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Short communication

Mucolipidosis II and III alpha/beta in Brazil: Analysis of the *GNPTAB* gene

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ABSTRACT

Mucolipidosis II and III (MLII and MLIII) alpha/beta are rare autosomal recessive lysosomal storage diseases (LSDs) caused by pathogenic variations in the *GNPTAB* gene. *GNPTAB* gene codes for the α and β subunits of phosphotransferase, the enzyme responsible for synthesis of the mannose-6-phosphate (M_6P) marker that directs lysosomal enzymes to the lysosome.

Objectives: The objective of this study is to identify sequence variations of the *GNPTAB* gene in Brazilian patients with MLII and MLIII alpha/beta.

Method: Sequencing of the *GNPTAB* gene was performed in samples of gDNA extracted from the peripheral blood of patients with MLII/III diagnosed at a national reference center for LSDs.

Results: Twelve unrelated patients, from several regions of Brazil, were included in this study. Only one was born of consanguineous parents. All patients were found to carry at least one nonpathogenic variation. Nine causal sequence variations were found: c.242G>T (p.W81L); c.1123C>T (p.R375X); c.1196C>T (p.S399F); c.1208T>C (p.I403T); c.1514G>A (p.C505Y); c.1759C>T (p.R587X); c.2808A>G (p.Y937_M972del, novel mutation); c. 2269_2273delGAAAC (p.E757KfsX2, novel mutation); and c.3503_3504delTC (p.L1168QfsX5). Both pathogenic variations were identified in 8 of 12 patients; in four patients, only one pathogenic variation was identified. Mutation c.3503_3504delTC, located in exon 19, was the most frequent pathogenic variation found (n = 11/24 alleles). The deleterious effect of the c.2808A>C mutation on splicing was confirmed by cDNA analysis.

Discussion/conclusions: Our findings confirm that the *GNPTAB* gene presents broad allelic heterogeneity and suggests that, in Brazilian ML II and III patients, screening for mutations should begin at exon 19 of the *GNPTAB* gene. Further analyses will be conducted on patients in whom both pathogenic mutations have not been found in this study.

Abbreviations: A, adenine; ARSA, arylsulfatase A; CAA, mRNA codon for amino acid glutamine; CAG, mRNA codon for amino acid glutamic acid; cDNA, DNA complementary to RNA; CNPq, Conselho Nacional de Pesquisa e Desenvolvimento; CUA, mRNA codon for amino acid leucine; CUG, mRNA codon for amino acid leucine; DBS, dried blood spot; dNTPs, deoxyribonucleoside triphosphate; EC, enzyme code; ELISA, enzyme-linked immunosorbent assay; FAPERGS, Fundação de Amparo à Pesquisa do estado do Rio Grande do Sul; FIPE, Fundo de Incentivo à Pesquisa e Eventos do Hospital de Clínicas de Porto Alegre; GAGs, glycosaminoglycans; gDNA, genomic deoxyribonucleic acid; GlcNAc-PT, UDP-N-acetylglucosamine:lysosomal hydrolase N-acetyl_1-phosphotransferase; *GNPTAB*, official symbol for the gene "N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits"; *GNPTG*, official symbol for the gene "N-acetylglucosamine-1-phosphate transferase, gamma subunit"; HCPA, Hospital de Clínicas de Porto Alegre; IDS, iduronate-sulfatase; IDUA, α-L-iduronidase; IEM, inborn errors of metabolism; kDa, kilodaltons; LC, liquid chromatografy; MS, mass spectrometry; LREIM-HCPA, Laboratório de Erros Inatos do Metabolismo Hospital de Clínicas de Porto Alegre; LSD, lysosomal storage disease; $MgCl_2$, magnesium chloride; ML II, mucolipidosis type II; ML III, mucolipidosis type III; mM, millimolar; mRNA, messenger RNA; M_6P, mannose-6-phosphate; PCR, polymerase chain reaction; Pmol, picomol; PolyPhen, polymorphism phenotyping; PRONEX, Programa de Apoio a Núcleos de Excelência; RNA, ribonucleic acid; SIFT, sorting intolerant from tolerant; *taq* DNA polymerase, thermostable DNA polymerase.

