Received: 14 March 2023 Revised: 17 April 2023 Accepted : 20 Mei 2023
DOI:10.29244/primatology.2.01.37-48 E-ISSN: 2964-5441

INAJP
INDONESIAN JOURNAL OF PRIMATOLOGY

RESEARCH ARTICLE

Single Nucleotide Polymorphism of LDLR Gene as Atherogenesis Markers on *Macaca fascicularis* and *Macaca nemestrina*

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Abstract
Long-tailed macaques (*Macaca fascicularis*) and pig-tailed macaques (*Macaca nemestrina*) are non-human primate species most commonly used as animal models in atherosclerosis. Genetic variation in the low-density lipoprotein receptor gene (LDLR) has been associated with normal variations in plasma lipid profile and the risk of coronary heart disease (CAD) in humans. In this study, the screening of nucleotide polymorphisms on LDLR genes as molecular markers of atherogenesis in *M. fascicularis* and *M. nemestrina* was performed. The LDLR gene of exon region 6 is amplified with specific primers. The sequencing technique determines the nucleotide sequences of the amplicons, and the results were bioinformatically analyzed. Analysis of the exon 6 region LDLR gene in *M. fascicularis* and *M. nemestrina* revealed no SNP in this exon. Based on the alignment results, the entire sample has a type of haplotype I. The type of haplotype owned by the six animals relates to the hyper-response. Both species are the potential for animal models to study atherosclerotic disease. The animal selection of hypo- from hyper-responder is more efficient using exon 6 as a genetic marker of *M. fascicularis* and *M. nemestrina* on a fat cholesterol diet.

Key words: atherosclerosis, LDLR gene, *Macaca fascicularis*, *Macaca nemestrina*, single nucleotide polymorphism

1. Introduction

Long-tailed monkeys (*Macaca fascicularis*) and pig-tailed macaques (*Macaca nemestrina*) are non-human primates often used as animal models in biomedical research. *Macaca fascicularis* has been used as an animal model to study human atherosclerosis because it is responsive and sensitive to dietary cholesterol and fat (Clarkson 1998). There are three animal groupings in the analysis of plasma cholesterol diet responses. Hyper-responder individuals are sensitive to dietary cholesterol and fat and show a marked progression of atherosclerosis. Hypo-responder individuals are less or insensitive to dietary cholesterol and fat, so they do not experience hypercholesterolemia (Beynen *et al*. 1987). Hypo-responder individuals do not show an obvious progression of atherosclerosis (Clarkson 1998).

The low-density lipoprotein receptor (LDLR) is a protein on the cell surface that mediates the endocytosis of LDL and other cholesterol-carrying particles. The human LDLR gene on chromosome 19p13.2 consists of 18 exons and 17 introns spanning 45 kilobases (kb) (Südhof *et al*. 1985). Mutations in the LDLR gene cause familial hypercholesterolemia, resulting in changes in the structure and function of the receptors that bind plasma low-density lipoprotein cholesterol (LDL cholesterol). These results lead to an increase in LDL cholesterol levels that can provide a wide range of the clinical spectrum, from the accumulation of cholesterol in the skin and connective tissue to atherosclerosis in coronary arteries that will cause death (Goldstein *et al*. 2001).

Atherosclerosis is a vascular disease characterized by the formation of an atheroma that

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narrows the lumen of the artery and causes lumen obstruction. This impaired blood flow can lead to ischemia and tissue death, especially in areas of arterial flow in organs with very few collaterals, such as the heart and brain (Suryohudoyo 2000). *M. fascicularis* has similar symptoms of atherosclerosis to humans. In addition, *M. fascicularis* fed an experimental diet also showed a progression corresponding to the disease's clinical stages, including ischemia with coronary artery stenosis and sudden death resulting from occlusive thrombosis and myocardial infarction (Shelton *et al*. 2012).

