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Niemann-Pick Disease Type C: Spectrum of *HE1* Mutations and Genotype/Phenotype Correlations in the NPC2 Group

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In Niemann-Pick disease type C (NPC), a genetic heterogeneity with two complementation groups—NPC1, comprising ⩾95% of the families, and NPC2—has been demonstrated. Mutations in the *NPC1* gene have now been well characterized. *HE1* was recently identified as the gene underlying the very rare NPC2. Here we report the first comprehensive study of eight unrelated families with NPC2, originating from France, Algeria, Italy, Germany, the Czech Republic, and Turkey. These cases represent essentially all patients with NPC2 who have been reported in the literature, as well as those known to us. All 16 mutant alleles were identified, but only five different mutations, all with a severe impact on the protein, were found; these five mutations were as follows: two nonsense mutations (E20X and E118X), a 1-bp deletion (27delG), a splice mutation (IVS2+5G→A), and a missense mutation (S67P) resulting in reduced amounts of abnormal HE1 protein. E20X, with an overall allele frequency of 56%, was established as the common mutant allele. Prenatal diagnosis was achieved by mutation analysis of an uncultured chorionic-villus sample. All mutations except 27delG were observed in a homozygous state, allowing genotype/phenotype correlations. In seven families (with E20X, E118X, S67P, and E20X/27delG mutations), patients suffered a severe and rapid disease course, with age at death being 6 mo–4 years. A remarkable feature was the pronounced lung involvement, leading, in six patients, to early death caused by respiratory failure. Two patients also developed a severe neurological disease with onset during infancy. Conversely, the splice mutation corresponded to a very different clinical presentation, with juvenile onset of neurological symptoms and prolonged survival. This mutation generated multiple transcripts, including a minute proportion of normally spliced RNA, which may explain the milder phenotype.

Introduction

Niemann-Pick disease type C (NPC [MIM 257220 and MIM 601015]) is an autosomal recessive lysosomal lipid-storage disorder with a protean clinical presentation (Patterson et al. 2001). The disease is most commonly characterized by hepatosplenomegaly and a severe progressive neurological dysfunction with varying age at onset and varying later course. The cellular hallmark of NPC is a late-endosomal/lysosomal accumulation of endocytosed unesterified cholesterol. In tissues, there is a complex pattern of accumulating lipids that differs between nonneural organs and brain, with no overall increase of sphingomyelin and cholesterol in the latter (Vanier and Suzuki 1996; Blanchette-Mackie 2000; Patterson et al. 2001).

A genetic heterogeneity was established in NPC by somatic-cell hybridization and linkage analysis, defining two different genetic complementation groups, NPC1 and NPC2 (Steinberg et al. 1994; Vanier et al. 1996). Because identical cellular and biochemical phenotypes were observed in patients belonging to the two genetic complementation groups (Vanier et al. 1996; Christomanou et al. 2000), it has been concluded that both gene products may function either in tandem or sequentially (Vanier et al. 1996). The *NPC1* gene (mapped to 18q11), mutated in 95% of families with NPC, was isolated by positional cloning and has been fully characterized (Carstea et al. 1997; Morris et al. 1999). The 4.7-kb *NPC1* cDNA sequence encodes a 1,278-amino-acid protein, shown to contain 13 transmembrane domains, three large luminal loops, four small luminal loops, six small cytoplasmic loops, and one cytoplasmic tail (Davies and Ioannou 2000). It also contains a sterol-