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1. Introduction

Mucolipidosis type II alpha/beta (MLII; MIM#252500) and type III alpha/beta (MLIII; MIM#252600) are inherited autosomal recessive diseases caused by deficient activity of UDP-N-acetylglucosamine: lysosomal hydrolase N-acetyl-1-phosphotransferase (UDP-GlcNAc-phosphotransferase, GlcNAc-PT or phosphotransferase; EC 2.7.8.17) (Brooks et al., 2007; Tiede et al., 2005; Zarghooni and Dittakavi, 2009). Phosphotransferase plays a role in the synthesis of mannose-6-phosphate (M_6P), a marker molecule responsible for directing lysosomal hydrolases to the lysosome (Braulke et al., 2008; Cathey et al., 2008; Tiede et al., 2005). In the absence of M_6P residues, correct targeting of lysosomal hydrolases is impaired, which results in massive secretion of these enzymes in both the extracellular space and body fluids, as well as a decrease of their activity in cells such as fibroblasts (Braulke et al., 2008; Cathey et al., 2008; Encarnação et al., 2009). MLII is the most severe form of the disease, and is often apparent at birth; progression is rapid, leading to death as early as the first decade of life due to cardiorespiratory complications. Conversely, MLIII follows a slower clinical course, and patients have been known to survive until the eighth decade of life (Cathey et al., 2010; Encarnação et al., 2009; Kornfeld and Sly, 2001). MLII and III correspond to the extreme phenotypes associated with phosphotransferase, deficiency, and patients with intermediate clinical manifestations have also been described (Cathey et al., 2010).

Phosphotransferase is a hexameric protein composed of two α subunits, two β subunits, and two γ subunits (Bao et al., 1996; Tiede et al., 2005), where the α and β subunits are encoded by the *GNPTAB* gene, located in chromosome 12q23.3, and the γ subunits encoded by the *GNPTG* gene, located in chromosome 16p13.3 (Cathey et al., 2008; Encarnação et al., 2009; Kudo et al., 2005; Zarghooni and Dittakavi, 2009). Patients who are homozygous or compound heterozygous for pathogenic mutations in *GNPTAB* (ML alpha/beta) exhibit a phenotype consistent with MLII or MLIII, whereas patients who are homozygous or heterozygous for pathogenic mutations in *GNPTG* (ML gamma) exhibit a phenotype compatible with MLIII (Bargal et al., 2006; Cathey et al., 2010; Encarnação et al., 2009; Persichetti et al., 2009; Tappino et al., 2009).

The main objective of the present study was to conduct an analysis of the *GNPTAB* gene in Brazilian patients with MLII and III alpha/beta.

2. Materials and methods

Twelve unrelated patients with a biochemical diagnosis of MLII or MLIII were included in the study. They were recruited from the cohort of patients (approximately 40,000) investigated for inborn errors of metabolism (IEM) at the Reference Laboratory for IEM at Hospital de Clínicas de Porto Alegre, Brazil (LREIM-HCPA), from 1983 to 2011. The LREIM-HCPA is a national reference laboratory for the diagnosis of lysosomal diseases, and its database of diagnoses probably includes most cases of MLII/III diagnosed in Brazil. Biochemical diagnosis of MLII/III at LREIM-HCPA includes analysis of arylsulfatase A (ARSA; EC 3.1.6.8) and several other lysosomal hydrolases, including α-L-iduronidase (IDUA; EC 3.2.1.76), iduronate-sulfatase (IDS; EC 3.1.6.12), β-glucuronidase (GUSB; EC 3.2.1.31), and β-hexosaminidase (EC 3.2.1.30), in plasma. Measurement of the activity of these enzymes in fibroblasts, as well as analysis of glycosaminoglycans (GAGs) and sialyloligosaccharides in urine, are also performed whenever samples are available. If only filter paper samples are available, the activity of IDS, GUSB, and hexosaminidases is analyzed.

