Niemann-Pick type C disease: mutations of NPC1 gene and evidence of abnormal expression of some mutant alleles in fibroblasts

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Abstract We analyzed Niemann-Pick type C disease 1 (NPC1) gene in 12 patients with Niemann-Pick type C disease by sequencing both cDNA obtained from fibroblasts and genomic DNA. All the patients were compound heterozygotes. We found 15 mutations, eight of which previously unreported. The comparison of cDNA and genomic DNA revealed discrepancies in some subjects. In two unrelated patients carrying the same mutations (P474L and nt 2972del2) only one mutant allele (P474L), was expressed in fibroblasts. The mRNA corresponding to the other allele was not detected even in cells incubated with cycloheximide. The promoter variants (−1026T/G and −1186T/C or −238 C/G), found to be in linkage with 2972del2 allele do not explain the lack of expression of this allele, as they were also found in control subjects. In another patient, (N1156S/Q922X) the N1156S allele was expressed in fibroblasts while the expression of the other allele was hardly detectable. In a fourth patient cDNA analysis revealed a point mutation in exon 20 (P1007A) and a 56 nt deletion in exon 22 leading to a frameshift and a premature stop codon. The first mutation was confirmed in genomic DNA; the second turned out to be a T→G transversion in exon 22, predicted to cause a missense mutation (V1141G). In fact, this transversion generates a donor splice site in exon 22, which causes an abnormal pre-mRNA splicing leading to a partial deletion of this exon. In some NPC patients, therefore, the comparison between cDNA and genomic DNA may reveal an unexpected expression of some mutant alleles of NPC1 gene.—Tarugi, P., G. Ballarini, B. Bembi, C. Battisti, S. Palmeri, F. Panzani, E. Di Leo, C. Martini, A. Federico, and S. Calandra. **Niemann-pick type C disease: mutations of NPC1 gene and evidence of abnormal expression of some mutant alleles in fibroblasts.** *J. Lipid Res.* **2002.** 43: 1908–1919.

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Niemann-Pick type C disease (NPC, OMIM 257220) is a fatal autosomal recessive neuro-visceral disease characterized by progressive neurological deterioration and hepato-splenomegaly, with varying age of onset and ensuing course (1). One of the hallmarks of the NPC disease is the intracellular accumulation of unesterified cholesterol and other lipids in various tissues (2). In NPC fibroblasts, delayed homeostatic responses towards the regulatory effects of plasma LDL-derived cholesterol have been demonstrated (3). In these cells, the movement of LDL-derived cholesterol from the cytoplasm to the cell membrane is defective and, as a consequence, unesterified cholesterol accumulates in the lysosomes (2, 3). Complementation analysis using cultured skin fibroblasts indicated the presence of two groups of NPC: i) Niemann-Pick type C disease 1 (NPC1) (the major group that comprises >90% of NPC patients) and ii) Niemann-Pick type C disease 2 (NPC2) (the minor group) (4, 5). The first gene responsible for NPC, referred to as the Niemann-Pick C1 gene (NPC1 gene), encodes a membrane protein which has 13 predicted membrane-spanning domains (1, 2), five of which share sequence homology with the putative sterol-sensing domains identified in other integral membrane proteins that respond to cell cholesterol content (1, 2, 6). Although the function of the NPC1 protein is not completely understood, several lines of evidence indicate that it is required for the intracellular trafficking of LDL derived cholesterol and endogenously synthesised cholesterol, as well as plasma membrane-derived glycolipids (7–9). More than 50 mutations of NPC1 gene have been reported in the last couple of years in NPC1 patients of different ethnic groups (10–16). A second gene, designated HE1, was found to be responsible for NPC2 (17). It

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Abbreviations: NPC1, Niemann-Pick type C disease 1; NPC2, Niemann-Pick type C disease 2; RT-PCR, reverse transcription and PCR amplification.
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encodes a 151 amino acid lysosomal glycoprotein which is present in many tissues (17). Several mutations of this gene have been reported in NPC patients of different ethnic groups (18). The function of this protein in the intracellular cholesterol traffic and its functional relationship with the NPC1 protein are unknown at present.

In the present study we report the survey of mutations of the NPC1 gene in a group of Italian NPC1 patients and describe in detail some mutations characterised by a discrepancy between cDNA and genomic DNA sequence, which are associated with an unexpected expression of the mutant alleles in cultured fibroblasts.

MATERIALS AND METHODS

Patients

We studied 12 patients (seven males and five females) in whom the diagnosis of Niemann-Pick type C disease was made at an age ranging from 4 months to 33 years. The diagnosis of Niemann-Pick type C disease was based on well established clinical and biochemical criteria (1). Classification of patients with respect to their clinical and biochemical characteristics was made as reported by Vanier et al. (19) and Millat et al. (12).

All the patients' families were unrelated and no consanguinity was reported. According to the type and age of onset of the first neurological symptoms, patients were stratified into infantile form (onset at age <2 years), late infantile (onset at age 5–16 years), and adult form (onset at the age >16 years). The classical biochemical phenotype refers to patients with a massive accumulation of free cholesterol in lysosomes, as revealed by a strongly positive filipin staining. The variant biochemical phenotype refers to patients who, despite the presence of classical neurological symptoms, showed a weakly positive filipin stain.

Informed consent was obtained from all subjects, and, in the case of children, from their parents. The study protocol was approved by the human investigation committee of each participating institution.

A skin biopsy was taken from all probands and from some healthy control subjects after informed consent. Explants were cultured in 25 cm² flasks in DMEM (Dulbecco's modification of Eagle's medium), 100 IU/ml of penicillin, and 50 μg/ml of streptomycin, 2 mM glutamine, 15% fetal calf serum, and 95% air-5% CO_2 as previously reported (20).

Reverse transcription and PCR amplification

Total cellular RNA was isolated from cultured fibroblasts by extraction with RNAzol™ B (Tel-Test, Inc. Friendswood, TX) according to the manufacturer's instructions. Total RNA (5 μg) from cultured fibroblasts was retro-transcribed in a 20 μl reaction mixture containing 0.5 μg of oligo (dT)$_{12-18}$ and 200 units of RNase H Reverse Transcriptase (Super Script ™ II, Gibco BRL, Life Techologies) according to the manufacturer's instructions. NPC1 cDNA was amplified in 10 partially overlapping fragments using the primers listed in **Table 1**. For all PCR amplifications, 3 μl of cDNA were added to a 50 μl mixture containing 0.2 mM of each deoxynucleoside triphosphate (dNTP), 25 pmol of each primer, and 2.6 units of Taq DNA polymerase (Expand High Fidelity, Roche Diagnostics, GmbH, Mannheim, Germany) in PCR buffer (50 mM KCl, 10mM Tris-HCl pH 9, and 1.5 mM $MgCl_2$). The amplification conditions were 30 cycles at: *a*) 95°C for 1.5 min/65°C for 1 min/68°C for 1.5 min (cDNA fragments 1, 4, 5, and 6); *b*) 95°C for 1.5 min/68°C for 1.5 min (cDNA fragments 2, 3, 8 and 10); *c*) 95°C for 1.5 min/63°C for 1 min/68°C for 1.5 min (cDNA fragments 7 and 9) followed by a final extension at 68°C for 10 min.

