A novel large deletion (exons 12, 13) and a missense mutation (p.G46R) in the *PAH* in a Japanese patient with phenylketonuria

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Case report

Background: **Phenylketonuria (PKU) is caused by a defect in phenylalanine hydroxylase (*PAH*). More than 500 mutations have been reported for the gene encoding *PAH*. However, approximately 1%–5% of these include large deletions and large duplications that cannot be detected by conventional methods.**

Methods: **In this report we tried to fully characterize a *PAH*-deficient patient. The patient was a 2-year-old Japanese boy who was diagnosed with classical PKU at the time of neonatal screening, which was confirmed by the tetrahydrobiopterin-loading test. PCR-related direct sequencing and multiplex ligation-dependent probe amplification (MLPA) were used to analyze of the *PAH* of the patient.**

Results: **Using PCR-related direct sequencing method, we could detect only a heterozygous novel missense mutation: p.136G>C (p.G46R). A second mutation was detected by MLPA. The patient was heterozygous for a novel large deletion of exons 12 and 13: c.1200-?_1359+?del (EX12_13del). For genetic counseling, an accurate genetic diagnosis is often necessary.**

Conclusions: **Through a combination of MLPA and conventional methods, the success rate of *PAH* mutation identification can be close to 100%.**

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Key words: **large deletion; multiplex ligation-dependent probe amplification; phenylalanine hydroxylase; phenylketonuria**

Introduction

Phenylketonuria (PKU; MIM 261600), an autosomal recessive disease, is an important inborn error of amino acid metabolism.[1] The incidence of PKU is 1 in 120 000 individuals in the Japanese population. PKU is caused by a defect in phenylalanine hydroxylase (*PAH*: EC 1.14.16.1). This enzyme catalyzes the hydroxylation of phenylalanine to tyrosine with a tetrahydrobiopterin (BH4) cofactor. *PAH* is the rate-limiting enzyme for the catabolism of phenylalanine, and a defect in this enzyme results in elevation of serum phenylalanine levels and the development of PKU and benign hyperphenylalaninemia.[1] Increase of the serum phenylalanine level induces the accumulation of neurotoxic secondary metabolites. In order to ensure normal neuromotor development in children with PKU, it is essential to conduct a newborn screening program for early diagnosis and initiation of diet therapy.

The *PAH* gene is located on chromosome 12 (12q22-q24.2) and contains 13 exons, which spans about 90 kb.[1] Thus far, more than 500 mutations have been reported in this gene (http://www.pahdb.mcgill.ca/), and approximately 60% of which are missense mutations. Nonsense, deletion, insertion mutations and splice-site mutations have also been reported, although they are few. Most mutations are detectable by conventional methods for genetic analysis, such as denaturing gradient gel electrophoresis, direct sequencing. However, approximately 1%-5% of mutations are large deletions and large duplications that cannot be detected by conventional methods.[2]

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Recently, some novel methods for analyzing the genome have been developed. Multiplex ligation-dependent probe amplification (MLPA) is useful for detecting large deletions and duplications in the genome.[3] This method has helped to detect large deletions in the *PAH* gene in patients with PKU who lack a *PAH* mutation in one or two alleles. We report a novel large deletion which was detected using the MLPA method in a 2-year-old boy.

Case report

Case report

The patient was a 2-year-old Japanese boy. He was the first child born to non-consanguineous parents. He was born at term (weight: 3064 g) by normal vaginal delivery after an uncomplicated pregnancy. A newborn screening program at 5 days of life showed that his serum phenylalanine level was above 900 μmol/L (14.8 mg/dL). He was admitted to our hospital at 14 days of life. At the time of admission, his serum phenylalanine level was 2202 μmol/L (36.37 mg/dL). Since a tetrahydrobiopterin-loading test (10 mg BH4/kg) did not improve his serum phenylalanine level, he was diagnosed as having classical PKU. After the start of the diet therapy, his serum phenylalanine level was maintained between 22.4-290.0 μmol/L (0.37-4.79 mg/dL). His physical and neuromotor development was normal. After obtaining informed consent from the parents, blood samples were collected from the patient and his parents for genetic analysis.

