2) a triple peak profile detected in three DNAs with a heterozygous c.3161C>G/p.Pro1054Arg polymorphism. *Fragment 2* is *RBI* exon 8, used as external control for LGR detection. Only one peak profile was detected. *Fragment 3* is *ATM* exon 28. Two profiles were identified: (1) a single peak profile detected in 92 DNAs, and (2)

a double peak profile detected in 1 DNA with the heterozygous IVS28, c.3993+40G>A variant. **b** Individual DNA profiles of *ATM* 24 and 28 amplicons obtained with Emmalys<sup>®</sup> software. Regarding exon 24, 5 DNAs lead to a normal profile (2a–6a). The 1a profile has three peaks and corresponds to the superimposition of the c.3161C/p.Pro1054 and the c.3161G/p.Arg1054 alleles. Regarding exon 28, 5 DNAs lead to a normal profile (1b, 3b–6b). The 2b profile has two peaks and corresponds to the superimposition of the IVS28, c.3993+40G and IVS28, c.3993+40A alleles

sequencer (Applied Biosystems). Seqscape® (Applied Biosystems) software was used for sequence analysis.

The *ATM* coding sequence reference is U33841. The first translated nucleotide is the reference nucleotide.

Control DNAs for analysing the population frequency of sequence variants identified in the 122 BC cases

The frequency of DNA variants detected in the series of 122 BC women was determined in a series of 93–186 unrelated controls. Controls were children tested for *RBI* gene mutation due to the presence of retinoblastoma, a disease unrelated to the *ATM* gene and for which our laboratory routinely performs mutation screening.

The arbitrary threshold of 1% was selected according to the definition of polymorphism in population genetics: a DNA variant with a frequency of 1% or above was rather considered with no functional effect.

Splice site score predictions

The genomic sequence environment of a DNA variant was analysed using Splice Site Prediction by Neural Network (NNSPLICE available at [http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html)), Splice Site Finder (SSF available at <http://violin.genet.sickkids.on.ca/~ali/splicesitefinder.html>), Max EntScan (MES available at [http://genes.mit.edu/burgelab/maxent/Xmaxentscan\\_scoreseq.html](http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html)) [30].

Protein conservation and A-GVGD score

The A-GVGD algorithm developed by Tavtigian et al. for the analysis of various genes, including the *ATM* gene, was used to contribute to discriminate disease-causing missense mutations from neutral changes (<http://agvgd.iarc.fr/>) [31, 32]. This algorithm combines the biophysical characteristics of amino acid changes (GV) and alignments of protein

**Table 1** *ATM* germline variants and their characteristics in 122 BC patients with a family history of breast and haematological malignancy

