GENETIC VARIATION OF cDNA OF LDLR GENE IN HYPORESPONDER CYNONOMULUS MACAQUES (Macaca fascicularis)

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ABSTRACT

The study aimed to identify genetic variation of cDNA sequence from low density lipoprotein receptor (LDLR) gene of hyporesponder cynomolgus macaques. The animal used in this study was one hyporesponder cynomolgus macaque obtained from selection result in Primate Research Center-Bogor Institute of Agriculture (PSSP IPB). Amplification of cDNA from LDLR gene was performed using polymerase chain reaction (PCR) method with 4 pairs of walking primer. Alignment of amplification result sequence from 4 pairs of walking primer generated target sequence of 2353 bp which located on position 188-2540. Analysis of target sequence alignment on reference sequence in GenBank found 8 single nucleotide polymorphism (SNP), namely c.408C>T; c.1200C>T; c.1497C>T; c.1644T>C; 1791T>C; 1804A>G; 2088C>T; and 2377G>A. Of 8 SNPs, c.1804A>G and 2377G>A caused changing of amino acids composition namely p.K602E (lysine → glutamate) and p.V793I (valine → isoleucine). This result proved the potential use of genetic variation of cDNA sequence from LDLR gene as genetic marker for selection of hyporesponder cynomolgus macaques.

Key words: hyporesponder, LDLR, Macaca fascicularis, SNP

INTRODUCTION

Cynomolgus macaque (Macaca fascicularis) is one of the most common Indonesian primate animal used as experimental animal in biomedical research related to atherosclerosis (Sajuthi et al., 2015). As a model in understanding atherosclerotic disease in humans, cynomolgus macaques are made hypercholesterolemia through atherogenic diet interventions. Provision of atherogenic diets in these animals will generally increase plasma cholesterol levels, but the level varies between individual. Animals with hyper-responsive (hyper-responder) properties are very sensitive to atherogenic diets that are susceptible to hypercholesterolemia. In contrast, the animals with the hypo-responsive character (hypo-responder) are not or less sensitive, therefore they are not experience the state of hypercholesterolemia (Beynen et al., 1987).

Nowadays, the selection of hyper or hyporesponder cynomolgus macaque for metabolic heterogeneity of dietary cholesterol and atherogenesis studies was performed through atherogenic dietary interventions for two months, then the responses were grouped base on increasing of plasma cholesterol concentrations (Clarkson et al., 1988; Turley et al., 1997). This selection method is inefficient due to the high cost of maintenance in captivity and the high price of commercial atherogenic food (Astuti et al., 2014). Therefore, it is necessary to develop more efficient and effective method of selecting hypo and hyper-responder animals. Genetic marker-based selection is a viable alternative because it is stable and unaffected by the environment.

Similar to human, cynomolgus macaque also has a high genetic diversity of polymorphisms as previously reported (Street et al., 2007; Ebeling et al., 2011; Higashino et al., 2012). Polymorphism in the gene encoding obesity character (Putra, 2009) and various functional gene polymorphisms involved in cross-metabolic and inflammation (Flynn et al., 2009; Uno et al., 2010; Blancher et al., 2012; Wu and Adkins, 2012) showed that genetic variations are also related to the physiological condition of cynomolgus macaque. However, the data focusing on genetic variation of low density lipoprotein receptor (LDLR) gene does not yet exist.

The LDLR gene is a LDL receptor protein gene (LDL-R) that plays a very important role in cholesterol homeostasis (Brown and Goldstain, 1986). The human LDLR gene consists of 18 exons and 17 introns, with the length of about 45 kb, and is located at...
chromosome 19 p13.2. This gene produces mRNA of 5.3 kb and protein with 860 amino acids and consists of the regulator region (promotor), the signal peptide (exon 1), the ligand binder region (exon 2-6), the epidermal growth epidermal precursor region (7-14), the bounded O-sugar region (exon 15), transmembrane area (exon 16,17), cytoplasmic area (exon 17,18), and 3’UTR (3-untranslated region) (Sudhof et al., 1985).

This study aimed to identify genetic variations of cDNA sequences of LDLR genes in hyporesponder cynomolgus macaque. This identification is expected to be useful for the selection of hyper and hypo-responder cynomolgus macaque which is molecularly through genetic markers.

MATERIALS AND METHODS

Laboratory Animal
Experimental animal used was one adult male hyporesponder cynomolgus macaque, one of collection from Primate Research Center-Bogor Institute of Agriculture (PSSP IPB). Animal had undergone IPB-1 atheregenic dietary interventions with a cholesterol content of 0.29 mg/cal for one year and had total plasma cholesterol concentrations below 200 mg/dL (Astimu et al., 2014).

Blood Sample Collection
Animal was sedated with ketamine HCl (10 mg/kg body weight) intramuscularly and taken its blood from the femoral vein as much as 3 mL in the tube with anticoagulant EDTA. All animal procedures have been approved by the Welfare and Laboratory Animal Laboratory of PSSP IPB with protocol number 12-B009-IR.

