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Taher Achmad

*Graduate School, Institut Pertanian Bogor, Dramaga, Bogor 16680, Indonesia, a.taher@unipa.ac.id*

Dedy Duryadi Solihin

*Departmen of Biology, Faculty of Mathematics and Natural Science, Institut Pertanian Bogor, Dramaga, Bogor 16680, Indonesia*

Sulistiyani Sulistiyani

*Departmen of Biochemistry, Faculty of Mathematics and Natural Science, Institut Pertanian Bogor, Dramaga, Bogor 16680, Indonesia*

Dewi Apri Astuti

*Departmen of Animal Nutrition, Faculty of Animal Husbandry, Institut Pertanian Bogor, Dramaga, Bogor 16680, Indonesia*

Dondin Sajuthi

*Primate Research Center, Institut Pertanian Bogor, Lodaya, Bogor 16151, Indonesia*

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### Cover Page Footnote

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## Single Nucleotide Polymorphism within the Promoter Region of Cynomolgus Monkey *LDLR* Gene

Achmad Taher<sup>1\*</sup>, Dedy Duryadi Solihin<sup>2</sup>, Sulistiyan<sup>3</sup>, Dewi Apri Astuti<sup>4</sup>, and Dondin Sajuthi<sup>5</sup>

1. Graduate School, Institut Pertanian Bogor, Dramaga, Bogor 16680, Indonesia

2. Departmen of Biology, Faculty of Mathematics and Natural Science, Institut Pertanian Bogor, Dramaga, Bogor 16680, Indonesia

3. Departmen of Biochemistry, Faculty of Mathematics and Natural Science, Institut Pertanian Bogor, Dramaga, Bogor 16680, Indonesia

4. Departmen of Animal Nutrition, Faculty of Animal Husbandry, Institut Pertanian Bogor, Dramaga, Bogor 16680, Indonesia

5. Primate Research Center, Institut Pertanian Bogor, Lodaya, Bogor 16151, Indonesia

\*E-mail: a.taher@unipa.ac.id

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### Abstract

The cynomolgus monkey (*Macaca fascicularis*) is one of the most common nonhuman primates used as an animal model in biomedical research related to atherosclerosis. However, little is known about genetic variation in the low density lipoprotein receptor (*LDLR*) gene, its effect on cholesterol levels, and associated risks of atherosclerosis. Therefore this study aimed to identify genetic polymorphisms, namely single nucleotide polymorphisms (SNPs), within the promoter region of *LDLR* and their relationship with animal responsiveness to hypercholesterolemia due to an atherogenic diet. In this research, SNPs were studied using DNA isolated from 22 cynomolgus monkeys obtained from a previous study, consisting of two hyporesponders, 19 hyperresponders, and one extreme case. The result showed that two SNPs existed in the promoter region, namely g.-169T>C and g.-265G>A. SNP g.-265G>A showed linkages with extreme responsiveness and can be used as a potential genetic marker for extreme animals. The result of this study has extended our knowledge of polymorphism in the *LDLR* gene and its use in the selection of cynomolgus monkeys as animal models in research on hypercholesterolemia and atherosclerosis.

### Abstrak

**Polimorfisme Nukleotida Tunggal (SNP) dalam Daerah Promotor Gen *LDLR* Monyet Ekor Panjang.** Monyet ekor panjang (*Macaca fascicularis*) adalah salah satu satwa primata yang paling umum digunakan sebagai model hewan dalam penelitian biomedis yang terkait aterosklerosis. Meskipun demikian, masih sedikit informasi mengenai variasi genetik pada gen reseptor lipoprotein densitas rendah (*LDLR*) dan pengaruhnya terhadap kadar kolesterol dan risiko aterosklerosis. Penelitian ini bertujuan mengidentifikasi polimorfisme genetik berupa polimorfisme nukleotida tunggal (SNP) dalam daerah promotor gen *LDLR* dan keterkaitannya dengan sifat respons hewan terhadap hiperkolesterolemia akibat diet aterogenik. Pada penelitian ini SNP dikaji menggunakan DNA yang diisolasi dari 22 monyet ekor panjang. dari penelitian sebelumnya, terdiri atas dua individu yang bersifat hipo-respons, 19 hiper-respons dan satu ekstrem. Hasil penelitian menunjukkan adanya dua SNP pada daerah promotor, yaitu g.-169T>C dan g.-265G>A. SNP g.-265G>A menunjukkan keterkaitan dengan sifat respons yang ekstrem dan berpotensi digunakan sebagai penanda genetik terhadap hewan ekstrem. Hasil studi ini menambah pemahaman kita mengenai polimorfisme dalam gen *LDLR* dan penggunaannya dalam seleksi monyet ekor panjang sebagai model hewan dalam penelitian mengenai hiperkolesterolemia dan aterosklerosis.

