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SHORT REPORT

Six novel *NPC1* mutations in Chinese patients with Niemann–Pick disease type C

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In patients with Niemann–Pick disease type C (NPC), an autosomal recessive lipid storage disorder, neurodegeneration can occur in early life. Vertical ophthalmoplegia and extrapyramidal signs may be seen. Cholestatic jaundice and hepatosplenomegaly occur frequently in patients with early onset disease, with bone marrow biopsies showing diffuse infiltration of foamy histiocytes. Cholesterol esterification in skin fibroblasts is reduced, resulting in intracellular accumulation of cholesterol. *NPC1* mutations are responsible for the disease in ~95% of patients. *NPC1* encodes a 1278 amino acid protein which contains 13 transmembrane domains. Over 130 mutations have been identified in *NPC1*, with over a third present within an *NPC1* specific cysteine-rich domain positioned in a large extracellular loop. It has been proposed that the defect in cholesterol homeostasis is the cause of neuronal apoptosis, but the precise role of the NPC1 protein and the functional implications of its mutations remain unknown. Although NPC is routinely diagnosed by biochemical analysis, identification of molecular defects helps confirm the diagnosis and enables family studies, and rapid, accurate prenatal diagnosis. This report describes the analysis of the *NPC1* gene in five Taiwanese/Chinese patients with NPC. Six novel *NPC1* mutations (N968S, G1015V, G1034R, V1212L, S738Stop, and I635fs) were identified of which three are missense mutations located in the cysteine-rich domain. These are the first *NPC1* mutations reported from Chinese patients with NPC.

Niemann–Pick disease type C (NPC) is a rare autosomal recessive neurodegenerative disorder characterised by intracellular accumulation of unesterified cholesterol and gangliosides within the endosomal–lysosomal system.¹ The incidence is estimated to be about 1 in 100 000 live births.^{2–3} NPC is clinically heterogeneous and there is a broad spectrum of phenotypes with respect to the age of onset, organomegaly, and neurological disease. In most cases, neurological symptoms appear between the ages of 4 and 10 years,⁴ although the timing of onset can range from perinatal to adulthood. The neurological abnormalities develop insidiously and are manifested as ataxia, mental deterioration, psychomotor retardation, and seizures. Pyramidal tract signs, cerebellar ataxia, and extrapyramidal features such as dystonia are common. Supranuclear ophthalmoplegia with paralysis of down gaze is characteristic in patients with NPC. Cataplexy and various types of seizure may also occur during the evolution of the disease.⁵

Cultured fibroblasts from patients with NPC show a significant reduction in the rate of cholesterol esterification.⁶ Filipin staining of these cells reveals the abnormal accumulation of intracellular cholesterol, as evidenced by intense

perinuclear punctate staining. In addition, cerebral neurones show the presence of neurofibrillary tangles, which are immunologically and ultrastructurally similar to those found in the brains of patients affected by Alzheimer's disease.⁷ Some patients have cholestatic jaundice, and the infiltration of foamy histiocytes (Niemann–Pick cells) can be identified in the bone marrow.

NPC is genetically heterogeneous with two complementation groups.⁸ Mutations of the *NPC1* gene have been identified in ~95% of the patients with NPC.^{9–10} The *NPC1* gene, located on chromosome 18q11–12, spans more than 47 kb and contains 25 exons.¹¹ It encodes a 1278 amino acid protein with 13 transmembrane domains, three large luminal hydrophilic loops, six cytoplasmic loops, and a cytoplasmic tail with a dileucine motif.^{12–13} The third luminal loop contains an *NPC1* specific cysteine-rich domain (residues 855–1098). The NPC1 protein also contains a sterol sensing domain (residues 616–797), which is homologous to those found in 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-R) and sterol regulatory element binding protein cleavage activating protein.¹⁴ Disease causing mutations are spread throughout the *NPC1* gene, although there is a concentration of mutations within the cysteine-rich domain, with an apparent hot spot for mutations between residues 927 and 958.¹⁵ In addition, the region between amino acids 1038 and 1253, which shares 35% identity with a portion of the Patched 1 (PTC1) protein, also contains a significant proportion of mutations.¹⁶ Most molecular defects of *NPC1* involve only a few nucleotides such as small deletions, small insertions, or missense mutations (see the human mutation database¹⁷). The functional implications of the mutations to *NPC1* are not yet clear; however, sterol homeostasis dysfunction, especially the blockage of cholesterol transport between the lysosome and sterol regulatory machinery leading to the excessive accumulation of cholesterol, has been proposed to be the major cause of neuronal apoptosis in NPC.¹⁸

Based on an analysis of 134 cases, Vanier *et al* showed that diagnosis of NPC is best reached by the combined demonstration of a deficient induction of esterification and intravesicular cholesterol storage by filipin staining.¹⁹ However, identification of the molecular defect is important for confirming the diagnosis of NPC as well as enabling rapid and accurate prenatal diagnosis. Here, we describe the analysis of the *NPC1* gene in five Taiwanese/Chinese patients with NPC, resulting in the detection of six novel *NPC1* mutations.