PBMC Preparation
The blood sample was centrifuged at 1000 g for 15 min at room temperature to separate the plasma. The cell fraction is suspended in sterile phosphate buffer saline (PBS) of the same volume. Cynomolgus macaque suspension cells were mixed with one-fifth volume of dextran 5% T-2000 and T-500, and then incubated at 37° C for 30 min to separate erythrocytes. The supernatant obtained was leukocyte fraction, which was centrifuged at 400 g for 30 min at room temperature. Peripheral blood mononuclear cells (PBMC) accumulated above the Ficoll-Conray layer. Then the PBMC fraction was washed twice with a cold sterile PBS to remove the contaminating platelets. All solutions for the preparation of PBMC were pyrogen free.

RNA Isolation and Reverse Transcription
Total RNA was isolated from PBMC using RNeasy micro kit (Qiagen, Santa Clarita, CA, USA) in accordance with manufacturer protocol of on-column DNase treatment (DNase-Free RNaase Set, Qiagen). cDNA was synthesized from 1 μg of total RNA through reverse transcription with AML reverse transcriptase XL (Takara Bio, Otsu, Japan) and primers with oligo (dT) 12-18 primers (Invitrogen, Carlsbad, CA, USA).

Primer Designing, Amplification, and Sequencing
The primers to amplify cDNA of LDLR gene were designed based on M. fascicularis mRNA (GenBank access code XM_005587996.1) using Primer3 program (http://primer3.wi.mit.edu/). The primers used were listed in Table 1. Amplification was done by Gene Amp® PCR System 9700 (Applied Biosystem). The reaction components for cDNA amplification consisted of: 2 μL cDNA mold, 1 μL forward primer and 1 μL reverse primer with concentration 10 pmol/μL, 12.5 μL KAPA HotStart ready mix kit (KAPA Biosystem, MA, USA) and 8.5 μL nuclease free water. PCR consisted of an initial denaturation temperature of 94° C for 5 minutes and 40 cycle stages. Each cycle was carried out with the following reaction steps: the sample was denatured at 94° C for 30 seconds, followed by annealing on each primer for 30 seconds, and extension at 72° C for 30 seconds. After that the PCR process continued with post extension at 72° C for 7 minutes, and ended at 25° C for 4 minutes. Confirmation of PCR results was performed using 1.8% electrophoresis gel containing 10 μg/mL of ethidium bromide, and ran with 100 Volts for 45 minutes. The ladder DNA size used was 100 pb (Invitrogen). The gel electrophoresis results were observed under GelDoc UV light with the Quantity One (Biorad) program. PCR products which had been confirmed of its tape size were purified and sequenced using forward and reverse primers were also used for PCR. The PCR product purification process and sequencing were done by 1st BASE Laboratories Malaysia through PT Genetika Science Indonesia (http://www.ptgenetika.com).

Sequence Analysis
All sequence data, both forward and reverse were aligned to obtain target sequences using the ClustalW

<table>
<thead>
<tr>
<th>Target fragment</th>
<th>Primer pairs</th>
<th>Size of PCR product</th>
<th>Nucleotide position in cDNA</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA_1</td>
<td>F: 5’-GCTTGTCTGTCACCTGCAA-3’ R: 3’-TTGAATGGTTCATCTGACCA-5’</td>
<td>745 pb</td>
<td>188 – 932</td>
<td>57° C</td>
</tr>
<tr>
<td>cDNA_2</td>
<td>F: 5’-GGCCCAACAATGCTAAGTG-3’ R: 5’-AACCCATGACAGATCCAC-3’</td>
<td>757 pb</td>
<td>833 – 1589</td>
<td>54° C</td>
</tr>
<tr>
<td>cDNA_3</td>
<td>F: 5’-TTACAGCAACCCAGCTTGAA-3’ R: 5’-GGTGAGGTGTTGGAAAGGAG-3’</td>
<td>624 pb</td>
<td>1354 – 1977</td>
<td>56° C</td>
</tr>
<tr>
<td>cDNA_4</td>
<td>F: 5’-TTTTGTTGGCCGGAAAACCT-3’ R: 5’-TAGCTGTAAGCCGTCTTGTT-3’</td>
<td>621 pb</td>
<td>1920 – 2540</td>
<td>56° C</td>
</tr>
</tbody>
</table>
MEGA6 program (Tamura et al., 2013). The target sequences were further aligned with the reference sequence in GenBank and genetic variation was analyzed using the same program.

RESULTS AND DISCUSSION

Amplification and Sequencing
The use of 4 pairs of running primers (primer of cDNA-1 to cDNA-4) successfully amplified cDNA of LDLR gene of hyporesponder cynomolgus macaque with base pair (bp) size as expected (Figure 1 A, B, C, and D). The alignment of the amplified sequences of the four running primers produced a 2353 bp target sequence located at 188-2540 position (nucleotide A on ATG as +1). This indicated that the primers designed in this study had the potential to be used as diagnostic primers for cDNA of LDLR gene of cynomolgus macaque.

