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# Characterisation of two deletions involving *NPC1* and flanking genes in Niemann–Pick Type C disease patients

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ABSTRACT

Niemann–Pick type C (NPC) disease is an autosomal recessive lysosomal disorder characterised by the accumulation of a complex pattern of lipids in the lysosomal-late endosomal system. More than 300 disease-causing mutations have been identified so far in the *NPC1* and *NPC2* genes, including indel, missense, nonsense and splicing mutations. Only one genomic deletion, of more than 23 kb, has been previously reported. We describe two larger structural variants, encompassing *NPC1* and flanking genes, as a cause of the disease. QMPSF, SNP inheritance and CytoScan® HD Array were used to confirm and further characterise the presence of hemizygous deletions in two patients. One of the patients (NPC-57) bore a previously described missense mutation (p.T1066N) and an inherited deletion that included *NPC1*, *C18orf8* and part of *ANKRD29* gene. The second patient (NPC-G1) had a 1-bp deletion (c.852delT; p.F284Lfs*26) and a deletion encompassing the promoter region and exons 1–10 of *NPC1* and the adjacent *ANKRD29* and *LAMA3*. This study characterised two novel chromosomal microdeletions at 18q11–q12 that cause NPC disease and provide insight into missing *NPC1* mutant alleles.



## 1. Introduction

Niemann–Pick type C (NPC) disease (OMIM ID: 257220, 607625) is a rare autosomal recessive disorder characterised by the defective lysosomal storage of multiple lipids, such as cholesterol and glycosphingolipids [1]. The clinical phenotype of NPC is extremely heterogeneous, with an age of onset ranging from the perinatal period until well into adulthood [2]. Affected individuals present hepatosplenomegaly and progressive neurodegeneration.

NPC is caused by mutations in two genes, *NPC1* (MIM ID: 607623) or *NPC2* (MIM ID: 601015) [3,4]. Approximately 95% of patients have mutations in the former, which encodes a transmembrane protein residing in late endosomes/lysosomes [5], whereas the remaining 5% of the cases result from defects in the latter, a small soluble lysosomal protein [6].

To date over 300 disease-causing mutations have been reported [2], most being missense mutations [7] while the remaining ones are splicing mutations, indels and nonsense mutations. Only one genomic deletion, encompassing intron 4 to exon 12 of the *NPC1* gene, was reported [8].

Here we describe two heterozygous deletions at 18q11–q12 involving *NPC1*. In a previous survey we briefly described one of these deletions [9]. Here we characterised both deletions using three different methods: the segregation analysis of several SNPs within the patients' families, in which Mendelian errors were consistent with the presence of a deletion, quantitative multiplex PCR of short fluorescent fragments (QMPSF) [10] and CytoScan® HD Array.

The deletions described in this study were in compound heterozygosity. The other mutations found were: a previously described missense mutation (p.T1066N) in patient NPC-57, and a single-nucleotide deletion (c.852delT) causing a frame shift and a premature stop codon (p.F284Lfs*26) in patient NPC-G1. This mutation was previously described in Italian patients [11]. The two patients described in this report,

*Abbreviations:* NPC, Niemann–Pick type C; *NPC1*, *NPC1* gene; *NPC2*, *NPC2* gene; QMPSF, quantitative multiplex PCR of short fluorescent fragments; NMD, nonsense-mediated mRNA decay; MAF, minor allele frequency; MLPA, multiplex ligation probe amplification; CNVs, copy number variants; JEB, junctional epidermolysis bullosa; LOCS, laryngo-onycho-cutaneous syndrome; DGV, Database of Genomic Variants.

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together with the one described by Bauer et al. [8], indicate that rare structural variants encompassing *NPC1* are responsible for NPC disease.

## 2. Materials and methods

### 2.1. Patients

General information and clinical details of the patients NPC-57 and NPC-G1 are shown in Table 1.

### 2.2. Genomic DNA amplification and sequencing

Primers were designed to amplify each *NPC1* exon and corresponding intronic flanking regions (primers available upon request). The PCR products were sequenced in forward and reverse directions using ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

### 2.3. Reverse transcription of NPC1 mRNA and cDNA sequencing

Total cellular RNA was isolated and cDNA was synthesised as previously described [12]. The *NPC1* cDNA was then amplified using primers 5′TGTCCAGATGTCCATCCTGTT3′ and 5′ATTGCTATCGATGGGAGTGT3′ to confirm the c.852delT mutation. The resulting amplification product was sequenced as described above. To assay nonsense-mediated mRNA decay (NMD), fibroblasts from the patient and from a control individual were cultured in the presence of 500 μg/ml of cycloheximide for 6 hours.

