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Allelic and non-allelic heterogeneities in pyridoxine dependent seizures revealed by *ALDH7A1* mutational analysis

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Abstract

Pyridoxine dependent seizure (PDS) is a disorder of neonates or infants with autosomal recessive inheritance characterized by seizures, which responds to pharmacological dose of pyridoxine. Recently, mutations have been identified in the ALDH7A1 gene in Caucasian families with PDS. To elucidate further the genetic background of PDS, we screened for *ALDH7A1* mutations in five PDS families (patients 1–5) that included four Orientals. Diagnosis as having PDS was confirmed by pyridoxine-withdrawal test. Exon sequencing analysis of patients 1–4 revealed eight *ALDH7A1* mutations in compound heterozygous forms: five missense mutations, one nonsense mutation, one point mutation at the splicing donor site in intron 1, and a 1937-bp genomic deletion. The deletion included the entire exon 17, which was flanked by two *Alu* elements in introns 16 and 17. None of the mutations was found in 100 control chromosomes. In patient 5, no mutation was found by the exon sequencing analysis. Furthermore, expression level or nucleotide sequences of *ALDH7A1* mRNA in lymphoblasts were normal. Plasma pipecolic acid concentration was not elevated in patient 5. These observations suggest that *ALDH7A1* mutation is unlikely to be responsible for patient 5. Abnormal metabolism of GABA/glutamate in brain has long been suggested as the underlying pathophysiology of PDS. CSF glutamate concentration was elevated during the off- pyridoxine period in patient 3, but not in patient 2 or 5. These results suggest allelic and non-allelic heterogeneities of PDS, and that the CSF glutamate elevation does not directly correlate with the presence of *ALDH7A1* mutations.

Keywords: Vitamin B_6; Convulsion; ALDH7A1 gene; Pipecolic acid; Genetic heterogeneity; Private mutations; A large deletion; *Alu* repeats; Lymphoblasts; RT-PCR analysis

Pyridoxine dependent seizure (PDS) is a disorder of neonates or infants with autosomal recessive inheritance (MIM 266100) characterized by intractable seizures, which is responsive to administration of pharmacological dose of pyridoxine, but refractory to ordinary anticonvulsants [1,2]. The diagnosis as having PDS can be confirmed by

Abbreviations: PDS, pyridoxine-dependent seizures; GAD, glutamate decarboxylase; GABA, γ-amino butyric acid; CSF, cerebrospinal fluid; P6C, L-Δ^1-piperidine-6-carboxylate; PLP, pyridoxal phosphate; EEG, electroencephalogram; RT-PCR, reverse transcription-mediated PCR; PA, pipecolic acid.

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the withdrawn test of pyridoxine. In the case of PDS, seizures resume within 7–10 days. Patients with PDS are dependent on pharmacological dose of pyridoxine for the rest of their lives to remain seizure-free. Development delay is not rare, even with early diagnosis and treatment [3]. Linkage analysis of PDS families with multiple affected members has mapped a PDS gene on chromosome 5q31 [4]. Biochemical analysis has revealed that pipecolic acid concentration was elevated in plasma and CSF in PDS patients [5]. L-Pipecolic acid (PA) is converted by PA oxidase into L-Δ^1-piperidine-6-carboxylate (P6C). Accumulated P6C can bind and inactivate pyridoxal phosphate (PLP) *in vivo* by Knoevenagel condensations, which is supposed to cause depletion of intracellular PLP. Based on these biochemical findings, Mills et al., hypothesized that PDS has a lesion in P6C dehydrogenase encoded by ALDH7A1 gene on chromosome 5q31 [6]. They have successfully identified causative *ALDH7A* mutations in PDS families. Plecko et al have subsequently reported the *ALDH7A1* mutational analysis in 18 patients, and found that a missense mutation, E399Q, was most prevalent in Caucasian patients [7].

The purpose of this study is to elucidate further the genetic background of PDS by mutational analysis of PDS families, especially in Oriental patients. We screened *ALDH7A1* mutations in five PDS families, which included one family with elevated CSF glutamate level [8] and two families with normal CSF glutamate level [9,10] during pyridoxine-withdrawal period. The mutational analysis of the ALDH7A1 gene revealed complex etiological nature of PDS.

Methods

Subjects

We studied five apparently non-related patients, patients 1–5 (Table 1). All of the patients were products of parents with non-consanguineous marriage. Patient 4 had an affected younger sister while in the other families there was no history of affected siblings. Diagnosis as having PDS was confirmed by pyridoxine withdrawal test in all the patients. Profiles of patients 1–5 were summarized in Table 1, including onset of convulsive seizures, findings of electroencephalogram (EEG), status of psychomotor developments. This study was approved by Ethical Committee of Tohoku University School of Medicine.

