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Five Haplotypes Account for Fifty-Five Percent of ATM Mutations in Brazilian Patients With Ataxia Telangiectasia: Seven New Mutations

Gabriela Coutinho,[1,2] Midori Mitui,[1] Catarina Campbell,[1] Beatriz T. Costa Carvalho,[3] Shareef Nahas,[1] Xia Sun,[1] Yong Huo,[1] Chih-hung Lai,[1] Yvonne Thorstenson,[4] Robert Tanouye,[1] Salmo Raskin,[5] Chong A. Kim,[6] Juan Llerena Jr,[7] and Richard A. Gatti[1]*

[1]*Department of Pathology and Laboratory Medicine, The David Geffen School of Medicine, Los Angeles, California*
[2]*Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, RJ, Brazil*
[3]*Divisão de Alergia, Imunologia Clínica e Reumatologia, Departamento de Pediatria, Universidade Federal de São Paulo, SP, Brazil*
[4]*Stanford Genome Technology Center, Stanford University, Palo Alto, California*
[5]*Laboratório Genetika, PR, Brazil*
[6]*Departamento de Genética Clínica, Instituto da Criança do Hospital das Clínicas, USP, SP, Brazil*
[7]*Instituto Fernandes Figueira, FIOCRUZ, RJ, Brazil*

We have studied the molecular genetics of 27 Brazilian families with ataxia telangiectasia (AT). Five founder effect haplotypes accounted for 55.5% of the families. AT is an autosomal recessive disorder of childhood onset characterized by progressive cerebellar ataxia, ocular apraxia, telangiectasia, immunodeficiency, radiation sensitivity, chromosomal instability, and predisposition to cancer. The *ATM* gene spans more than 150 kb on chromosome region 11q23.1 and encodes a product of 3,056 amino acids. The ATM protein is a member of the phosphatidylinositol 3-kinase (PI-3K) family of proteins and is involved in cell cycle control and DNA repair pathways. DNA was isolated from lymphoblastoid cell lines and haplotyped using four STR markers (D11S1818, NS22, D11S2179, D11S1819) within and flanking the *ATM* gene; all allele sizes were standardized in advance. In addition to the STR haplotypes, SNP haplotypes were determined using 10 critical polymorphisms. The entire gene was screened sequentially by protein truncation testing (PTT), single strand conformation polymorphism (SSCP), and then denaturing high performance liquid chromatography (dHPLC) to identify the disease-causing mutations. Of the expected 54 mutations, 50 were identified. All mutations but one, led to a truncated or null form of the ATM protein (nonsense, splice site, or frameshift). Five families (18.5%) carried a deletion of 3450nt (from IVS28 to Ex31), making this one of the two most common Brazilian mutations. Mutations were located throughout the entire gene, with no clustering or hotspots. Standardized STR haplotype analysis greatly enhanced the efficiency of mutation screening.

KEY WORDS: ataxia telangiectasia; ATM haplotypes; ATM mutations; Brazilian families

INTRODUCTION

Ataxia telangiectasia (AT) is an autosomal recessive disorder of childhood onset with an incidence of 1:40,000 to 1:100,000 live births. It is characterized by progressive cerebellar ataxia with degeneration of the Purkinje cells, conjunctival telangiectasia, ocular motor apraxia, elevated serum alphafetoprotein, immunological deficiencies with recurrent sino-pulmonary infections, chromosomal instability, sensitivity to ionizing radiation, and predisposition to cancer, especially lymphomas and leukemias [Boder and Sedwick, 1958; Taylor and

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*Correspondence to: Richard A. Gatti, The David Geffen School of Medicine, Department of Pathology, Los Angeles, CA 90095-1732. E-mail: rgatti@mednet.ucla.edu

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Harnden, 1975; Metcalfe et al., 1996; Gatti, 2002]. Additional characteristics of AT include premature aging, thymic and ovarian degeneration, growth retardation, and telomere shortening. The carrier frequency in the Caucasian population has been variously estimated at 1–1.8% and carriers have an increased susceptibility to cancer, more specifically, breast cancer in women [Swift et al., 1987; Easton, 1994; Gatti et al., 1999; Concannon, 2002].

