

Short Report

Molecular analysis of 30 Niemann–Pick type C patients from Spain

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Mutations in the *NPC1* or *NPC2* gene are responsible for Niemann–Pick type C (NPC) disease (OMIM #257220), an autosomal recessive neurodegenerative lysosomal storage disorder caused by an incorrect regulation of intracellular lipid trafficking. A molecular analysis carried out in 30 unrelated patients identified 43 distinct mutations in the *NPC1* gene, 12 of which had not been previously described. The novel *NPC1* alleles were four amino acid substitutions (p.F995L, p.F1079S, p.L1106P and p.G1209E), a nonsense mutation (p.E1089X), a 1-bp insertion (p.L1117PfsX4), an in-frame deletion (p.N916del), four intronic changes (c.58-3280C>G, c.882-28A>T, c.2604+5G>A and c.3591+5G>A) that affect the splicing mechanism, and the first deletion including the whole gene described in NPC disease. In all the splice site mutations, the formation of abnormal spliced transcripts was confirmed by cDNA analysis, and mRNA degradation by the nonsense-mediated mRNA decay process was also assessed. As it has been previously reported in this disease, genotype–phenotype correlations are limited due to the large number of private mutations. We describe for the first time one homozygous patient for p.I1061T mutation, who presented the severe infantile clinical onset, and another patient with the variant biochemical phenotype, whose clinical presentation was the neonatal form of the disease.

Conflict of interest

The authors declare that they have no conflict of interest.

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Niemann–Pick type C (NPC) disease (OMIM #257220) (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM>) is an autosomal recessive lipid storage disorder. At the cellular level, the disease produces a late-endosomal/lysosomal accumulation of endocytosed unesterified cholesterol that leads to the accumulation of a complex pattern of lipids in non-neural tissues and in the brain (1). The clinical manifestations are characterized by the presence of hepatosplenomegaly and severe progressive neurological dysfunction with varying age at onset and later course (2).

Genetic and allelic heterogeneity was established for this disease by the identification of two different genes, *NPC1* (MIM 607623) and *NPC2* (MIM 601015) (3–5). Over 294 and 19 different disease-causing mutations have been reported worldwide in *NPC1* and *NPC2* gene, respectively (<http://www.hgmd.cf.ac.uk/ac/index.php>).

A number of studies point towards a key role for the NPC1 and NPC2 proteins in modulating vesicular trafficking of cholesterol and glycolipids (6–8), although whether they work in concert, interacting at the functional and potentially at the structural levels, is unclear. It is worth mentioning that direct protein–protein interactions for NPC2 with NPC1 have not been reported so far (9).

In a previous survey by our group, we reported the mutational analysis of the *NPC1* gene in a cohort of 40 Spanish patients and we suggested a unique origin for p.C177Y and p.G993EfsX4 mutations in contrast to p.I1061T mutation that showed different origins. Here, we present the molecular analysis in 30 unrelated NPC patients, which allowed the identification of 12 novel mutations in the *NPC1* gene including a large deletion and several splice site mutations that have been characterized at RNA level.

Materials and methods

Patients

Samples were obtained from 31 patients belonging to 30 unrelated families. Most of them were

of Spanish origin, except for seven patients from other ethnic backgrounds: Moroccan, Costa Rican, Ecuadorian and Dutch. Nine patients (NPC02, 03, 07, 13, 21, 29, 36, 38 and 40) had been previously described by our group (10), but one of the mutant alleles in each of them had remained unidentified. Diagnosis of NPC disease was determined by cytochemical demonstration of pathologically enriched cholesterol via filipin staining in cultured skin fibroblasts as described by Vanier et al. (11). Classification of patients with respect to their clinical characteristics was as previously proposed (12).

Mutation screening: analysis of cDNA and genomic DNA

To identify mutations in *NPC1* gene, sequence analysis of its cDNA was performed. The changes identified were confirmed by sequencing the corresponding genomic DNA region. When only one mutation was found in the cDNA sequence, all exons and intron boundaries were sequenced.

To detect possible mutations whose mRNAs are candidate to suffer nonsense-mediated mRNA decay (NMD) process (13), cells were treated with cycloheximide (CHX) (Sigma, St. Louis, MO) according to the protocol previously described (14). RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) were performed using standard methods. *NPC1* cDNA was amplified in 10 overlapping PCR fragments using either specific primers previously described (15) or self-designed primers, which are available upon request. To analyse the occurrence of exon 11 skipping in CHX-treated or untreated fibroblasts from the NPC29 patient and from a healthy individual, cDNA was amplified using the forward primer specific for the skipped transcript (overlapping exons 10 and 12) 5'TTGGGAGGCTATGATGGTTTA3' and the reverse primer in exon 14 5'GGTTTCCCC TTGAAGACGTT3'. The real-time PCR method used to quantify the mRNA levels in this patient was previously described (14). To determine the intronic change that causes exonization of 374

bp in the NPC59 patient, primers based on the sequence of intron 1 were used (primer sequences available upon request). PCR products were screened for mutations by DNA sequencing using the Big Dye Terminator Cycle Sequencing v3.1 kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. The sequencing reactions were run on an ABI Prism® 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA).

