NPC1 silent variant affecting splicing (p.Val562Val) and unfold protein response in an NPC patient

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Abstract

Niemann-Pick type C (NPC, MIM #257220) is a neuro-visceral disease, caused predominantly by variants in NPC1 gene. Here we studied patients with clinical diagnosis of NPC but inconclusive results regarding molecular analysis. We used a NGS-panel followed by cDNA analysis. Latter, we used massively parallel single cell RNA-seq (MARS-Seq) to address gene profiling changes and finally the effect of different mutations on the protein and cellular levels. We have identified novel mutations and cDNA analysis allowed us to stablish the functional effect of a silent variant. We demonstrated that this mutation induces complete skipping of exon 11 and premature stop codon and identified it in NPC patients from unrelated families. MARS-Seq analysis showed that a number of upregulated genes were related to the unfold protein response (UPR) and endoplasmic reticulum (ER) stress and for all analyzed mutations, the NPC1 protein was partially retained in the ER.

KEYWORDS

Niemann-Pick type C, *NPC1* , silent variant, exon skipping, RNA-seq, unfold protein response

Main text

Niemann-Pick type C (NPC, MIM #257220) is an autossomal recessive lysosomal storage disease (LSD) with varying age of onset and progression (Patterson et al., 2017). One of its hallmarks is the intracellular accumulation of unesterified cholesterol and other lipids in various tissues (Lloyd-Evans & Platt, 2010). NPC disease also affects central nervous system (CNS) with the major clinical symptoms being vertical supranuclear gaze palsy (VSGP), cognitive impairment, ataxia, dystonia, dysarthria and or dysphagia and progressive neurological deterioration (Garver et al., 2010; Patterson et al., 2017; Wijburg et al., 2012). The vast majority (˜95%) of NPC patients have mutations in the *NPC1* gene but in a few cases the defective gene is *NPC2* (Vanier et al., 1996; Carstea et al., 1997; Millat et al., 1999). The *NPC1* gene encodes for NPC1, a transmembrane protein, which localizes in late endosomes (LE) and lysosomes (Naureckiene et al., 2000). It is highly glycosylated and its biosynthesis and trafficking pathways implicate co-translational*N* -glycosylation and correct folding in the endoplasmic reticulum (ER), transported to the Golgi apparatus

and processing to a mature complex glycosylated protein and finally targeted to the lysosomes (Alfalah, Jacob, & Naim, 2002; Watari et al., 1999). Over 400 mutations have been found in the *NPC1* gene, the majority of which encode missense alleles (Schultz, Krus, & Lieberman, 2016). In recent years, a number of *NPC1* missense pathogenic mutations have been categorized with regard to their trafficking ability and it was shown that most of them result in conformational changes disrupting NPC1 protein trafficking to lysosome (Shammas, Kuech, Rizk, Das, & Naim, 2019). As consequence, the misfolded mutants are retained in the ER and can trigger ER-association degradation (ERAD) and unfold protein response (UPR). UPR, along with heat-shock (HS) responses are cytoprotective for the toxic effects of misfolded protein aggregates (Cox & Cachón-González, 2012). UPR has been reported in other LSD, namely in fibroblasts from patients with neuropathic Gaucher's disease (Cox & Cachón-González, 2012) but not in a Gaucher murine model (in which Glucocerebrosidade - Gcase was effectively absent), suggesting that UPR is not activated by the disturbance of glycosphingolipid metabolism. The same was reported for NPC disease: UPR was not observed in *NPC1* deficient mice neither in NPC cell-based model generated by knocking down the expression of *NPC1* (Klein et al., 2011). However, it has been shown that the most frequent *NPC1* mutant, p.Ile1061Thr, is recognized by ERAD and targeted to the proteosome (Schultz et al., 2018).

