Novel variations in Exon 4 of the iduronate 2-sulfatase gene in six Indonesian patients with mucopolysaccharidosis type II

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Abstract. Mucopolysaccharidosis type II (MPS II) is an X-linked lysosomal storage disorder caused by the inability to produce iduronate 2-sulfatase (IDS). We genotyped exon 4 of the IDS gene in Indonesian patients with MPS II. To detect IDS gene mutations, DNA samples from 6 patients with MPS II and 49 normal individuals were analyzed with direct sequencing of exon 4. One novel mutation (c.489G>A) was identified in four of six patients. Protein analysis of these mutations revealed no amino acid sequence changes (silent mutation). Another 20 variations were found in normal individuals, including missense, nonsense, and silent mutations. The discovery of this novel mutation provides new mutational data for MPS II, whereas the identified variations strengthen the single nucleotide polymorphism database for the IDS gene. These mutation and variation data should be useful for identifying biomarkers for diagnosing MPS II.

1. Introduction
Mucopolysaccharidosis (MPS) comprises a group of syndromes arising from genetically determined deficiencies of enzymes involved in glycosaminoglycan (GAG) degradation. GAGs, long-chain complex carbohydrates that are linked to proteins to form proteoglycans, are abundant in connective tissue. Enzymes degrade GAGs by cleaving the terminal sugars from the polysaccharide chains disposed along the polypeptide or core protein. The absence of such enzymes leads to the accumulation of these polysaccharide chains in the lysosomes of all cell types, tissues, and organs in the body. The GAGs that accumulate in patients with MPS are dermatan sulfate, heparan sulfate, keratan sulfate, and chondroitin sulfate. There is MPS type IX, it should be MPSs range from MPS type I to MPS type IX [1].

MPS type II (MPS II) is characterized as an X-linked recessive inherited disorder, differentiating it from other MPSs, which are inherited as an autosomal recessive trait. MPS II is also characterized by dermatan sulfate and heparan sulfate accumulation in lysosomes due to iduronate 2-sulfatase (IDS) deficiency. This accumulation results in the specific progressive pathological lysosomal storage of
GAGs in all cell types, tissues, and organs [2]. As the disorder progressively develops, patients with MPS II experience various clinical manifestations, including skeletal and cardiac abnormalities, hepatosplenomegaly, hernia, hearing difficulty, and occasional neurological involvement [3].

As an X-linked recessive disorder, MPS II was believed to only affect males. However, a case report by Tuschi et al. in 2005 described female patients diagnosed with MPS II [4]. The case report revealed that affected females generally exhibited low IDS activity and attenuated clinical phenotypes, although the somatic abnormalities can be severe in some individuals. In affected heterozygous females, skewed inactivation of the X-chromosome prevents expression of the normal allele.

IDS is encoded by the IDS gene located at Xq28. The IDS gene is 28,365 bp in length and features nine exons. Its mRNA length is 7595 bp. According to Mashima and Okuyama, a genotype–phenotype correlation exists between MPS II disease severity and pathogenic mutations in the IDS gene.5 IDS gene mutations can cause significant changes in the enzyme structure if the alteration is substantial (gross deletion, splicing, and complex rearrangement) or occurs in a conserved region. In particular, Asn144 and His145 in exon 4 are consistently found in vertebrate IDS sequences.6

Previous studies revealed that MPS II is common in Asian countries, such as Taiwan, China, Korea, Japan, and the Philippines [7–11]. Although the genotypes of patients with MPS II in these countries have been reported, no data exist for Indonesian patients. In this study, we identified variations in exon 4 of the IDS gene to provide genetic data about Indonesian patients with MPS II.

2. Materials and Methods
2.1 DNA samples and isolation
DNA was obtained from the blood samples of 6 patients with MPS II as well as 49 normal individuals from Cipto Mangunkusumo Hospital. DNA isolation was performed using a Genomic DNA Mini Kit for Blood/Cultured Cell GB004 (Geneaid). The isolated DNA was then quantified spectrophotometrically to determine its concentration and purity.

