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CASE REPORT

R208X Mutation in CLN2 Gene Associated with Reduced Cerebrospinal Fluid Pterins in a Girl with Classic Late Infantile Neuronal Ceroid Lipofuscinosis

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Clinical picture of neuronal ceroid lipofuscinosis with late infantile onset (LINCL) is characterized by myoclonic seizures and psychomotor regression. We present a case of classic LINCL and reduced cerebrospinal fluid (CSF) pterins in a girl of normal psychomotor development and born to non-consanguineous parents. She first presented with febrile seizures at the age of four. At that time, brain computed tomography finding was normal, but electroencephalogram showed hypsarrhythmia. At the age of five, tremor, generalized ataxia, and motor and mental regression appeared. Brain magnetic resonance imaging showed cerebellar atrophy. Electron microscopy examination showed storage of intracytoplasmic curvilinear inclusions in neurons, fibroblasts, and secretory cells of the skin and rectal mucosa. Tripeptidyl peptidase I (TPP-I) activity in leukocytes was very low (5.4 nmol/h/mg protein; range in homozygote cases of LINCL, 0.4–26.0). Molecular genetic studies showed a homozygous mutation, R208X, in exon 6 of CLN2 gene. CSF analysis revealed very low neopterin (7.3 nmol/L; normal range, 9-30) and biopterin (4.1 nmol/L; normal range, 10–30), reduced homovanillic acid (266 nmol/L; normal range, 211-871), and low homovanillic acid/5-hydroxyindoleacetic acid ratio (1.21; normal ratio, 1.5-3.5). Treatment with L-Dopa/Carbidopa (4 mg/kg) and antiepileptics was introduced, but without significant effect. It seems that low CSF pterins and impaired dopamine turnover are secondary manifestations of classical LINCL caused by homozygous inheritance of the R208X mutation in CLN2 gene.

Key words: *aminopeptidases; biopterin; cerebrospinal fluid; dopamine; enzyme activation; epilepsies, myoclonic; genes; neuronal ceroid-lipofuscinosis*

Neuronal ceroid lipofuscinoses are autosomal recessive progressive lysosomal disorders. At least eight genes (CLN1–8) are thought to be responsible for this group of disorders. The incidence is estimated at 0.1-7 per 100,000 live births (1,2). Neuronal ceroid lipofuscinoses are classified by the age of patient at the onset of symptoms, clinical signs, and pathologic findings. There are four classic forms and four variants of neuronal ceroid lipofuscinoses of late infantile onset. The four classic forms comprise the following diseases (3-9): 1) infantile or Haltia-Santavouri disease, with the onset between 6 months and 2 years, characterized by the ultrastructural appearance of granular osmiophilic deposits (GROD); 2) neuronal ceroid lipofuscinosis of late infantile onset, or Jansky–Bielshowski disease, with the onset between 2 and 4 years, characterized by the ultrastructural appearance of curvilinear bodies; 3) juvenile neuronal ceroid lipofuscinosis, or Spielmeyer-Vogt, with the onset between 4 and 10 years and fingerprint inclusions; and 4) the adult form, or Kufs disease, with the onset between 15 and 50 years. The four variants of neuronal ceroid lipofuscinoses of late infantile onset are the Finnish variant, caused by mutations in CLN5; Portuguese variant, caused by mutations in CLN6; Turkish variant, with gene designation CLN7; and progressive epilepsy with mental retardation, caused by mutations in CLN8 (2,3,10). CLN1 and CLN2 genes encode lysosomal hydrolytic enzymes (5,6,9), whereas CLN3, CLN5, and CLN8 are believed to code for transmembrane proteins (2,3).

