

RESEARCH ARTICLE

NPC1: Complete Genomic Sequence, Mutation Analysis, and Characterization of Haplotypes

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Communicated by William Sly

Niemann-Pick type C disease (NP-C) is a rare, autosomal recessive lipid storage disorder. At least 96% of all NP-C patients link to NPC1 which encodes for a lysosomally-targeted protein. We describe the complete genomic sequence of 57,052 kb corresponding to the transcribed region of human NPC1 including several exonic and intronic single nucleotide polymorphisms (SNPs). Sequencing of all exons, splice sites, and the promoter region of NPC1 in 12 unrelated Caucasian NP-C patients revealed nine novel and four known most likely disease-causing mutations. Ten unique mutations found only once in 24 disease alleles were observed in patients being compound heterozygous for two different mutations. Two of the three missense mutations identified more than once were observed in a total of four patients homozygous for the respective mutation along with homozygosity for the underlying haplotype. The patients were offspring of most likely nonconsanguineous couples. Based upon genotyping exonic SNPs c.2572A>G (I858V; g.45020A>G) and c.2793C>T (N931N; g.45686C>T) and segregation analysis we characterized the haplotype of all 24 NPC1 alleles and of 138 alleles of healthy Caucasian control subjects. All four permutations between the two SNPs were identified in the control alleles: 2572A-2793C (50%), 2572G-2793T (41%), 2572G-2793C (5%), and 2572A-2793T (4%). These data are suggestive for an ancestral intragenic recombination within a genomic fragment of <666 bp. While 17 of 24 NP-C alleles (71%) shared haplotype 2572G-2793T, this haplotype accounted for only 41% in the controls ($p=0.007$; 2-sided Fisher exact test) suggesting the possibility of an influence of the haplotypic background on expression of missense mutations in NPC1. Hum Mutat 19:30–38, 2002. © 2001 Wiley-Liss, Inc.

KEY WORDS: Niemann-Pick disease type C; NP-C; NPC1; haplotype; genomic sequence; cholesterol; LDL; SNP

DATABASES:

NPC1 – OMIM: 257220; GDB: 138178; GenBank: AF338230, AF123045, AF123046, NM_008720 (*Mus musculus*), AF315034 (*Canis familiaris*), AF258783 (*Felis catus*), AF169635 (*Sus scrofa*), AJ249606 (*D. melanogaster*) and AL035539 (*A. thaliana*); [http://www.nhgri.nih.gov/CONF/Niemann Pick](http://www.nhgri.nih.gov/CONF/Niemann%20Pick) (First International NPC Workshop)

INTRODUCTION

Niemann-Pick type C (NP-C; MIM# 257220) is a rare neurovisceral disorder, usually with clinical onset in late childhood and death in the second decade (for review see Patterson et al., 2001). Leading clinical signs are moderate to severe hepatosplenomegaly and vertical supranuclear gaze palsy. Other signs like pronounced neonatal jaundice, ataxia, or seizures regularly accom-

Received 14 June 2001; accepted revised manuscript 20 September 2001.

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Contract grant sponsors: Deutsche Niemann-Pick Selbsthilfegruppe; Genzyme Corp. (Alzenau, Germany).

The Supplementary Material referred to in this article can be viewed at http://www.interscience.wiley.com/humanmutation/suppmat/19_1/v19_1.html

pany the disease. If foam cells are present in the bone marrow, clinical diagnosis of NP-C is likely. A decreased rate of cholesterol esterification in fibroblasts of NP-C patients [Pentchev et al., 1986; Pentchev et al., 1987] and abundant staining of free, lysosomally-localized cholesterol by filipin serve as histochemical hallmarks for NP-C diagnosis [Vanier et al., 1991, 1992; Patterson et al., 2001]. Biochemically, a block in the translocation of LDL-derived cholesterol from lysosomes to the endoplasmic reticulum and the Golgi apparatus seems to be the specific alteration in NP-C [Sokol et al., 1988; Pentchev et al., 1994; Patterson et al., 2001; Vanier and Suzuki, 1998].

