MUTATION IN BRIEF

A Point Mutation in the Lariat Branch Point of Intron 6 of *NPC1* as the Cause of Abnormal Pre-mRNA Splicing in Niemann-Pick Type C Disease

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The lariat branch point sequence (BPS) is crucial for splicing pre-mRNA even if BPS mutations have infrequently been reported in human disease. In two siblings with Niemann-Pick type C (NPC) disease we identified two mutations of the *NPC1* gene: i) one in exon 20 (c.2932C>T) (p.R978C) previously reported in NPC patients; ii) the other (c.882-28A>G) unreported, in the highly conserved adenosine of a putative lariat BPS of intron 6. Using RT-PCR we found that, besides the normally spliced mRNA, patients' fibroblasts contained minute amounts of an mRNA devoid of exon 7. The exon 6 - exon 8 junction in this mRNA causes a frameshift and a premature stop codon, predicted to result in a truncated protein. To assess the effect of c.882-28A>G mutation we constructed two minigenes (wild type and mutant), spanning from intron 5 to intron 8, which were inserted into a pTarget vector and transfectd in COS1 cells. The wild type minigene generated an mRNA of the expected size and sequence; the mutant minigene generated only an mRNA devoid of exon 7. This is the first example of a splicing defect due to a mutation in the lariat BPS in an intron of *NPC1* found in NPC patients. © 2004 Wiley-Liss, Inc.

KEY WORDS: Niemann-Pick tipe C disease; NPC1; splicing defect.

INTRODUCTION

Niemann-Pick type C disease (NPC) is a fatal autosomal recessive neuro-visceral disease with varying age of onset and ensuing course (Vanier and Millat, 2003). One of the hallmarks of the NPC disease is the intracellular accumulation of unesterified cholesterol and other lipids in various tissues. In NPC fibroblasts unesterified cholesterol (derived from both the internalisation of plasma low density lipoproteins and endogenous synthesis) accumulates in the late-endosome/ lysosomal compartment (Garver and Heidenreich, 2002; Wojtanik and Liscum, 2003). Complementation analysis using NPC fibroblasts indicated the presence of two complementation groups: i)

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Niemann-Pick type C disease 1 (NPC1, MIM# 257220) (the major group that comprises more than 95% of cases) and ii) Niemann-Pick type C disease 2 (NPC2, MIM# 607625) (the minor group) (Vanier and Millat, 2003). NPC1 is due to mutations of NPC1 gene (MIM# 607623) located on chromosome 18q11. The product of this gene (GenBank accession number NP_000262.1) is an integral membrane protein containing 1278 amino acids, which is highly conserved among animal species. It is localized in the late endosomal membrane and is intimately involved in cholesterol trafficking. (Vanier and Millat, 2003). More than 100 mutations of NPC1 gene with a large majority (71%) of missense mutations have been reported in NPC patients (Vanier and Millat, 2003; Park et al., 2003). Only few mutations in introns have been reported in NPC1 patients as putative pathogenic mutations (Millat et al., 2001; Ribeiro et al., 2001; Sun et al., 2001; Park et al., 2003). At present most databases contain annotation data that are primarily or exclusively derived from genomic DNA analysis and the effect of an intronic mutation on mRNA is usually predicted from the genomic DNA sequence rather than by experimentally determining mRNA expression and splicing pattern.

In the present study we describe the identification of a novel point mutation in the putative lariat branch point of intron 6 of NPC1 gene. This mutation disrupts pre-mRNA splicing in patient's fibroblasts and in COS1 cells transfected with a mutant NPC1 minigene.

MATERIALS AND METHODS

NPC Patients

The proband is 31 years-old male, born from non-consanguineous parents. Since the age of 10 he has been suffering from progressive impairment of fine movements and dysarthria. At the age of 28 he was found to have vertical gaze paralysis, severe dysarthria and dysphagia, ataxia, tremor and intellectual impairment. The proband's 25 year-old sister showed gait ataxia and dysarthria at the age of 20 and vertical gaze paralysis at 24.The result of filipin stain in skin fibroblasts of both siblings was consistent with a "variant" form of NPC (Millat et al., 2001). Informed consent was obtained from siblings' parents. The study protocol was approved by the human investigation committee of each participating institution. Skin biopsy was taken from both siblings.

