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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