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SHORT REPORT

Subclinical course of adult visceral Niemann–Pick type C1 disease. A rare or underdiagnosed disorder?

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Summary We present the third case of Niemann–Pick disease type C without neurological symptoms. The patient was a 53-year-old woman without significant prior health problems who died of acute pulmonary embolism. Autopsy findings of hepatosplenomegaly, lymphadenopathy and ceroid-rich foam cells raised the suspicion of the visceral form of acid sphingomyelinase deficiency (Niemann–Pick disease type B; NPB) or a much rarer disorder, variant adult visceral form of Niemann–Pick disease type C (NPC). To verify the histopathological findings, *SMPD1*, *NPC1* and *NPC2* genes were analysed. Two novel sequence variants, c.1997G > A (S666N) and c.2882A > G (N961S) were detected in the *NPC1* gene. No pathogenic sequence variants were found either in the *SMPD1* gene mutated in NPB or in *NPC2* gene. The pathogenicity of both *NPC1* variants was supported by their location in regions important for the protein function. Both variations were not found in more than 300 control alleles. Identified sequence variations confirm the diagnosis of the extremely rare adult visceral form of Niemann–Pick disease type C, which is otherwise dominated by neurovisceral symptoms. Although only three patients have been reported, this (most probably underdiagnosed) form of NPC should be considered in differential diagnosis of isolated hepatosplenomegaly with foam cells in adulthood.

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References to electronic databases: OMIM (http://www.ncbi.nlm.nih.gov/): Niemann–Pick disease, type C1 #257220; Niemann–Pick disease, type C2 #607625; Niemann Pick disease type A/B #257200, #607616; Gaucher disease, type I #230800. GenBank reference sequences (http://www.ncbi.nlm.nih.gov/), accession numbers: *SMPD1*, NC_000011.8 REGION: 6368302..6372783; *NPC1*, NC_000018.8, REGION: complement (19365461..19420426); *NPC2 (HE1)*, NC_000014.7, REGION: complement(74016397..74029837). Uniprot database (http://www.expasy.uniprot.org/). The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Databases used for SNP analysis: GenBank (http://www.ncbi.nlm.nih.gov/), Ensembl (http://www.ensembl.org/), HGVD (http://hgvbase.cgb.ki.se/).

Enzymes: SMPD1 (EC 3.1.4.12), HMG CoA reductase (EC 1.1.1.88)

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Abbreviations

FFPE	formalin fixed paraffin-embedded
LDL	low-density lipoprotein
NP A/B	Niemann–Pick disease type A/B
NPB	Niemann–Pick disease type B
NPC	Niemann–Pick disease type C
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
SSD	sterol sensing domain

Introduction

Niemann–Pick disease type C (NPC; OMIM 257220, 607625) is an autosomal recessive lysosomal disorder caused by deregulation of the cellular lipid trafficking due to the molecular defects in either of the two late endosomal/lysosomal proteins (NPC1 and NPC2). NPC1, a transmembrane protein localized in a subset of late endosomes, is endowed with a sterol sensing domain exerting control of cholesterol content of late endosomal membranes (Liscum and Sturley 2004). Excess cholesterol is translocated for

esterification to other cellular membranes, including Golgi apparatus. When mutated (as it is in the majority of NPC patients), NPC1 protein impairs control of cholesterol membrane content and leads to a cascade of events, not yet fully defined, resulting in an accumulation of a variable set of lipids (e.g. glucosyl and lactosylceramide, GM_2 ganglioside, and sphingomyelin) in late endosomes/lysosomes. This process resembling classical lysosomal storage progresses despite unhindered degradative capacity of lysosomal enzymes. The cellular changes described are found mainly in neurons and macrophages, and to a lesser extent in other cell types (Mukherjee and Maxfield 2004).

The second protein (NPC2) engaged in intracellular cholesterol trafficking is localized in the lysosomal lumen and functions in close association with NPC1 (Vanier et al 2004; Zhang et al 2003). Its dysfunction leads to the phenotype that is clinically and/or biochemically indistinguishable from NPC1 phenotype with the exception of a tendency to more severe lung involvement (Vanier and Millat 2003).

