

Antisense Oligonucleotide Treatment for a Pseudoexon-Generating Mutation in the *NPC1* Gene Causing Niemann-Pick Type C Disease



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ABSTRACT: Niemann-Pick type C disease is an autosomal recessive disorder caused by mutations in either the *NPC1* or *NPC2* gene. While most of the mutations are missense, a few splicing mutations have also been described. We identified and characterized a novel point mutation c.1554-1009G>A located in intron 9 of the *NPC1* gene in a Spanish patient. Sequencing of the cDNA from the patient showed that this intronic mutation creates a cryptic donor splice site resulting in the incorporation of 194 bp of intron 9 as a new exon (pseudoexon) in the mRNA. This new transcript bears a premature termination codon and is degraded by the nonsense-mediated mRNA decay mechanism. Experimental confirmation that the point mutation generates the inclusion of a pseudoexon in the mRNA was obtained using a minigene. A specific antisense morpholino oligonucleotide targeted to the cryptic splice site was designed and transfected into fibroblasts from the patient. Using this approach, normal splicing was restored. These results demonstrate the importance of screening deep intronic regions and support the efficacy of antisense therapeutics for the treatment of diseases caused by pseudoexon-generating mutations. © 2009 Wiley-Liss, Inc.

KEY WORDS: Pseudoexon-generating mutation, Niemann-Pick type C disease, *NPC1* gene, morpholino treatment

INTRODUCTION

Niemann-Pick type C disease (NPC; MIM# 257220) is an autosomal recessive lipid storage disorder that is characterized by the accumulation of unesterified cholesterol and glycosphingolipids in lysosomes as a

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consequence of a defect in intracellular lipid trafficking. The clinical course is heterogeneous and the initial manifestations may be hepatic, neurological or even psychiatric [Patterson et al., 2001]. Usually the onset of the disease occurs in childhood and patients typically die before adulthood. Two genes have been identified as being responsible for NPC disease [Steinberg et al., 1994; Vanier et al., 1996]. The majority of affected individuals have mutations in the *NPC1* gene (MIM# 607623), while a few bear mutations in the *NPC2* gene (MIM# 601015). The *NPC1* gene is located on chromosome 18q11-q12, spans 57 kb and contains 25 exons [Carstea et al., 1997; Patterson et al., 2001]. It encodes a large glycoprotein with 13 transmembrane domains that resides in late endosomes [Higgins et al., 1999; Neufeld et al., 1999]. In contrast, *NPC2* has only five exons encompassing 13.5 kb and is situated on chromosome 14q24.3 [Naureckiene et al., 2000]. It encodes a small soluble protein identified as a cholesterol-binding protein [Friedland et al., 2003; Ko et al., 2003]. Even though the precise role of both proteins is still unclear, data indicate that they are involved in the egress of lipids from lysosomes and that they may function cooperatively in the same pathway [Sleat et al., 2004].

Many different mutations have been described over the whole sequence of the *NPC1* gene. The vast majority of them are missense mutations (<http://npc.fzk.de>). Only a few intronic disease-causing mutations have been reported in *NPC1* patients [Di Leo et al., 2004; Millat et al., 2001; Park et al., 2003; Ribeiro et al., 2001; Sun et al., 2001; Fernandez-Valero et al., 2005] and in many cases, the effect on RNA splicing was predicted from genomic DNA without experimental confirmation.

Although most splicing mutations affect canonical splice-sites, pathogenic changes that disrupt sequences known as exonic and intronic splicing enhancers or silencers may also occur. These sequences are recognized by specific factors, such as the SR proteins, and are involved in the generation of correct splicing [Cartegni et al., 2002]. Moreover, other mutations create novel splice sites that are used by the splicing machinery, resulting in the aberrant inclusion of intron sequences known as “pseudoexons” in the mRNA. These mutations are not always detected because they are usually located deep within introns, in regions that are not normally analyzed. In addition, they often lead to premature stop codons that trigger degradation through the nonsense-mediated mRNA decay (NMD) mechanism. Antisense oligonucleotides offer the possibility of therapeutic correction of these “pseudoexon”-generating mutations, by blocking the aberrant splice sites and permitting the restoration of normal splicing and the generation of a wild-type protein. This strategy has been used in several disease models, such as Duchenne muscular dystrophy [Gurvich et al., 2008] and congenital disorder of glycosylation type IA [Vega et al., 2009].

