

Phenotypic Variability and Newly Identified Mutations of the *IVD* Gene in Japanese Patients with Isovaleric Acidemia

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Isovaleric acidemia (IVA) is an autosomal recessive inborn error affecting leucine metabolism. It is caused by a deficiency in isovaleryl-CoA dehydrogenase (IVD), a mitochondrial matrix enzyme that catalyzes the oxidation of isovaleryl-CoA to 3-methylcrotonyl-CoA. IVD is a FAD-containing enzyme, consisting of four identical subunits. Clinical features of IVA include poor feeding, vomiting, lethargy, developmental delay, metabolic acidosis, and a characteristic "sweaty foot" odor. IVA is one of the target disorders for newborn screening by tandem mass spectrometry (MS/MS). The human *IVD* gene is located on chromosome 15q. To date, over 50 disease-causing mutations have been reported worldwide. In this study, we searched for *IVD* mutations in five Japanese patients with IVA (neonatal type, two patients; chronic intermittent type, two patients; and mild biochemical type, one patient). The diagnosis of IVA was confirmed by urinary organic acid analysis using gas chromatography and mass spectrometry. All coding exons and the flanking introns in the *IVD* gene were amplified by PCR and were directly sequenced. We thus identified six hitherto unknown mutations (p.G94D, p.E116K, p.M167T, p.L243P, p.L246P, and c.696+1G>T) and four previously reported (p.R53P, p.R395C, p.Y403C, and p.E411K) pathogenic mutations. All patients were compound heterozygotes, and each mutation was identified in a single patient. Pathogenicity of newly identified mutations was validated using computational programs. Among them, the p.M167T is believed to influence FAD binding, as the position 167 is present in one of the FAD-binding sites. Our results have illustrated the heterogeneous mutation spectrum and clinical presentation of IVA in the Japanese patients.

Keywords: isovaleric acidemia; isovaleryl-CoA dehydrogenase; *IVD*; newborn screening; tandem mass spectrometry
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Introduction

Isovaleric acidemia (IVA; MIM 243500) is caused by a deficiency in isovaleryl-CoA dehydrogenase (IVD; E.C.1.3.99.10), which catalyzes the third step in the catabolism of leucine (Vockley and Ensenauer 2006). IVD is a FAD-containing mitochondrial matrix enzyme, consisting of four identical subunits. The lengths of its precursor protein and mature sequence are 426 and 394 amino acid residues, respectively (Matsubara et al. 1990). IVA is clinically characterized by lethargy, vomiting, and an odor of "sweaty feet"; biochemically, IVA is characterized by accumulation of isovaleryl-CoA derivatives that are associated with hyperammonemia and ketoacidosis. IVA can be diagnosed

pre-symptomatically as a result of the implementation of newborn screening (NBS) by tandem mass spectrometry (MS/MS). Recent NBS reports have set the prevalence of IVA at 1:67,000 in Germany (Ensenauer et al. 2011), 1:130,000 in North Carolina, USA (Frazier et al. 2006), and 1:365,000 in Taiwan (Lin et al. 2007). In Japan, expanded NBS by MS/MS was introduced in 2011 as a public health program, following over a decade of pilot studies conducted in selective regions (Yamaguchi 2008).

IVD is encoded by a single gene (*IVD*) mapped to 15q14-15. *IVD* consists of 12 exons spanning 15 kb. To date, over 50 disease-causing mutations have been reported in the human *IVD* gene (Hertecant et al. 2012; Ozgul et al. 2014). However, a clear genotype-phenotype correlation

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has not yet been established. Some reports have indicated the occurrence of specific mutations among various ethnic groups, such as the p.A314V mutation (originally reported as A282V, based on the mature protein) in Caucasians (Ensenauer et al. 2004) and the p.G123R mutation in Caucasians colonized in South Africa (Dercksen et al. 2012). Among the East and Southeast Asian populations, the mutations p.Y403C (originally reported as Y371C) and c.466-3_2CA > GG (originally reported as c.457-3_2CA > GG, according to NM_002225.2 (previous version)) are frequently observed in Taiwan (Lin et al. 2007), and Korea (Lee et al. 2007) and Thailand (Vatanavicharn et al. 2011), respectively.

In this study, we analyzed the mutations in five Japanese patients with IVA to examine the spectrum of mutations.

Methods

Patients

Five Japanese patients with IVA were studied (Table 1). The diagnosis of IVA was confirmed by urinary organic acid analysis using gas chromatography and mass spectrometry. Patients 1, 2, 3, and 4 displayed symptoms such as “sweaty foot” odor during acute attacks.