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Table 1

Clinical and Biochemical Survey of Families with NPC2

Patient ID (Ethnicity)	Age at Death	Clinical Summary	Consanguinity?	LDL-Induced Cholesteryl-Ester Formation[a]	Reference(s)
Case 1 (French)	6 mo	Female; hydramnios, cesarean section at 32 wk gestation, respiratory distress from birth, moderate neonatal cholestatic icterus at age 4–30 d, hepatosplenomegaly, respiratory failure (two affected siblings were diagnosed prenatally, and the pregnancies were terminated)	No	<10	
Case 2 (French)	6 mo	Female; fetal ascites (that recessed) at 23 wk gestation, hepatosplenomegaly at birth, cholestatic icterus at age 1 mo, severe respiratory insufficiency from age 3 mo, respiratory failure	No	50	Pin et al. (1990), Vanier et al. (1996) (case 16)
Case 3 (Italian)	6 mo	Female; neonatal cholestatic icterus, hepatosplenomegaly, respiratory failure (three further affected siblings were diagnosed prenatally, and the pregnancies were terminated)	No	75	Steinberg et al. (1994), Vanier et al. (1996) (case 19), Naureckiene et al. (2000)
Case 4 (German)	7 mo	Female; neonatal period uneventful, severe interstitial pneumonia/hepatosplenomegaly/respiratory insufficiency/failure at age 3 mo (a brother with "neonatal hepatitis" and severe lung involvement had died at age 6 wk)	Yes	320	Schofer et al. (1998), Vanier et al. (1996) (case 28)
Case 5 (Algerian)	19 mo	Male; neonatal cholestatic icterus until age 4 mo, then pulmonary involvement; severe motor development delay from age 7–9 mo (able to sit only at age 15 mo, no further progress); growth retardation, increasing pulmonary fibrosis, oxygen dependency, respiratory failure	Yes	10	Vanier et al. (1996) (case 2)
Case 6 (Czech)	4 years	Female; failure to thrive (with poor feeding), hepatosplenomegaly and mild delay of motor milestones at age 1 year, severe lung involvement, oxygen dependent from age 20 mo, quadruspasticity	No	45	Christomanou et al. (2000), Elleder et al. (2001)
Case 7 (Turkish)	Alive (at 20 mo)	Female; no neonatal cholestatic icterus; respiratory distress and pulmonary infiltration at age 11 mo, (hepato-)splenomegaly; new acute respiratory problems at age 13 mo, at which psychomotor development was normal; hypotonia and gait problems from age 18 mo (an elder brother had splenomegaly and died, of respiratory distress, at age 6 mo in Turkey)	Yes	10	
Case 8 (Algerian)	Alive (at 21 years)	Female; splenomegaly at age 6.5 years, school problems, epileptic fits at age 11 years, mental retardation, slow progression, still able to walk normally at age 21 years (an affected sister with quite similar course was still alive at age 27 years)	Yes	380	Vanier et al. (1996) (case 27)

[a] After 4.5 h, expressed as pmol/mg cell protein, studied as described by Vanier et al. (1991) (normal values 2,950 ± 1,200).

sensing domain with the same orientation as that of HMG-CoA reductase and SCAP (sterol regulatory element binding protein [SREBP] cleavage activation protein) (Davies and Ioannou 2000). Approximately 100 different *NPC1* mutations have already been identified in patients with NPC1 (Carstea et al. 1997; Greer et al. 1998, 1999; Yamamoto et al. 1999, 2000; Millat et al. 2001; Ribeiro et al. 2001; Sun et al. 2001); they have been shown to affect all domains of the protein—with some predilection, however, for a conserved cysteine-rich luminal loop (Greer et al. 1999; Millat et al. 2001). Only three of these mutations—I1061T, G992W, and P1007A—appear to occur quite frequently (Greer et al. 1998; Millat et al. 1999, 2001). The NPC1 protein resides in late endosomes and interacts transiently with lysosomes and with the *trans*-Golgi network (Higgins et al. 1999; Neufeld et al. 1999). The exact cellular function of NPC1 and the role that it has in intracellular trafficking of lipids remain unclear (Liscum and Munn 1999; Blanchette-Mackie 2000; Cruz et al. 2000; Davies et al. 2000; Ory 2000). A number of studies suggest that NPC1 has a key role in the modulation of vesicular trafficking of cholesterol and of glycolipids (Neufeld et al. 1999; Blanchette-Mackie 2000; Zhang et al. 2001), but recent data suggest that NPC1 is a permease acting as a transmembrane efflux pump (Davies et al. 2000).