PATIENTS AND METHODS

Patients

Five index patients and their family were studied. All families were of Taiwanese/Chinese origin. Ethical approval for

Abbreviations: DHPLC, denaturing high performance liquid chromatography; NPC, Niemann–Pick disease type C

Table 1 Clinical manifestations of the patients with *NPC1* mutations

Family	Mutation (type)	Age (years) /sex	Age at onset	Neurological manifestations	Neuroimage (MRI) findings	Filipin stain	CE (pmol/h/mg protein)	Histiocyte infiltration in bone marrow
1	G1015V V1212L (Het)	12/F *	8 years	Psychomotor retardation, downward gaze palsy, dysarthria, progressive intellectual decline, dysphagia, pyramidal tract signs	High SI bilaterally in centrum semiovale (T2-weighted image)	Positive	0.83	Yes
		7/M	4 years	Downward gaze limitation, psychomotor retardation in writing and finger tapping, frequent choking	ND	Positive	0.73	ND
2	G1034R (Het)	9/F *	3 months	Developmental delay, failure to thrive, ataxia, psychomotor retardation, right preaxial polydactyly, grand mal seizure, myoclonus, quadriplegia	Dysgenesis of corpus callosum, diffuse cerebral atrophy	Positive	0.1	Yes
3	S738Stop (Hom)	5/M *	3 years	Developmental delay, psychomotor retardation, slow in speech	Unremarkable	Positive	0.2	Yes
4	V1212L (Het)	13/M *	5 years	Vertical gaze limitation, ataxia, mental deterioration	Cerebral and cerebellar atrophy, dysgenesis of corpus callosum	Positive	2.1	Yes
		Dec'd/M	5 years	Ataxia, vertical gaze limitation, psychomotor retardation	ND	ND	ND	ND
5	N968S I635fs (Het)	20/M *	11 years	Psychomotor retardation, ataxia, spasticity, dysphagia, dysarthria, cognitive decline, urine and stool incontinence, bilateral optic atrophy, partial seizure, myoclonus, dystonia, akathisia	Cerebral atrophy, high SI in the thoracic cord (T2-weighted image)	Positive	1.4	Yes
		Dec'd/M	13 years	Psychomotor retardation, dysarthria, dysphagia, cognitive decline, spasticity	ND	ND	ND	ND

*Index patient.

CE, cholesterol esterification specific activity; dec'd, deceased; F, female; Het, compound heterozygous mutation; Hom, homozygous mutation; M, male; ND, not done; SI, signal intensity.

genetic studies in Niemann–Pick disease type C (NPC) was obtained from the National Taiwan University Hospital Ethics Committee.

All index cases were diagnosed as having NPC based on clinical features, pathology in bone marrow, and biochemical analysis of cultured fibroblasts using filipin staining and cholesterol esterification. None of the families had a history of consanguinity. In all cases the disease started early in life, usually in the first decade (table 1). Hepatosplenomegaly was noted in all patients. The neurological features were highly variable among the families. However, patients within the same family had a similar neurological presentation (see table 1, patients 1, 4, and 5). The neurological manifestations included cerebellar ataxia, cerebral atrophy with psychomotor retardation, speech and mental function deterioration, and pyramidal as well as extrapyramidal signs (see table 1). Neuroimaging studies demonstrated dysgenesis of the corpus callosum with cerebellar or cerebral atrophy in patients 2 and 4. Non-specific high signals in T2-weighted images were seen in the white matter of brain and spinal cord of patients 1 and 5, respectively (fig 1C). Bone marrow biopsies of all index patients showed histiocyte infiltration (fig 1A). Filipin staining of skin fibroblasts and cholesterol esterification assays were performed in the National Referral Laboratory, Adelaide, Australia (Dr M J Fietz). All patients had accumulation of intracellular cholesterol similar to that shown in fig 1B. The rate of cholesterol esterification was also significantly reduced in these patients (table 1, normal range >3 pmol/hour/mg protein).