### 2.4. Polymorphism analysis

As first approach to define the two deletions we analysed the inheritance of several polymorphisms located in *NPC1* and flanking genes *(CABLES1, C18orf45, RIOK3, C18orf8, ANKRD29, LAMA3, TTC39C, OSBPL1A, IMPACT, ZNF521)*. Polymorphisms were selected from the CEU panel of the HapMap database (www.hapmap.org, release 28), choosing only those with a minor allele frequency (MAF) >0.2 in order to increase the chance of heterozygosity in our sample. The list of polymorphisms is shown in Supplementary Table 1.

### 2.5. QMPSF assay

QMPSF assay was used to detect the deletions and to narrow down their extension. Short genomic fragments (between 100 and 250 bp) located in *NPC1* and flanking genes were simultaneously PCR-amplified in a single tube using dye-labelled primers. An additional fragment corresponding to exon 7 of the *RNF20* gene, located on chromosome 9q, was co-amplified as a reference [13]. Three distinct multiplex PCR reactions were designed to delimit the breakpoints of the deletions in the two patients (Set 1, 2 and 3). Primers, location and length of the fragments are shown in Supplementary Table 2. One μL of the PCR product was resuspended in a mix containing 10.9 μL of deionised formamide and 0.1 μL of GeneScan 600 LIZ size standard (Applied Biosystems). PCR products were run on an ABI PRISM sequencer and data were analysed using Peak Scanner v.1.0 software (Applied Biosystems). The analysis was based on the comparison of peak heights between each patient and an average of healthy controls. The copy number of each tested fragment was expressed as the following ratio: (height of the peak corresponding to the tested fragment for the patient / height of the peak corresponding to *RNF20* for the patient) / (height of the peak corresponding to the tested fragment for the average of controls / height of the peak corresponding to *RNF20* for the average of controls). Ratios<0.65 were indicative of deletion. Positive results were confirmed in a second independent QMPSF assay.

### 2.6. CytoScan® HD Array

The high-resolution genome-wide DNA copy number analysis was performed in both patients using the CytoScan® HD Array (Affymetrix, Santa Clara, CA, USA). Data was analysed with Affymetrix Chromosome Analysis Suite software. The CytoScan® HD Array includes more than 2.67 million copy number markers of which 1.9 million are non-polymorphic probes and 750,000 are SNP probes that genotype with 99% accuracy and provides confident breakpoint determination.

## 3. Results

In the present study we performed an exhaustive characterisation of mutations borne by two severe cases of Niemann–Pick C disease, NPC-G1 and NPC-57, with a wide variety of clinical features (Table 1).

Sequencing the 25 *NPC1* exons and intronic flanking regions allowed the identification of a mutation in exon 6, c.852delT, in patient NPC-G1. This defect was inherited from the mother. cDNA analysis revealed that the c.852delT allele was partially subjected to NMD (data not shown). The affected patient was apparently homozygous for this mutation and no additional mutation was found, either at the gDNA or cDNA level. Surprisingly, the father did not carry the c.852delT mutation (Fig. 1). Moreover, Mendelian inconsistencies were observed at this first stage for two *NPC1* polymorphisms, rs12970899 and rs1805081 (see "•" in Supplementary Table 1). This evidence suggested the potential presence of a paternal deletion encompassing the *NPC1* gene.

In a previous study we briefly described another patient (NPC-57) consistent with the presence of a heterozygous deletion including the whole *NPC1* gene [9].

Initially, QMPSF and SNP inheritance analyses were used to confirm the deletions in patients NPC-57 and NPC-G1.

Regarding the QMPSF assay, three sets of probes were designed (Fig. 2). Initially, set 1 (probes A, C, E, I, J) was used for both patients. Based on the results of set 1, two additional sets of probes, set 2 (probes B, C, D, E) and set 3 (probes E, F, G, H, I), were designed for patients NPC-57 and NPC-G1, respectively. In sets 2 and 3 we also included probes from set 1 as a control for the presence (set 2: C; set 3:

