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Single Nucleotide Polymorphism within the *LDLR* gene and responsiveness of cynomolgus macaque (*Macaca fascicularis*) to atherogenic diet

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Abstract. *Taher A, Solihin DD, Sulistiyani, Sajuthi D, Astuti DA. 2016. Single Nucleotide Polymorphism within the LDLR gene and responsiveness of cynomolgus macaque (*Macaca fascicularis*) to atherogenic diet. Biodiversitas 17: 430-434.* Genetic variation within low density lipoprotein receptor (*LDLR)* gene has been associated with normal variation of plasma lipid profiles and risks of coronary heart diseases (CHD) in human. Although cynomolgus macaque *(Macaca fascicularis)* is one of non-human primates commonly used as models in atherosclerosis research, little is known about the extent of polymorphism within the *LDLR* gene and its consequences on responsiveness to atherogenic diet. In this study, two regions of *LDLR* gene, namely exon 6 and intron 5, were sequenced in a sample of 22 male cynomolgus macaques which had differences in responsiveness to atherogenic diet. The objective of the study was to identify single nucleotide polymorphism (SNP) within the *LDLR* gene and to evaluate the kinds of haplotypes in relation to the responsiveness of the cynomolgus macaque to atherogenic diet. Sequence analysis revealed that there were two SNPs at exon 6, i.e. IVS5-6C > G and 825C > G, which were distributed in 3 haplotypes, and five SNPs at intron 5, i.e. g.IVS5+99T > C, g.IVS5+173G > T, g.IVS5+327A > G, g.IVS5−96C > T, and g.IVS5−6C > G, which were distributed in 6 haplotypes. It was found that haplotype II (GC) at 6 base pairs prior to the exon 6 and haplotype III (CGGTG) within the intron 5 were associated with hyporesponsiveness to atherogenic diet. The results showed that potential SNP existed within the exon 6 and intron 5 can be used as genetic markers for selecting hypo- from hyperresponders.

Keywords: Hyporesponsiveness, *LDLR* gene, *Macaca fascicularis*, SNP

INTRODUCTION

Cynomolgus macaque (*Macaca fascicularis*), also commonly known as the crab-eating or long-tailed macaque, has a lengthy history of being used as a nonhuman primate model for the study of human atherosclerosis because this species is responsive to dietary cholesterol (Shelton et al. 2012). Even though cynomolgus macaques are responsive, feeding of atherogenic diets to this species results in marked inter individual differences in the response of plasma cholesterol. Certain animals show only small responses (*hyporesponders*) whereas others develop high degrees of hypercholesterolemia (*hyper-responders*) (Beynen et al. 1987). Hyporesponders are constrained in the provision of hypercholesterolemia animals to be used as models to evaluate the effect of diet on plasma lipid profile and its association with the progression or regression of atherosclerosis.

Low density lipoprotein receptor (LDL-R) is a cell membrane glycoprotein that plays a key role in maintaining normal plasma cholesterol levels, mediating the endocytosis of LDL and other cholesterol-carrying particles (Goldstein et al. 1995). Human *LDLR* gene consists of 18 exons and 17 introns with the length of approximately 45 kb, and is mapped onto chromosome 19p13.2. Mature mRNA is 5.3 kb long and encodes a protein of 860 amino acids. Mature receptor (without the signal peptide) is a 839-amino acid protein which can be divided into five functional domains: (i) a 292-amino acid ligand-binding region, (ii) a 400-amino acid region which is homologous to the precursor for epidermal growth factor and is required for dissociation of the receptor from the ligand in lysosomes and for recycling of the receptor to plasma membrane, (iii) a 58-amino acid domain which is extensively glycosylated, (iv) a transmembrane region, and (v) a cytoplasmic domain which is required for targeting the protein to clathrin-coated pits for internalization (Sudhof et al. 1987). Mutations in the *LDLR* gene that disturb the normal functions of the LDL-R protein can cause familial hypercholesterolemia (FH), which is associated with elevated total and LDL-cholesterol and premature coronary heart diseases (CHD) (Hoobs et al. 1990). FH, however, accounts for only about 5% of patients with CHD, and the contribution of genes to CHD in the remaining 95% of cases is still unknown (Ahn et al. 1994). Common single nucleotide polymorphisms (SNPs)

in genes involved in lipid metabolism are potentially important genetic markers in affecting normal variation in plasma or serum lipid profiles and thus determining susceptibility or resistance to CHD in a general population (Kathiresan et al. 2008; Shandu et al. 2008; Talmud et al. 2013).