Patient 1 was delivered uneventfully after a 39 week-gestation period, with a birth weight of 2,916 g. The baby showed symptoms of dyspnea and hypothermia two days after birth and was diagnosed with metabolic acidosis (pH 7.15, HCO_3^- 4.7 mEq/L) and hyperammonemia (279 $\mu\text{g/dL}$). Therefore, oral feeding was stopped and the baby was subjected to an intravenous infusion of glucose, L-carnitine, and water-soluble vitamins. MS/MS also detected extremely high levels of C5-acylcarnitine (8.27 nmol/mL; cut off, 1.0 nmol/mL) in the dried blood spot; urinary metabolite screening by gas chromatography-mass spectrometry detected an increased level of isovaleryl-glycine excretion. The patient was treated with leucine-free formula and L-glycine after a diagnosis of IVA, and was discharged on the 48th day.

Patient 2 was delivered after a 39 week-gestation period, and discharged 6 days after birth. The patient displayed symptoms such as poor feeding and lethargy 10 days after birth. Biochemical laboratory examinations indicated elevated levels of ammonia (1,356 $\mu\text{g/dL}$); therefore, he was subjected to an intravenous administration of benzoate and arginine, as well as continuous hemodiafiltration. Following a diagnosis of IVA, the patient was administered with leucine-free formula, L-carnitine, and L-glycine. The blood ammonia level decreased considerably in 12 hours and was maintained within the normal range; however, severe mental retardation persisted in the patient.

Patient 3 developed symptoms such as petechiae and poor feeding within 10 days after birth. Based on the results of a complete blood count, the patient was diagnosed with anemia (Hb 8.1 g/dL) and thrombocytopenia (3,000 per μL). The patient was subjected to a blood transfusion, and treated with antibiotics. Mild mental retardation was identified at the age of 3 years. The patient showed symptoms such as repetitive vomiting with mild hyperammonemia (187 $\mu\text{g/dL}$) at the age of 4 years. A diagnosis of IVA was made upon observation of recurrent vomiting with metabolic acidosis (pH 7.243, HCO_3^- 9.4 mEq/L) at the age of 5 years.

Patient 4 was a 6 year-old boy, who was admitted to the emergency ward with recurrent vomiting symptoms. The patient was diagnosed with metabolic acidosis (pH 7.11, HCO_3^- 7.0 mEq/L) and hypoglycemia (50 mg/dL). He was hospitalized for 6 days and treated with an intravenous infusion of glucose, L-carnitine, and vitamin B₁.

Patient 5 was delivered after a gestation period of 41 weeks, with a birth weight of 3,442 g. NBS demonstrated slightly high levels of C5-acylcarnitine (1.29 nmol/mL; cut off, 1.0 nmol/mL) in the dried blood spot. She was biochemically diagnosed as IVA by urinary organic acid analysis at the age of one month. The patient was treated with protein-restricted diet and L-carnitine supplementation and remained without symptoms until last examination (four years old). The residual IVD activity was 28% of that of the control, as determined by high-performance liquid chromatography (Tajima et al. 2005).

Direct sequencing of the IVD gene

Genomic DNA was extracted from peripheral blood. The IVD gene was directly sequenced using a previously described method (Vatanavicharn et al. 2011). All coding exons, including the flanking introns in the IVD gene, were amplified via polymerase chain reaction (PCR), using 11 primer pairs. The PCR conditions were 40 cycles of 94°C for 20 s, 57°C for 20 s, and 72°C for 20 s. The PCR products were purified and directly sequenced using a Big Dye Primer Cycle Sequencing kit and an ABI 310 or 3500 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA). The potential pathogenic effects of these mutations on IVD protein were assessed using multiple online prediction software (PolyPhen2, SIFT, PhD-SNP, and Splicing Finder program Version 2.4.1).

The protocols used in this study were approved by the Ethics Committee of the Tohoku University School of Medicine.

Results and Discussion

Sequencing of the entire coding region of the IVD gene of the 5 patients revealed the presence of two nucleotide substitutions (Table 1, Fig. 1). All patients were compound heterozygotes, and displayed no common mutation.

Four missense mutations (p.R53P, p.R395C, p.Y403C, and E411K) were previously reported. The mutations p.R53P and p.R395C are generally found pan-ethnically in the Caucasian, Taiwanese, and Turkish (Mohsen et al. 1998; Ensenauer et al. 2004; Lin et al. 2007; Ozgul et al. 2014) population. This suggests that the c.158G and c.1183C regions in the IVD gene might be mutational hot spots. So far, the mutation p.E411K (reported as p.E408K (c.1222G>A)) has been found only in one Egyptian. The mutation p.Y403C, on the other hand, is the proposed founder mutation, which has been discovered only in the East and Southeast Asian regions, such as Taiwan (Lin et al. 2007), China (Lee et al. 2010), and Thailand (Vatanavicharn et al. 2011). Unexpectedly, c.466-3_2CA > GG, which is the most common mutation characterized in Korean and Thai patients (Lee et al. 2007; Vatanavicharn et al. 2011), was not observed in our (Japanese) patients.