**Table 1**
Clinical features of patients NPC57 and NPC-G1.

| Patient | Origin | Clinical phenotype | Biochemical phenotype | Age at diagnosis | Diagnosis method | Age of death | Clinical features |
|---|---|---|---|---|---|---|---|
| NPC57 | Spain | Severe Infantile | Classical | 3 years | Filipin test | 6 years | Ascitis, neonatal jaundice, hepatosplenomegaly, hypotonia, delay in motor and mental development, vertical ophthalmoplegia, dystonia, dysarthria, cataplexy, dysphagia, respiratory failure and epilepsy |
| NPC-G1 | Greece | Severe Infantile | Classical | 7 months | Liver biopsy Filipin test | 26 months | Foetal ascites resolved until birth, neonatal jaundice, hepatosplenomegaly, ascites, severe dystrophy and malnutrition, hypotonia, dystonia, developmental delay, able to sit at 18 months, unable to walk and sit up at 2 years, vertical gaze palsy, pulmonary alveolar proteinosis and respiratory failure |

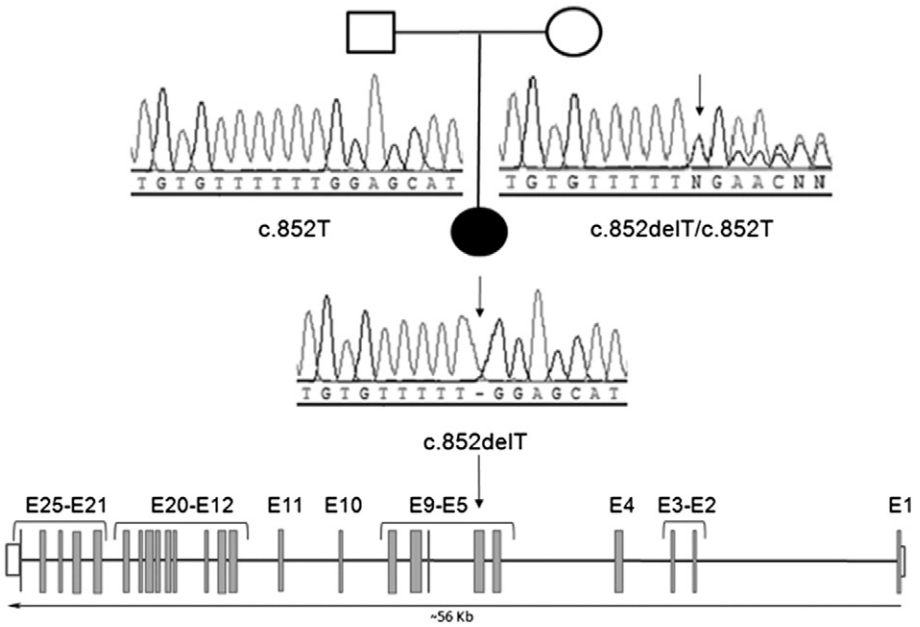


**Fig. 1.** Pedigree of NPC-G1 family showing the chromatograms for mutation c.852delT. The *NPC1* gene scheme, indicating the exon where this mutation was found, is shown at the bottom.

E) or absence (set 2: E; set 3: I) of the deletion. Both patients, their parents, and a sample of 80 healthy controls were analysed with the three sets of probes. The results of the QMPSF analysis are shown in Fig. 2 and in Supplementary Table 2.

In addition, the segregation analysis of 149 polymorphisms was used to map the deletions across *NPC1* and flanking genes. The results for both patients and their respective parents are summarised in Supplementary Table 1.

The combination of these two assays confirmed the presence of the deletions in the two patients and their carrier parents (Fig. 2 and Supplementary Table 1). The NPC-57 deletion encompassed a minimum of 89.7 kb (chr18: 21097460–21187167) and included the whole *NPC1* gene and at least part of *C18orf8* and *ANKRD29*. Unfortunately, both parents of patient NCP-57 shared the same haplotypic block in the distal and the proximal breakpoint regions (Supplementary Table 1), thus precluding further delimitation of the deletion boundaries using allelic information. Thus, using these techniques, the deletion in this patient remained not completely characterised, because it spanned from a minimum of 90 kb to a maximum of 376 kb likely including other genes such as part of *RIOK3* or *LAMA3* (Fig. 2).