A single nucleotide polymorphism (SNP) in the LDLR gene is reported to affect normal variation in plasma lipid profiles. Polymorphism studies in the LDLR gene are commonly conducted in humans to identify associations between SNPs and normal blood lipid profiles or susceptibility to hypercholesterolemia (Knoblauch *et al*. 2002). Most of the SNPs identified were non-functional, indicating they were in linkage disequilibrium with other functional SNPs. The first functional SNP in the human LDLR gene associated with a normal blood lipid profile was the 1773T allele (rs688) in exon 12 (Zhu *et al*. 2007). This SNP is functional as it causes transcription without exon 12. Transcription without exon 12 changes the reading frame and early termination proteins, increasing total cholesterol and LDL-C levels. The presence of SNPs in genes involved in lipid metabolism affects susceptibility to coronary heart disease (CHD) (Kathiresan *et al*. 2008). The LDLR gene region of exon 6 can be used as a molecular marker for atherogenesis in *M. fascicularis* (Taher *et al*. 2016).

Based on this issue, this study aimed to screen single nucleotide polymorphisms (SNP) in the exon 6 region of the LDLR gene as a molecular marker of atherogenesis in *M. fascicularis* and *M. nemestrina*. The screening was carried out by analyzing haplotypes based on SNPs in the exon 6 region of the LDLR gene and analyzing the relationships by constructing a phylogenetic tree based on sample groups according to their response to dietary fat cholesterol (hyper-responder, hypo-responder, extreme).

2. Materials and Methods

2.1 Sample Collection and Genomic DNA Extraction

Whole blood was obtained from the archives samples of the Primate Research Center, IPB University, Bogor, Indonesia, referring to the research of Taher *et al*. (2016) (Table 1).

Table 1. List of Individuals and reference samples of *Macaca fascicularis* and *M. nemestrina* used in the analysis of SNP LDLR gene

No	Species	Gender	Number of Individuals	Group
1	*M. fascicularis*	Male	J200110A	In group
2	*M. fascicularis*	Male	J210110A	
3	*M. fascicularis*	Female	J090909A	
4	*M. nemestrina*	Male	B140401	
5	*M. nemestrina*	Female	B140531	
6	*M. nemestrina*	Female	B131025	
7	*M. fascicularis* (Taher *et al.* 2016)		T4927, FC8501, 9695, C0631, T3049, T3536, T3700, C4939, T4278, T3303, Fc9015, FE7777, T3307, T3300, FG7909, C0750, FG7998, C2480, FC9113, T3707, K30, T3535	
8	*Homo sapiens, Pan paniscus, Gorilla gorilla, Pongo pygmaeus*			Out group

According to the manufacturer's instructions, genomic DNA was extracted from blood samples using a QiaAmp™ DNA blood mini kit (Qiagen, Hilden, Germany).

2.2 Amplification, Visualization, and Sequencing

The primers used for amplification of exon 6 of the LDLR gene were as follows: F: 5'-CCTTCCTCCTTCCTCTCTCT-3', R: 5-'ACTCTGCAAGCCGCCTGCAC-3' (Taher *et al.* 2016). The size of the amplification product was 184 bp. The reaction was carried out in a volume of 25 µL and contained 5 µL genomic DNA [400–1000 ng/µL], 1 µL each of forward and reverse primers [10 pmol/µL], 12.5 µL Gotaq Green Mastermix (buffer solution, dNTPs and Taq polymerase enzyme) and 5.5 µL of nuclease-free water. Amplification was performed using a GeneAmp® PCR System 9700 machine under the following conditions: Pre-PCR at 94°C for 5 minutes followed by 40 cycles. Denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 7 minutes. The PCR products were run on 1.8% agarose gel electrophoresis. The results of agarose gel electrophoresis were observed under UV Gel Doc 2000. Band size was calculated using a 100 bp DNA ladder (Biorad). The amplification results were determined by sequencing techniques at First BASE Laboratories Sdh Bhd (Malaysia).