Genomic DNA was extracted from cultured skin fibroblasts by standard methods. *NPC1* and *NPC2* exons and their intron boundaries were amplified using primers and PCR conditions previously described (10, 16). PCR products were purified and sequenced as described in the preceding paragraphs. Novel mutations were analysed in 50 healthy controls.

Characterization of the mutations of patient NPC57

To confirm the presence of p.T1066N mutation in exon 21 of the *NPC1* gene in samples from the NPC57 patient and from their parents, gDNA was amplified.

To analyse the deletion, quantitative real-time PCR experiments were performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). All PCR reactions, with a final volume of 10 µl, were run in triplicate and contained 50 ng of DNA. Exons 1, 21 and 25 of the *NPC1* gene and exon 5 of the *PMM2* gene, which was used as endogenous control, were run in separate wells. The reagents were SYTO®9 green fluorescent nucleic acid stain (Invitrogen Molecular Probes, Eugene, Oregon) and Ampli Taq® Gold DNA Polymerase with Gene Amp® 10× PCR Gold buffer and MgCl₂ solution (Applied Biosystems, Foster City, CA). Primers used to amplify the *PMM2* fragment were forward 5'AGGCTGTTTATCTATGTTGCC3' and reverse 5'CACCAGGCCATATCTTATTT3'. PCR conditions were 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. The runs were monitored via the STEPONE Software v2.0 (Applied Biosystems, Foster City, CA). Levels of gDNA were relatively quantified by evaluating C_t values according to the comparative C_t (ΔΔC_t) method (Applied Biosystems, Foster City, CA).

A total of 13 polymorphisms located in the *NPC1* gene were analysed by PCR amplification and sequencing. In particular, three of the polymorphisms were in the 5'UTR region, four were in intron 1, and the remaining six were in exons 4, 6, 12, 17, 18 and 25 (last exon of the gene),

respectively. Parents' samples were also analysed for these polymorphisms.

Mutation nomenclature

All mutations were described according to the recommended nomenclature (17) (the updates found at the web page: <http://www.HGVS.org/mutnomen/>). Gene nucleotide numbering was according to the GenBank sequence NM_000271.3 (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>), with +1 as A of the start codon. The ATG codon represents +1 for the amino acid numbering according to NPC1 protein sequence NP_000262.1.

Results

Clinical and biochemical phenotype

The clinical and biochemical phenotype distribution of the unrelated NPC patients is summarized in Table 1. Clinical phenotypes were classified according to the age at onset of neurological symptoms, except for the neonatal fatal systemic form. The group of 30 patients included 3 (10%) neonatal, 10 (33.3%) severe infantile (onset at age <2 years), 6 (20%) late infantile (onset at age 3–5 years), 6 (20%) juvenile (onset at age 5–16 years) and 2 (6.6%) adult (onset at age >16 years) clinical forms. In three patients, the clinical phenotype could not be established due to the lack of data relating to disease progression.

Regarding the biochemical phenotype, 21 (70%) of the patients presented the classical phenotype (a massive unesterified cholesterol accumulation) and 8 (26.6%) of them showed the variant phenotype (a moderate unesterified cholesterol accumulation). No biochemical classification was possible in one patient because the filipin staining could not be performed.

Mutation analysis

Table 1 shows the *NPC1* genotypes of the patients included in this study. Molecular analysis of *NPC1* gene allowed the identification of 43 different mutations, 12 of which had not been described before. The novel mutations were four amino acid substitutions [p.F995L (c.2983T>C), p.F1079S (c.3236T>C), p.L1106P (c.3317T>C) and p.G1209E (c.3626G>A)], a nonsense mutation [p.E1089X (c.3265G>T)], a 1-bp insertion [p.L1117PfsX4 (c.3349dupC)], an in-frame deletion [p.N916del (c.2746_2748delAAT)], four intronic changes that affect the splicing mechanism (c.58-3280C>G, c.882-28A>T, c.2604+5G>A

Table 1. Clinical, biochemical and mutational features of 30 Niemann–Pick C disease patients

