Identification of PRODH Mutations in Korean Neonates with Type I Hyperprolinemia

Mi-Ae Jang¹, Byung Cheol Kim³, Chang-Seok Ki¹, Soo-Youn Lee¹, Jong-Won Kim¹, Tae Youn Choi³, Dong Hwan Lee⁴, Junghan Song⁵, Yong-Wha Lee², and Hyung-Doo Park¹

¹Department of Laboratory Medicine & Genetics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea; ²Department of Laboratory Medicine and Genetics, Soonchunhyang University Bucheon Hospital and Soonchunhyang University College of Medicine, Bucheon, Korea; ³Department of Laboratory Medicine, Soonchunhyang University Hospital, Soonchunhyang University College of Medicine, Seoul, Korea; ⁴Department of Pediatrics, Soonchunhyang University College of Medicine, Seoul, Korea; ⁵Department of Laboratory Medicine, Seoul National University College of Medicine, Seoul, Korea

Abstract. Background: Hyperprolinemia is a rare inherited metabolic disorder characterized by a high proline level in blood and/or urine and various neuropsychiatric symptoms. Type I hyperprolinemia is caused by a proline oxidase deficiency, which is encoded by the PRODH gene on chromosome 22q11. Herein, we present a study of Korean patients with type I hyperprolinemia who were diagnosed during newborn screening by tandem mass spectrometry and confirmed by molecular analysis.

Methods: Four neonates were referred to our hospital for workup of high proline levels in newborn screening test. We analyzed the biochemical findings and the PRODH gene was amplified by long-range PCR to confirm molecular genetic abnormalities.

Results: All patients had high plasma proline levels, ranging from 742 to 1192 μmol/L (reference range, 77.4 - 244.6 μmol/L). In molecular analysis, 4 disease-associated mutant alleles were identified: c.1414G>A (p.A472T), c.1279G>A (p.V427M), c.1357C>T (p.R453C) and c.1562A>G (p.Q521R). All mutations were missense and c.1279G>A included the majority of mutant alleles. No relationships between type of mutation and clinical outcomes were observed.

Conclusion: We found that distinct molecular alterations of the PRODH gene result in abnormal proline levels. Newborn screening and molecular analysis are necessary to identify patients before clinical expression of metabolic disease.

Key words: Korean, mutation, PRODH, type I hyperprolinemia

Introduction

Type I hyperprolinemia (HPI; MIM 239500) is an autosomal recessive disorder caused by a deficiency of the enzyme proline oxidase (POX, also called proline dehydrogenase). POX is a mitochondrial inner-membrane enzyme that catalyzes the degradation of proline into delta-1-pyrroline-5-carboxylate, the first step of proline metabolism [1]. Thus, HPI is biochemically defined by high plasma proline levels, ranging from 2 to 10 times above normal values, without urinary excretion of delta-1-pyrroline-5-carboxylate [2,3]. POX is encoded by the proline dehydrogenase (PRODH) gene, which spans ~23.77 kb of genomic DNA with 15 exons and is located on chromosome 22q11 [4].

HPI is a rare inherited metabolic disorder and its prevalence is uncertain. The diagnosis of HPI remains a challenge and the clinical features of HPI are not well characterized. Some individuals with HPI are asymptomatic, while others have neurologic, renal, and/or auditory impairments [1,3,5]. High serum proline levels have been described in patients with various phenotypes and PRODH mutations [2]. Another problem is that a PRODH pseudogene (ΨPRODH) is located 1.4 Mb telomeric to the functional gene. Although ΨPRODH
has an internal deletion that removes a 13.1-kb segment containing exons 2 through the 5' half of exon 7 of PRODH, it has >95% homology with PRODH in some of the exons [4,6]. Several missense mutations in ΨPRODH have been transferred to PRODH by gene conversion. Therefore, molecular confirmation of HPI is complicated and further study is required to ensure recognized variants are unique to PRODH.

The current study presents the first biochemically and genetically confirmed Korean cases of HPI using a long-range polymerase chain reaction (PCR) and sequencing method. In addition, we compared the relationships between PRODH gene mutations and hyperprolinemia.

Materials and Methods

Clinical and biochemical analysis. The patients included in the study were referred to our hospital for further evaluation of hyperprolinemia after being diagnosed by a newborn screening test performed 3 days after birth at a local hospital. The patients underwent various biochemical tests, including aspartate aminotransferase, alanine aminotransferase, ammonia, lactate, urine proline, and glycine. The results of the newborn screening test were confirmed by liquid chromatography-tandem mass spectrometry and amino acid analysis. Organic acids in the blood and urine were also analyzed.