As with humans, cynomolgus macaques have a diverse genetic background as evidenced by number of genetic polymorphisms that have been reported (Ebeling et al. 2011; Yan et al. 2011; Higashino et al. 2012). Some polymorphisms are functional in gene that involves in metabolic and inflammatory pathway (Uno et al. 2010; Wu and Adkins 2012), and others are related to malaria susceptibility (Flynn et al. 2009). However, very few studies have focused on polymorphism within *LDLR* gene and its consequences on responsiveness to atherogenic diet. In this study, two regions of *LDLR* gene, namely exon 6 and intron 5, were sequenced in a sample of 22 adult male cynomolgus macaques which had differences in responsiveness to atherogenic diet. The primary objective of the study was to identify single nucleotide polymorphism (SNP) within the *LDLR* gene. In addition, a secondary objective of the study was to evaluate kinds of haplotypes in relation to the responsiveness of cynomolgus macaque to atherogenic diet.

MATERIALS AND METHODS

Animals

Blood samples were obtained from 22 adult males of cynomolgus macaques (*M. fascicularis*) from captivity in Primate Research Center of Institut Pertanian Bogor, West Java, Indonesia. The animals were housed in individual cages that were positioned as such so that they can see and hear each other. They were fed with 100-180 g per animal twice a day (08.00 am and 02.00 pm) plus one piece of 70 g banana (12.00 pm). Water was given *ad libitum*. Blood samples were collected by femoral venipuncture about 2 ml using standard techniques while the monkeys were sedated by Ketamin HCl (10 mg/kg body weight, given intramuscular). All treatment procedures applied on the animals had been approved by Institutional Animal Care and Use Committee (IACUC) with protocol number 12-B009-IR. The 22 cynomolgus macaques were devided into 3 groups based on their responsiveness to atherogenic diet (Table 1). They were classified as hyporesponse, hyperresponse, or extreme following a feeding regime of a high cholesterol diet for three months. Animals that had plasma cholesterol concentrations within range of 1.5 SD from mean were classified as hyperresponse (250 to 900 mg/dL), whereas animals with cholesterol levels below or above this range (< 250 mg/dL or > 900 mg/dL) were classified as hyporesponse or extreme.

Genomic DNA extraction

Genomic DNA was extracted from all whole-blood samples using a QIAamp™ DNA Mini Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions.

Table 1. Animals used in this study and their groupings based on responsiveness to atherogenic diet

Animals (Tattoo no.)	Respon-siveness	Animals (Tattoo no.)	Respon-siveness
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-	C0613	Extreme
C4939	Hyper-	T3535	Extreme

Table 2. Primers used and length in base pairs of expected PCR products

Regions	Primers	PCR product	*Annealing* Temp.
Exon 6	F: 5'-CCTTCCTCCTTCCTCTCTCT-3' R: 5'-ACTCTGCAAGCCGCCTGCAC-3'	184 bp	56°C
Intron 5	F: 5'-AAAATCAACACACTCTGTCC-3' R: 5'-ACTCTGCAAGCCGCCTGCAC-3'	1010 bp	56°C

PCR and sequencing

Two regions within *LDLR* gene of cynomolgus macaque were amplified based on primers from previous study (Hummel et al. 1990). Primers used are shown in Table 2. Reactions were conducted in a 25 µL volume and contained 5µL genomic DNA, 1 µL of each primer 10 pmol, 12.5 µL KAPA HotStart readymix Kit (buffer solution, dNTP and Taq polymerase enzyme) and 5.5 µL nuclease free water. Amplification was performed by using a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA) 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 post extension was at 25°C for 4 min. Amplicons was visualized on a transilluminator following agarose gel electrophoresis to check band specificity and sufficiency for subsequent sequence analysis. DNA fragments were then purified using the MinElute Qiagen Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions, and sequencing was performed in First BASE Laboratories Sdh. Bhd. (Malaysia).