By using positional cloning and genomic complementation, Carstea et al. [1997] identified *NPC1*, the gene encoding NPC. This gene has positive linkage in at least 96% of NP-C patients. Recently, NPC has been characterized functionally as a transmembrane molecular pump mobilizing lipophilic molecules but not cholesterol out of the endosomal-lysosomal system [Davies et al., 2000]. Five percent of all NP-C patients, the NP-C2 complementation group, are considered to carry mutations in *NPC2*, the gene encoding for an ubiquitously expressed lysosomal protein (HE2) with cholesterol-binding properties (MIM 601015) [Vanier et al., 1996; Naureckiene et al., 2000]. NP-C1 and NP-C2 cannot be differentiated with the histochemical methods currently available for diagnostics.

Several exonic mutations and polymorphisms in *NPC1* are known [Carstea et al., 1997; Greer et al., 1998, 1999; Millat et al., 1999; Ribeiro et al., 1999; Snow et al., 1999; Yamamoto et al., 1999, 2000]. In 1999 the genomic structure of *NPC1*, including all intron/exon boundaries, was published [Morris et al., 1999]. The genomic size of the gene was estimated at approximately 50 kb but the sequence of 11 out of 24 introns remained undetermined.

Here we describe the complete genomic sequence of 57,052 kb corresponding to the transcribed region of human *NPC1* based upon genomic sequencing by "primer walking." Thereby, novel and known SNPs could be characterized and common haplotypes in Caucasians could be worked out. Through sequencing of cDNA and genomic DNA we identified 13 different mutations in *NPC1* of 12 patients affected by NP-C1.

MATERIALS AND METHODS

Study Subjects

Clinical diagnosis of NP-C was confirmed by bone marrow cytology and histochemical demonstration of pathologically enriched cholesterol by filipin staining. Peripheral blood leucocytes (PBL) and cultured fibroblasts were collected from 12 unrelated patients along with PBL of their parents. DNA samples of 146 unrelated healthy control subjects were used to assess genotype counts and allelic frequencies of SNPs and to exclude novel mutations identified in the patients. Informed consent was obtained from all subjects. The study has been approved by the Ethics Committee of the University of Rostock.

DNA and RNA Extraction

RNA and DNA were prepared within 24 hr after blood sampling. Total PBL RNA of 1 ml EDTA-anticoagulated blood was extracted using RNeasy™ spin columns (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and stored at -80°C. Genomic DNA was isolated from 5–10 ml anticoagulated blood using salting out standard procedure [Miller et al., 1988]. RNA and DNA from cultured fibroblasts was extracted by means of HighPure™ extraction kits (Roche Diagnostics, Mannheim, Germany).

RT-PCR and Sequencing

Up to 1 µg RNA was incubated with 100ng random hexanucleotides for 10 min at 70°C and cooled down to 4°C. After adding a reaction mix containing 100U Superscript MMLV reverse transcriptase (Life Technologies, Heidelberg, Germany), 3mM MgCl₂, 50mM Tris-HCl (pH 8.3), 75mM KCl, 10mM DTT, 0.5mM dNTPs, and 40U RNasin (MBI Fermentas, St. Leon-Rot, Germany) the sample (20 µl) was incubated at 42°C for 50 min followed by denaturation at 95°C for 5 min. *NPC1* cDNA-specific fragments covering the entire open reading frame (ORF) were amplified by PCR in 25µl reaction volumes containing 1/20 of the RT product as template, 10 pmol of each primer (Table S1, available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat/19_1/v19_1.html), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 100 µM deoxynucleoside triphosphates, 1.0U Taq polymerase (Amersham Pharmacia

Biotech, Freiburg, Germany) using standard thermocycling profiles with the respective primer annealing temperatures outlined in Table S1. PCR fragments were purified with "Qiaquick PCR purification" filter centrifugation (Qiagen, Hilden, Germany) according to the manufacturer's instructions, sequenced by BigDye™ chemistry (Qiagen, Hilden, Germany), and analyzed by an ABI 377 automated DNA sequencer.

For clarifying *NPC1* genomic sequence a modified long-range PCR primer-walking technique was applied [Szybalski, 1993] by using an initial primer design based upon published sequences of *NPC1* intron/exon boundaries [Morris et al., 1999]. A commercially available, pooled human genomic DNA served as PCR template (Roche Diagnostics, Mannheim, Germany). *NPC1*-specific PCR products were designed to span at least one complete intron. Primer 5' extensions, PCR conditions, product purification, and sequencing reactions were as described above.