Clinical presentation of NPC is highly variable. An acute form presents usually at birth and is associated with severe hepatopathy and splenomegaly. Surviving patients develop psychomotor retardation during infancy and slowly progressive neurodegeneration with vertical supranuclear gaze palsy, seizures and spasticity later in childhood. Patients with the juvenile-onset form develop slowly progressive ataxia, spasticity, seizures and supranuclear gaze palsy during childhood and dementia later in the teenage years. NPC individuals with later-onset forms have similar clinical symptoms appearing in adolescence or adulthood. Adult-onset NPC patients can be especially difficult to diagnose, as only about 50% of them have palpable hepatosplenomegaly and the dominant symptoms can be psychiatric (psychosis or bipolar disorder) (Vanier and Millat 2003).

The proof of lysosomal storage of free cholesterol, detected in cultured skin fibroblasts by filipin staining and reduced rate of LDL-derived cholesterol esterification, is diagnostic for NPC. The residual rates of cholesterol esterification tend to some extent to correlate inversely with the severity of the phenotype.

Some patients do not fulfil the clinical criteria of the three common phenotypes described above (Klunemann et al 2002). It is not unusual for the patients to present initially with isolated splenomegaly. The majority of the patients develop the neurological symptoms later during the course of the disease. Only two of the reported cases were free of neurological symptoms in their fourth and sixth decades, respectively, suggesting that most likely they would never develop them (Fensom et al 1999; Frohlich et al 1990).

In this report we present the third case of this rare purely visceral NPC1 variant, evaluated at both the tissue and molecular levels, and argue that a substantial number of these patients can easily escape diagnosis.

Clinical report

The clinical history of the patient was unremarkable. She was employed as a clerk. She had varicose veins of the lower extremities and from 48 years of age she had been treated for arterial hypertension. She never complained of neurological symptoms and spleen enlargement was not noted. During the 6 months before her admission to hospital she became easily fatigued and developed occasional mild dyspnoea. Shortly after arrival from a prolonged coach trip she suffered acute pulmonary embolism, and after admission to the hospital she suffered acute myocardial infarction. In spite of early and adequate hospital care she died the next day due to cardiac failure at the age of 53 years. Splenomegaly was noted on examination and laboratory work-up showed unexplained moderately severe microcytic anaemia (haemoglobin 87 g/L, reference range 116–183; red blood cells 4.25×10^{12}/L, reference range 3.54–5.18; mean cell volume 64 fl, reference range 82.3–100) with normal counts of white blood cells and platelets.

Her mother and two sons are without any clinical abnormalities; family history was uneventful and was not suggestive of a hereditary disorder. Informed consents were obtained from the living relatives of the patient.

Methods

Histology and immunohistology

An autopsy and a histopathological examination were performed according to the standard protocols including immunohistochemical detection of cathepsin D with rabbit polyclonal antibody (DAKO Cytomation, Copenhagen, Denmark), and of CD68 (PGM1) and Tau protein with mouse monoclonal antibodies purchased from DAKO and Novocastra (Newcastle Upon Tyne, UK), respectively. The primaries (after overnight incubation at 4°C) were detected with appropriate Envision systems (DAKO).

DNA analysis

Sequence analysis of the *SMPD1* gene and the acid sphingomyelinase activity measurements were performed as described previously (Pavlu-Pereira et al 2005; Sikora et al 2003).

Genomic DNA of the proband was extracted from the formalin fixed paraffin embedded (FFPE) tissues (spleen and lymph node) using heat deparaffinization, Proteinase K

digestion and phenol–chloroform extraction as no other biological materials were available. Genomic DNA from the proband's living relatives (her mother and two sons) was isolated from peripheral blood leukocytes using standard phenol–chloroform extraction procedure. The entire gene-coding region as well as 5′ and 3′ untranslated regions and exon–intron boundaries of *NPC1* and *NPC2* genes were examined by direct sequencing of PCR products. Primers for both genes, *NPC1* and *NPC2 (HE1)*, were designed according to GenBank reference sequences (NC_000018.8 and NC_000014.7). PCR product sizes, primers and PCR reaction conditions are available from the authors upon request. The substitution c.1997G > A was verified by PCR/RFLP using restriction endonuclease *Dde*I (NEB, Ipswich, MA, USA). As the c.2882A > G did not alter a restriction site, we designed a modified primer (5′ GCATTGCAGAACTGGTCAGGGATA 3′) that produced a new recognition site for *Bfu*I (Fermentas, Burlington, ONT, Canada) in the variant allele. This PCR/RFLP protocol was used for patients' samples and control samples.