Genetic Variation
The results of pairwise distance analysis between the target sequence and reference cDNA sequence of cynomolgus macaque in GenBank (access number: XM-005587996.1) found 8 SNPs in the target sequence (using model number of differences) with a genetic distance of 0.3% (using p-distance model). The eight SNPs were identified, namely: c.408C>T; c.1200C>T; c.1497C>T; c.1644T>C; c.1791T>C; c.1804A>G; c.2088C>T; and c.2377G>A. The Six of the eight SNPs, including c.408C-T, c.1200C-T, c.1497C-T, c.1644T-C, c.1791T-C, and c.2088C-T, did not alter the amino acid sequence as they were located at the 3rd position of the codon. Two other SNPs, c.1804A-G and c.2377G-A, caused amino acid sequence changes as they were located at the 2nd position of the codon. SNP c.1804A>G, which was located on exon 12, converted the codons that produce amino acids at position 602 from lysine to glutamate (p.602E), whereas SNP c.2377G-A, which was located on exon 16, changed the codons that produce amino acid at position 793, i.e. from valine to isoleucine (p.V793I). The polymorphism of p.K602E was located in the amino acid region of the homologous precursor domain of the epidermal growth factor, whereas p.V793I polymorphism was located in the amino acid region of the transmembrane domain.

Table 2 showed the identified SNP and the changes in amino acid produced.

In primate animals, the presence of nucleotide polymorphisms in cDNA that cause amino acid changes was first reported in Rhesus monkeys (Macaca mulatta). In this polymorphism, the codon of exon 6 producing the amino acid tryptophan turns into a stop codon (TGG to TAG) therefore the formation of the LDL receptor protein stops at the 283rd amino acid. As result, the number of LDL receptors is reduced and the rhesus monkeys undergo “natural” hypercholesterolemia (Hummel et al., 1990).

In human genetic, a common variation in the LDLR gene has been widely reported to be associated with cholesterol levels. The study by Willer et al. (2008) and Linsel-Nitschke et al. (2008), respectively, indicate the presence of SNP in intron 1 and exon 2 LDLR genes. Although they were not causing amino acid sequence changes, but the SNP mentioned is associated with low cholesterol levels in normal conditions thus increasing individual resistance to coronary heart disease (CHD). Similarly, Franceschini et al. (2009) reported the presence of SNP in promoter areas which was associated with CHD risk. Another study by Long et al. (2011) demonstrated the presence of a long polymorphism of restriction fragments (RFLP) in exon 3 which was also associated with serum lipid levels. The successful identification of genetic polymorphisms in the LDLR gene of the hyporesponder cynomolgus macaque suggests a link between genetic variation and the nature of response to the atherogenic diet, although it still requires further confirmation of using more number of hyporesponder animals and an explanation of its underlying mechanism. This supports an important principle in animal modeling that common genetic variations in experimental animals can influence the results of biomedical experiments (Haus et al., 2014).

Thus, the identified SNP is potentially used as a genetic marker in the selection of hyporesponder animals from hyperresponder. The use of genetic markers will greatly reduce the costs required for initial selection of experimental animals. Selection of experimental animals prior to use in research related to atherosclerosis is of great importance because it can improve the accuracy and efficiency of scientific studies, hence greatly support the success of the study. It also reduces the number of experimental animals used to support the 3R principle (reduction, refinement and replacement) (Rogers, 2013).

<table>
<thead>
<tr>
<th>SNP</th>
<th>Exon</th>
<th>Amino Acids changes</th>
<th>Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.408C&gt;T</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1200C&gt;T</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1497C&gt;T</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1644T&gt;C</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1791T&gt;C</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1804A&gt;G</td>
<td>12</td>
<td>p.K602E</td>
<td>Growth epidermal factor precursor homolog</td>
</tr>
<tr>
<td>c.2088C&gt;T</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.2377G&gt;A</td>
<td>16</td>
<td>p.V793I</td>
<td>Transmembrane</td>
</tr>
</tbody>
</table>

Table 2. Genetic variation of SNP in cDNA of LDLR gene of hyporesponder cynomolgus macaque
CONCLUSION

The result of sequence characterizing cDNA of LDLR gene of hyporesponder cynomolgus macaque showed polymorphism, in the form of SNP, which potentially used as a genetic marker in selecting experimental animals based on their response to atherogenic diets. Out of the 8th SNPs, there were 6 SNPs, namely: c.408C>T, c.1200C>T, c.1497C>T, c.1644T>C, c.1791T>C, and c.2088C>T, did not change the amino acid structure while another two SNPs, namely c.1804A>G and c.2377G>A, caused amino acid sequence changes.

ACKNOWLEDGEMENT

We expressed our gratitude to Primate Research Center, Agency of Research and Community Empowerment, Bogor Institute of Agriculture for the use of experimental animals and research facilities.

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