Original Article

Identification of a Novel Variant in Exon 5 of Galactosamine (N-acetyl)-6-Sulfatase Gene in Mucopolysaccharidosis IVA Patients in Indonesia

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Abstract

Objective: Mucopolysaccharidosis IVA (MPS IVA), or Morquio A syndrome, is a lysosomal storage disorder caused by a deficiency of galactosamine (N-acetyl)-6-sulfatase (GALNS) enzyme that leads to the accumulation of keratan sulfate and chondroitin-6-sulfate in the lysosome and eventually in the tissue or organ damaged. This enzyme deficiency occurs because of mutations in the galactosamine (N-acetyl)-6-sulfatase (*GALNS*) gene located at locus 16q24.3. *GALNS* comprises 14 exons, has a size of ~43 kb, and encodes 522 amino acids. Currently, 47 of 368 mutations have been detected in exon 5, indicating that this region is a hotspot of mutations. The objective of this study was to analyze the mutations in exon 5 of *GALNS* in MPS IVA patients in Indonesia. **Materials and Methods:** Genomic DNA was isolated from fresh blood samples obtained from patients with MPS IVA and normal individuals at Cipto Mangunkusumo Hospital. Exon 5 of *GALNS* was amplified using a pair of specific primers, and polymerase chain reaction products were sequenced using an automated sequencing technique. **Results:** We found a novel missense mutation c.503G>T that alters the amino acid at position 168 from glycine to valine (G168V). Three previously reported variations identified in this study are c.510T>C (Y170), c.566 + 5T>C, and IVS5 + 134G>A. **Conclusion:** This finding provides new data about variants in exon 5 of *GALNS*. Further, research is needed to identify variations in other exons and to map the mutation profile in MPS IVA patients in Indonesia.

Keywords: *GALNS,* mucopolysaccharidosis IVA, mutation, variation

Introduction

Mucopolysaccharidosis IVA (MPS IVA; OMIM 253000), or Morquio A syndrome, is an autosomal recessive disorder caused by loss of activity of a lysosomal enzyme, N-acetylgalactosamine-6-sulfatase (*GALNS*).[1] This enzyme hydrolyzes sulfate ester groups from N-acetylgalactosamine-6-sulfate in keratan sulfate (KS) to chondroitin-6-sulfate (C6S).[2] The deficient activity of *GALNS* enzyme causes progressive accumulation of KS and C6S in the ligaments, bone, and cartilage, which leads to the most common clinical manifestation, such as skeletal immobility, respiratory, and cardiac abnormalities.[1,3] *GALNS* enzyme deficiency occurs because of mutations in the galactosamine (N-acetyl)-6-sulfatase (*GALNS*) gene located at locus 16q24.3. *GALNS* comprises 14 exons, has a size of ~43 kb, and encodes 522 amino acids from 1566 base pairs coding sequence of mRNA transcript.[4] Morrone *et al*. documented 277 identified gene alterations and developed the *GALNS* Mutation Database (http://galns.mutdb.org/) as a locus-specific database.[5] To date, 368 mutations have been reported in the *GALNS* mutation database, and 47 of them have been identified in exon 5. The identified mutations include 39 different base substitutions and eight frameshift mutations. A recent report suggests that exon 5 is a hotspot region for the mutation.[6] Exon 5 contributes to the synthesis of amino

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acid residues located at domain 1, and about 35 of 47 gene alterations identified in exon 5 are reported to be pathogenic. This finding is consistent with the report by Rivera-Colón *et al*., who found that domain 1 is a critical scaffold for the overall structure and functionality of *GALNS* enzyme.[2]

The distribution of the mutation and allele frequency differs between various ethnic groups. The most common allele found in Chinese patients is M318R, whereas I113f alteration is the most prevalent in Irish patients.[5] This fact suggests that mutation profile analysis is needed, especially in exon 5 of *GALNS* in MPS IVA patients in Indonesia. Hence, in this study, we analyzed the mutations in exon 5 of *GALNS* in MPS IVA patients in Indonesia. We also performed bioinformatics analyses to assess the pathogenicity of the identified missense variant.

Materials and Methods

Sample collection and DNA isolation

Four previously diagnosed patients with MPS IVA and 50 healthy individuals agreed to participate and signed an informed consent form. Blood was collected by a health-care professional who followed the procedure based on the approval of the FMUI-RSCM Research Ethical Committee. Genomic DNA was isolated from fresh blood samples using a blood/cell DNA mini kit (Geneaid) according to the manufacturer's protocol. The concentration and A_{260}/A_{280} purity of eluted genomic DNA were measured using Varioskan Microplate Reader (Thermo Fisher Scientific).