Neuronal ceroid lipofuscinosis of late infantile onset is characterized by great clinical and neuropathological variability and molecular heterogeneity. Typically, initial symptoms of the classic form of neuronal ceroid lipofuscinosis of late infantile onset appear at the age of 2 to 4 years, and curvilinear inclusions can be seen in biopsy material by electron microscopy. Clinical symptoms include cerebellar ataxia, extrapyramidal symptoms, myoclonic seizures, retinal degeneration, and rapidly progressive psychomotor regression resulting in early death at the end of the first decade (3,4). The accumulation of a low-molecular-weight protein, with the appearance of lysosomal curvilinear inclusions, has been identified as the subunit c of the mitochondrial adenosine tripho-

sphate synthase complex, which is responsible for shuttling protons across the mitochondrial wall. CLN2, the gene underlying this classic neuronal ceroid lipofuscinosis of late infantile onset, is localized on chromosome 11p15. The cloning of the gene revealed that it consists of 13 exons and introns, spanning 6.65 kb of genomic DNA. Molecular genetic studies have disclosed 32 mutations in CLN2 gene: 5 small deletions, one 1-bp insertion, 14 missense, 4 nonsense, 8 splicing errors, and 14 polymorphisms (2,9). The most common mutations in CLN2 are IVS5-1G>C, accounting for 33% of mutations, and R208X, estimated to account for 26% of mutations in neuronal ceroid lipofuscinoses of late infantile onset (10). The CLN2 gene product is tripeptidyl peptidase I (TPP-I), whose activity levels in the classic type of the disease are below 5% of normal (4). TPP-I is a lysosomal enzyme, which acts as an aminopeptidase removing proteolytic tripeptides from the free α-amino-N termini of the proteins (6). No clear genotype/phenotype correlation has been established regarding CLN2 mutations.

Biopterins are cofactors catalyzing mixed oxidation reactions in different metabolic pathways essential for neurotransmitters dopamine and serotonin (11,12). Tetrahydrobiopterin is also an important cofactor for inducible nitric oxide synthase (13). Monoamine metabolite levels in the cerebrospinal fluid reflect the neurotransmitter turnover in the central nervous system. Low concentrations of dopamine and serotonin, the respective products of the tyrosine and tryptophan metabolic pathways, are clinically associated with symptoms of Parkinsonism, truncal hypotonia, limb hypertonus, extensor plantar responses, swallowing difficulties, convulsions, and intermittent hyperthermia, which are similar to clinical features of neuronal ceroid lipofuscinoses (12,14). Recently, Aberg et al (7) reported a favorable response to antiparkinsonian treatment in juvenile neuronal ceroid lipofuscinosis based on decreased dopamine transporter density. A reduced concentration of biopterin in cerebrospinal fluid associated with R208X mutation in CLN2 gene has not been previously reported.

We present a case of a girl with classic neuronal ceroid lipofuscinosis of late infantile onset and biopterin deficiency in the central nervous system, resulting in altered dopamine metabolism.

Methods

Preparation of Cell Homogenates

Leukocytes were isolated as described by Whiteman and Young (8). Buffy coat was isolated from heparinized blood (10 mL) by centrifugation at 1,000 G for 10 min, and washed twice with 1 mL of cold solution of 0.155 mol/L NaCl. After resuspension of the leukocyte pellet in 0.75 mL cold water for 90 s, isotonic conditions were restored by addition of 0.25 mL of 0.62 mol/L NaCl. The leukocyte pellet was finally sonicated in water and the whole sonicate was used in the assay. Protein was assayed on the whole sonicate. Sonication amplitude of 8 μmol/L of pellet was performed in water for 10 s in a Soniprep 150 W sonicator (MSE Scientific Instruments, Crawley Sussex, UK). The sonicate was inspected for uniform consistency. The volume of sonicate of white cells obtained from 10 mL sample of blood was approximately 100 μL.

