

CLN5 mutations are frequent in juvenile and late-onset non-Finnish patients with NCL

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ABSTRACT

Objectives: To explore a potential expansion of the phenotypic and genotypic characteristics of Finnish variant late-infantile neuronal ceroid lipofuscinosis (NCL), we screened a collection of 47 patients with clinically diagnosed NCL in whom no molecular diagnosis had been made.

Methods: We used PCR amplification of genomic DNA, followed by fluorescent-labeled dideoxy-nucleotide chain termination sequencing and multiplex ligation-dependent probe amplification, to screen our cohort of patients for mutations in *CLN5*. We collected ethnic background, clinical, and pathologic information, as available, to clarify the breadth of *CLN5* disease expression and to explore possible genotype-phenotype correlations.

Results: We identified 10 patients with pathogenic *CLN5* mutations, including 11 mutations not previously described: 4 missense, 5 out-of-frame insertion/deletion mutations, and 2 large intra-genic deletions. We also documented 3 previously reported *CLN5* mutations. The age at disease onset in this cohort is predominantly juvenile rather than late infantile. Importantly, we have identified 2 adult-onset patients who share a common pathogenic allele. The majority of patients presented with motor and visual impairments and not seizures. In those patients with available longitudinal data, most had progressed to global neurodevelopmental and visual failure with seizures within 1 to 4 years.

Conclusions: Our study suggests that *CLN5* mutations 1) are more common in patients with neuronal ceroid lipofuscinosis (NCL) than previously reported, 2) are found in non-Finnish NCL patients of broad ethnic diversity, and 3) can be identified in NCL patients with disease onset in adult and juvenile epochs. *CLN5* genetic testing is warranted in a wider population with clinical and pathologic features suggestive of an NCL disorder. ***Neurology*® 2010;74:565-571**

GLOSSARY

EM = electron microscopy; **MLPA** = multiplex ligation-dependent probe amplification; **mRNA** = messenger RNA; **NCL** = neuronal ceroid lipofuscinosis; **PTC** = premature termination codon.

The neuronal ceroid lipofuscinoses (NCLs) are a group of genetically inherited neurodegenerative diseases exhibiting both locus and phenotypic heterogeneity. The incidence of NCL varies widely but has been estimated to be between 1:56,000 to 1:67,000 in the United States.¹ The common characteristics of this group of diseases include progressive cognitive and motor deterioration, seizures, visual loss, dementia, and early death. The disease phenotype usually presents during infancy or early childhood and rarely in adolescence and adulthood.^{2,3}

Three classic childhood forms of NCL exist (infantile NCL, *CLN1*; late-infantile NCL, *CLN2*; and juvenile NCL, *CLN3*), with broad ethnic diversity.⁴⁻⁶ Four variant late-infantile forms have been genetically mapped (*CLN5*, *CLN6*, *CLN7*, and *CLN8*) and were originally reported to be restricted to specific ethnic groups.⁷⁻¹² Mutations in *CLN5* were originally reported in the Finnish and Northern European populations, where a major mutation (*CLN5* Fin major) was present in 17 families, a minor mutation (*CLN5* Fin minor) was present in 1 family, and a mutation of non-

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Finnish origin (*CLN5* European) was present in 1 Dutch family.⁷ Consistent with this ethnic distribution, Holmberg et al.¹³ reported 8 patients of Finnish, Dutch, or Swedish ethnicity with identifiable mutations in the *CLN5* gene. *CLN5* mutations were also later found in a Colombian family,¹⁴ a single Portuguese patient,¹⁵ and an Italian family.¹⁶ More recently, molecular studies in both a Pakistani and an Afghan family have been described.¹⁷ These case reports argue against *CLN5* being exclusively a Finnish or Northern European variant.

CLN5 is a 46-kDa soluble lysosomal protein that is glycosylated and transported to lysosomes in a mannose 6-phosphate–dependent manner, although its function remains unclear.^{2,18,19} Reported interactions among heterogeneous NCL proteins,²⁰ some of which are transmembrane as well as soluble, suggest a possible common functional pathway. Recent gene expression profiling studies support this hypothesis.²¹

To further characterize the clinical and molecular features of variant late-infantile NCL due to *CLN5*, we screened a collection of patients with clinically diagnosed NCL but in whom no molecular genetic diagnosis had been made. We report 11 new, as well as 3 previously described, *CLN5* mutations in patients with adult and juvenile age at disease onset and representing a wide variety of ethnic backgrounds. This report significantly expands our understanding of the ethnic diversity, the genotypic-phenotypic variability, and the mutation spectrum in *CLN5*.

