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Identification and characterization of *SMPD1* mutations causing Niemann-Pick types A and B in Spanish Patients

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Abstract

Niemann-Pick disease (NPD) types A/B are both caused by a deficiency of lysosomal acid sphingomyelinase and display autosomal recessive inheritance. These two types of the disease were described according to the presence (type A) or absence (type B) of neurological symptoms. We present a molecular analysis of 19 Spanish NPD A/B patients and two from Maghreb. Eight of the patients had type A and 13 had type B NPD. All mutant *SMPD1* alleles were identified, including 17 different mutations, 10 of which were novel. The only frequent mutations in the 21 NPD patients were c.1823_1825delGCC (p.R608del) (38%) and c.1445C>A (p.A482E) (9%). Genotype-phenotype correlations were established for most of the mutations and, in particular, the p.R608del-type B association was confirmed. This mutation accounts for 61.5% of the mutant alleles in the type B subgroup of patients. Expression studies performed on six of the identified mutations confirmed them to be disease-causing due to their low enzyme activity. An allele with a mutation affecting a non-canonical donor splice site produced only aberrant mRNAs corresponding to previously reported non-functional *SMPD1* minor transcripts. This study is the first exhaustive mutational analysis of Spanish Niemann-Pick A/B disease patients.

Keywords

Niemann-Pick A/B; Acid sphingomyelinase deficiency; *SMPD1*; heterologous expression

Introduction

Niemann-Pick disease (NPD) types A/B is an autosomal recessive sphingolipidosis caused by lysosomal acid sphingomyelinase (ASM, E.C. 3.1.4.12) deficiency. Type A (MIM# 257200) is an infantile neurovisceral fatal form characterized by massive hepatosplenomegaly and rapidly progressive neurological deterioration, and type B (MIM# 607616) presents a purely visceral form and survival till adulthood. In addition, some

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patients are described as presenting an intermediate phenotype that includes neurological involvement, cherry-red maculae and mental retardation (Schuchman and Desnick, 2001). Note that Niemann-Pick type C (MIM# 257220), which is outside the scope of this study, is a different disease caused by mutations in other (*NPC1* or *NPC2*) genes. For this reason, Niemann-Pick types A and B are sometimes referred as acid sphingomyelinase deficiency (ASMD).

The acid sphingomyelinase gene (*SMPD1*; MIM# 607608, GenBank accession number M81780.1) is composed of 6 exons and is located on chromosome 11p15.1-11p15.4 (Schuchman and Desnick, 2001). Three types of human ASM transcripts have been identified. The type 1 transcript is the major ASM species and encodes a catalytically active enzyme. Type 2 and 3 ASM transcripts result from alternative splicing, most likely due to the presence of a weak donor splice site in intron 3. Type 2 and 3 transcripts do not encode functional enzymes (Schuchman and Desnick, 2001).

The human ASM protein is synthesized as a 75 KDa pre-polypeptide, which is converted into a precursor form of 72 KDa. The precursor is subjected to two different processing events. A minor part is cleaved in the endoplasmic reticulum-Golgi complex, yielding a 57 KDa form, whilst a major part is processed to a 70 KDa mature form (Ferlinz, et al., 1994).

To date, nearly one hundred different mutations have been described (see Human Gene Mutation Database: <http://www.hgmd.org/>), but only a few have been found with a relatively high frequency in specific populations. This is the case, for example, of mutation c.1823_1825delGCC (p.R608del), which was first described by Levran et al. (1991) and is always associated with the type B phenotype. This mutation has been found with a low frequency in Europe, for instance, 9.4% in Italy (Pittis, et al., 2004). In contrast, it was observed in more than 85% of the mutant alleles from Northern-African NPD type B patients (Vanier, et al., 1993). Mutation spectra for specific populations have been published for only a few countries, such as Italy (Ricci, et al., 2004, Pittis, et al., 2004) or the Czech Republic and Slovakia (Pavlu-Pereira, et al., 2005). In some cases, expression studies have been performed (Dardis, et al., 2005, Pavlu-Pereira, et al., 2005, etc.).

Here we present clinical and molecular findings for 21 unrelated NPD A/B patients diagnosed in the last twenty years in Spain. In particular, the identification of 17 *SMPD1* mutations, ten of which are novel, is reported. To confirm their pathogenicity, eight of the mutant alleles were characterized by *in vitro* expression in COS-7 cells.