Enzyme Assay

TPP-I was assayed in leukocytes as described by Young et al (15). The protein was assayed on the whole sonicate. The sample was further diluted to a concentration of 1 mg protein/mL in a solution of 0.1% triton X-100 in 50 mmol/L sodium acetate buffer pH 4.0 (Triton/acetate buffer). TPP-I activity was measured in duplicate in a mixture containing 22.2 mmol/L ethylenediaminetetraacetic acid (EDTA)-Na2 in Triton/acetate buffer (90 μL), 10 μL homogenate or sonicate (10 μg protein), and 500 μmol/L Ala-Ala-Phe-7-amido-4-methylcoumarin (A3401; Sigma, St. Louis, MO, USA) in 0.1 mol/L sodium acetate buffer pH 4.0 (100 μL). After an hour of incubation at 37 °C, the reaction was stopped by addition of 1.0 mL of 100 mmol/L sodium chloroacetate in 30 mmol/L sodium acetate buffer pH 4.3, and the fluorescence was measured (excitation 365 nmol/L, emission 450 nm).

Normal TPP-I activity reference range is 42-339 nmol/h/mg protein, whereas in neuronal ceroid lipofuscinoses of late infantile onset, TPP-I ranges between 0.4 and 26 nmol/h/mg protein (5,6).

Mutation Detection

Primers (Sigma-Genosys, Cambridgeshire, UK) to amplify exon 6 and surrounding intronic sequences were constructed according to Sleat et al (9) and the genomic sequence of CLN2 (16). The IVS5-1G>C mutation was excluded by restriction enzyme digestion (SfcI or BsrI; New England Biolabs, Hitchin, UK). A further artificially created restriction site (ACRS) primer was designed specifically for the R208X mutation (5'GTAACCCCCTCT GTGATCCGTAGG3'). The new, artificially created restriction site CLN2 exon 6 forward primer used primes adjacent to the R208X mutation site and had a single base-pair mismatch to the normal sequence, an A to G, one nucleotide from the 3' end. This mismatch creates an HphI restriction endonuclease site in the presence of the R208X mutation. The artificially created restriction site CLN2 exon 6 produced by polymerase chain reaction (PCR) is 155 base pairs long when uncut. The HphI-digested PCR-fragment sizes were as follows: R208X mutation allele – 155bp cut to 67bp plus 53bp plus 35bp; wild type allele – 155bp cut to 102bp plus 53bp; and wild type/R208X heterozygote – 155bp cut to 102bp plus 63bp plus 53bp plus 35bp, as both alleles were present (Fig. 1).

Capillary sequence electrophoresis was carried out on a 310 Genetic Analyser (Applied Biosystems, Warrington, UK) and the sequences were analyzed with Sequencing Analysis Software™ (Applied Biosystems) to detect mutations. The PCR diges-

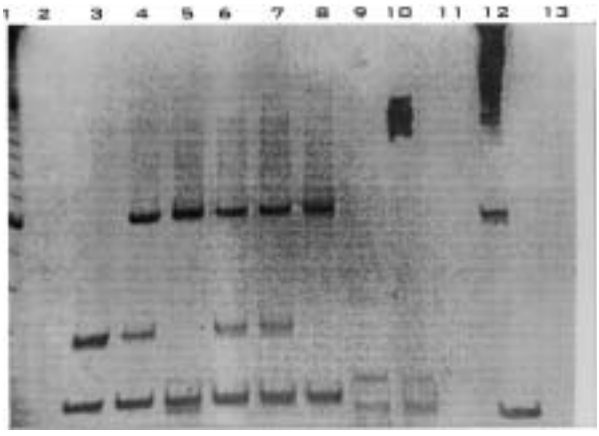

Figure 1. R208X mutation detection by 10% ATTO acrylamide gel electrophoresis (ethidium bromide stained UV image) of the patient and parents with mutation controls. Lanes: 1 – 10 bp ladder; 2 – blank; 3 – R208X homozygous patient (proband); 4 – R208X heterozygous patient (mother); 5 – P202L homozygous control; 6 – R208X heterozygote control; 7 – R208X heterozygous patient (father); 8 – normal control; 9 – water control; 10 – undigested polymerase chain reaction control; 11 – blank; 12 – 50 bp ladder; 13 – blank.

