Mutational analysis of exon 4 of the 6-pyruvoyl-tetrahydropterin synthase gene in an Indonesian population

D C Putri1,2, R Priambodo1, Y Ariani1,2,3, R Amaani3, K Yuliarti1,2, R Lestari4 and D R Sjarif1,2*

1Human Genetic Research Center, Indonesian Medical Education and Research Institute, Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia
2Department of Pediatric, Cipto Mangunkusomo National Referral Hospital, Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia
3Department of Medical Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia
4Department of Biology, Faculty of Math and Sciences, Universitas Indonesia, Jakarta, 10430, Indonesia
*E-mail: ukk.npm.idai@gmail.com

Abstract. The enzyme 6-pyruvoyl-tetrahydropterin synthase (PTPS), which is coded by the PTS gene, plays a key role in the biosynthesis of tetrahydrobiopterin (BH4). BH4 deficiency causes hyperphenylalaninemia (HPA). The PTS gene comprises six exons and five introns. There are several types of mutations to the PTS gene in patients with PTPS enzyme deficiency that cause functional changes in the resulting protein. Primers for mutational analysis of exon 4 of the PTS gene were designed using BioEdit and Chromas softwares. Mutations to exon 4 (56 bp in length) were identified in patients with PTPS enzyme deficiency and normal healthy patients as controls. The primers used to identify mutations to the PTS gene were optimal at 57 °C. Six new mutations and 27 variations were identified in exon 4, which included five variations that were previously described.

1. Introduction
The enzyme 6-pyruvoyltetrahydropterin synthase (PTPS) plays a key role in the biosynthesis of tetrahydrobiopterin (BH4), which is an essential cofactor for neurotransmitter synthesizing the enzymes tyrosine hydroxylase (which catalyzes the conversion of tyrosine to l-dopa), tryptophan hydroxylase (which catalyzes the conversion of tryptophan to 5-hydroxytryptophan), and phenylalanine hydroxylase (which converts phenylalanine to tyrosine). In addition, BH4 is necessary for the biosynthesis of the neurotransmitters catecholamine and serotonin [1,2]. PTPS is coded by the PTS gene, which contains six exons located on the chromosomal region 11q22.3-11q23.3. Exon 4 of the PTS gene is only 56 bp in length [3]. To date, 53 various missense and nonsense mutations, as well as deletions and insertions have been described in the PTS gene [4]. Approximately 1%–2% of hyperphenylalaninemia (HPA) cases are caused by a deficiency of the BH4 cofactor, which affects the activity of the PTPS enzyme. Mutational analysis of the PTS gene has been conducted in Taiwan, China, Malaysia, Japan, Korea, Thailand, and the Philippines. In total, 43 mutations have been observed in these East Asian countries, including the missense mutations c.155A4G, c.259C4T, c.272A4G, and 286G4A, which are a new mutations [4]. However, no mutational analysis of the PTS
gene has been conducted in Indonesian patients with PTPS enzyme deficiency. The purpose of this study was to determine the types of mutations occurring in exon 4 of the PTS gene in Indonesian patients with PTPS enzyme deficiency.

2. Materials and Methods

DNA was extracted from whole blood 2 samples of PTPS deficiency patients and 33 normal individuals as controls using the Genomic DNA Blood Mini Kit (Geneaid Biotech, Ltd. New Taipei City, Taiwan). The purity and concentration of the DNA samples were determined using a spectrophotometer.

Primers for amplification of exon 4 of the PTS gene were designed using the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and verified using the PrimerQuest Tool (Integrated DNA Technologies, Inc., Coralville, IA, USA) and NetPrimer web-based tool (PREMIER Biosoft, Palo Alto, CA, USA). Polymerase chain reaction (PCR) amplification was performed with the following cycling conditions: denaturation at 94 °C for 20 s, followed by 40 cycles of annealing for optimization at 50 °C, 52 °C, 54 °C, 56 °C, 58 °C, and 60 °C for 20 s, and extension at 72 °C for 30 s, with a final extension step at 72 °C for 10 min. The amplified DNA was visualized by agarose gel electrophoresis. The DNA samples were sequenced by 1st Base Sequencing Services, Singapore. Mutation and variation analyses of the generated sequences of exon 4 of the PTS gene were performed using the BioEdit Sequence Alignment Editor (Ibis Therapeutics, Carlsbad, CA, USA) and Chromas software (Technelysium Pty Ltd., South Brisbane, QLD, Australia).