To date, only eight families belonging to the minor, NPC2 complementation group (MIM 601015), estimated to account for 5% of families with NPC, have been documented (Steinberg et al. 1994, 1996; Vanier et al. 1996; Christomanou et al. 2000; Naureckiene et al. 2000; Elleder et al. 2001). Recently, by a proteomic approach, the *HE1* gene (mapped to 14q24) was identified as the disease-causing gene in patients with NPC2 (Naureckiene et al. 2000). For practical reasons and by analogy with *NPC1,* we propose henceforth to use the name "*HE1/NPC2*." *HE1/NPC2* is ~13.5 kb long and is composed of five exons of size 78–342 bp (Naureckiene et al. 2000). Analysis of the 822-bp cDNA sequence showed an open reading frame of 453 bp, followed by a 3′ UTR of 327 bp comprising most of exon 5 (Kirchhoff et al. 1996). Translation of *HE1/NCP2* leads to a mature protein of 132 amino acid residues, with a theoretical molecular mass of 14.5 kD.

The HE1/NPC2 glycoprotein (GenBank) was first characterized as a major secretory protein in the human epididymis (Kirchhoff et al. 1996) and was also shown to bind cholesterol (Baker et al. 1993; Okamura et al. 1999). It is now known to be a small, soluble, ubiquitously expressed lysosomal protein, the functional alteration of which leads to an NPC phenotype (Naureckiene et al. 2000). In the original study establishing *HE1* as the gene underlying NPC2, severe mutations leading to absence of HE1 protein were reported in two patients (Naureckiene et al. 2000). One patient carried a homozygous E20X mutant allele, and the other patient was a compound heterozygote for the E20X mutation and for a single-nucleotide deletion (311delA) in exon 2. In the present report, the molecular defect has been characterized in a further seven unrelated families with NPC2 and has been applied to rapid first-trimester prenatal diagnosis of the condition. Furthermore, the clinical and biochemical phenotypes in the eight families can now be better understood in terms of the underlying genetic mutations.

Table 2

Oligonucleotide Sequences

Oligonucleotide	Sequence[a] (5′→3′)
NPC2-ex1s	gctgtggttactggtgacagg
NPC2-ex1as	tccaaggctcagcctggccg
NPC2-ex2s	ctaaatgggagagcagagcac
NPC2-ex2as	ccctccattcccatgcttattc
NPC2-ex3s	atgcttgcactgtgctagtac
NPC2-ex3as	tgatgcctaacaccgcaccta
NPC2-ex4s	ggagttaggaagtttgctactg
NPC2-ex4as	tggcactgatttagtttcagtc
NPC2-ex5s	gccctgacagtggaggaaag
NPC2-ex5as	ggcctgccttccatggttct
NPC2-ADNcs(3)[b]	$^{-21}$CTTGGAACTTCGTTATCCGCG^{-1}
NPC2-ADNcas(2)[b]	^{535}TGTTGAAGCAGCAGAGCTGG^{516}

[a] Exonic sequences are given in uppercase, and intronic sequences are given in lowercase.
[b] Counted from the adenosine residue of the initiation codon.

Subjects, Material, and Methods

Subjects and Biological Material

Fibroblast strains from eight unrelated patients with NPC2 (six previously documented cases and two from newly diagnosed families) were used in the study. The diagnosis of NPC disease had been assessed by filipin staining and by measurement of LDL-induced cholesteryl-ester formation, as described elsewhere (Vanier et al. 1991) (table 1). Complementation analysis by somatic-cell hybridization and by filipin staining had previously been documented in a majority of the cases (Steinberg et al. 1994; Vanier et al. 1996; Schofer et al. 1998) and, in the remaining three families (cases 1, 6, and 7), was performed by the simplified procedure described by Schofer et al. (1998). Genomic DNA was also available from parents of cases 1, 3, and 6–8 and from four affected fetuses who would have been siblings of cases 1 and 3. The main clinical and biochemical features of the patients with NPC2 are summarized in table 1.