To validate all sequence changes obtained by sequencing the respective cDNA of the NPC patients, a set of exon-flanking primers was designed (Table S2, available online at http://www.interscience.wiley.com/humanmutation/suppmat/19_1/v19_1.html) and genomic DNA was amplified and sequenced according to the methods outlined above. The *NPC1* promoter region was amplified and sequenced using the primers PromFP and PromRP (Table S2). Nomenclature of sequence alterations followed the recommendations for a nomenclature system for human gene mutations [Den Dunnen and Antonarakis, 2000].

Genotyping of SNPs

In order to describe individual haplotypes within *NPC1*, LightCycler™ (Roche Diagnostics, Mannheim, Germany) PCR systems were established for the exonic polymorphisms c.644a>g (g.24,149 A>G; H215R), c.2572a>g (g.45,020 A>G; I858V), and c.2793c>t (g.45,686 C>T; N931N). The LightCycler™ is a rapid real-time PCR cyclor with an integrated three-channel fluorescence photometer for fluorometric site-specific sequence information through sequence- and temperature-dependent melting curve analysis of a labeled sensor probe hybridizing to the PCR product. Melting off of the sensor probe eliminates fluorescence energy

resonance transferred by a labeled anchor probe hybridizing in close proximity to the sensor probe. PCR primers, sensor, and anchor probes are listed in Table S3 (available online at http://www.interscience.wiley.com/humanmutation/suppmat/19_1/v19_1.html). PCRs and melting curves were performed under equal cycle conditions for all assays: 1×95°C 120s and 50×(95°C 0s, 55°C 10s, 72°C 10s) at transition rates of 20°C/s. Melting curve analysis was performed after 1×(95°C 0s, 45°C 20s, 20°C/s transition rate), and then ramping to 95°C at a transition rate of 0,1°C/s. For the melting curves the fluorescence channel F2/1 was used and the fluorescence gains were F1/1, F2/15, F3/30.

Statistics

Linkage disequilibrium was analyzed by standard Fisher's exact test. Deviation of Hardy-Weinberg equilibrium was tested by goodness of fit χ^2 test.

RESULTS AND DISCUSSION

NPC1 Genomic Sequence and SNPs

Sequencing genomic long range PCR fragments of *NPC1* using primer walking revealed a contig of 57,052bp (Fig. 1). The interrupted protein coding elements are distributed over 53,296 bp, flanked by 3,043 bp 5'-untranslated region UTR and 713 bp 3'-UTR sequences. The genomic structure of *NPC1* characterized previously [Greer et al., 1999; Morris et al., 1999] could be confirmed. 25 exons ranging in size from 74 to 788 bp are interrupted by introns ranging from 97 to 12,434 bp (Table 1). Analysis of both DNA strands of the contig by BLAST searching through GenBank (data sets nr, htgs, and dbEST) did not reveal additional ORFs or significant homologies to anonymous ESTs. Comparison of the intronic sequences with fragments of *NPC1* reported previously [Morris et al., 1999] revealed few mismatches of single nucleotides distributed in introns 5, 13, and 17. Since Morris et al. [1999] used a PCR-cloning system in order to fill short intronic gaps, these non-matching nucleotides may reflect either mismatched PCR amplification or cloning artifacts [Sambrook et al., 1989; Hooft van Huijsduijnen, 1998], or represent rare SNPs. The template we used for sequencing consisted of a commercially available pool of genomic DNA samples of 20 European subjects, suitable to detect the major-