The amplification products were purified using the High Pure PCR product purification kit (Roche Diagnostics, GmbH, Mannheim, Germany) analyzed by 2% agarose gel electrophoresis and sequenced with the BigDye Terminator Cycle Sequencing kit (PE Applied Biosystems, Warrington, UK) on an Applied Biosystem 377 DNA sequencer using appropriate primers. Once a sequence variant was identified in a sample, its presence was confirmed by fresh re-amplification and re-sequencing of the culprit exon.

Densitometric scanning of multiple RT- PCR products was performed using the LKB Ultrascan Densitometer.

PCR amplification of genomic DNA

Genomic DNA was extracted from peripheral blood leukocytes or cultured fibroblasts by a standard procedure (21). For all PCR amplifications, 0.5 μg of genomic DNA was added to a 50 μl mixture containing 0.2 mM of each dNTP, 25 pmol of each primer, and 2.6 units of Taq DNA polymerase (Expand High Fidelity, Roche Diagnostics, GmbH, Mannheim, Germany) in PCR buffer (50 mM KCl, 10mM Tris-HCl pH 9, and 1.5 mM $MgCl_2$). The primer pairs used are listed in **Table 2**. The amplification

TABLE 1. PCR amplification of NPC1 cDNA fragments

Fragment	Location in cDNA	Size of PCR Product (bp)	Oligonucleotide Primers
1	−87–887	974	F: 5′-CTGAAACAGCCCGGGGAAGTAG-3′ R: 5′-CGTTTTCTGTAGCACCACACTGCA-3′
2	501–1137	637	F: 5′-AAGTAATGACAAGGCCCTGGGACTC-3′ R: 5′-GTCAACTGGATTGGTTGTGACCCG-3′
3	794–1336	543	F: 5′-TGGACGCCATGTATGTCATCATGTG-3′ R: 5′-AGTCAAGAACCTGGTGCAGTATCTG-3′
4	1051–1617	567	F: 5′-GGCTGTGTCATTTTCTTCTCGCTGG-3′ R: 5′-TCCACCAAACGTACCCAGACAAG-3′
5	1501–2034	534	F: 5′-GACGACTTCTTTGTGTATGCCGAT-3′ R: 5′-AATGTAGCTGAAGACACCCAAGGAG-3′
6	1793–2359	567	F: 5′-ATCTGACCATTTCCTTCACTGCTGA-3′ R: 5′-TGTCTAACCCCAAGAGACTCACG-3′
7	2208–2738	531	F: 5′-CCTGTCATCCTTTTCTGAGACTG-3′ R: 5′-CCCATGCCGCCGCACACCATGTT-3′
8	2431–3034	604	F: 5′-CAGGCCTCAGAGAGCTGTTTGTTTC-3′ R: 5′-CACACTTGGGGGTTAGGGTTATC-3′
9	2828–3705	878	F: 5′-TCGACGATTATTTCGACTGGGTG-3′ R: 5′-CAGTAAGACCATGGCCAAATACATC-3′
10	3368–3882	515	F: 5′-CTGCAGTCATCATGTGTGCCACCA-3′ R: 5′-GACCGACCCTTAGACACAGTTCAG-3′

conditions were 30 cycles at: *a*) 95°C for 1.5 min/65°C for 1 min/68°C for 1.5 min (exons 1, 7, 11, 14, and 17); *b*) 95°C for 1.5 min/63°C for 1 min/68°C for 1.5 min (exons 2, 6, 8, 9, 12, 13, 15+16, 21, and 24); *c*) 95°C for 1.5 min/67°C for 1 min/68°C for 1.5 min (exons 3, 5, 18, 19, 23, and 25); *d*) 95°C for 1.5 min/51°C for 1 min/68°C for 1.5 min (exons 4 and 10); *e*) 95°C for 1.5 min/59°C for 1 min/68°C for 1.5 min (exon 20); *f*) 95°C for 1.5 min/61°C for 1 min/68°C for 1.5 min (exon 22) followed by a final extension at 68°C for 10 min. The amplification products were purified, analysed by 2% agarose gel electrophoresis, and sequenced as specified above.

The 5′ flanking region of the NPC1 gene (from nt −1750 to +201 from the translation start site +1) was amplified in four overlapping fragments using the primers indicated below. Fragment P1 (551 bp, from nt −1750 to nt −1200): 5′-TCTCCTGC-CCTCCTGTCTCCAGC-3′ (forward primer) and 5′-AGGTCCTC-CACGACGACCTCGTG-3′ (reverse primer); Fragment P2 (535 bp, from nt −1318 to nt −784): 5′-CTGCATGAAGGCATACAC-

AGGACT-3′ (forward primer) and 5′-AGTTCTACGTGAAGGCA-GATAAAGA-3′ (reverse primer); Fragment P3 (593 bp, from nt −928 to nt −336): 5′-ATGCTTTTGAGTGGTCTGAATACTTG-3′ (forward primer) and 5′-GGAGAGTATCTGTGGAGTTGTGC-3′ (reverse primer); Fragment P4 (646 bp, from nt −445 to nt +201): 5′-CAGAACAGCACTACCCTTCGCTGA-3′ (forward primer) and 5′-ACAAGTGAGGAACCTCCGAGCTC-3′ (reverse primer). The amplification conditions were 30 cycles at: *a*) 95°C for 1.5 min/68°C for 1.5 min (fragment P1); *b*) 95°C for 1.5 min/65°C for 1 min/68°C for 1.5 min followed by a final extension at 68°C for 10 min (fragments P2, P3, and P4). Once a sequence variant was identified in a sample, its presence was confirmed by fresh re-amplification and re-sequencing of the culprit DNA region.

Screening of NPC1 gene mutations

Rapid screening methods were developed for the detection of four new NPC1 gene mutations that introduce or eliminate a site