2.2 Genetic analysis
Specific primers (F: 5’ TCATCAGGGCTTAGGGACCA 3’; R: 5’ CCAGCTTCACAGAACATGCAG 3’) were designed using NCBI’s Primer-Blast. These primers were optimized using gradient PCR, which was performed using six different annealing temperatures (55 °C, 57 °C, 59 °C, 61 °C, 63 °C, and 65 °C). The PCR mixture included 5.3 µL of ddH2O, 0.1 µL each of the forward and reverse primers, 2.5 µL of My Taq mix, and 2 µL of DNA template from one normal individual in a total volume of 10 µL. The PCR program was as follows: initial denaturation at 95 °C for 60 s; 40 cycles of denaturation at 95 °C for 15 s, annealing for 15 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 10 min. Then, 5 µL of the PCR reaction products were visualized via agarose gel electrophoresis (AGE) and stained with Gel Red.

A PCR mixture with a top-up volume of 50 µl per tube was created for sequencing. The exon 4 sequence was amplified with an annealing temperature of 59.5 °C. PCR was performed as described for the optimization step. After verification using AGE, the samples were then sent to First Base Sequencing in Singapore, and the results were analyzed.

3. Results
3.1 DNA isolation result
The isolation process yielded DNA at concentrations of 1.40–2939 ng/µL. The purity of DNA calculated using wavelengths of 260/280 nm ranged 1.35–1.90. Conversely, the purity of DNA at wavelengths of 260/230 nm ranged 0.13–1.12. Most of the samples had an OD260/OD280 ratio of 1.7–2.0 and a small absorbance value at 230 nm. These data indicate that the isolated DNA is sufficiently pure to serve as a template [12].

3.2 PCR result
Exon 4 gradient PCR produced single bands for the target sequence at each annealing temperature as
shown in Figure 1. The result at 55–63 °C revealed bright and clear bands, but the brightness began to dim at 65 °C. On the basis of this result, it can be concluded that the optimal annealing temperature for exon 4 primers is 63 °C.

Amplification of exon 4 was performed using uniform thermal PCR at 59.5 °C. The data revealed no differences in the band at 59–63 °C, and thus, amplification at 59 °C was expected to produce the same result. Figure 2 shows a clear single band at the specific location of the target sequence (389 bp).

Figure 1. Visualization of the result of gradient PCR of exon 4 (optimization)

Figure 2. Visualization of the result of PCR of exon 4 (top-up volume)

3.3 Mutation analysis

The alignment data revealed several variations in exon 4 of the IDS gene in both patients with MPS II and normal individuals (control). The identified variations are listed in Table 1.

Six mutations have previously been reported in exon 4 of the IDS gene, including three missense mutations, one silent mutation, one small deletion, and one gross deletion. Meanwhile, 21 novel exon 4 variations were found in samples from Indonesian subjects in this study, including 11, 9, and 1 missense, silent, and nonsense mutations, respectively. These variations were mostly found in normal individuals, whereas only one variation (c.489G>A) was identified in patients with MPS II. There were two alterations at the same site (c.460T>G and c.460T>C) that led to a change in the amino acid sequence (missense). Although the identified missense and nonsense mutations can lead to structural changes to the enzyme, these mutations were considered normal variation because they were also found in healthy subjects and were thus considered to have no effect.

Table 1. Variation in exon 4 of the iduronate 2-sulfatase gene

| Sample Code | Position | Mutation | Reference          |
|-------------|----------|----------|--------------------|
| c.438C>T    | Silent   |          | Li et al. 1999     |
| c.425C>A    | Missense |          | Amartino et al. 2014|
| c.469C>T    | Missense |          | Amartino et al. 2014|
| c.425C>T    | Missense |          | Christiakov et al. 2014|
| c.500del    | Deletion |          | Vafiadaki et al. 1998|
Table 1 Continue