Whole blood samples were collected for gDNA and RNA extraction. Determination of clinical severity (ML II or III) took into account the criteria usually reported in the literature, such as age at diagnosis, survival, and extent of skeletal involvement (and, consequently, patient height) (Cathey et al., 2010). The patients included in this study were under the care of different physicians, but a summary of their clinical records was always sent alongside each patient's blood sample, so that the classification assigned by the attending physician was always reviewed and confirmed by the investigators. Whenever possible, a sample of gDNA from the parents was also obtained for confirmation of the presence of the mutations found in the patient.

Genomic DNA was extracted from peripheral blood leukocytes with the DNeasy Blood and Tissue Kit (Qiagen, Germany). The 21 exons that comprise the *GNPTAB* gene, as well as the intron-exon boundaries and part of the 5′ and 3′ untranslated regions, were amplified from the specific sequences of oligonucleotides projected for this study. Amplification was performed by the polymerase chain reaction (PCR) using 50 ng of gDNA, 16 pmol of each oligonucleotide, 0.6 mM of dNTPs, 2.4 mM of $MgCl_2$, 1× reaction buffer, and 1 unit of *taq* DNA polymerase. The annealing temperatures and oligonucleotide sequences are available as supplementary data.

Sample sequencing was performed using the automatic ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). The reference sequence of the *GNPTAB* gene was GenBank accession nº. NM_024312.3. Each mutation found was confirmed by sequencing performed with a new amplicon and the oligonucleotide inverse to that used in the first stage.

In silico analysis of the potential effect of missense mutations was done by means of PolyPhen2 (Polymorphism Phenotyping)

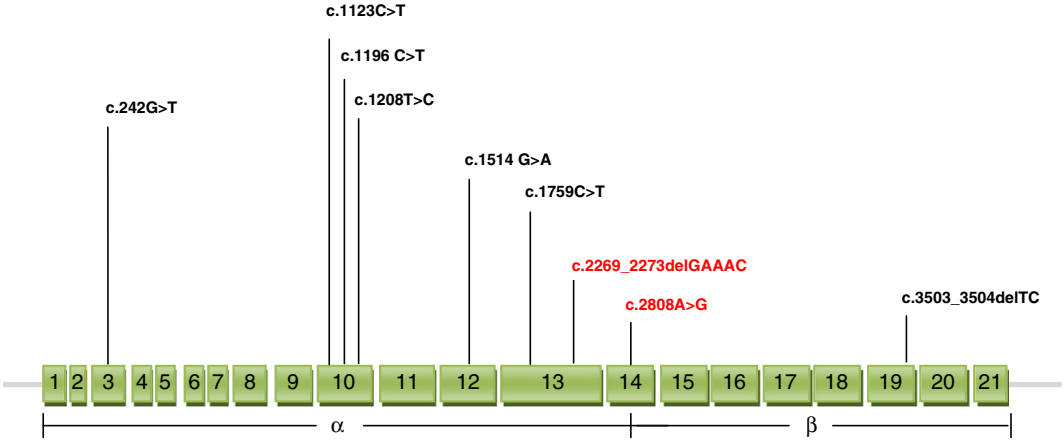

Fig. 1. *GNPTAB* gene: localization of pathogenic mutations found in the present study (modified from Tappino et al. (2009)). In red: mutations described for the first time in the present study. In black: mutations previously described in the literature. The α- and β-subunits are represented.

