Single Nucleotide Variation in the Promoter Region of the APOA1 Gene as a Candidate Biomarker for Dyslipidemia

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Abstract
Dyslipidemia is a lipid profile abnormality that can escalate the risk of cardiovascular disease. The rate of cardiovascular events in Indonesia is very high. One of the causes of dyslipidemia is due to polymorphisms in genes associated with lipid metabolism. The APOA1 gene encodes the APOA1 protein which functions to regulate HDL protein synthesis. The objective of this study is to determine APOA1 gene polymorphisms in patients with dyslipidemia. Samples of healthy controls and dyslipidemia patients were used in this investigation. The lipid profiles of the patients and normal controls are determined at the onset of the study. Following DNA extraction, the APOA1 gene was amplified and sequenced using the serum sample. MEGA X and BLAST were used to analyze the sequencing results. The study's findings demonstrated that the APOA1 gene length in samples of dyslipidemic patients and normal controls was 433 bp. While the normal control samples have the same sequence as the database, the dyslipidemic patient samples have an APOA1 gene polymorphism in the promoter region. APOA1 gene polymorphism results in disturbances in lipid profiles, particularly HDL which is at risk of developing dyslipidemia. The APOA1 gene has the potential to be developed as a biomarker for diagnosing dyslipidemia involving a larger number of samples.

Keywords: APOA1 gene, Dyslipidemia, Polymorphism.

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1. **INTRODUCTION**

Dyslipidemia is a disorder of lipid metabolism characterized by abnormal lipid profiles (triglycerides, LDL (low-density lipoprotein), HDL (high-density lipoprotein), and cholesterol) (Xu, Song, Mao, & Xu, 2022). Dyslipidemia patients possess total cholesterol ≥ 200 mg/dL, LDL ≥ 160mg/dL, HDL cholesterol <40 mg/dL, or triglycerides ≥200 mg/dL (Rojas et al., 2018). One of the potential factors for cardiovascular disease is dyslipidemia. Approximately, 17.9 million people die from cardiovascular disease each year, accounting for 32% of all deaths worldwide (WHO, 2019). The cardiovascular event rate is evaluated to be 15 out of 1,000 people, or around 2.8 million individuals in Indonesia (Kementerian Kesehatan Republik Indonesia, 2018).

Dyslipidemia is affected by genetic, lifestyle, diet, and environmental factors (Kopin & Lowenstein, 2017). An imbalance in the genes involved in lipid metabolism leads to genetic factors. Polymorphisms in genes cause variations in the expression of proteins related to lipid metabolism. Genome-wide association Studies have discovered over a hundred genes that affect lipid levels and have correlated dyslipidemia to a genetic component (Asselbergs et al., 2012). The genes that play a role in influencing HDL lipid levels are the APOA-1, ABCA1, LCAT, SAR1B, and ABCG1 genes. These genes are identified to influence the risk of early atherosclerotic cardiovascular disease (García-Giustiniani & Stein, 2016).

One of the genes that codes for the production of the APOA-1 protein is APOA-1 (Apolipoprotein A-I), promotes cholesterol efflux from tissues and serves as a cofactor for the enzyme lecithin cholesterol acyltransferase (LCAT), which is involved in the reverse transport of cholesterol from tissues to the liver for excretion. One of the main proteins discovered in plasma HDL, APOA-1, is crucial for moving extra cholesterol from the peripheral blood to the liver. Furthermore, APOA-1 functions as an antioxidant and an anti-inflammatory (Georgila, Vyrla, & Drakos, 2019). APOA-1-related studies have illustrated that APOA-1 levels can be employed as a predictor and prognostic tool in ischemic stroke patients (Eldeeb et al., 2020).

The expression of proteins involved in HDL formation is disrupted by the polymorphism in the APOA-1 gene. The amounts of HDLC and APOA-1 proteins in a serum drop as a result of variations in the APOA-1 gene sequence on the gene promoter (Al-bustan, et. al., 2013). Several single-nucleotide polymorphisms (SNPs) have been identified in the APOA-1 gene which is situated on the long arm of chromosome 11. The transition from G to A which is located at -75 bp upstream from the transcription start site of the APOA-1 gene predisposes a person to coronary artery disease (Xu et al., 2017). Individuals with the APOA1-75A allele possess a low risk of suffering from coronary artery disease and T2DM as the serum contains high concentrations of APOA-1 and HDL-C (Hedayatnia et al., 2020; Liao, et. al., 2015; Rashad et al., 2021).