Sequence and data analysis

Consensus sequences were obtained by combining forward and reverse strands for each amplicon and aligning them to a reference sequences of *Macaca fascicularis* (Genbank accession number XM_005587996.2). We used Geneious 7.0.2 (http://geneious.en.softonic.com, the 30-day trial version) to edit and assess the quality of sequence data, and to generate final consensus sequence for each amplicon. Multiple sequence alignments were obtained by using CLUSTAL W 1.8 in Mega-6, and phylogenetic tree

were conducted in the same program (Tamura et al. 2013). Haplotypes analyses was conducted in DnaSP (Rozas et al. 2003).

RESULTS AND DISCUSSION

Amplification and sequencing of exon 6 region

The amplification of exon 6 region gave results as expected, that was the amplicon with a size of 184 bp. Aligning the consensus sequences to the reference showed that the amplicon contained not only the base nucleotide of exon 6 (125 bp), but also 30 bp of intron 5 and 29 bp of intron 6.

SNP and Haplotype within exon 6 region

The analysis of the nucleotide sequences for the amplicon of 22 cynomolgus macaques had identified two single nucleotide polymorphisms (SNPs), those were IVS5-6C > G (within the intron 5, 6 nucleotides before the beginning of exon 6 nucleotide) and 825C > G (within the exon 6). The identified polymorphic sites as presented in Table 3 show that only 3 out of the 22 animals have nucleotide base sequences possessing polymorphism, i.e. T3535, T3707 and K30. The T3535 animal has two SNPs, i.e. IVS5-6C > G and 825C > G, whereas each of the T3707 and K30 animals have one SNP, i.e. IVS5-6C > G. The 825 polymorphic site in *LDLR* cDNA is located on the third position of codon triplet and it has no effects on the encoded amino acids. Also, the SNP IVS5-6C > G gives no effects on the encoded amino acids because it is located on intron region.

The two sites of polymorphic sites produced three haplotypes i.e. haplotype I (CC), II (GC) and III (GG) with a diversity of 0.255 ± 0.01347. Haplotype I is similar to the one that belongs to the reference cynomolgus macaque in the GenBank and contained in almost all animal's blood samples (Table 3). Animal grouping based on the haplotypes shows an interesting fact when it is related to the responsiveness of cynomolgus macaque to atherogenic diet in Table 1. The fact is that the haplotype II (GC) is a haplotype that belongs to the cynomolgus macaque that possesses hyporesponsiveness i.e. animals T3707 and K30. The similarity of the grouping of these two species based on the haplotype types and the responsiveness to atherogenic diet shows a relationship between the kinds of haplotype and the responsiveness. Electropherogram results from the three kinds of haplotypes are shown in Figure 1.

Amplification and sequencing of Intron 5 region

Amplification using forward and reverse primer for intron 5 results in product sizing of 1010 bp. The sequencing results show that the amplification product consists of 30 bp as part of intron 4, 126 bp is exon 5, 704 bp is intron 5, 126 bp is exon 6 and 24 bp as part of intron 6.

SNP and haplotype within Intron 5 region

An analysis that was performed on the intron 5 sequences of the 22 cynomolgus macaques, had identified 5 polymorphic sites which produce 6 haplotypes. The identified polymorphic sites consists of variables of four parsimony sites (IVS5+99, IVS5+327, IVS5−96 and IVS5−6) and one singleton site (IVS5+173). The resulting haplotype diversity was 0.680 ± 0.095. Six identified haplotypes were haplotypes I (TGACC), II (CGGCC), III (CGGTG), IV (CGACC), V (TGATG) and VI (TTACC). Haplotype I was similar to the reference haplotype belongs to rhesus macaque as in the GenBank and had the highest number of individuals. The rhesus macaque was used as a reference because the intron 5 sequences data of the cynomolgus macaque was not yet available in the Genbank. The Haplotype IV was singleton haplotype and it belonged only to T3535 animals. The total haplotypes and individual groupings based on the haplotypes are shown in Table 4.