2.3 Data Analysis

The nucleotide base sequences were edited using Bioedit version 7.2.6 (Hall 1999). Nucleotide sequence alignment was performed using the ClustalW program on GenomeNet (Thompson *et al.* 1994). The results of the DNA sequence alignment of the LDLR gene in the exon 6 region were analyzed for homology through the NCBI website (https://blast.ncbi.nlm.nih.gov) by selecting the BLAST-N option. Phylogenetic tree analysis was performed using the Mega-6 program (Tamura *et al.* 2013). The phylogenetic tree was formed based on Neighbour-joining by bootstrapping 1000 repetitions and adding outgroup sequences. Outgroup sequences were obtained from the BLAT website (Kent 2002). These sequences are the *Homo sapiens*, *Pan paniscus*, *Gorilla gorilla*, and *Pongo pygmaeus sequences*.

3. Results

3.1 Amplification and Sequencing of the Exon 6 Region

Genomic DNA amplicons from *M. fascicularis* and *M. nemestrina* blood produced a single clear band product with an amplicons band size of approximately 184 bp (Figure 1). The LDLR gene exon 6 amplicons showed a high DNA concentration (Table 2). Alignment of the consensus sequence with the reference *M. fascicularis* sequence (accession number XM_005587996.2) showed that the amplification product contained not only the nucleotide bases of exon 6 (125 bp) but also several nucleotide bases of the exon-clamping introns (30 bp in introns 5 and 29 bp on introns 6).

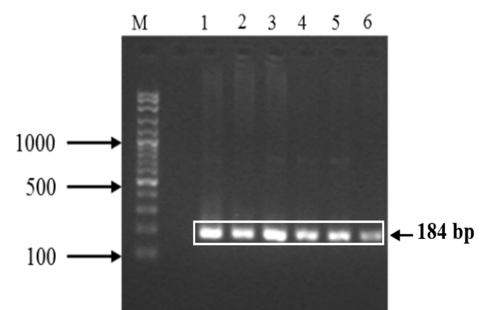

Figure 1. Visualization of exon 6 LDLR gene PCR amplification product resulted in 184 bp DNA bands indicated by white rectangle, run in 1.8% agarose gel electrophoresis stained with ethidium bromide and observed under UV Gel Doc 2000. M is 100 bp DNA marker, 1-3 are *M. fascicularis* samples and 4-6 are *M. nemestrina* samples.

Table 2. Quantification of DNA concentration extracted from *M. fascicularis* and *M .nemestrina* blood samples using Nanodrop spectrophotometer at λ260/280 nm

Animals ID	DNA Concentration (ng/µL)
J200110A	257.0
J210110A	167.3
J090909A	120.4
B140531	75.6
B131025	112.0
B140401	70.6

3.2 Haplotypes and Response to Dietary Cholesterol

All samples' LDLR gene sequences in the exon 6 region were edited using BioEdit software (Hall 1999). Homology analysis of the LDLR gene DNA sequence in the exon 6 region of *M. fascicularis* and *M. nemestrina* was performed using BLAST-N.

3.3 Phylogenetic Tree Construction

Phylogenetic tree construction of the LDLR gene exon from *M. fasicularis* and *M. nemestrina*, divided into three clusters (Hyper-response, Hypo-response, Extreme). The number on the branch indicates the bootstrap value. The entire individual sample of *M. fascicularis* and *M. nemestrina* was compared with the reference sequences, divided into three major clusters: the hyper-responder individual group with a bootstrap value of 99, and the hypo-responder and extreme individual group with bootstrap values of 66 and 31, respectively (Figure 2). The other samples using comparators from BLAT were contained in one group.

4. Discussion

4.1 Haplotypes and Response to Dietary Cholesterol

Alignment analysis aims to determine the level of homology of the DNA base sequences being analyzed (Kemena and Notredame 2009). The alignment results showed a high level of homology among the studied samples (Figure 3). The existence of homology is indicated by the number of regions with the same sequence (conserved). The alignment results showed three different types of haplotypes (Figure 4). A haplotype is a group of markers (polymorphisms) on a single chromosome to be inherited together. Haplotypes refer to a combination of alleles or a single set of nucleotide polymorphisms (SNPs). The grouping of individuals by haplotype type is shown in Table 3.