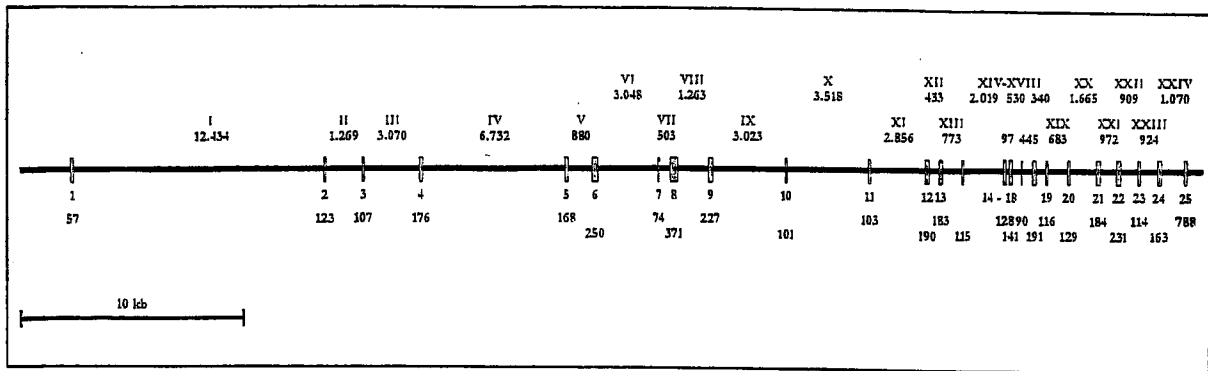


FIGURE 1. A pictorial, scaled presentation of the genomic organization of *NPC1*. Exon numbers are assigned in Arabian, intron numbers in Roman numerals. Their respective length is given in base pairs.

ity of SNPs occurring at an allelic frequency greater than 0.3. We identified one synonymous SNP, three nonsynonymous exonic SNPs, 11 intronic SNPs, and one SNP in the promoter region not reported previously (Table 2, Fig. 2). In 24 alleles of our NP-C patients we did not observe the nonsynonymous SNPs for G333D, L472P, V757A, I1220T, and R1266Q reported in Caucasians by Morris et al. [1999] or Millat et al. [1999], suggesting a low frequency of these SNPs in Caucasians. Including the data presented here 10 nonsynonymous SNPs are known in *NPC1* [Millat et al., 1999; Morris et al., 1999; Yamamoto et al., 2000]. Eight of the 10 nonsynonymous SNPs affect amino acids fully conserved among mammals suggesting the possibility of functional allelic variability in humans. Indeed, allelic variant c.2572G (V858) diverging of mammalian consensus was associated with the NP-C phenotype in our patients (see below).

Mutations in NP-C Patients

Sequencing of cDNA and genomic DNA of 12 Caucasian NP-C patients revealed 13 most likely disease-causing mutations (Table 2). Four patients were homozygous for *NPC1* mutations predicting amino acid changes C670W, I825C, or P1007A (2x). Four patients were compound heterozygous for two different *NPC1* mutations predicting the combined changes H512R/P1007A, D874V/D948Y, R958L/R1032fs1049X, or R607X/genomic deletion from intron 4 to > = exon 12. In each of four further patients only one heterozygous mutation could be detected, predicting S849I/?, S954L/?, I1061T/?, or P1007A/?, though the complete open reading frame and a relevant part of the promoter re-

gion had been sequenced (Fig. 3). All mutations of this study, including nine not previously described (Table 2) are most likely pathogenic, as they are associated with loss of expression (1), predict premature translational stops through nonsense or frameshifting characteristics (2), or relevant changes of amino acids (7). None of the seven missense mutations were observed in a total of over 100 human subjects screened for mutations by single strand conformation polymorphism polyacrylamide gel electrophoresis (SSCP-PAGE) techniques covering the respective sites [Carstea et al., 1997; Greer et al., 1999; Millat et al., 1999; Yamamoto et al., 1999, 2000], though SSCP-PAGE screening can not exclude the presence of sequence alterations with a 100% sensitivity.

Though Millat et al. [1999] characterized I1061T as the most common mutation in Afro-Caucasian samples, we could detect this mutation only once in our collective. Y825C, D874V, and P1007A, the latter a redundant mutation in our study, have been described by others [Greer et al., 1999; Millat et al., 2001; Snow et al., 1999; Sun et al., 2001] underscoring the impact of these mutations in NP-C. The novel mutation C670W, found homozygously in one patient, affects the domain homologous to the sterol-sensing domain (SSD) of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-R) and sterol regulatory element binding-protein cleavage-activating protein (SCAP). Topological and functional studies characterized this SSD as functionally important in *NPC1* [Davies and Ioannou, 2000; Watari et al., 2000]. One patient was found to be hemizygous for a nonsense mutation in Exon 12 (g.40,415C>T, c.1819, R607X) as segregation analysis in this family