Subject and Methods

Patients

Nineteen of the twenty-one unrelated NPD patients were of Spanish origin. Patients 2 and 17 were from Maghreb. According to the clinical presentation and age of onset, eight patients were classified as type A and thirteen patients as type B. Patients were diagnosed at the Institut de Bioquímica Clínica, Hospital Clínic, Barcelona.

DNA extraction and mutation identification

Genomic DNA was extracted from patients' leucocytes or cultured fibroblasts using standard protocols. Each exon and the intron boundaries of the *SMPD1* gene were PCR-amplified using self-designed oligonucleotides (primer sequences are available upon request). PCR products were directly sequenced in the forward and reverse directions using the Big Dye Terminator Cycle Sequencing v3.1 Kit (PE Applied Biosystems, Foster City, CA, USA), in an ABI PRISM 3700 DNA analyzer (PE Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions.

Gene nucleotides were numbered according to sequence RefSeq NM_000543.3, with +1 as A of the ATG start codon. The ATG codon represents +1 for amino acid numbering, according to the preprotein sequence NP_000534.3.

cDNA and NMD analyses for mutation c.1257+4_1257+7delAGGG

RNA was isolated from patient and control skin fibroblast cultures using QIAshredder (Qiagen, Hilden, Germany) to homogenize the sample, followed by the RNeasy mini kit (Qiagen, Hilden, Germany). RNA integrity was verified by 1% agarose gel electrophoresis, and the concentration was determined spectrophotometrically at OD 260nm. Single-stranded cDNA was synthesized using an oligo-dT primer and the M-MLV reverse transcriptase, RNase H Minus (Promega, Madison, WI, USA). The polymerase chain reaction was performed using Expand High Fidelity Taq polymerase (Roche, Mannheim, Germany) and the following primers: NPB14F1 (5'GGCGAATACAGCAAGTGTGA3') in exon 2 and NPB14R1 (5'TGAGTGTGGCCAAAGAACTG3') in exon 5, for the non-specific PCR; NPB14F2 (5'CTGGCTCTATGAAGCGATGG3') in exon 2 and NPB14R2 (5'ATCTGGCATCAGGTGGCGG3') in exon 6, for the specific amplification of the c. 1257+4_1257+7delAGGG allele. The resulting PCR fragments were purified with GFX PCR DNA and Gel Band purification kit (Amersham Pharmacia Biotech, Amersham, UK) and sequenced. For nonsense-mediated mRNA decay (NMD) studies, fibroblasts from the patient and control were cultured in the presence of two different concentrations of cycloheximide (500 and 1000 microg/ml) for 6h.

Vector construction

The entire coding region of the *SMPD1* cDNA was amplified by PCR in two fragments. These two fragments were digested, ligated and cloned in a pBluescript vector to obtain the whole cDNA, which was confirmed by direct sequencing. Mutagenesis was performed on the *SMPD1*-pBluescript plasmid. For protein expression, the mutant and wild-type cDNAs were subcloned into the pcDNA3.1 expression vector.

Site-directed mutagenesis

All mutations were introduced in the wild-type full length *SMPD1* cDNA cloned in pBluescript by PCR-based site-directed mutagenesis using the QuickChange II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The oligonucleotides used to generate the mutations are listed in Supp. Table S1. All constructs were resequenced to ensure that no spurious mutation had been introduced by the mutagenesis procedure.

Cell culture and transient transfection

COS-7 cells were grown in 100mm tissue culture dishes with Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, UK) supplemented with 10% fetal bovine serum and antibiotics (Gibco, Grand Island, NY). For the transfection, 30×10^5 cells were split in antibiotic-free medium and transfected twenty-four hours later (when cells were at 90% confluence) with 3 microg of wild-type or mutant plasmid, using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). As a negative control, an empty pcDNA3.1 vector was transfected. Forty-eight hours after transfection, cells were harvested by scraping and centrifuging. Cellular extracts were washed twice with PBS and stored at -80°C until acid sphingomyelinase analysis was performed. Approximately 10% of the cellular pellets volume was put aside for Western blotting.

Enzymatic analysis

Acid sphingomyelinase activity was measured in COS-7 transfected cells using the fluorogenic substrate 6-hexadecanoylamino-4-methylumbelliferyl-phosphorylcholine (Moscerdam Substrates, Rotterdam, the Netherlands). Protein concentration was determined by the Lowry method. In every expression experiment, transfections were performed three times for each mutation as well as for the wild-type construct and for an empty vector used as a negative control. Two independent experiments were carried out for each mutant allele. The residual enzymatic activity of the mutant alleles was expressed as a percentage of the mean of the activity of the wild-type construct transfected in the same experiment. The activity of the negative control was subtracted.