Patient	Origin	Clinical phenotype ^a	Biochemical phenotype ^b	Nucleotide change (allele 1)	Amino acid change ^c (allele 1)	Nucleotide change (allele 2)	Amino acid change ^c (allele 2)	Ref.
NPC13 ^d	Spanish	Neonatal	Classical	c.2804+5G>A	p.I839_D868del	c.2604+5G>A	p.I839_D868del	N
NPC55	Spanish	Neonatal	Classical	c.3591+5G>A	p.[S1160_S1197del, V1177_S1197del, S1197_V1198ins15]	c.3591+5G>A	p.[S1160_S1197del, V1177_S1197del, S1197_V1198ins15]	N
NPC61	Spanish	Neonatal	Variant	c.[2932C>T; 3467A>G]	p.[R978C; N1156S]	c.3019C>G	p.P1007A	18, 4, 19
NPC02 ^d	Spanish	Severe infantile	Classical	c.3245+1dupG	p.[S1082RfsX15, S1082_Q1090delinsR]	c.3265G>T	p.E1089X	10, N
NPC07 ^d	Spanish	Severe infantile	Classical	c.894dupT	p.V299GfsX9	c.2612A>G	p.Y871C	10, 20
NPC40 ^d	Spanish	Severe infantile	Classical	c.1935T>A	p.C645X	c.3236T>C	p.F1079S	10, N
NPC47	Ecuadorian	Severe infantile	Classical	c.530G>A	p.C177Y	c.530G>A	p.C177Y	18
NPC51	Spanish	Severe infantile	Classical	c.1436G>A	p.C479Y	c.3160G>A	p.A1054T	10, 21
NPC53	Spanish	Severe infantile	Classical	c.352_353delAG	p.Q119VfsX8	c.2830G>A	p.D944N	22, 21
NPC57	Spanish	Severe infantile	Classical	c.3197C>A	p.T1066N	Deletion of the whole NPC1 gene	-	10, N
NPC62	Moroccan	Severe infantile	Classical	c.3182T>C	p.I1061T	c.3182T>C	p.I1061T	23
NPC63	Spanish	Severe infantile	Classical	c.2324A>C	p.Q775P	c.2830G>A	p.D944N	21
NPC64	Moroccan	Severe infantile	Classical	c.3317T>C	p.L1106P	c.3317T>C	p.L1106P	N
NPC03 ^d	Spanish	Late infantile	Classical	c.530G>A	p.C177Y	c.2604+5G>A	p.I839_D868del	18, N
NPC27 ^d	Spanish	Late infantile	Classical	c.882-28A>T	p.K295RfsX6	c.1274C>A	p.S425X	N, 10
NPC36 ^{d,e}	Spanish	Late infantile	Classical	c.1554-1009G>A	p.A519QfsX1	c.2883_2897del	p.I962_F966del	24, 10
NPC38 ^d	Spanish	Late infantile	Variant	c.1554-1009G>A	p.A519QfsX1	c.3754G>C	p.V1198GfsX4 ^f	24, 10
NPC45	Costa Rican	Late infantile	Classical	c.2201G>T	p.S734I	c.2201G>T	p.S734I	25
NPC54	Spanish	Late infantile	Variant	c.352_353delAG	p.Q119VfsX8	c.2974G>T	p.G992W	22, 19
NPC29 ^d	Spanish	Juvenile	Variant	c.1436G>A	p.C479Y	?	?	10
NPC46	Spanish	Juvenile	Classical	c.1990G>A	p.V664M	c.3104C>T	p.A1035V	25, 18
NPC49	Spanish	Juvenile	Classical	c.2292G>A	p.A750_G765del	c.3425T>C	p.M1142T	18, 21
NPC52	Spanish	Juvenile	Variant	c.2932C>T	p.R978C	c.2983T>C	p.F995L	18, N
NPC58	Spanish	Juvenile	Classical	c.2746_2748delAAT	p.N916del	c.3451G>A	p.A1151T	N, 26
NPC60	Spanish	Juvenile	Variant	c.1554-1009G>A	p.A519QfsX1	c.3182T>C	p.I1061T	24, 23
NPC50	Spanish	Adult	Variant	c.2974G>C	p.G992R	c.2983T>C	p.F995L	21, N
NPC56/NPC56 ^g	Spanish	Adult	Classical	c.3349dupC	p.L1117PfsX4	c.3672C>G	p.F1224L	N, 10
NPC48	Dutch	Unknown	Unknown	c.2861C>T	p.S954L	c.3626G>A	p.G1209E	19, N
NPC59	Moroccan	Unknown	Variant	c.58-3280C>G	p.V200fsX6	c.2819C>T	p.S940L	N, 19
NPC65	Moroccan	Unknown	Classical	c.3557G>A	p.R1186H	c.3557G>A	p.R1186H	4

?, not found; N, novel mutation identified in this report; NMD, nonsense-mediated mRNA decay.

^aClassification of clinical phenotypes by age at onset of neurological symptoms (12), except for the neonatal fatal systemic form that includes those patients who died from liver failure in the first months of life. We included in the unknown form those patients who were diagnosed at the neonatal period on the systemic signs but not presented any neurological symptom yet.

^bDefined by the degree of severity of alterations of intracellular cholesterol processing.

^cNote that in some cases, the putative protein might not be synthesized due degradation of the mRNA by NMD.

^dPatients previously reported (10), but they had only identified one mutated allele.

^eNote that this patient was mentioned as NPC11 in the article in which the c.1554-1009G>A mutation was first described (24).

^fNote that this mutation was previously described as p.G1252R (10).

^gPatient 56 and 56' are affected siblings.

and c.3591+5G>A), and a large deletion that includes the whole *NPC1* gene.

We also found a novel mutant allele carrying two *in cis* mutations, p.[R978C;N1156S], as shown by analysis on the NPC61 parents' DNA (not shown).

Our analysis of *NPC1* gene allowed completion of 29 genotypes. In one patient (NPC29), after sequencing both cDNA and genomic DNA, one allele still remains unknown (see subsequent sections).