In the case of patient NPC-G1, the combination of the two approaches allowed us to refine the proximal and distal boundaries. In this patient, the deletion spanned approximately 432 kb (chr18: 21129874–21561790, Supplementary Table 1). The proximal breakpoint was found to be between the markers rs1652344 and rs34491171 (intron 10 of *NPC1*, see "->" in Supplementary Table 1). The heterozygosity for the first marker and the apparent Mendelian error in the second were consistent with the deletion status. Thus, the promoter region and exons 1 to 10 of the *NPC1* gene were deleted. The distal breakpoint occurred between the markers rs1258143 and rs17187360 (see "=>" in Supplementary Table 1), located in the intergenic region between *LAMA3* and *TTC39C*, which are at 5′ of *NPC1*. The candidate region could be narrowed down by the analysis of the loss of heterozygosity for rs1652344 (proximal region) and rs17187360 (distal region) in several PCRs of different length. As seen in Supplementary Fig. 1, the proximal limit of the candidate region was defined by the positions of primer 4

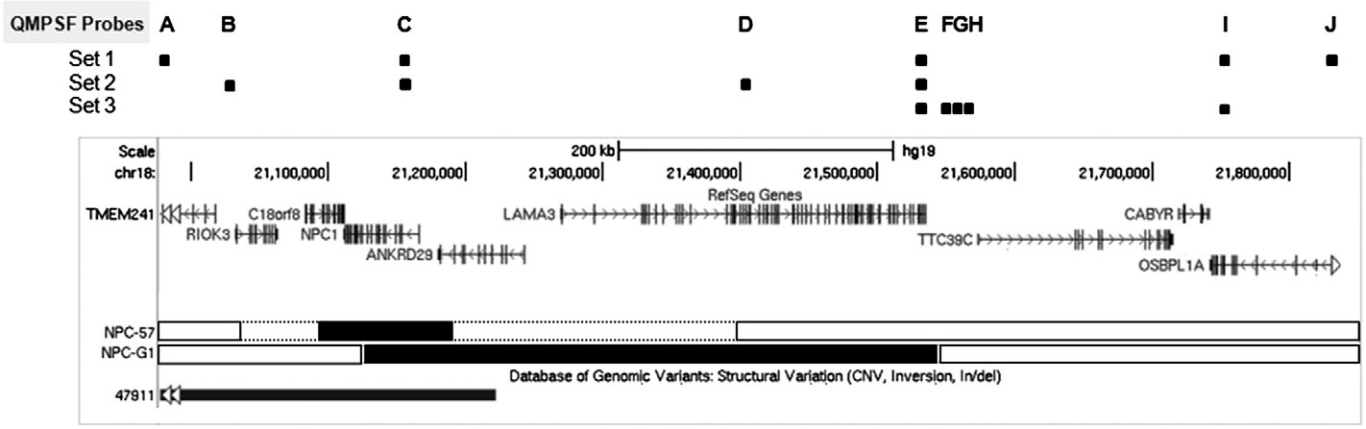


**Fig. 2.** Schematic representation of the deletions identified in the NPC-57 and NPC-G1 patients in their genomic context, according to the results obtained by QMPSF. At the top, the QMPSF probes designed for sets 1, 2 and 3 are shown as black squares. The genes in the region are shown below. At the bottom: black boxes indicate the minimum length of the deletions. Dashed lines correspond to regions for which the status could not be determined by this technique. A CNV found in the Database of Genomic Variants (DGV) is also shown.

(that yield PCR products in which rs1652344 is heterozygous) and primer 5 (PCR products with lack of heterozygosity for marker rs1652344). Similarly, the distal border was placed between the positions of primers 8 and 9. This approach allowed narrowing down the candidate region to 272 bp (proximal breakpoint) and 453 bp (distal breakpoint).

The QMPSF assay and the analysis of SNPs inheritance allowed us define the breakpoint boundaries of the deletion in patient NPC-G1. However, these methods were of limited use for patient NPC-57, due to the homozygosity of the region across the deletion and the high number of QMPSF probes necessary to achieve the coverage of this larger deletion. The Affymetrix CytoScan® HD Array was finally used to address these issues. The high density of probes of this array allowed confirmation of the previously defined NPC-G1 deletion boundaries and also narrowing down of the NPC-57 deletion from a maximum of 376 kb to 130.707 kb (Fig. 3). The NPC-57 deletion includes *C18orf8, NPC1* and part of *ANKRD29*. The proximal breakpoint occurred between the positions chr18:21072726 and 21074676 (between genes *RIOK3* and *C18orf8*) and the distal breakpoint was found to be between the positions chr18:21203179 and 21203433 in the *ANKRD29* gene. Thus, the *RIOK3* and *LAMA3* genes were not included.

On the basis of these results, we performed two prenatal diagnoses in the NPC-G1 family (not shown). The first foetus carried both *NPC1* mutations (pregnancy was interrupted), whereas the second inherited the wild-type allele from the parents.