Genomic DNA was extracted from blood leukocytes by using standard techniques. The genomic DNA sequence and cDNA sequences were based on the human genome database (NCBI accession numbers CCDS9092.1 and U49897). All 13 *PAH* exons and their flanking intronic sequences were amplified by PCR using 10 pairs of appropriate primers designed by us (Table) and an MJ Mini™ Personal Thermal Cycler (Bio-Rad, Hercules, CA). PCR products were purified using the NucleoSpin Gel and PCR clean-up Kit (TaKaRa, Kyoto, Japan), and the sequences of the amplified DNA fragments were determined directly using the BigDye® Terminator version 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3130Xl Genetic Analyzer (Applied Biosystems). The PCR products, including novel missense mutations in *PAH*, were subcloned into pCR® 2.1 vectors using the TA Cloning® Kit (Invitrogen, San Diego, CA) to determine changes in the nucleotide sequence.

The SALSA MLPA Kit P055 *PAH* (MRC-Holland, Amsterdam, The Netherlands) was used to detect large deletions in the *PAH* gene. The kit contained the probe mix for all of the 13 coding exons and was

Table. PCR and sequencing conditions for the *PAH* gene

PCR primers		Anti-sense primer	Melting temperature (ºC)	PCR product (base pairs)	Sequencing primers	
Target exons	Sense primer				Exon	Primers
1	5'-CCACGTGCTGTTTGCAAACC-3'	5'-AGGAAGCACCAGCAGTCTTC-3'	60	215	1	PCR primer (sense side)
2	5'-CCTTAAGCTGATAACTGACCCAGTAG-3'	5'-CAAAGTTCCTGTTGAAACTGACAAGG-3'	60	575	2	PCR primer (sense side)
3	5'-TGCGTTAGGTTTTCCTGTTCTGGT-3'	5'-ATATACCAGGCACTTGCCTAGGTC-3'	62	504	3	PCR primer (sense side)
4	5'-ACCGGTTTCTAAGGAAAATGGAGTT-3'	5'-GCTCCAAGTAGAGAAGGTAAGAGGA-3'	60	348	4	PCR primer (sense side)
5	5'-AGACATGCACTGTCATGGCT-3'	5'-ACACGCATGCACATGAACAC-3'	60	318	5	PCR primer (sense side)
6	5'-AGTGATGGCAGCTCACAGGTTCTGG-3'	5'-TTGGCACCATCCCCGAAAATAGCAC-3'	64	710	6	PCR primer (sense side)
7, 8	5'-CCCTGGGCAGTTATGTGTACTACTC-3'	5'-TGGGTATTTAGCTTCCCAGAACCAC-3'	62	1532	7	PCR primer (sense side)
					8	5'-CCATTCTTTCTGCCCATTCC-3'
9	5'-GGGTCTATGTGGGCTGTTCTGAAGG-3'	5'-ATAACCTGGCTTCCAGGGGAGTAGG-3'	64	275	9	PCR primer (sense side)
10, 11	5'-GTCCACTGACTCACATGCCAATCC-3'	5'-TGACTTGGTGGTTGCGTTGAACAG-3'	62	1189	10	PCR primer (sense side)
					11	5'-AGAGAAGGGGCACAAATGGC-3'
12, 13	5'-AAATGCCACTGAGAACTCTCTTAAGACTAC-3'	5'-GAGTTCTCTGCAAAGCATATATGAAGCTTG-3'	62	1763	12	PCR primer (sense side)
					13	5'-TCCAAGAAGCCCACTTATCC-3'

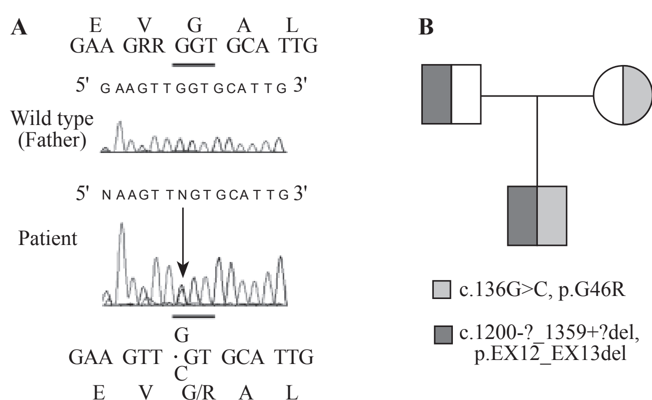

Fig. 1. Family pedigree and a novel missense mutation. **A:** Part of the direct sequenced data for exon 2. The arrow shows the nucleotide substitution. The patient was heterozygous for c.136G>C. This mutation changes codon 46 from arginine (R) to glycine (G); **B:** The patient was compound heterozygous for c.136G>C and c.1200-?_1359+? del. The father was heterozygous for c.1200-?_1359+?del and the mother was heterozygous for c.136G>C.