Genomic DNA Extraction

Genomic DNA was extracted from cultured skin fibroblasts, peripheral blood leukocytes, and uncultured

Table 3

Spectrum of Mutations Identified in Patients with NPC2

Patient (Cell Line)	Genotype	Nucleotide Change(s)[a]	Location(s)	Predicted Protein Alteration(s)
Case 1 (20082)	E20X/E20X	G58T	Exon 1	Stop at codon 20
Case 2 (88082)	E20X/27delG	G58T/27delG	Exon 1/exon 1	Stop at codon 20/frameshift from codon 9; premature termination codon + 24 amino acids
Case 3 (88057/NIH99.04[b])	E20X/E20X	G58T	Exon 1	Stop at codon 20
Case 4 (93104)	E118X/E118X	G352T	Exon 3	Stop at codon 118
Case 5 (89095)	E20X/E20X	G58T	Exon 1	Stop at codon 20
Case 6 (96059)	E20X/E20X	G58T	Exon 1	Stop at codon 20
Case 7 (21033)	S67P/S67P	T199C	Exon 3	S67P
Case 8 (82017/NIH94.85[b])	IVS2+5G→A/IVS2+5G→A	…	Intron 2	Multiple mRNAs (see text and fig. 1)

[a] Counted from the adenosine residue of the initiation codon.
[b] New code, assigned after the strains were transferred to the National Institutes of Health (NIH) within the frame of a collaboration between either A.H.F. or M.T.V. and Dr. P. G. Pentchev.

chorionic-villus samples, by standard procedures (Jeanpierre 1987; Miller et al. 1998)

Sequencing

After amplification, PCR products were purified and directly sequenced in both directions by the Thermosequenase cycle sequencing kit and were primer labeled with $\gamma[^{33}P]$-ATP at 3,000 Ci/mmol (Amersham Pharmacia Biotech).

RNA Isolation and cDNA Synthesis

Total RNA was isolated from fibroblast monolayers by Trizol reagent (Life Technologies), and reverse transcription was performed with oligo-dT primer, by the First Strand cDNA synthesis kit (Amersham Pharmacia Biotech) according to the manufacturers' instructions.

Subcloning

cDNA from case 8 was amplified by primers NPC2-ADNcs(3) and NPC2-ADNcas(2) (table 2). PCR products were purified and subcloned into pGEM-T vector (Promega). After transformation, 32 isolated subclones were sequenced in both directions.

Mutation Detection of the E20X Nonsense Mutation in Genomic DNA

Exon 1 of *HE1/NPC2* was PCR amplified by the primers NPC2-ex1s and NPC2-ex1as (table 2). The 40 PCR cycles each included steps of 1 min at 92°C, 1 min at 62°C, and 1 min at 72°C. Because the mutation created a *Stu*I restriction site, the 325-bp PCR product was digested for 2 h at 37°C by *Stu*I and was analyzed on 3% Nusieve-GTG gels (BioWhitaker Molecular Applications).

Western Blot Studies

For western blot studies, fibroblast lysates were prepared as described by Naureckiene et al. (2000), and soluble protein (10 μg protein/lane) was separated on 12% SDS-acrylamide gels. The blot was probed with rabbit antibodies to HE1/NPC2 (Naureckiene et al. 2000), followed by chemiluminescence by the ECL detection kit (Amersham Pharmacia Biotech).