Table 2. PCR amplification of NPC1 gene from genomic DNA

Exon	Oligonucleotide Primers	Size of PCR Product (bp)
1	F: 5′-AGCCGACGACGCCTTCTTCCTT- 3′ R: 5′-ACAAGTGAGGAACCTCCGAGCTC-3′	383
2	F: 5′-GAAGTTTCTGTGATTGTACTTGAGT-3′ R: 5′-TCCACCTCCACCCTGCAATAACAT-3′	310
3	F: 5′-GTGTCTTAGTTCACTGAGGAATGTTG-3′ R: 5′-GAAAGCTGAGCATTACCAGTTCACA-3′	253
4	F: 5′-TGGACACAATAAATGCAGAAAGTAAT-3′ R: 5′-TGACAGGACAACTAAAAGGAACAAT-3′	475
5	F: 5′-AGCATGGTGCATATGGAGTTCGTG-3′ R: 5′-CAAGCACTGGTGAGCCACTGTGC-3′	369
6	F: 5′-GTATTTCAGTGGGCTTTTCTTTGAGT-3′ R: 5′-CATGGAGGTATTTGTTTCTTGTCCTA-3′	457
7	F: 5′- ACCTCACTGTGATGAAGTCCACTA-3′ R: 5′-CATGACAGACAGCATCATCTGAAC-3′	178
8	F: 5′-TGATTCCTGCCATGAGATAGCAACT-3′ R: 5′-CCCATCTAGCAGTAGTCAACATGTA-3′	556
9	F: 5′-ATGTGACGTGTTTCTGGGTTTGC-3′ R: 5′-GTCTTGTTGTTTGCTCACCTCTG-3′	384
10	F: 5′-AGGTGAGTGCTGAGCTGTATTA-3′ R: 5′-AGGAGATACTATTCTGGGATTCA-3′	403
11	F: 5′-AGATACAGTCCATAGCTCCAGTGAG-3′ R: 5′-TAAGTGCTTGCTGCAAGTGTCTAGC-3′	288
12	F: 5′-AGTTTCTTACTTAGCTGTCAGTTAG-3′ R: 5′-TGACGTTACACTGTGCACTGCTG-3′	312
13	F: 5′-TTTAGTAACAAGTGGGACAGACAAC-3′ R: 5′-AGGTCACACTCACGAATGCGGAG-3′	339
14	F: 5′-CACAAGGCAGCAAGAAATGGCGT-3′ R: 5′-CAGGCTCAGCAGACTACAGGACT-3′	233
15+16	F: 5′-GCTGTAAACAGAAGTGACGCAGA-3′ R: 5′-CTGGCTTCTTAGAAGGCATGTGAT-3′	480
17	F: 5′-CCTGTACTCCCTATTAGCCTGTCAT-3′ R: 5′-ACTTGCTTGAAACACCTACGTGCATG-3′	322
18	F: 5′-TGCTTAGTTACTATCAGAGTGTTCAC-3′ R: 5′-CCTCCTCCGCTGCTTCTGAAGTA-3′	291
19	F: 5′-CTGTGGAGCAGGTCAGTAACCCT-3′ R: 5′-GTATAAACTGAGGCACGATGCAAATG-3′	245
20	F: 5′-CTTCTAACAGTCCTCCCTGCA-3′ R: 5′-CTGTCTTAGCCCAGTCCTCTC-3′	247
21	F: 5′-TGCTTAGCCTCAAGTGCTCAGAT-3′ R: 5′-ACCCAGTGTAGGCCCTTTGCTG-3′	337
22	F: 5′-GCGAGCTTTAATGAGGCCTCC-3′ R: 5′-GACAAACTGAGACTGTATGAGGA-3′	295
23	F: 5′-CAGGGTGCCCTGGGTAATTAGCA-3′ R: 5′-GATCCAGACTCTTCAGTCACTGAG-3′	292
24	F: 5′-TTCAATTACAGGTTGGTAAAAGTGGTT-3′ R: 5′-CTTGAAAAGAATGCCTCAGGATAGA-3′	297
25	F: 5′-TTCCAAAGTGGGATTACAGGCGTG-3′ R: 5′-GACCGACCCTTAGACACAGTTCAG-3′	221

TABLE 3. Screening of novel point mutations of NPC1 gene

Exon	Mutation	PCR Product (bp)	Oligonucleotide Primers	Restriction Enzyme
9	nt 1351 G→A (E541K)	384	F: 5′-ATGTGACGTGTTTCTGGGTTTGC-3′ R: 5′-GTCTTGTTGTTTGCTCACCTCTG-3′	*Taq*I
9	nt 1421 C→T (P474L)	384	F: 5′-ATGTGACGTGTTTCTGGGTTTGC-3′ R: 5′-GTCTTGTTGTTTGCTCACCTCTG-3′	*Hph*I
18	nt 2669 A→G (Y890C)	279	F: 5′-ACTGGTGGTGTTAGGAATCATGAGTC-3′ R: 5′-CCCATGCCGCCGCACACCATGTT-3′	*Rsa*I
22	nt 3422 T→G (splicing defect)	138	F: 5′-CTGCAGTCATCATGTGTGCCACCA-3′ R: 5′-GACAAACTGAGACTGTATGAGGA-3′	*Mnl*I

for commonly available restriction enzymes. The nucleotide primers and the restriction enzymes used in these screenings are shown in **Table 3**. The amplification conditions of each exon were the same as the ones specified above. Digestion products were separated by either 2% agarose gel or 4% Metaphor (FMC, Bio-Products, Rockland, ME).

Northern blot analysis

Total RNA was isolated from cultured fibroblasts as specified above. A human NPC1 probe, spanning from the 3′ half of exon 5 to the 5′ half of exon 8 (cDNA fragment 2 in Table 1), was synthesized by reverse transcriptase-PCR using the appropriate primers (Table 1). The cDNA clone pHFβA1 of human β-actin was used to normalize the RNA filters. Northern blots were performed as described previously (20).

Restriction fragment analysis of NPC1 cDNA and genomic DNA

cDNA regions encompassing exons 9, 20, and 22, respectively, were amplified by PCR using the following pairs of primers: 5′-AGCTGATGTACCCTTTGGACCTC-3′ (forward primer) and 5′-CTTGTATCATTCAGAGAGGCAGG-3′ (reverse primer) (exon 9 region); 5′-TCGACGATTATTTCGACTGGGTG-3′ (forward primer) and 5′-CACACTTGGGGTTAGGGTTATC-3′ (reverse primer) (exon 20 region); 5′-CTGCAGTCATCATGTGTGCCACCA-3′ (forward primer) and 5′-CAGTAAGACCATGGCCAAATACATC-3′ (reverse primer) (exon 22 region).

Genomic DNA regions encompassing exons 9, 20, and 22 respectively were amplified by PCR using the pairs of primers listed in Table 2.

Purified PCR products of both cDNA and genomic DNA were digested with the following restriction enzymes: *Hph*1, *Ava*I, and *Hind*II (for exons 9, 20, and 22 respectively). Digestion products were separated by either 2% agarose gel or 3–4% Metaphor gel (FMC, Bio-Products, Rockland, ME).

RESULTS

Analysis of mutations in cDNA and genomic DNA

A summary of the main clinical features of the patients is given in **Table 4**.

Table 5 shows the results of the analysis of NPC1 cDNA and genomic DNA. In two cases (probands C.N. and G.G./G.M. siblings) the analysis of cDNA was not carried out because the fibroblasts were not available. All the mutations detected in cDNA by sequencing were confirmed in genomic DNA with four exceptions (probands L.L., R.F., Z.M., and S.A.), as described in detail in the following sections.