Splicing mutations

The electrophoretic profile of RT-PCR products obtained from total RNA isolated from several patients' fibroblasts, which bear novel splice site mutations (c.58-3280C>G, c.882-28A>T, c.2604+5G>A and c.3591+5G>A) or previously described changes (c.1554-1009G>A, c.2292G>A and c.3754G>C), showed multiple bands (Fig. 1). Therefore, these mutations are worth to be described in more detail.

The presence of the intronic mutation c.58-3280C>G promotes a pseudoexon insertion that corresponds to 374 bp of intron 1, which was detected by direct sequencing (c.57_58ins374) (Fig. 1a). This change presumably creates a novel acceptor region and activates a cryptic donor splice site (splicing score 0.92), whereas the naturally used adjacent splice sites of the surrounding exons remain functional, resulting in the generation of an aberrant transcript. The inserted intronic sequence led to a premature termination codon (PTC), but the extra band was present in both CHX-treated and untreated samples, suggesting no degradation by the NMD mechanism (not shown).

The c.882-28A>T intronic change, which involves the conserved adenosine residue of the lariat branch point in intron 6, causes an abnormally spliced cDNA with the complete skipping of exon 7 (c.882_954del173) (Fig. 1b). The loss of exon 7 disrupts the reading frame leading to a PTC, which activates mRNA degradation by the NMD process (not shown).

The c.2604+5G>A splice mutation, which is located in a conserved position of the donor splice site of intron 17, promotes skipping of exon 17 (c.2515_2604del190) (Fig. 1c).

The analysis of c.3591+5G>A products reveals three abnormally spliced cDNAs: one with an insertion of the first 45 bp of intron 23 (c.3591_3592ins45), another with a deletion of the last 63 bp of exon 23 (c.3529_3591del63) and a third one with the skipping of exon 23 (c.3478_3591del114) (Fig. 1d).

Among the previously described mutations, the deep intronic change c.1554-1009G>A (24) is located in intron 9 and creates a cryptic donor splice site resulting in the incorporation of 194 bp of the intron 9 (c.1553_1554ins194) as a pseudoexon (Fig. 1e).

The c.2292G>A mutation leads to the creation of an acceptor splice site in exon 15, generating a transcript with an in-frame deletion of the first 48 bp of exon 15 (c.2246_2293del48) (Fig. 1f).

The c.3754G>C mutation affects the last nucleotide of exon 24, which plays a key role in governing the splicing efficiency, and generates skipping of exon 24 (c.3592_3754del163) (Fig. 1g). In a previous report (10), the effect on NPC1 protein of this change was indicated as p.G1252R; nevertheless, skipping of exon 24 disrupts the reading frame, and the predicted translation product of this mRNA, which is not degraded by the NMD process (not shown), is a truncated protein.

Different transcripts found in the sample from patient NPC02

In a previous study (10), one of the mutant alleles, c.3245+1dupG, of this patient was found. Here, we report the identification of a mutation in the second allele, p.E1089X. Thus, the two disease-causing mutations of the patient were now identified. However, the analysis of the cDNA from the patient showed the appearance of four different transcripts (Fig. 2) as a result of the combination of the presence/absence of the dupG and of an alternative pattern of splicing that skipped the first 25 nucleotides of exon 22 (from position c.3246 to c.3270). In wild-type individuals, this alternative splicing was also observed but at very low levels and was only detectable upon CHX treatment (not shown). In the patient, one abundant transcript was that bearing the extra G and in which the alternative acceptor site within exon 22 had been used (Fig. 2, transcript D). This is consistent with the fact that this transcript would not be affected by NMD because the frameshift caused by the addition of the G nucleotide would be corrected by the elimination of the 25 nucleotides of exon 22 due to the alternative splicing. The analysis of the genomic and cDNA samples from the parents confirmed this hypothesis. The father carries the duplication of the G and the mother the nonsense p.E1089X mutation. At the cDNA level, the p.E1089X mutation was not observed, suggesting degradation by NMD.