Reverse Transcription of NPC1 mRNA and cDNA Sequencing

Total RNA extracted from cultured fibroblasts was retro-transcribed and NPC1 cDNA (GenBank accession number NM_000271.1) was amplified by PCR in 10 partially overlapping fragments as previously described (Tarugi et al., 2002). For the amplification of the exon 6 – 8 region of NPC1 cDNA, the following primers were used: 9SN (forward primer in the exon 6) 5'-TGGACGCCATGTATGTCATCATGTG-3' and 9ASN (reverse primer in the exon 8) 5'-GTCAACTGGATTGGTTGTGACCCG-3' under the following conditions: 30 cycles at 95°C for 1.5 min, 69°C for 1 min and 68°C for 1.5 min followed by a final extension at 68°C for 10 min. The purified amplification products were sequenced on an ABI PRISM ®3100 genetic analyzer.

PCR Amplification of Genomic DNA

Genomic DNA (gDNA) was extracted from cultured fibroblasts by a standard procedure. NPC1 gene was amplified and sequenced as previously described (Tarugi et al., 2002).

Construction of NPC1 Minigenes

To investigate the effects of the c.882-28A>G mutation in intron 6 of NPC1 gene (GenBank accession number AF157371.1) on pre-mRNA splicing, we constructed two minigenes designated MTgene (mutant) and WTgene (wild-type) containing the 3' end of intron 5, exon 6 (GenBank accession number AF157370.1) followed by the abridged intron 6, exon 7, intron 7, exon 8 and a truncated intron 8. The minigene constructs were generated from gDNA of the proband and of a control subject respectively, by ligating two PCR fragments designed F1 and F2, using EcoRI sites. F1 contained the 3'end of intron 5 (169 bp) followed by exon 6 and the 5'end of intron 6 (145 bp); F2 contained the 3' end of intron 6 (130 bp), exon 7, intron 7, exon 8 and the 5' end of intron 8 (27 bp).

F1 was amplified using the following primer pairs: ex6S (forward primer) 5'-CAT AGG ACG AAG CAG CAA AAC ATA-3' and 6AS (reverse primer) 5'-CGG AAT TCG ACA CAA TAA TCC AT-3', with the first eight nucleotides modified to introduce a EcoRI site. F2 was amplified using primer pairs: 7S (forward primer) 5'-CGG

AAT TCA GTA ATT AGG GAG GA-3' with the first eight nucleotides modified to introduce a EcoRI site and ex8AS (reverse primer) 5'-GAC TTA TTT CTT CAA ACA GCA GG-3'. The amplification conditions were 10 cycles at 95°C for 3 min, 95°C for 45 sec, 59°C for 45 sec, and 68°C for 7 min, followed by 20 cycles at 95°C for 45 sec, 59°C for 45 sec and 68°C for 7 min.

These minigenes were cloned in the pTargeT expression vector that contains the Neomycin resistance gene (NeoR gene) under the control of the SV40 early promoter. Plasmids of the minigene constructs were prepared using Qiagen Plasmid maxi kit (Qiagen) and sequenced. The two minigenes had the same sequence, with the exception of the c.882-28A>G mutation.

Expression of NPC1 Minigenes in Transfected Cells

COS1 cells were maintained in the DMEM containing 5-10 % FBS and used for transfection. Cells 6-7 x 105 were plated in 60 mm ∅ dishes. After 24 h, 4 μg of plasmids containing WTgene and MTgene were transfected using LipofectaMINE Plus reagent (Invitrogen, Life Technologies, Paisley, UK). Forty-eight hours after transfection, total RNA was extracted with Eurozol (Euroclone Ltd, Paignton, Devon, UK) and treated with RNase-free DNase (Promega). RT-PCR amplification of NPC1 minigene mRNA was performed using primers 9SN and 9ASN (see above). These primers did not amplify the endogenous NPC1 mRNA from COS1 cells (see Fig.3). The RT-PCR products were sequenced as described above. To provide an internal control for transfection efficiency, the mRNA of Neomycin-resistance gene (NeoR gene) was reverse transcribed and PCR amplified using primers previously described (Altilia et al., 2003).

Restriction Fragment Analysis of NPC1 Gene Mutation

The c.882-28A>G mutation of NPC1 gene introduced a new site for the restriction enzyme MaeI thus allowing a rapid screening of this mutation by restriction fragment analysis. The digestion of the region encompassing exon 7 of gDNA by MaeI generates two fragments of 156 bp and 22 bp in the case of the mutant allele, as opposed to a single fragment (178 bp) in the case of the wild type allele.