As shown in Table 5, all the patients were compound heterozygotes at the genomic DNA level. P474L and P1007A substitutions were found in three and in two unrelated patients, respectively. The novel missense mutations, detected in cDNA, and confirmed in genomic DNA (P474L, G910S, Y890C, Y899D) or found in genomic DNA (E451K), cause non-conservative amino acid substitutions. They were not found in 100 randomly selected control subjects. Northern blot analysis of RNA isolated from fibroblasts of proband C.D. (P474L/G910S), proband C.M. (Y890C/G992W), and proband B.T. (Y899D/?) did not show a significant reduction of the NPC1 mRNA content as compared to control fibroblasts (**Fig. 1**).

In one proband (B.T.), only one mutant allele was iden-

TABLE 4. Clinical features of NPC1 patients

Proband (Sex)	Present Age/ Age of Death (ad)	Age at Onset	Age at Diagnosis	Filipin Test	Dystonia	Dementia	Ataxia	Hepatomegaly	Seizures	Vertical Gaze Paralysis
C.D. (m)	18 years	10 years	13 years	Classic	Severe	Severe	Severe	Yes	Yes	Yes
C.M. (m)	20 years	13 years	15 years	Classic	Mild	Mild	Severe	No	Yes	Yes
L.L. (m)	20 years	6 years	15 years	Variant	Severe	Mild	Severe	Yes	Yes	Yes
C.N. (f)	24 years ad	10 years	23 years	Classic	Mild	Severe	Severe	No	Yes	Yes
S.A. (f)	19 years	4 years	15 years	Variant	No	Mild	Mild	Yes	No	Yes
R.F. (m)	13 years	6 years	11 years	Classic	No	Mild	Severe	Yes	Yes	Yes
Z.M. (m)[a]	9 years	5 years	5 years	Classic	No	Severe	Severe	Yes	Yes	Yes
Z.G. (f)[a]	11 years ad	5 years	10 years	Classic	Mild	Severe	Severe	Yes	Yes	Yes
B.T. (f)	1 years	1 months	4 months	Classic	No	No	No	Yes	No	No
A.A. (m)	38 years ad	25 years	33 years	Classic	Mild	Severe	Mild	Yes	No	Yes
G.G. (m)[b]	14 years	2 months	5 years	Variant	No	No	No	Yes	No	No
G.M. (f)[b]	9 years	6 months	2 years	Variant	No	No	No	Yes	No	No

[a] Siblings.
[b] Sibling.

TABLE 5. Mutations in NPC1 cDNA and NPC1 gene

Patient	Exon	Mutation in NPC1 cDNA	Effect on NPC1 Protein	Mutation in NPC1 Gene	Previously Reported
C.D.	9	C1421T ht	P474L ht	C1421T ht	New
	18	G2728A ht	G910S ht	G2728A ht	New
C.M.	18	A2669G ht	Y890C ht	A2669G ht	New
	20	G2974T ht	G992W ht	G2974T ht	ref. 10
L.L.	9	C1421T hz	P474L hz	C1421T ht	New
	20	none		nt 2972(del2) (AG) ht *(Fs. from codon 991→Stop 1005)*	ref. 11
C.N.	19	NA		C2861T ht *(S954L ht)*	ref. 11
	24	NA		nt 3734(del2) (CT) ht *(Fs. from codon 1245→Stop 1256)*	New
S.A.	20	C3019G ht	P1007A ht	C3019G ht	ref. 11
	22	nt 3422–3477 (del56) ht	Fs. from codon 1141→Stop 1238 ht	T3422G ht *(V1141G)*	New
R.F.	9	C1421T hz	P474L hz	C1421T ht	New
	20	none		nt 2972(del2) (AG) ht *(Fs. from codon 991 →Stop 1005)*	ref. 11
Z.M./Z.G. Siblings	18	none		C2764T ht *(Q922X)*	New
	22	A3467G hz	N1156S hz	A3467G ht	refs. 10, 15
B.T.	18	T2695G ht	Y899D ht	T2695G ht	New
	?	?	?	?	
A.A.	20	C3019G ht	P1007A ht	C3019G ht	ref. 11
	21	T3182C ht	I1061T ht	T3182C ht	ref. 11
G.G./G.M. Siblings	9	NA		G1351A ht *(E451K)*	New
	20	NA		G2974T ht *(G992W)*	ref. 11

NA, cDNA not available; ht, heterozyote; hz, homozygote. The effect on the NPC1 protein predicted on the basis of the mutations found in genomic DNA (NPC1 gene) is indicated in italics.

tified in cDNA and genomic DNA. Both NPC1 alleles of this proband were expressed in cultured fibroblasts, as demonstrated by the presence of heterozygosity for some common polymorphisms in the coding region: C1926G (I642M), A2572G (I858V), and C2793T (N931N) (see below).

Sequence polymorphisms detected in cDNA and genomic DNA

The following common polymorphisms were found in cDNA (and confirmed in genomic DNA) of both NPC1 patients and controls: T387C (Y129Y), A644G (H215R), C1926G (I642M), A2572G (I858V), C2793T (N931N), and G3797A (R1266Q). All of them were previously reported (16, 22). We found another silent mutation, C540T (D180D), previously unreported. The following unreported sequence variants were observed in the 5′ flanking region

(designated promoter region) in patients and control subjects: −238C/G, −887C/T, −1026T/G, −1186T/C, −1435A/T.

Discrepancy between cDNA and genomic DNA sequence

Monoallelic expression of some mutant alleles in fibroblasts

PROBAND L.L. The analysis of cDNA showed that proband L.L. was homozygous for two nucleotide substitutions: *i*) C1421T leading to the rare mutation P474L (Table 5); *ii*) C1926G leading to the common amino acid variant I642M (22). The analysis of genomic DNA revealed that the patient was in fact heterozygous for: *i*) both these substitutions; *ii*) two additional common polymorphisms [A644G (H215R) and C2793T (N931N)]; *iii*) a dinucleotide deletion in exon 20 [AG deletion at position 2972-2973, indicated as 2972(del2)]. The latter muta-
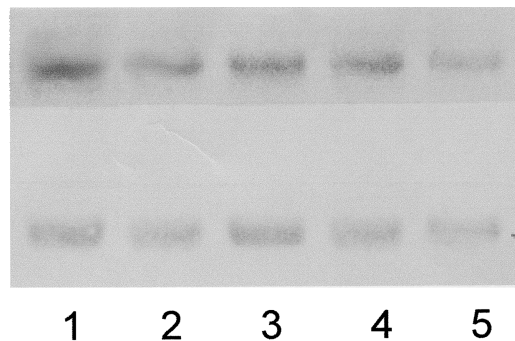

Fig. 1. Northern blot analysis of Niemann-Pick type C disease 1 (NPC1) mRNA isolated from fibroblasts. Lane 1, proband C.D. (P474L/G910S); lane 2, proband C.M. (Y890C/G992W); lane 3, proband B.T. (Y899D/?); lanes 4 and 5, control subjects.

tion is predicted to cause a frameshift leading to a premature stop codon at position 1005 (Table 5). This unexpected result was confirmed in two samples of genomic DNA extracted from fibroblasts and peripheral blood leukocytes of this patient. The patient was also a carrier of two nucleotide substitutions (-1026T/G and -1186T/C) in the promoter of the NPC1 gene that were also found in control subjects. We constructed the intragenic haplotypes of the proband's parents and investigated their segregation in the family (**Fig. 2**). This analysis confirmed that the proband was a compound heterozygote. However, in his fibroblasts only the maternal derived NPC1 allele (haplotype *x*) (Fig. 2) is expressed and the patient appears to be homozygous for the P474L mutation. To ascertain whether trace amounts of the mRNA corresponding to the mutant allele carrying the AG deletion were detectable, the proband's fibroblasts were incubated in the presence of cycloheximide, on the assumption that the inhibition of protein synthesis might prevent the rapid degradation of this mRNA (23). The cDNA sequencing failed to detect the cDNA corresponding to the allele harbouring the AG deletion in exon 20 [2972(del2)] (data not shown).