## 4. Discussion

In this study we report two deletions encompassing *NPC1*, thereby confirming that rare structural variants are also involved in NPC disease. Only one case of a large deletion was previously described by another group [8].

A total of three different techniques were applied in order to confirm and characterise the deletions in the two patients: QMPSF, SNP inheritance and the Affymetrix CytoScan® HD Array. We initially considered using the multiplex ligation probe amplification (MLPA) methodology since a commercial kit, that allows inspection of *NPC1* and *NPC2*, was available from MRC Holland (probemix P193-A2). However, this option was discarded because a SNPs inheritance analysis indicated that both deletions extended over the *NPC1* gene. The QMPSF method represented a more flexible approach for our study. The QMPSF and the SNPs inheritance patterns were sufficient to finely map the deletion in patient NPC-G1, whereas they were not adequate for patient NPC-57 due to the fact that the deletion spanned across a homozygosity region and too many QMPSF probes were necessary to define the unknown region. The CytoScan® HD Array allowed a better refinement of the proximal and distal breakpoints in the NPC-57 patient. The deletion boundaries for NPC-G1 were also confirmed.

Repetitive sequence elements and short repeats are known to be involved in genomic rearrangements [14]. In this regard, non-homologous recombination mediated by *Alu* elements has been described in other lysosomal disorders [15–18]. For patient NPC-57, several repetitive elements mapped in the regions flanking the deletion. This observation suggests that a number of combinations of these elements may be at the origin of the rearrangement observed. Regarding patient NPC-G1, the candidate regions for the proximal and distal breakpoints were finely mapped, resulting in small regions of 272 bp and 453 bp, where two repetitive elements, an AluSx3 (intron 10 of *NPC1*) and an L2a element (intergenic region between *LAMA3* and *TTC39C*) may be involved in the recombination event (Supplementary Fig. 1). However, although the small region between the two repetitive elements was expected to be no longer than 725 bp, no PCR product was amplified after several attempts under different conditions. One possible explanation for this is that a complex chromosomal rearrangement associated with the deletion prevents this amplification. Complex chromosomal rearrangements have been described in other diseases [19,20]. The finding that the patient's cytogenetic study showed a normal karyotype led us to rule out the presence of a large chromosomal recombination. The possibility of a duplication event, flanking the proximal and distal breakpoints, was