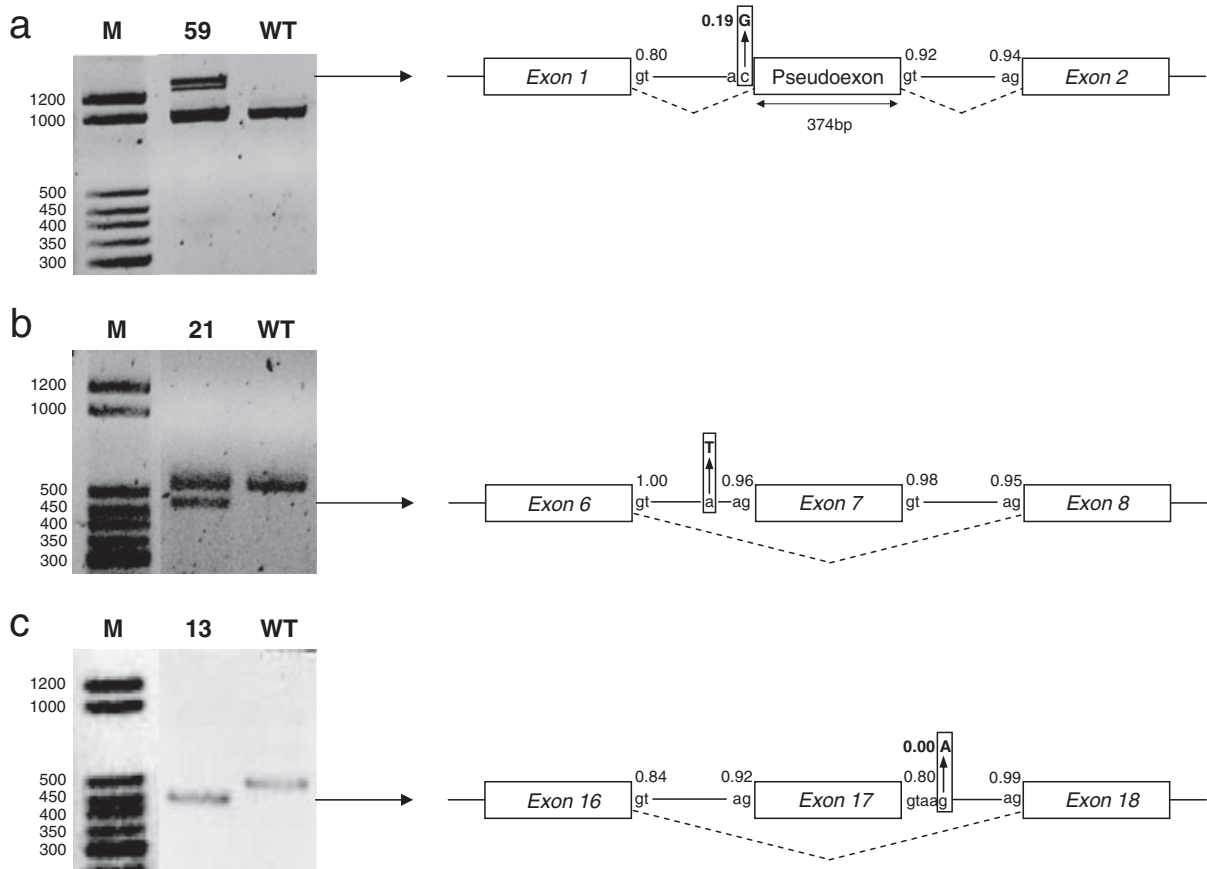


Fig. 1. Abnormal *NPC1* mRNAs in cases with splice site mutations. Agarose gel electrophoresis of reverse transcription-polymerase chain reaction products and schematic depictions of samples of *NPC1* cDNA isolated from the patients' fibroblasts treated with cycloheximide. The splice scores (calculated with BDGP software) are shown above the corresponding 5' and 3' splice sites. The mutant scores are in bold letters. Mutations are indicated by arrows. M, molecular weight of 50-bp DNA marker (expressed in bp); WT, wild-type individual. **(a)** Patient 59 with c.58-3280C>G mutation. The intermediate band corresponds to heteroduplex. **(b)** Patient 21 with c.882-28A>T mutation. **(c)** Patient 13 with c.2604+5G>A mutation in homozygosity. **(d)** Patient 55 with c.3591+5G>A mutation in homozygosity. **(e)** Patients 36, 38 and 60 with c.1554-1009G>A mutation. **(f)** Patient 49 with c.2292G>A mutation. **(g)** Patient 38 with c.3754G>C mutation. The upper band corresponds to heteroduplex.

Large deletion allele carried by patient NPC57

The first analysis of samples from patient NPC57 revealed homozygosity for mutation p.T1066N. However, while the father was heterozygous for this change, the mother did not carry the mutation. The analysis of 13 polymorphisms within the *NPC1* gene (three in the 5'UTR region: rs1620047, rs1788774 and rs1652354; four in intron 1: rs1788781, rs1788783, rs1788826 and rs7226548; and six in the coding region: p.Y129Y, p.H215R, p.M642I, p.I858V, p.N931N and p.R1266Q) in the patient and parents suggested a deletion of the maternal allele, because the polymorphisms covered all the gene from the 5'UTR to the last exon. Quantitative PCR analyses revealed that the patient and the mother have half of the amount of *NPC1* gDNA. The relative quantification (RQ) of the *NPC1* DNA was normalized to *PMM2* DNA levels (endogenous

control) using the comparative C_t ($\Delta\Delta C_t$) method. The control sample (wild-type individual) was set as the reference value (RQ = 1). Sample from the father showed RQ around 1, whereas the patient and the mother showed RQ around 0.50 for the exon 21 and also the first and the last exons of the *NPC1* gene. To determine the limits of the NPC57 deletion, additional polymorphisms located in other chromosome 18 flanking genes will be analysed.