Mutation Nomenclature

All mutations are described according to mutation nomenclature (den Dunnen and Antonorakis, 2000; den Dunnen and Paalman, 2003; and http://www.hgvs.org/munomen/). Nucleotide numbers are derived from cDNA NPC1 sequences (GenBank accession number NM_000271.1). For cDNA numbering +1 corresponds to the A of the ATG translation initiation codon.

RESULTS

The strategy of investigation of NPC1 mutations we adopted implies the sequencing of both cDNA and gDNA. In a previous study this strategy allowed us to detect a point mutation in the middle of exon 22 resulting in a splicing defect, and to show monoallelic expression of a mutant allele in compound heterozygous NPC patients (Tarugi et al., 2002).

The first run of sequence of NPC1 cDNA isolated from proband's fibroblasts indicated that he was homozygous for a rare point mutation in exon 20 (c.2932C>T) predicted to result in a missense mutation (p.R978C), and for the rare alleles of common sequence variants: c.1926C>G (p.I642M); c.2572A>G (p.I858V); c.2793C>T (p.N931N); c.3797G>A (p.R1266Q) (Ribeiro et al., 2001; Park et al., 2003; Vanier and Millat, 2003) (data not shown). However, when we sequenced gDNA, we found that the proband was heterozygous for the rare mutation and all other sequence variants. This discrepancy between cDNA and gDNA sequence had been previously documented in other patients' heterozygotes for NPC1 gene mutations leading to mRNAs harbouring premature termination codon (Tarugi et al., 2002). The sequence of gDNA also revealed that the proband was heterozygous for a A>G transition at position –28 of intron 6 (c.882-28A>G) within a putative branch point sequence (reviewed but not shown). In view of the latter finding we thought that the patient was heterozygous for a splicing defect leading to a complete block in pre-mRNA processing and/or the formation of minute amounts of abnormally spliced mRNAs.

To test this hypothesis we amplified a restricted region of cDNA encompassing exons 6 – 8 so as to ascertain whether we could detect minute amounts of abnormally spliced mRNA that might have been overlooked in the

analysis of a longer fragment (637 bp) (Tarugi et al., 2002). Agarose gel electrophoresis of the exon 6- exon 8 fragment showed the presence of a major band of 344 bp (as expected) and a minor band of 270 bp (Fig. 1).

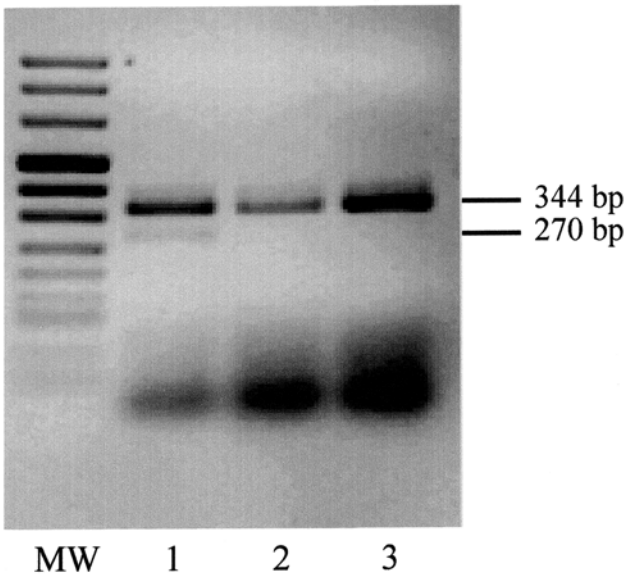

Figure 1. Agarose gel electrophoresis of NPC1 cDNA obtained by RT-PCR from RNA isolated from fibroblasts of the proband (1) and a control subject (2 and 3). Exons 6-8 region was PCR amplified and separated by 2% agarose gel electrophoresis. MW, molecular size markers.

Sequence analysis showed that in the 270-bp band exon 6 was followed by exon 8, with the complete skipping of exon 7 (Fig. 2). The exon 6-exon 8 junction disrupts the reading frame leading to a premature termination codon. The predicted translation product of this mRNA is a truncated protein of 299 amino acids (Fig. 2).

The gDNA sequence of proband's sister showed that she carried the same rare mutations and common sequence variants found in the proband. The analysis of cDNA isolated from her fibroblasts also confirmed the presence of minute amounts of the mRNA devoid of exon 7 (data not shown).