METHODS Patients. We examined 47 patients collected through our clinical molecular diagnostic laboratory who presented with an NCL phenotype and in whom we were able to exclude mutations in the *CLN1*, *CLN2*, *CLN3*, *CLN6*, and *CLN8* genes by full sequencing of the coding and surrounding noncoding regions. All 47 patients were from the United States except for 1 from Sweden (patient 6). Patient samples were collected under institutional Human Studies Committee approval, and signed written consent forms were obtained from all patients or guardians.

Preparation of genomic DNA. Genomic DNA was extracted from patients' peripheral leukocytes, cultured fibroblasts, or frozen brain tissue, using the PureGene DNA Purification System (Gentra, Minneapolis, MN). Standard protocols provided by the manufacturer were followed. Genomic DNA was stored in DNA hydration buffer at 4°C.

PCR and DNA sequencing. Patient DNA was PCR amplified using 4 primer pairs as described (table e-1 on the *Neurology*[®]

Web site at www.neurology.org). PCR products were purified using the QIAquick Multiwell PCR Purification System (Qiagen, Valencia, CA), and the purified products were sequenced bidirectionally on an ABI 3130x1 capillary gel electrophoresis system (Applied Biosystems Inc., Foster City, CA). Positive mutations were identified by comparison of bidirectional sequence data against normal control sequence and were further confirmed by independent reamplification and bidirectional sequencing from the patients' original DNA.

Multiplex ligation-dependent probe amplification. Synthetic multiplex ligation-dependent probe amplification (MLPA) probes were designed using general MLPA guidelines and probe design criteria outlined by Schouten et al.²² and the manufacturer (MRC-Holland, Amsterdam, The Netherlands). Variable probe lengths were achieved by altering the target hybridization probe sequence instead of by using stuffer sequences. Two probe pairs were designed to target each of the 4 *CLN5* coding exons so that gene dosage changes could be measured for a total of 8 probes. Three control probe pairs were designed to the *SOD1* (21q22.1), *PARKIN* (6q25.2–q27), and *GCHI* (14q22.1–q22.2) genes, all of which have gene map loci distinct from *CLN5* (13q21.1–q32). The probe sequences are presented in table e-2. Amplified samples were run on an Applied Biosystems 3730XL DNA Analyzer under standard fragment analysis conditions, and the raw output data were analyzed using GeneMarker software (SoftGenetics, State College, PA). Three independent analyses were performed, and the average gene dosage ratio for each sample was compared with that of control DNA.

***CLN5* polymorphism evaluation.** Each *CLN5* exon was PCR amplified and sequenced bidirectionally for more than 80 non-NCL DNA samples. The exonic sequencing data from more than 160 chromosomes allowed calculation of polymorphism frequency (table e-3).

RESULTS Clinical analysis. NCL had been diagnosed clinically in the 47 patients studied based on phenotypic features including seizures, visual loss, motor impairment, and cognitive regression, as well as ultrastructural studies of biopsy samples when available. Table 1 summarizes the clinical information gathered from the 10 patients in whom we identified pathogenic *CLN5* mutations. The age at clinical symptom onset was surprisingly variable and ranged from 4 to 17 years. Seventy percent (7 of 10) had a juvenile mean age at onset of 5.6 (range 4–8) years. Remarkably, 2 patients had an adult onset with symptom appearance not until age 17 years (patients 3 and 5; table 1). No late-infantile onset was noted in this *CLN5* cohort. Clinical information was not available for the now 12-year-old patient 4 (table 1).