METHODS

DNA was extracted from leucocytes using a standard method. All the coding exons (26 amplicons) and the flanking regions of the *NPC1* gene were amplified by polymerase chain reaction (PCR). The primer sequences are available on request. Heteroduplexes were screened by denaturing high performance liquid chromatography (DHPLC) using a Transgenomic Wave Nucleic Acid Fragment Analysis System (Transgenomic Inc, San Jose, CA). The protocol for this has been described elsewhere.²⁰ Equal amounts of PCR product from the patient and a normal control were mixed and then denatured and renatured by cooling from 95 °C to 25 °C over 70 minutes. Exons showing the presence of a heteroduplex were sequenced using the BigDye Terminator sequencing chemistry (ABI) and the ABI3100 automatic DNA sequencer (Applied Biosystems, Foster City, CA).

Both filipin staining and cholesterol esterification assays were performed using modified versions of the methods of Vanier *et al.*¹⁵

RESULTS

All 26 exons of the *NPC1* gene were amplified from each of the five index patients and subjected to DHPLC. This revealed the presence of heteroduplexes in all five patients. Sequencing demonstrated a total of six different mutations, 2213C>A (S738Stop), 2903A>G (N968S), 3044G>T (G1015V), 3100G>A (G1034R), 3761G>T (V1212L), and 1905del T/insATG (I635fs) (see table 1). The missense mutations were located in exons 19 (N968S), 21 (G1015V,

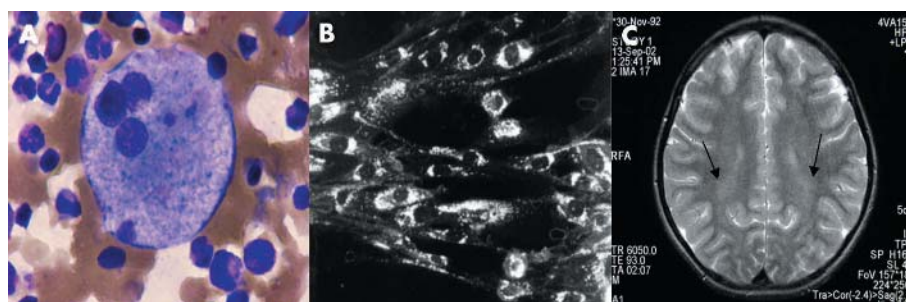


Figure 1 Pathological and neuroimaging manifestations in patients with NPC. (A) Light micrograph of a bone marrow aspirate showing the presence of a foamy histiocyte. (B) Presence of perinuclear non-esterified cholesterol deposits in cultured fibroblasts visualised by fluorescence microscopy after filipin staining. (C) Axial T2-weighted magnetic resonance image showing non-specific hyperintense areas in the white matter.

G1034R), and 24 (V1212L). The nonsense mutation (S738Stop) resulted in a premature stop codon at position 738. Likewise, the frameshift mutation (I635fs) encoded a truncated protein with a premature stop codon at position 638. One of the missense mutations (V1212L) occurred in two index patients (patients 1 and 4). Patient 3 was homozygous for S738Stop while the other four mutations each accounted for a single allele in the cohort. Only one mutant allele each was identified in patients 2 and 4, G1034R and V1212L, respectively. The six mutations were absent in 100 Taiwanese controls. In addition to the mutations, six polymorphisms (H215R, I642M, I858V, N931N, R1266Q, and R1314R) were also identified.

DISCUSSION

As is seen in many lipid storage disorders, the clinical features of NPC are highly variable. There is considerable variation in the age at which symptoms first appear as well as in the progression and neurological manifestations of the disease. Generally, the later the neurological symptoms appear, the slower the progression of the disease. In our patients, the younger the age of onset, the more severe was the phenotype.

