Mutation identification of unreported and reported mutation profile in exon 7 of N-Acetylgalactosamine-6-Sulfates (GALNS) gene of mucopolysaccharidosis type IV A (MPS IVA) patients in Indonesia

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Abstract. Mucopolysaccharidosis type IVA (MPS IVA), is an autosomal recessive genetic disorder because of N-acetylgalactosamine-6-sulfate deficiency which causes keratan sulfate and chondroitin sulfate to not degrade in lysosome. MPS IVA was caused of GALNS gene which located in chromosome 16q24.3 with the most frequent mutation occurrence in exon 7. There are no report or publication about MPS IVA or GALNS gene mutation in Indonesia, therefore this research is aimed to analyze the mutation profile in exon 7 of the GALNS gene in MPS IVA patients in Indonesia. The DNA from blood samples of four patients and three control samples from RSUPN Cipto Mangunkusumo were analyzed. Amplification by polymerase chain reaction was done after designing the primer. Furthermore, electrophoresis and sequencing analysis has been performed. The result shows that there is silent mutation c.708C>T and nonsense mutation c.751C>T. The silent mutation is categorized as a benign variant, while the nonsense mutation is categorized as a pathogenic variant because it may affect protein features and cause neuromuscular disorder. The silent and nonsense mutation that were found were already reported by Laradi and Morrone, but has not been reported in Indonesia. Further experiment was needed to find other mutation in other genes.

1. Introduction
MPS IVA (Morquio A Syndrome) is an autosomal recessive disease due to N-acetylgalactosamine-6-sulfate deficiency which causes keratan sulfate and chondroitin sulfate compounds to not degrade in lysosomes, making these compounds accumulate in the lysosomes [1]. Mucopolysaccharidosis IVA
results from the mutation of the \textit{GALNS} gene with a cytogenetic location of chromosome 16q24.3. The \textit{GALNS} gene has a size of 43237 bp which is composed of 14 exons with a coding sequence length of 1568 bp. The \textit{GALNS} gene produces 1566 nucleotides of mRNA and 522 amino acids [2].

There are several types of mutations in the \textit{GALNS} gene such as missense, nonsense, deletion, gross deletion, small insertion, duplication, splice site, and silent mutation [3]. The most common mutations are missense & small deletion. There are a total of 328 \textit{GALNS} gene mutations recorded in 2016 [1]. Symptoms of MPS IVA ranges from decreased motor function, stunted posture and growth, hypoplasia odontoid, skeletal abnormalities, typical cranial facial features, hepatosplenomegaly, joint hypermobility, and respiratory and hearing disorders. Depression of the spinal cord by the vertebrae, the presence of respiratory disorders, and heart valve problems are causing high morbidity and mortality in MPS IVA patients [2]. The prevalence of MPS IVA globally ranges from 1: 76000 to 1: 640000. The severity of MPS IVA is vary from mild, moderate, and severe [4].

The exon 7 was the most frequent mutation location after exon 8 based on research of Bidchol et al. There are 24 mutations in exon 7 based on \textit{GALNS} Variants Database [5]. There are deletion (6), missense [7],[8], and one common polymorphism that were found in exon 7 [9],[10]. There are no records of mutation profile of MPS IVA in Indonesia. This research is aimed to analyze mutation in exon 7 of the \textit{GALNS} gene from Indonesian MPS IVA patients to record a new database in Indonesia.

2. Methods

2.1. DNA isolation and quantification
Four blood samples of MPS IVA patients and three blood samples of non-MPS IVA patients from Indonesia were collected in RSUPN Cipto Mangunkusumo and the DNA was isolated using Genomic DNA Mini Kit GB 100 [Geneaid] according to the manufacturer’s protocol. The DNA concentration and purity were measured using NanoDrop plate in Varioskan LUX Microplate Reader [Thermo Scientific].

2.2. Primer design and polymerase chain reaction
The primer was designed using Primer3, BioEdit, and NCBI Primer BLAST. Primer design was done by identifying some characteristic such as melting temperature (Tm), and secondary structure. Primers were optimized through PCR to identify the optimum annealing temperature (Ta).

2.3. Electrophoresis and sequencing
Agarose gel 1.5% was used in electrophoresis. 10 µl of PCR products were visualized through electrophoresis. 40 µl of PCR products and forward primer were sent to 1st Base in Singapore to be sequenced through Sanger sequencing.

2.4. Bioinformatics analysis
Sequencing results were analyzed using BioEdit and FinchTV. BioEdit was used to align the sequences. \textit{GALNS} gene sequence (NC 000016.10) was used as reference sequence, to detect mutations that occurred. FinchTV was used to see the quality of the chromatogram.