(http://genetics.bwh.harvard.edu.pph2/) and SIFT (Sorting Intolerant From Tolerant) (http://sift.jcvi.org/www.SIFT_seq_submit2.html). The MaxEntScan software was used to evaluate the effect of point mutations on generation or exclusion of splice sites (http://http://genes.mit.edu/burgellab/maxent/Xmaxentscan_scoreseq.html). The frequency of synonymous codon usage was evaluated according to the database of codon usage in *Homo sapiens* (http://www.kasuza.or.jp/codon/cgi-bin/showcodon.cgi?species=9606). To evaluate the pathogenicity of the novel exonic point mutation found (c.2808A>C), a hundred control alleles were analyzed using 0.3 U of *Hyp 188I* restriction enzyme (New England Biolabs, USA), 2 μl of buffer 4 (supplied with the restriction enzyme) and 17.7 μl of amplicon; samples were incubated for 3 h at 37 °C. Total RNA extraction was performed on a whole blood sample from the patient presenting this mutation using the Paxgene blood RNA Kit (Qiagen, Germany), and conversion to cDNA was made using the Superscript II conversion kit (Invitrogen, USA), according to manufacturer instructions. cDNA was subsequently sequenced as described above.

This study was approved by the Hospital de Clínicas de Porto Alegre (HCPA) Research Ethics Committee.

3. Results

Of the patients included, eight were classified as having MLII. Only one (patient 12) was born of consanguineous parents. Regarding biochemical investigation, fibroblast samples were unavailable for three patients (patients 6, 7 and 12); patient 6 was investigated only through a dried blood spot (DBS) sample. Urinary GAG measurements were normal in 10 of 10 patients, thin-layer chromatography of GAGs was abnormal in 4 of 10 patients (patient 4, dermatan/keratan sulfate; patients 5 and 7, dermatan sulfate; patient 9, keratan sulfate), while thin-layer chromatography of sialyloligosaccharides was abnormal in 1 of 3 patients (patient 10). Detailed data on the clinical and biochemical findings presented by the patients are available as supplementary material.

Nine causal sequence variations were found, two of which were novel: c.2808A>G (p.Y937_M972del) and c. 2269_2273delGAAAC (p.E757KfsX2) respectively (Fig. 1, Table 1). Both pathogenic mutations were identified in 8 of 12 patients, and only one pathogenic mutation was identified in four patients (Table 1). Mutation c.3503_3504delTC, located in exon 19, was the most frequent pathogenic mutation (n = 11/24 alleles; Table 1).

At least one nonpathogenic variant was found in each patient. The mutations c.365+145C>T in intron 4 (n = 17/24) and c.-41_-39delGGC in the 5′UTR (n = 16/24) were the most frequent nonpathogenic variants found, and the nonpathogenic mutation c.323+20delT (intron 3) has not been reported elsewhere. Additional information on nonpathogenic mutations is available as supplementary material.

3.1. The c.2808A>G mutation

As the c.2808A>G mutation (exon 14) was apparently not associated with an amino acid change, we decided to investigate its effect on splicing. According to MaxEntScan software (http://http://genes.mit.edu/burgellab/maxent/Xmaxentscan_scoreseq.html) results, this mutation would strongly interfere with *GNPTAB* mRNA splicing (Fig. 2). In fact, this mutation creates a similar sequence to the canonical donor splice site. cDNA analysis for this patient showed the presence of an abnormal transcript in which the final 108 bp of exon 14 is absent (Fig. 3). We did not perform expression studies, but this isoform is predicted to generate a truncated protein (p.Y937_M972del), with deletion of 36 amino acids of the phosphotransferase β-subunit. This mutation was not found in any of the 100 control alleles analyzed (Fig. 4).

Table 1
Pathogenic mutations found in the *GNPTAB* gene of 12 unrelated Brazilian patients with Mucolipidosis II and III alpha/beta.