In the present study, we investigated the molecular basis of disease in two unrelated NPC patients, who were referenced for analysis in a NGS-targeted gene panel for LSD. As a result from the targeted sequencing workflow, we identified two heterozygous variants in the*NPC1* gene (NM_000271.5, Transcript ENST00000269228.10) in each patient (Table S1, Group I): a novel missense mutation (c.T1514G, p.Val505Gly) in compound heterozigosity with a silent variant (c.1686G>A; p.Val562Val) in patient P1; and two known disease-causing mutation (c.7G>A; p.Ala3Thr and c.2882A>G; p.Asn961Ser) (Dvorakova et al., 2006; Runz et al., 2008) in patient P2. Still, if the molecular diagnosis of P2 offered no doubts, the same would not apply for the analysis of P1. In fact, even though the novel variant p.Val505Gly seemed to be very rare (only one heterozygous individual identified in gnomAD) and occurs in a conserved part of the protein, the silent variant p.Val562Val had previously been reported as a polymorphism in Spanish patients with clinical and biochemical diagnosis of NPC (Fernandez-Valero et al., 2005). Therefore, additional studies were necessary to address the role of these variants in the disease pathogenicity.

The analysis of gDNA by Sanger sequencing confirmed that the patient P1 was heterozygous for both mutations. Still, when we analysed the patient's cDNA, one of the alleles seemed virtually absent, suggesting that one allele was more expressed than the other (figure 1A). In fact, the allele harbouring the p.Val562Val generated a less abundant transcript (figure 1A) and that observation raised the question of whether the p.Val562Val could lead to an unstable transcript most likely degraded by nonsense-mediated decay mechanism (NMD). Upon treatment of P1 primary fibroblasts with CHX for 5h, on the assumption that the inhibition of protein synthesis would prevent the rapid degradation of this mRNA (Carter et al., 1995), that transcript became more expressed (figure 1A, lower panel). In fact, at the boundary between exon 10 and 11, it was possible to visualize the presence of a second transcript with the skipped exon 11 in the sequencing electropherogram (figure 1B). Coincidently, the same pattern was observed in the mother´s cDNA (p.Val562Val carrier) (figure 1B, lower panel). This allowed us to establish the functional effect of the silent p.Val562Val, previously described as a polymorphism (Fernandez-Valero et al., 2005) in the*NPC1* splicing process. The silent p.Val562Val actually leads to exon 11 skipping, frameshift and premature stop codon (PTC) at the amino acid 551 (full length protein of 1278 amino acids).

Having realized this, we then revisited other families with clinical diagnosis of NPC but whose definitive molecular had not been achieved since they presented with a sole *NPC1* -causing mutation in heterozigosity, assuming that some of them could harbour the silent p.Val562Val in the second allele and this would fully justify the associated phenotype. And, in fact the p.Val562Val variant was present in two siblings from another unrelated family (F2:P3 and F2:P3' -, figure 1C and table S1, Group II).

To identify the gene expression profiles of the NPC patients versus controls, the expression pattern of more than 11,000 genes was analysed. MARS-Seq was performed on RNA samples isolated from cultured skin fibroblasts from controls and NPC patients (P1, P4 and P5 – the last two had previously been diagnosed