Primer design and polymerase chain reaction amplification

A pair of specific primers to amplify exon 5 of *GALNS* was designed using Primer3 (http://primer3.ut.ee/) software with the predetermined primer melting temperature (Tm) and complementarity conditions [Table 1]. NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was then used to check the specificity before the amplification of the whole region of exon 5 and adjacent intronic sequences. Selected primers were optimized using gradient polymerase chain reaction (PCR) (Applied Biosystems) with annealing Tms in the range of 53°C–63°C. PCR amplification using MyTaq™ HS Red Mix PCR mixture was performed under optimized annealing conditions with the following stages: initial denaturation at 95°C for 60 s, 40 cycles at 95°C for 15 s, annealing at 64°C for 15 s, extension at 72°C for 30 s, and completion by final extension at 72°C for 10 min. A PCR product which sized 443 bp was visualized using 1.5% agarose gel electrophoresis, and then, processed for sequencing.

DNA sequencing

About 40 µL of PCR products were shipped to First Base Sequencing Service, Kuala Lumpur, Malaysia. DNA was sequenced using an automated sequencing technique, which was initiated by PCR cleanup and followed by cycle sequencing using PCR forward primer and BigDye Terminator v3.1 sequencing chemistry (Applied Biosystems) [California, USA]. Extension products were then purified and loaded onto capillary electrophoresis for separation and base calling.

Bioinformatics analysis

All sequencing results were aligned with the *GALNS* reference sequence (NC_000016.10) using BioEdit 7.0.5.3 to detect any possible substitutions, insertions, or deletions in the region of exon 5 and adjacent intronic sequences. DNA sequences were then translated to amino acid sequences to detect amino acid alterations. Bioinformatics analyses were performed using MutPred, Provean, and PMUT and to predict the pathogenicity of the variants identified. CUPSAT was also used to measure the destabilizing potential of the detected variants on *GALNS* enzyme. The potential of splicing site alterations was also analyzed using Human Splicing Finder.

Results and Discussion

Sample collection and DNA isolation

The concentration and A_{260}/A_{280} purity of isolated genomic DNA were ranged from 43.02–977.90 ng/µ L to 1.76–1.94, respectively. Purity was measured at a wavelength of 260 nm, because nucleic acids have a maximum absorbance at this wavelength. The A_{260}/A_{280} purity of nucleic acids is typically in the ratio of ~1.8 and ~2.0 for pure DNA and RNA, respectively.[7] Purity values below 1.8 indicate the presence of contaminants such as phenol. The A_{260}/A_{280} purity may be critical, because the presence of excessive contaminants can inhibit downstream application.[8]

Primer design and polymerase chain reaction amplification

A pair of specific primers was selected with ideal sequence length, guanine-cytosine (GC) content, Tm, and with no possible secondary structure. The Tm was determined by GC content and sequence length. To ensure specificity, a good primer will have a Tm in the range of 50°C–65°C, 40%–60% CG content, and 18–30 nucleotides in length. Self-complementarity and cross-complementarity should be avoided to ensure no interference during primer annealing to the DNA template.[9]

Gradient PCR showed that a specific target with a size of 443 bp was amplified at the annealing Tm of 55°C–63°C [Figure 1].

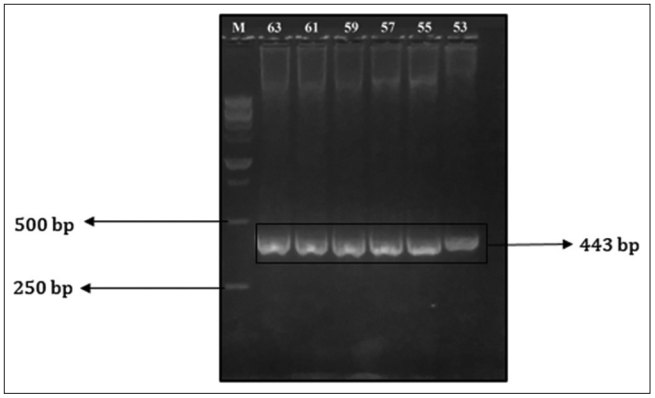

Figure 1: Polymerase chain reaction optimization result for GALNS-ID Exon 5 primers

However, we verified an annealing Tm at 64°C, because a 1°C increment did not significantly reduce the copy number of the PCR product. Therefore, we considered the optimum annealing Tm to be 64°C. Visualization of the PCR products showed consistent results as a single band [Figure 2].

