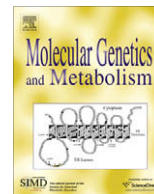




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Nonsense-mediated mRNA decay process in nine alleles of Niemann-Pick type C patients from Spain

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ABSTRACT

Mutations in *NPC1* or *NPC2* genes are responsible of Niemann-Pick type C disease (OMIM #257220), an autosomal recessive neurodegenerative lysosomal storage disorder caused by a non-regulation of intracellular lipid trafficking.

Alterations such as nonsense or frame shift mutations generate a premature termination-codon (PTC). Nonsense-mediated mRNA decay (NMD) is a natural cellular process that degrades mRNAs that encode a prematurely truncated protein.

In this study we have analyzed 9 *NPC1* mutations which generate a PTC (p.R116X, p.Q119VfsX8, p.W260X, p.S425X, p.A558GfsX12, p.Q775X, p.G993EfsX4, p.R1059X and p.I1061NfsX4), in order to determine if their mRNAs suffer NMD process. To achieve this objective we compared fibroblasts of patients carrying these alleles with and without cycloheximide (CHX) treatment using conventional PCR and real-time PCR.

The results of conventional PCR of untreated fibroblasts showed a reduction of the amount of *NPC1* mRNA compared to control in all patients. After CHX-treatment, a recovery of mRNA was detected but not in all the alleles. However, when real-time PCR was used, the recovery was observed including those alleles that qualitatively showed no apparent increase in mRNA level. In conclusion, we confirmed that NMD process is responsible for the mRNA decay for all the analyzed *NPC1* PTC-encoding mutations.

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Introduction

Mutations in the *NPC1* (RefSeq NM_000271.3) [1] (95%) and *NPC2* (RefSeq NM_006432.3) [1] (5%) genes are responsible for Niemann-Pick type C disease (OMIM #257220) [2], a neurodegenerative autosomal recessive lysosomal disorder. Over 270 different mutations have been described worldwide in *NPC1* gene [3], which is located in 18q11-q12 chromosome [4]. It spans 47 Kb, has 25 exons and encodes a NPC1 transmembrane protein composed by 1278 amino acids. Although most of the pathogenic described alterations are missense, other types of mutations, which generate a premature termination-codon (PTC) such as nonsense and frame shift, have also been reported. It is known that these kinds of mutations may cause mRNA degradation by a post-transcriptional mechanism called nonsense-mediated mRNA decay (NMD). NMD is used by eukaryotic cells to control the quality of the mRNA in order

to prevent the expansion of truncated polypeptides [5]. It has been described that NMD process takes place when PTC occurs more than 50–55 nucleotides upstream of the 3'-most exon-exon junction [6]. The protein synthesis inhibitor cycloheximide (CHX) is known to suppress NMD [7] and therefore, is used as proof of NMD pathway involvement.

In this study, we wanted to determine whether NMD mechanism affected the stability of *NPC1* mRNAs bearing a PTC. For this purpose, we performed the analysis of 9 selected nonsense or frame shift *NPC1* mutations. We analyzed and compared the results obtained using conventional PCR and real-time PCR.

Materials and methods

Patients and controls

Samples were obtained from 11 unrelated patients. All of them are of Spanish origin except two patients, one Moroccan (NPC41) and one Portuguese (NPC42). Diagnosis of NPC disease was determined by cytochemical demonstration of pathologically enriched cholesterol via filipin staining [8].

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The control group consisted of four samples from non-related wild-type subjects. To perform the experiments, we prepared a pool of control cDNAs.

Cell culture

Fibroblasts from skin biopsies of control individuals and patients were cultured in monolayer at 37 °C under 5% CO₂ conditions in T75 flasks with MEM medium (Sigma, St. Louis, MO, USA) containing 12% newborn calf serum (Invitrogen, Carlsbad, CA, USA), penicillin and streptomycin.