To date, more than 130 disease causing mutations have been reported for NPC and most of these are missense mutations (71%).¹⁵ Only a small number of frequently occurring mutations have been described, namely, I1061T and P1007A (in individuals of western European descent), R518Q (Japanese), and P474L (Italian). None of these was detected in the present study. More than a third of the *NPC1* mutations are concentrated within the cysteine-rich luminal loop of NPC1. The cysteine-rich loop contains a ring-finger motif and is a likely site for functionally significant protein-protein interactions.^{22–23} Three of the missense mutations detected in this study are located within the cysteine-rich loop (N968S, G1015V, and G1034R). However, none occurs in the hot spot region (residues 927–958). The fourth missense mutation, V1212L, occurs in transmembrane domain XII (TM XII), which is located in the mutation-rich region homologous to PTC1. Mutations I635fs and S738Stop introduce premature stop codons at amino acids 638 (TM IV) and 738 (TM VI), respectively. None of the six novel mutations was detected in 100 Taiwanese controls indicating that it is likely that these six mutant alleles are pathogenic.

For two of the five patients examined in this study only one mutation to the *NPC1* gene was detected. Given the clinical and biochemical presentations of these patients, it is highly unlikely that both are true carriers of NPC. Therefore, it remains possible that a second point mutation in these patients was beyond the resolution of DHPLC and sequencing analysis. Alternatively, these mutations could have resulted from a large deletion or other genomic changes affecting either the amplification of the relevant exons or the overall structure of the gene. Amplification of the *NPC1* cDNA or genomic Southern analysis could be used to examine these options further.

NPC is a complex neurological disorder with a broad range of clinical presentations, routinely diagnosed using biochemical analysis. Mutation screening provides an alternative means of confirming the diagnosis of NPC. In addition, the detection of both causative mutations allows carrier screening of family members as well as enabling rapid and accurate prenatal testing by mutation analysis of DNA from fetal cells.

In summary, we have described the mutation screening of five Taiwanese/Chinese patients with NPC, resulting in the detection of six novel *NPC1* mutations. This is the first report of *NPC1* mutations in patients from this ethnic background.

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NEUROLOGICAL PICTURE

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Capsular lingual paresis

A 68 year old hypertensive woman was referred to the neurology department having experienced a sudden weakness in mastication and mild dysarthria. When the patient was asked to protrude her tongue, a left lingual paresis became evident (fig 1); however, the tongue was normal while at rest in the mouth. Neurological examination was otherwise normal.

A brain computed tomogram gave evidence of a small haematoma limited to the posterior limb of the right internal capsule (fig 2). No other abnormal radiological findings were shown to be present on successive brain magnetic resonance imaging.

Facial and lingual hemiparesis with mild limb involvement have been previously described in capsular genu syndrome, suggesting that motor corticopontine and corticobulbar fibres are situated more in the genu of the internal capsule. Each hypoglossal nucleus receives impulses from both sides of the cortex but the genioglossus muscle probably has crossed unilateral innervation, thus an isolated right sided lesion of the upper motor neurone, in the internal capsule, is capable of causing lingual paresis that is evident only when the tongue is protruded. Differential diagnosis of sudden lingual hemiparesis, if unilateral, include lesions of the 12th nerve; atrophy and fasciculations of the tongue will also be present subsequently. This nerve can be damaged in the medulla (by conditions such as ischaemia or neoplasm) or after it leaves the brainstem and the skull through the hypoglossal foramen. Acute involvement of the hypoglossal nerve may arise with carotid dissection in the neck.



Figure 1 Left lingual paresis evident on tongue protrusion.

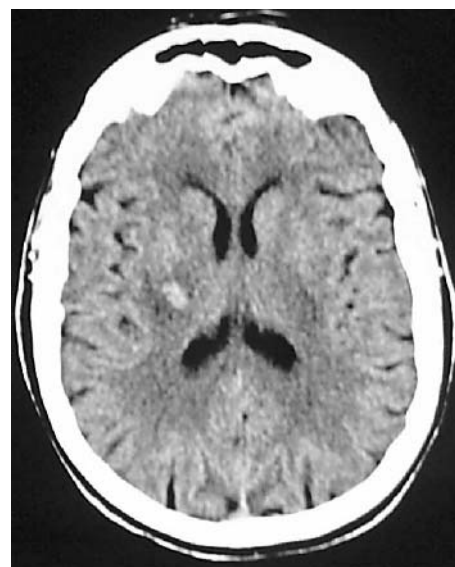


Figure 2 Haematoma visible in right internal capsule, CT scan.

Sudden bilateral sopranuclear lingual paresis has been described with a bilateral cerebral infarction involving the anterior opercular region, and manifests itself in facial and pharyngeal diplegia.

The condition of this patient was unique because the lingual paresis manifested itself without having any association with other neurological findings and the lesion had occurred in the posterior limb of the internal capsule.

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Consent has been obtained for fig 1

Reference

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