The majority of the patients (5 of 9) first presented with motor impairment or regression, as has often been described in this disorder. Seizures were noted as presenting symptom in only 2 patients. Visual loss was the first recognized symptom in 2 of the patients. All 9 of the patients for whom clinical information was obtained developed all of the classic hallmark features of NCL (seizures, visual loss, motor

Table 1 Patient information^a

Patient	Clinical onset, y	Ethnicity	Presenting symptom	Seizure onset, y	Visual loss onset, y	Motor difficulty onset, y	Cognitive regression onset, y	Biopsy/autopsy (EM findings)
1	5	French Canadian, Irish, UK, Dutch	Motor difficulty and visual loss	7	5	5	7	NA
2	5-6	Non-Finnish, mixed Caucasian	Seizures	5	6	6	6	FP, CL
3	17	Non-Finnish, mixed Caucasian	Cognitive regression and visual loss	23	20	23	17	GR, FP, CL (neurons, glial); CL, RL (muscle); CL, FP (skin); CL (spleen)
4	NA	Hispanic	NA	NA	NA	NA	NA	NA
5	17	Non-Finnish, mixed Caucasian	Motor difficulty	18	18	17	25	GR (blood); FP (brain)
6	8	Swedish	Motor difficulty	8	12	8	11	FP, CL (skin)
7	4	Chinese	Visual loss	6	4	5	5	NA
8	5-6	Asian Indian	Motor difficulty	NA	Abnormal ERG	5.5	6	Normal (conjunctiva)
9	6	Egyptian	Motor difficulty	7	7	6	7	Vacuoles (muscle)
10	5	Pakistani	Motor difficulty	5	24	5	5	FP, CL

Abbreviations: CL = curvilinear inclusions; EM = electron microscopy; ERG = electroretinogram; FP = fingerprint inclusions; GR = granular inclusions; NA = not available; RL = rectilinear inclusions.

^aSummary of clinical data for patients in whom we identified pathogenic DNA changes in *CLN5*.

difficulty, and cognitive regression) within 1 to 8 years from their initial clinical presentation.

In the NCL disorders, ultrastructural studies may reveal the accumulation of membrane-bound, intralysosomal material with inclusion characteristics often suggestive of a specific NCL diagnosis. Biopsies are, however, often negative. The relationships among genetic defect, inclusion characteristics, and cell dysfunction are not well understood. The electron microscopy (EM) data available in the patients reported showed significant variation in inclusion type and included fingerprint, curvilinear, and granular patterns.

A diverse ethnic background was noted in these 10 patients (table 1), including 3 of non-Finnish Caucasian and others of French Canadian, Hispanic, Swedish, Chinese, Asian Indian, Egyptian, and Pakistani ancestry.

***CLN5* mutation spectrum.** *CLN5* molecular genetic analysis in our patient cohort identified 11 new mutations, including 4 missense mutations, 4 small deletions, 1 small insertion, and 2 large deletions, as well as 3 previously reported mutations (table 2 and figure 1) (<http://www.ucl.ac.uk/ncl/CLN5.shtml>).

Of the 20 disease alleles identified in this study, more than half (12 of 20) resulted in a premature termination codon (PTC) through small deletions (patients 1, 4, 7, and 9), small insertions (patients 6 and 10), or nonsense changes (patients 1, 2, and 6). All 4 small deletions occurred in exon 4 and predict a frame shift with resultant PTC anywhere from 4 to 29 amino acids downstream (table 2). The 2 small

exon 3 insertions resulted in a PTC 11 and 30 amino acids downstream.

Fewer missense changes were identified in these patients. Patient 3 had 2 missense mutations (exon 2, c.377G>A, p.Cys126Tyr and exon 4, c.1121A>G, p.Tyr374Cys). The c.377G nucleotide is highly conserved throughout evolution, as is the corresponding amino acid cysteine (up to *Treaodon* genus and considering 9 species). The p.Cys126Tyr missense mutation also results in a large physicochemical difference (Grantham distribution = 194 on a scale of 0–215). Similarly, the c.1121A nucleotide and the corresponding tyrosine at amino acid 374 are also highly conserved throughout evolution. Comparison of 9 species indicates that this tyrosine is conserved up to *Xenopus*. The p.Tyr374Cys change also results in a large physicochemical difference (Grantham distribution = 194). Targeted mass spectrometry (patient 3, frozen brain tissue) documented the absence of the *CLN5* protein and confirmed mutation pathogenicity.²³ Interestingly, both adult-onset patients (3 and 5) share the Tyr374Cys pathogenic allele (tables 1 and 2).