The *ATM* gene is mutated in AT patients; most patients are compound heterozygotes. The gene maps to chromosome region 11q23.1 [Gatti et al., 1988; Lange et al., 1995]. It is composed of 66 exons spread over 150 kb of genomic DNA [Savitsky et al., 1995a,b] and encodes a 13 kb mature transcript with an open reading frame (ORF) of 9168 nucleotides. The 370 kDa ATM protein, located predominantly in the cell nucleus, is ubiquitously expressed and is a member of the phosphatidylinositol 3-kinase (PI-3K) family with its kinase domain at the C-terminus region [Hartley et al., 1995; Chen and Lee, 1996]. The ATM protein shows strong similarities to several checkpoint/damage response proteins, such as the *Saccharomyces cerevisiae* Tel1p and Mec1p, Rad 3 in *Schizosaccharomyces pombe*, mei-41 in *Drosophila melanogaster*, and human DNA-PK_{CS} and ATR [Savitsky et al., 1995a,b; Lavin and Shiloh, 1997; Platzer et al., 1997].

Although the phenotype of AT is distinct, the age of onset and rate of progression is variable. The initial observation of telangiectasias may be late, the severity of pulmonary infections varies, and approximately 5% of patients do not show increased levels of alphafetoprotein. Although the incidence of cancer in AT patients is about 35%, the occurrence of certain tumors types in some families suggest that this predisposition may not be uniform and may relate to the types of mutations found in different families [Swift et al., 1987, 1991; Gatti, 2002]. About 70% of the reported mutations are predicted to truncate the ATM protein [Gilad et al., 1996; Telatar et al., 1996, 1998a; Castellvi-Bel et al., 1999; Teraoka et al., 1999; Eng et al., 2003]. Mutations in the conserved GT and AG splice sites regions, leading to exon skipping are frequently observed while missense mutations account for only about 10% of all ATM mutations in AT patients (http://www.benaroyaresearch.org/bri_investigators/atm.htm).

Previous studies have shown that specific short tandem repeat (STR) haplotypes observed in ethnic populations can be associated with founder effect mutations, i.e., "affected haplotypes" [Uhrhammer et al., 1995; Telatar et al., 1996; Ejima and Saaki, 1998; Laake et al., 1998; Telatar et al., 1998a,b]. Single nucleotide polymorphism (SNP) haplotyping across the region of the *ATM* gene is of limited informativeness [Bonnen et al., 2000; Thorstenson et al., 2001]. Despite this, when SNP and STR haplotyping were combined with mutation analysis, valuable insights were discovered as to the age of some mutations; ancestral relationships extend beyond conventional ethnicities [Campbell et al., 2003].

We have studied the spectrum of haplotypes and mutations in the *ATM* gene in Brazilian patients from 27 unrelated AT families. This work extends our previous report comparing the haplotypes and mutations of Iberian AT families [Mitui et al., 2003]. To facilitate mutation identification in such a complex gene, we prescreened all patients by STR haplotyping. We have identified 50 of the expected 54 mutations and 7 novel mutations were found. Five affected haplotypes accounted for 55.5% of Brazilian ATM mutations. Haplotype prescreening greatly enhanced the efficiency of mutation detection especially when mutations were encountered that would have been missed by conventional screening strategies.

MATERIALS AND METHODS

Subjects

Twenty-seven Brazilian patients with clinical symptoms and laboratory results (alphafetoprotein levels, western blot, and colony survival assay (CSA)) compatible with classical AT were selected for this study. Lymphoblastoid cell lines (LCLs) were established on 18 of 27 patients. DNA and RNA were isolated from LCLs and the diagnosis was confirmed by immunoblotting [Becker-Catania et al., 2000; Chun et al., 2003] and by CSA [Huo et al., 1994; Sun et al., 2002]. For the remaining nine patients, only DNA was available for analysis. At least one ATM mutation was eventually identified in each of the 27 patients.