Analysis of NMD

To determine whether any observed reduction of the mutant transcript was due to NMD, cells were subdivided into two cultures. One of these was treated with cycloheximide (CHX) (Sigma, St. Louis, MO, USA) added to the medium at a concentration of 500 µg/ml and the other was untreated. After 6 h of incubation, fibroblasts were harvested after trypsin treatment [9].

RNA extraction and reverse transcription-PCR

Total RNA was extracted from patient and control fibroblasts using suitable kits (Qiagen, Hilden, Germany). Single-stranded cDNA was obtained using oligo-dT primers and M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Conventional PCR

NPC1 and *Cyclophilin A* cDNA were amplified using 100 ng of cDNA, 20 µM each of the self-designed specific primers and 1 U of Taq DNA Polymerase (Roche, Mannheim, Germany). Primer sequences for the *NPC1* fragment (514 bp) were: *NPC1* forward 5'-CTGCAGTCATCATGTGTGCCACCA-3' and *NPC1* reverse 5'-ACCGACCTTAGACACAGTTTCAGTCAG-3'. Primers used to amplify the *PPIA* fragment (138 bp) were provided by Pre-Developed TaqMan Assay Reagent for *PPIA* (Applied Biosystems, Foster City, CA, USA). The PCR conditions were 45 s at 94 °C, 1 min at 64 °C, 1 min at 72 °C for 35 cycles. The amplified products were analyzed by electrophoresis in 2% agarose gel.

Real-time PCR

Quantitative real-time PCR experiments were performed using the StepOnePlus real-time PCR System (Applied Biosystems, Foster City, CA, USA). All PCR reactions, with a final volume of 10 µl, were run in triplicate and contained 100 ng of cDNA. *NPC1* (target gene) and the endogenous controls *GAPDH* (Glyceraldehyde-3-Phosphate Dehydrogenase), *PPIA* (Cyclophilin A) and *β2M* (β-2-Microglobulin) (housekeeping genes) were run in separate wells. The reagents were TaqMan Gene Expression Master Mix, Human *NPC1* TaqMan Gene Expression Assay (Hs00975249_m1) and Pre-Developed TaqMan Assay Reagents for *GAPDH*, *PPIA* and *β2M* (Applied Biosystems, Foster City, CA, USA). PCR conditions were 50 °C for 2 min, 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, USA). Levels of cDNA were relatively quantified by evaluating C_t values according to the Comparative C_t (ΔΔC_t) method (Applied Biosystems, Foster City, CA, USA).

Results

Table 1 reports *NPC1* gene mutations of the 11 unrelated patients of the present study. Among the identified changes, there

are 3 novel mutations (p.G535V (c.1604G>T), p.Q775X (c.2323C>T) and p.I1061NfsX4 (c.3181dupA)) and 11 previously described, 6 of which by our group [10].

The suspicion that NMD could degrade the mRNA encoded by these mutations was given by NPC20 patient. In this case, p.I1061T mutation was found in homozygosis in the cDNA, while actually, this mutation was observed in heterozygosis together with p.G993EfsX4 mutation in genomic DNA. The cDNA sequence analysis of CHX-treated fibroblasts confirmed the loss of the p.G993EfsX4-bearing mRNA by the NMD cellular process (data not shown).

In all studied patients, at least one of the alleles carries a mutation which creates a PTC (p.R116X (c.346C>T), p.Q119VfsX8 (c.352_353delAG), p.W260X (c.780G>A), p.S425X (c.1274C>A), p.A558GfsX12 (c.1672dupG), p.Q775X (c.2323C>T), p.G993EfsX4 (c.2978delG), p.R1059X (c.3175C>T) and p.I1061NfsX4 (c.3181dupA)). All of them follow the NMD-rules suggested for this process [17].

To determine whether the transcripts encoded by these mutations were targeted by the NMD process, we analyzed the level of the different PTC-bearing mRNAs by conventional PCR (qualitative) and real-time PCR (quantitative) before and after cycloheximide (CHX) treatment.