Results

Spectrum of HE1/NPC2 *mutations*

Sequencing of all five exons of *HE1/NPC2* and of their boundaries was performed on genomic DNA isolated from all patients except proband 3, who was included in the initial, "*HE1*" study (Naureckiene et al. 2000; case NPC2-99-04). All 14 new mutant alleles were characterized. As shown in table 3, only five different mutations were identified—two nonsense mutations, E20X and E118X; a 1-bp deletion, 27delG, leading to early termination of protein; a missense mutation, S67P; and a splice mutation, IVS2+5G→A, in the consensus sequence of the 5′ donor site of intron 2. The homozygous status of the mutation in probands 7 and 8 was confirmed by sequencing of genomic DNA of both parents. In two independent experiments, the electrophoretic profile of reverse-transcriptase PCR (RT-PCR) products from RNA isolated from proband 8 showed multiple bands (fig. 1). RT-PCR products therefore were subcloned, and 32 subclones were analyzed by sequencing. This analysis revealed 30 abnormally spliced cDNAs and 2 normally spliced cDNAs. Among the 30 mutant cDNAs, only three classes were observed—cDNA with a deletion of the last 76 bp of exon 2 (in 15/30), cDNA with a total deletion of exon 2 (in 6/30), and cDNA with a 10-bp insertion between exons 2 and 3 (in 9/30). The 10-bp

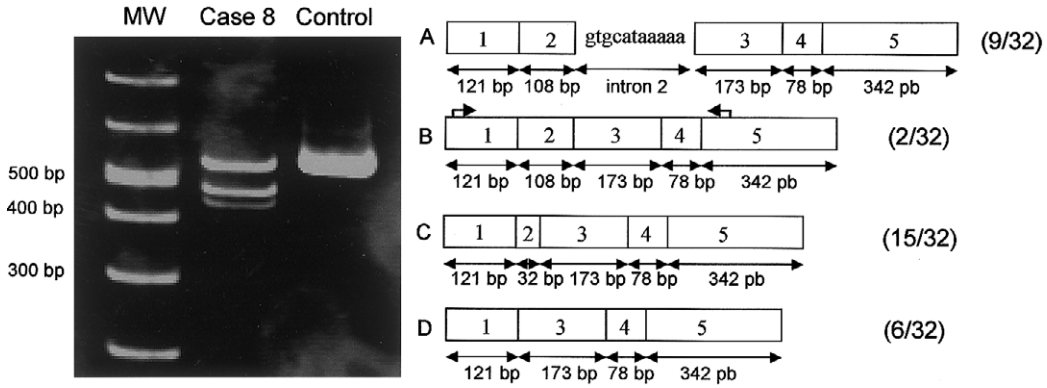

Figure 1 Multiple abnormal *HE1/NPC2* mRNAs in patient 8—PAGE analysis of RT-PCR products and schematic representations of samples of *HE1/NPC2* cDNA isolated from the patient's fibroblasts.

insertion (gtgcataaaa) corresponds to the 5′ end of intron 2 (fig. 1).

Western Blot Studies of HE1/NPC2 in Patients with NPC2

HE1/NPC2 was studied in cultured fibroblasts from a normal subject and from patients with the S67P, IVS2+5G→A, and E20X mutations. As shown in figure 2, no detectable protein was observed in fibroblasts with either IVS2+G→A or E20X, whereas S67P protein appeared as a weak single band—instead of as a doublet, as observed in normal fibroblasts.

The E20X Nonsense Mutation—a Frequent Mutant Allele in Patients with NPC2

The most striking finding was that E20X was a highly recurrent mutation, observed in five of the eight patients included in the study. Interestingly, the ethnic origin of the patients was quite varied (i.e., Italian, French, Algerian, and Czech). Because the E20X mutation creates a *Stu*I restriction site, a simple screening test was devised. Whereas the normal allele remains uncut (325 bp), the mutant allele generates two shorter bands (177 bp and 148 bp) (fig. 3). Parents of probands 1, 3, and 6 were studied by this test, confirming the homozygous status of the mutation in these patients. The study of skin fibroblasts from the abortus also provided ultimate confirmation of four prenatally diagnosed affected siblings in two families. In the eight families studied, the allele frequency of E20X amounted to 56%.