We report here the identification and characterization of a novel intronic point mutation located in intron 9 of the *NPC1* gene. This mutation alters the splicing pattern, leading to the insertion of a 194-bp intronic sequence in the mRNA. This was observed in the patient’s fibroblasts and, also, in HeLa cells transfected with a mutant, but not with a wild type, *NPC1* minigene. The cryptic splice-site activated by the mutation was targeted with a specific antisense morpholino oligonucleotide (AMO). This form of treatment prevented the aberrant splicing in favour of the correct one, indicating that this could be a promising therapeutic approach.

MATERIAL AND METHODS

Patient

The patient was diagnosed at the Institut de Bioquímica Clínica, Barcelona. He is a compound heterozygote for the mutation described here and the in-frame deletion insertion p.N961_F966delinsS, which was described in a previous study [Fernandez-Valero et al., 2005] along with the main clinical data of the patient (patient #36). Samples from the parents were not available.

Reverse transcription of *NPC1* mRNA and cDNA sequencing

Total cellular RNA was isolated from patient 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 its concentration was determined by OD 260 nm. Single-stranded cDNA was synthesized using an oligo-dT primer and M-MLV Reverse Transcriptase, RNase H Minus (Promega, Madison, WI, USA) according to the manufacturer’s instructions. *NPC1* cDNA was then amplified in nine partially overlapping cDNA fragments that cover the entire open reading frame of *NPC1*

(information on the primers for the cDNA amplification and for the specific amplification of the exon 8–exon 9 and exon 9–exon 10 regions of the cDNA are available on request). For all PCR amplifications, 1 μ l of cDNA was added to a 25 μ l mixture containing 200 μ M of dNTPs, 0.4 μ M of each primer and 1 unit of Taq DNA Polymerase (Promega, Madison, WI, USA) in a PCR buffer containing 2 mM MgCl₂. The resulting amplification products were purified with the GFX PCR DNA and Gel Band purification Kit (Amersham Pharmacia Biotech, Amersham, UK) and sequenced in forward and reverse direction using the Big Dye Terminator Cycle Sequencing v3.1 Kit (PE Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions in an ABI PRISM 3700 DNA analyzer (PE Applied Biosystems, Foster City, CA, USA).

Analysis of intron 9 and mutation description

Genomic DNA was extracted from harvested skin fibroblasts using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). *NPC1* intron 9 was amplified in eight fragments (primer sequences available upon request) and sequenced as described above. Numbering for the mutation nomenclature was based on the cDNA sequence ENST00000269228 (Ensembl), in which +1 corresponds to the A of the ATG translation initiation codon. Control samples were analyzed by *MluI* digestion. Splice scores of natural and cryptic donor and acceptor sites were determined using the Splice Site Score Calculation website (http://rulai.cshl.edu/new_alt_exon_db2/HTML/score.html), and the presence of exonic splicing enhancer or silencer sequences was predicted using ESEfinder.

Cycloheximide treatment of patient's fibroblasts

Fibroblasts from the patient and from a control individual were cultured in the presence of three concentrations of cycloheximide (100, 500 and 1000 μ g/ml) for 4 or 6 hours and the *NPC1* cDNA was obtained as described above. A specific PCR amplification was performed and, after electrophoretic separation, the band whose intensity was enhanced by the CHX treatment was purified using GFX PCR DNA and the Gel Band purification Kit (Amersham Pharmacia Biotech, Amersham, UK) and then sequenced.

Minigene construction and *in vitro* splicing analysis

For the *in vitro* splicing evaluation, human genomic DNA was amplified from normal and mutant (c.1554-1009G>A) *NPC1* intron 9 to generate a fragment containing the pseudoexon along with 418 bp of the 5' and 369 bp of the 3' intronic flanking regions using the forward oligonucleotide, 5' GACGAGCCATGGGTAATTTG 3' and the reverse oligonucleotide, 5' GAAGGAGGGGGCCAGTCT 3'. The resulting product was cloned into a previously produced minigene plasmid pGLB1, which consists of a pcDNA3.1 vector containing exons 7, 8 and 9 and introns 7 and 8 of the *GLB1* gene [Santamaria et al., 2008]. Intron 7 of the *GLB1* gene has a *PmeI* restriction site, which was used to clone amplified *NPC1* fragments from the wild-type and the mutant alleles. The hybrid plasmids were named pGLB1-NPCwt and pGLB1-NPCmut.