and reported (Ribeiro et al., 2001) (Table S1). After normalization of the counts and differential expression analysis and considering only genes with fold change > 1.5 and p<0.05, we found up-regulation of 43 genes and down-regulation of 58 genes in P1, compared with P4, P5 and controls. Comparing P4 and P5 with controls, 87 genes were up-regulated and 77 were down-regulated (figure 2A). We decided to focus on a group of clustered genes, which were exclusively up-regulated in P1. Ingenuity pathway analysis (IPA) revealed that the genes differentially expressed in P1, compared with the other NPC patients and controls were associated with the following cellular pathways: UPR; Response to incorrect protein; Response to ER stress; Protein folding; Protein refolding and ERAD (figure 2B). The most pronounced relative fold-change was observed for the *HSPA5* and *HSPA1A* genes, and confirmed by qtRT-PCR (figure 2C). The *HSPA5* and *HSPA1A* genes encode the chaperones BiP and Hsp72, respectively, both of which belong to the HSP70 family. BiP has important functions in protein folding and quality control in the ER and is also a master regulator of UPR. We next sought to determine the consequences of the identified mutations at cellular and biochemical levels. We analysed the endogenous NPC1 protein trough WB and IF. The anti-NPC1 antibody detected two bands on the control, a major band at ˜170 kDa and a minor at ˜130 kDa, which represent glycosylated and non-glycosylated forms of the protein, respectively (figure 2D). We observed that, unlike the pattern observed for controls, there was a part of the protein that is retained in the ER (as observed in the band marked as EndoH resistant that is weaker in the patients' samples - lanes 6, 9, 12 and 15). However, since all patients are compound heterozygous for *NPC1* mutations, the potential interaction between the two mutations in the NPC1 protein is difficult to assess. Interestingly, since the second mutation harboured by patient P1 is the silent mutation p.Val562Val, which leads to a truncated protein of ˜55 kDa, the observed bands at ˜170 kDa and ˜130 kDa are most likely result of the translation of only one *NPC1* allele: that harbours the p.Val505Gly mutation. Immunostaining followed by confocal laser microscopy of NPC1 in control cell lines showed that the wild type NPC1 localizes predominantly in lysosome, as confirmed by its co-localization with LAMP1 (figure 2E, panel 1). Slight colocalization was also observed with the ER marker calnexin. In NPC1 mutants, nevertheless, no colocalization was found between NPC1 and LAMP1. This IF result corroborated the WB studies, confirming the partial ER retention of the protein (figure 2E).

The wide range of clinical phenotypes, in age of onset and the rarity of NPC disease, makes the diagnosis a significant challenge. Therefore, the study of novel putative missense *NPC1* mutations at the protein and cellular levels is very important, namely the effect on the intracelular processing, trafficking and localization of NPC1 protein. In the case of silent mutations as the one here reported, the cDNA analysis on the trio (índex case and parents) is also essential to adress the possible impact on the mRNA processing. In this study, we have demonstrated that the p.Val562Val alteration, which had previously been reported and classified as a polymorphism (Fernandez-Valero et al., 2005) is in fact a disease-causing mutation and was found in three Portuguese patients (two siblings and one unrelated patient). In the same protein region (luminal loop between TM2 and TM3) a second (novel) mutation in compound heterozigosity was identified (p. Val505Gly) in patient P1. Once we had successfully achieved the molecular diagnosis of these challenging cases, and being aware that NPC disease is characterized by several different pathogenic cascades, we wanted to further understand the different pathomechanisms related to different *NPC1* mutations combinations but all leading to the juvenile type of the disease. Interestingly, we found that P1 has massive upregulation of genes related to UPR and ERAD, namely *HSPA5* and *HSPA1A* . ERAD has been described for the mutation p. Ile1061Thr, since this mutated protein is retained in ER (Schultz et al., 2018). In fact, immunoprecipitation of overexpressed and endogenous control and NPC1- p.Ile1061Thr demonstrate interactions with the molecular chaperones Hsp70 (encoded by *HSPA1A*), Hsc70, Hsp90 and the ER-localized chaperone calnexin (Schultz et al., 2016). Interestingly, the upregulation of genes encoding these chaperones is much higher in the patient currently studied (P1) than in P4 and P5, which reserves future studies. Our results suggest that UPR activation changes among the different NPC patients (harbouring different mutations), as previously described for Krabbe disease (Irahara-Miyana et al., 2018), but we cannot exclude other players in this process. Since we observed UPR, our next hypothesis was whether NPC1 mutants are retained in ER or not. As recently described, there are several *NPC1* disease-causing mutations leading to ER retention (Shammas et al., 2019) besides p.Ile1061Thr (Gelsthorpe et al., 2008; Nakasone et al., 2014). All the mutations studied herein lead to partial retention in the ER, even though only P1 triggers UPR. This last observation requires

further studies in order to better understand its impact on cellular dysregulation and ultimately shed light in the pathomechanisms underlying NPC disease.