Unidentified allele

Regarding NPC29 patient, apart from the identification of p.C479Y mutation in one allele, an extra band corresponding to the skipping of exon 11 (lost of 103 bp) was detected. However, no mutation that could explain this alternative splicing was found after cDNA and gDNA

Molecular analysis of 30 NPC patients

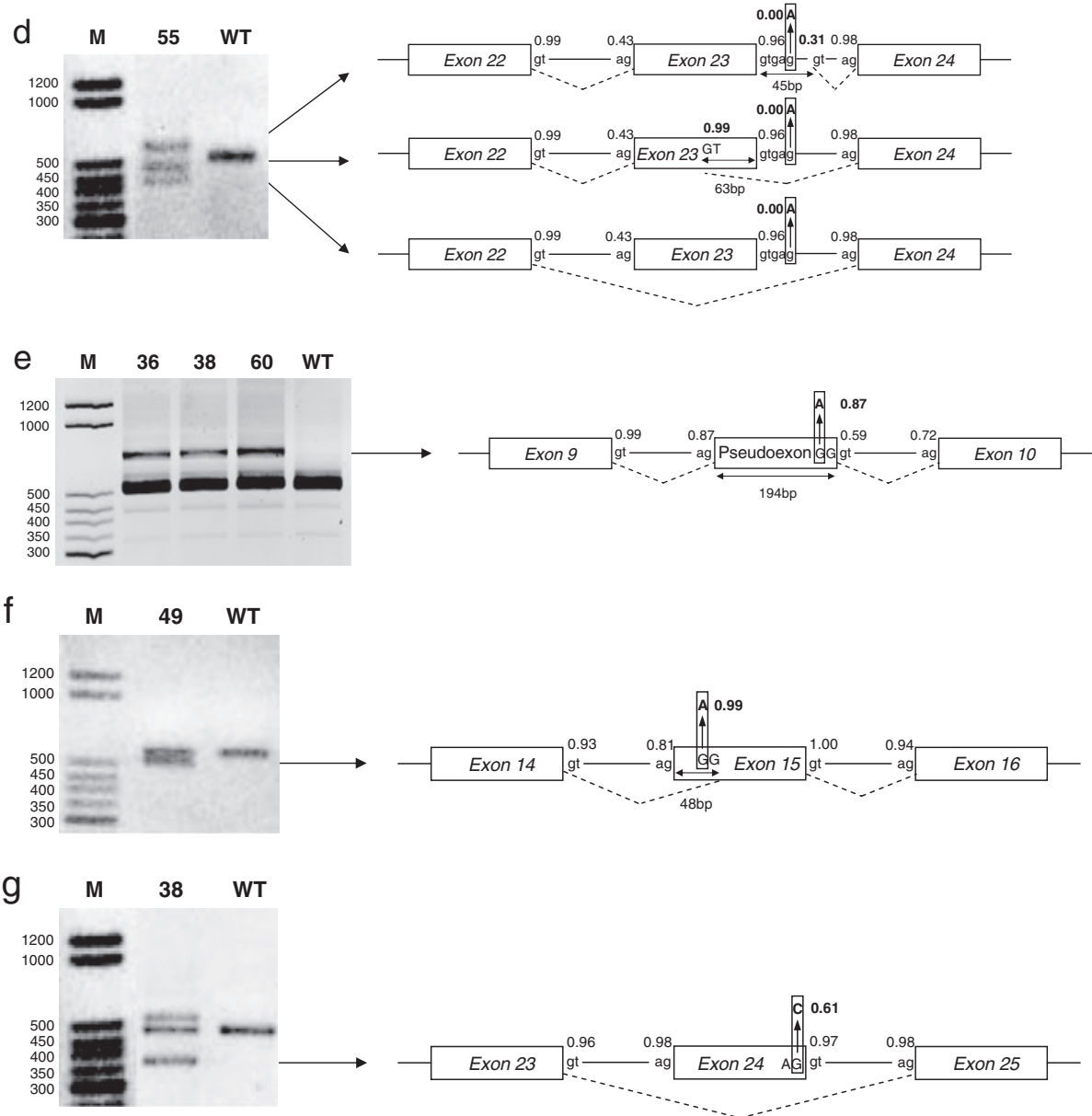


Fig. 1. Continued

sequencing. To analyse whether this transcript was naturally produced, we performed the same PCR amplification in cDNA from wild-type fibroblasts. The extra band was also detected in the wild-type sample, although the intensity was lower than in that of the patient (Fig. 3a). CHX treatment showed that this transcript was partly subjected to NMD. To further confirm these results, an additional PCR using a forward primer specifically designed for the amplification of the transcript lacking exon 11 was carried out. As shown in Fig. 3b, in all cases, a band of the expected size (416 bp) was obtained, suggesting that this alternative product is not related with the disease in

the patient. The analysis of polymorphic markers in the coding region of the *NPC1* gene and the normal mRNA levels observed by real-time PCR (RQ around 1) in this patient did not suggest the presence of a whole *NPC1* deletion or a mutation targeted by the NMD process. *NPC2* gene was also analysed in this patient but no change was detected.