The exon 3 missense mutation found in patient 7 (c.620G>C, p.Trp207Ser) affects the same codon of an unpublished c.619T>C, p.Trp207Arg mutation previously reported in the NCL mutation database for patients with Finnish variant late-infantile NCL (<http://www.ucl.ac.uk/ncl/cln5.shtml>). The affected nucleotide and corresponding tryptophan are highly conserved throughout evolution. The tryptophan-to-serine change produces an amino acid substitution

Table 2 *CLN5* mutations^a

Patient	Mutation 1	Mutation 2	Other sequence alterations
1	E3, c.671G>A	E4, c.1103_1106delAACA	None
	p.Trp224X	p.Lys368SerfsX15	
2	E3, c.671G>A	E4 deletion by MLPA	None
	p.Trp224X	c.907_1094del	
3	E2, c.377G>A	E4, c.1121A>G	None
	p.Cys126Tyr	p.Tyr374Cys	
4	E4, c.1083delT	No deletion by MLPA	E1, c.72A>G, p.Gly24Gly
	p.Phe361LeufsX4 homozygous		Int1, c.320+8C>T; E4, c.1103A>G, p.Lys368Arg; 3'UTR c.1224+33A>G; (all homozygous)
5	E4, c.1121A>G	E4 deletion by MLPA	None
	p.Tyr374Cys	c.907_1094del	
6	E1, c.225G>A	E3, c.669insC	E3, c.528T>G, Thr176Thr
	p.Trp75X	p.Trp224LeufsX30	
7	E3, c.620G>C	E4, c.1071_1072delCT	None
	p.Trp207Ser	p.Leu358AlafsX4	
8	E3, c.575A>G	No deletion by MLPA	None
	p.Asn192Ser Homozygous		
9	E4, c.919delA	NA	None
	p.Arg307GlufsX29		
10	E3, c.527insA	NA	None
	p.Thr176AsnfsX11 Homozygous		

Abbreviations: MLPA = multiplex ligation-dependent probe amplification; NA = not available.

^aSummary of mutations from 10 patients identified to carry pathogenic *CLN5* alleles.

with a large physicochemical difference (Grantham distribution = 177). These characteristics support a pathogenic role for this mutation.

The fourth missense change identified was in patient 8 (exon 3, c.575A>G, p.Asn192Ser). The c.575A nucleotide and the corresponding asparagine are both highly conserved. However, there is only a small physicochemical difference between asparagine and serine (both polar with neutral side chains; estimated Grantham distribution = 46). Further evaluation of this change using RESCUE-ESE software²⁴ suggests that the high conservation of this nucleotide might function as an exonic splicing enhancer and thereby dictate exon 3 inclusion during pre-messenger RNA (mRNA) splicing. Together, these data support the pathogenicity of this change.

Initial results from our sequencing study identified 2 presumed pathogenic mutations in only 4 of the 10 patients (patients 1, 3, 6 and 7; table 2). Only a single pathogenic mutation was identified by sequence analysis in each of the remaining 6 patients. Patients 9 and 10 had a positive family history of consanguinity, and we hypothesized that the

c.919delA and the c.527insA mutations represented homozygous mutations in these patients. The parents of these patients were not available for carrier status confirmation. In addition to the apparently homozygous 1-bp deletion (c.1083delT), patient 4 had a number of additional homozygous nucleotide polymorphisms and noncoding changes (tables 2 and e-3). Parents of patient 4 were unavailable, and we could not confirm allelic homozygosity.