To perform the splicing assay, 1 μ g of the wild-type or mutant minigenes was transfected into 2×10^5 HeLa cells, grown in six-well plates, with 5 μ l of Lipofectamine 2000 Reagent (Life Technologies, Basel, Switzerland). Total RNA was isolated from cultured HeLa cells 24 hours after transfection and RT-PCR analysis was performed using the pcDNA3.1 specific primers T7-F 5' AATACGACTCACTATAGGGA 3' and SP6-R 5' CATTTAGGTGACACTA 3'.

Oligonucleotide treatment and analysis

The antisense morpholino oligonucleotide (AMO) targeted to the donor cryptic splice site in the pre-mRNA was designed, synthesized and purified by Gene Tools. AMO treatment was carried out using the Endo-Porter peptide delivery system (Gene Tools, Inc., Philomath, OR). Between 4 and 5×10^5 fibroblasts from the patient were grown in six-well plates and, after overnight culture, different concentrations of AMO (10, 20 or 30 μ M) were added with 9 μ l/ml of Endo Porter to the culture medium. Cells were harvested at different times, total cellular mRNA was extracted and RT-PCR was performed as described above.

RESULTS

Identification of a deep intronic *NPC1* mutation

In a previous study, the analysis of the 25 exons and adjacent intronic regions of the *NPC1* gene from the patient's DNA allowed the identification of mutation p.N961_F966delinsS [Fernandez-Valero et al., 2005] in heterozygosity. No other mutation was identified in the exons or intron–exon boundaries.

In the present study RT-PCR was performed on the whole RNA isolated from the patient's fibroblasts to screen for a possible *NPC1* splicing error. Agarose gel electrophoresis of one of the cDNA fragments, fragment 4, showed the presence of a larger DNA product in addition to the band of the expected size (Fig. 1A). The extra DNA fragment was very faint, suggesting degradation by the nonsense-mediated decay mechanism (NMD). Since the forward and reverse primers for the amplification of fragment 4 were located in exon 8 and exon 10 respectively, we hypothesized that the larger product was possibly a new transcript formed due to a splicing error that included a region of intron 8 or of intron 9. To test this hypothesis specific primers for the amplification of both regions were designed, which allowed the exclusion of intron 8. Afterwards, the entire intron 9 (approximately 3 kb) was amplified and sequenced and a G-to-A transition, located 1009 bp upstream of the 3' end of intron 9, was identified in heterozygosity. The c.1554-1009G>A change was not present in 50 unrelated control individuals.