The analysis of NPC1 gDNA in the family members revealed that the rare mutation c.2932C>T (p.R978C), as well as all the rare alleles of the common sequence variants: c.1926C>G (p.I642M), c.2572A>G (p.I858V), c.2793C>T (p.N931N) and c.3797G>A (p.R1266Q), were transmitted by the father. The rare mutation in intron 6 (c.882-28A>G) was transmitted by the mother. Neither of the two rare mutations was found in 50 randomly selected individuals of our population.

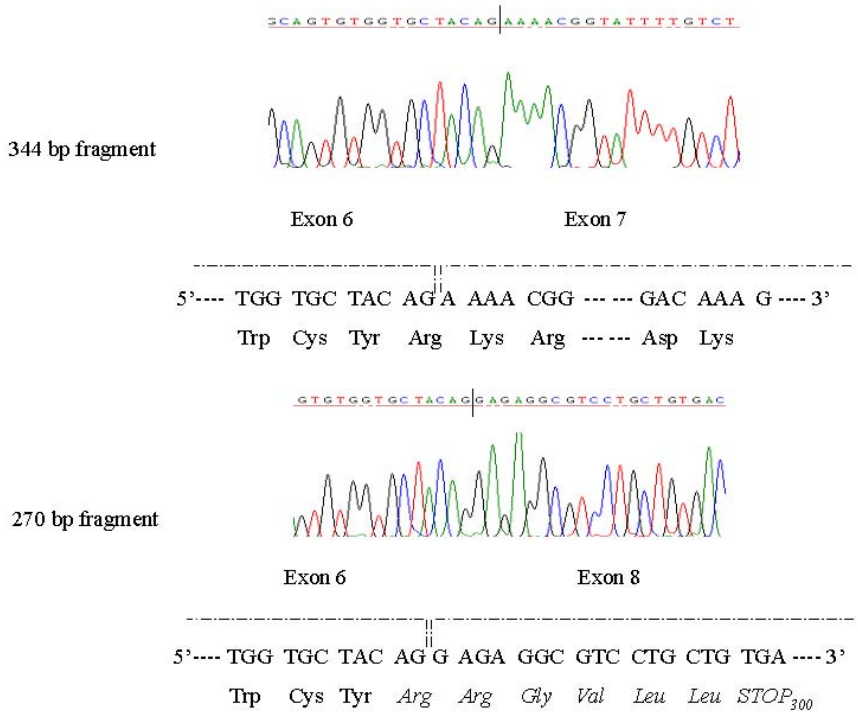

Figure 2. Nucleotide sequence of NPC1 cDNA (GenBank accession number NM_000271.1) fragments obtained by RT-PCR from RNA isolated from proband'fibroblasts and the corresponding amino acid sequence. The upper panel shows the sequence of the normal 344-bp fragment resulting from the PCR amplification of exon 6-exon 8 region (lane 1 of Fig. 1). In this fragment exon 6 was followed by exon 7, as in the corresponding fragment of the control subject (lanes 2 and 3 of Fig. 1). The lower panel shows the sequence of the abnormal 270-bp fragment observed in proband's fibroblasts (lane 1 of Fig. 1). In this fragment exon 6 is followed by exon 8, with the skipping of exon 7. The exon 6/exon 8 junction causes a frameshift which leads to a stretch of six novel amino acids (in italics) preceding a premature termination codon.

In Vitro Expression of NPC1 Minigene Harbouring c.882-28A>G

NPC1 minigene harbouring the mutation c.882-28A>G (MTgene) and its wild type counterpart (WTgene) were transfected in COS1 cells and the corresponding mRNAs were reverse transcribed and PCR amplified. Figure 3 shows that both minigenes were transcribed in transfected cells. The size of the transcription product of the WTgene (344 bp) was comparable to that of the NPC1 gene observed in control human fibroblasts (Fig.1). The size of the transcription product of MTgene (270 bp) was superimposable onto the abnormal one found in proband's fibroblasts (Fig. 1). The sequence of the two minigene cDNAs confirmed that of the corresponding cDNA fragments found in proband's fibroblasts (data not shown).