Discussion

Mutation profile

As previously reported (27), molecular results in NPC patient series of this study show a broad

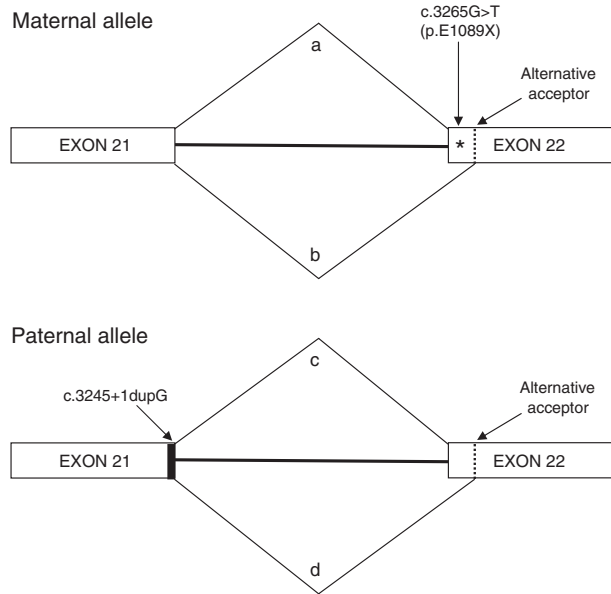


Fig. 2. *NPC1* transcripts observed in samples from patient NPC02. The maternal allele generates one transcript (a), which uses normal splice sites but contains the nonsense mutation c.3265G>T (p.E1089X) in exon 22, and another transcript (b) that uses an alternative acceptor site within exon 22 and skips the first 25 nucleotides of exon 22. The paternal allele bears an extra G nucleotide at the end of exon 21 and generates two transcripts depending on the usage of the normal (c) or alternative (d) acceptor site.

spectrum of disease-causing mutations in the *NPC1* gene.

The mutation profile of the studied cohort mainly shows mutant alleles containing missense alterations; however, it is worth mentioning the relevance of splicing defects. The true prevalence of this type of mutation is probably underestimated because deep intronic sequences are not conventionally sequenced, and in mRNA analysis, the aberrant transcripts (usually with a frameshift and a PTC) are usually prone to degradation by NMD (28).

Mutation p.I1061T is the most common *NPC1* mutation in patients of Western European descent (23). In our cohort of 55 Spanish NPC patients, including those of this study together with the ones reported in our preceding survey (10), this mutation accounts for 8% of the mutant alleles. The frequency of p.I1061T mutation is lower than that reported by other authors (23, 25, 29); however, it is similar to that described in the Portuguese and Italian populations (18, 30). Mutation p.P1007A was the second most frequent allele, with a frequency of 4.5%, in agreement with previous studies (20). The intronic change c.1554-1009G>A, first described by our group in the Spanish patient NPC36 (24), which causes a splicing error,

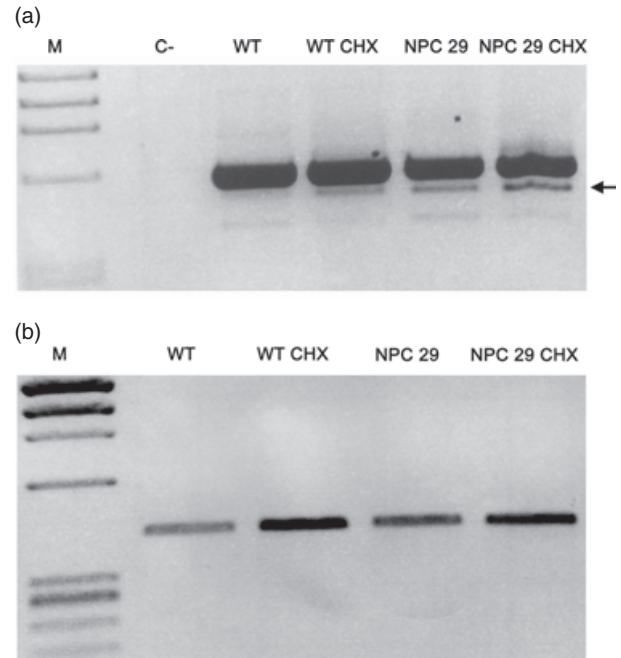


Fig. 3. Reverse transcription-polymerase chain reaction (RT-PCR) amplification of fragments of the *NPC1* cDNA in samples from a wild-type (WT) individual and patient 29 (NPC29), in the absence or presence of cycloheximide (CHX). M, molecular weight marker; C-, negative control (PCR without DNA). (a) Amplification of fragment 5 of the *NPC1* cDNA, including exons 9–13. The arrow indicates the band corresponding to the skipping of exon 11. (b) Amplification of the skipped transcript using a specific forward primer overlapping exons 10 and 12.

appeared to be relatively common in our country, because three patients (NPC36, NPC38 and NPC60) were compound heterozygous for this mutation (2.7%). There is a great allelic heterogeneity, because few mutations are present in more than one patient.

Referring to the novel mutations, the missense changes (p.F995L, p.F1079S, p.L1106P and p.G1209E) are probably pathogenic mutations due to the fact that they were not observed in 100 healthy control alleles; after sequencing all *NPC1* gene, no other mutations were found, and the affected amino acid residues are conserved throughout species. Moreover, functional effect prediction programs such as POLYPHEN (<http://genetics.bwh.harvard.edu/pph/>) or PANTHER (<http://www.pantherdb.org/>) confirmed the possible pathogenesis of these novel changes (not shown). Although to verify these predictions, it should be necessary to perform expression studies on these alleles.