Genetic analysis. Mutation analyses of the PRODH gene were performed in all patients. Blood samples were collected from each patient after obtaining informed parental consent. Genomic DNA was isolated from peripheral blood leukocytes using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. We screened all of the coding exons and the flanking introns of the PRODH gene. Exons 7-14, which are known to have high homology with ΨPRODH [6], were pre-amplified by a single long-range PCR. Exons 2-6 and exon 15 have low sequence homology and were amplified directly from genomic DNA with primers unique to PRODH.

The current study presents the first biochemically and genetically confirmed Korean cases of HPI using a long-range polymerase chain reaction (PCR) and sequencing method. In addition, we compared the relationships between PRODH gene mutations and hyperprolinemia.

Materials and Methods

Clinical and biochemical analysis. The patients included in the study were referred to our hospital for further evaluation of hyperprolinemia after being diagnosed by a newborn screening test performed 3 days after birth at a local hospital. The patients underwent various biochemical tests, including aspartate aminotransferase, alanine aminotransferase, ammonia, lactate, urine proline, and glycine. The results of the newborn screening test were confirmed by liquid chromatography-tandem mass spectrometry and amino acid analysis. Organic acids in the blood and urine were also analyzed.

Genetic analysis. Mutation analyses of the PRODH gene were performed in all patients. Blood samples were collected from each patient after obtaining informed parental consent. Genomic DNA was isolated from peripheral blood leukocytes using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. We screened all of the coding exons and the flanking introns of the PRODH gene. Exons 7-14, which are known to have high homology with ΨPRODH [6], were pre-amplified by a single long-range PCR on a thermal cycler 9700 (Applied Biosystem, Foster City, CA, USA). The long-range PCR (expected size: 9,837 bp) was performed using primer pairs that are not complementary to any pseudogene sequences: forward (5’-ctgcctccatgcacctct-3’) and reverse (5’-cccagcaccgtaccccatc-3’) [6]. Subsequently, exons 7-14 were amplified from the long-range PCR product for mutation analysis. Exons 2-6 and exon 15 with low sequence homology were amplified directly from genomic DNA with primers unique to PRODH. The PRODH gene was amplified by PCR using appropriate primers designed by the authors (available upon request) and the Thermal Cycler 9700 (Applied Biosystem, Foster City, CA, USA). Direct sequencing was performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on ABI Prism 3130 Genetic Analyzer (Applied Biosystem). Obtained sequences were analyzed using the Sequencher program (Gene Codes Corp., Ann Arbor, MI, USA) and were compared to reference sequences. The GenBank accession number of the reference sequence was NM 016335.4. Sequence variation was described according to the recommendations of the
**Results**

**Clinical and biochemical findings.** The clinical, biochemical, and genetic findings of the patients tested are summarized in Table 1. All patients were born at term and pregnancy, delivery, and perinatal periods were unremarkable. No patient had a family history of genetic abnormalities. High plasma proline levels were found in all patients, ranging from 742 to 1192 μmol/l (reference range: 77.4-244.6 μmol/L). Two patients were diagnosed with hyperprolinemia in dried blood spot analyses by liquid chromatography-tandem mass spectrometry: patient 1 (588.8-602.8 μmol/L; cut-off, <308.3 μmol/L) and patient 2 (495.2-553.8 μmol/L; cut-off, <308.3 μmol/L). We were unable to obtain the initial proline levels from the newborn screen for patients 3 and 4. Urine proline concentrations were elevated in all patients (0.5-39.9 mmol/g creatinine; reference range, 0-0.1 mmol/g creatinine). Glycine levels were also elevated compared to the normal range except for patient 1, who showed a near upper-normal limit. The level of delta-1-pyrroline-5-carboxylate was not determined in this study, which is the first metabolite from the proline catabolism and is not excreted in urine of the patients with HPI. However, other laboratory investigations, such as aspartate aminotransferase, alanine aminotransferase, ammonia, and lactate, were all within normal range for the patients. All patients have been growing normally and have been continuously followed without any specific medical treatment.