The CDS region is part of a nucleotide sequence composed of exons that code for a protein. A 125 bp CDS region sequence of LDLR gene exon 6 in hyper-responder, hypo-responder, and extreme individuals was translated into amino acids. The translated results in the three groups were the same, totalling 40 amino acids (Figure 5). Two single nucleotide polymorphisms (SNP), 25C>G and 39C>G (in exon 6), were identified in the nucleotide base sequence of the amplification product (184 bp) of 22 *M. fascicularis* (Taher *et al*. 2016). Different types of haplotypes in the exon 6 region of the LDLR gene sequences can be used as a reference in identifying animal responses to dietary cholesterol and fat. The more diverse types of haplotypes in a population, the higher level of genetic diversity and vice versa. The haplotype type in hypo-responder individuals has a polymorphism in the nucleotide base sequence, which is G (guanine) at position 25. Extreme individuals also have a polymorphism in the nucleotide base sequence: G (guanine) at position 25 and G (guanine) at base 39. These differences in nucleotide positions were not found in the hyper-responder individual haplotype types.

The polymorphic site 39 in the nucleotide sequence of exon 6 of the LDLR gene is located at the third position of the codon triplet and does not cause a change in the encoded amino acid. Similarly, SNP 25C > G is located in the intron region (Figure 5). Nucleotide changes that cause amino acid