To ascertain whether minute amounts of this mRNA were present in proband's fibroblasts, we took advantage of the fact that the AG deletion introduces a new *Ava*I site in exon 20. The *Ava*I digestion of genomic DNA generated a single fragment (247 bp) in controls and three fragments (predicted size: 245, 154, and 91 bp) in the proband, who is heterozygous for this mutation (Table 5) (**Fig. 3**, lanes 5 and 6). In view of this finding we reasoned that the *Ava*I digestion of the cDNA region containing exon 20 (207 bp in control and 205 bp in the proband) might allow the detection of the transcript corresponding to the mutant allele harbouring the AG deletion. Figure 3 (lanes 8 and 9) shows that *Ava*I digestion of proband's cDNA did not reveal the presence of the fragments (predicted size: 142 and 63 bp) expected to result from the introduction of the *Ava*I site in the transcript of the allele harbouring the AG deletion. This is a further evidence that this allele was not expressed in fibroblasts. This result was confirmed by using a complementary strategy based on the fact that the other mutation present in the proband (C1421T) (P474L) abolishes the *Hph*1 restriction site in exon 9. The *Hph*1 digestion of cDNA region encompassing exon 9 (304 bp), generated two fragments
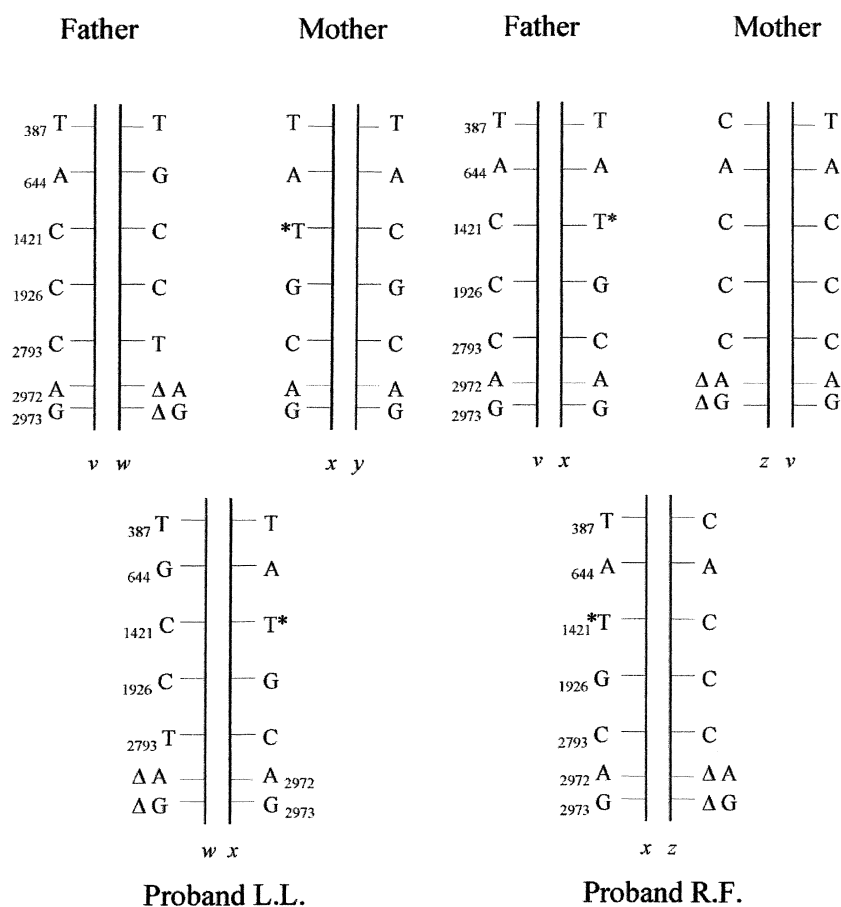

Fig. 2. Co-segregation analysis of the NPC1 gene haplotype in the pedigrees of proband L.L. and proband R.F. The rare mutation on the *x* allele (C1421T), which converts proline at position 474 into leucine (P474L), is indicated by a star. The two nucleotides (AG) at position 2972/2973, which are deleted in the *w* and *z* alleles respectively, are indicated by an arrowhead. The only mRNA species present in the fibroblasts of both probands was that corresponding to the *x* allele.
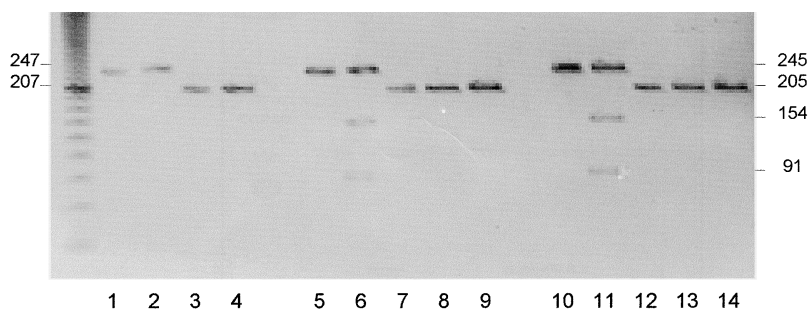
Fig. 3. *Ava*I restriction fragments of genomic DNA (gDNA) and cDNA of probands L.L. and R.F. The region encompassing exon 20 of NPC1 gene (containing nt 2972del2 mutation) was amplified by PCR from gDNA (247 bp in control and 245 bp in the probands) and cDNA (207 bp in control and 205 bp in the probands), as specified in Materials and Methods. Lanes 1–4, control subject; lane 1, undigested gDNA ; lane 2, *Ava*I digested gDNA; lane 3, undigested cDNA; lane 4, *Ava*I digested cDNA. Lanes 5–9, proband L.L.; lanes 10–14, proband R.F. Lanes 5 and 10, undigested gDNA ; lanes 6 and 11, *Ava*I digested gDNA ; lanes 7 and 12, undigested cDNA; lanes 8, 9 and 13, 14, *Ava*I digested cDNA (10 and 15 ng respectively). The size of the gDNA and cDNA fragments is given in base pairs.

(predicted size: 160 and 144 bp) in the control subject but only one fragment (304 bp) in the proband (data not shown). The 304 bp fragment corresponds to the transcript of the mutant allele (C1421T) (P474L), the only transcript present in proband's fibroblasts.