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Contributors

MTC, PC and ELT phenotyped the patient P1. ME and SA processed and analyzed the NGS-data. ME, MFC and IR performed the Sanger sequencing. ME performed RNA extractions, cDNA synthesis, qtRT-PCR, WB and IF. MFC and JIS performed the CHX treatments, some RNA extractions and cell culture maintenance. IR performed filipin staining of all patients. SMC performed and analysed RNA-seq. ME, MFC, IR and SMC analysed and interpreted the data. SA, LV, DQ, LL and AHF obtained funding support. ME wrote the first version of the manuscript and prepared the figures. MFC helped in the first version of the manuscript. SA coordinated the work and corrected the manuscript. All authors read the final version of the manuscript and gave their permission for publication.

Competing interests

None declared.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Legends

Figure 1 The silent variant p.Val562Val leads to the skipping of exon 11 and premature stop codon (PTC) and was found in two unrelated NPC families . (A) The discrepancies between patient P1 gDNA (heterozygous for two nucleotide substitution: c.1514T>G; p.Val505Gly and c.1686G>A; p.Val562Val) and the cDNA (one allele more expressed than other) led to the hypothesis of an unstable transcript. After segregation studies (chromatograms not shown) we observed that the allele inherited from the mother, that carries the variant c.1686G>A; p.Val562Val is expressed in low abundance. Treatment with cycloheximide (CHX) partially stabilized the transcript (Fig.1A, right and lower panel), suggesting NMD. (B) Agarose gel (ethidium-bromide 1%) electrophoresis and sequencing chromatograms of cDNA-derived PCR products (using PAXgene blood RNA) comprising exons 9-13. The 697 bp amplicon represents the normal splice product and the 594 bp represents the aberrant transcript, with the skipping of exon 11. CTRL= control cDNA; negative = water control. At the boundaries of exon 10 and 11 it is possible to observe the second transcript, where the exon 11 is missing (Fig. 1B, right) and the PTC, marked in red. The same transcript was observed in mother cDNA (Fig. 1B, lower panel) (C) gDNA analysis of exons 9 and 11 in the siblings P3/P3' (Family 2 – F2), showing the mutations in heterozigosity. Subsequent/additional analysis on the parents and non-affected sister, shows the segregation of the mutations (Fig. 1C, right).

Figure 2 Patient P1 has differentially expressed genes (upregulated) associated with UPR, but other NPC1 mutants exhibit delayed trafficking to lysosome (A) List of genes with altered expression ($p<0.05$, Fold change >1.5) in P1 patient compared with P4, P5 and controls and P4 and P5 vs controls. In red are marked those that are upregulated exclusively in P1. (B) After cluster analysis, the results shown that the most part of the genes are associated with ER stress and UPR. (C) Relative expression levels of mRNA for *HSPA1A* and *HSPA5* genes (two of the obtained hits). Error bars represent standard deviation (SD) from three independent experiments. ***$p<0.05$ P1 vs CTRL by one-way ANOVA with Bonferroni's multiple comparison post-hoc test (n=3 in each group); ns=non significant (D) CTRL and NPC1 primary human fibroblasts were treated with PNGaseF to deglycosylate the NPC1 protein (all N-linked oligosaccharides) and Endo H to cleave all sugars except complex-sugars and subjected to Western blotting (WB) using anti-NPC1 antibody. In CTRL the NPC1 protein is Endo H-resistant, but in all patients the EndoH-sensitive form is more abundant suggesting that part of the protein was retained in the ER, and did

not go for complex sugars formation, a process that only happens in the Golgi; (E) Immunostaining followed by confocal laser scanning microscopy of CTRL fibroblasts revealed that endogenous NPC-1 protein localizes with LAMP1 and partially to calnexin. In primary human fibroblasts of P1 patient, NPC1 colocalizes much less with LAMP1. Instead, it presents a higher colocalization with calnexin. Scale bar 25 μm.