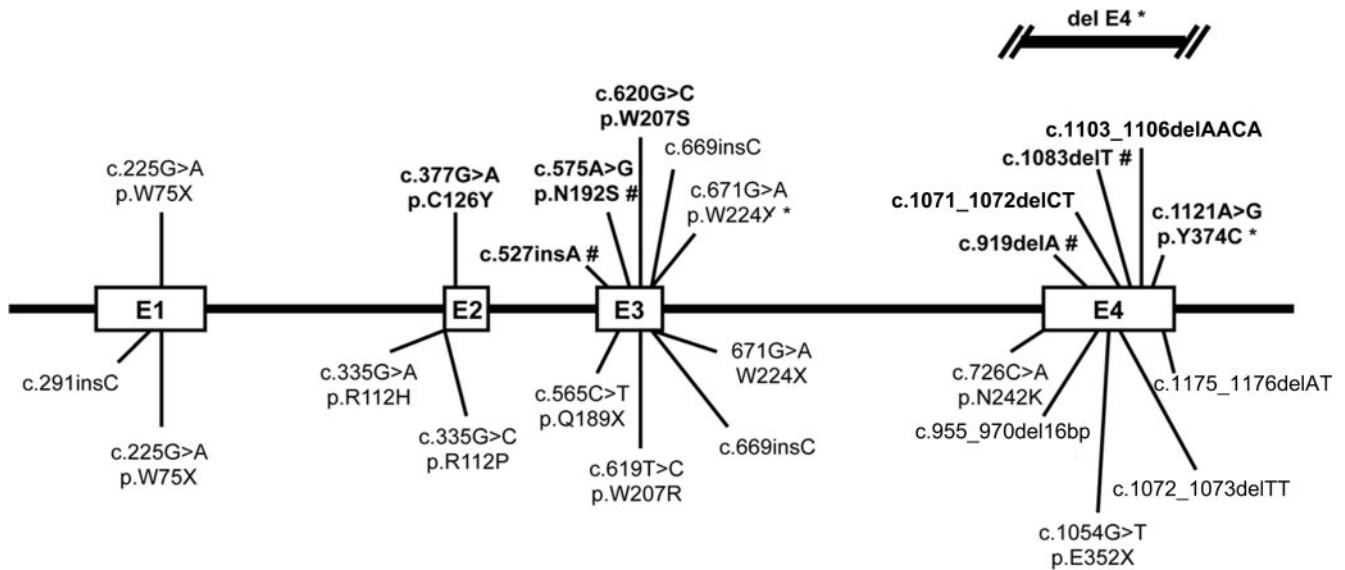
To extend our molecular analysis in those patients with unidentified pathogenic mutations (patients 2, 4, 5, and 8) and the one with presumed homozygosity (patient 9), we developed an MLPA assay to assess gene dosage (figure 2). Patients 2 and 5 both showed reproducible exon 4 deletions. Although the exact deletion boundaries in these patients have not yet been determined, probe design data suggest that the deletion extends at least between the 2 independent MLPA E4 probes (c.907–1094). It is not known whether the exon 4 deletions in both patients are identical, but we suspect the deletion to be much larger in patient 5 given failure to identify a normal allele at position c.1121A>G by sequencing. These deletions presumably alter the pre-mRNA splicing and mRNA product and are presumed pathogenic. This is the first report of large exonic deletions in the *CLN5* gene.

We failed to detect a deletion in either patient 4 or patient 8. It is possible that these patients are indeed homozygous, as we suspect, for their mutations. Patient 4 showed lack of heterozygosity for all *CLN5* polymorphisms and nucleotide changes identified, and the parents of patient 8 are from the same local area in India. No second mutation was identified by MLPA in patient 9, supporting presumed homozygosity given known parental consanguinity.

***CLN5* polymorphisms.** In addition to the 2 reported common polymorphisms in exons 1 and 4 (c.4C>T, p.Arg2Cys and c.1103A>G, p.Lys368Arg), we have identified 3 silent synonymous changes and calculated general population frequencies ranging from 0.5% to 2.6%. None of the 9 new point mutations found in our patients were observed in the polymorphism study control samples.

DISCUSSION Historically, *CLN5* disease has been thought to be a rare variant late-infantile NCL with predominant incidence in Finnish and Northern European populations. A previous *CLN5* phenotype-genotype correlation study of 8 European patients revealed a relatively homogeneous phenotype and age at disease onset.¹³ Despite the recent reports of *CLN5* disease in single families outside of Northern Europe,^{14–17} *CLN5* disease is still generally considered a Finnish or European late-infantile onset vari-

Figure 1 Mutation spectrum



CLN5 mutations identified in our study are shown above the exon cartoon and previously reported *CLN5* mutations are below. Novel mutations are in bold. * Change observed in at least 2 of our patients. # Change found as a homozygous mutation.

ant. Our clinical and molecular report of *CLN5* gene mutations in 10 NCL patients with age at onset from juvenile to adult and all of non-Finnish ethnic background significantly broadens our understanding of the age at onset and ethnic diversity of a disease caused by mutations in *CLN5*.