The Indonesian population has a high incidence and mortality rate from cardiovascular disease. Dyslipidemia-related APOA-1 gene polymorphisms can be uncovered to monitor cardiovascular events. Since dyslipidemia can cause other comorbidities involving atherosclerotic cardiovascular disease, stroke, and subcortical infarction if it is not detected early, the APOA-1 gene polymorphism has the potential to be used as an initial screening in patients with dyslipidemia (de Grooth et al., 2004; Liu, et al., 2019; Smach et al., 2012; Wang et al., 2016; Westfall et al., 2012; Xu et al., 2017). This study aims to identify variations in the apoA1 gene sequence in the promoter region, 5'UTR, introns, exon 1, and exon 2.

2. **RESEARCH METHOD**

The materials required are 5 whole blood samples of dyslipidemia patients, Merck lysis buffer, proteinase K Promega, phenol Smart Lab, Merck 96% ethanol, Merck 70% ethanol, Vivantis TE buffer, fluorvue Promega 500 µL, PCR Kit (Green Taq Promega), running buffer,
Agarose Gene Direx, NFW Promega, loading dye Promega, isolate DNA from PCR sample, Smobio DNA Marker 100 bp, primer forward 5'- AGG GAC AGA GCT GAT CCT TGA ACT CTT AAG-3' dan primer reverse 5' - TTA GGG GAC ACC TAC CCG TCA GGA AGA GCA - 3'.

This type of study is referred to as exploratory design. Patients at the Semarang City Health Center who were dyslipidemic provided the research sample. For the dyslipidemia group, there were 25 patients whose samples fulfilled the criteria of having triglycerides ≥200 mg/dL, HDL cholesterol <40 mg/dL, LDL cholesterol <160 mg/dL, or cholesterol ≥200 mg/dL to be tested for the APOA1 gene. Five individuals without a history of abnormal lipid profiles served as controls.

Each group (dyslipidemia and control) received 4 mL of venous blood. A nurse at the Puskesmas (Primary Health Unit) utilized the venous blood of patients with dyslipidemia and controls. Through research licensing procedures, specifically ethical clearance and approval of research subjects with informed consent number: 320/KEPK/EC/2021, the retrieval process was performed.

Peripheral blood is drawn from 25 dyslipidemic patients and 5 controls, and 3 mL of each is put into a 15 mL conical tube to begin the DNA isolation process. Proteinase K was added in an amount of 20µL and buffer lysis in a ratio of 1:1. For sixty minutes, the solution was vigorously shaken. After adding phenol up to a 1:1 ratio, homogenize for ten to fifteen minutes. The samples were centrifuged for 20 minutes at 3000 rpm. Using a micropipette, the supernatant was extracted and then placed into a microtube. The supernatant was gradually stirred with a 1:1 addition of cold 96% ethanol. After being removed, the DNA threads were placed in a different microtube. Three 500µL 70% ethanol washes were performed on the DNA threads. Washing was performed by centrifugation for 10 min at 14000 rpm. The supernatant was discarded the pellet was air-dried. The pellet was added with 50µL of TE buffer.

DNA purity was evaluated using the MaestroGen MN-913A Nanodrop Spectrophotometer with a wavelength of 260nm to assess DNA double bands and a wavelength of 280nm to measure protein or phenol contaminants. 2µL of DNA sample was inserted into the sample well on the tool.

DNA amplification using PCR must be use sterile materials. The PCR components to be reacted were Master Mix Green Taq Promega 12.5µL, template DNA adjusted for DNA concentration (total concentration 120ng), primer APOA1 forward 5' - AGG GAC AGA GCT GAT CCT TGA ACT CTT AAG-3' 2µL , primer APOA1 reverse 5'-TTA GGG GAC ACC TAC CCG TCA GGA AGA GCA-3' (Al-bustan et al., 2013) in the amount of 2µL and Nuclease Free Water to adjust the volume of the DNA template. The APOA1 gene primer owns a product length of 433bp.