STR Haplotype

STR haplotypes were identified using four microsatellite markers spanning an interval of 1.4 cM: A4 (D11S1819) [Rotman et al., 1994], NS22 [Udar et al., 1999], D11S2179 [Vanagaite et al., 1995], and A2 (D11S1818) [Rotman et al., 1994; Lange et al., 1995]. Primers were designed to flank each STR and used for PCR amplification by γ^{33}P-ATP end-radiolabeled fragments [Campbell et al., 2003; Mitui et al., 2003]. Alleles were standardized using a control DNA, CEPH 1347-2. Phases were defined by haplotyping the parents of propositi, whenever available, or from other homozygous patients carrying the same mutation.

SNP Haplotype

SNP haplotypes were identified by single strand conformation polymorphism (SSCP) at 10 polymorphic sites in the *ATM* gene [Castellvi-Bel et al., 1999; Campbell et al., 2003]. These are in complete linkage disequilibrium over >133 kb [Thorstenson et al., 2001].

Mutation Identification

DNAs were sequentially screened for mutations by protein truncation testing (PTT) [Telatar et al., 1996], SSCP [Castellvi-Bel et al., 1999], and denaturing high performance liquid chromatography (dHPLC) [Thorstenson et al., 2001]. Regions that were identified as abnormal were sequenced using an automated method (ABI 3700) or manual radiolabeled sequencing

(USB). Mutations were confirmed in both forward and reverse directions.

Identifying Phase

The fragment sizes obtained for each marker were compared between patients and respective parents to establish the allelic segregation. Next, we compared the STR haplotypes of all patients included in our database to identify possible recurring haplotypes.

RESULTS

Haplotypes

Thirty-two different STR haplotypes were observed in 27 Brazilian AT patients and 5 of them were seen more than once (i.e., recurring) in 55.5% of the families (Fig. 1). Although most recurring haplotypes were identical between patients, some variation among STR alleles was notable (especially for the marker S1818) and this may be a consequence of polymerase slippage or uneven crossing-over during cell division.

The SNP haplotypes H2, H3, H4, and H7 (Fig. 1) were identified in our patients, the first three being common worldwide. The H7 haplotype (BRAT20-3) has not been identified previously outside of African populations [Thorstenson et al., 2001]. We found approximately the same proportions of SNP haplotypes in our population as in the global population, with H2 being the most prevalent (67.9%) followed by H4 (24.5%).

Specific STR haplotypes were found to segregate with specific mutations (Table I). This is in accordance with our previous observations, which suggest that STR haplotypes and specific ATM mutations have evolved on specific ancestral SNP haplotypes [Campbell et al., 2003].

We observed a one repeat (CA) difference with regard to the marker D11S1819 for haplotype [5] (AT121LA and BRAT25-3) and haplotype [I] (BRAT26-3). In the case of haplotype [5], the two patients gained one repeat, whereas for haplotype [I] one repeat was lost. This most likely reflects microsatellite instability. We also observed instability with marker D11S2179 (BRAT26-3).

Mutations

Twenty-eight distinct mutations were identified on 50 of 54 chromosomes, and all but one would theoretically lead to a truncated form of the ATM protein (nonsense, splice site, or frameshift) (Table I). Mutations were located throughout the entire gene, without the occurrence of a mutation hotspot (Fig. 2).