Table 1. Biogenic amines/folates (metabolites, nmol/L) and pterins in the cerebrospinal fluid of a 5-year-old patient*

	5HIAA	HVA	HVA/5HIAA	DOPAC	MHPG	3OMD	50HTrp	L-Dopa	5MTHF	Neo	Bio	% Bio
Patient	189	266	1.21	12.6	30.0	43.5	5.4	< 5	54	7.3	4.1	36
Reference range	105-299	211-871	1.5-3.5	8-18	39-73	< 50	< 10	< 25	41-117	9-20	10-30	

*Abbreviations: 5HIAA – 5-hydroxyindoleacetic acid; HVA – homovanillic acid; DOPAC – 3,4-dihydroxyphenylacetic acid; MHPG – 3-methoxy-4-hydroxyphenylglycol; 3OMD – 3-O-methyl-Dopa; 5OHTrp – 5-hydroxytryptophan; 5MTFH – 5-methyltetrahydrofolic acid; Neo – neopterin; Bio – biopterin; % Bio = 100B/(N + B).

tion products were analyzed by electrophoresis on a 10% acrylamide gel in an ATTO gel tank (ATTO Corp., Tokyo, Japan) and stained with ethidium bromide.

Cerebrospinal Fluid Biogenic Amine and Pterins

Homovanillic acid is the end-metabolite of dopamine. The ratio between homovanillic and 5-hydroxyindoleacetic acid is an indicator for the shortage (depending on its value) of one of the two pathways, dopamine or serotonin. Homovanillic and 5-hydroxyindoleacetic acids in cerebrospinal fluid were measured by using high-performance liquid chromatography with electrochemical detection, and pterins were measured with fluorescence detection (15).

Separation was achieved on an YMC Pack-Pro C18 (4.6 x 250 mm) column (YMC, Inc., Wilmington, NC, USA) with a 50 mmol/L sodium phosphate buffer, pH 2.0, containing 5 mmol/L octansulphonic acid, 0.05 mmol/L EDTA, and 25% (v/v) ethanol as the mobile phase. The flow rate was 1.1 mL/min and the analytical cell (Model 5011, ESA, Bedford, MA, USA) was adjusted to +0.45 V (ESA Coulochem Model 5100A, ESA) with a response time of 2 s.

Guanosine Triphosphate Cyclohydrolase Assay

Guanosine triphosphate cyclohydrolase activity was measured in cytokine-stimulated fibroblasts as described elsewhere (11). The assay monitors the conversion of the substrate guanosine triphosphate under saturating conditions to neopterin triphosphate, which is detected as neopterin, the oxidized and dephosphorylated product. Fibroblasts were analyzed immediately after 24-h incubation with cytokines. The 150 μL of supernatant from the lysed cells were desalted on a Sephadex G50 column and 50 μL of filtrate were added to 148 μL of reaction buffer (50 mmol/L Tris-HCl, pH 7.5 containing 0.1 mol/L KCl, and 1 mmol/L EDTA) and 2 μL of 100 mmol/L guanosine triphosphate. Half of the mixture (100 μL) was incubated for 60 minutes at 37 °C, whereas the remaining 100 μL were immediately oxidized (blank with cell extract). The reaction was stopped by cooling the sample on ice and adding 10 μL of oxidizing solution (0.5% w/v iodine/1% w/v potassium iodide in 1 mol/L HCl). After oxidation in the dark for 60 minutes, the reaction was stopped by adding 10 μL of 2% ascorbic acid (w/v, freshly prepared). The mixture was adjusted to pH 8.5 by adding 14 μL of 1 mol/L NaOH and the sample was incubated with 20 μL of alkaline phosphatase solution (300 U/mL of calf intestine alkaline phosphatase in 0.1 mol/L Tris-HCl, pH 8.0 containing 1 mmol/L $MgCl_2$, and 0.1 mmol/L $ZnCl_2$) for 60 minutes at 37 °C. After deproteinization through an Ultrafree-MC filter, neopterin was measured by high pressure liquid chromatography. One unit of guanosine triphosphate cyclohydrolase produces 1 μmol neopterin per minute at 37 °C.