The next step was DNA amplification utilizing the PCR BIO-RAD T100™ Thermal Cycler. The PCR cycle was generated for 35 cycles, then the hot start temperature was set at 95°C for 4 min, denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 4 min and extension finally at 72°C for 10 min, and cooling down at 12°C for ∞.

Using DNA gel electrophoresis, the outcomes of DNA amplification of patient samples with dyslipidemia were observed. Utilizing a set of Mupid electrophoresis instruments for DNA electrophoresis. Weighing 2.5 grams of agarose is the first step in making agarose gel. Erlenmeyer was filled with a solution of agarose powder and 100 milliliters of 1x TAE buffer. The temperature and duration were adjusted before the Erlenmeyer containing the solution was placed inside the microwave. After adding up to 4µL of Fluorvue via pipette to the agarose solution, homogenize the mixture. A comb is installed after the solution is poured into a gel mold. The gel is moved to the electrophoresis chamber once it has solidified, and 1x TBE buffer is added until the gel sinks. Pipette 2µL of loading dye and add 10µL of PCR product. The
mixture was put into the electrophoretic gel wells. Samples were run at 50 volts for 60 min. The sample was read in the MUV21-312 UV transilluminator and then the DNA bands formed were detected.

PT. Indonesian Science Genetics will be sequencing the amplified sample. The DNA baser application was used to sequence the DNA and produce a consensus fasta file or contig analysis. The BLAST NCBI application was subsequently utilized to align the results, and changes in the nucleotide base sequence and amino acid composition of the sample were examined. Using the BLAST (Basic Local Alignment Search Tool) online bioinformatics tool, the analysis results validated their presence and degree of similarity with the gene database.

3. RESULTS AND DISCUSSION

ApoA1 gene amplification in dyslipidemic patients and controls.

Figure 1. Products of APOA-1 gene amplification in dyslipidemic patients and controls. A single DNA band was formed which displayed a size of about 433 bp, this indicated a specific apoA1 gene amplification product. Figures A, B, C show M (Ladder Smobio DNA Marker 100 bp), C (Control), D1-25 (Dyslipidemia)

The APOA-1 gene amplification results revealed a single, parallel band with a marker measuring 433 bp between the dyslipidemic samples (D1 to D25) and control samples (C1 to C6) (Figure 1). APOA-1 gene amplification with a 435 bp product length in the promoter region, 5'UTR, introns, exon 1, and exon 2 (Al-bustan et al., 2013). The APOA-1 promoter region is located at nucleotides 1-142. APOA-1 is part of the APOA1/CIII/IV/V cluster gene located on chromosome 11q23-24 functioning to encode a protein component of HDL which is responsible for regulating the amount of serum lipids.

B. Sequencing of the APOA-1 gene in the dyslipidemia and control groups

The APOA-1 gene amplification results can be sequenced to perform a polymorphism analysis. Using BLAST NCBI, the acquired APOA-1 sequences were examined for similarities with the APOA-1 gene in the database. The homo sapiens apolipoprotein A1 gene, promoter region, exons 1, 2, and partial CDS accession number JX438706.1 are similar to the sequence data for samples D1–D5 and C1 (Figure 2).
Figure 2. BLAST results of APOA-1 samples.

The process of ascertaining a gene’s specific nucleotide base order is referred to as DNA sequencing. The degree of similarity between the DNA sequencing results and the genes in the GenBank BLAST NCBI database was initially determined. The E-Value, or expected value, which demonstrates how many sequences are anticipated to be found in the database using Bit Score, indicates the degree of similarity. It is simpler to determine the degree of similarity when the e-value is lower because it indicates a higher likelihood of similarity with the previously identified gene (Stover & Cavalcanti, 2017). The degree of similarity between the sample sequences and the previous gene sequences can be perceived from the e-value (figure 2). E value which demonstrates a low value (0.0) so that the sample can be identified to be similar to the NCBI database gene sequence (figure 2).

Alignment analysis of the APOA-1 gene with the database available at NCBI. Sequences of samples D1-D5 and C1 were aligned with the database available at NCBI to see differences in nucleotides that lead to polymorphism.
Figure 3. Alignment Results of the NCBI BLAST Program Sample D1.