Patients	Genotype (cDNA)	Genotype (protein)	Phenotype	In silico analysis of the effect in the protein*			Parental genotype**
				Polyphen2	SIFT	MaxEntScan	
1	c.[1123C>T]+[?]	p.[R375X]+[?]	ML II	NA	NA	NI	Mother: c.[1123C>T]+[N]
2	c.[1514G>A]+[1759C>T]	p.[C505Y]+[R587X]	ML III	Probably deleterious	Intolerable	NI	Mother: c.[1514G>A]+[N] Father: c.[1759C>T]+[N]
3	c.[1208T>C]+[3503_3504delTC]	p.[I403T]+[L1168QfsX5]	ML III	Probably deleterious	Intolerable	NI	Mother: c.[1208T>C]+[N] Father: c.[3503_3504delTC]+[N]
4	c.[3503_3504delTC]+[?]	p.[L1168QfsX5]+[?]	ML II	NA	NA	NA	Mother: c.[N]+[N] Father: NA
5	c.[242G>T]+[?]	p.[W81L]+[?]	ML II	Probably deleterious	Intolerable	NI	NA
6	c.[2269_2273delGAAAC]+[2269_2273delGAAAC]	p.[E757KfsX2]+[E757KfsX2]	ML II	NA	NA	NA	Mother: c.[2269_2273delGAAAC]+[N]
7	c.[2808A>G]+[3503_3504delTC]	p.[Y937_M972del]+[L1168QfsX5]	ML II	NA	NA	Probably splice site interference (exon 14)	Mother: c.[3503_3504delTC]+[N] Father: [c.2808A>G]+[N]
8	c.[1196C>T]+[3503_3504delTC]	p.[S399F]+[L1168QfsX5]	ML III	Probably deleterious	Intolerable	NI	Mother: c.[1196C>T]+[N] Father: c.[3503_3504delTC]+[N]
9	c.[3503_3504delTC]+[?]	p.[L1168QfsX5]+[?]	ML III	NA	NA	NA	NA
10	c.[3503_3504delTC]+[3503_3504delTC]	p.[L1168QfsX5]+[L1168QfsX5]	ML II	NA	NA	NA	NA
11	c.[3503_3504delTC]+[3503_3504delTC]	p.[L1168QfsX5]+[L1168QfsX5]	ML II	NA	NA	NA	Mother: c.[3503_3504delTC]+[N] Father: c.[3503_3504delTC]+[N]
12	c.[3503_3504delTC]+[3503_3504delTC]	p.[L1168QfsX5]+[L1168QfsX5]	ML II	NA	NA	NA	Mother: c.[3503_3504delTC]+[N] Father: c.[3503_3504delTC]+[N]

?: Unidentified mutation; NA: not analyzed; NI: no interference; N: normal allele. Polyphen2 is available at http://genetics.bwh.harvard.edu/pph2 and classifies variations as benign/possibly deleterious/or probably deleterious. SIFT is available at http://sift.jcvi.org/www/SIFT_seq_submit2.htm and classifies variation as tolerable or intolerable. MaxEntScan is available at http://genes.mit.edu/burgeldo/maxent/Xmaxentscan_scoreseq.html.

* Conducted only in case of missense mutations.

** Parents were analyzed only in relation to the mutations found in the proband.

4. Discussion

This is the first study to describe the pathogenic/nonpathogenic mutation spectrum of the *GNPTAB* gene in Brazilian patients with MLII and III alpha/beta. As in other populations, this analysis confirmed that *GNPTAB* exhibits great allelic heterogeneity, that there are few recurrent mutations, and that c.3503_3504delTC is the most prevalent pathogenic mutation (Bargal et al., 2006; Cathey et al., 2010; Kudo et al., 2006; Paik et al., 2005; Tiede et al., 2005). Furthermore, three novel sequence variations (two pathogenic) are described herein.