**Genetic analysis.** The PRODH mutational analyses of patients with HPI are summarized in Table 2. All patients had a distinct molecular alteration of the PRODH gene: Patient 1 c.1279G>A (p.V427M) and c.1562A>G (p.Q521R), patient 2 c.1279G>A (p.V427M) and c.1414G>A (p.A472T), patient 3 homozygous for

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Gestational age (weeks)</th>
<th>Birth weight (g)</th>
<th>Clinical Delay</th>
<th>Developmental delay</th>
<th>Plasma proline (μmol/L)</th>
<th>Urine proline (mmol/g Cr)</th>
<th>Urine glycine (mmol/g Cr)</th>
<th>Organic acid analysis</th>
<th>PRODH genotype (allele1/allele2)</th>
<th>Clinical outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>F</td>
<td>41</td>
<td>3,200</td>
<td>-</td>
<td>-</td>
<td>998.3</td>
<td>1.8</td>
<td>4.3</td>
<td>WNL</td>
<td>c.1279G&gt;A/c.1562A&gt;G</td>
<td>Well, 29 months</td>
</tr>
<tr>
<td>Patient 2</td>
<td>F</td>
<td>40</td>
<td>3,100</td>
<td>-</td>
<td>-</td>
<td>1,133</td>
<td>4.36-28.3</td>
<td>11.5-19.4</td>
<td>WNL</td>
<td>c.1279G&gt;A/c.1414G&gt;A</td>
<td>Well, 42 months</td>
</tr>
<tr>
<td>Patient 3</td>
<td>F</td>
<td>39</td>
<td>2,700</td>
<td>-</td>
<td>-</td>
<td>272.6</td>
<td>39.4</td>
<td>25.6-140</td>
<td>WNL</td>
<td>c.1357G&gt;C/p.453C&gt;T</td>
<td>Well, 38 months</td>
</tr>
<tr>
<td>Patient 4</td>
<td>F</td>
<td>40</td>
<td>3,200</td>
<td>-</td>
<td>-</td>
<td>1,192</td>
<td>0.5-9.9</td>
<td>3.6-26.1</td>
<td>WNL</td>
<td>c.1279G&gt;A/p.472T</td>
<td>Well, 52 months</td>
</tr>
</tbody>
</table>

Reference range: Plasma proline (77.4-244.6 μmol/L), urine proline (0.4-4.3 mmol/g Cr) and urine glycine (0.4-4.3 mmol/g Cr).

Abbreviation: WNL, within normal limits.
c.1357C>T (p.R453C), and patient 4 homozygous for c.1279G>A (p.V427M). Every identified mutation was a single base-pair substitution in coding regions and all have been reported previously [4,7]. In the current study, the most common mutant allele was c.1279G>A (p.V427M), which had a frequency of 4/8 (50%). The next was c.1357C>T (p.R453C) harboring 2/8 (25%). Mutations c.1414G>A (p.A472T) and c.1562A>G (p.Q521R) were each seen in one patient. All mutations were located on exon 12 except for c.1562A>G (p.Q521R), which was located on exon 14. Three of the four mutant alleles are also registered in the SNP database and the Human Gene Mutation Database (http://www.hgmd.org). Guilmatre et al. showed that none of the 114 normal controls have the c.1279G>A (p.V427M) mutation [8]. In substitutions of c.1357C>T (p.R453C) were each seen in one patient. All mutations were located on exon 12 except for c.1562A>G (p.Q521R), which was located on exon 14. Three of the four mutant alleles are also registered in the SNP database and the Human Gene Mutation Database (http://www.hgmd.org). Guilmatre et al. showed that none of the 114 normal controls have the c.1279G>A (p.V427M) mutation [8]. In substitutions of c.1357C>T (p.R453C), it has been reported that none of the controls were homozygous for CC, although the heterozygous genotype had a 2% allele frequency [8]. The allele frequency for c.1414G>A (p.A472T) has not yet been determined.

**Discussion**

Hyperlactatemia, could be possible cause to hyperprolinemia, was not observed in our patients [9]. Although delta-1-pyrroline-5-carboxylate was not determined in our study, type II hyperprolinemia (HPII) was not considered as a primary genetic defect. Because the degree of increased proline level was less than 5 times over the normal range, which is not consistent with the findings that plasma proline levels 10 to 15 fold above normal in HPII. The clinical phenotype of HPII included an increased frequency of seizures and mild mental retardation, while the most of the HPI patients have no clinical symptom like our patients [9].