TABLE 1. Complete Genomic Structure of the NPC1 Gene

	Length	Position DNA	
		Start	End
5' UTR		1	3043
Exon 01	57	3044	3100
Intron 01	12434	3101	15534
Exon 02	123	15535	15657
Intron 02	1269	15658	16926
Exon 03	107	16927	17033
Intron 03	3070	17034	20103
Exon 04	176	20104	20279
Intron 04	6732	20280	27011
Exon 05	168	27012	27179
Intron 05	880	27180	28059
Exon 06	250	28060	28309
Intron 06	3048	28310	31357
Exon 07	74	31358	31431
Intron 07	503	31432	31934
Exon 08	371	31935	32305
Intron 08	1263	32306	33568
Exon 09	227	33569	33795
Intron 09	3023	33796	36818
Exon 10	101	36819	36919
Intron 10	3518	36920	40437
Exon 11	103	40438	40540
Intron 11	2856	40541	43396
Exon 12	190	43397	43586
Intron 12	433	43587	44019
Exon 13	183	44020	44202
Intron 13	773	44203	44975
Exon 14	115	44976	45090
Intron 14	2019	45091	47109
Exon 15	128	47110	47237
Intron 15	97	47238	47334
Exon 16	141	47335	47475
Intron 16	530	47476	48005
Exon 17	90	48006	48095
Intron 17	445	48096	48540
Exon 18	191	48541	48731
Intron 18	340	48732	49071
Exon 19	116	49072	49187
Intron 19	683	49188	49870
Exon 20	130	49871	50000
Intron 20	1665	50001	51665
Exon 21	204	51666	51869
Intron 21	972	51870	52841
Exon 22	232	52842	53073
Intron 22	909	53074	53982
Exon 23	114	53983	54096
Intron 23	927	54097	55023
Exon 24	163	55024	55186
Intron 24	1070	55187	56256
Exon 25	83	56257	56339
3' UTR	713	56340	57052

proved a genomic deletion spanning >23 kb on the maternal allele ranging at least from intron 04 to exon 12 (Fig. 3, pedigree 5). Analysis of the common SNPs in exon 17 and 18 showed heterozygosity in the mother's sample. Therefore, we expect the 3' breakpoint between exons 12 and 17. To our knowledge, this represents the first demonstration of a larger genomic deletion in *NPC1*. In another patient, in addition

to heterozygosity for 3182T>C (11061T), a heterozygous nucleotide transversion -238C>G in the *NPC1* promoter region was found on the same (maternal) allele (Fig. 3, pedigree 4). Sequencing of genomic DNA of this patient revealed heterozygosity for the exonic SNPs c.2572A>G (g.45,020A>G), and c.2793C>T (g.45,686C>T), while sequence data of the patient's cDNA suggested exclusive expression of the allele bearing c.2572G-2793T-3182C. We could not detect a mutation in the paternal allele of this patient. We speculate that the promoter alteration -238C>G represents a nonpathogenic polymorphism, as reporter gene constructs of the *NPC1* promoter were fully active, when 232 bp of the *NPC1* 3'-upstream sequence were included [Watari et al., 2000]. Moreover, using MatInspector [Mathcom Inc., Seattle; Quandt et al., 1995] we did not find significant regulatory elements in the region flanking nucleotide position -238. A cryptic mutation of the paternal allele might have caused the loss of expression of the paternal allele in this patient, possibly explaining the finding of exclusive maternal sequence information on the cDNA level.

At the moment, ~100 *NPC1* mutations have been reported [Patterson et al., 2001], the majority being missense and nonsense mutations, three splicing mutations [Millat et al., 2001; Yamamoto et al., 1999], and several small deletions or insertions. Gross deletions, insertions, duplications, or complex rearrangements have not been described so far. Our findings correspond to this mutational spectrum and add the notion of at least one gross genomic deletion in *NPC1*. This observation suggests the possibility that deletions or duplications in *NPC1* may be one of the major sources of mutations escaping detection by PCR-based techniques, as reported here and elsewhere [Millat et al., 1999, 2001; Sun et al., 2001; Yamamoto et al., 1999]. Concerning the coding region we have neither seen altered splice sites nor any aberrantly sized cDNA amplicons as a possible marker of exon-skipping or deletions.