| Sample Code | Position     | Mutation | Reference |
|-------------|--------------|----------|-----------|
|             | whole exon   | gross deletion | Zhang et al. 2011 |
| 13, 28, 33, 46, 57 | c.422T>A | Missense deletion | This study |
| 35          | c.429T>A | Silent | This study |
| 35          | c.431A>C | Missense | This study |
| 13          | c.434A>G | Missense | This study |
| 22          | c.438C>G | Silent | This study |
| 35          | c.442G>A | Missense | This study |
| 13, 14, 20, 28, 31, 33, 35, 44, 46, 47, 52, 57 | c.447T>C | Silent | This study |
| 33          | c.450G>C | Silent | This study |
| 13, 28, 33, 46, 57 | c.453T>A | Nonsense | This study |
| 35          | c.455G>C | Missense | This study |
| 13, 14, 20, 28, 31, 33, 46, 55, 57 | c.460T>G | Missense | This study |
| 28, 33      | c.468A>C | Silent | This study |
| 35          | c.477T>C | Silent | This study |
| 13, 20, 28, 33, 44, 46, 47, 57 | c.484T>C | Missense | This study |
| 24, 27, 43  | c.487G>A | Missense | This study |
| 2, 4, 5, 51, 7, 8, 9, 13, 14, 15, 16, 18, 20, 21, 22, 24, 25, 27, 28, 29 | c.489G>A | Silent | This study |
| 13, 28, 31, 33, 44, 46, 57 | c.492G>A | Silent | This study |
| 35          | c.496G>A | Missense | This study |
| 13, 28, 44  | c.502A>C | Missense | This study |

4. Discussion
Using genetic analysis of exon 4 of the IDS gene, we identified 21 novel variations, one of which was found in four of six patients with MPS II. The c.489G>A variation changed the codon from GAG to GAA. However, both codons encode glutamic acid. The c.489G>A mutation was also found in 16 of 49 normal samples. The silent mutation occurred at frequencies of 42.1% in males (8/19) and 26.7% in females (8/30). These results provide evidence that c.489G>A is an intragenic polymorphism (variation) carried by Indonesians.

Silent mutations were also identified at eight other sites in normal samples. The c.447T>C variation was the second most common mutation, being detected in 12/49 normal samples, followed by c.492G>A (7/49). Meanwhile, the remaining variations were only detected in one sample each. The only silent mutation that has been reported in exon 4 of the IDS gene in patients with MPS II is c.438C>T [13]. This mutation changes the codon from ACC to ACT, both of which encode threonine. Similarly, a silent
mutation was also found at the same site (c.438C>G) in the IDS gene in normal subjects in this study. The cysteine base was changed into guanine instead of thymine without changing the amino acid sequence.

In addition to the silent mutations, several other mutations have been reported in exon 4 of the IDS gene. The previously reported missense mutations include c.425C>A, c.469C>T, and c.425C>T [14,15]. The c.469C>T mutation was found in patients with MPS II and a severe phenotype, and it changes the amino acid from proline (nonpolar) to serine (polar) [14]. A single base deletion (c.500del) has also been detected [16]. Small deletions usually cause frameshift mutations that can significantly alter the structure of the resulting enzyme. Conversely, deletion of an entire exon [16,17] can cause the loss of a large part of the IDS enzyme complex. Thus, it is associated with a severe MPS II phenotype, although this gross deletion was also found in a patient with an attenuated phenotype [17].

In this study, we identified 11 new missense and 1 nonsense mutation. These mutations were only detected in samples from normal subjects. Some of these variations (c.431A>C and c.434A>C) alter the conserved amino acids Asp144 and His145, indicating that variants exist for these sites despite their conserved status in vertebrates [16]. Missense and nonsense mutations are known to alter the amino acid sequences of gene products, but the discovery of these mutations in normal samples illustrates that these alterations are polymorphisms of the IDS gene in Indonesians because the phenotype was not altered.

5. Conclusion
The one novel (c.489G>A) mutation identified in exon 4 of the IDS gene in patients with MPS II is a silent mutation, providing additional mutational data about the disease. Furthermore, the 20 variations found in a normal individual contribute new information about single nucleotide polymorphisms in the gene. These data should be useful for identifying biomarkers for diagnosing MPS II.