Seven mutations have not been previously described: two nonsense (1520T > A, 4198A > T); three frameshifts (1330insCC, 8372ins16, 9080insA); one aberrant splicing (IVS11-2A > G), and one missense (8726G > C). The latter mutation (R2909T) was identified in BRAT5, in the highly conserved region in the kinase domain. A particularly interesting new mutation, 8372ins16, identified in BRAT29-3 duplicates 16 nucleotides immediately adjacent to the mutation site. The mutation IVS11-2A > G (BRAT[T]) disrupts the 3′ acceptor splice site and activates a new 3′acceptor site nine nucleotides downstream of the mutation site, causing the deletion of the first 7nt of exon 12.

Two large deletions were observed in our patients. The mutation IVS28 + 1711del 3450nt was observed in five families and deleted exons 29, 30, and part of 31. Like most large deletions, it is missed by conventional screening techniques in heterozygous patients, as the normal allele masks the deleted allele in PCR-based assays. In one such patient, because the second allele was located within the same region and, thus, occurred in hemizygous form, it was also missed by dHPLC [Mitui et al., 2003]. A homozygous deletion of ~17 kb was observed in BRAT3 between two nearly identical LINE repeats in the *ATM* gene. It was not possible to precisely define the break points. This mutation was previously identified in Costa Rican AT patients and also in patients of Iberian origins [Telatar et al., 1996; Mitui et al., 2003].

Polymorphisms and Variants

Nine variants were seen in the Brazilian AT patients in the course of searching for mutations, all of which have been reported previously [Hacia et al., 1998; Shayeghi et al., 1998; Castellvi-Bel et al., 1999; Li et al., 1999; Sandoval et al., 1999; Sommer et al., 2002; Buzin et al., 2003]. We identified the frequencies of each variant observed among AT patients in 100 chromosomes obtained from normal Brazilian controls (Table II). The missense variant, 2119T > C (S707P), identified in BRAT11-3, has been associated with breast cancer patients in a cohort study and deemed to be a polymorphism [Sommer et al., 2002]; mutagenesis studies are underway to functionally evaluate this DNA change. This variant was not idenfied in any of the Brazilian controls.

DISCUSSION

The large size of the *ATM* gene makes the screening for mutations expensive and labor intensive. The approach of initially haplotyping the propositi and relatives (to identify phases) has been shown to be a successful strategy in screening ethnic populations for mutations in the *ATM* gene [Fukao et al., 1998; Telatar et al., 1998a,b] (Fig. 3). This approach may be particularly important in characterizing mutations in other large genes. In developing countries, it may lower the costs of mutation detection.

The lack of detectable ATM protein levels by Western blot analysis and the abnormal CSA results (i.e., radiosensitivity) (data not shown) offer strong support for a clinical diagnosis of AT. All propositi in this study met these criteria. The diagnoses were also supported by finding mutations in all patients. Most nonsense mutations were associated with the classical AT phenotype in Brazil, suggesting that patients with milder phenotypes may exist who have non-truncating types of mutations as well.

Among the novel mutations identified in this study, six were predicted to truncate the ATM protein, either