**Keywords:** *Cynomolgus monkey, LDLR, promoter, responsiveness, SNP*

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### Introduction

Macaques are commonly used in scientific research as animal models of human behavior and disease. Although

less expensive and more abundant animal models, such as rodents and zebrafish, overshadow macaque use in research in part due to their easier husbandry, such animal models do not always provide the best

translational medicine when trying to elucidate human pathologies [1]. The long-tailed macaque (*Macaca fascicularis*), also known as the crab-eating or cynomolgus monkey, one of the monkeys (or macaques) most commonly used in biochemical research, particularly as an animal model for human atherosclerosis research, due to the general response of its plasma cholesterol to an atherogenic diet [2]. Even though most cynomolgus monkeys show a strong response to dietary cholesterol (*hyperresponder*), there are individuals in the population that show only a small response (*hyporesponder*) and still others that show a particularly strong response (*extreme*) [3]. A previous study on the development of an atherogenic diet to induce lipid plasma, conducted at the Primate Research Center of Bogor Agricultural University (PSSP IPB), reported that two of 22 cynomolgus monkeys fed an atherogenic diet for three months were hyporesponders and that one was extreme. It is hypothesized that variations in responsiveness to an atherogenic diet may be caused by genetic variability [4].

As with humans, cynomolgus monkeys have a diverse genetic background, as evidenced by a number of genetic polymorphisms reported previously [5,6], including several functional polymorphisms in genes involved in metabolic and inflammatory pathways [7,8] and in the immune system [9]. However, few studies have focused on polymorphisms in genes that are involved in cholesterol metabolism, particularly the *LDLR* gene, which codes for the low density lipoprotein receptor protein (LDL-R), and its implications for cholesterol levels and risk of atherosclerosis. Our previous study showed the existence of common genetic polymorphisms within the exon 6, intron 5, and 3'UTR regions of the cynomolgus monkey *LDLR* gene [10,11] but did not focus on the promoter region.

The *LDLR* gene encoding human LDL-R is 45 kb and is localized on chromosome 19 p13.1–13.3. The gene is composed of 18 exons and 17 introns, encoding an mRNA of 5.3 kb and a protein of 860 amino acids. The *LDLR* gene encodes a signal peptide (exon 1), a ligand binding domain (exons 2–6), an epidermal growth factor

homology domain (exons 7–14), an O-linked sugar domain (exon 15), a transmembrane domain (exons 16, 17), and a cytoplasmic domain (exons 17 and 18) [12]. LDL-R, a transmembrane cellular protein, plays a crucial role in the receptor-mediated pathway of lipoprotein metabolism. LDL-R modulates plasma levels of low density lipoprotein cholesterol (LDL-C) by regulating the liver's uptake of LDL particles and by delivering cholesterol to the adrenal glands and gonads for steroid hormone synthesis and to the liver for bile acid synthesis [13].

In this study, an attempt was made to identify polymorphisms within the promoter region of the cynomolgus monkey *LDLR* gene. The objective of this study was to identify single nucleotide polymorphisms (SNPs) within the promoter region of *LDLR* and their relationship with animal responsiveness to hypercholesterolemia due to an atherogenic diet. Results from this study on polymorphism are expected to improve efficiency in the use of cynomolgus monkeys as animal models in research on hypercholesterolemia and atherosclerosis.

## Methods

**Animals.** The 22 cynomolgus monkeys (*Macaca fascicularis*) used in this study were adult males held at the animal facility of the Primate Research Center of Bogor Agricultural University, Indonesia. The monkeys ranged from six to eight years old, as estimated through their dentition, their body weights averaged between four and five kg. All monkeys were subject to the standards set forth by the Institutional Animal Care and Use Committee (ACUC) with protocol number 12-B009-IR. Blood samples were obtained from each of the 22 monkeys, which, as previously described, were identified as hyporesponder, hyperresponder, or extreme after following a three-month, high cholesterol diet [4]. Animals that had plasma cholesterol concentrations within a range of 1.5 standard deviations from the mean (250 to 900 mg/dL) were classified as hyperresponders, whereas animals with cholesterol levels below or above this range (<250 mg/dL or > 900 mg/dL) were classified as hyporesponder or extreme, respectively (Table 1).