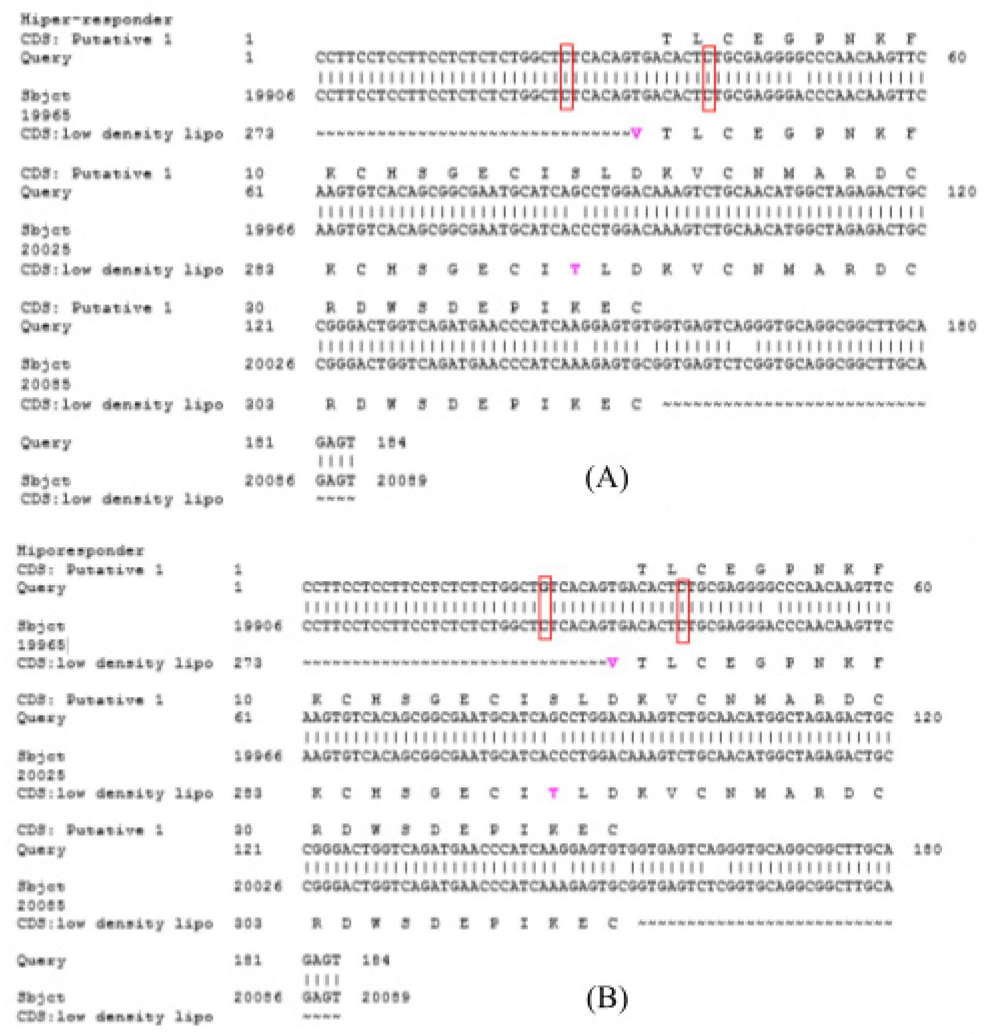

Figure 2. Two single nucleotide polymorphisms (SNP) of 25C>G and 39C>G in exon 6 of the LDLR gene of *M. fascicularis* were identified in the nucleotide base sequence of the amplification product (184 bp) grouped as hyper-responder (A), hypo-responder (B), and extreme (C). Translation of these three groups using the Blast-N program through NCBI resulted from the same amino acid sequences

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J210110A   CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
K3535      CCTTCCTCCTTCCTCTCTCTGGCTGTCACAGTGACACTGTGCGAGGGGCCCAACAAGTTC 60
FE7777     CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
C0613      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
FC9015     CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
C0750      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
C4927      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
T3300      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
FG7909     CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
T3303      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
C2480      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
T3536      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
C4939      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
9695       CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
FC9113     CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
T3278      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
T3700      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
T3307      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
FG7998     CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
T3049      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
FC8501     CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
B140531    CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
B140401    CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
B131025    CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
J200110A   CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
J090909A   CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
T3707      CCTTCCTCCTTCCTCTCTCTGGCTGTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
K30        CCTTCCTCCTTCCTCTCTCTGGCTGTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
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J210110A   CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
K3535      CCTTCCTCCTTCCTCTCTCTGGCTGTCACAGTGACACTGTGCGAGGGGCCCAACAAGTTC 60
FE7777     CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
C0613      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
FC9015     CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
C0750      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
C4927      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
T3300      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
FG7909     CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
T3303      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
C2480      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
T3536      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
C4939      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
9695       CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
FC9113     CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
T3278      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
T3700      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
T3307      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
FG7998     CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
T3049      CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
FC8501     CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
B140531    CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
B140401    CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
B131025    CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
J200110A   CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
J090909A   CCTTCCTCCTTCCTCTCTCTGGCTCTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
T3707      CCTTCCTCCTTCCTCTCTCTGGCTGTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
K30        CCTTCCTCCTTCCTCTCTCTGGCTGTCACAGTGACACTCTGCGAGGGGCCCAACAAGTTC 60
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J210110A  AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
K3535     AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
FE7777    AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
C0613     AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
FC9015    AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
C0750     AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
C4927     AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
T3300     AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
FG7909    AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
T3303     AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
C2480     AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
T3536     AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
C4939     AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
9695      AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
FC9113    AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
T3278     AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
T3700     AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
T3307     AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
FG7998    AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
T3049     AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
FC8501    AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
B140531   AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
B140401   AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
B131025   AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
J200110A  AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
J090909A  AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
T3707     AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
K30       AAGTGTCACAGCGGCGAATGCATCAGCCTGGACAAAGTCTGCAACATGGCTAGAGACTGC 120
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J210110A  GAGT 184
K3535     GAGT 184
FE7777    GAGT 184
C0613     GAGT 184
FC9015    GAGT 184
C0750     GAGT 184
C4927     GAGT 184
T3300     GAGT 184
FG7909    GAGT 184
T3303     GAGT 184
C2480     GAGT 184
T3536     GAGT 184
C4939     GAGT 184
9695      GAGT 184
FC9113    GAGT 184
T3278     GAGT 184
T3700     GAGT 184
T3307     GAGT 184
FG7998    GAGT 184
T3049     GAGT 184
FC8501    GAGT 184
B140531   GAGT 184
B140401   GAGT 184
B131025   GAGT 184
J200110A  GAGT 184
J090909A  GAGT 184
T3707     GAGT 184
K30       GAGT 184
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Figure 3. The alignment of LDR gene sequences in exon 6 of Macaca *fascicularis* (J210110A, J200110A, J090909A) and *Macaca nemestrina* (B140531, B140401, B131025) compared to other sequences samples showed a high level of homology (conserved region indicated by stars symbol).