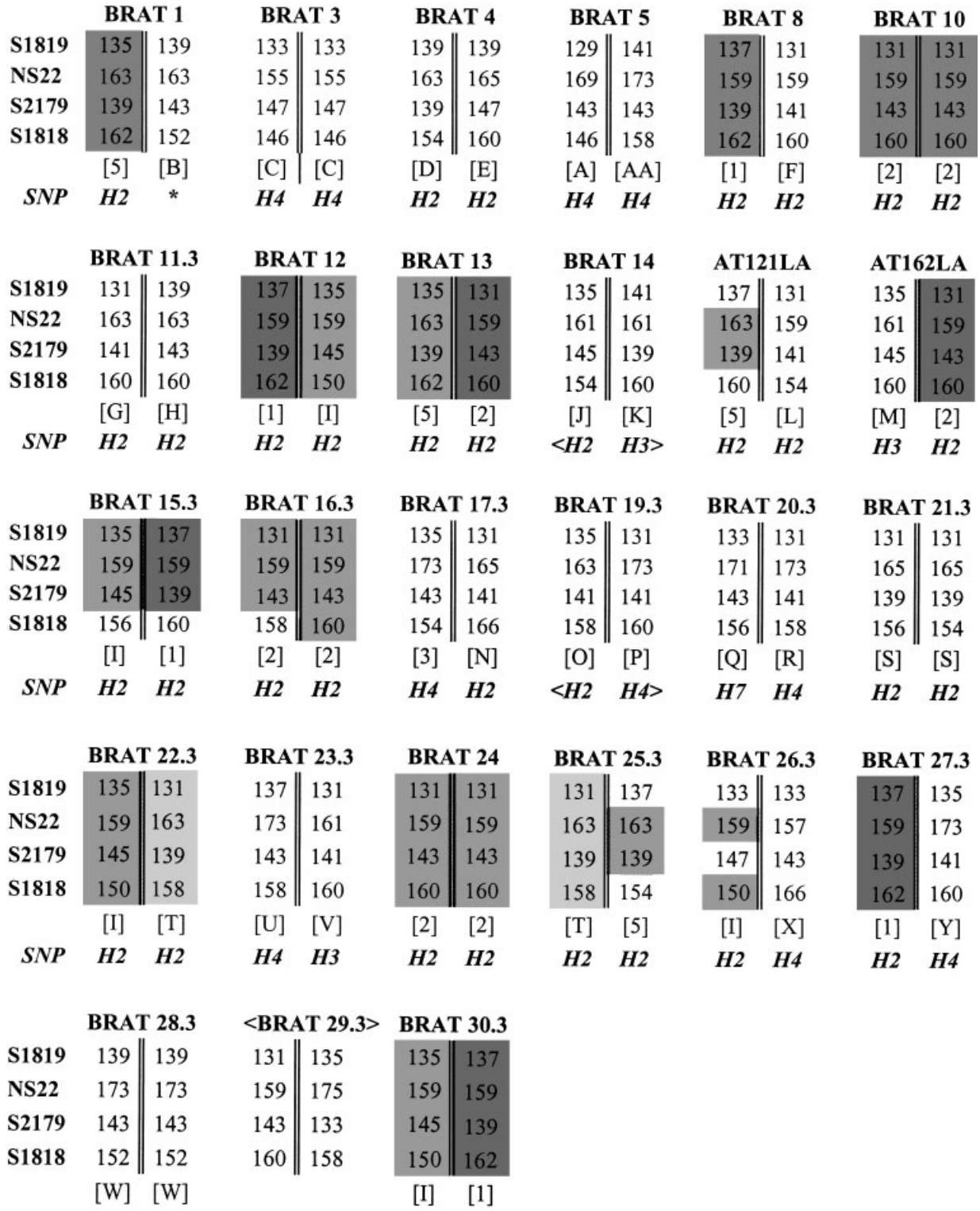

Fig. 1. STR and SNP haplotype distribution in Brazilian AT patients. The colors (shading) indicate those haplotypes that recur in at least two families. The *indicates insufficient DNA and <> indicates that the haplotype phase was not defined.

by frameshifts or by introducing a premature termination codon. A particularly interesting mutation, 8372ins16, identified in patient BRAT29-3, duplicates 16 nucleotides immediately adjacent to the mutation site. This duplication might be a consequence of unequal crossing-over. The haplotype phase associated with this mutation could not be established due to the absence of genetic material on the parents of the patient.

The only missense mutation identified in our case study, R2909T, which substitutes a conserved amino acid in the PI-3K domain, underscores the importance of this amino acid to the biological activity of the protein. More interestingly, this patient also carries a nonsense mutation, and in contrast to what was anticipated for a patient with one nonsense and one missense mutation [Becker-Catania et al., 2000], our patient does not show any detectable ATM protein. This suggests that the C-terminus end of the protein may also participate in the stabilization of the ATM protein [Becker-Catania et al., 2000].