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References
[1] Kumar V, Abbas A K and Aster J C 2015 Robbins and Coltran Pathologic basis of disease ed. V Kumar, A K Abbas and J C Aster (Philadelphia: Elsevier Saunders) p 154-155.
[2] Wraith J E, Scarpa M, Beck M, Bodamer O A, De Meirleir L and Guffon N 2008 Mucopolysaccharidosis type II (Hunter syndrome): Clinical review and recommendations for treatment in the era of enzyme replacement therapy Eur. J. Pediatr. 167 267-77
[3] Muenzer J, Beck M, Eng C M, Escolar M L, Giugliani R, Guffon N H, et al. 2009 Multidisciplinary management of Hunter syndrome Pediatrics 124 18-8
[4] Tuschl K, Gal A, Paschke E, Kircher S and Bodamer O A 2005 Mucopolysaccharidosis type II in females: Case report and review of literature Pediatr. Neurvol. 32 270-2
[5] Mashima R and Okuyama T 2016 Molecular diagnosis of 65 families with mucopolysaccharidosis type II (Hunter syndrome) characterized by 16 novel mutations in the IDS gene: Genetic, pathological, and structural studies on iduronate-2-sulfatase J. Rare Dis. Res. Treat. 2 43-6
[6] Holmes R S 2017 Comparative studies of vertebrate iduronate 2-sulfatase (IDS) genes and proteins: Evolution of a mammalian X-linked gene. 3 Biotech. 7 22
[7] Lin S P, Chang J H, Lee-Chen G J, Lin D S, Lin H Y and Chuang C K 2006 Detection of hunter syndrome (mucopolysaccharidosis type II) in Taiwanese: Biochemical and linkage studies of the iduronate-2-sulfatase gene defects in MPS II patients and carriers Clinica Chimica Acta 369 29-34
[8] Zhang H, Li J, Zhang X, Wang Y, Qiu W, Ye J, et al. 2011 Analysis of the IDS gene in 38 patients with Hunter syndrome: The c.879G>A (p.Gln293Gln) synonymous variation in a female create exonic splicing PLoS ONE 6 1-8

[9] Cho S Y, Sohn Y B and Jin D K 2014 An overview of Korean patients with mucopolysaccharidosis and collaboration through the Asia Pacific MPS Network Intractable Rare Dis. Res. 3 79-86

[10] Kosuga M, Mashima R, Hirakiyama A, Fuji N, Kumagai T, Seo J H, et al 2016 Molecular diagnosis of 65 families with mucopolysaccharidosis type II (Hunter syndrome) characterized by 16 novel mutations in the IDS gene: Genetic, pathological, and structural studies on iduronate-2-sulfatase Mol. Genet. Metab. 118 190-7

[11] Chiong M A D, Canson D M, Abacan M A R, Baluyot M M P, Cordero C P and Silao C L T 2017 Clinical, biochemical, and molecular characteristics of Filipino patients with mucopolysaccharidosis type II–Hunter syndrome Orphanet J. Rare Dis. 12 1-11

[12] Green M R and Sambrook J 2012 Molecular cloning a laboratory manual vol 4 (New York: Cold Spring Harbor Laboratory Press) p 541.

[13] Li P, Bellows A B and Thompson J N 1999 Molecular basis of iduronate-2-sulphatase gene mutations in Hunter syndrome J. Med. Genet. 36 21-7

[14] Amartino H, Ceci R, Masllorens F, Gal A, Arberas C and Bay L 2014 Identification of 17 novel mutations in 40 Argentinean unrelated families with mucopolysaccharidosis type II (hunter syndrome) Mol. Genet. Metab. Rep. 1 402-6

[15] Chistiakov D A, Kuzenkova L M, Savost’anov K V, Gevorkyan A K, Pushkov A A, Nikitin A G, et al 2014 Genetic analysis of 17 children with Hunter syndrome: Identification and functional characterization of four novel mutations in the iduronate-2-sulfatase gene J. Genet. Genomics 41 197-203

[16] Vafiadaki E, Cooper A, Heptinstall L E, Hatton C E, Thornley M and Wraith J E 1998 Mutation analysis in 57 unrelated patients with MPS II (Hunter’s disease) Arch. Dis. Child 79 237-41

[17] Zhang H, Li J, Zhang X, Wang Y, Qiu W, Ye J, et al 2011 Analysis of the IDS gene in 38 patients with Hunter syndrome: The c.879G>A (p.Gln293Gln) synonymous variation in a female create exonic splicing PLoS ONE 6 1-8