**Table 1. Study Animals' Responsiveness to Atherogenic Diet**

Animals (Tattoo No.)	Responsiveness	Animals (Tattoo No.)	Responsiveness
T3707	Hypo-	FE7777	Hyper-
K30	Hypo-	T3536	Hyper-
FC8501	Hyper-	C2480	Hyper-
T3049	Hyper-	T3303	Hyper-
FG7998	Hyper-	FG7909	Hyper-
T3307	Hyper-	T3300	Hyper-
T3700	Hyper-	C0750	Hyper-
T3278	Hyper-	FC9015	Hyper-
FC9113	Hyper-	C4927	Hyper-
9695	Hyper-	T3535	Hyper-
C4939	Hyper-	C0613	Extreme

**Genomic DNA Extraction.** Genomic DNA was extracted from 22 whole-blood samples using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions.

**PCR and Sequencing.** The promoter regions within the cynomolgus monkey *LDLR* genes were amplified based on primers from a previous study [14]. The primers were F: 5'-GGATCCCGACCTGCTGTGTCCTAGC TGGAAA-3' and R: 5'-GGATCCAATCAAGTCGCCT GCCCTG GCGACA-3'. PCR reactions were conducted in a 25 µL volume and contained 5 µL of genomic DNA, 1 µL of each primer (10 pmol), 12.5 µL of KAPA HiFi HotStart ReadyMix PCR Kit (KAPA Biosystems, Boston, USA) and 5.5 µL of nuclease-free water. Amplification was performed using a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Foster City, USA), and the cycling parameters were as follows: denaturation at 94 °C for 5 min, followed by 40 cycles. Each cycle consisted of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 7 min. The final extension was at 72 °C for 4 min. Amplicons were visualized on a transilluminator following 1.8% agarose gel electrophoresis containing ethidium bromide 10 µg/mL to check band specificity and sufficiency for subsequent sequence analysis. DNA fragments were then purified using the MinElute PCR Purification Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions, and sequencing was performed at First BASE Laboratories Sdn Bhd (Malaysia).

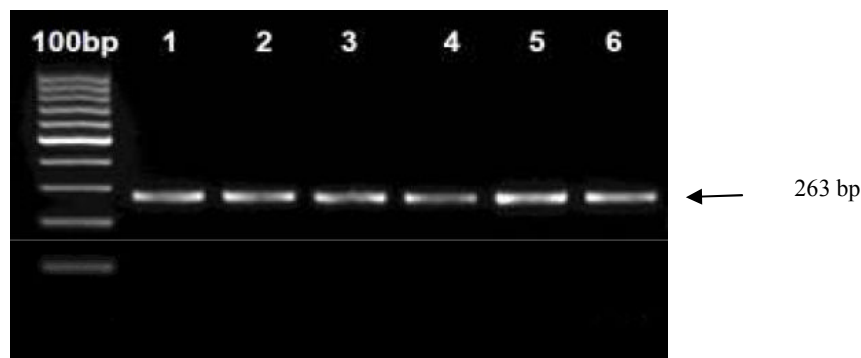
**Sequence and Data Analysis.** Consensus sequences were obtained by combining forward and reverse strands for each amplicon and aligning them to reference sequences of *Macaca fascicularis* (GenBank accession number XM\_005587996.2) using MEGA-6. Geneious 7.0.2 (<http://geneious.en.softonic.com>, 30-day trial version) was used to edit and assess the quality of sequence data and to generate a final consensus sequence for each amplicon. Multiple sequence alignments were obtained using CLUSTAL W 1.8 in MEGA-6, and haplotype analysis was conducted in

DnaSP. The program TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>) was used to identify transcription factor binding sites within the vicinity of SNPs. The accession numbers of our nucleotide data in GenBank are: KY962394 (T3707), KY962395 (K30), KY962396 (FC8501), KY962397 (T3049), KY962398 (FG7998), KY962399 (T3307), KY962400 (T3700), KY962401 (T3278), KY962402 (FC9113), KY962403 (9695), KY962404 (C4939), KY962405 (FE7777), KY962406 (T3536), KY962407 (C2480), KY962408 (T3303), KY962409 (FG7909), KY962410 (T3300), KY962411 (C0750), KY962412 (FC9015), KY962413 (C4927), KY962414 (T3535), and KY962415 (C0613).