Prenatal Diagnosis by Uncultured Chorionic-Villus Sampling in a Family with E20X

In family 1, which had a homozygous E20X mutation, the third pregnancy was monitored by study of genomic DNA extracted from uncultured chorionic-villus sampling by the *Stu*I restriction-enzyme test described above. The fetus was found to be affected, and the pregnancy was terminated at 11 wk gestation. The diagnosis was later confirmed on cultured chorionic-villus samples, by the conventional biochemical strategy (Vanier et al. 1992).

Genotype/Phenotype Correlations

Six of the eight patients with NPC2 who were included in the study had a short life span; four (cases 1–4) did not survive longer than ~6 mo, owing to respiratory or hepatic failure, and the remaining two (cases 5 and 6) developed a neurological disease and died at age 19 mo and 4 years, respectively. In all six of these patients, the *HE1/NPC2* mutation was obviously very deleterious, leading to early termination of the HE1/NPC2 protein (cases 1–3 and 6, E20X or frameshift; case 4, E118X). All four unrelated patients homozygous for the recurrent E20X mutation presented with hepatosplenomegaly and lung involvement, and three of the four presented with a neonatal cholestatic icterus. Two patients died, of respiratory failure, at age <6 mo, whereas two lived longer and developed a severe neurological disease with onset during infancy. Somewhat unexpectedly, the two affected sibs with a homozygous S67P missense mutation also showed a severe clinical course. Both had marked splenomegaly and less-severe hepatomegaly. The first-born child died early, of respiratory failure, and the other child (case 7), also showing severe respiratory problems, already had obvious neurological involvement by age 18 mo.

Conversely, the two affected sisters in family 8 suffered a quite different disease course, with onset of neurological symptoms during late childhood, slow progression of the disease with long survival, and no prominent pulmonary involvement. The splice mutation underlying the disease in this family was found to produce multiple

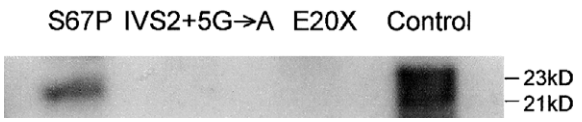

Figure 2 Western blot of HE1/NPC2 in cultured fibroblasts from three patients with NPC2 and from one normal subject. The patients with NPC2 were homozygous for the indicated mutations.

mRNAs, with very small amounts (2/32 clones) of normal message, which may explain the definitely milder disease course in these patients.

Discussion

To date, *HE1/NPC2* mutations have been studied in nine unrelated families with NPC2. All mutant alleles have been identified. In the original study establishing *HE1* as the gene underlying NPC2, three mutant alleles corresponded to the E20X nonsense mutation, and one allele corresponded to a 1-bp deletion leading to a frameshift (Naureckiene et al. 2000). The present study, of seven additional families, has led to the identification of only four novel mutations—comprising one nonsense mutation (E118X), one frameshift (27delG), one missense mutation (S67P), and one splice mutation (IVS2+5G→A)—as well as to the finding of 7/14 E20X alleles (table 3). E20X, with an overall allele frequency of 56%, was thus established as the common mutant allele in patients with NPC2. The variable ethnic origin of the families suggests that a founder effect is unlikely. Five of the six mutations thus far described lead to either premature termination of the protein or severe alterations of the transcripts, and the single missense mutation shows a clearly diminished amount of HE1/NPC2 (table 1 and fig. 2). The situation observed with *HE1/NPC2* thus is in strong contrast with the mutational pattern described for *NPC1*—for which a wide variety of mutations have been documented, a majority of which are missense mutations (Carstea et al. 1997; Greer et al. 1999; Yamamoto et al. 2000; Millat et al. 2001; Ribeiro et al. 2001; Sun et al. 2001). Of the missense mutations, several have been shown to result in an essentially normal amount of NPC1 (Yamamoto et al. 2000; Millat et al. 2001; Ribeiro et al. 2001).