Case Report

A girl of normal psychomotor development, born to non-consanguineous parents, presented with febrile seizures at the age of 4 years. Brain computed tomography (CT) findings at that time were normal, but electroencephalographic (EEG) examination showed focal abnormality. At the age of 4.5 years, she developed recurrent generalized tonic-clonic and myoclonic seizures. EEG showed hypsarrhythmia. At the age of 5, tremor, generalized ataxia, motor and mental regression developed. The examination of the optic fundi showed bilateral atrophy of the optic nerves. Brain magnetic resonance imaging (MRI) showed cerebellar atrophy. Electron microscopy examination of biopsy samples revealed storage of intracytoplasmic curvilinear inclusions within neurons, fibroblasts, and secretory cells of the skin and rectal mucosa.

TPP-I activity in her leukocytes was very low (5.4 nmol/h/mg protein) and consistent with the diagnosis of classical neuronal ceroid lipofuscinoses of late infantile onset according to the TPP-I reference range in homozygote cases of neuronal ceroid lipofuscinoses of late infantile onset (5,6). Her parents' leukocyte TPP-I activities were normal. Molecular genetic studies showed a homozygous mutation, R208X, in exon 6 of CLN2 (Fig. 1). Her parents and sister were heterozygous for R208X, confirming their carrier status. The nucleotide change was a C to T at nucleotide 622 in the cDNA sequence (nucleotide 3670, genomic DNA), which changes an arginine to a STOP codon, causing the premature termination of the CLN2 protein.

Electromyoneurography showed a myopathic pattern with signs of mild neurogenic lesion, lower compound muscle action potentials, and decreased motor nerve conduction velocity in the lower extremities. Muscle biopsy showed myopathic pattern. Cerebrospinal fluid analysis revealed reduced homovanillic acid (266 nmol/L; normal 211-871), low homovanillic acid/5-hydroxyindoleacetic acid ratio, and very low neopterin and biopterin levels (Table 1). Screening for mutations in the GCH1 gene was negative in the whole family. Guanosine triphosphate cyclohydrolase in the patient's fibroblasts showed normal activity, as well as pterin production (data not shown). Organic acids in urine, amino acid high-performance liquid chromatography blood ammonia, and acylcarnitine were normal. She was treated with valproic acid and clobazam. Improved seizure control was obtained when lamotrigine was introduced. Treatment with L-Dopa/Carbidopa (4 mg/kg) and tetrahydrobiopterin was commenced, in addition to the antiepileptics (valproic acid, clobazam, and lamotrigine), but without any significant improvement of her symptoms. Trihexyphenidyl improved sialorrhea in the patient. Worsening of dystonia occurred when L-Dopa/Carbidopa was reduced and stopped. Now, at the age of 8, she manifests severe psychomotor retardation, and occasionally partial motor and brief myoclonic seizures.

Discussion

We described an association of homozygous R208X mutation in CLN2 gene with reduced cerebrospinal fluid neopterin and biopterin levels. The R208X mutation is a C to T change at genomic position g3670C>T, cDNA position c622 C>T within the arginine amino acid 208. The C to T mutation is a nonsense mutation causing a STOP codon, which

prematurely terminates the CLN2 protein. The same CLN2 gene may be responsible not only for neuronal ceroid lipofuscinoses of late infantile onset, but also for a juvenile onset form of the disease (17). Clinical diagnosis is first confirmed by TPP-I activity analysis. Patients with neuronal ceroid lipofuscinoses of late infantile onset show either absent or severely reduced TPP-I activity in lymphocytes, fibroblasts, brain chorioid villi, and amniotic fluid (18). Initial symptoms occurring in our patient after the age of 4, which is later than usual, may be the result of higher TPP-I activity efficiency, which prevented an earlier build-up of storage material. Probably other unknown factors also influenced the development of clinical symptoms. Different ages of onset can be observed in affected siblings of the same family (10). Our patient suffered from progressive myoclonic epilepsy – a syndrome consisting of myoclonus, generalized and focal seizures, progressive neurological dysfunction, and psychomotor regression, including cerebellar ataxia. Beside neuronal ceroid lipofuscinoses of late infantile onset, which caused these clinical manifestations in our patient, the differential diagnosis includes Lafora's disease, Unverricht-Lundborg, and mitochondrial encephalomyopathy of the myoclonus epilepsy and ragged fibres.