Nucleotide change	Amino acid change	Exon/IVS	Freq. in patients	Freq. in controls	FAT or PI3K domain	GV score	GD score	A-GVGD combined score and prediction	Signification proposed
c.4852C>T	p.Arg1618X	exon 34	1/122	0/186	–	–	–	–	Deleterious
c.7789G>T	p.Asp2597_Lys2643>LysfsX3	exon 55	1/122	0/186	–	–	–	–	Deleterious
c.749G>A	p.Arg250Gln	exon 9	1/122	0/186	–	0.00	42.81	C35	Likely deleterious
c.6067G>A	p.Gly2023Arg	exon 43	1/122	1/186	FAT	0.00	125.13	C65	Likely deleterious
c.7187C>G	p.Thr2396Ser	exon 51	1/122	0/93	FAT	111.69	6.18	C0	Likely deleterious
c.7305C>G	p.Asn2435Lys	exon 51	1/122	0/93	FAT	0.00	93.88	C65	Likely deleterious
c.8560C>T	p.Arg2854Cys	exon 60	1/122	0/93	PI3K	42.81	154.23	C35	Likely deleterious
c.9023G>A	p.Arg3008His	exon 65	1/122	0/186	PI3K	0.00	28.82	C25	Likely deleterious
c.1952T>C	p.Leu651Pro	exon 15	1/122	4/186	–	0.00	97.78	C65	Unknown
c.2119T>C	p.Ser707Pro	exon 15	3/122	0/186	–	73.35	0.00	C0	Unknown
c.2254C>G	p.Leu752Val	exon 17	1/122	0/186	–	0.00	30.92	C25	Unknown
c.2289T>A	p.Phe763Leu	exon 17	2/122	0/186	–	157.56	0.00	C0	Unknown
c.2476A>C	p.Ile826Leu	exon 19	1/122	0/186	–	141.80	4.86	C0	Unknown
c.4556T>C	p.Val1519Ala	exon 32	1/122	8/186	–	0.00	65.28	C65	Unknown
c.4802G>A	p.Ser1601Asn	exon 34	1/122	0/186	–	115.58	0.00	C0	Unknown
c.4975A>G	p.Ile1659Val	exon 35	1/122	0/93	–	93.66	0.00	C0	Unknown
c.5018G>C	p.Ser1673Thr	exon 36	1/122	0/186	–	111.49	0.00	C0	Unknown
c.6844A>C	p.Asn2282His	exon 49	1/122	1/186	FAT	64.77	36.57	C0	Unknown
c.6919C>T	p.Leu2307Phe	exon 49	1/122	1/186	FAT	0.00	21.82	C15	Unknown
c.1899-24A>G	–	IVS14	1/122	0/186	–	–	–	–	Unknown
c.3993+40G>A	–	IVS28	1/122	0/186	–	–	–	–	Unknown
c.4237-54T>C	–	IVS30	4/122	1/186	–	–	–	–	Unknown
c.4437-7A>C	–	IVS31	1/122	0/186	–	–	–	–	Unknown
c.6807+39C>T	–	IVS48	2/122	0/185	–	–	–	–	Unknown
c.7516-32T>G	–	IVS52	1/122	0/93	–	–	–	–	Unknown
c.7927+69T>C	–	IVS55	1/122	0/186	–	–	–	–	Unknown
c.3342G>A	p.Lys1114Lys	exon 25	1/122	1/149	–	–	–	–	Unknown
c.685C>T	p.Leu229Leu	exon 9	1/122	5/186	–	–	–	–	Likely neutral

sequences of different and phylogenetically distant species (GD), from human to zebrafish. The output from Align-GVGD is an ordered series of grades ranging from C65 (most likely deleterious) to C0 (most likely neutral). Missense mutations with a score below C15 are considered as probably neutral. When the score is at or above C15 and below C35, missense mutations remain unclassified, at or above C35 they are likely deleterious [32].

## Results

### EMMA validation for the *ATM* gene study

All of the 49 nucleotide substitutions located in 49 different amplicons of the *ATM* gene and selected regardless of their biological significance were detected (Fig. 1 and data not shown, see supplementary data for the list of mutations studied, Table S2). The deletion of exons 26–65 was detected in all corresponding amplicons.

EMMA analysis in the series of 122 women with familial BC and one relative with haematological malignancy

A total of 28 different monoallelic sequence variants were identified in 35 of the 122 BC women screened for *ATM* gene mutation. These variants were initially interpreted as follows: 1 nonsense mutation, 18 missense mutations, 2 silent mutations, 7 intronic variants (Table 1). No LGR was detected.

Twenty-two heterozygous polymorphisms were also detected and were classified as polymorphisms on the basis of previous reports in the literature, in databases, especially the NCBI SNP database, or in A–T patients studied in our laboratory (data not shown) (Table 2).

A nonsense mutation: c.4852C>T/p.Arg1618X was identified in one patient. This mutation, leading to a putative truncated and non-functional protein was considered to be deleterious.