Mutational analysis of the ALDH7A1 gene

Peripheral leukocyte DNA was obtained from patients and their parents. All of the 18 protein-coding exons in ALDH7A1 gene were amplified by PCR as described [6]. PCR products were size-separated on 2.5% agarose gel electrophoresis. Then the band with expected size was excised, purified, and subjected to direct sequencing analysis as described [11].

Reverse transcription-mediated PCR (RT-PCR)

Lymphoblast cell lines were established from blood samples from two control subjects and patients 4 and 5 by infection of Epstain–Barr virus. Total RNA was isolated from 5×10^6 cells of lymphoblast cells using RNeasy kit (Qiagen, Germany). Complementary DNA was synthesized from 10 μg of total RNA in a 20 μl mixture according to the manufacturer's instruction (Superscript II, Invitrogen, Carlsbad, Calif.). Subsequently, an entire coding region of *ALDH7A1* cDNA was amplified by three sets of PCR using 1 μl of the first-strand synthesis mixtures. The most 5′ part of cDNA with 504 bp in size containing exons 1–6 was amplified with forward primer (AASA-cDNA-5 F), 5′-TGTAAAACGACGGCCAGTTTGGAGCAGGCCTGCCGCCTTC-3′ and reverse primer (AASA-cDNA-5R), 5′-CAGGAAACAGCTATGACCCCAACCAGGCCTACGGGATTCC-3′. The middle part of cDNA (343 bp) corresponding to exons 6–9 was amplified with forward primer (AASA-cDNA-6F), 5′-TGTAAAACGACGGCCAGTTGGCCATGCACTGATTGAGCAGT-3′ and reverse primer (AASA-cDNA-6R), 5′-CAGGAAACAGCTATGACCCTCCTGCACCATCAGGCCCACC-3′. The most 3' part fragment with 974 bp in size containing exons 8–18 was amplified with forward primer (AASA-cDNA-3F), 5′-TGTAAAACGACGGCCAGTGTTCCTTGACTTGTGGTGGAGC-3′ and reverse primer (AASA-cDNA-4R), 5′-CAGGAAACAGCTATGACCAATGCATTTATTCAGGGAAAACTT-3′. Singly and doubly underlined sequences were universal M13 and reverse sequences, respectively, for further sequencing analysis. Long range PCR was performed with LA-PCR kit (Takara Bio Inc., Tokyo, Japan). Primers for long-range PCR were forward primer, AASA-E16F, 5′-TGTAAAACGACGGCCAGTGGGGCTGAGATTGGAGGTGCC-3′ and reverse primer, AASA-cDNA-4R as mentioned above. The PCR products were size-separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

Multiplex RT-PCR analysis

A human β-actin cDNA with 1206 bp was co-amplified with the 3′ part of the *ALDH7A1* cDNA (974 bp) using Multiplex PCR kit (Qiagen, Germany). Nucleotide sequences of PCR primers for β-actin cDNA were reported previously [12]. The multiplex PCR products were subjected to 2.5% agarose gel electrophoresis. Intensity ratio of the two bands was measured by NIH image software for evaluation of *ALDH7A1* mRNA level.

Plasma PA concentration

Plasma samples were collected in patient 2, 4, and 5, and stored at −20 °C until analysis. Their PA concentrations were determined using gas chromatography mass spectrometry (GCMS-QP Model 2010, Shimadzu Biotech, Kyoto, Japan) as described [13,14].

Results

Sequencing analysis of ALDH7A1 genes

Sequencing analysis of entire coding regions of the ALDH7A1 gene in patients 1–4 revealed seven point mutations. In patient 1, two missense mutations were identified: G378R mutation in exon 14 and D449N mutation in exon 16 (Fig. 1a). In patient 2, P403L missense mutation was found in exon 14 in a heterozygous form. Additional single base substitution from A to T was observed at the splicing-donor site of intron 1, IVS1+3A>T (Fig. 1b). In patient 3, two missense mutations, G174V and V367G (Fig. 1c) were identified in exon 6 and exon 13, respectively, both of which have been reported [7]. In patient 4, a nonsense mutation (W31X) in exon 1 was found in the paternal allele, but no mutation was detected in the maternal allele by the sequencing analysis (Fig. 1d). Amino acid residues at these missense mutations, Gly^{174}, Val^{367}, Gly^{378}, Pro^{403}, and Asp^{449}, are highly conserved among vertebrae, higher plant, and bacteria, suggesting their evolutional importance. Any of the seven base substitutions described