This study is the first report of *CLN5* in the American population. We have documented it as pan-ethnic, present in individuals with non-Finnish Caucasian, Chinese, Asian Indian, Hispanic, Pakistani, and Egyptian ancestry. The age at disease onset for the majority of these patients was in childhood (average 5.6 years), not late infantile as originally reported. Most patients' initial symptoms were of motor impairment, as is commonly appreciated, but a number had early significant visual failure. Only 2 patients presented with seizures, although the majority went on, within 1 to 8 years, to have motor and cognitive impairment, visual loss, and seizures. There are reports that juvenile NCL patients exhibit marked psychiatric and behavioral problems, and these changes are reliably present before the more classic phenotypic hallmarks, such as seizure onset and motor and visual impairment, are observed.^{25,26} Our clinical history methodology failed to gather psychiatric and behavioral information. We are unable, unfortunately, to determine the extent of behavioral and psychiatric changes in this cohort. We appreciate our oversight, and emphasize that such history should be a consideration and gathered in the clinical setting.

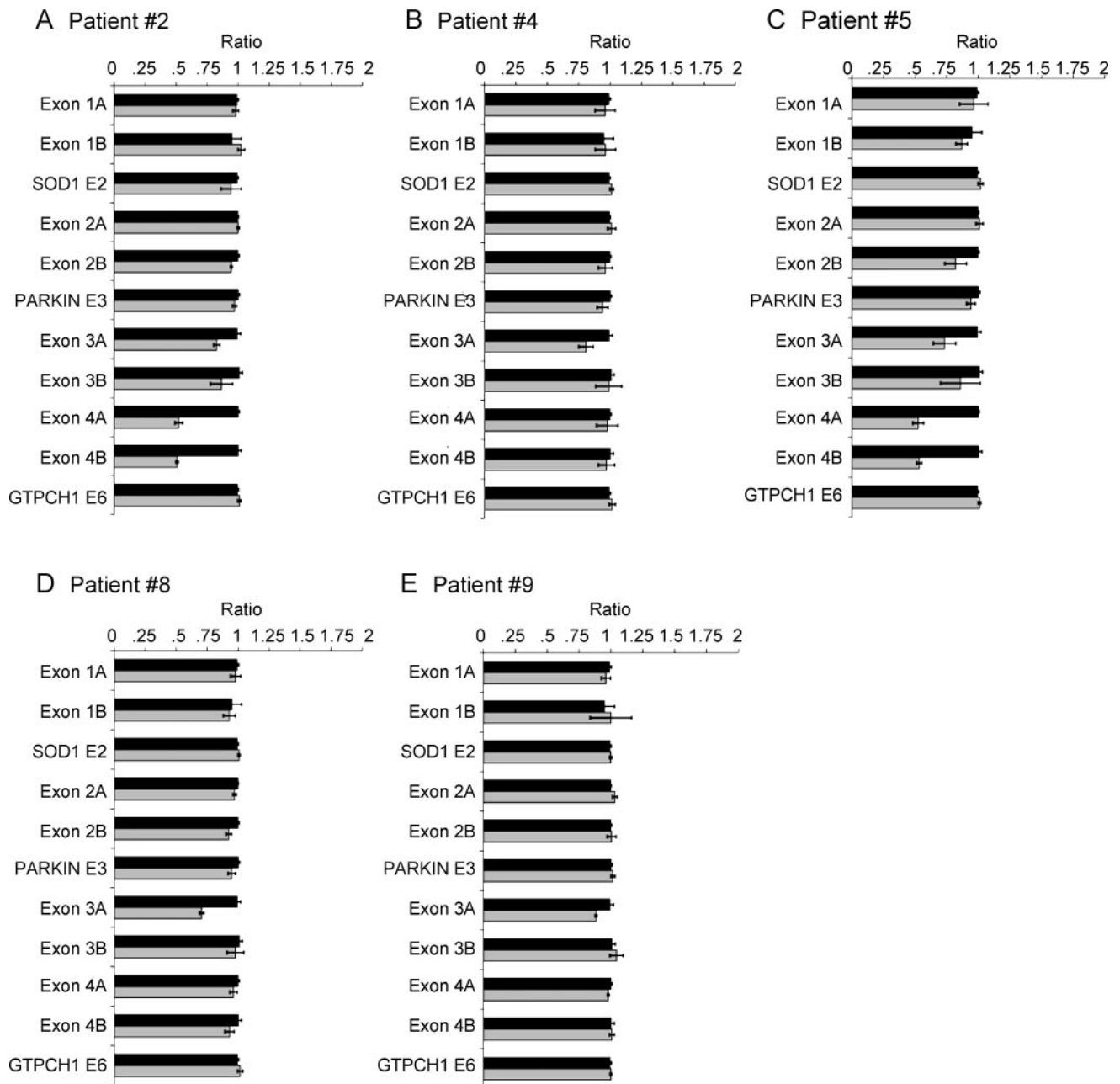
Pathogenic *CLN5* mutations underlying adult onset NCL have not been previously reported. Both