The biological function of the 18 missense mutations identified was unknown, as they have not been previously reported as deleterious in databases. The following five criteria were used to assess the significance of their functional effect: (1) a carrier frequency <1% in a series of controls, (2) identification of the missense mutation in an A–T child carrying no more than one deleterious *ATM* mutation, (3) location in an *ATM* protein domain known to be functionally important, especially the PIK3 and FAT domains, (4) the importance of the amino acid (aa) change and aa species conservation analysed by the A-GVGD algorithm, and (5) a putative splice effect by using in silico splice site score prediction models. These criteria are listed

in order of increasing relevance with respect to a putative deleterious effect.

In one case, a splicing defect was suspected on in silico analyses, as the c.7789G>T mutation, initially interpreted as p.Asp2597Tyr, is actually a splicing mutation. c.7789G is the first nucleotide of exon 55. The G > T substitution leads to disruption of the acceptor site of intron 54 and a putative truncated protein of 2,598 aa (c.7789\_7927del139/p.Asp2597\_Lys2643>LysfsX3). This splicing defect was confirmed by RNA analysis of patient lymphoblasts showing loss of exon 55 (Fig. 2). This mutation was identified in only one patient.

The remaining 17 missense mutations were classified into three categories according to the above criteria: likely deleterious, unknown variants (UV), and likely neutral.

Six missense mutations were classified as likely deleterious: p.Arg250Gln, p.Gly2023Arg, p.Thr2396Ser, p.Asn2435Lys, p.Arg2854Cys, and p.Arg3008His (Table 1). They were considered as “likely deleterious” according to the A-GVGD algorithm except for p.Thr2396Ser and p.Arg3008His which were respectively “neutral” or “unclassified”. These missense mutations were located in the FAT

**Table 2** Identified *ATM* variants classified as polymorphisms in 122 BC patients with a family history of breast and haematological malignancy

Nucleotide change	Amino acid change	Exon/IVS	Het. freq. (%)
c.544G>C	p.Val182Leu	exon 8	2.0
c.2442C>A	p.Asp 814Glu	exon 18	1.3
c.2572T>C	p.Phe858Leu	exon 19	1.5
c.3161C>G	p.Pro1054Arg	exon 24	1.9
c.4258C>T	p.Leu1420Phe	exon 31	3.1
c.4578C>T	p.Pro1526Pro	exon 32	4.4
c.5557G>A	p.Asp1853Asn	exon 39	10.6
c.5558A>T	p.Asp1853Val	exon 39	1.1
c.1899-55T>G	–	IVS14	2.4
c.2125-68T>C	–	IVS15	3.7
c.2250+22A>C	–	IVS16	1.2
c.2250+34A>C	–	IVS16	1.2
c.2250+78G>A	–	IVS16	1.9
c.2839-90T>G	–	IVS20	38.0
c.3403-14insA	–	IVS25	49.0
c.3285-9del	–	IVS24	11.0
c.3747-34A>G	–	IVS27	1.0
c.3993+48A>G	–	IVS28	1.3
c.4777-20A>G	–	IVS33	1.0
c.5497-8T>C	–	IVS38	1.5
c.5497-15G>C	–	IVS38	1.0
c.8786+8A>C	–	IVS62	1.1