Watari et al. [1999a, 1999b, 2000] introduced a functional *NPC1*-GFP expression system, which allows the morphological characterization of distinct *NPC1* mutants in NP-C fibroblasts and CT60 cells, both characterized by the absence of functional *NPC1* transcripts. So far, their work elucidated the importance of the N-

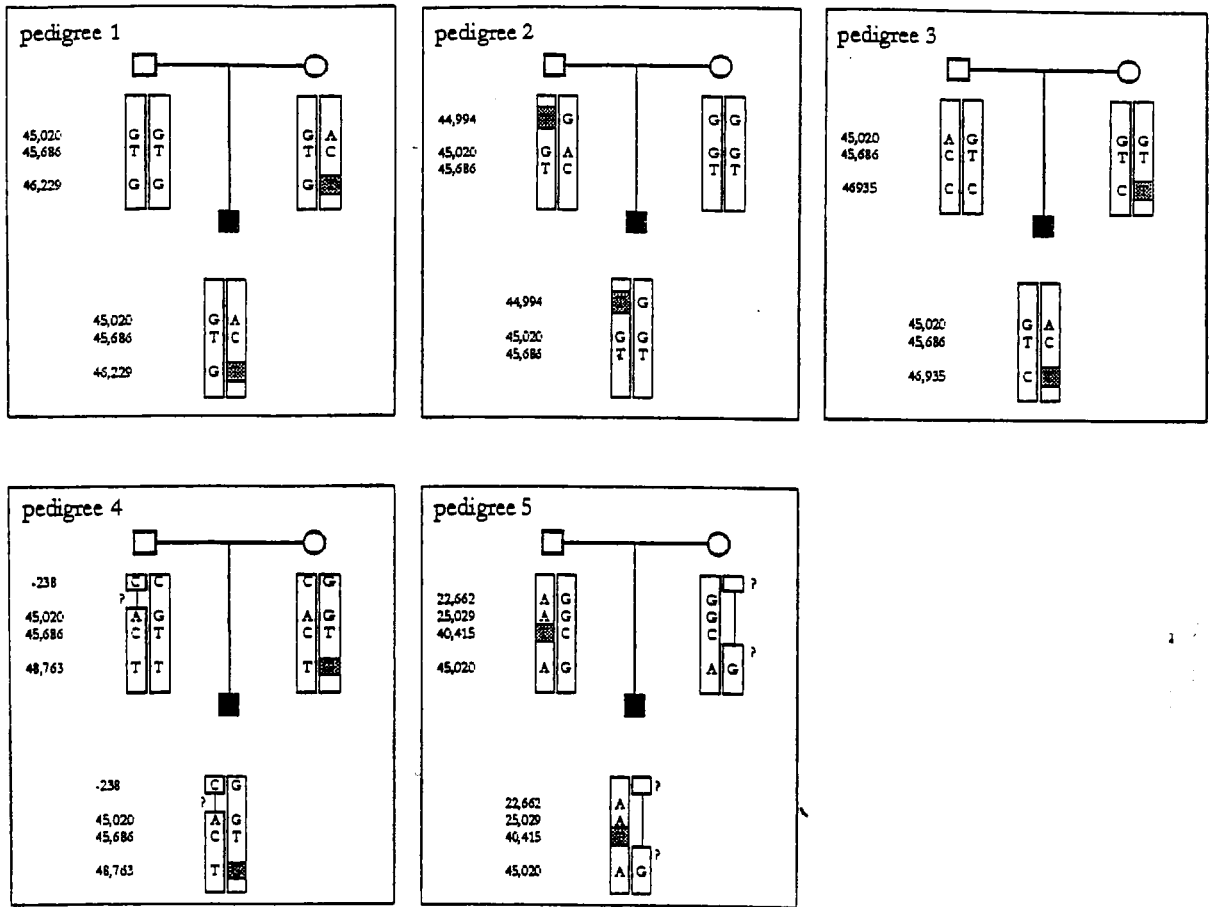


FIGURE 3. Pedigrees in five Caucasian NP-C families with unusual mutational pattern. Locus-specific data are given for family-specific mutations in *NPC1* and for some common polymorphisms. Pathogenic *NPC1* mutations are labeled by a shaded box, numbers refer to the genomic position. Pedigrees 1-4 display NP-C patients where the second disease-causing mutation could not be characterized. In pedigree 4 a disease-causing paternal deletion cannot be ruled out (question mark). Pedigree 5 displays segregation of a >23kb genomic deletion within the maternal *NPC1* allele. Genomic breakpoint is not defined for the 5' breakpoint. The 3' breakpoint must be located between exon 12 and exon 17 (question marks).

C>T) were genotyped in 146 unrelated healthy Caucasian control subjects and 12 NP-C patients. Genotype counts of controls did not deviate significantly from those expected under Hardy Weinberg equilibrium. The frequency of

the respective rarer allele was $F_{2572G} = 0.469$ and $F_{2793T} = 0.474$. Through observation of homozygosity in one of the two SNPs or genotyping parental samples (if available) we were able to determine unambiguous haplotypes in a total of

TABLE 3. *NPC1* Haplotype Frequencies*

Haplotype	Ctrl (n=138 alleles)		NP-C (n=24 alleles)		Haploidentic mutation
	Alleles (n)	Frequency (%)	Alleles (n)	Frequency (%)	
2572A_2793T	6	4.3	1	4.2	D874V or D948Y ^a
2572A_2793C	69	50.0	6	25.0	R607X, H512R, V971G, R958L, deletion>23kb, missing
2572G_2793C	7	5.1	0	0	C650W, P1007A, Y825C, S848I, I1061T, D874V or D948Y ^a , R1032fs1049X, missing (3x)
2572G_2793T	56	40.65	17	70.8	

*Based on exonic polymorphisms c.2572a>g (g.45,020, 1858V) and c.2793c>t (g.45,686, N931N) in 146 healthy Caucasian subjects (Ctrl) (138 alleles (40.3%) could be defined definitely, 154 alleles (59.7%) were heterozygous for both loci) and 12 NP-C patients (7 familial and 5 isolated patients).