The number of patients included in this study (n = 12) is significant, considering that all patients were Brazilian and that ML is quite rare and severe (http://www.geneclinics.org). Unfortunately, not all patients had undergone biochemical investigation in plasma and/or fibroblasts. It bears stressing, however, that all patients exhibited a clinical phenotype consistent with MLII or MLIII. Patient 6 was investigated using filter paper only, as described by Chamoles et al. (2001); as this patient was later confirmed as having ML II alpha/beta through DNA analysis, this finding corroborates the hypothesis that DBS samples can also be used for MLII/III screening. Interestingly, four patients exhibited an abnormal pattern on urinary thin-layer chromatography of GAGs, although their levels of total urinary GAGs were normal. Abnormal catabolism of GAGs is expected to occur in patients with MLII/III, as lysosomal enzymes involved in the catabolism of GAGs are deficient in these disorders. Levels of keratan sulfate (determined by ELISA), as well as dermatan sulfate and heparan sulfate (determined by LC/MS/MS), were found to be increased in plasma of some patients with MLII/II (Tomatsu et al., 2005, 2010). However, secretion of abnormal GAGs is usually too low in MLII/III patients to be detected by thin-layer chromatography.

The methodology used in the present study was able to identify 20 of 24 pathogenic alleles (roughly 83%). This rate is not very different from that reported in the literature (approximately 95%) (http://www.geneclinics.org). The most frequently found pathogenic mutation was c.3503_3504delTC, which was present in homozygosity in three MLII patients (patients 10, 11 and 12) and in heterozygosity in another two MLII patients (patients 4 and 7) and three MLIII patients (patients 3, 8 and 9); the prevalence found for this mutation in our study (45%; 11 of 24 alleles) is similar to that described in the literature. Bargal et al. (2006) found this same mutation in 13 of 24 MLII patients, most of Arab-Muslim origin (11 homozygous; 2 compound heterozygous; 10 born to consanguineous couples). Encarnação et al. (2009) found this microdeletion in five patients with MLII (n = 9 of 18 alleles); four of these patients were homozygous for the mutation. Tappino et al. (2009) identified this mutation in 47 of 92 alleles; it was found in homozygosity in 14 patients with MLII, most of whom were Italian and born to non-consanguineous couples. Mutation c.3503_3504delTC was the only pathogenic mutation identified in the sample studied by Plante et al. (2008).

4.1. Effect on phenotype

Our data suggest that nonsense and frameshift mutations are associated with the severe phenotype (MLII alpha/beta), whereas missense mutations are associated with the attenuated phenotype (MLIII alpha/beta). These findings are in agreement with those previously described in the literature (Bargal et al., 2006; Encarnação et al., 2009; Tiede et al., 2005).

Thus far, c.3503_3504delTC, located at the region that codes for the β subunit of phosphotransferase, has been associated with the severe phenotype when found in homozygosity or when found in heterozygosity with nonsense mutations or frameshift mutations. Three of our patients with MLII alpha/beta presented this deletion in homozygosity (patients 10, 11 and 12). However, two patients with MLIII alpha/beta (patients 3 and 8, respectively) presented this deletion in heterozygosity with the missense mutations c.1208T>C (p.I403T) and c.1196C>T (p.S399F), both located at the region that codes for the α subunit of phosphotransferase. Other authors have also reported cases of compound heterozygosity for mutations in the α and β subunits (Cathey et al., 2010; Tappino et al., 2009), and Bargal et al. (2006) suggest that there is no intragenic complementation between these subunits. The genotype c.[1196C>T]+[3503_3504delTC] (patient 8) was also reported in a MLIII French patient by Bargal et al. (2006). In that same study, the authors reported that this patient exhibited a severe MLIII phenotype and that fibroblast testing showed cytoplasmic inclusions typical of patients with MLII. Our patient may also be considered a patient with severe MLIII alpha/beta, given the severity of his skeletal compromise, which is reflected by his present height. The c.[1208T>C]+[3503_3504delTC] genotype, on the other hand, is being described for the first time herein (patient 3, MLIII alpha/beta). Mutation c.1208T>C (p.I403T) has already been described by Tappino et al. (2009) in homozygosity in an Italian patient with MLIII, as well as by Encarnação et al. (2009) in a Portuguese patient. Expression studies of the mutant p.I403T protein in COS cells conducted by Tappino et al. (2009) showed that this mutation had an expected molecular mass of 170 kDa, and the authors presumed that the resulting protein would be partially dysfunctional, which could explain the attenuated phenotype exhibited by these patients.