## Results and Discussion

**Amplification and Sequencing.** Amplification using forward and reverse primers for the promoter region resulted in product sizing of 263 bp (Figure 1). Aligning the consensus sequences to the reference showed that the amplicon contained part of the promoter region of the cynomolgus monkey *LDLR* gene from position -296 to -34, relative to the major transcriptional initiation site (nucleotide +1 is the A of the ATG-translation codon, and nucleotide 5' of the ATG-translation codon is -1). This region contains three 16-bp repetitions which have been shown to be important for basal level expression and sterol regulation of human *LDLR* and transcription initiation sites [15].

**SNP and Haplotype.** Nucleotide sequence analysis of the amplicons of 22 cynomolgus monkeys identified two SNPs, namely g.-169T>C and g.-265G>A. The results of the alignment analysis showed that only two out of the 22 animals have nucleotide base sequences possessing polymorphism, i.e. C2480 (GenBank accession number KY962407) and C0613 (GenBank accession number KY962415). Animal C2480 has one SNP, i.e. g.-169T>C, whereas animal C0613 has one SNP, i.e. g.-265G>A (Figure 2). Analysis using the TFSEARCH program revealed that there was no binding site of transcription factors in the vicinity of the polymorphic sites. This result indicated that the identified polymorphisms



**Figure 1.** Agarose Gel Electrophoresis Image of PCR Products of Samples. Lane 100 bp is Marker Ladder; Lanes 1-6 are Samples; 263 bp is Product Sizing

20 animals	-34 >GGATCCCGACCTGTCGTGTCCTAGCTGGAAACTCTGGCTTCCCGCGATTGCACTCGGGGGCCCCACG > -99
C2480	-34 >GGATCCCGACCTGTCGTGTCCTAGCTGGAAACTCTGGCTTCCCGCGATTGCACTCGGGGGCCCCACG > -99
C0613	-34 >GGATCCCGACCTGTCGTGTCCTAGCTGGAAACTCTGGCTTCCCGCGATTGCACTCGGGGGCCCCACG > -99
20 animals	-100 >TCATTTACAGCATTTCAACGTGAGGTTTCTAGCAGGGGGAGGAGTTTGCAGTGGGGTGATTTTCAA > -165
C2480	-100 >TCATTTACAGCATTTCAACGTGAGGTTTCTAGCAGGGGGAGGAGTTTGCAGTGGGGTGATTTTCAA > -165
C0613	-100 >TCATTTACAGCATTTCAACGTGAGGTTTCTAGCAGGGGGAGGAGTTTGCAGTGGGGTGATTTTCAA > -165
20 animals	-166 >ATGTCTTCACCTCACAGTGAGAGGAGGAGTTTCGAACGGCTGATGTGACATCGGCTTTTAAACCCG > -231
C2480	-166 >ATGCTTCACCTCACAGTGAGAGGAGGAGTTTCGAACGGCTGATGTGACATCGGCTTTTAAACCCG > -231
C0613	-166 >ATGTCTTCACCTCACAGTGAGAGGAGGAGTTTCGAACGGCTGATGTGACATCGGCTTTTAAACCCG > -231
20 animals	-232 >TGAAGCTCTGATTCCCAGTCCAGTCTTTTGAAAGTGTGCCAGGGCAGGCGACTTGATTGGATCC > -296
C2480	-232 >TGAAGCTCTGATTCCCAGTCCAGTCTTTTGAAAGTGTGCCAGGGCAGGCGACTTGATTGGATCC > -296
C0613	-232 >TGAAGCTCTGATTCCCAGTCCAGTCTTTTGAAAGTGTGCCAGGGCAGGCGACTTGATTGGATCC > -296

**Figure 2. Alignment of the Promoter Sequence from 22 Cynomolgus Monkeys. The Single Nucleotide Polymorphisms (SNPs) in Animals C2480 and C0613 are Indicated by the Gray Shadow**

**Table 2. Identified SNPs within the Promoter Region, Aligned to Reference and Kind of Haplotype**

Haplotypes	Nucleotides base position		No. of Individuals	Tattoo No.
	-169	-265		
Ref	T	G	-	-
I	T	G	20	FE7777, T3700, T3536, FC9015, T3303, T3278, FC8501, T3535, T3307, C4927, 9596, T3049, FG7909, FG7998, C0750, T3300, C4939, FC9113, T3707, K30
II	C	G	1	C2480
III	T	A	1	C0613

were non functional; a functional polymorphism is defined as a site that causes binding site appearance or disappearance of new transcription factors [16].