Fig. 1A shows the NMD analysis of 11 patients and control individuals by conventional PCR. We observed that there were differences between untreated and CHX-treated control samples, probably due to the rescue of other mRNAs encoded by alternative spliced transcripts of *NPC1* gene [15]. When fibroblasts cultures of homozygous patients (NPC14, NPC17, NPC35, NPC41 and NPC42) were untreated (0 h) a clear decay of the *NPC1* cDNA was observed. The same phenomenon was seen in the case of untreated fibroblasts culture of NPC20 heterozygous compound for p.G993EfsX4 mutation. The rest of heterozygous patients (NPC11, NPC22, NPC26, NPC28 and NPC43) only showed a slight reduction in the intensity of *NPC1* band. After CHX-treatment (6 h), important recoveries in the *NPC1* cDNA levels were observed for the homozygous (NPC14, NPC17, NPC41 and NPC42) and NPC20 heterozygous patients. Only slight recovery was showed for NPC22, while no obvious changes were observed for the rest of the heterozygous patients (NPC11, NPC26, NPC28 and NPC43) and the homozygous NPC35.

In order to confirm the qualitative results, we quantified the differences at the cDNA level of each NPC patient with respect to *PPIA*, *GAPDH* and *β2M* genes by real-time PCR. *GAPDH* and *β2M* genes showed significant differences in C_t values between the control samples tested (data not shown) and they were discarded as endogenous controls.

Fig. 1B shows relative quantification (RQ) of the *NPC1* cDNA normalized to *PPIA* cDNA levels (endogenous control) for the different patients using the Comparative C_t (ΔΔC_t) method. The control samples (untreated and CHX-treated) were set as the reference value (100%). When untreated, samples from homozygous patients showed very low mRNA levels, ranging from 94% (RQ NPC41 = 5.97) to 74% (RQ NPC35 = 26.00) reduction respect to control. With regard to heterozygous patients, in all cases decay of mRNA was also observed, ranging from 61% (RQ NPC20 = 38.66) to 20% (RQ NPC26 = 80.18). After CHX-treatment, the five homozygous patients (NPC14, NPC17, NPC35, NPC41 and NPC42) increased the cDNA levels between 2.4 (NPC14) and 8.2 (NPC41) times compared to untreated samples, but still the cDNA levels were around 50% of those observed in treated control sample. Concerning heterozygous patients, all them presented recoveries between 1.3 (NPC22) and 2.2 (NPC11) times in comparison with untreated samples, reaching similar cDNA levels as treated control. Exceptions were patients NPC20 and NPC26. In the case of NPC20, it showed low percentage of recovery in relation to untreated sample, sur-

Table 1

Clinical phenotype and genotype of the studied NPC patients.

Patient ^a	Clinical phenotype ^b	Nucleotide change ^c (allele 1)	Amino acid change (allele 1)	Nucleotide change ^c (allele 2)	Amino acid change (allele 2)	Reference ^d
NPC11	Late infantile	c.3175C>T	p.R1059X	c.2594C>T	p.S865L	[10]; [10]
NPC14	Severe infantile	c.1672dupG	p.A558GfsX12	c.1672dupG	p.A558GfsX12	[10]
NPC17	Severe infantile	c.2978delG	p.G993EfsX4	c.2978delG	p.G993EfsX4	[10]
NPC20	Late infantile	c.2978delG	p.G993EfsX4	c.3182T>C	p.I1061T	[10]; [11]
NPC22	Late infantile	c.780G>A	p.W260X	c.3182T>C	p.I1061T	[10]; [11]
NPC26	Juvenile	c.1274C>A	p.S425X	c.1421C>T	p.P474L	[10]; [12]
NPC28	Severe infantile	c.346C>T	p.R116X	c.2324A>C	p.Q775P	[13]; [14]
NPC35	Neonatal	c.3175C>T	p.R1059X	c.3175C>T	p.R1059X	[10]
NPC41	Severe infantile	c.3181dupA	p.I1061NfsX4	c.3181dupA	p.I1061NfsX4	Novel mutation
NPC42	Unknown	c.352_353delAG	p.Q119VfsX8	c.352_353delAG	p.Q119VfsX8	[15]
NPC43	Severe infantile	c.2323C>T	p.Q775X	c.1604G>T	p.G535V	Novel mutations