TABLE I. Genotype and Haplotype/Mutations Correlation in the *ATM* Gene in Brazilian Patients With Classical A-T Phenotype

Patient	Genotype	STR haplo	Mutation	Ex/In	Consequence
BRAT1	Compd	[5]	8264delATAAG	58	Deletion of Ex 58
	Htz	[B]	*		
BRAT3	Hom	[C]	IVS63del~17 kb	IVS63	Frameshift
BRAT4	Compd	[D]	IVS28 + 1G > A	IVS28	(?) Deletion of 120nt
	Htz	[E]	1563delAG	12	Frameshift
BRAT5	Compd	[A]	**4198A > T**	30	Nonsense
	Htz	[AA]	**8726G > C**	62	R2909T
BRAT8	Compd	[1]	3802delG	28	Frameshift
	Htz	[F]	7884delTATAT	55	Frameshift
BRAT10	Hom	[2]	IVS28 + 1711del3450 bp	IVS28	del Ex 29–31
BRAT11-3	Compd	[G]	ND		
	Htz	[H]	7517delGAGA	53	(?) del of Ex 53
BRAT12	Compd	[1]	3802delG	28	Frameshift
	Htz	[I]	7913G > A	55	Nonsense
BRAT13	Compd	[2]	IVS28 + 1711del3450 bp	IVS28	del Ex 29–31
	Htz	[5]	8264delATAAG	58	Deletion of Ex 58
BRAT14	Compd	[J]	1110C > G	11	Nonsense
	Htz	[K]	IVS54-3T > G	IVS54	(?) del of Ex 55
AT121LA	Compd	[5]	8264delATAAG	58	Deletion of Ex 58
	Htz	[L]	3485T > G	26	Nonsense
AT162LA	Compd	[M]	4002delCTTA	29	Frameshift
	Htz	[2]	IVS28 + 1711del3450 bp	IVS28	del Ex 29–31
BRAT15-3	Compd	[I]	7913G > A	55	Nonsense
	Htz	[1]	3802delG	28	Frameshift
BRAT16-3	Hom	[2]	IVS28 + 1711del3450 bp	IVS28	del Ex 29–31
BRAT17-3	Compd	[3]	5644C > T	39	Nonsense
	Htz	[N]	8395del10	59	Frameshift
BRAT19-3	Compd	[O]	8620C > T	61	Nonsense
	Htz	[P]	4303A > T	31	Nonsense
BRAT20-3	Compd	[Q]	7792C > T	55	Nonsense
	Htz	[R]	4732C > T	33	Nonsense
BRAT21-3	Hom	[S]	**1520T > A**	12	Nonsense
BRAT22-3	Compd	[I]	7913G > A	55	Nonsense
	Htz	[T]	**IVS11-2A > G**	IVS11	del first 7nt of Ex 12
BRAT23-3	Compd	[V]	IVS53-2A > C	IVS53	del Ex 54
	Htz	[U]	**9080insA**	65	Frameshift
BRAT24	Hom	[2]	IVS28 + 1711del3450 bp	IVS28	del Ex 29–31
BRAT25-3	Compd	[T]	**IVS11-2A > G**	IVS11	del first 7nt of Ex 12
	Htz	[5]	8264delATAAG	58	Deletion of Ex 58
BRAT26-3	Compd	[I]	7913G > A	55	Nonsense
	Htz	[X]	**1330insCC**	12	Frameshift
BRAT27-3	Compd	[1]	3802delG	28	Frameshift
	Htz	[Y]	ND		
BRAT28-3	Hom	[W]	640delT	8	Frameshift
BRAT29-3	Compd	<>	ND		
	Htz	<>	**8372ins16**	59	Frameshift
BRAT30-3	Compd	[I]	7913G > A	55	Nonsense
	Htz	[1]	3802delG	28	Frameshift

ND, not yet detected.
*Insufficient DNA.
<>Phase not defined.
New mutations in bold.
(?) Theoretical consequence.