The two promoter variants −1026T/G and −1186T/C found in the proband's genomic DNA were transmitted from the father together with the 2972(del2) mutation.

PROBAND R.F. The cDNA sequence revealed that proband R.F. was homozygous for: *i*) the C1421T transition leading to the rare mutation P474L (Table 5); *ii*) the C1926G transversion leading to the common variant I642M, a situation identical to that found in cDNA of proband L.L. (see above). At genomic DNA level, proband R.F. was in fact heterozygous for: *i*) both these substitutions; *ii*) an additional T387C substitution (Y129Y) (15); *iii*) the dinucleotide deletion (AG at position 2972-2973) 2972(del2) also observed in proband L.L. (see above). The haplotype segregation in the pedigree (Fig. 2) is consistent with the finding that the proband is a compound heterozygote at the genomic DNA level. However in his fibroblasts only the paternal derived NPC1 allele is expressed and the patient appears to be homozygous for the rare missense mutation P474L. The search for the transcript corresponding to the allele harbouring the AG deletion in exon 20 was performed following the strategies outlined above (in the case of proband L.L.). No transcript corresponding to this allele was observed in proband's fibroblasts (Fig. 3, lanes 13 and 14).

Proband R.F. was found to be homozygous for a nucleotide substitution in the promoter region (−238C/G); his parents were heterozygotes.

PROBAND Z.M. The cDNA sequence revealed that proband Z.M. was homozygous for: *i*) an A3467G transition leading to the missense mutation N1156S (Table 5) (14); *ii*) a C1926G transversion leading to the common variant I642M (22). At genomic DNA level, proband Z.M. was in fact heterozygous for: *i*) both these substitutions; *ii*) a C2764T transition introducing a nonsense mutation at

codon 922 (Q922X); *iii*) the common variants: A644G (H215R), A2572G (I858V) (22), and the silent mutations C540T (D180D) and C2793T (N931N).

The analysis of the NPC1 gene of the proband's parents revealed that the A3467G mutation was transmitted from the father while the C2764T was transmitted from the mother. However, in proband's fibroblasts, only the transcript of paternal derived mutant allele (N1156S) was detected by cDNA sequencing (Table 5).

To ascertain the presence of minute amounts of the transcript of the mutant allele harbouring the stop codon in exon 18 (C2764T) (Q922X), we adopted an indirect strategy based on the fact that the other mutation carried by the proband (A3467G) (N1156S) eliminates a *Hind*II restriction site in exon 22. We reasoned that the *Hind*II digestion of the cDNA region encompassing exon 22 could allow the detection of the "missing" transcript. **Figure 4** (lanes 2 and 6) shows that *Hind*II digestion of genomic DNA (295 bp) resulted in three fragments (predicted size: 198, 57, and 40 bp) in the control subject and in four fragments (predicted size: 198, 97, 57, and 40 bp) in the proband, confirming that the latter was heterozygote for the A3467G (N1156S) substitution. In view of this finding, if the only transcript present in proband's fibroblasts is that generated by the allele harbouring the A3467G mutation (as indicated by cDNA sequencing, Table 5), the *Hind*II digestion of cDNA region encompassing exon 22 (338 bp) would produce two fragments with the predicted size of 297 and 41 bp as opposed to three fragments (240, 57, and 41) in control cells. In fact, the *Hind*II digestion of proband's cDNA revealed besides the expected 297 and 41 bp fragments (corresponding to the transcript harbouring the A3467G mutation), minute amounts of a 240 bp fragment (Fig. 4, lanes 4, 8, and 9). This fragment results from the presence of the transcript generated by the allele harbouring the C2764T (Q922X) in exon 18, in which the *Hind*II site is maintained. Densitometric analysis of the gel indicated that in proband's fibroblasts the content of the transcript containing the premature stop
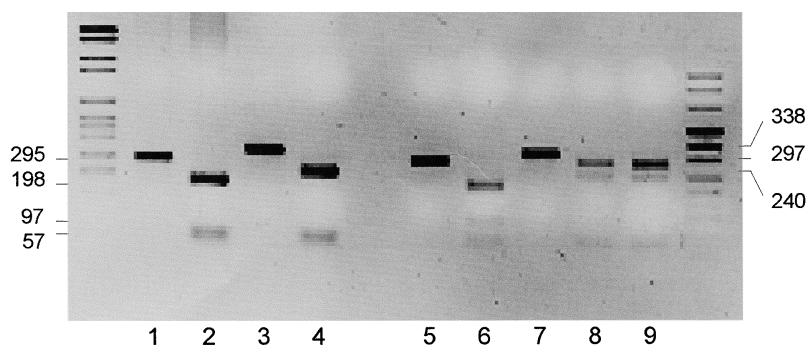
Fig. 4. *Hind*II restriction fragments of genomic DNA (gDNA) and cDNA of proband Z.M. The region encompassing exon 22 of NPC1 gene (containing the mutation A3467G) was amplified by PCR from gDNA (295 bp) and cDNA (338 bp) as specified in the Materials and Methods. Lanes 1–4, control subject; lane 1, undigested gDNA; lane 2, *Hind*II digested gDNA (198 and 57/40 bp fragments); lane 3, undigested cDNA; lane 4, *Hind*II digested cDNA (240 and 57/41 bp fragments). Lanes 5–9, probands Z.M.; lane 5, undigested gDNA; lane 6, *Hind*II digested gDNA (198, 97, and 57/40 bp fragments); lane 7, undigested cDNA; lanes 8 and 9, *Hind*II digested cDNA (297, 240, and 57/41 bp fragments) (10 and 15 ng respectively). The 57/40 bp and 57/41 bp fragments migrate as a single broad band. The size of the gDNA and cDNA fragments is given in base pairs.

codon (C2764T) (Q922X) is less than 10% of that containing the missense mutation (A3467G) (N1156S).

Point mutation in the coding sequence and activation of a donor splice site

PROBAND S.A. In proband S.A. the amplification of the cDNA fragment 10 (Table 1) (spanning from the 3′ half of exon 22 to the 5′ half of exon 25) generated two products of 515 bp and 460 bp, respectively, as opposed to a single PCR product of 515 bp found in a control subject (**Fig. 5**). Densitometric analysis of the gel demonstrated that the normal (515 bp) and the abnormal band (460 bp) were in