3. Results and Discussion

3.1. DNA isolation and quantification
The DNA concentration range is 43.02-55.51 ng/µl and categorized as pure because the range of pure DNA samples through A260/A280 ratio is ranged from 1.88-1.94. The value of A260/A280 ratio less than 1.8 shows that there is RNA contaminant and the value more than 1.8 shows that there is protein contaminant [11].
3.2. Primer design and polymerase chain reaction

The primer sequences can be seen in Table 1. The primer pair are categorized as a good primer because it contains guanine and cytosine in the sequence with the range of 40%-60%, with the melting temperature still ranged inside 50°C-60°C, and no secondary structure were found [12]. Although the primers contain cross dimer of -4.54 kcal/mol, it still can be tolerated because the value is bigger than -6 kcal/mol.

| Primer Name | Nucleotide Sequence | Length (bp) | GC Content (%) | Tm (°C) | Secondary Structure (kcal/mol) | PCR Product (bp) |
|-------------|---------------------|-------------|----------------|---------|-------------------------------|------------------|
| GALNS Exon 6-7 F | TCAGGGAGAAGGGG GACTTT | 19          | 52.63          | 56.68   | -4.54                         | 802              |
| GALNS Exon 6-7 R | ATGAAGGACAGAG CCAGCAC | 20          | 55             | 57.26   |                               |                  |

3.3. Electrophoresis and sequencing

Electrophoresis result can be seen in (Figure 1a, 1b, and 1c). It shows that the optimum annealing temperature is 63°C. The blocking band is caused by too much DNA concentration, the electrical current used is too high, and/or there may be a dimer primer formed. Multiband and smear appeared due to annealing temperature is too low, besides contaminants, too many PCR cycles, and/or the primer concentration. The curvature may occur because the PCR reagent has not been completely homogenized and the electrical current may be unstable when electrophoresis were performed [13].

![Figure 1. Optimization Result of PCR](image)

3.4. Bioinformatics analysis

All samples were analyzed by identifying the chromatogram quality using FinchTV. The chromatogram quality from P1, P2, P3, N4, N5, and N6 are categorized as good. There is one sample (P4) that has a bad chromatogram quality, so P4 was removed from bioinformatics analysis. The GALNS reference sequence (NC_000016.10) was aligned with the sample sequences using BioEdit. The alignment results (Figure 2) shows that there are two reported mutations; silent mutation
(c.708C>T) from P1, P2, P3, and N4, which is not altered the 236th amino acid histidine (p.H236H) and nonsense mutation (c.751C>T) from P3 which altered arginine to be early stop codon (R251*).

Figure 2. DNA alignment result of GALNS reference sequence and samples (P1, P2, P3, N4, N5, N6)

Figure 3. Amino acid alignment result of GALNS reference sequence and samples (P1, P2, P3, N4, N5, N6)

The software result shows that the pathogenicity probability value of c.751C>T is 6 based on MutationT@ster and -9.695 based on Provean. The silent mutation is an unreported mutation in the database, but was reported in Laradi et al. in 2006 [10] and Pajares et al. in 2012 [14]. The report from Laradi showed that the MPS IVA is caused of consanguineous mating remains frequent. This mutation was considered as a benign mutation based on MutationT@ster [15]. The nonsense mutation is reported in Morrone et al. in 2014, severely affect the GALNS protein function by introducing early stop codon, may affect protein features, causing neuromuscular disease (NMD), and considered as a pathogenic variant based on MutationT@ster [15] and Provean [16]. The results were supported because P1 is a severe MPS IVA patient and P3 is not able to walk.

Table 2. Mutation list

| Coding Sequence | Event | Individu | Mutation Type | Amino Acid |
|-----------------|-------|----------|---------------|------------|
| c.708C>T        | Exon 7| P1, P2, P3, N4 | Silent | p.H236H |
| c.751C>T        | Exon 7| P3       | Nonsensse    | p.R251*    |

Table 3. Pathogenicity prediction

| Allele Name | MutationT@ster | Provean | Status            |
|-------------|----------------|---------|------------------|
| R251*       | 6              | -9.695  | Pathogenic (deleterious) |
4. Conclusion
Mutation identification in exon 7 of the GALNS gene in MPS IVA patients and non-patients in Indonesia were successfully identified. There are silent and nonsense mutation, there is also silent variant present. The silent mutation is benign and the nonsense mutation is pathogenic, may affected protein features and can cause neuromuscular disease. Further research of mutation analyzing about other gene is needed to get more detailed, so it can be full database mutation profile about MPS IVA patients in Indonesia.

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