Animal grouping based on the haplotypes showed an interesting fact when it is related to the responsiveness of the cynomolgus macaque to atherogenic diet as seen in Table 1. The fact is that the haplotype III (CGGTG) is a haplotype that belongs to the cynomolgus macaque that possesses hyporesponsiveness i.e. animals T3707 and K30. Reconstruction of the phylogenetic tree showed that individuals with haplotype III formed their own groups with high bootstrap value, that is 81% (Figure 2). The high Bootstrop values are benchmarks for the determination of grouping confidence level. This means that T3707 and K30 animals are in separate groups based on the haplotypes owned. The similarity of the groupings of these two species based on the haplotype types and the hyporesponsiveness to atherogenic diet showed a relationship between the types of haplotype and the responsiveness to atherogenic diet.

Table 3. Identified SNPs within exon 6 region aligned to reference GenBank (access number XM_005587996.2).

Haplo-types	Nucleotides base position		No. of ind.	Tattoo no.
	IVS5-6	825		
Ref	C	C	-	-
I	C	C	19	FE7777, T3700, T3536, FC9015, T3303, T3278, FC8501, C0613, T3307, C4927, 9596, T3049, FG7909, FG7998, C0750, T3300, C2480, C4939, FC9113
II	G	C	2	T3707, K30
III	G	G	1	T3535

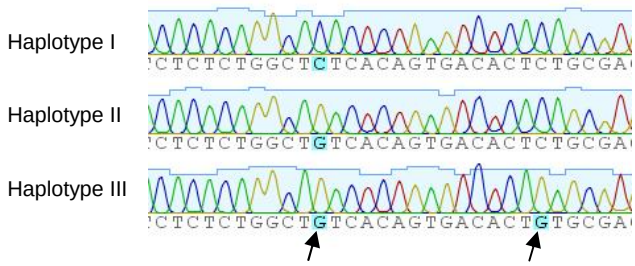

Figure 1. Electropherogram of three patterns of haplotypes

Table 4. Identified polymorphic sites within intron 5 *LDLR* gene and haplotypes, and aligned to the rhesus macaque as a reference (GenBank accession number AY466854).

Haplo-type	Position within the intron 5					No. of ind.	Tattoo no.
	IVS5+99	IVS5+173	IVS5+327	IVS5−96	IVS5−6		
Ref	T	G	A	C	C	-	-
I	T	G	A	C	C	12	9695, C0613, C0750, C2480, FC8501, FC9113, FG7998, T3049, T3278, T3303, T3700, FC9015
II	C	G	G	C	C	4	C4927, C4939, FG7909, T3300
III	C	G	G	T	G	2	T3707, K30
IV	C	G	A	C	C	2	FE7777, T3307
V	T	G	A	T	G	1	T3535
VI	T	T	A	C	C	1	T3536

Discussion

This research showed the existence of common genetic polymorphisms within the regions of exon 6 and intron 5 of the cynomolgus macaque *LDLR* gene. The existence of haplotype II (GC) at 6 base pairs prior to the exon 6 and haplotype III (CGGTG) within intron 5 which are solely possessed by hyporesponders show that common genetic polymorphisms within these regions might be linked to the response variations of the cynomolgus macaque to atherogenic diet. In fact, these evidences have not been reported previously. Generally, reports on identifications of SNPs or haplotypes of the cynomolgus macaque were mainly related to the origin or geographical distribution (de Groot et al. 2011; Fawcett et al. 2011). Investigations on individual genetic variations that affect susceptibility to diseases or other disorders are still less compared to total number of biomedical researches conducted on cynomolgus macaque. Some of them were genetic variations against malaria susceptibility (Flynn et al. 2009), drug safety (Ebeling et al. 2011) and neurobiology reactivity due to stress (Rogers et al. 2013). In humans, the presence of SNP in the *LDLR* gene has been reported to affect normal variation in plasma lipid profile lies in intron 1 and exon 2 (Linsel-Nitschke et al. 2008; Willer et al. 2008).