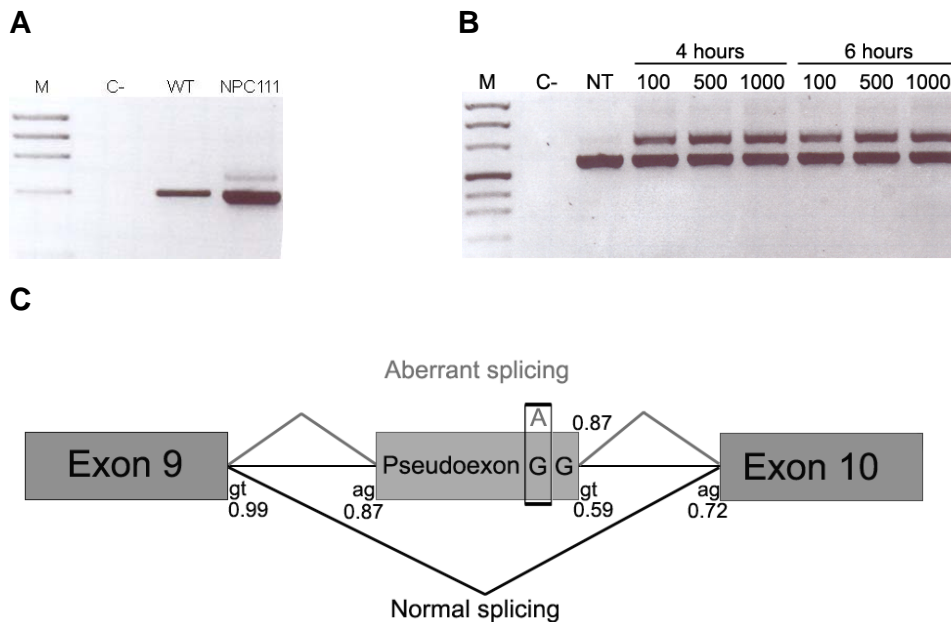


Figure 1. A: Agarose gel electrophoresis of the amplified fragment 4 of the *NPC1* cDNA. M: molecular weight marker, C-: negative control, WT: wild-type, NPC111: patient. (The amount of cDNA from the NPC111 patient was larger than that of wild type to allow detection of the upper band). B: RT-PCR amplification of RNA extracted from patient's fibroblasts treated with three different concentrations of cycloheximide (100, 500 and 1000 µg/ml) for 4 or 6 hours. M: molecular weight marker, C-: negative control, NT: untreated. C: Scheme of part of the *NPC1* gene, showing the pseudoexon inclusion found in the patient. Calculated splice scores are indicated below the corresponding 5' and 3' splice sites. The mutant score for the G-to-A mutation, denoted by a box, is stated above.

To avoid nonsense-mediated mRNA decay and to characterize the inserted sequence, patient's fibroblasts were treated with cycloheximide under different conditions followed by RT-PCR amplification. In all conditions

tested, the larger fragment was recovered for the proband (Fig. 1B) but not for the WT individual (data not shown). Direct sequencing of the extra band revealed that the insertion corresponded to 194 bp of intron 9. Thus, the presence of the intronic mutation c.1554-1009G>A promotes a pseudoexon insertion, whereas the naturally used splice sites remain fully functional. As predicted, the inserted intronic sequence led to a premature termination codon. The scores of the pseudoexon 3' and 5' cryptic splice sites were 0.87 and 0.59, respectively, in the wt allele, and the mutation increased the score of the cryptic donor splice site to 0.87 (Fig. 1C). Prediction of the presence of exonic splicing enhancers or silencers using the ESEFinder algorithm showed only a slight increase for an SRp40 splicing factor site.

In vitro expression of *NPC1* minigene harbouring c.1554-1009G>A

To provide further evidence that the change produces aberrant splicing causing the inclusion of the pseudoexon in the patient's mRNA, we performed a minigene assay using the p.GLB1 plasmid in which the *NPC1* intron 9 pseudoexon and flanking regions, from the wild-type (pGLB1-NPCwt) or c.1554-1009G>A (pGLB1-NPCmut) allele, was cloned (see Materials and Methods). Figure 2 shows the result of the splicing analysis after transfection in HeLa cells. Control lanes (2) and (3) show the result of RT-PCR amplifications using the T7 and SP6 primers in cells transfected with the intact pcDNA3.1 plasmid (2) or the pGLB1 plasmid (3) where bands of the expected size (154 and 301 bp, respectively) were obtained. The wild-type *NPC1* minigene construct (4) showed total absence of the pseudoexon inclusion while the mutant allele (5) produced an additional band of 495 bp corresponding to the pseudoexon inclusion. Thus, the minigene experiment confirmed that the c.1554-1009G>A transition affects normal splicing and can be considered pathogenic.

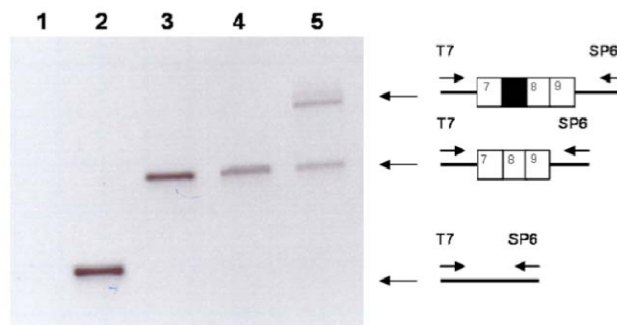


Figure 2. Minigene experiment showing the *in vitro* effect of the c.1554-1009G>A mutation. RT-PCR amplification from RNA extracted from HeLa cells untransfected (1) or transfected with intact pcDNA3.1 (2), pGLB1 plasmid (3), pGLB1-NPCwt (4) and pGLB1-NPC mut (5). Schemes of the different amplification products are represented on the right. White boxes: GLB1 exons; black box: pseudoexon insertion due to c.1554-1009 G>A change. Primers used for amplification (T7 and SP6) are indicated.