^aParental genotypes not available. Fisher's exact test analyzing differences in frequency of the 2572G_2793T haplotype between both groups (two-tailed test) gave a p-value of 0.007.

138 control chromosomes. All four permutations between the two SNPs were identified: 2572A-2793C (50%), 2572G-2793T (41%), 2572G-2793C (5%), and 2572A-2793T (4%) (Table 3). These data are suggestive for an ancestral intragenic recombination within a genomic fragment of <666 bp earlier in the evolution of the human population, although linkage disequilibrium between the SNPs was still significant (data not shown). While the majority of the NPC1 alleles of our patients displayed the haplotype 2572G-2793T (72.7%), this haplotype only accounted for 40.6% in the control group ($p=0.007$, Fisher's exact test). In three of the four cases where only one pathogenic mutation was found, the haplotype with a "missing" mutation was 2572G-2793T (Fig. 3). Among mammals the corresponding residue for variable amino acid I858V (due to c.2572A>G) seems to be a fully conserved isoleucine. This suggests the possibility of a modifying effect of this semiconservative amino acid change (or its underlying haplotype) on expression of NP-C associated mutations. As long as there are no functional assays of full length wild type and mutated NPC1 in vitro we can neither prove nor exclude the possibility of distinct allelic variants acting in cis with mutations, particularly missense mutations.

Our data broaden the perspective onto NPC1's diversity. It may be necessary to evaluate intragenic linkage disequilibria in other ethnic populations, especially in Japanese, as their mutational pattern in NPC1 seems to differ from Caucasians.

ACKNOWLEDGMENTS

We are indebted to all NP-C families who participated patiently in this study. Jill Morris and Peter Pentchev (NIH, Washington) generously provided preliminary data onto NPC1 genomic structure. Finally, we wish to thank Marion Graf-Effenberg for excellent technical assistance and Olaf Rieß, Rejko Krüger (Bochum, Germany), and Eilhard Mix for helpful and critical comments on the manuscript.

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