The D1 sample's alignment results correspond to the APOA-1 gene JX438706.1, which has a 435 bp total molecular length and a 433 bp specific target sample. Nucleotide bases in Sample D1 differed from those in the database. The APOA-1 gene database indicates adenine in Sample D1, which has a deletion at nucleotide 42 (Figure 3). The promoter region of sample D1 contains the sequence variation. Nucleotides 1-142 in the targeted sequence contain the APOA-1 promoter region.
The APOA-1 gene JX438706.1, which has a total molecular length of 435 bp and a specific target sample of 433 bp, is aligned with the alignment results of the D2 sample. Nucleotide bases in Sample D2 differed from those in the database. In the APOA-1 gene database, adenine is the 43rd nucleotide; sample D2 contains a deletion (Figure 4). Sample D2's sequence variation is located in the promoter region.

**Figure 4.** Alignment Results of the NCBI BLAST Program Sample D2.
Homo sapiens apolipoprotein A1 gene, promoter region, exons 1, 2 and partial cds

Sequence ID: JX438706.1  Length: 435  Number of Matches: 1

Range 1: 2 to 435  GenBank  Graphics

<table>
<thead>
<tr>
<th>Score</th>
<th>Expect</th>
<th>Identities</th>
<th>Gaps</th>
<th>Strand</th>
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<tr>
<td>793 bits(429)</td>
<td>0.0</td>
<td>434/436(99%)</td>
<td>2/436(0%)</td>
<td>Plus/Plus</td>
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Query 1  GGGACAGAGCTGATCCTTGAACTCTTAAATTCCACATTGCCAGGACAGATGGAGGACGAA | 60 |
Sbjct 2  GGGACAGAGCTGATCCTTGAACTCTTAAATTCCACATTGCCAGGACAGATGGAGGACGAA | 60 |

Query 61  CAGGGCGGGGCTGGGCTTAATCAGCCTCCACGGCCAGAAGGCCTGGTCGAAATGATAAATA | 120 |
Sbjct 61  CAGGGCGGGGCTGGGCTTAATCAGCCTCCACGGCCAGAAGGCCTGGTCGAAATGATAAATA | 119 |

Query 121  GGCCTCGAAGCAGCTGGCTTAGAAGACTGCAGAAGAGGAGGGCTGCTGCTGGCC | 180 |
Sbjct 120  GGCCTCGAAGCAGCTGGCTTAGAAGACTGCAGAAGAGGAGGGCTGCTGCTGGCC | 179 |

Query 181  CGGTCACACTGGCTCCAGGACCTCACGGCTTGGCCCCAGGGCCGGCCTCTGGGT | 240 |
Sbjct 180  CGGTCACACTGGCTCCAGGACCTCACGGCTTGGCCCCAGGGCCGGCCTCTGGGT | 239 |

Query 241  ACCGTAGCTTCTCTCCGGCTTCTTCTCTCTCTTCTCTTAATGAGTGGGGGAG | 300 |
Sbjct 240  ACCGTAGCTTCTCTCCGGCTTCTTCTCTCTCTTCTCTTAATGAGTGGGGGAG | 299 |

Query 301  CACGGGCTTCTGGCTAGTGAAGGCACCACACTCAGGACGGCCCTTCTCTTCTGGAGT | 360 |
Sbjct 300  CACGGGCTTCTGGCTAGTGAAGGCACCACACTCAGGACGGCCCTTCTCTTCTGGAGT | 359 |

Query 361  CCCGCCGCCCCTCTAGGATGAAAGCTGCGCTGCTGCACTTCTGGCTTCTCTTCTGGAGT | 420 |
Sbjct 360  CCCGCCGCCCCTCTAGGATGAAAGCTGCGCTGCTGCACTTCTGGCTTCTCTTCTGGAGT | 419 |