SDS-PAGE and Western blot analysis

Protein extracts from transfected COS-7 cells (35 microg of protein/lane) were subjected to SDS-PAGE (12.5% polyacrylamide) and electrophoretically transferred onto nitrocellulose membranes. Each membrane was blocked with 5% non-fat milk in PBS, containing 0.2% Tween 20 (PBST). The blotted membranes were probed with polyclonal anti-human acid sphingomyelinase antibody (He, et al., 1999) for 4 h in 3% non-fat milk in PBS with 0.1% Tween and then washed 4 times with PBST and another 4 times with PBS. Anti-rabbit IgG antibody (Sigma-Aldrich, UK) was used as a secondary antibody, and the immunoreactive bands were detected by incubation of the membrane for 2 minutes in the following solution: 10 ml of 100mM Tris-HCl pH 9, 50 microl of 45 mM p-coumaric acid, 50 microl of luminol and 10 microl of 30% H₂O₂. Anti-alpha-tubulin monoclonal antibody (Sigma-Aldrich, UK) was used to detect alpha-tubulin as loading control.

Statistical analysis

The Mann-Whitney U test was used to analyze significant differences in enzyme activities between pairs of mutant and/or wild-type enzymes.

Results

Mutation analyses

We analysed samples from 21 unrelated NPD patients (8 type A and 13 type B) to identify the molecular defects. We identified seventeen different mutations, ten of which were novel. The genotypes and clinical features of the patients are shown in Supp. Table S2. Four of the novel mutations were missense changes [c.1100A>G (p.Y367C), c.1400A>C (p.Y467S), c.1445C>A (p.A482E) and c.1456A>G (p.T486A)], two were nonsense changes [c.503G>A (p.W168X) and c.939C>A (p.Y313X)], and four were small deletions [c.1159delC (p.R387VfsX7), c.1169_1171delTCT (p.F390del), c.1257+4_1257+7delAGGG, and c.1774_1776delACT (p.T592del)]. Of the new mutations, the most frequent was p.A482E, identified in 9.5% (4/42) of the alleles (Table 1). None of the new missense mutations were found in one hundred control alleles. Of the seven previously reported mutations, only p.R608del had a high frequency: 38% (16/42) of total alleles. All the patients carrying this mutation presented with type B of the disease and p.R608del accounted for 61.5% of type B patient alleles.

Haplotype analyses

Haplotype analyses were performed to determine whether frequent mutations had a unique origin. We analysed twenty previously reported polymorphisms: five in exon 1, seven in exon 2 and eight in exon 6. Only four of them were polymorphic in our patient series: the hexanucleotide repeat polymorphism c.103CTGG(T/C)(G/T)[4_9] (exon 1) reported by Wan and Schuchman (1995) in the signal peptide, and the SNPs c.107C>T (exon 1), c.

1516G>A (exon 6), and c.*45G>A (exon 6, 3'UTR). In our series of patients, five out of the six patients homozygous for the p.R608del mutation were also found to be homozygous for the haplotype [7-C-G-G] (alleles of the four polymorphisms were in the order described above). The only exception was patient 13 who was homozygous for [8-C-G-G]. It should be mentioned that in the original work, Wan and Schuchman did not find the 8-repeat allele for the hexanucleotide polymorphism. Patient 11, homozygous for mutation p.A482E, was also homozygous for the haplotype [6-T-G-G]. The haplotypes of patients 6 and 20, with genotype p.[R608del] + [A482E], were consistent with those associated with each mutation alone, although the phases could not be confirmed as samples from the parents of these patients were not available.

Expression and enzyme activity of wild-type and mutant acid sphingomyelinase in COS-7 cells