Het. freq. Heterozygote frequency according to NCBI

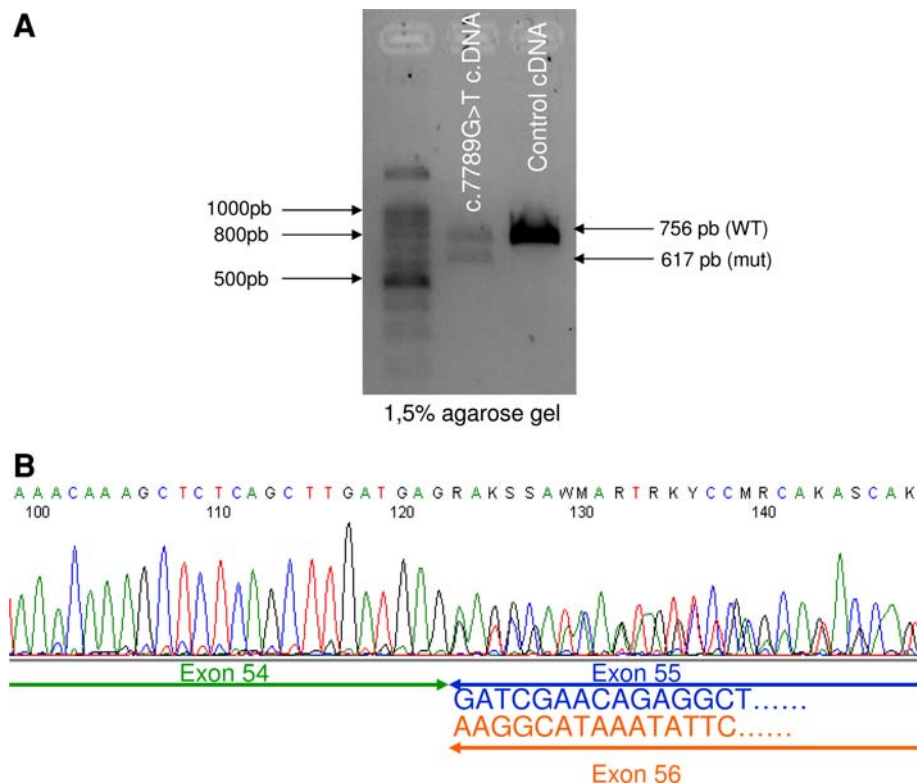
protein domain and were not detected in a set of control DNAs. They have also been previously reported in a T-PLL, a feature that may argue in favour of their deleterious effect as they could have been selected by the tumour process [16]. In addition, the p.Arg3008His missense mutation has been detected in an A–T child whose second mutation was introducing an *ATM* stop codon (Micol et al., in preparation). These six missense mutations were identified in six patients.

Eleven missense mutations were classified as UV: p.Leu651Pro, p.Ser707Pro, p.Leu752Val, p.Phe763Leu, p.Ile826Leu, p.Val1519Ala, p.Ser1601Asn, p.Ile1659Val, p.Ser1673Thr, p.Asn2282His, and p.Leu2307Phe (Table 1). The majority of these UV were not located in the PI3K or FAT domains, and the A-GVGD scores were in favour of unclassified or neutral effect except in two cases. Regarding the p.Leu651Pro and p.Val1519Ala missense mutations, although their A-GVGD score was in favour of a likely deleterious effect, they were classified as UV as their carrier frequency amongst controls was high, respectively above 2

and 4% (Table 1). These 11 missense mutations were identified in 14 patients.

Two silent and seven intronic variants were also detected. Only criteria (1), (2), and (5) quoted above can be used. The in silico analyses did not suggest a splicing defect in any of these nine variants. The frequency of the seven intronic variants was <1% in our series of controls; they were therefore classified as UV: IVS14, c.1899-24A>G; IVS28, c.3993+40G>A; IVS30, c.4237-54T>C; IVS31, c.4437-7A>C; IVS48, c.6807+39C>T; IVS52, c.7516-32T>G; IVS55, c.7927+69T>C. The p.Lys1114Lys variant was also classified as UV, as its frequency in the control group was <1%. The p.Leu229Leu silent variant was classified as likely neutral, since its frequency in controls was >1%.

In summary, a total of eight patients carrying an *ATM* mutation, two patients with a truncating mutation and six patients with a likely deleterious *ATM* mutation, were identified in this series of 122 patients. The frequency of *ATM* heterozygote mutation carriers is 6.56% (95% CI: 2.16–10.95).