3. Results

DNA purity was verified by an optical density (OD) ratio of 260/280 nm of 1.85–1.90. The sequences of the forward and reverse primers for amplification of exon 4 of the PTS gene were 5'GCT TGT ATG TTG CTA ACT TGT GCT-3' and 5'GAGATA ACT GGT TGG GGA GGT AG-3', respectively. The length of the amplification products were 624 bp. A summary of designed primers is shown in Table 1. The optimum annealing temperature for PCR amplification was tested at 50 °C–60 °C and determined to be 57 °C. Primer optimization and PCR visualization of exon 4 of the PTS gene are shown in Figure 1.

| Name | Sequence | Length (bp) | Tm (°C) | Ta (°C) | XX | XX | XX | XX |
|------|----------|-------------|---------|---------|----|----|----|----|
| 3-4 F | GCTTGATATGTTGCTAACTTTGTGCT | 24 | 55.66 | 41.67 | 0 | 0 | -3.56 | 643 |
| 3-4 R | GAGATAAAGTTGGGAGGTTAG | 23 | 59.96 | 52.17 | 0 | 0 | | |

**Figure 1.** Visualization of PCR results of exon 4 of the PTS genes PTPS enzyme deficiency patients and normal controls at 60 °C (A) and 57 °C (B).
The sequences of the PTS genes of all samples were aligned to identify mutations and variations. Six mutations and 27 variations were identified in the PTS genes of PTPS deficiency patients and normal individuals. The variations and mutations are summarized in Table 2 and Table 3, respectively.

### Table 2. Variations in the sequences of exon 4 of the PTS gene.

| Mutant Type | Location in Gene | Predicted Protein | Reference |
|-------------|------------------|-------------------|-----------|
| Silent      | c.243G>A         | p.E81E            | (Thonny & Blau 2006) |
| Silent      | c.195T>C         | p.P65             | SNP       |
| Substitution| c.200C>T         | p.Y67M            | SNP       |
| Substitution| c.216T>A         | p.N72K            | SNP       |
| Substitution| c.217C>A         | p.L73M            | SNP       |
| Silent      | c.228G>A         | p.L76             | SNP       |
| Substitution| c.188T>C         | p.I63T            | This Study|
| Substitution| c.190G>T         | p.D64Y            | This Study|
| Substitution| c.194C>T         | p.P65L            | This Study|
| Silent      | c.198T>C         | p.A66A            | This Study|
| Silent      | c.204A>T         | p.G68G            | This Study|
| Deletion    | c.201_202delGG   | p.G3104X          | This Study|
| Substitution| c.206T>C         | p.M69T            | This Study|
| Substitution| c.207G>T         | p.M69I            | This Study|
| Substitution| c.210T>C         | p.V70V            | This Study|
| Substitution| c.212T>C         | p.M71T            | This Study|
| Substitution| c.215A>C         | p.N72T            | This Study|
| Silent      | c.219G>T         | p.L73L            | This Study|
| Substitution| c.220G>A         | p.A74T            | This Study|
| Substitution| c.221C>T         | p.A74V            | This Study|
| Silent      | c.222T>G         | p.A74A            | This Study|
| Substitution| c.225T>A         | p.D75E            | This Study|
| Substitution| c.226C>A         | p.L76I            | This Study|
| Substitution| c.227T>G         | p.L76R            | This Study|
| Substitution| c.232A>C         | p.K78Q            | This Study|
| Substitution| c.239T>C         | p.M80T            | This Study|
| Substitution| c.240G>A         | p.M80I            | This Study|

### Table 3. Mutations in exon 4 of the PTS gene of patients with PTP enzyme deficiency.

| Mutant Type | Location in Gene | Predicted Protein | Reference |
|-------------|------------------|-------------------|-----------|
| Silent      | c.189T>A         | p.I63I            | This Study|
| Substitution| c.196G>T         | p.A66S            | This Study|
| Substitution| c.213G>C         | p.M71I            | This Study|
| Substitution| c.218T>C         | p.L72P            | This Study|
| Substitution| c.235T>A         | p.Y79N            | This Study|
| Silent      | c.241G>A         | p.E81E            | This Study|
4. Discussion

In this study, DNA purity varied from 1.7 to 2.0 at a ratio of OD 260/280 nm, suggesting that the extracted DNA was free of contamination of RNA and proteins, and of sufficient quality for amplification.

Primers used for optimization of exon 4 are specific. Primer used for optimization of exon 4 has the good primary requirement. Specific primer conditions were categorized by product length, primary melting temperature (Tm), primary annealing temperature (Ta), primary melting temperature difference, GC content, GC clamp, secondary structure, repeats, and runs [6].

The lengths of the forward and reverse primers of exon 4 were 24 and 23 bp, respectively. If the product length exceeds 30 bp, the primer becomes nonspecific. The Tm ranged from 55 °C to 60 °C, suggesting specificity. The GC content should not be <40% or >60% to ensure primer specificity [5].