The functional effect on enzymatic activity of some of the *SMPD1* mutations reported in Spanish patients was evaluated by in vitro expression in COS-7 cells. Wild type *SMPD1* cDNA or cDNAs bearing the following mutations: p.V130A (c.389T>C), p.L225P (c.674T>C), p.R228C (c.682C>T), p.Y367C (c.1100A>G), p.Y467S (c.1400A>C), p.A482E (c.1445C>A), p.T486A (c.1456A>G) and p.R608del (c.1823_1825delGCC) were transiently transfected in COS-7 cells. Mutations p.Y367C, p.Y467S, p.A482E and p.T486A were the four novel missense changes, while p.R228C and p.R608del had been described before. The previously studied p.V130A and p.L225P were expressed for comparative purposes. As a negative control, cells were transfected with an empty pcDNA3.1 vector. The mean of wild-type acid sphingomyelinase activity in COS-7 cells was 291.33 ± 48.94 nmol/h/mg, whereas the average endogenous enzyme activity (COS-7 cells transfected with an empty pcDNA3.1) was 5.29 ± 2.79 nmol/h/mg. The residual enzymatic activities for each protein, given as a percentage of that of the wild-type enzyme, are shown in Figure 1. Alleles bearing mutations p.R228C, p.Y367C, p.Y467S, p.A482E and p.T486A resulted in low levels of activity, ranging from 1.81 to 4.75%. The changes p.V130A, p.L225P and p.R608del produced enzymes with 33.26, 11.03 and 21.46% of wild type activity respectively. Table 2 shows the statistical analysis of the activity values between all pairs of mutant enzymes with detectable levels of activity and between each of them and the wild-type protein. All changes show residual enzymatic activities that were statistically different from those of the wild-type enzyme. No significant differences were detected for p.Y367C, p.Y467S and p.A482E in all pair-wise comparisons. The p.R228C allele activity differed significantly from all the mutants except for p.T486A.

To confirm the correct synthesis of recombinant proteins in COS-7 cells, immunoblot analysis of the wild-type and mutant proteins was performed. A unique band of 70 KDa, corresponding to the mature ASM protein, was detected in all cases using polyclonal anti-human acid sphingomyelinase antibody (Fig. 2).

Characterization of the c.1257+4_1257+7delAGGG mutation

A novel mutation consisting of a 4-bp deletion in the donor splice site of intron 3 was identified in the sample from patient 14. In order to test whether this mutation affected the splicing process, RNA from the patient was analysed. Since the mutation could probably generate an mRNA with a premature stop codon and thus be susceptible to degradation by the nonsense-mediated mRNA decay (NMD) mechanism, fibroblasts from the patient were cultured in the absence or presence of cycloheximide (CHX), an inhibitor of the NMD process. Three transcripts were detected in the patient's sample, the smallest of which was only visible after CHX treatment (Fig. 3A). Sequencing of the patient cDNA from CHX non-treated and treated fibroblasts showed that the two additional transcripts corresponded to the type 2 and 3 ASM transcripts described by Schuchman et al. (1991). These authors

demonstrated the existence of three human ASM transcripts due to alternative splicing. Type 1 transcript is the major ASM species and is the only one that encodes a catalytically active enzyme. Type 2 and 3 ASM transcripts result from alternative splicing. Exon 3 is skipped in both of them. In type 2 transcripts, a donor site located 40 nucleotide downstream of the canonical exon 2 donor site is used. In type 3 transcripts, exon 2 is directly joined to exon 4. Mutation c.1257+4_1257+7delAGGG precludes the use of the exon 3 donor site and causes exon 3 to be skipped, leading to the formation of type 2 and 3 ASM transcripts. To confirm that no functional transcript was produced by the c.1257+4_1257+7delAGGG allele, an allele-specific PCR reaction was performed. For this purpose, one of the primers included the region of the p.R608del mutation, present in the other allele of patient 14. Thus, only alleles that did not bear the R608del mutation would be amplified (the c.1257+4_1257+7delAGGG allele of the patient and any wild type allele). As shown in Figure 3B, no normal-size transcript was observed for the patient. The band present in the CHX lane is actually a double band, which contains the amplified products of transcripts 2 and 3, as made evident by the digestion of the PCR products with the *HinfI* restriction enzyme. The band patterns for patient NPB14 indicate the presence of transcripts 2 and 3, the latter only visible after CHX treatment (Fig. 3C).

Discussion

The mutation analysis of 21 NPD patients from Spain, 8 with the type A and 13 with the type B, allowed all of the mutant alleles to be identified. Seventeen different mutations were detected, 10 of which had not been previously described. Only one mutation (p.R608del) was frequent in this patient population (16 alleles, 38%). It is also one of the most frequently reported mutations in other populations and it was found to be always associated with the type B of the disease (Levrán, et al., 1991). Reported frequencies of mutation p.R608del in type B NPD patients were: 9.4% in Italy (Pittis, et al., 2004), 12% in 324 patients of multiethnic origin (Simonaro, et al., 2002), 86.6% in Northern-Africa (Vanier, et al., 1993) and 100% in four patients from the Canary Islands (Fernandez-Burriel, et al., 2003). In our series, it was also only found in type B patients and occurred at a very high frequency (61.5%). Moreover, due to the mild phenotype associated with homozygosity for this mutation, a number of cases might remain undiagnosed.