**Fig. 2** cDNA analysis of the c.7789G>T mutation. **a** Initially interpreted as a missense mutation, the c.7789G>T substitution is a splicing mutation as shown by cDNA analysis of a lymphoblast cell line established from the patient carrying this mutation. Exons 54–59 were amplified from primers located in the coding sequence (sequence available on request). The amplified product was analysed by 1.5% agarose gel electrophoresis. One 756 bp long fragment was

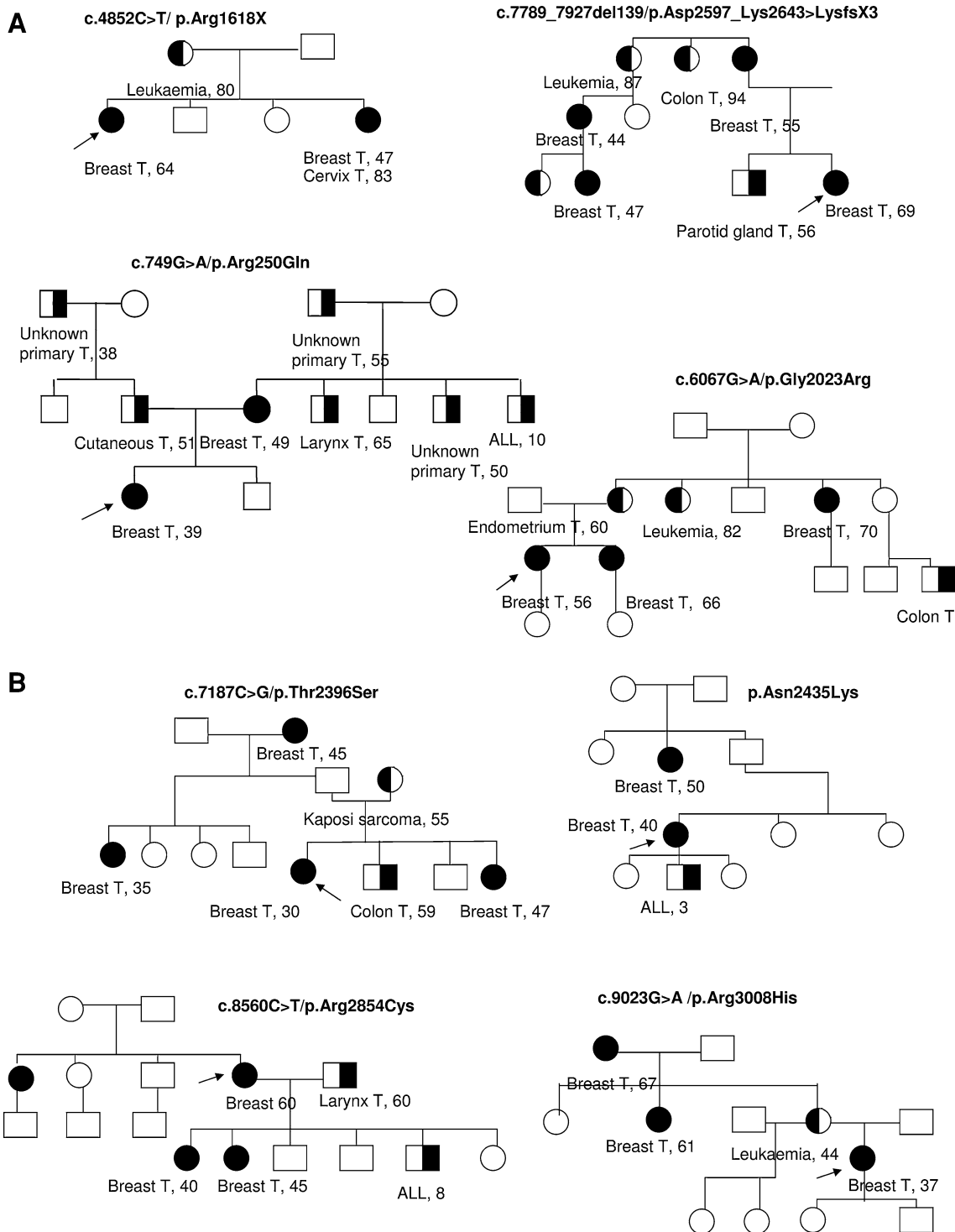
obtained from a control lymphoblast cell line; an additional fragment, 617 bp long, was detected in the c.7789G>T mutant cell line, suggesting that exon 55 deletion has occurred. **b** Sequence of the 54–59 amplified exons from the c.7789G>T mutant cell line. Superimposition of the 54–55 and 54–56 exon junctions confirms the exon 55 deletion. The c.7789G>T substitution leads to the putative truncated p.Asp2597\_Lys2643>LysfsX3 protein



