Variations on exon 3 of 6-pyruvoyl-tetrahydropterin synthase gene in Indonesian population

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Abstract. Pyruvoyltetrahydropterin synthase (PTPS) is one of the several enzymes involved in tetrahydrobiopterin (BH4) biosynthesis. The deficiency of BH4 can cause hyperphenylalaninaemia (HPA). Mutations in PTS gene can alter protein function. We conducted genetic analysis of exon 3 of PTS gene of an Indonesian patient with PTPS-deficiency and healthy individuals. Direct sequencing of DNA samples was performed from an Indonesian patient with PTPS deficiency and 33 healthy individuals. No mutation was identified in the patient sample. However, a total of 6 novel variations were recorded in exon 3 of PTS. All these variations were found to be single-base alterations. The 6 novel variations identified in exon 3 of healthy individuals in this study were missense mutations. This variation data is expected to contribute to the development of the new database of Single Nucleotide Polymorphism (SNP) on PTS as well as to serve as a biomarker for the diagnosis of patients with PTPS deficiency.

1. Introduction

6-Pyruvoyl-tetrahydropterin synthase (PTPS) is an important enzyme involved in tetrahydrobiopterin (BH4) biosynthesis. BH4 is a natural cofactor of several enzymes including phenylalanine hydroxylase. Deficiency of BH4 or phenylalanine hydroxylase is the main cause of hyperphenylalaninemia (HPA)-related diseases [1]. BH4 also catalyzes the biosynthesis of catecholamine and serotonin from tryptophan [2,3].

Production of PTPS is regulated by PTS gene, which constitutes 6 exons located on the chromosome 11q22.3-11q23.3 [3]. Among the 6 exons of PTS gene, exon 3 is the smallest in size (23-bp long).3 Based on previous research, mutations occurring in PTS can be distributed uniformly across 6 exons [4].

Mutation analysis in PTS gene has been performed in Taiwan, China, Malaysia, Japan, Korea, Thailand, and the Philippines [1]. However, it has not yet been analyzed in Indonesian PTPS enzyme-deficiency patients. Therefore, we undertook the assessment of any mutations occurring in PTS gene, especially on exon 3 in PTS-deficient patients in Indonesia.
2. Materials and Methods

2.1 Subject
The samples used in this study were DNAs extracted from the blood of a patient with PTPS-deficiency and healthy individuals (control).

2.2 Isolation of DNA
DNA samples were isolated by adopting the following steps: tissue isolation, destruction of cell walls (lyses), DNA extraction, DNA precipitation, DNA purification, and DNA preservation. Blood samples were collected from PTPS-deficiency patients as well as healthy controls. DNA was then isolated from the blood samples using the Genomic DNA Blood Mini Kit (Geneaid).

2.3 Primer design
A specific primer pair was designed and verified using reference websites, such as the National Center for Biotechnology Information (NCBI), NetPrimer, and IDTDNA. NCBI was used for PTS sequence reference for designing the primer pairs, while specific primer pair verification process was conducted using NetPrimer and IDTDNA.

2.4 Optimization and verification primer
Primer pair optimization using PCR gradient was performed to determine the appropriate annealing temperature for exon 3-4, followed by verification using PCR. The result of primer-optimization process was visualized using gel electrophoresis.

2.5 Sequencing and analysis
The primer and DNA samples from the patient and controls were dispatched to the 1st Base Sequencing Services in Singapore for sequencing. The possibility of mutations in exon 3 of PTS was analyzed on sequence obtained using the software BioEdit and Chromas.

3. Results

3.1 DNA isolation
DNA isolated from the patients and controls using the Genomic DNA Blood Mini Kit (Geneaid) were tested for their purity and concentration. The purity of the DNA samples used in this study were within the range of 1.7–2.0.

3.2 Primer design
A primer pair used to analyze the mutation occurring on exon 3 of PTS was designed for exon 3-4 because exon 3 is only 23-bp long. A good and specific primer pair for exon 3-4 of PTS were thus obtained. The forward primer was 24-bp long, while the reverse primer was 23-bp long.

3.3 Optimization and verification primer
Optimization of the primer pair was performed using PCR Gradient at an annealing temperature range of 50°C–60°C (Figure 1). The verified optimum annealing temperature for exon 3-4 was 57°C, which was used as the annealing temperature to amplify DNA samples from the patient and the controls. Secondary band and single, thick target band sized between 500 and 750 bp obtained at 50–60°C. Single DNA of band size 500–750 bp was obtained at 57°C as the annealing temperature.

3.4 Sequencing and analysis
Sequence obtained from the 1st Base Sequencing Services in Singapore. Description: (M) 1-kbp marker; (1–8) well samples DNA sequences obtained from the 1st Base Sequencing Services in Singapore were analyzed for the possibility of mutations in exon 3 of PTS using the software BioEdit and Chromas. No
mutation was identified in exon 3 of PTS. However, 6 novel variations (c.167T>G, c.170T>G, c.176T>A, c.177A>C, c.179A>T, and c.184G>A) were identified in exon 3 in this study. All variations identified were due to missense mutations (Table 3).