DNA sequencing results and bioinformatics analysis

Unclear peaks and low-quality chromatograms were omitted to provide high-quality DNA sequences. Based on the alignment results, four base substitutions were identified. A newly identified missense mutation c.503G>T, which alters the amino acid at position 168 from glycine to valine (G168V), was found in patient 2 [Figure 3]. Three different amino acid alterations have been previously reported at the same position. Replacement of two guanines by two thymines c.502_503GG>TT (G168 L) was identified by Wang *et al*. in Chinese patients with the severe growth phenotype.[10] Base substitution at c.503G>A (G168E) was reported by EGL genetics, but the significance is unknown.[11] Another amino acid alteration such as G168R was also detected by Bunge *et al*. using allele-specific oligonucleotides analysis but was also classified as having uncertain significance.[12,13]

Gly168 is not located in the active site of *GALNS* enzyme;[2] however, the presence of previously identified amino acid alterations from glycine (G23R, G47R, G96C, G96V, G116S, G155E, G155R, G168R, G247D, G290S, G301C, G309R, G340D, and G421E) correlates with the severe growth phenotype.[4] MutPred, Provean, and PMUT have predicted G168V allele as pathogenic. CUPSAT has predicted that allele G168V also has a potential destabilizing effect on the *GALNS* enzyme [Table 2].[14] This prediction is consistent with Rivera-Colón *et al*., who found that MPS IVA is caused primarily by disruption of the hydrophobic core of the protein, which causes the protein to fold incorrectly.[2]

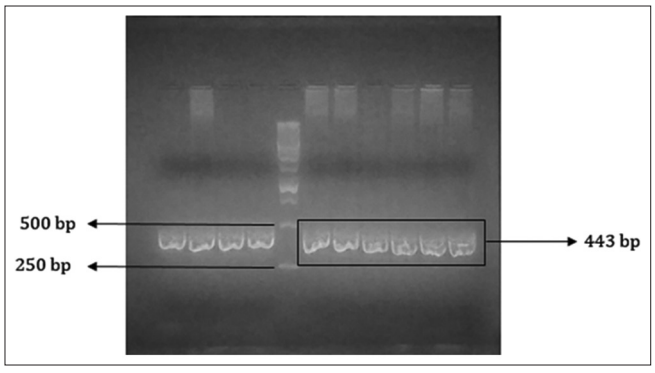

Figure 2: Visualization of polymerase chain reaction products

Another variant was identified in patient 1 as c.510T>C (Y170) and has been previously described by Tomatsu *et al*. in 1998. This variant has been reported as benign/likely benign, because it does not cause amino acid alterations in the enzyme.[15,16] In this study, an intronic variation IVS5 + 134G>A was identified in patient 1, patient 4, and 17 normal individuals and was previously described by Tomatsu *et al*. This base transversion from G to A produces a *StyI* restriction site and has been reported as a common polymorphism in Japanese people with an allele frequency of 0.58.[17] Another intronic variation c.566 + 5T>C was also detected in two normal individuals in our study. We conducted splicing site analysis using Human Splicing Finder, because this variant is located close to the donor splicing site.[18] However, the analysis suggested that this variant probably has no impact on the splicing site. The prediction is consistent with a report from Illumina CSL that classified this variant as likely benign.[19]

Conclusion

We report one novel variant G168V in exon 5 of *GALNS* in blood samples from MPS IVA patients in Indonesia. A silent mutation and two intronic variants were also identified. Although bioinformatics analyses and protein stability predictions suggest that this novel variant could be pathogenic, further investigation is needed to determine its real significance. This finding provides supplementary data in exon 5 of *GALNS*. Further, research is needed to analyze variations in other exons and to map the mutation profile in MPS VIA patients in Indonesia.

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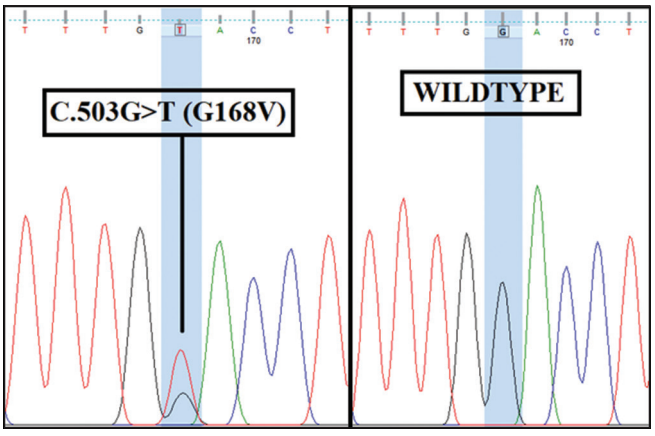

Figure 3: Chromatogram view of G168V and the wild type

Table 1: Polymerase chain reaction primer properties

Name	Sequence (5′ → 3′)	Length (bp)	GC (%)	Tm (°C)	Secondary structure (kcal/mol)	PCR product (bp)
GALNS-ID E×5 F	CTGGAGGGTGCTCGTCTTAC	20	60	57.08	-	443
GALNS-ID E×5 R	ATAGGCTCTGGGGCAAAACT	20	50	58.2		

PCR: Polymerase chain reaction, Tm: Temperature, GC: Guanine-cytosine

Table 2: Prediction of the pathogenicity effect and destabilizing potential

Allele name	MutPred	Provean	PMUT	CUPSAT
G168V	0.939	−3.620 (deleterious)	0.74 (disease)	Destabilizing

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Conflicts of interest

There are no conflicts of interest.

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