We also observed five novel missense mutations and one novel intronic mutation (p.G94D, p.E116K, p.M167T,

Table 1. Clinical and molecular analysis of Japanese IVA patients.

Patient	Onset	Clinical presentation	C5 (cut off < 1.0 nmol/mL)	Changes in the nucleotide (Consequence)	
1	2 d	tachypnea metabolic acidosis hyperammonemia	8.27	c.728T>C (p.L243P)	c.1183C>T (p.R395C)*
2	10 d	lethargy hyperammonemia	9.03	c.158G>C (p.R53P) [†]	c.737T>C (p.L246P)
3	42 d	42 d: anemia, thrombocytopenia 3 y: mild mental retardation 4 y: recurrent vomiting 5 y: recurrent vomiting, metabolic acidosis (diagnosed)	4.94	c.1208A>G (p.Y403C) [§]	c.1231G>A (p.E411K) [‡]
4	6 y	recurrent vomiting metabolic acidosis hypoglycemia	data not available	c.281G>A (p.G94D)	c.696+1G>T (splicing error)
5	NBS	asymptomatic	1.29	c.346G>A (p.E116K)	c.500T>C (p.M167T)

The numbering of nucleotides that showed changes was based on the cDNA sequence, in accordance with the GenBank entry NM_002225.3.

The amino acid numbers were designated according to NP_002216.2, which corresponds to the sequence of the precursor protein.

*previously reported as R363C based on the mature protein by Ensenauer et al. 2004, Lin et al. 2007, Ozgul et al. 2014.

[†]previously reported as R21P based on the mature protein by Mohsen et al. 1998, Lin et al. 2007, Ozgul et al. 2014.

[§]previously reported as Y371C based on the mature protein by Lin et al. 2007, Lee et al. 2010, Vatanavicharn et al. 2011.

[‡]previously reported as E408K based on NP_002216.1 by Hertecant et al. 2012.



Fig. 1. Schematic representation of the IVD protein and the locations of the missense mutations identified in this study.

Table 2. Novel missense mutations.

Nucleotide change	Exon	Effect of mutation	Position on the mature protein	PolyPhen-2	SIFT	PhD-SNP
c.281G>A	Ex3	p.G94D	62	probably damaging	Affects protein function	Disease-related
c.346G>A	Ex4	p.E116K	84	probably damaging	Affects protein function	Disease-related
c.500T>C	Ex5	p.M167T	135	possibly damaging	Affects protein function	Disease-related
c.728T>C	Ex7	p.L243P	211	probably damaging	Affects protein function	Disease-related
c.737T>C	Ex7	p.L246P	214	probably damaging	Affects protein function	Disease-related

p.L243P, p.L246P, and c.696+1G>T). These substitutions were not observed in dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) and the Human Genetic Variation Browser

(<http://www.genome.med.kyoto-u.ac.jp/SnpDB/>). PolyPhen2, SIFT, and PhD-SNP predicted that these novel missense mutations imparted potential pathogenic effects

(Table 2). As the position 167 is known to exist in the FAD binding site (<http://www.uniprot.org/uniprot/P26440>), p.M167T is believed to influence FAD binding. The c.696+1G>T substitution weakened the natural splicing donor site of intron 6. The splicing consensus values calculated by Splicing Finder program were 97.89 (c.696+1G) and 71.05 (c.696+1G>T).

Patient 5 showed a mild elevation in C5-carnitine expression and a relatively high residual IVD activity (28% of that of the control). Using NBS, the p.A314V (A282V) missense mutation has been previously identified as being common to asymptomatic Caucasian IVA patients with mild metabolite elevations (Ensenauer et al. 2004). The enzyme activity of the expressed p.A314V-mutant IVD was 19% of that of the wild type (Mohsen et al. 1998). Patient 5 was a compound heterozygote (p.E116K and p.M167T); therefore, the mutation responsible for the relatively high residual IVD activity could not be identified.

In conclusion, Japanese patients with IVA have been shown to express mutations in the *IVD* gene; however, the mutation spectrum is different from that seen previously in other ethnic groups. The number of mutations suggests the highly heterogeneous nature of IVA in Japan.

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Conflict of Interest

The authors declare no conflict of interest.

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