Genotype c.[1514G>A]+[1759C>T] was found in one patient with MLIII alpha/beta (patient 2). The first is a missense mutation (p.C505Y), while the second is a nonsense mutation (p.R587X). This

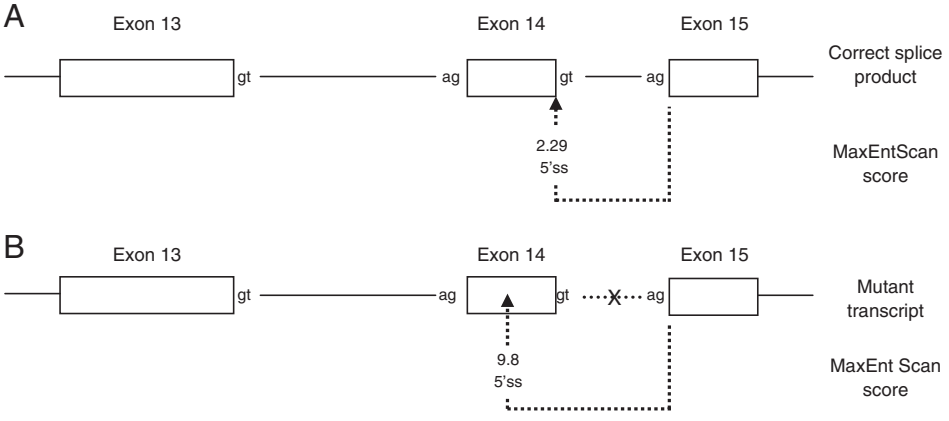

Fig. 2. Schematic representation of exons and introns 13, 14 and 15 of the *GNPTAB* gene and scores obtained with the MaxEntScan program for wild (A) and mutated (B) forms obtained in the presence of c.2808A>G. Dotted line: new splicing. X: splicing replaced.

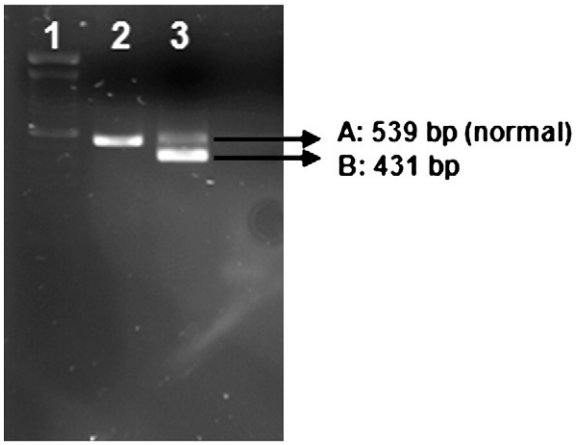

Fig. 3. Electrophoresis of RT-PCR for the c.2808A>G mutation (patient 7) showing the normal transcript (A) and the alternative transcript (B). 1: Standard molecular weight; 2: negative control for c.2808A>G; 3: patient sample.

genotype is being reported herein for the first time, but the mutations have already been reported by Cathey et al. (2010), in compound heterozygosity, in a patient with MLII (p.R587X) and in one patient with MLIII (p.C505Y).

Genotype c.[242G>T]+[?] was found in a patient with ML II alpha/beta (patient 5). Encarnação et al. (2009) described this mutation, in homozygosity, in one of their Portuguese patients with MLIII. One hypothesis that might justify the phenotype of patient 5, with only one pathogenic mutation identified, would be the presence of two silent mutations in exon 1 of the second allele: c.18G>A and c.27G>A. The first induces a change from the sixth CUG codon (frequency of codon usage per thousand = 39.6) to CUA (frequency of codon usage per thousand = 7.2). The second mutation changes the ninth CAG codon (frequency of codon usage per thousand = 34.2) to CAA (frequency of codon usage per thousand = 12.3) (http://www.kasuza.or.jp/codon/cgi-bin/showcodon.cgi?species=9606). This could cause a greater change in the translation kinetics of the protein, generating a protein with an altered conformation (Komar, 2007; Sauna and Kimchi-Sarfaty, 2011). Unfortunately, parental DNA and patient fibroblasts were unavailable, and, therefore, we are not able to confirm whether these mutations are in *cis* or in *trans*. Additional studies will be conducted to confirm this hypothesis.