**Discussion**

Epidemiological studies designed to elucidate the role of *ATM* mutations in cancer risks are limited by the large size of the gene and the large number of mutations spread

throughout the coding sequence. To simplify the extensive laboratory work required for *ATM* screening, a new DNA analysis method was applied. Using similar analysis conditions for each amplicon, all of the 49 different control mutations previously identified by direct sequencing in



**Fig. 3** Family history of the eight patients carrying a deleterious or likely deleterious *ATM* mutation. The BC women tested for *ATM* mutation are indicated by an arrow. ALL: acute lymphoid leukaemia

A–T children and a large gene deletion were detected by EMMA. Multiplex analysis including the search for LGR allowed screening of the 10 kb *ATM* coding sequence in 3 weeks in a large set of 94 patients.

In the series of 122 BC women with a relative with haematological malignancy, two deleterious mutations, leading to a putative truncated protein were identified, a nonsense mutation, and a splicing mutation not previously reported and characterised. The remaining 17 missense mutations were classified into three categories: six likely deleterious mutations, nine mutations of unknown significance, and two likely neutral mutations. The main criterion used to classify these missense mutations was the A-GVGD score. Moreover, p.Gly2023Arg, p.Thr2396Ser, and p.Arg3008His variants have been reported as somatic events in T-PLL or B-CLL and associated with loss of the second *ATM* allele by 11q deletion, features that argue strongly in favour of their deleterious effect as they could have been selected by the tumour process [16, 33]. The multiplicity of criteria to assess the significance of the functional effect of a DNA variant underlines that none a criterion alone is sufficient and that they have to be altogether taken into account with in some cases when they are leading to divergent conclusions the need to make some arbitrary decisions. The availability of functional testing and in vitro functional assays allowing characterisation of missense mutations would be very useful [7].

For each of the eight BC women carrying a deleterious or likely deleterious *ATM* mutation, only one relative presented a haematological malignancy. In six cases, this relative was a first degree relative and in two cases a second degree relative (Fig. 3). The disease was leukaemia in seven cases and Kaposi sarcoma occurring at age 55 in the remaining case. Three acute lymphoid leukaemia cases occurred during childhood at the ages of 3, 8, and 10 years; one leukaemia case occurred at 44 years and the three other cases were late-onset leukaemia, occurring after the age of 80. No blood samples which would have been required for *ATM* mutation co-segregation study from both cases with haematological malignancies and with breast cancer were obtained, most of the cases being deceased or lost of follow-up.

The estimated frequency of A–T heterozygotes in our series is 6.56% (95% CI: 2.16–10.95), a significantly higher figure than that observed in the general population which is estimated to be between 0.3 and 0.6% [12, 14]. Although a trend towards an increased frequency of *ATM* carriers was observed in our series, it was not different from that observed in a population of familial BC women not selected for haematological malignancy in relatives, as the frequency of *ATM* carriers was 2.70%, a value situated in the confidence interval 2.16–10.95 estimated in our study [14]. It is noteworthy that, although the RR of haematological

malignancies in *ATM* heterozygote carriers may be high—SIR 37 in Olsen’s study—[11], the absolute risk remains low, as the incidence of haematological malignancies is low in the general population. Thus the availability of familial aggregations of both breast cancer and haematological malignancies is low, limiting the power of such study. Finally, although the expected final classification of some UVs in the deleterious mutation group and that of some rare polymorphic allele will increase this frequency, a larger set of patients with a control group of BC women with no family history of haematological malignancy needs to be studied to determine whether the presence of relatives with leukaemia and/or lymphoma in BC families is a predictive factor for *ATM* mutation.

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