Electrophysiological abnormalities may be the most sensitive method for the detection of early central nervous system involvement in neuronal ceroid lipofuscinoses of late infantile onset (19). A focal EEG abnormality was first registered at the age of 4 years in our patient, and subsequently progressed to hypsarrhythmia. EEG characteristically shows focal discharges in the occipital region in response to photic stimulation (17).

Neuronal ceroid lipofuscinoses of late infantile onset should also be included in differential diagnosis of patients with leading symptoms of developmental delay, cognitive impairment, attention deficit, or autism, which are associated with other clinical features of neuronal ceroid lipofuscinoses besides epilepsy.

Various therapeutic approaches to neuronal ceroid lipofuscinoses have been introduced. Lamotrigine as monotherapy has been shown 100% effective in improving seizure control in patients with juvenile neuronal ceroid lipofuscinosis (20). Lamotrigine significantly decreased seizure frequency in our patient, but only in combination with valproic acid and clobazam. Association of the same homozygous R208X mutation and severe caudate atrophy has been recently reported in a boy with neuronal ceroid lipofuscinoses of late infantile onset and pronounced dystonia at the age of 3 years and 9 months (21). Brain MRI in our patient revealed cerebellar atrophy, which is the common finding associated with neuronal ceroid lipofuscinoses of late infantile onset.

The genetic defect in neuronal ceroid lipofuscinoses of late infantile onset could be responsible for the abnormalities in dopamine synthesis and extrapyramidal symptoms. Measurement of biogenic amine metabolites in cerebrospinal fluid is important in detection of inborn errors affecting catecholamine and serotonin biosynthesis and defects in tetrahydrobiopterin metabolism. Investigation should be carried out in children with motor retardation and extrapyramidal signs (14). Low homovanillic acid concentration in the cerebrospinal fluid is the result of decreased catecholamine synthesis. Illnesses with low pterin levels are very rare. The most well known condition is Dopa-responsive dystonia (DRD, or Segawa disease), which is due to autosomal dominant mutations in the guanosine triphosphate cyclohydrolase gene (GCH1) (11,12). So far, around 200 patients have been described in the literature. Autosomal recessive mutations in the GCH1 gene are very rare, with only 17 patients reported to date. In both variants of tetrahydrobiopterin deficiency, neopterin and biopterin are extremely low in almost all body fluids (12). Very low concentrations of neopterin and biopterin in cerebrospinal fluid of our patient initially suggested reduced guanosine triphosphate cyclohydrolase activity in the brain, thus reflecting reduced dopamine turnover. However, normal guanosine triphosphate cyclohydrolase activity in fibroblasts and normal production of neopterin and biopterin after stimulation with cytokines indicated that depletion of cerebrospinal fluid pterins may rather be of the secondary origin. Almost 40% of patients with guanosine triphosphate cyclohydrolase deficiency have no detectable gene mutations (12). Although mutations may occur within some regulatory regions of GCH1 gene, the fact that no GCH1 mutations could be detected in this patient support the hypothesis that reduced cerebrospinal fluid pterins are, probably, of the secondary, degenerative origin. Previous reports suggest that the monoamine neurone system may be involved in the pathophysiology of other diseases (22).

The R208X mutation, and possibly other mutations, in CLN2 gene may result in low cerebrospinal fluid pterin production and impaired dopamine metabolism in classical neuronal ceroid lipofuscinoses of late infantile onset. Studies of neurotransmitter abnormalities in different genetic disorders provide a step forward in better understanding of cell physiology in health and disease.

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