approximately 1:1 ratio (data not shown). The nucleotide sequence of the 460 bp fragment revealed a deletion of the last 56 nucleotides of exon 22 (Fig. 5), which leads to a frameshift and a premature stop codon at position 1238. The sequence of the normal cDNA fragment revealed that proband S.A. was a carrier of a C3019G transversion in exon 20, leading to the missense mutation P1007A. The sequence of genomic DNA confirmed that the proband was heterozygous for the mutation C3019G in exon 20 and revealed that he was a carrier of a T3422G transversion in exon 22 (**Fig. 6**). The latter mutation is predicted
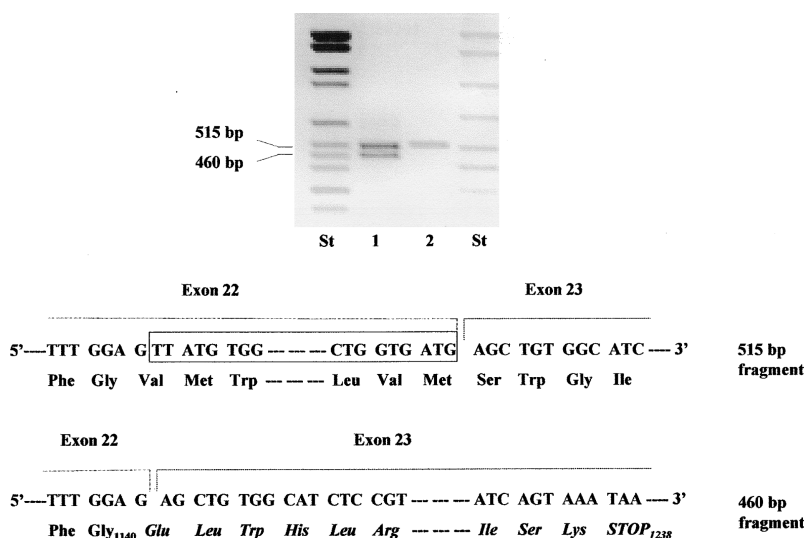

Fig. 5. NPC1 mRNAs in fibroblasts of proband S.A. The upper panel shows the agarose gel electrophoresis of the reverse transcription and PCR amplification (RT-PCR) fragment 10 (Table 1) spanning from the 3′ half of exon 22 to the 5′ end of exon 25. Lane 1, proband S.A.; lane 2, control subject; St, DNA size markers. The middle panel shows a partial sequence of the normal 515 bp RT-PCR fragment encompassing the junction between exon 22 and exon 23. The box demarcates a sequence of 56 nt that is deleted in the abnormal 460 bp RT-PCR fragment observed in the patient. The lower panel shows that the joining of partially deleted exon 22 with exon 23 causes a frameshift, resulting in a sequence of 97 novel amino acids (indicated in italics) preceding a premature stop codon.

to cause a glycine for valine substitution at position 1141 (V1141G). The discrepancy between cDNA and genomic DNA is explained in Fig. 6. The T→G substitution at position 3422 converts the wild type sequence (GAGTTAT, from nt 3419 to nt 3425) into a novel sequence (GAG**G**-TAT), which is similar to the consensus sequence of a donor splice site. The activation of this new donor splice site causes an abnormal splicing of the pre-mRNA leading to a mature mRNA in which the 3′ end of exon 22 is deleted. The joining of the partially deleted exon 22 to exon 23 causes a frameshift and the occurrence of a premature stop codon (Fig. 5). The predicted translation product of this abnormal mRNA is a truncated NPC1 protein of 1237 amino acids containing a stretch of 97 novel amino acids at its carboxy-terminal end.

The analysis of the genomic DNA of proband's parents showed that the C3019G (P1007A) mutation was transmitted from the father and the T3422G (activation of a donor splice site in exon 22) from the mother.

Rapid screening of some point mutations of NPC1 gene. The G1351A substitution in exon 9 (E451K) eliminates a *Taq*I site. Two fragments (315 and 69 bp) are generated by *Taq*I digestion of the normal allele, while a single fragment (384 bp) is obtained from the mutant allele.

The C1421T substitution in exon 9 (P474L) eliminates a *Hph*I site; the digestion of PCR-amplified exon 9 with this enzyme generates 2 fragments (128 and 256 bp) in the normal allele and a single 384 bp fragment in the mutant allele.

The A2669G substitution in exon 18 (Y890C) eliminates a *Rsa*I site; the digestion of PCR-amplified exon 18 with this enzyme generates three fragments (24, 70, and 185 bp) in the normal allele and two fragments (94 and 185 bp) in the mutant allele.

The T3422G substitution in exon 22 introduces a *Mnl*I site; the digestion of PCR-amplified exon 22 generates two fragments (11 and 127 bp) in the normal allele and three fragments (11, 44, and 83 bp) in the mutant allele.

DISCUSSION

In this work we report a spectrum of mutations of the NPC1 gene in a group of Italian NPC1 patients. The search for mutations in the NPC1 gene was carried out on both cDNA obtained from RNA isolated from skin fibroblasts and genomic DNA isolated from peripheral blood leukocytes and/or fibroblasts. Those results of cDNA analysis that were confirmed in genomic DNA (Table 5) lead to the identification of several mutations, some of which have not been previously reported. Recent studies have shown that some mutations of the NPC1 gene (P1007A and I1061T) are frequent in NPC patients of European descent (11, 12, 14, 15). This appears to be the case in our series for P1007A, an amino acid substitution that has been associated with a less severe NPC phenotype (variant phenotype) in homozygotes (16) and appears to reduce the effect of a severe mutation in some compound heterozygotes (e.g., P1007A/I1061T) (14, 16). Indeed, our proband A.A. (P1007A/I1061T genotype) had relatively mild late onset clinical manifestations. Although the I1061T mutation was reported in 14% of patients of European descent (12), we found only one patient with this allele, a situation similar to that recently documented in NPC1 patients from Portugal (16). These results suggest that the I1061T mutation might be less frequent in southern Europe.

In our patients we found five novel missense mutations (E451K, P474L, Y890C, Y899D, and G910S) that are likely to be the cause of NPC disease for the following reasons: *i*) they are non-conservative substitutions of amino acids which, according to the multiple NPC1 protein alignment, are highly conserved in mammals (mouse, hamster, rabbit, and pig) and are located in a context of highly conserved amino acid sequences; *ii*) they were not found in 100 control subjects; and *iii*) they are not associated with a reduced content of NPC1 mRNA in fibroblasts of three compound heterozygotes carrying four of these mutations (P474L, Y890C, Y899D, and G910S). Among these missense mutations, two (E451K and P474L) are located in the second hydrophilic loop and three (Y890C, Y899D, and G910S) in the third hydrophilic loop (6). These results, together with those of other recent large surveys (12–16), indicate that the missense mutations of NPC1 gene are widely distributed on the NPC1 protein and not clustered in the cysteine rich loop as suggested in an early report (11).