Antisense oligonucleotide treatment of patient's fibroblasts

An antisense morpholino oligonucleotide (AMO) complementary to the cryptic donor splice site created by the mutation was used in order to block the access of the splicing machinery to the pre-mRNA and to avoid the formation of the aberrantly spliced transcript. In all conditions tested, RT-PCR analysis showed the disappearance of the abnormally spliced mRNA when cells were treated with the specific AMO (AMO 111), indicating a

correction in favour of normal splicing. The effect of the oligonucleotide treatment was sequence-specific since a non-specific AMO (AMO C-) did not prevent inclusion of the pseudoexon (Fig. 3A).

As c.1554-1009G>A produced a transcript that was degraded by NMD, the AMO treatment was carried out in the presence of cycloheximide to emphasize the effect (Fig. 3B). As before, the band corresponding to the aberrant splicing was not detected, indicating restoration of the correct splicing.

The experiments described above demonstrated that AMO treatment prevents inclusion of the pseudoexon, but did not reveal whether or not the correctly spliced mRNA was produced for this allele. To prove that the AMO was able to restore normal splicing in the allele bearing the c.1554-1009G>A mutation, the RT-PCR band corresponding to the normal spliced mRNAs from untreated (i.e., treated with AMO C-) and treated patient cells was purified and sequenced in a region that included a single nucleotide polymorphism (rs1788799 in exon 12) for which the patient was heterozygous. Using this strategy it was found that without AMO treatment, the correctly spliced product (lower band) bears the C variant of the polymorphism, whereas after AMO treatment, both alleles of the polymorphism (C and G) are present (see the double peak, of similar height, in Fig. 3C). This confirms that the normal splicing of the allele bearing the c.1554-1009G>A mutation (and the G variant of the polymorphism) was recovered after treatment.

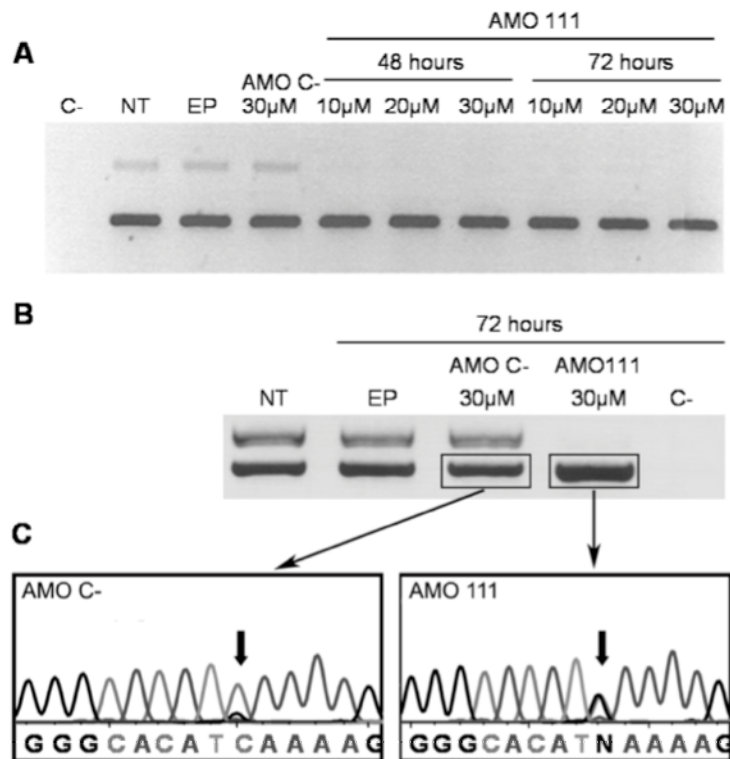


Figure 3. Antisense morpholino oligonucleotide treatment of patient's fibroblasts. A: RT-PCR amplification of RNA extracted from untreated patient's fibroblasts (NT) or those treated with a non-specific AMO (AMO C-) or different amounts of an AMO targeted to the cryptic splice site (AMO 111) for 48 or 72 hours. C-: PCR negative control, EP: transfection agent control. B: The same experiment shown in A but carried out in the presence of cycloheximide. C: Sequences of the RT-PCR band corresponding to the normal spliced mRNA from patient cells treated with AMO C- or AMO111.

A similar result was obtained when HeLa cells transfected with the minigene constructs were treated with AMO 111 (not shown).

A time course experiment showed that the effect of the treatment lasted for several days, since levels of the upper band that were similar to those of the untreated cells were not observed until around day 7 (data not shown).