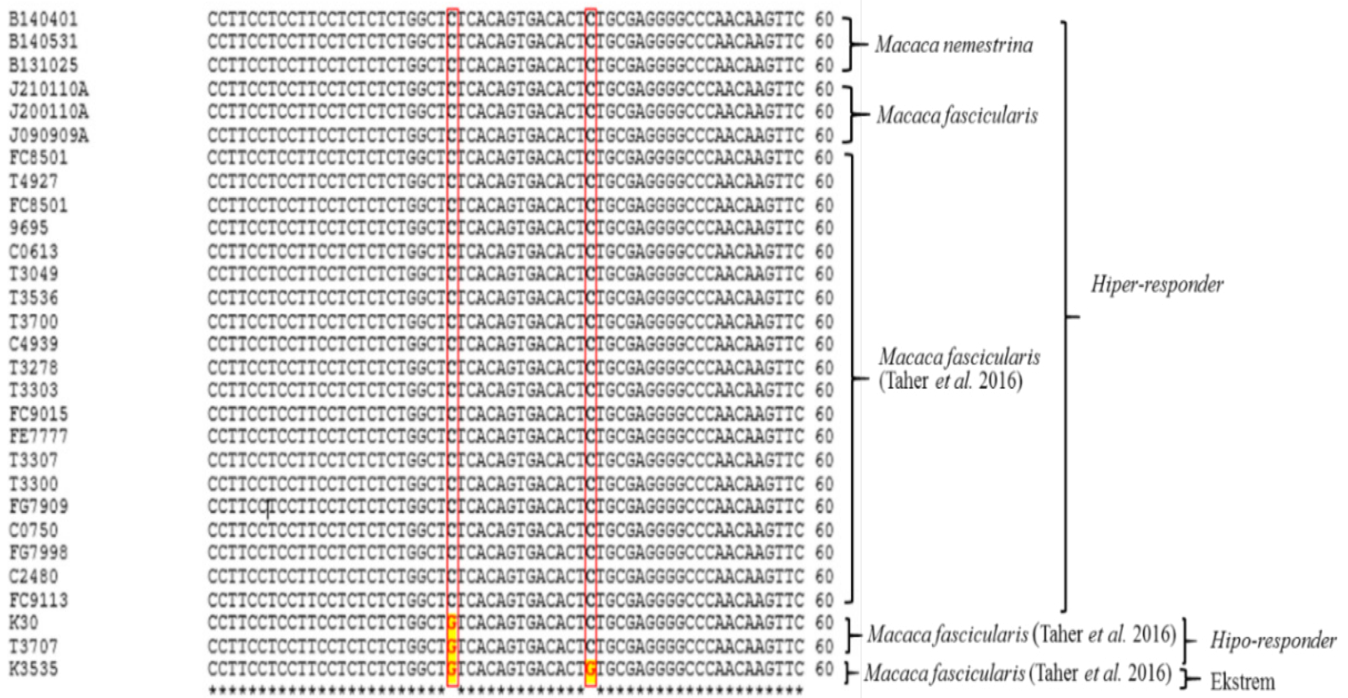

Figure 4. The alignment result of the exon 6 LDL-R gene of *M.fascicularis* and *M.nemestrina* compared to references sequencing (Taher *et al*. 2016) showed that the sequences in this study clustered together as hyper-responder. The different haplotypes (Two single nucleotide polymorphisms of 25C>G and 39C>G) are indicated by a red rectangle column highlighting the nucleotide bases in yellow. The nucleotide sequence alignment was conducted using the ClustalW program (https://www.genome.jp/tools-bin/clustalw).