Table 1. Information on Patients and Controls for Genetic Analysis at Exon 3 of PTS gene from PTPS-Deficiency Patients in Indonesia

| Code | Concentration (ng/µl) | Purity 260/280 | Sex | Information   |
|------|-----------------------|----------------|-----|---------------|
| R-1  | 1374                  | 1.85           | P   | PTPS Patient |
| K-2  | 575.50                | 1.86           | L   | Control       |
| K-3  | 92.24                 | 1.87           | L   | Control       |
| K-4  | 308.10                | 1.84           | L   | Control       |
| K-5  | 638.20                | 1.85           | L   | Control       |
| K-6  | 257.20                | 1.85           | L   | Control       |
| K-7  | 1335                  | 1.89           | P   | Control       |
| K-8  | 276.80                | 1.87           | P   | Control       |
| K-9  | 185.90                | 1.85           | P   | Control       |
| K-10 | 492.10                | 1.87           | P   | Control       |
| K-12 | 105                   | 1.82           | L   | Control       |
| K-13 | 1753                  | 1.87           | L   | Control       |
| K-14 | 2939                  | 1.86           | L   | Control       |
| K-15 | 1998                  | 1.86           | P   | Control       |
| K-16 | 660.30                | 1.86           | P   | Control       |
| K-17 | 252.10                | 1.82           | L   | Control       |
| K-19 | 818.50                | 1.87           | L   | Control       |
| K-22 | 818.50                | 1.86           | P   | Control       |
| K-23 | 1.40                  | 1.35           | P   | Control       |
| K-24 | 827.30                | 1.84           | L   | Control       |
| K-25 | 103.30                | 1.83           | L   | Control       |
| K-26 | 282.70                | 1.85           | P   | Control       |
| K-27 | 137                   | 1.80           | P   | Control       |
| K-28 | 527.40                | 1.84           | P   | Control       |
| K-29 | 346.20                | 1.82           | P   | Control       |
| K-30 | 172.70                | 1.90           | P   | Control       |
| K-31 | 289.50                | 1.85           | L   | Control       |
| K-32 | 271.70                | 1.79           | P   | Control       |
| K-33 | 486.90                | 1.87           | P   | Control       |
| K-34 | 308.20                | 1.88           | P   | Control       |
| K-35 | 225.40                | 1.83           | P   | Control       |
| K-36 | 632.10                | 1.87           | L   | Control       |
| K-37 | 253.30                | 1.84           | L   | Control       |
| K-38 | 309.90                | 1.83           | L   | Control       |

Figure 1. Result of Primer Optimization for Exon 3-4
Table 2. Primer Design Result for Exon 3-4

| Name | Primer (5’→3’) | Length (bp) | Tm (°C) | %GC | Hairpin Self dimer | Cross dimer | Product size (bp) |
|------|----------------|-------------|---------|------|-------------------|-------------|------------------|
| 3-4 F | GCTTGTATGTGCTAACTTTGTGCT | 24 | 55.66 | 41.67 | 0 | 0 | -3.56 | 643 |
| 3-4 R | GAGATAACTGTTGGGGAGGTAG | 23 | 59.96 | 52.17 | 0 | 0 | - | |

4. Discussion

A primer is a single-stranded oligonucleotide molecule ~30 bp long. A specific primer attaches only to a particular DNA sequence which it is designed to anneal with. Determination of the primer pair quality can be performed on the basis of the analysis of product length, primer melting temperature (Tm), primary annealing temperature (Ta), primary melting temperature difference, GC content, GC clamp, secondary structure, and repeats and runs [5].

The quality of the primer pair used for exon 3-4 mutation analysis was qualified as good based on the above-mentioned properties [6]. The forward primer of exon 3-4 is 24-bp long, while reverse is 23-bp long. In addition, the primer pair for exon 3-4 also showed a melting temperature (Tm) of 55 °C–60 °C, as well as GC content ≥40%. The primer has no hairpin secondary structure and self-dimer, and its cross dimer possessed a low energy (<−7 kcal/mol).

The primer optimization result of exon 3-4 using gradient PCR with annealing temperature range 50–60 °C revealed a curved thick band even at the highest temperature of 61°C (Figure 1). Determination of the optimum annealing temperature of exon 3-4 was performed by further verification processes. Based on the study results, 57 °C was selected as the optimum annealing temperature for exon 3-4 and used as the annealing temperature for further DNA sampling of the patient and controls by PCR.

In this study, no mutation was identified in the PTPS-deficient patients, but 6 variations were identified in the healthy individuals (c.167T>G, c.170T>G, c.176T>A, c.177A>C, c.179A>T, and c.184G>A). These variations were different from any previously recorded mutation; for example, c.166G>A [7] and c.169_171delGTG [8,9] were reported in studies on East Asian population. All of the 6 variations identified in the Indonesian normal patient (K-34) were because of missense mutation. The T>G transition at nucleotide 167 caused a Val to Gly substitution at codon 56; the same transition occurred at nucleotide 170 and caused the same amino acid substitution at 57. Meanwhile the T>A transition at nucleotide 176 and A>C transition at nucleotide 177, both caused a Val to Asp substitution at codon 59. The A>T transition at nucleotide 179 led to the replacement of His with Leu at codon 60. The last transition, G>A at nucleotide 184 caused substitution of Glu to Lys. Because all the transitions were obtained from a normal individual (control), it was ascertained that all variations had normal phenotypes.

5. Conclusion

No mutation was identified in an Indonesian PTS-deficient patient, but 6 novel variations were identified on exon 3 of a normal individual (control) in this study. All variations identified were because of missense mutation. Information regarding these variations is expected to contribute to the development of a new database of Single Nucleotide Polymorphism (SNP) on PTS as well as to serve as a biomarker for the diagnosis of PTS-deficient patients.

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