DISCUSSION

Mutation analysis for monogenic diseases normally identifies a high percentage of the mutant alleles but a few may remain undetected. Current methods include the screening of all exons, flanking intronic regions and, sometimes, part of the promoter. Thus, deep intronic mutations, which usually generate pseudoexon insertions, are often missed. Strategies as those described in this work, including cDNA analysis and cycloheximide treatment to avoid NMD, could help the identification of these elusive mutations. In recent years an increasing number of these genetic alterations have been reported for disorders such as neurofibromatosis [Raponi et al., 2006], Duchenne muscular dystrophy [Gurvich et al., 2008] propionic and methylmalonic acidemia [Rincon et al., 2007], Marfan syndrome [Guo et al., 2008], and autosomal recessive polycystic kidney disease [Michel-Calemard et al., 2009]. Here we report the first pseudoexon mutation found in a Niemann-Pick type C patient.

Analysis of the genomic DNA from this patient revealed only one mutant allele [Fernandez-Valero et al., 2005]. To search for the other mutation, the cDNA was amplified in nine fragments, one of which showed an additional larger band. Since it included exons 8, 9 and 10, a specific PCR experiment was designed and the result indicated that an insertion had occurred in intron 9. The low intensity of the new band suggested that the allele was subjected to NMD degradation. Treatment with cycloheximide led to a clear increase in the amount of the enlarged cDNA fragment, allowing its sequencing. The intron 9 insertion was 194 bp long. The genomic sequence of intron 9 revealed a G-to-A transition 1900 bp upstream of the end of this intron, located within a potential cryptic donor splice site. The change was not present in controls. The *in silico* analysis of this site showed that the GG/gtgggtca sequence had a score of 0.59, which increased to 0.87 when the mutation was present (AG/gtgggtca). Therefore, the presence of the intronic mutation c.1554-1009G>A promotes a pseudoexon insertion, whereas the naturally used adjacent splice sites remain functional. In addition, a minigene experiment confirmed that the mutation generated the aberrantly spliced product. Overall, these results indicate the pathogenicity of the mutation.

It should be noted that, in the minigene context, the G>A transition does not fully prevent normal splicing. A small amount of normal spliced product was also detected in the patient's fibroblasts (see Fig. 3C). Whether this fact could have an impact on the clinical presentation of this patient (a juvenile case) remains an open question.

Alleles bearing intronic mutations that activate cryptic splice sites are good candidates for therapeutic correction using antisense oligonucleotides that block the aberrant splice site. This strategy has been used successfully to restore correct splicing in several disease models such as cystic fibrosis [Friedman et al., 1999], ocular albinism type I [Vetrini et al., 2006], afibrinogenemia [Davis et al., 2009], and congenital disorder of glycosylation type IA [Vega et al., 2009].

In this paper, we have reported the use of such antisense therapeutics to correct aberrant splicing in Niemann-Pick type C disease. A specific antisense morpholino oligonucleotide (AMO) targeted to the donor cryptic splice site created by the mutation c.1554-1009G>A, was transfected into patient's fibroblasts in order to prevent the inclusion of the pseudoexon. Due to the NMD process triggered by c.1554-1009 G>A, the experiment was also carried out in the presence of cycloheximide in order to emphasize the effect. In both cases, the band corresponding to the aberrant splicing disappeared, suggesting a correction in favor of normal splicing. This was confirmed by the presence of both allele products, as shown by the use of a polymorphism for which the patient was heterozygous, in the band corresponding to the normal splicing. However, the effect of the treatment could not be analyzed at a functional level since the NPC1 protein is not an enzyme, and so, has not activity to be measured. Another practical limitation is that, since the patient is heterozygous for the mutation, the protein generated from the other allele would probably mask the increment in the amount of NPC1 protein produced by the corrected allele.

These are promising results that, together with those obtained for other diseases, support the use of an antisense therapeutic strategy for correction of pseudoexon-generating mutations. Although several issues should

be addressed before applying this therapeutic approach to patients, such as possible secondary effects, development of an efficient delivery method, or a longer lasting effect, there are several advantages of this type of treatment. First, it could be applied to any gene and disease involving a pseudoexon-generating mutation. Second, the small size of the morpholinos would probably allow them to cross the blood–brain barrier, allowing treatment of neurological diseases. Finally, it is worth mentioning that in a recent study, Aouadi et al. [2009] showed efficient oral delivery of siRNAs in mice. Since siRNAs and morpholinos are similar in size, it is possible that this delivery method could also be applicable for morpholinos.

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