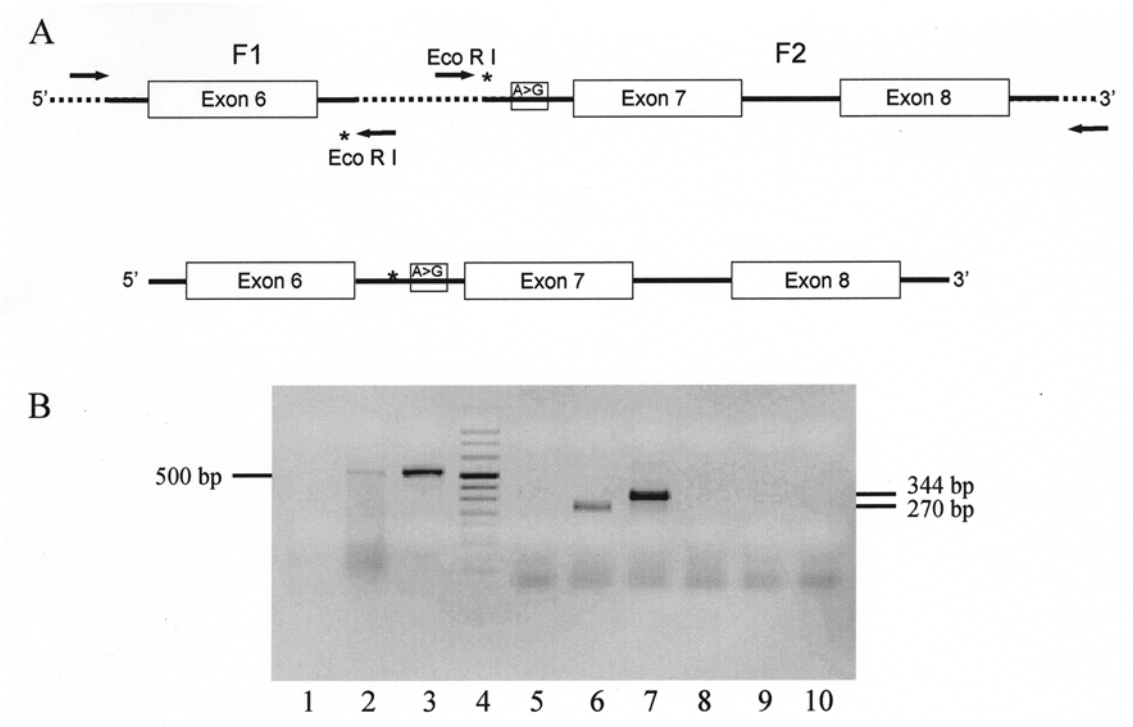

Figure 3. A: Strategy of mutant NPC1 minigene construction. Two fragments (F1 and F2) were PCR amplified from gDNA of the proband and a control subject by using modified primers to introduce a EcoRI site. Minigenes were obtained by ligation of F1 and F2. The boxed A>G substitution indicates the mutation in the putative BPS in intron 6. Minigenes were inserted into a pTarget vector and transfected into COS1 cells. **B:** Analysis of minigene transcripts. RNA isolated from COS1 cells was reverse transcribed and the exon 6/exon 8 region was amplified by PCR. Untransfected cells (1); cDNA of neomicyn resistance gene (2 and 3); molecular size markers (4): cells transfected with the empty vector (5); NPC1 cDNA in cell transfected with mutant minigene (6); NPC1 cDNA in cell transfected with wild type minigene (7); mock RT-PCR in cells transfected with empty vector (8); mutant (9) and wild type minigene (10).

DISCUSSION

In this work we report the characterization of two rare mutations of NPC1 gene in two siblings with NPC disease. The first mutation is a rare missense mutation (p.R978C) previously reported in other NPC1 patients (Sun et al. 2001; Ribeiro et al. 2001).

The second mutation (c.882-28A>G), previously unreported and undetected in 50 healthy subjects, involves the highly conserved adenosine residue of a putative lariat branch point in intron 6. It appears to cause a splicing defect which we discovered when we detected minute amounts of an mRNA devoid of exon 7 in proband's fibroblasts.