In the past, the ability to diagnose HPI has been limited, because POX activity is not readily measured as it is found primarily in liver, kidney, and brain and not in leukocytes or cultured fibroblasts. After the identification of PRODH, the gene associated with hyperprolinemia, in 1997, HPI diagnosis becomes clearer [10]. Moreover, Bender et al. established functional consequences of PRODH gene mutations in vitro [4]. They measured POX activity radioisotopically using transfection into cells (CHO-K1-C9) lacking endogenous POX activity on 16 known PRODH mutations. As a result, the missense mutations divided into three groups according to the effect on POX activity: those leading to mild (< 30%), moderate (30-70%), and severe (> 70%) reduction. Group I contained 4 alleles (p.R185Q, p.L289M, p.A455S, and p.A472T) with mild effects; group II contained 6 alleles (p.Q521R, p.A167V, p.R185W, p.D426N, p.Q19P, and p.R185W) with moderate effects; group III contained 5 alleles (p.P406L, p.L441P, p.R453C, p.T466M, and p.Q521E) with severe reduction of POX activity. One allele, p.Q521R, increased POX activity (120% of wild type) in reverse. In the same way, Guilmatre et al [8] measured the functional activities of other PRODH missense...
mutations. They showed that p.G444D and p.P8L substitutions lead to severe and moderate reduction on POX activity, respectively. In the present study, all of the patients with hyperprolinemia showed distinct molecular alterations. The identified variants of p.V427M, p.R453C, and p.A472T were reported in schizophrenia patients at first [11]. Later, they have been also documented in severe HPI patients as causable mutations [2,12], and functional deficiency was also clarified [4].

Various phenotypes and gene mutations were found in patients with hyperprolinemia [7,8,13]. Prospective studies of HPI probands, recognized through newborn screening and families with hyperprolinemia, noted that the metabolic disorder is not necessarily associated with clinical manifestations, suggesting that hyperprolinemia is a benign condition [1,5,14]. Later, however, hyperprolinemia with severe neurologic impairment was reported, and raised the question of whether HPI really was a benign trait [15]. In the present study, although the patients revealed persistent hyperprolinemia during the follow-up period, none of them had any clinical symptoms. The results of the present study may support the idea that HPI recognized by abnormal newborn screening may not be necessarily associated with neuropsychiatric problems [1,5,14]. However, considering that the age of disease onset was variable in previous studies, 5 months to 10 years, it is too early to draw any conclusions [2,12]. Our patients with HPI were identified by abnormal newborn screening tests and thus they may have been too young to present significant clinical features at the time of the molecular study. Close follow-up and careful monitoring may be needed even though they were growing and developing normally at the time of study.

Although there have been several studies on genotype-phenotype correlations in HPI patients, the relationship remains poorly understood. Some authors suggested that patients with more severe psychomotor and behavioral phenotypes had severe hyperprolinemia and a highly deleterious PRODH genotype. Rosa et al. [12] described four children with neuropsychiatric disorders consisting of epilepsy and mental retardation, and presented HPI and PRODH gene mutations. One patient with a complete deletion in one allele and four missense mutations exhibited moderate to severe hyperprolinemia (422-1883 μmol/L, normal value < 271 μmol/L) and had uncontrolled epilepsy and severe developmental delay. Another patient with a heterozygous missense mutation, not a complete deletion nor homozygous inactivation, had moderate hyperprolinemia (403-862 μmol/L, normal value < 271 μmol/L) and a milder cognitive and behavioral phenotype [12]. Jacquet et al. [16] also documented a HPI patient with severe psychomotor delay and a very high level of plasma proline (2246 μmol/L, normal value 133-227 μmol/L) who had a homozygous deletion of the entire PRODH gene. In the present study, a rather poor relationship between the functional consequences of mutation and proline levels was observed. Patient 3, who was homozygous for p.R453C, was expected to exhibit a very severe reduction of POX activity (< 30%), but the patient’s proline levels were the lowest compared to other patients. Although the p.Q521R mutation is known to increase POX activity, patient 1, who was a compound heterozygote for p.Q521R and p.V427M, showed higher proline levels than that of patient 3. Patient 4, who was homozygous for p.V427M/p.V427M, which is known to moderately decrease POX activity, showed higher proline levels than the p.R453C/p.R453C homozygous patient. This poor correlation has led to the suggestion that previous functional experiments were limited because they were in vitro studies. The expected POX activity was not confirmed by direct demonstrations of POX deficiency and thus, the functional consequences in vivo remain unknown. Other variables, such as dietary intake, metabolic state, and drugs may also affect plasma proline levels. Further development of enzyme activity measurement and an additional study on a large cases series are needed to resolve this problem.

In conclusion, we observed distinct molecular alterations of the PRODH gene resulting in abnormal plasma proline levels. This is the first report of biochemically and genetically confirmed HPI in Korea and will provide additional opportunities to observe HPI in Korean patients in order to gain a better understanding of the disease.
Acknowledgements

This study was supported by a grant from the Korea Health technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A120030), and a Samsung Biomedical Research Institute grant, SBRI C-B1-304-1.

References