Query 421  GTAGGTTGCTCCCCCTAA  436 |
Sbjct 420  GTAGGTTGCTCCCCCTAA  435 |

**Figure 5.** Alignment Results of the NCBI BLAST Program Sample D3.

The APOA-1 gene JX438706.1, which has a total molecular length of 435 bp and a specific target sample of 433 bp, is aligned with the alignment results of the D3 sample. Nucleotide bases in Sample D3 differed from those in the database. Nucleotides 51 and 103nt in the APOA-1 gene database are guanine, but sample D3 has a deletion (Figure 5). The promoter region of the D3 sample contains the sequence variation.
Figure 6. Alignment Results of the NCBI BLAST Program Sample D5

The APOA-1 gene JX438706.1, which has a total molecular length of 435 bp and a specific target sample of 433 bp, is aligned with the alignment results of the D5 sample. Positions 55 and 56nt of the apoA1 gene contain the nucleotides guanine and adenine in the database, but sample D5 has a deletion (Figure 6). The nucleotide bases of the APOA-1 gene varied between Sample D5 and the database.
Figure 7. NCBI BLAST Program Alignment Results Sample DA6 (normal).

The alignment results of sample C are aligned with the APOA-1 gene JX438706.1 which posses a total molecular length of 435 bp with a specific target sample of 433 bp. Sample C and did not experience changes in nucleotide bases compared to the APOA-1 gene database contained in NCBI (Figure 7).

The bands of DNA that are visible on the band vary in thickness (figure 1). This results from varying sample DNA concentrations during the PCR mixing procedure. Adding the DNA template concentration, which needs to be the same for every PCR reaction, is a crucial first step in optimizing the PCR reaction. While excessive DNA concentrations can result in DNA
bands that smear and non-specific amplification results, low template DNA concentrations can cause DNA bands to become faint, not clearly visible, and even amplification may not occur (Asif et al., 2021). The thickness of DNA bands in this study cannot be utilized as a reference for individuals with dyslipidemia or not because the control sample has the same band thickness as the sample. Thus, to determine more specifically the differences in APOA-1 gene polymorphism in the sample, a further process was performed, which is DNA sequencing.

After analyzing the level of similarity, the analysis process is continued by perceiving the DNA sequence alignment utilizing the BLAST program (Figure 3 to figure 7). It is presented that the APOA-1 gene with accession number JX438706.1 had specifically targeted dyslipidemia and control samples at 433 bp in length. Al bustan's research targeting the APOA-1 gene illustrated a PCR product with a length of 435 bp. A 433 bp molecule was discovered in a Chinese study that employed PCR to examine the connection between the G75A polymorphism of the APOA-1 gene and lipid regulation in hyperlipidemic patients implementing pravastatin. The study's findings suggest that in hyperlipidemic patients, the presence of different APOA-1 SNP G75A genotypes may have an impact on how well pravastatin and policosanol regulate lipid levels (Liu et al., 2016).

Variations in the APOA-1 gene sequence in samples D1 to D5 are located in the promoter area. Variations of Rs670 has been reported to enhance promoter function with minor A alleles associated with abnormal variations in serum lipid levels in some populations (Al-bustan et al., 2013). Variations in the upstream region on the transcriptional side have also been reported to be essential for regulating APOA-1 expression. Variations in the promoter region result in reduced APOA-1 expression in serum proteins. The promoter region that undergoes polymorphism is in accordance with low HDL-C concentrations (Al-bustan et al., 2013). Sequence integrity of the upstream region on the transcription site is also identified to influence APOA-1 expression. Various mutations in the promoter region result in decreased expression of the APOA-1 gene and the amount of APOA-1 protein in serum (Pagani et al., 1990; Smith, Brinton, & Breslow, 1992) demonstrated a heterozygosity component in mutations in the promoter region (-27 and -5) affecting the reduction of the amount of APOA-1 expression, hence, it would reduce the APOA-1 protein and HDLC in serum. Sequence variations and allelic frequencies in the APOA-1 promoter region can influence variations in APOA-1 expression (França, Alves, & Hutz, 2005; Haase, et al., 2010; Wang, et al., 2017) which will affect the amount of lipids in serum.