of our late-onset patients (3 and 5), as well as a sibling of patient 5 with symptom onset at age 20 years, share a common pathogenic allele (c.1121A>G, p.Tyr374Cys). We presume that this missense mutation may allow for some maintained protein expression and function and thereby underlie the later-onset phenotype. However, we cannot reconcile this with documented absence of the *CLN5* protein at autopsy in 1 case²³ (patient 3). It is possible that the p.Tyr374Cys change results in altered secondary and tertiary protein structure and that despite some initial residual function there may be eventual protein destabilization and destruction. The second mutation in patient 3 (p.Cys126Tyr) also alters a cysteine and presumably disulfide bond formation essential for proper protein structure and function. These findings extend the molecular basis of adult-onset NCL. Further study of protein expression may yield better understanding of the molecular pathobiology and later disease onset.

The available EM results, from 7 of 10 patients, indicate 3 types of inclusions: fingerprint, curvilinear, and granular. They are variably present and of mixed type in many of the patients. It is important to note that specific inclusion characteristics are not necessary, nor should specific type limit diagnostic consideration of *CLN5* disease. As in many NCL cases, a number of our patients did not have EM-identified inclusions. Negative biopsy data should neither exclude NCL as potential diagnosis nor limit further molecular diagnostic testing.

The 11 mutations identified in the 10 patients of our study represent a wide range of molecular lesions.

Figure 2 Multiplex ligation-dependent probe amplification analysis



Cases with a single *CLN5* allele mutation identified were evaluated for gene dosage defect (deletion/duplication) using multiplex ligation-dependent probe amplification (A-E). Raw data at each interrogated locus are represented as a peak height ratio of patient DNA (gray bars) compared with control DNA (black bars). The mean ratio of 3 independent experiments is represented, and standard deviations between experiments are depicted as vertical bars.

The exon 4 intragenic deletions, detected by MLPA, are the first reported for this gene. The majority of mutations in our series represent small deletions/insertions resulting in a shift in the reading frame and consequential PTC. PTCs are generally pathogenic changes and presumably generate loss of function in 1 of 2 ways: 1) production of a truncated C-terminus that is unstable or missing a functionally important domain integral to protein function and posttranslational processing or 2) production of mRNA that is sensitive to the nonsense-mediated decay pathway with mRNA

degradation and failed protein production. Further investigation is warranted to determine the mRNA and protein effects of PTCs in the terminal exon of the *CLN5* gene. The results of our polymorphism study expand the existing *CLN5* polymorphism database (<http://www.ucl.ac.uk/ncl/cln5.html>) and should be helpful in future clinical patient test interpretation.

Our high mutation detection rate (21%), identification of 11 new mutations, clinical analysis of variable age at onset and phenotypic features, and broad

spectrum of pathologic EM inclusions described in this study are significant contributions to the description of CLN5 disease. Our study suggests that CLN5 is more common than originally expected and can be identified in late-onset cases and in patients from diverse ethnic backgrounds. *CLN5* genetic testing is warranted in a wider population whose clinical features are suggestive of NCL, including those with motor and cognitive regression and visual failure with or without seizures, and independent of age at onset and ethnic background.

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