The novel mutation p.A482E was the second most frequent change in Spanish NPD patients (9.5%). Our results indicate that this new missense mutation is associated with the severe form of the disease, since patient 11, the only one homozygous, was diagnosed as NPD type A due to the early onset and severity of the clinical presentation.

Six out of the eight type A patients were homozygous for a different mutation each. Thus, it can be concluded that mutations p.W168X, p.Y313X, p.F390del, p.Y467S and p.T592del (as well as the aforementioned p.A482E), found in homozygosity, are associated with this severe form of the disease. The same correlation can be established for mutations p.G245S, p.Y367C and p.H421R, found in compound heterozygotes. Although the effect of the small-deletion mutations p.F390del and p.T592del was not confirmed by expression studies, the fact that they were both found in homozygous type A patients suggests that residues F390 and T592 are more relevant for the ASM protein than R608, responsible for type B disease.

In the three type B patients not bearing the p.R608del mutation, the mutations responsible for the mild phenotype seemed to be p.R376H, p.R474W and p.R228C, since either the accompanying allele was null or were found in homozygosity. These three mutations had previously been associated with type B disease (Simonaro, et al., 2002).

Mutation p.T486A is the only novel missense mutation for which no association can be determined since it was found in a patient who was a compound heterozygote with p.R608del, responsible for the B phenotype. Only the expression analysis could provide some information (see below).

Haplotype analyses were performed in patients carrying the most frequent mutations, p.R608del and p.A482E. This analysis suggests a common origin for most of the p.R608del alleles in Spain. Similarly, the results are consistent with a possible unique origin for mutation p.A482E.

Regarding the *in vitro* expression study, 6 mutations found in Spanish patients, 4 of which are novel, were analysed and a significant reduction in the enzyme activity was found for all of them. Thus, all can be considered disease-causing mutations. Only mutation p.R608del has relatively high residual activity (21.5% of wild-type activity), consistent with the less severe B phenotype with which it is associated. The other type B mutation, p.R228C presents a much lower activity (4.75%) than that of p.R608del. In fact, this result is in agreement with other studies that showed a wide spectrum of residual activity of NPB associated mutations (ranging from 0 to about 30% of the wild type enzyme) when expressed *in vitro*. Mutation p.T486A was found in heterozygosity with p.R608del in a type B patient and no genotype-phenotype correlation could be established. The fact that its activity (4.15%) was similar to that of p.R228C suggests that it could be associated with type B phenotype. Mutations p.Y367C, p.Y467C and p.A482E were associated with type A, according to the clinical features of the patients (see above). These mutations consistently showed negligible residual activity. It should be noted that all mutant proteins, including those with very low residual activity, were synthesized at normal levels, as shown by Western blot analysis. These *in vitro* results are in agreement with what was observed in fibroblasts of NPD A/B patients homozygous for p.R608del and other *SMPD1* mutations, where the amount of mutant protein in the patients' fibroblasts was found to be similar to that of the wild type (Jones, et al., 2008).

Mutations p.V130A and p.L225P were not found in our series of patients but were expressed for comparison. We found 33.3% and 11.0% of wild-type activity respectively for these mutations. Dardis et al. (2005) had already expressed these mutations, identified in Italian type B patients, and found 13% and 0% of activity. The differences between the two studies could be due to the fact that Dardis et al. used COS-1 cells, whereas we used COS-7 cells. However, it is clear that p.V130A retained some residual activity in both studies. Moreover, the levels of activity found in the present work seem consistent with the type B phenotype presented by the Italian patients.

Mutation c.1257+4_1257+7delAGGG lies in the intron 3 donor site, but does not affect the canonical GT dinucleotide. Thus, the effect on RNA processing should be analysed. *In silico* predictions using the Splice Site Score Calculation website (http://rulai.cshl.edu/new_alt_exon_db2/HTML/score.html) showed a score reduction from 8 to 1.7 when the mutation was present, indicating a possible drastic effect in the splicing process. To confirm this hypothesis, cDNA analyses were performed. The results undoubtedly showed that no normal mRNA was produced from the mutant allele. In contrast, two mRNAs lacking exon 3 were produced, one of them including 40 pb of intron 2. These mRNAs have been previously described as minor transcripts of the *SMPD1* gene, found in wild-type cells, which do not code for active enzymes. One of these transcripts was degraded by the NMD mechanism, as shown in the present study by CHX treatment. All these results showed that the 4-bp deletion was a severe, disease-causing mutation.