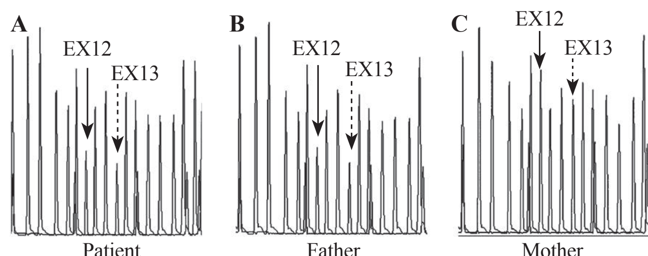

Fig. 2. Multiplex ligation-dependent probe amplification analysis. A section of the electrophoresis chart shows heterozygosity for a large deletion of exons 12 and 13. In the patient (**A**) and the father (**B**), peaks for exon 12 (arrows) and exon 13 (dashed arrows) were lower than those of the unaffected mother (**C**).

used according to the manufacturer's instructions. The amplification products were separated by capillary electrophoresis on an ABI 310 PRISM® Genetic Analyzer (Applied Biosystems). Raw data were analyzed using GeneMapper® Software Version 4 (Applied Biosystems). Four normal control samples were used to normalize the allele dosage. Deletions of the exon were detected when the height ratios of the fluorescent peaks were lower than the normal height ratio at 0.7.[3]

The patient was heterozygous for a novel missense mutation. Substitution of guanine for cytosine at nucleotide 136 in exon 2 changed the codon from glycine to arginine at position 46 of the corresponding protein (c.136G>C: p.G46R, Fig. 1A). We detected no additional mutations in exon 1, 3-13 or in the exon-intron boundaries of the patient. The mutation analysis showed that his mother was heterozygous for this mutation, while his father had no mutation (Fig. 1B).

The sequence data for the *PAH* mutation c.136G>C:p.G46R have been submitted to the DDBJ/EMBL/GenBank databases under the accession number AB778766.

MLPA analysis revealed that the peaks for exons 12 and 13 were lower than the normal height ratio of 0.7 (Fig. 2). This result indicated that the patient was heterozygous for a large deletion in exons 12 and 13 (c.1200-?_1359+?del: p.EX12_EX13del). His father was also heterozygous for the deletion. The breakpoint of the large deletion could not be detected by long-range PCR.

Discussion

Conventional mutation analysis and long-range PCR alone could not help to detect a novel missense mutation (p.G46R) in one of the alleles. Because the patient had classical PKU, another mutation should have been detected in the second allele. Previous studies have shown that most mutations (missense mutations, nonsense mutations, small deletions and small insertions) can be detected by conventional methods. However, in some patients, mutant alleles (1%-5%) were not detected by using conventional methods. In 2006, Desviat et al[4] identified large exonic deletions by using the MLPA method in 19 of the 22 patients who had undetectable mutant alleles. Thereafter, some studies revealed the existence of a large exon deletion as a cause of PKU by using the MLPA method. In certain European studies, the frequency of large exon deletions was 0.5%-3% of the mutations (Danish-German, 0.5%; Italian, 1.7%; Slovenia, 1.7%; Polish, 2-3%; Czech, 3%).[5,6] A Korean study by Lee et al[7] revealed that 7.5% of *PAH* mutations were large exon deletions. The deletions noted in their patients were Ex3del, Ex5del, Ex 6del, Ex1_2del, Ex5_6del, Ex4_7del, and Ex4_8del.[7] The frequency of the large deletions was low and varied according to race, but it was very important for clinical diagnosis.

A previous report on a Japanese patient with PKU suggested the possibility of a large deletion at the 3′ end of the *PAH* gene.[8] However, another study on Japanese patients showed only a large deletion of Ex5_6del in *PAH*.[9] In this study, we could detect a large deletion of exons 12 and 13 (EX12_13del) in our patient by using the MLPA method. The long PCR method could not detect breakpoints in the gene because there is a length limit of amplification with genomic DNA.

The patient had a second novel missense mutation (c.136G>C, p.G46R) in exon 2. Previously, a c.136G>A mutation was reported in a Danish PKU patient. This nucleotide change from A to G altered the glycine codon to a serine codon (p.G46S). Thus, it is thought that the position around codon 46 may have an important role in regulating enzyme activity.[10]

For genetic counseling, an accurate diagnosis is

always necessary, though conventional methods cannot identify some mutation including large deletions or duplications. Through a combination of MLPA and conventional methods, the detection rate for *PAH* mutations can be close to 100%.

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