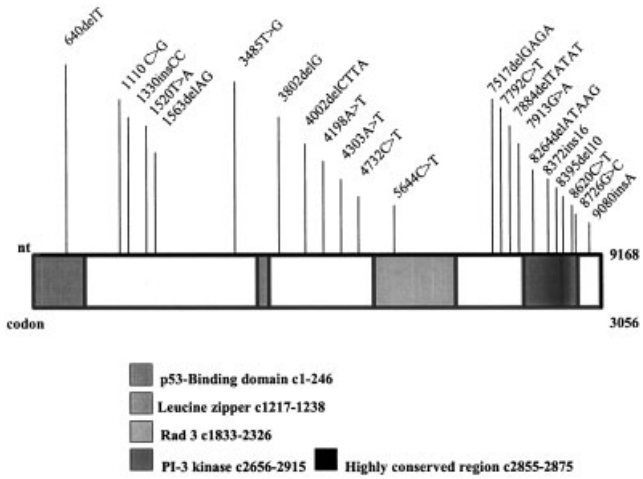

Fig. 2. Distribution of ATM mutations identified in Brazilian AT patients within the coding region.

Five mutations identified in the studied group had been previously observed in different European populations: Spain (640delT, 5644C > T, 8264delATAAG), Germany (3802delG), and Italy (7517delGAGA) (Gilad et al., 1996; Mitui et al., 2003]. These data corroborate the genetic findings of other authors that a variety of European backgrounds contribute to Brazilian lineages [Salzano and Freire-Maia, 1967; Raskin et al., 1999; Carvalho-Silva et al., 2001].

TABLE II. Frequencies of Polymorphisms Identified in Brazilian AT Patients Among 50 Normal Brazilians

Ex/In	Variant	Amino acid change	Allelic frequency (wt:variant) (N = 100 chromosomes)
IVS4	IVS4 + 37insAA		40:60
15	2119T > C	S707P	100:0
22	IVS22-77T > C		16:84
IVS24	IVS24-9delT		78:22
IVS25	IVS25-12insA		20:80
32	4578C > T	P1526P	81:19
41	5793T > C	A1931A	99:1
48	IVS48-69insATT		87:13
62	IVS62-65G > A		100:0

Despite the recent colonization of Brazil by Europeans, Africans, and Amerindians, two unique founder effect mutations were found (haplotypes [I] and [T]). The mutation 7913G > A, associated with the recurring haplotype [I], introduces a premature termination codon. Although most of the nonsense mutations were identifiable by the protein truncation test, this one mutation failed, on repeated testing, to show a truncated band and was detected by SSCP instead. Because the truncation would occur in two overlapping PTT regions, we think that it should have been detected by PTT in at least one of the two regions.