5. Conclusions

This was the first DNA analysis-based study conducted in Brazilian patients with ML alpha/beta. Its findings suggest that analysis of *GNPTAB* in these patients should begin by exon 19, thus optimizing the investigation and reducing costs. The results obtained herein emphasize the need for further studies, such as application of other techniques that will enable completion of genotyping of the patients whose genotype has not been fully characterized (e.g. sequencing of promoter and intronic regions, exclusion of large deletions/rearrangements), and for determination of the frequency of recurrent variants in healthy Brazilian individuals.

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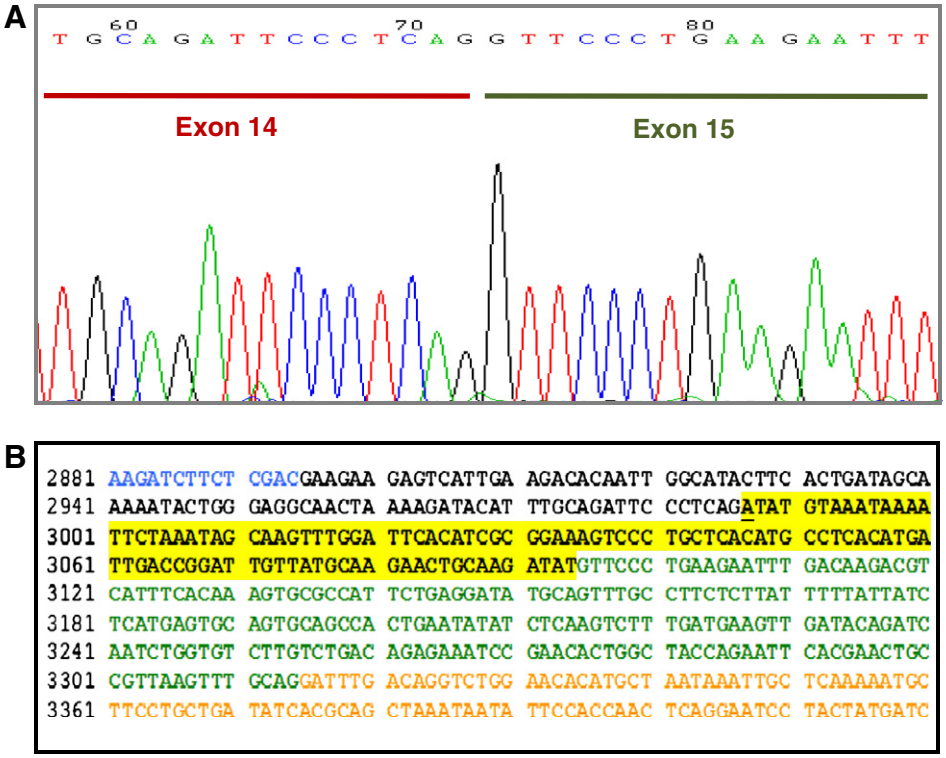

Fig. 4. A) Patient 7 (genotype: c.2808A>G; p.Y937_M972del): Sequencing of the abnormal fragment amplified by RT-PCR. B) Normal cDNA sequence of the *GNPTAB* gene. In blue, part of exon 13; in black, exon 14; in green, exon 15; in orange, exon 16; underlined, mutation site; in yellow, 108 bp lost due to the c.2808A>G mutation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2013.03.105.

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