Among the other novel changes, there is the mutation p.N916del that causes an in-frame deletion. Although the effect of this deletion was not

confirmed by expression studies, the previous programs suggest that residue 916 is relevant for the NPC1 protein.

Mutations p.E1089X and p.L1117PfsX4 are undoubtedly disease-causing mutations, as they create a PTC that triggers mRNA degradation by the NMD mechanism (not shown).

The analysis of the novel splicing mutations (c.58-3280C>G, c.882-28A>T, c.2604+5G>A and c.3591+5G>A) using splicing prediction software (http://www.fruitfly.org/seq_tools/splice.html) indicates that they disrupt natural splice sites. The effect of each mutation on the splicing score is shown in Fig. 1. In all cases, the formation of abnormal spliced transcripts has been confirmed by cDNA analysis. Nevertheless, to provide evidence that the observed intronic changes are disease causing, it should be necessary to evaluate them using minigene transfection assays. In the case of the novel deep intronic change, c.58-3280C>G, the fact that after CHX treatment no difference was observed in the cDNA from the patient NPC59 may be due to the escape of the NMD process. Termination codons are recognized by the NMD apparatus as premature if they are located more than 50–55 nucleotides upstream of the 3'-most exon–exon junction, but a number of apparent exceptions have been previously reported. The mechanisms involved in these individual cases of NMD resistance include the proximity of nonsense mutations to the natural initiation codon, translation re-initiation downstream of the nonsense codon and the presence of a sequence *cis*-acting element that confers immunity to the 50–55 nucleotide boundary rule (31). The same as the c.1554-1009G>A mutation (24), this intronic point mutation resulting in pseudoexon insertion can be effectively targeted with antisense therapy. In the case of c.882-28A>T mutation, the appearance of skipping of exon 7 coincides with what it was described for the c.882-28A>G mutation (32), because both of them affect the same nucleotide. Regarding the previously described c.2292G>A mutation, the observed effect in cDNA PCR product is the same as the one reported by other authors (18, 25).

We also describe a large deletion in one NPC patient, including the whole *NPC1* gene. To our knowledge, this is the first large deletion described in a NPC patient.

One allele remained unidentified, in the case of patient NPC29, in spite of the comprehensive analysis at the genomic and cDNA levels. The facts that a mutation on one *NPC1* allele was identified and that no mutation in *NPC2* was found strongly suggest that the missing mutation is in the *NPC1*

gene. An aberrant splicing including the skipping of exon 11 was observed; however, it also occurred in wild-type individuals, indicating that it is not related to the disease. No other aberrant splicing was detected, which rules out the presence of a deep intronic mutation. The mutation (in exon 9) and polymorphisms in exons 4 and 12 were found in heterozygosity, both at the genomic and at the cDNA levels. These data rule out a complete deletion of the gene or a mutation in a regulatory region that prevents the transcription of the gene from one allele. Moreover, no differences at the RNA level were detected by real-time PCR. A complex rearrangement, a small deletion, or another alteration could exist, but it is difficult to understand how it was detected neither by the genomic nor by the cDNA analyses performed.

Genotype–phenotype correlation

As we previously reported, this NPC series shows a great prevalence of the severe infantile form (around 30%), whereas this clinical presentation represents 20% of cases in a European survey (27).

Genotype–phenotype correlations are limited, due to the large number of private mutations and because most of the patients' samples were found to be heteroallelic. Among these correlations, we would like to introduce several considerations relating to some mutations. The p.I1061T mutation has been suggested to correlate with juvenile clinical presentation (23). Conversely, we describe for the first time one homozygous patient for p.I1061T mutation (NPC62), who presented the severe infantile clinical onset. This last fact is in disagreement with the previous conclusion that one p.I1061T allele is sufficient to exclude the most severe infantile neurological form (20). In this patient, the *NPC1* gene was entirely sequenced and it shared the haplotype [–, –, –, –, –, –] with respect to the six polymorphic markers p.Y129Y, p.H215R, p.M642I, p.I858V, p.N931N and p.R1266Q as we previously reported for all chromosomes bearing the p.I1061T mutation (10). This patient presented neonatal cholestasis but absence of neurological signs until second year of life. At that time, he presented with retarded psychomotor development, followed rapidly by the appearance of other symptoms as ataxia, dystonia and cataplexy. The parents are consanguineous and a brother died at 1 year by hepatopathy of unknown origin.

According to published data, the presence of p.P1007A mutation led to a juvenile or adult clinical onset (10, 18, 21). Patient NPC61, heterozygous compound for this mutation and biochemical variant, presented the neonatal systemic clinical presentation of the disease. This patient presented hepatosplenomegaly and cholestasis at birth. He died from liver failure at 6 months of life before onset of neurological disease.

In conclusion, we have established the mutation profile in a large number of NPC patients, which contributes to a greater knowledge of this uncommon disorder. Moreover, we want to emphasize the need to analyse the cDNA in presence of CHX in those patients with one unidentified allele after performing the mutation screening in the genomic DNA. It is essential to detect splicing defects, which have relevance (20% of the mutated alleles in this cohort) as disease-causing mutations.

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Molecular analysis of 30 NPC patients

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