From a clinical point of view, the classic signs and symptoms of NPC were present in all cases. Yet, in the population of patients studied, the distribution of the various clinical subtypes was very different from that observed in NPC1. In the latter, patients with a juvenile onset of neurological symptoms constitute ~50% of the cases, patients with an onset of neurological symptoms during infancy constitute ~20%, and patients dying, of liver or respiratory failure, during the 1st year of life, constitute, at most, 10% (Vanier et al. 1991; Vanier 2000). A first remarkable feature of the patients with NPC2 was pronounced lung involvement, which was prominent in six of the seven families for which detailed clinical data were available (Pin et al. 1990; Schofer et al. 1998; Elleder et al. 2001; table 1) and which led to early death in six patients. The possible elective lung involvement in patients with NPC2 has been described elsewhere (Vanier et al. 1996; Schofer et al. 1998). Nevertheless, this feature is not specific to NPC2. Although the complementation group of some published cases with severe lung involvement has not been reported (Kovesi et al. 1996), we have recently definitively shown one patient who died, of acute respiratory failure, at age 2.5 mo, to belong to the NPC1 complementation group (G.M. and M.T.V., unpublished data). Interestingly, similar to the situation for neonatal cholestatic icterus and neurological disease in families with NPC1 (Vanier et al. 1991; Millat et al. 2001), the study of family 7 indicates that fatal respiratory failure and a neurological course can be seen in the same sibship. A second remarkable observation is that, in three of the four families in which patients lived long enough to have neurological symptoms, the neurological disease showed onset during infancy and a rapid course of decline.

The E20X mutation represents a null allele, because all but the signal sequence of HE1/NPC2 is deleted. This logically results in a severe disease phenotype, with all homozygous patients dying at age <4 years. As discussed above, neonatal respiratory manifestations were frequent and could lead to early death at age <6 mo and, thus, before onset of neurological symptoms. Patients who had less-acute or no neonatal systemic manifestations showed an onset of neurological symptoms at age 1–2 years and did not survive past early childhood, as is the rule in the infantile-neurological-onset form (Vanier and Suzuki 1996; Vanier 2000). The 27delG and E118X mutations are also associated with severe disease and thus are likely to represent null alleles. It is difficult to predict a priori the impact of missense mutations. On the basis both of the neurological symptoms already observed, at age 18 mo, in case 7 and of the early death of her brother, it appears that the S67P mutation markedly affects the function of HE1/NPC2. This missense mutation is a nonconservative (polar-to-hydrophobic) substitution that generates a proline, an imino acid known to be particularly important for protein folding. The low level of S67P protein detected by western blot analysis suggests that, in addition to having a possibly deleterious effect on function, this mutation may also affect biosynthesis and/or stability. The IVS2+5G→A mutation is associated with a milder clinical course, since the two siblings homozygous for this mutation both showed a juvenile onset of the neurological disease and a prolonged survival. A more detailed study showed that this splice mutation generated

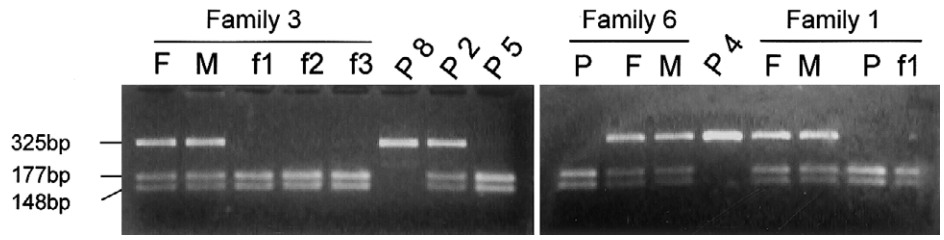

Figure 3 Restriction analysis of E20X. E20X creates a *Stu*I restriction site. For families 1, 3, and 6 (tables 1 and 3), besides the proband (P) or affected fetuses (f), the study included the father (F) and the mother (M). Lane P5 corresponds to case 5. Probands with other *HE1/NPC2* mutations (cases 8 and 4 [lanes P8 and P4, respectively]) showed a normal pattern and served as controls.