The primers were specific at temperatures between 50 °C and 60 °C, and optimal at 56 °C to 60 °C. Multiple bands appeared at a temperature of 61 °C. The shape of the band was curved, suggesting that the mixture was not homogeneous because the reagents were not mixed well. But, when the primers for patient and normal samples were used at 57 °C, the results may have been biased. Single DNA bands appeared at 57 °C, which was determined as the optimal temperature. Amplification of exon 4 at 57 °C yielded products of 645 bp in length.

Sequencing analysis was conducted using the BioEdit Sequence Alignment Editor and Chromas software. BioEdit software is used for sequence alignment, whereas Chromas software is used to generate graphs of DNA sequences [8].

As shown in Table 2, there were 27 variants between patients with PTPS deficiency and normal controls. Mutation analysis identified six silent mutations, 20 substitutions, and one deletion. Five of these 27 variations were described previously by Thony and Blau in 2006 based on SNP data collected from The National Center for Biotechnology Information website (https://www.ncbi.nlm.nih.gov/snp/). One of the variants was a deletion of the base sequence 92769277 with notation c.201_202delGG, which caused phenotypical changes.

As shown in Table 3, there were six mutations, which were presumed to be newly discovered. These mutations were either silent or substitutions. A silent DNA mutation is defined as a change in one amino acid codon, but does not change the order of the amino acids. A substitution is a DNA mutation in which there is a change in the codon sequence that results in a change in the order of the amino acid sequence. Substitution mutations include point and missense mutations. A point mutation is a change of one codon that can change the order and yield of amino acids, thereby affecting protein function [9]. However, it remains uncertain as to whether an amino acid change will affect the activities of the PTPS enzyme [2].

5. Conclusion

Mutations to exon 4 of the PTS gene in 2 patients with PTPS enzyme deficiency were identified. The optimal temperature for amplification of exon 4 was determined to be 57 °C. The purity of the extracted DNA was 1.7–2.0 at OD 260/280 nm, which was sufficient for analysis with BioEdit and Chromas software. Six new mutations and 27 variations were identified in exon 4, which included five variations that were previously described.

Acknowledgement

This research was funded by PITTA Grant from Direktorat Riset dan Pengabdian Masyarakat (DRPM) UI 2017. We thank the team of Human Genetic Research Cluster, Indonesian Medical Education and Research Institute (IMERI), Universitas Indonesia for their support and cooperation. We also thanked the academic counselor of the Department of Biology, Faculty of Medicine, Universitas Indonesia, for technical advice.
References
[1] Thöny B and Blau N 2006 Mutations in the BH4-metabolizing genes GTP cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase, sepiapterin reductase, carbinolamine-4a-dehydratase, and dihydropteridine reductase *Hum. Mutat.* 27 870-8
[2] Thöny B and Blau N 1997 Mutations in the GTP cyclohydrolase I and 6-pyruvoyl-tetrahydropterin synthase genes *Hum. Mutat.* 10 11-20
[3] Kluge C, Brecevic L, Heizmann C W, Blau N and Thony B. 1996 Chromosomal localization, genomic structure and characterization of the human gene and a retropseudogene for 6-pyruvoyl-tetrahydropterin synthase *Eur. J. Biochem.* 240 477-84
[4] Chiu Y H, Chang Y C, Chang Y H, Niu D M, Yang Y L, Ye J, Jiang J, Okano Y, Lee D H, Pangkanon S, Kuptanon C, Hock N L, Chiong M A, Cavan B V, Hsiao K J and Liu T T 2012 Mutation spectrum of and founder effects affecting the PTS gene in East Asian populations *J. Hum. Genet.* 57 145-52
[5] Sambrook J and Green M R 2012 *Molecular Cloning: A Laboratory Manual* vol 4. (New York: Cold Spring Harbour Laboratory Press)
[6] Sasmito K D E, Kurniawan R and Muhimmah I. 2004 Seminar Nasional Informatika Medis (SNIMed) V 2004
[7] GenScript 2017 Molecular Cloning Handbook: Past, Present, and Future Techniques of Molecular Cloning (New York: GenScript USA Inc)
[8] Electropherogram Software Guide 2017 Micromon DNA Sequencing: Viewing & Editing (Australia: Monash University) https://platforms.monash.edu/micromon/index.php?option=com_content&view=article&id=68&Itemid=170
[9] Electropherogram Trace Files 2017 https://platforms.monash.edu/micromon/images/stories/forms-and-userguides/electropherogram%20software.pdf.
[10] Chamary J V and Laurence H D 2009 The price of silent mutations *Sci. Am.* 300 46-53.