The change in amino acid sequence from guanine to adenine at base pairs at the onset of transcription (-75G/A) indicates that variations in the APOA-1 gene polymorphism occur and play a role in the occurrence of lipid regulation (Liao et al., 2015). Point mutations are alterations in one or more nucleotide base pairs. Proteins may change slightly as a result of certain mutations. If the new amino acid is similar to the one being replaced in terms of properties or if it is still present in a protein region where the amino acid sequence serves no functional objective, then the amino acid change may not be significant (Urry et al., 2021).The APOA-1 gene polymorphism is believed to have an essential role in a number of other diseases such as Alzheimer's diabetes, breast cancer, and schizophrenia and others (Jian et al., 2013; Pandith et al., 2021; Smach et al., 2012).

Dyslipidemia is a disorder of lipid metabolism characterized by increased LDL, cholesterol, and triglycerides, and decreased HDL (Rojas et al., 2018). relationship between dyslipidemia in the family and CVD risk. Both genetics and epigenetics have an impact on CVD. The identification of novel molecules, gene mutations, and polymorphisms in the synthesis, transport, and metabolism of lipoproteins provides therapeutic targets and enables treatment. Genetic abnormalities have been associated with a higher risk of CVD and low HDL-c. Low HDL-c and early CVD have been associated with APOA-1-4 deletions,
inversions, and substitutions. Reduced HDLC and familial hypoalphalipoproteinemia are caused by polygenic defects in a variety of genetic variants, including deletions, inversions, and substitutions in the apolipoprotein gene associated with CAD, accounting for roughly 50% of HDLC alterations (Gene et al., 2016).

It has been possible to identify polymorphisms in the APOA-1 gene that influence the risk of developing DM through prior research. Due to their high serum HDL-C counts, people with APOA1 -75 G/A genotypes AA and allele A have a low risk of T2DM and CAD (Rashad et al., 2021). Mutations at the locus (G-75A) were also consistent in previous studies involved in dyslipidemia, but (C83T), resulted in differences in the hyperlipidemic group. In the hyperlipidemic group, genotype allele frequencies, APOA1 concentrations were significantly different (Pakdel, et al., 2018).

There are several mutations in specific sequences of the APOA-1 gene. One is that in Chinese women, the rs662799 polymorphism is significantly correlated with dyslipidemia and coronary heart disease; however, larger sample sizes and multi-ethnic validation are required for these findings to be validated (APOA5) (Wang et al., 2016). Furthermore, the APOA-1 gene mutations rs670, rs5069, and rs2070665 affect the incidence of dyslipidemia in the Kazakh population. The frequency of the CC genotype at rs1321085 was discovered in 7.2% of the obese group, 4.4% of the overweight group, 5.6% in the control group. The APOA-1 rs5070 A/G genetic polymorphism plays an essential role in the susceptibility of large arterial sclerosis in diabetic patients (Hsu & Lee, 2017).

APOA-1 rs5072 variant is associated with dysregulation of triglyceride metabolism in schizophrenic patients treated with APD Antipsychotic Drug-Induced Dyslipidemia allowing for a higher risk of dyslipidemia (Fan, et al., 2021). Mutations of APOA-1 and ApoB are presented to predict the development of cardiovascular disease and patients with schizophrenia have lower expression of APOA-1 than healthy subjects (refs 39, 40).

Analysis of the relationship between APOA-1 polymorphism and high-density lipoprotein (HDL) cholesterol level and myocardial infarction in Japan (Shioji et al., 2004). The findings revealed that the three APOE genotype polymorphisms, ABCA1 G (-273)C, APOA-1 T84C, and APOA-1 independently correlate with HDL-C levels. Hepatic lipase, phospholipid transfer protein, and cholesterol ester transferase protein are some of the substances that have been utilized to control HDL-C levels. The APOA1 T84C polymorphism is a risk factor for myocardial infarction in Japan and has a significant impact on HDL-C levels in the general Japanese population.

While normal controls did not experience any variation in the APOA-1 sequence, four dyslipidemic patients who had the APOA-1 gene amplified had a sequence with a single nucleotide variation in the promoter area. The polymorphism is located in the APOA-1 gene's promoter region, with base positions ranging from base 41 to base 55.

4. CONCLUSION

Approximately, 433 bp of APOA1 gene amplification products were produced by all dyslipidemia samples and the normal control sample. In the promoter region of the APOA1 gene, single nucleotide sequence variations were detected in the four dyslipidemia samples that underwent successful sequencing; no such variations were detected in the normal control samples.

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