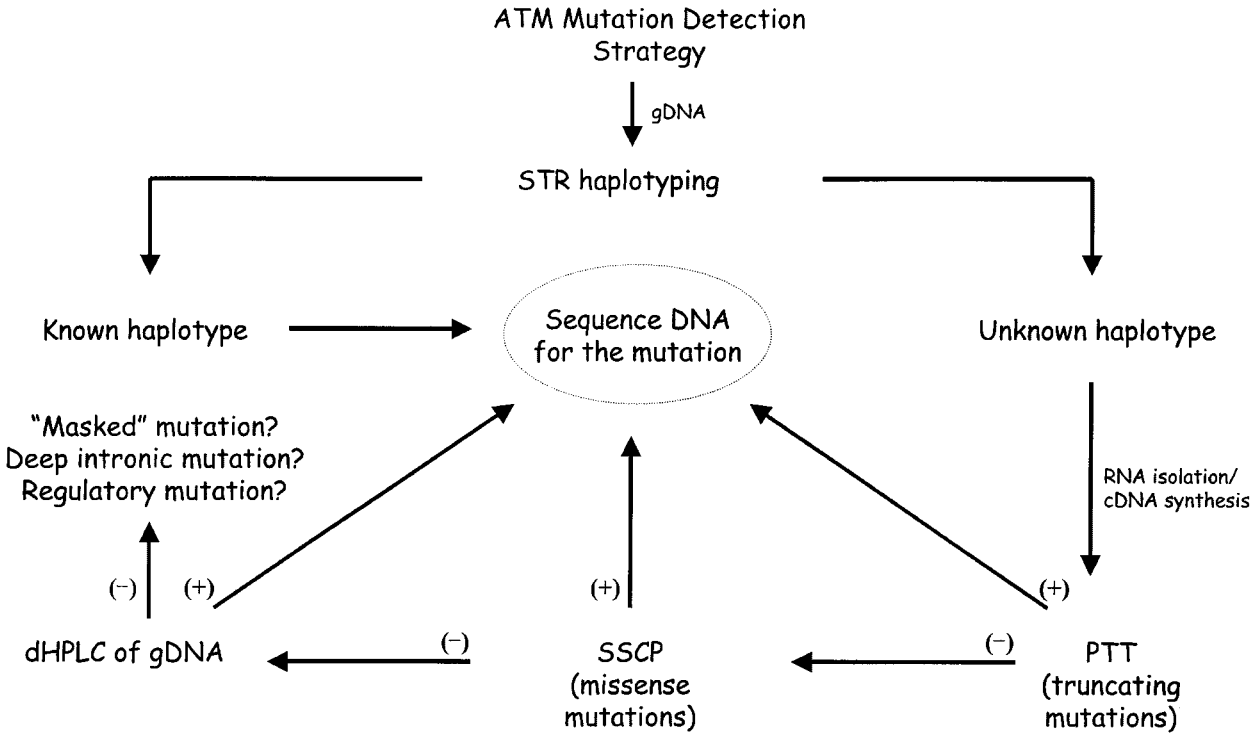

Fig. 3. ATM mutation detection strategy. After laboratory confirmation of the AT diagnosis standardized STR haplotypes are identified; if the haplotype is recognized, the associated mutation is confirmed by direct DNA sequencing. A database of STR haplotypes and associated mutations greatly simplifies the identification of ATM mutations. If the STR haplotype is not recognized, RNA is isolated, cDNA prepared, and protein truncation testing (PTT) performed to detect truncating mutations. If PTT fails to identify regions of mutations, either single strand conformation polymorphism (SSCP) or denaturing high performance liquid chromatography (dHPLC) is performed to screen for all types of mutations. Similarly, if abnormal regions are identified in either technique, DNA is sequenced for mutation identification.

PTT has been shown to be a very useful tool for the identification of nonsense mutations in the *ATM* gene [Telatar et al., 1996, 1998a,b; Teraoka et al., 1999] and in fact, most of our ATM mutations are detected by PTT. Nevertheless, premature termination codons should trigger the degradation of mRNAs by the nonsense mediated decay (NMD) pathway [Maquat and Carmichael, 2001; Byrd, 2002]. Despite this, we and others routinely detect a truncated RNA message but not a truncated protein. This calls into question whether the NMD pathway is intact in AT cells. ATX/hSMG1 has been shown to be one of the PI-3 kinases responsible for the phosphorylation of Upf1 [Yamashita et al., 2001], thereby participating directly in the NMD pathway. The catalytic similarities between ATX and ATM suggest that Upf1 may also be a phosphorylation target of ATM.

By establishing a Brazilian spectrum of haplotypes and associated mutations, we hope to provide valuable information towards the counseling of Brazilian families with AT and in the early diagnosis of very young patients. In general, we recommend prescreening new AT patients for haplotypes that are common to the ethnicity of the particular family before analyzing the entire *ATM* gene for mutations. Furthermore, the growing evidence that ATM mutations may account for susceptibility to various cancers in heterozygotes as well as homozygotes makes it important to understand the ATM mutation spectrum on a global basis.

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