Protein sequences mentioned in the Discussion were retrieved from the Uniprot database (http://www.expasy.uniprot.org/) and from The National Center for Biotechnology Information (http://www.ncbi. nlm.nih.gov/). Multiple alignments were performed using ClustalW in Blosum61 matrix (Thompson et al 1994).

Results

Autopsy findings of hepatosplenomegaly (spleen weight 1230 g, liver 3000 g) and lymphadenopathy together with histopathological signs of visceral lysosomal storage raised the pathologist's initial suspicion of Gaucher disease (OMIM 230800). Verification of the diagnosis required by the clinical geneticist was carried out using the paraffin-embedded autopsy samples. This showed spleen and bone marrow infiltration with histiocytic foam cells, many of them containing ceroid admixture (typical yellowish autofluorescence and sudanophilia in paraffin sections). Storage histiocytes were numerous in some of the lymph nodes and scarce in the liver. A large number of histiocytes (CD68 PGM1 positive) in mediastinal lymph nodes and liver sinusoids (Fig. 1, A–C) did not show any histologically detectable storage or ceroid accumulation. Hepatocytes were without vacuolization but displayed increased granular immunostaining for cathepsin D disproportional to lipofuscin pigmentation. There was extramedullary trilineage haematopoiesis in the liver and spleen. Neurons in the brain samples (basal ganglia) were remarkably distended, with numerous lipofuscin granules (Fig. 1D) that were in excess compared with the age-matched controls. Neither definite neuronal vacuolization nor neurofibrillary tangles were detectable by immunostaining.

The revision excluded Gaucher disease and suggested Niemann–Pick disease type B (OMIM 607616) as the most probable diagnosis with regard to the relatively high incidence of this disorder and to the visceral foam cell storage pattern.

In order to verify the histopathological findings, we analysed the acid sphingomyelinase gene (*SMPD1*), which is mutated in Niemann–Pick disease type A/B (NP A/B). As only FFPE tissues were available from the proband, we began the analyses using genomic DNA isolated from the peripheral blood leukocytes from the proband's living relatives, who were obligatory heterozygotes (the mother and two sons of the proband).

Direct sequencing of the *SMPD1* gene did not reveal any pathogenic sequence variations. Also, acid sphingomyelinase (EC 3.1.4.12) activity in leukocytes was in the normal range in all three relatives examined, while in NP A/B heterozygotes the activities are characteristically about 50% of control values.

Since neither biochemical assays nor mutation analyses supported the diagnosis of Niemann–Pick disease type B, we focused on the much less common disorder, variant adult visceral form of Niemann Pick disease type C. As noted in the introduction, NPC patients belong to either of the two complementation groups associated with pathogenic variations in *NPC1* or *NPC2* genes.

While no variations were detected in *NPC2*, the previously undescribed substitution c.1997G > A (S666N) was identified in exon 13 of the *NPC1* gene in all three living relatives and later in the DNA isolated from FFPE tissues from the proband.

In addition to common polymorphisms (Y129Y, H215R, I858V, N931N and IVS19 + 28T > C) and the c.1997G > A (S666N) variation, another novel nucleotide substitution was found in the *NPC1* gene of the proband: c.2882A > G (N961S) in exon 19. In order to minimize the artefacts due to previous formalin fixation (Williams et al 1999; Wong et al 1998) or translesion PCR synthesis from the low-quality DNA template (Quach et al 2004), we verified the c.2882A > G using an additional short PCR product. Direct sequencing of these 162 bp PCR products, obtained from DNA isolated from both tissues in a 35-cycle PCR reaction, confirmed the presence of c.2882A > G.

To exclude the possibility that c.1997G > A and c.2882A > G are common polymorphisms, we examined 318 control alleles from the Czech population by PCR/RFLP technique. Restriction analysis confirmed the substitution c.1997G > A in the proband and other three family members but in none of control alleles. Variation c.2882A > G was found only in the proband's samples.