Four mutations found in our patients (Table 5) are predicted to cause the formation of truncated NPC1 proteins
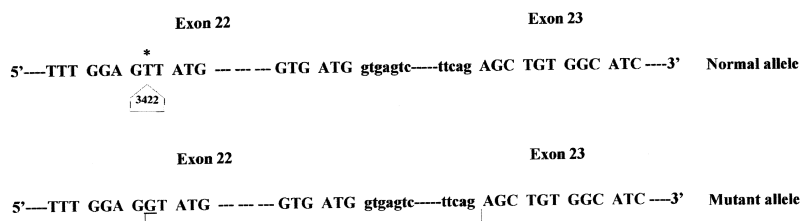

Fig. 6. Point mutation in exon 22 of the NPC1 gene in proband S.A. Upper case letters indicate exon 22 and 23 and lower case letters indicate the 5′ and 3′ ends of intron 22. The T3422G transversion in exon 22 generates a sequence for a donor splice site (in bold) within exon 22. The activation of this splice site produces the removal of the last 56 nucleotides of exon 22 together with the entire intron 22 (as indicated by the line) and the joining of the partially deleted exon 22 with exon 23, as indicated in Fig. 5.

of 921, 1004, 1237, and 1255 amino acids. These changes, which are expected to disrupt several trans-membrane domains of NPC1 protein (2, 6), probably prevent the proper integration of this peptide in the membrane of the endoplasmic reticulum as well as its localisation in late endosomes. It is also possible that these truncated proteins are either synthesised at a reduced rate (as mRNAs harbouring a premature stop codon may be degraded more rapidly than their normal counterparts) (see below) or are rapidly degraded in the cell shortly after their synthesis.

One unexpected finding of the present study was the discrepancy between the results of the sequence analysis of cDNA and that of genomic DNA we observed in four patients. Two types of discrepancies emerged: *i*) the expression of a single mutant allele (monoallelic expression) in fibroblasts of three patients who are compound heterozygotes; *ii*) the mutant mRNA and the corresponding mutant allele in genomic DNA bear different mutations.

Monoallelic expression

In two unrelated compound heterozygotes carrying the same mutations (P474L and nt 2972del2), cDNA sequence revealed that only the P474L allele was expressed in fibroblasts. Furthermore, we failed to demonstrate the "missing" transcript after specific PCR amplification and restriction enzyme digestion of the cDNA region harbouring the AG deletion (nt 2972del2) in exon 20 (Fig. 3). One simple explanation for this lack of expression is that the nt 2972del2 mutation is in linkage with another mutation in the promoter of the NPC1 gene, which prevents the transcription of the deletion carrying allele. The observation that one of these patients (proband L.L.) was a carrier of two single nucleotide substitutions in the promoter ($-1186T/C$ and $-1026T/G$), found to be in linkage with the nt 2972del2 mutation, might support this hypothesis. However, it is unlikely that these substitutions in the promoter abolish the expression of the mutant allele since they: *i*) represent common polymorphisms found in randomly selected healthy subjects; and *ii*) are not present in the promoter of the same mutant allele (nt 2972del2), which is not expressed in the fibroblasts of the second patient (proband R.F.) carrying this specific mutation. Of course, it cannot be excluded that in both patients nt 2972del2 is linked to another mutation elsewhere in the gene, outside the regions we have sequenced.

The third patient (proband Z.M.), in whom the cDNA sequence revealed the presence of the transcript of only one mutant allele (N1156S), was found to be a compound heterozygote for two mutations (N1156S and Q922X) different from the ones found in probands L.L. and R.F. In the case of proband Z.M. however, we were able to detect trace amounts of the transcript containing the premature stop codon by using PCR –amplification of the appropriate cDNA region followed by restriction enzyme digestion (Fig. 4). We found no sequence variations in the promoter in this proband that were in linkage with the mutant allele (Q922X).

The common feature of the three mutant alleles found in these three probands, which are not expressed or are

expressed at a very low level in fibroblasts, appears to be the presence of a premature termination codon in the corresponding mRNA. It is possible that these mRNAs containing a premature termination codon (PTC) undergo a rapid degradation (nonsense mediated RNA decay, NMD) as reported in other genetic disorders (24). NMD appears to be an ancient and evolutionary conserved surveillance strategy to protect cells from mutations that could yield truncated, potentially dangerous proteins. Several genes have been identified in human genome which are involved in this process (24–26). The cell compartment where mRNA harbouring PTC is destroyed is still a matter of intense investigation as there is evidence that it might occur in the cytoplasm (site of translation) as well as in the nucleus or close to the nuclear pore (24, 27–29). It is likely that the recognition of the PTC containing pre-mRNA and its destruction is linked with the splicing process. If the destruction occurs before the abnormal transcript leaves the nucleus, no mature mRNA is expected to accumulate in the cytoplasm. Not all pre-mRNAs (or mature mRNAs) of NPC1 gene that contain PTC are targeted for destruction. Notably, in the present study we found that in the fibroblasts of proband S.A. carrying a splicing defect (see below), the mature mRNA containing PTC was present in approximately the same concentration as the wild type mRNA (Fig. 5). This suggests that pre-mRNAs containing PTC loose their stability only when PTC has some specific location with respect to some recognition signal (for example the last intron, as suggested in the case of human triosephosphate isomerase and β-globin mRNA containing PTC) (30–31).

Regardless of the mechanism underlying the lack (or the substantial reduction) of the expression of some mutant alleles of NPC1 gene, it would be interesting to know whether this process is confined to fibroblasts or is present in other tissues more directly involved in the NPC syndrome (i.e., neurons, hepatocytes, etc.). It is tempting to speculate that the coexistence of bi-allelic and monoallelic expression in different tissues (if present) might affect the clinical expression of the disease and contribute to the phenotypic variability observed among NPC patients.

Activation of a new donor splice site by an exonic mutation

The other discrepancy between cDNA and genomic DNA is related to the presence of a point mutation (T3422G) in the middle of exon 22, which, instead of resulting in a missense mutation as predicted, causes the formation of a new donor splice site whose activation produces an abnormal pre-mRNA splicing (Fig. 5 and 6). The observation that the content of normally and abnormally spliced mRNA in the proband's fibroblasts was similar (Fig. 5) suggests that this new donor splice site in exon 22 completely supersedes the normal donor site in intron 22. It is unknown why the normal donor splice site in intron 22 is blocked while the novel site in exon 22 is mostly used by the spliceosome. The eight nucleotide sequence of the normal donor splice site in intron 22 (TGgtgagt) gives a

Shapiro and Senapathy's score (a system to find potential splice sites in a given sequence) of 88.8 (32). The splice site activated by the T3422G transversion in exon 22 (AG-gtatgt) has a Shapiro and Senaphaty's score of 88.6. In this respect the two donor sites should manifest the same activity, unless the presence of specific nucleotides at some crucial position (e.g., the last two nucleotides at the 3′ end of an exon) plays a key role in governing the splicing efficiency. The last two nucleotides of normal exon 22 (T and G at position −2 and −1 with respect to the first nucleotide of intron 22 indicated as +1) are present in 15% and 78% respectively of the cases of primate gene splice junctions. The nucleotides A and G present in mutant exon 22 at position −2 and −1 with respect to the new donor splice site, are present in 58% and 78% of the cases. This difference might increase the affinity of the new donor splice site for the spliceosomes.

In conclusion, the unexpected molecular findings in these four NPC1 probands underscore the fact that, in some instances, the predictions of the type of mRNA and protein changes made on the basis of the mutation found by sequencing genomic DNA should be taken with caution (33). A more systematic comparison between cDNA and genomic DNA might reveal mono-allelic expression or the presence of abnormal mRNA species not easily predictable from the mutations discovered in genomic DNA (33).

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