The two polymorphic sites produced three haplotypes, i.e. haplotype I (TG), II (CG), and III (TA). Haplotype I is similar to that belonging to the reference cynomolgus monkey in the GenBank; almost all (20 of 22) animal samples contained this haplotype (Table 2). Haplotype grouping becomes particularly interesting when the results are compared to the cynomolgus monkeys' responsiveness to an atherogenic diet, shown in Table 1. Namely, haplotype III (TA) belongs to cynomolgus monkey C0613, which is also the only study animal that demonstrates an extreme response to the atherogenic diet. The similarity of the SNP and responsiveness groupings shows a relationship between polymorphism and responsiveness to an atherogenic diet in cynomolgus monkeys.

This research demonstrated the existence of common genetic polymorphisms within the promoter regions of the cynomolgus monkey *LDLR* gene. Linking haplotype III to extreme responsiveness in the cynomolgus monkey gives the identified polymorphic sites the potential to be used as genetic markers, although the results still need to be confirmed using a larger sample of extreme responders. The existence of non functional SNPs within the promoter region suggests the possibility that these SNPs are exhibiting linkage disequilibrium

in relation to other functional SNPs, influencing the responsiveness to an atherogenic diet.

The function of polymorphisms identified in the *LDLR* gene of cynomolgus monkeys as a genetic marker for responsiveness has been studied before. Our previous study [10] reported the existence of a specific haplotype GCGC within the 3'UTR of the cynomolgus monkey *LDLR* gene that belonged only to an animal with extreme responsiveness. Moreover, within the regions of exon 6 and intron 5, the study found a specific haplotype associated with hyporesponders [11]. Thus, the present study supports the important principle that a common genetic variant can be used as a potential genetic marker of responsiveness. Previous reports identifying SNPs or haplotypes of the cynomolgus monkey have been mainly related to origin or geographical distribution [17,18]. Investigations of individual genetic variations that affect susceptibility to diseases or other disorders are still few when compared to the total number of biomedical research studies conducted on cynomolgus monkeys. Some such studies have included genetic variations related to malaria susceptibility [16], drug safety [19], and neurobiological reactivity due to stress [20].

Identification of genetic variations within the promoter region of the *LDLR* gene as genetic markers of responsiveness to an atherogenic diet is an important breakthrough, as it makes the preliminary selection of

animals simpler and more efficient. To date, in primate centers the selection of animals for research on responsiveness to diet requires an atherogenic diet intervention for two months [21,22]. This selection process is inefficient, because it requires strict control of the diet. Using the presence of genetic markers as a basis for the selection of extreme animals eliminates the need for treatment and control of the diet. In terms of time, the analysis of genetic variation is faster, while in terms of budget, genetic variation analysis is cheaper than animal feeding and care for the two month selection period in primate centers.

Selection of test animals prior to conducting research related to atherosclerosis is crucial, as it can improve the accuracy and efficiency of the scientific studies that will eventually support research success. Initial selection also reduces the number of test animals, thereby supporting the principles of the 3Rs (reduction, refinement, and replacement). Furthermore, the selection of animals based on genetic variation may reduce current limitations of using primates as models in genetic studies of complex diseases. Using animals that are genetically uniform will give greater power to the statistical analysis of tested variables, especially when using small samples [23]. The work presented here addresses the genetic variation in a functional gene and its influence on experimental outcomes. This result can also improve knowledge of biomarkers for disease, genetic disorders, and toxicology.

## Conclusion

This research has identified two SNPs in the promoter region of the cynomolgus monkey *LDLR* gene, which can be used as a potential genetic marker for animals with extreme responsiveness to an atherogenic diet. Results from this study contribute to the efficient use of the cynomolgus monkey as an animal model in research related to hypercholesterolemia and atherosclerosis.

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