Table 3. Types of haplotypes and polymorphic sites in the exon 6 region of the LDLR gene, and aligned to the *M. fascicularis* reference in GenBank (accession number XM_005587996.2) (Taher *et al*. 2016)

Haplotype	Position of Nucleotide		Number of Individuals	Species	Source	Responsiveness
	25	39				
Ref	C	C				
I	C	C	J210110A, J200110A, J090909A	*M. fascicularis*	This Study	Hyper-respons
I	C	C	B140401, B140531, B131025	*M. nemestrina*	This Study	Hyper-respons
I	C	C	T4927, FC8501, 9695, C0613, T3049, T3536, T3700, C4939, T3278, T3303, FC 9015, FE7777, T3307, T3300, FG7909, C0750, FG7998, C2480, FC9113	*M. fascicularis*	Taher *et al.* 2016	Hyper-respons
II	G	C	T3707, K30	*M. fascicularis*	Taher *et al.* 2016	Hypo-respons
III	G	G	T3535	*M. fascicularis*	Taher *et al.* 2016	Extreme

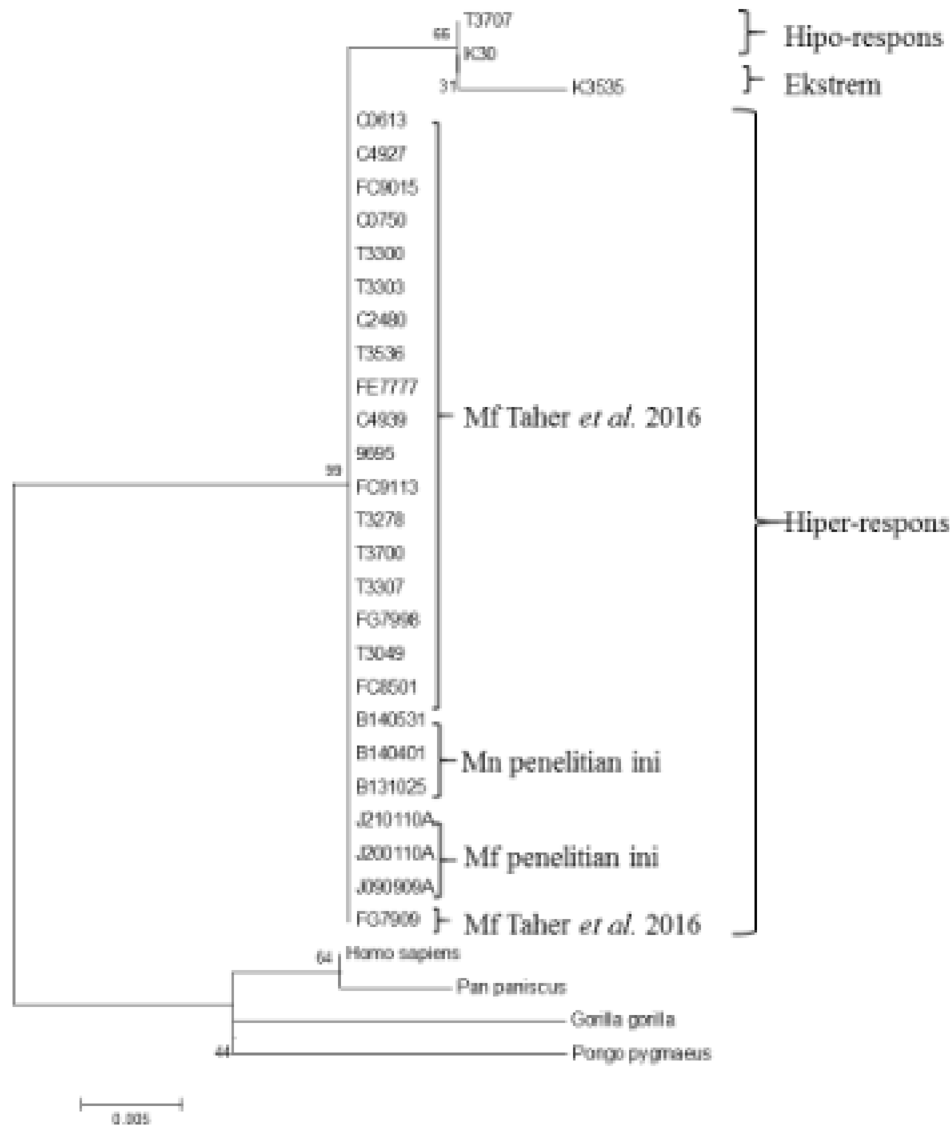

Figure 5. Phylogenetic tree construction based on exon 6 LDLR gene sequences constructed by the neighbour-joining method with bootstrap 1000 times. The numbers on the tree branches indicate the bootstrap value. The Mf and Mn sequences in this study were compared with reference sequences (Taher *et al*. 2016) and clustered as hyper-response groups.