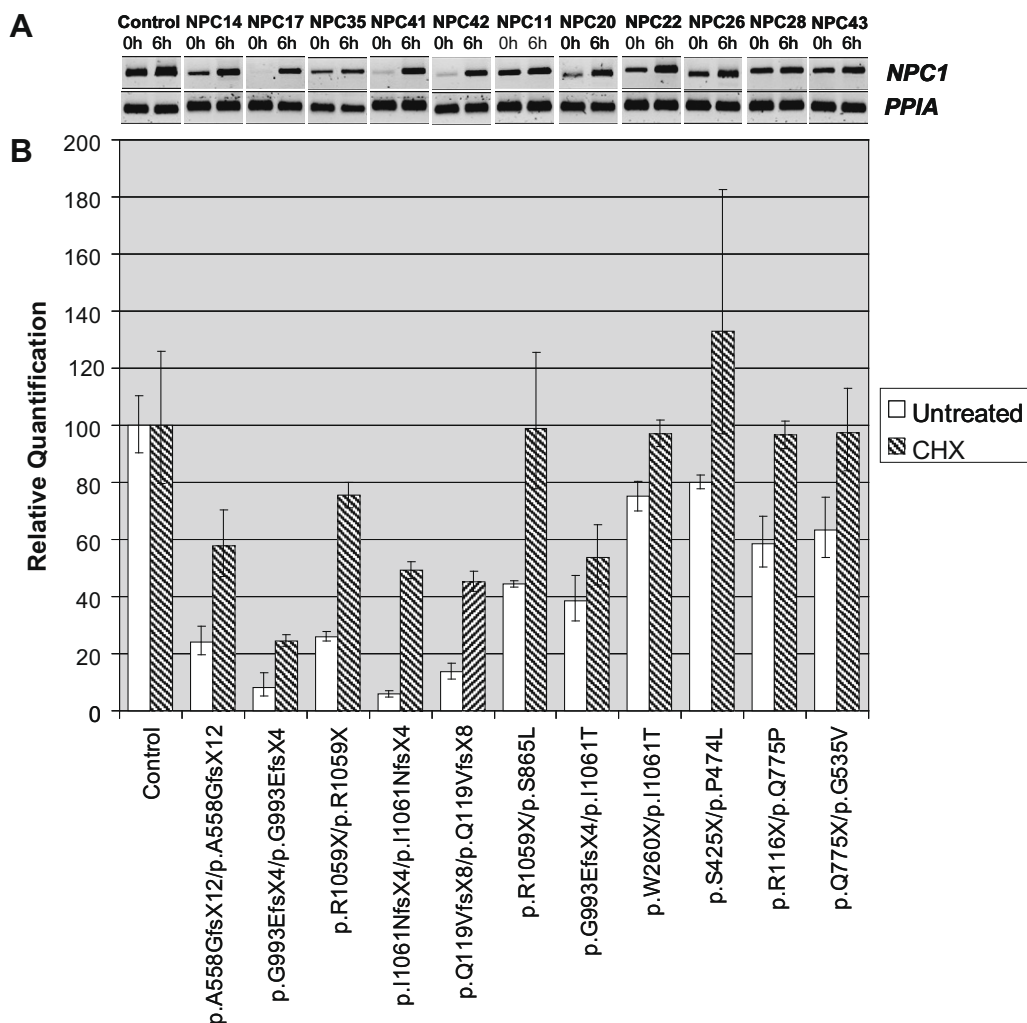
^a Patients NPC11 to NPC35 were previously reported in Fernández-Valero et al. 2005 [10].^b Clinical phenotype attending to the first neurological symptoms.^c Nomenclature according to den Dunnen and Antonarakis 2000 [16]. The A of the ATG of the initiator Met codon in cDNA is denoted nucleotide +1.^d For described mutations only the first citations are given.