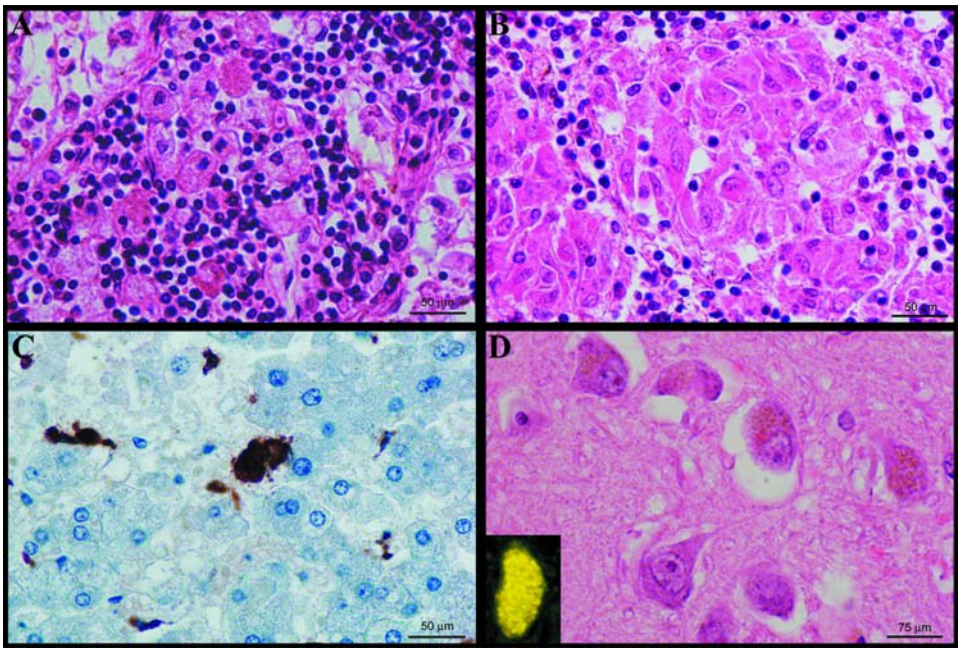

Fig. 1 Features of the storage process in the patient. (A) Lymph node infiltrated with ceroid-rich foam histiocytes (HE stain). (B) Mediastinal lymph node. Nonstorage histiocytes with compact (epitheloid) cytoplasm superficially resembling Gaucher cells (HE stain). (C) Liver sinusoidal histiocytes. Significant storage is present only in one of them (CD68 – clone PGM1). (D) Neurons in basal ganglia distended with an excess of lipopigment (HE stain). *Insert:* typical autofluorescence of the accumulated lipopigment

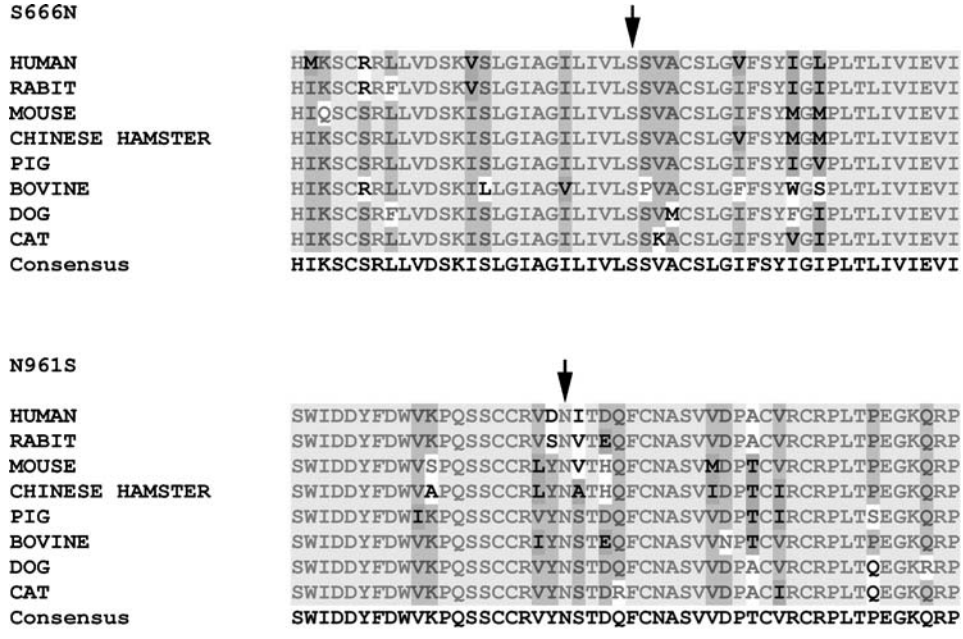

Fig. 2 Alignment of the amino acid sequence for the human NPC1 regions containing the mutation loci (S666N and N961S) with the corresponding regions from NPC1-related sequences of seven mammalian species. Arrow heads indicate the position of the found sequence variations.