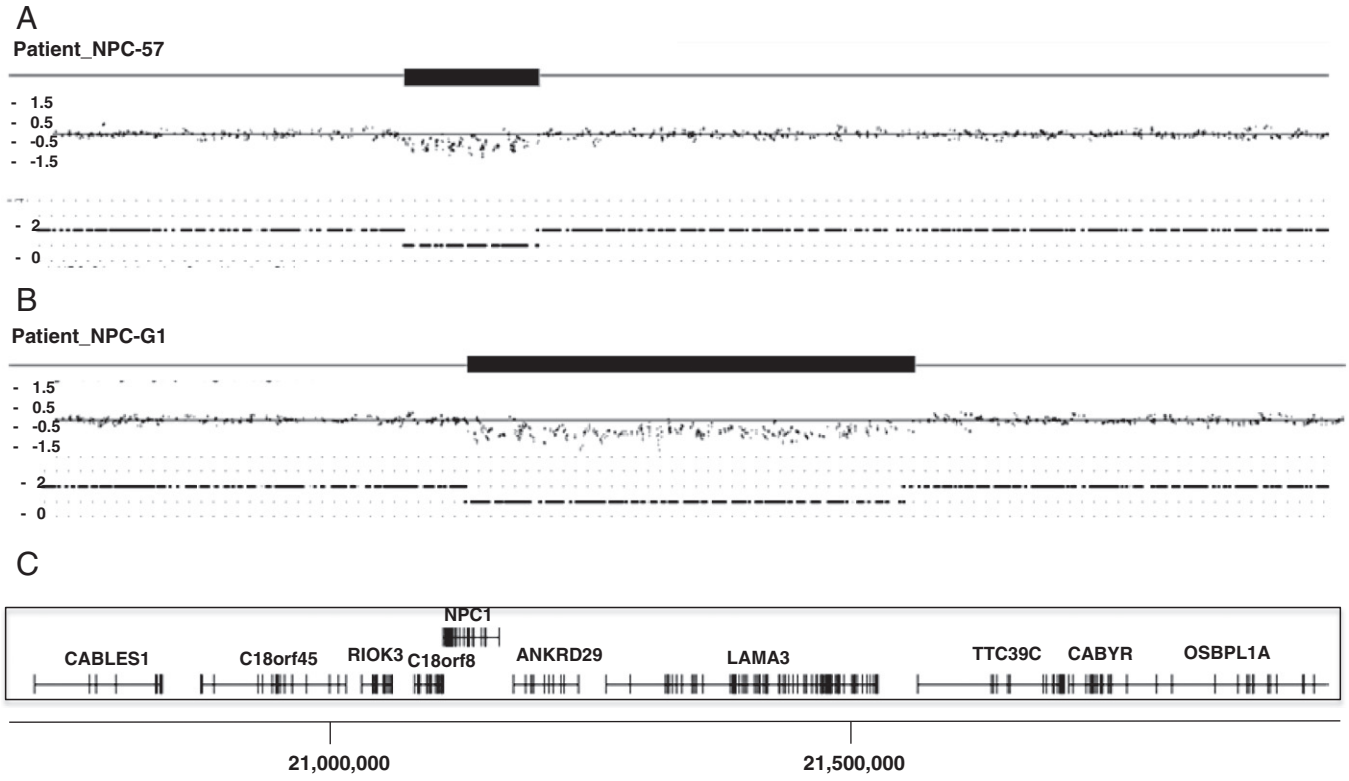


**Fig. 3.** Representation of the deleted regions for NPC-57 and NPC-G1 patients according to the results obtained by the CytoScan HD Array. For each patient, the deleted region is shown as a black bar (top) and the analysed markers and their dosage are represented as dots (medium) or dashed lines (bottom). A: patient NPC-57. B: patient NPC-G1. C: Genes in the region and nucleotide numbering based on UCSC Genome Browser February 2009 (GRCh37/hg19, http://genome.ucsc.edu).

also discarded as the QMPSF probes resulted in a normal dosage status. Thus, a plausible explanation is the occurrence of the deletion concomitantly with another type of rearrangement that escaped detection.

The Database of Genomic Variants was used to search for copy number variants (CNVs) at the *NPC1* locus (DGV, http://projects.tcag.ca/variation/). The only variation including *NPC1* was a deletion present in an isolated healthy population from Micronesia (variation_47911, [21] in Fig. 2). This deletion is different from those presented in this study, thus pointing to an independent recombination event in our patients.

Noteworthy, the deletion of patient NPC-G1 also encompassed *LAMA3*, a gene implicated in two autosomal recessive disorders, namely junctional epidermolysis bullosa (JEB) (OMIM ID: 226650) and laryngo-onycho-cutaneous syndrome (LOCS) (OMIM ID: 245660). Thus, the rearrangement described in this family, if present in the general population, might be involved not only in NPC but also in JEB and LOCS. The remaining genes included in these deletions are not reported to be responsible for any other known human disease.

The two patients included in this study had severe phenotypes (see Table 1). Although the structural variants described here are severe, the second allele should also be considered. In patient NPC-G1, the other allele bore a 1-bp deletion and did not produce functional protein. In the other patient, the second allele bore a missense mutation, p.T1066N. This mutation had been found only once before, in another Spanish patient, in compound heterozygosity with p.P1007A [22] but no genotype-phenotype correlation was established for the p.T1066N mutation. The case presented here suggests that p.T1066N is a severe mutation.

Using conventional sequencing some mutations can escape detection. For example, in our initial analysis on 40 Spanish patients, 10 alleles (12.5%) remained unidentified [22]. A more exhaustive analysis allowed the identification of 9 of the previously unidentified alleles [9]. These difficult-to-find alleles included, among other changes, deep intronic mutations [12] or large deletions as the ones described here. Our results highlight the need to search for unusual mutant alleles, such as structural variants, in NPC patients lacking characterised *NPC1* or *NPC2* mutations by conventional genomic DNA and cDNA screening methods. In our series, this deep mutational analysis allowed the identification of more than 99% of the mutant alleles (127/128).

Taking into account all the Spanish NPC patients analysed in our cohort, large deletion alleles represent less than 1% of the mutant alleles (1/128). In general large genomics deletions in the *NPC1* gene are very rare. The Human Gene Mutation Database (HGMD Professional, release 29 June 2012) only includes two *NPC1* deletions of more than 1 kb out of 320 different mutations. These mutations are the one described by Bauer et al. [8] and the one present in patient NPC-57 characterised here. This figure increases in one, up to three, by the description of the mutation borne by patient NPC-G1.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ymgme.2012.10.004.

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