In summary, this is the first report on the molecular analysis of Spanish Niemann-Pick type A/B patients, and one of the few relatively exhaustive studies on a specific population reported so far. Ten novel mutations were identified and some of them were characterized at the levels of enzyme activity or RNA processing. Genotype-phenotype correlations were established for most of the mutations and, in particular, the p.R608del-type B association was confirmed. These results provide useful information for the management of Niemann-Pick A/B patients. Moreover, they may help to establish criteria to select individuals with appropriate genotypes for a successful enzyme replacement treatment, which will be hopefully available in the near future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Residual acid sphingomyelinase activity of the proteins expressed in COS-7 cells from wild-type and mutant alleles, expressed as the percentage of wild-type (WT) activity. The data are shown as mean $\pm$ SD of two different experiments, each performed in triplicate. Error bars correspond to standard deviation. WT: wild-type.

Figure 2.

Western blot analysis of wild-type and mutant acid sphingomyelinase proteins expressed in COS-7 cells. The “COS” lane corresponds to cells transfected with an empty pcDNA3.1 plasmid. A total of 35µg of protein extract was loaded in each lane. ASM: acid sphingomyelinase; TUB: tubulin; WT: wild-type.

Figure 3.

A: RT-PCR amplification of RNA extracted from patient 14 (NPB14) or WT fibroblasts, untreated (NT) or treated (CHX) with cycloheximide, using non-specific primers (see Methods). **B:** specific RT-PCR amplification of alleles that do not bear the p.R608del mutation (the c.1257+4_1257+7delAGGG allele of patient 14 or wild-type alleles), on the same samples described in part A of the Figure. **C:** digestion with *HinfI* of the same RT-PCR products shown in B. The expected patterns after *HinfI* digestion for each transcript are: 394, 282 and 147 bp (transcript 1); 282, 262 and 147 bp (transcript 2); 282, 222 and 147 bp (transcript 3). The 222-bp band (arrowhead), specific for transcript 3, is only observed after CHX treatment. M: molecular weight marker.

Table 1
Mutations found in patients analysed in the present study

Exon / intron	Mutation	Coding effect	Number of alleles	Reference
2	c.503G>A	p.W168X	2	This report
2	c.682C>T	p.R228C	2	Simonaro, et al., 2002
2	c.733G>A	p.G245S	2	Simonaro, et al., 2002
2	c.939C>A	p.Y313X	2	This report
3	c.1100A>G	p.Y367C	1	This report
3	c.1128G>A	p.R376H	1	Simonaro, et al., 2002
3	c.1159delC	p.R387VfsX7	1	This report
3	c.1169_1171delTCT	p.F390del	2	This report
in3	c.1257+4_1257+7delAGGG	Splicing	1	This report
4	c.1262A>G	p.H421R	1	McGovern, et al., 2006
4	c.1321C>T	p.R441X	1	Schuchman, 1995
5	c.1400A>C	p.Y467S	2	This report
5	c.1420C>T	p.R474W	1	Simonaro, et al., 2002
5	c.1445C>A	p.A482E	4 (9.5%)	This report
5	c.1456A>G	p.T486A	1	This report
6	c.1774_1776delACT	p.T592del	2	This report
6	c.1823_1825delGCC	p.R608del	16 (38.1%)	Levrán, et al., 1991

Gene nucleotides were numbered according to sequence RefSeq NM_000543.3, with +1 as A of the ATG start codon. The ATG codon represents +1 for amino acid numbering, according to the preprotein sequence NP_000534.3.

Table 2
Significance of the differences in pair-wise comparisons between mutant enzymes with detectable levels of activity and the wild-type enzyme

V130A	L225P	R228C	Y367C	Y467S	A482E	T486A	R608del	Alleles	n
P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	Wild-type	18
	p=0.002	p=0.002	p=0.002	p=0.002	p=0.002	p=0.002	p=0.004	V130A	6
		p=0.002	p=0.002	p=0.002	p=0.002	p=0.002	p=0.009	L225P	6
			p=0.002	p=0.002	p=0.015	NS	p=0.004	R228C	6
				NS	NS	p=0.002	p=0.004	Y367C	6
					NS	p=0.002	p=0.004	Y467S	6
						p=0.041	p=0.004	A482E	6
							p=0.004	T486A	6
								R608del	5

N: number of replicates; NS: not significant.