Discussion

We feel fully justified in concluding, on the basis of the results of the analyses, that the patient presented here suffered from the visceral variant of Niemann–Pick disease type C1, although the filipin test could not be performed. The storage pattern with foamy ceroid-rich histiocytes was similar to that in Niemann–Pick disease type B (Elleder 1989). We consider the novel missense nucleotide changes found in the *NPC1* gene of the patient to be disease-causing for the following reasons:

1. The variations found do not occur commonly in the population, as we did not find them in any of 318 examined control alleles. In addition, SNP searches in the GenBank, Ensembl and HGVD databases did not reveal the substitutions.
2. The substitutions found are located in two out of the three functionally critical NPC1 domains (Vanier and Millat 2003). The S666N variant is situated in the highly hydrophobic and evolutionarily conserved region homologous to the sterol sensing domain (SSD) of the two key regulators of cholesterol homeostasis, HMG CoA reductase

(3-hydroxy-3-methylglutaryl coenzyme A reductase, EC 1.1.1.88) and SCAP protein (sterol regulatory element binding protein cleavage-activating protein). The serine residue at position 666 of NPC1 is conserved in the SSD of all three proteins. All mutations found so far in the SSD in the homozygous state, resulted in the lack of mature NPC1 protein and were associated with very severe biochemical and clinical phenotype (Millat et al 2001; Yamamoto et al 2000).The second variation, N961S, is situated in the cysteine-rich luminal loop between transmembrane domains VIII and IX. All but two mutations associated with the biochemical variant phenotype (mild alterations of cellular cholesterol trafficking) were located in the cysteine-rich loop (Vanier and Millat 2003).

3. The functional importance of both amino acid residues (S666 and N961) is supported by their conservation in seven other mammalian NPC1 homologous proteins.

As the NPC1 phenotype, despite its variability, is characterized by neurological involvement either closely associated with visceral symptoms or delayed (see Introduction), our case clearly belongs to the extremely rare visceral variant described so far only in two cases. The first described patient was a 63-year-old woman with a nonneuronopathic form homozygous for G992R localized in cysteine-rich domain (Millat et al 2001). The second patient came to attention at the age of 46 years because of splenomegaly found during splenectomy after a road traffic injury. The diagnosis of NPC1 was confirmed by biochemical analysis (Fensom et al 1999) and later by mutation analysis, when two heterozygous mutations were found (I1061T and V378A) (Millat et al 2001). Both patients were free of neurological symptoms at 66 and 50 years of age, respectively, suggesting that they may never develop neurological disease.

Dysfunction of the mutant NPC1 in the case described in this report became manifest within the lifespan of 53 years in the fraction of the histiocytic population with borderline involvement of hepatocytes and neurons. The patient had no neurological symptoms compatible with NPC during her life; moreover, the histological examination of her brain showed only increased amount of neurolipofuscin and none of the plethora of neuropathological signs that are abundant in NPC patients with neurological involvement (Vanier and Millat 2004). That allows us to assume that the patient had a visceral-only form of NPC1. The cause of microcytic anaemia in the patient remains unclear; no source of chronic bleeding was discovered at postmortem examination. It was apparently unrelated to the symptoms of NPC.

Although NPC1 patients as a group apparently represent a continuum of phenotypes with a wide range of severities, we feel that it is justified to distinguish the visceral-only adult phenotype as a separate form of the disease. This variant appears to be extremely rare, but its main clinical symptom, splenomegaly without neurological symptoms in adulthood, is nonspecific enough to raise suspicion for NPC only late if at all. At the tissue level, the presence of foam cells in an adult should alert the pathologist to this diagnosis. However, uneven expression of the storage in the histiocytic population may seriously complicate the diagnosis *in vivo*. We presume that the visceral variant of NPC may pass routine diagnosis undetected and therefore remain underdiagnosed. Whether these patients have subtle psychological or neurological symptoms remains to be established by repeated neuropsychological and neurophysiological evaluations. As patients with adult forms of NPC may benefit from future therapies—as suggested by preliminary results of a trial evaluating the effect of miglustat (Zavesca) in NPC patients (Patterson et al 2005)—adult physicians should be alert to the possibility of NPC in the absence of apparent neurological symptomatology.

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