At the stage of implementation, the similarity of grouping hyporesponder animals based on GC and CGGTG haplotypes allowed us to choose one of the two primer pairs used for genetic variation analysis. In practical aspects, the analysis of genetic variation wihin exon 6 (184 bp) is much more simple than intron 5 (1010 bp). Yet, both polymorphic sites on the exon 6 region differ only in a dozen of nucleotides which makes it easier to identify. Thus, the selection of exon 6 as a region used to identify genetic markers for hyporesponder the cynomolgus macaque becomes the primary choice. Linking the GC and CGGTG haplotypes to hyporesponsiveness in the cynomolgus macaque makes the identified polymorphic sites become potential to be used as genetic markers, although it still needs to be confirmed by using more samples of hyporesponders. The existence of the SNPs within the exon 6 and exon 5 regions that are not functional suggests the possibility of these SNPs to be in *disequilibrium linkage* with other functional SNPs in influencing the hyporesponsiveness to atherogenic diet.

Identification of genetic variation within the exon 6 and intron 5 regions of *LDLR* gene as genetic markers of responsiveness to atherogenic diet is an important breakthrough as it makes a preliminary selection of animals to be simpler and more efficient. Up to this time, the selection of responsiveness to diet in primate centers is done through atherogenic diet intervention in 2 months (Clarkson et al. 1988; Turley et al. 1997). This selection is inefficient as it requires large amount of animals, and strict control on the diet. By the presence of genetic markers as a basis for the selection of hyporesponder monkeys, then no treatment and control of the diet are needed. In terms of time span, the analysis of genetic variation is faster, while on the budget aspect, this technique is cheaper than costs of animal raising and feeding for two months during the selection period in the primate centers.

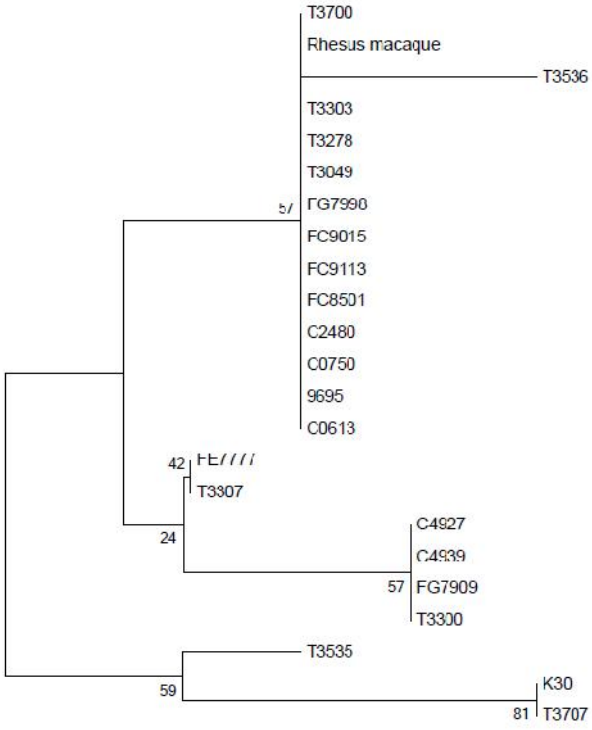

Figure 1. Phylogenetic tree based on intron 5 sequences reconstructed using *Neighbor Joining* method by a 1000-times bootstrap.

Selection of tested animals before conducting research related to atherosclerosis is crucial as it can improve the accuracy and efficiency of the scientific studies that eventually supports the success of the research. Initial selection will also reduces number of tested animals that supports the principles of 3Rs (*reduction*, *refinement* and *replacement*). Furthermore, the selection of animals based on genetic variation may reduce the limitation of using primates as models in genetic studies on complex diseases. The use of animals which are genetically uniform will give more power in statistical analysis of the tested variables, especially on small numbers of samples (Vallender and Miller 2013).

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