changes usually occur when the nucleotide changes are located in the first and second nucleotides that make up the triplet codon. Meanwhile, changes in the third nucleotide of the triplet codon are likely cause synonymous mutations. According to Nei (1987), the chance of synonymous mutations on the first and third codons is 5% and 72%, while nucleotide changes in the second codon will always cause amino acid changes (100%). The responsiveness possessed by the animals is related to genetic variation (Friedlander *et al*. 1999). The similarity of the grouping based on haplotype type makes the identified SNPs capable of showing the relationship between the haplotypes and the animals' responsiveness (Taher *et al*. 2016).

This study used 3 samples of *M. fascicularis* (J200110A, J210110A, J090909A) and 3 of *M. nemestrina* (B140401, B140531, B131025). Based on alignment results, all of the samples had haplotype I. The haplotype type possessed by the six animals was related to hyper-response. Previous studies have

analyzed the response of plasma cholesterol levels after dietary cholesterol and fat interventions. The analysis grouped the animals into three categories, which are hypo-responsive, hyper-responsive, and extreme. Twenty-two reference sequences with three haplotypes (I, II, and III) were used to compare animals based on their responsiveness. Haplotype II (GC) is a haplotype of animals with hypo-response properties, that are T3707 and K30. Haplotype III (GG) is a haplotype with extreme responsiveness, which is possessed by the animal with individuals number K3535, and Haplotype I is possessed by 19 samples of *M. fascicularis* with hyper-responder characteristics (Taher *et al*. 2016). In *M. fascicularis* and *M. nemestrina*, although the animal's responsiveness is confirmed to be related to haplotype type, the molecular mechanisms underlying this relationship are still unknown.

4.2 Phylogenetic Tree Construction

Phylogenetic tree construction in *M. fascicularis* and *M. nemestrina* is supported by bootstrap values. Bootstrap is a value that describes the confidence level from a branch point in a topology using a computer. If the bootstrap value is between 95-100%, it can be concluded that the branch has high confidence (Ubaidilah and Sutrisno 2009). A total of six individuals of *M. fascicularis* and *M. nemestrina* were analyzed in the same cluster. The result shows the same response to dietary cholesterol and fat in all six samples: hyper-response. The grouping of hyper-responder, hypo-responder, and extreme individuals is due to differences in the nucleotide bases found in the exon 6 region of the LDLR gene. Hyper-responders and hypo-responders differ in one nucleotide base, while hyper-responders and extreme differences in two nucleotide bases.

The SNP analysis of the LDLR gene in exon 6 indicated a high similarity between the exon 6 regions of the LDLR gene in the *M. fascicularis* and *M. nemestrina* samples studied. All samples of *M. fascicularis* and *M. nemestrina* had identical sequences of exon 6 regions of the LDLR gene. This result showed the absence of SNPs in all six sequences. Both individual samples can potentially be used as animal models for atherosclerotic research. The exon 6 regions of the LDLR gene can be used as a molecular marker for atherogenesis in *M. fascicularis* and *M. nemestrina*. Selection of animals based on their sensitivity to dietary cholesterol and fat becomes faster and more efficient and supports the ethical principles of animal welfare 3R (reduction, refinement, and replacement).

Exon 6 can be used as a molecular marker of hyper- and hypo-response of *M. fascicularis* and *M. nemestrina* to dietary cholesterol and fat. All of the study samples had haplotype I. The haplotypes possessed by the six animals were related to hyper-responsiveness. The six individuals of *M. fascicularis* and *M. nemestrina* were analyzed in the same group. Using exon 6 as a genetic marker makes the selection of hypo- from hyper-responder animals faster and more efficient.

Acknowledgements

This research was fully supported by Primate Research Center (PRC) at IPB University. All activities were conducted in the Laboratory of Biotechnology PRC IPB University. The authors thank Dr. Ahmad Taher from the University of Papua for sharing the data.

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