multiple abnormal mRNAs (fig. 3), a situation that has been described in other lysosomal diseases (Ohno and Suzuki 1988). In fibroblasts, a very small proportion (2/32) of correctly spliced transcripts also was demonstrated (fig. 3). Although this was not sufficient to produce enough HE1/NPC2 for detection by our western blot assay (fig. 2), the presence of low levels of functional protein presumably accounts for the milder clinical course. The question of whether different tissues could show a variability in the level of abnormally/normally spliced RNA transcribed from the IVS2+5G→A mutation also can be raised. Such a situation has been shown to occur in patients with cystic fibrosis who carry the 3,849+10-kb C→T mutation (Rave-Harel et al. 1997; Chiba-Falek et al. 1998) and has been considered as the basis of disease variability.

One potential explanation for the overall very severe clinical and mutational profile of the currently known families with NPC2 is that genetic complementation studies might have been preferentially performed in patients with a clinical presentation similar to that of the original case (Steinberg et al. 1994). Although this, in some respects, is true of earlier studies (Vanier et al. 1996), in recent years genetic complementation tests have been performed in a quite systematic way, in the laboratories of two of the authors (A.H.F. and M.T.V.) of the present article. Remarkably, the last three identified families with NPC2 (families 1, 6, and 7) still showed the most common phenotype. Severe alterations of cholesterol trafficking were present in six of the eight families reported in the present study, including the family with a missense mutation. A lesser degree of lysosomal cholesterol storage and impairment of LDL-induced cholesterol esterification was present in cells from patient 4 (Schofer et al. 1998) and in cells from the two siblings in family 8. Yet, no patient with the very mild impairment of cholesterol trafficking that we have described as the "variant" biochemical phenotype (Vanier et al. 1991) has been shown to belong to the NPC2 complementation group. In fibroblasts from these patients, alterations are demonstrated only after loading with pure LDL (Vanier et al. 1991; Sun et al. 2001). We agree with Sun et al. (2001) that genetic complementation tests using filipin staining are difficult and not always reliable in patients with the variant phenotype. In consideration of the fact that no *NPC1* mutations could be demonstrated in several of their variant NPC cell lines, Sun et al. (2001) suggested that such patients were good candidates for a systematic screening for *HE1/NPC2* mutations. In our view, excluding the presence of the most frequent *NPC1* mutant alleles—I1061T, P1007A, and G992W/ G992R—prior to *HE1/NPC2* sequencing is the proper strategy in newly diagnosed patients with variant NPC. In our survey of 53 variant cell lines, at least one allele carrying any of these four mutations was found in 31 cases. To date, complete sequencing of *HE1/NPC2* cDNA from 10/22 remaining cell lines was achieved, and no abnormality was detected (G.M. and M.T.V., unpublished data). With regard to systematic genetic testing of patients with "classic" NPC (i.e., with severe cholesterol-trafficking alterations), whether to test both for I1061T, on *NPC1*, as we had proposed elsewhere (Millat et al. 1999), and for E20X, on *HE1/NPC2*, should be considered before genetic complementation analysis is performed.

Proper identification of patients with NPC2 and their molecular characterization are important for two reasons. First, it has an essential, practical impact on genetic counseling; we have shown that the mutational approach greatly improves prenatal testing, resulting in significantly earlier diagnosis. Second, identification of more *HE1/NPC2* mutations may provide important new information about functional domains of HE1/NPC2 and advance the understanding of its exact function and of its potential interactions with NPC1.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for complete genomic DNA sequence of human *HE1* [accession number AC005479] and HE1/NPC2 [accession number Q15668])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for NPC [MIM 257220 and MIM 601015])

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