Fig. 1. NMD analysis in fibroblasts from NPC disease patients. (A) Conventional PCR: Fibroblasts from the different patients untreated (0 h) or treated with CHX (6 h) were processed. *NPC1* and *PPIA* cDNA PCR products (514 bp and 138 bp, respectively), were separated on a 2% agarose gel and stained with ethidium bromide. NPC patients are arranged first according to their genotypes (homozygous and heterozygous compounds) and second according to their numeration. (B) Real-time PCR: Relative Quantification of the *NPC1* cDNA using the comparative C_t method. Relative *NPC1* cDNA levels were normalized to *PPIA* cDNA levels (endogenous control). Pool of control cDNAs ($n = 4$) showed an untreated control range = [0.524–1.504] and a CHX-treated control range = [0.428–1.853]. Error bars represent standard deviations (patients $n = 3$ and control $n = 6$).

prisingly in disagreement with the qualitative observation. Patient NPC26 showed analogous recovery as the other heterozygous pa-

tients, but in this case, final cDNA level of treated fibroblasts was higher than those of control (133%).

Discussion

In mammals, the process of nonsense-mediated mRNA decay (NMD) is a quality-control mechanism to degrade mRNA harboring a premature termination-codon to prevent the synthesis of truncated proteins [5].

Here, we have presented 11 unrelated NPC patients, all of them bearing at least one PTC-encoding mutation in their *NPC1* gene.

From the series presented, we have described 3 novel mutations, two generating PTC (p.Q775X and p.I1061NfsX4) and one novel missense change (p.G535V). This last mutation is probably a pathogenic mutation due to the fact that it was not observed in 100 normal control alleles; after sequencing all genomic DNA no other mutations have been detected; the affected amino acid residue is conserved throughout species, and functional effect prediction programs as PolyPhen [18] or Panther [19] confirmed the possible pathogenesis of this novel change. Moreover, NPC43 patient's fibroblasts with p.G535V mutation associated with a nonsense mutation (p.Q775X) was studied by western blot, using a polyclonal NPC1 antibody raised against 19 amino acid residues (1256–1274) peptide located in the C-terminus of the human NPC1 protein [14]. No detectable protein was found in this patient in contrast to control fibroblasts (data not shown).

In this study, we were able to detect *NPC1* mRNA decay and its recovery after CHX-treatment of most of the studied alleles by conventional PCR (Fig. 1A). But it was essential to quantify mRNA by real-time PCR to observe recovery in all the alleles, including those that first qualitatively showed no apparent differences (p.R116X, p.S425X, p.Q775X and p.R1059X mutations) (Fig. 1B). We confirmed that NMD process is responsible for the mRNA decay for all here analyzed *NPC1* PTC-encoding mutations, in spite that only partial recoveries after CHX-treatment were detected for p.Q119VfsX8, p.A558GfsX12, p.G993EfsX4, p.R1059X and p.I1061NfsX4 alleles. CHX-treatment is presumed to stop the NMD process, so it makes possible to detect those mRNAs that this cellular mechanism degrades and otherwise would be undetectable.

The fact that after CHX-treatment no notable increase of the mRNA level was detected in patient NPC20 may be due to different factors: It has been suggested mechanistic basis for alternative NMD pathways [20]; the proximity of nonsense mutations to the natural initiation codon may cause the reinitiation phenomenon that may evade the NMD process [21]; this mRNA surveillance system has been reported to be an inherent character of cells and may vary among cells and tissues [22]; Isken and Maquat [23] suggested a delicate balance of metabolic events that can be influenced by different factors; and same authors gave more evidences that UPF and SMG proteins, which were originally discovered to function in NMD, also have roles in other pathways, including specialized pathways of mRNA decay, DNA synthesis and cell-cycle progression, and the maintenance of telomeres. Furthermore, several studies indicate that the post-transcriptional control of gene expression is much more complicated than expected with mRNA metabolism being subject to diverse regulatory mechanisms [24]. All these factors could explain the different recovery rate after CHX-treatment in different PTC mutations that fulfill the NMD-rules.

Our results give further evidence of the important role of the NMD process in the regulation of the cell transcripts, especially in the case of the disease-causing mutations.

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