The 3' end of an intron contains three sequence elements critical for the splicing, namely: the splice lariat branch point sequence (BPS), the polypyrimidine tract and the conserved dinucleotide AG at the 3' splice site (3' ss) (Cartegni et al., 2002; Black, 2003). Although several mutations at the 5' and 3' splice sites have been reported to affect exon definition and the splicing of pre-mRNA (Cartegni et al. 2002; Faustino and Cooper, 2003), only few mutations in BPS have been identified that result in genetic disease. BPS exhibits only a weak consensus in mammalian genes (YNYURAY; where Y = pyrimidine, R = purine, and N = any nucleotide) with the exception of the adenine (underlined) which, being involved in the lariat formation with the GU of the donor splice site (Cartegni et al., 2002), is highly conserved. It is likely that the adenine at position -28 of intron 6 of NPC1 gene represents the highly conserved adenine of BPS for the following reasons: i) it is in the context of a typical BP

consensus sequence (CACTAAT); ii) its distance from 3'ss is close to the average distance (-26 nt) of lariat BPS from 3'ss of an intron (Zhang, 1998); iii) it belongs to the only BP consensus sequence found in a segment of 50 nt upstream from the 3'ss of intron 6. It is conceivable that A>G conversion in this putative BPS is the cause of an abnormal pre-mRNA splicing. We used automated splice site analysis (https://splice.cmh.edu/) (Rogan et al., 1998) to ascertain whether the c.882-28A>G reduced the information content of 3'ss of intron 6. This mutation did not change the information content of the 3'ss of intron 6 (13.3 bits) but weakened (from 2.6 to 2.4 bits) a site located at -8 nt from the natural acceptor site. Whether this minute change is sufficient to induce the skipping of exon 7 in NPC1 pre- mRNA remains to be established.

The content of the abnormal mRNA devoid of exon 7 in fibroblasts of both siblings was very low as compared to the normal sized mRNA generated by the other mutant allele (carrying the mutation c.2932C>T). There are several reasons for this low content, namely: i) an almost complete block of the splicing process associated with a low efficiency alternative splicing, leading to trace amounts of an abnormally spliced mRNA; ii) the alternatively spliced mRNA is produced in the expected amount (i.e. in the same amount as the mRNA encoded by the other allele) but is rapidly degraded (nonsense mediated mRNA decay) (Cartegni et al., 2002); iii) the mutation in highly conserved adenine in the putative BPS of intron 6 has no substantial effect on the splicing process (apart from the formation of minute amounts of an alternatively spliced mRNA) because of the activation of a cryptic lariat branch point (Padgett et al., 1985; Reed and Maniatis, 1985; Ruskin et al., 1985). We tend to dismiss the last explanation in view of the results of cDNA sequencing. This analysis showed that the proband was homozygous for the rare alleles of some common sequence variants (c.1926C>G, c.2572A>G, c.2793C>T, c.3797G>A) in linkage with the pathogenic mutation (c.2932C>T, p.R978C), a situation inconsistent with the heterozygosity for these sequence variants observed in gDNA. The homozygosity found in cDNA indicates that no significant amount of normally spliced mRNA was generated from the mutant allele harbouring the mutation in the putative BPS. We concluded, therefore, that the normal sized mRNA detectable in proband's fibroblasts resulted only from the transcription of the allele harbouring the C>T transition in exon 20 (c.2932C>T) with no substantial contribution from mRNA generated from the allele containing the c.882-28A>G mutation through the possible activation of a cryptic branch point sequence.

To assess whether c.882-28A>G was the cause of a splicing defect we transfected COS1 cells with two minigenes (mutant and wild type respectively) encompassing the gDNA region harbouring the mutation (Fig. 3). We show that the mRNA generated by the mutant minigene was abnormally spliced as it was devoid of exon 7 like the mRNA generated by the mutant allele harbouring c.882-28A>G mutation in proband's fibroblasts. This result supports our hypothesis that the A>G transition at position -28 of intron 6 is the cause of a splicing defect whereby intron 6 and exon 7 are removed together, as an entire intronic sequence, from pre-mRNA. This mutation is the first mutation in the highly conserved adenosine residue of a putative BPS found in NPC1 gene.

Finally one can ask how the genotype found in our two siblings correlates with their clinical (juvenile form of NPC) and biochemical phenotype (variant type of filipin staining). One may assume that the c.882-28A>G mutation results in a null allele which prevents the formation of NPC1 protein. The c.2932C>T (p.R978C) (reported so far only in compound heterozygotes) has been included among the "variant" alleles (Ribeiro et al., 2001). In this context p.R978C might reduce but not abolish the function of NPC1 protein; a residual activity of NPC1 could explain the relatively slow progression of the disease in our siblings.

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