Association of CCL7 Promoter Polymorphism with Responsiveness to Allergen in Cynomolgus Macaque Model of Asthma

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B ACKGROUND: C-C motif Ligand 7 (CCL7) has been reported to be associated with asthma severity in humans. Cynomolagus macaques (Macaca fascicularis; Mf) are often used as animal model of asthma but little is known about Mf genetic profile such as polymorphism. Our aim was to identify CCL7 polymorphism in Mf as a potential surrogate marker for identification of allergen responsiveness in the Mf model of asthma.

M ETHODS: Real-time PCR was performed on archive of bronchoalveolar fluid samples previously collected from Mf that were exposed to allergen. Expression of CCL7 mRNA was evaluated, and sequencing technique was used to identify polymorphism in this gene.

R ESULTS: The results showed that CCL7 expression did not differ between Mf, despite a trend of lower expression in Mf that exhibited high response to allergen. By direct DNA sequencing of CCL7, 10 sequence variants were identified; three in promoter region (-460 G/A, -459 A/G, -456 -/A ), two in exon 1 (9 A/G, 65 G/C), four in intron 1 (135 T/C, 254 T/C, 420 T/C, 453 A/G), and one in intron 2 (1205 T/A).

C ONCLUSION: There was an association between Mf sensitivity to allergen with CCL7 promoter polymorphism at (-456 -/A). These results suggest that CCL7 may be a potential genetic marker to identify Mf sensitivity to allergen, which could be a useful tool to efficiently select for Mf model of asthma.

K EYWORDS: asthma, CCL7, allergy, Ascaris suum, nonhuman primate

responsive to allergen (12,13), the skin and airway responsiveness may vary between individuals (14), which could be important for study interpretation.

The pathogenesis of asthma has long been studied, and it was demonstrated that CC chemokines play an important role. They are a subfamily of chemokines containing 4 or 6 cysteins. CC chemokines and their receptors contribute in facilitating leukocyte function, and hence, developing immune response15. Protein C-C motif Ligand 7 (CCL7), previously known as Monocyte Chemotactic Protein-3 (MCP-3), is a secreted during inflammatory process of asthma. CCL7 is a member of C-C chemokine family located on chromosome 17q11.2-q12.(15) CCL7 polymorphisms in several sites in the human genome have been associated with asthma severity in certain populations.(16,17) Importantly, CCL7 expression was found high in asthmatic monkeys.(18,19) However, it is unknown if the nucleotide sequence profile of this gene is a determinant for allergen hypersensitivity in monkeys.

In this study, we identified mRNA expression and sequenced six regions of CCL7 gene in 8 adult Mfs. These animals were reported to exhibit different responses to Ascaris suum, a classic allergen used in asthma studies. The primary objective was to identify single nucleotide polymorphism (SNP) within the CCL7 gene in Mf as a potential genetic marker to identify allergen responsiveness.

Methods

Samples
This research used archived bronchoalveolar lavage (BAL) fluid samples collected from Mf that were enrolled in an asthma study.(14) Briefly, the asthma study was performed in Primate Research Center at Bogor Agricultural University and PT. Bimana Indomedical following approval from the Institutional Animal Care and Use Committee (IACUC) with ethical approval No. PTBI IACUC 05-07-1R. The results have been reported wherein Mf showed differential skin and airway responses to A. suum, and were defined as high and low responders. High and low responders were Mf that showed asthma response following exposure to low and high dose of A. suum, respectively.(14) Here, we used 8 archive samples from both groups (n=4 from high responders and n=4 from low responders).

Gene Expression Of CCL7, CCR1, and CCR3
The isolation of mRNA from BALf samples was performed using the Qiagen RNAeasy mini kit (Qiagen, Hilden, Germany). The isolated mRNA concentration was measured using NanoDrop (Thermo Fisher, Waltham, Massachusetts, USA). A total of 0.7 ng/μL mRNA was used as a template for reverse transcription process. The cDNA synthesis was performed with Superscript III RT enzyme from Invitrogen (Life Technology, Eugene, Oregon, USA) according to the company instructions. The molecular expression level of CCL7, CCR1, and CCR3 was evaluated using Realtime PCR technique (qRT-PCR). qRT-PCR was done using SsoFast™ EvaGreen® Supermix (Biorad, Hercules, California, USA) according to the company manual. The primer used is presented in Table 1. The qRT-PCR data were evaluated using delta Ct method using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the reference gene, and statistical analysis was performed using SPSS 18 (IBM Corporation, Armonk, New York, USA).

Polymerase Chain Reaction (PCR) and Sequencing
The same subset of BALf samples used for gene expression analysis was also used for sequencing purposes. DNA isolation from BALf was performed using DNeasy Blood and Tissue Kit Qiagen (Qiagen). Amplification of 5 μL DNA was performed by Hotstart PCR technique using KAPA Hotstart Master Mix PCR (KAPA Biosystem, Wilmington, North Carolina, USA). The primers for CCL7 used in this study were amplified from promoter to terminator region (Figure 1) referred to prior publication 17 with modification (Table 2). The PCR technique was performed in Thermo cycler Applied Biosystem Perkin Elmer 9700 with program incubation 94°C for 5 minutes, 94°C denaturation for 30 seconds, 60°C annealing for 30 seconds, extension of DNA at 72°C for 30 seconds, and post PCR 72°C for 5 minutes. The denaturation stage until the extension of the DNA strands

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL7</td>
<td>GAGCTACAGAAGGCCACCCAGT</td>
<td>AAGTCCTGGACCCACTTCT</td>
<td>Human 17</td>
</tr>
<tr>
<td>CCR1</td>
<td>CTGTTGGAAAACATCCTGGT</td>
<td>GGAAGGGTGAACGGAGAAGAG</td>
<td>Human 29</td>
</tr>
<tr>
<td>CCR3</td>
<td>GTGGTCACTGTGGGCCTCTT</td>
<td>GTGACGGAGAAGACGGATC</td>
<td>Human 28</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CGGATTTGGTCTATTGG</td>
<td>TCAAGGGAGGGAGTGG</td>
<td>Human 34</td>
</tr>
</tbody>
</table>
was repeated 40 cycles. The PCR results were visualized by agarose gel electrophoresis 1.8% and read using GelDoc 2000 (Biorad, Hercules, California, USA). DNA sequencing was performed at 1st BASE (1st BASE, Selangor, Malaysia). DNA sequencing results were analyzed using BioEdit program (Ibis Biosciences, Carlsbad, California, USA). Analysis of sequencing results was carried out using Clustal W program of MEGA 5.2 (Pennsylvania State University, Pennsylvania, USA). Analysis of haplotype was carried out using DnaSP program (Universitat de Barcelona, Barcelona, Spain).

Table 2. Primer sequence for SNP analysis CCL7 in Mf.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Region</th>
<th>Amplicon</th>
<th>Annealing Temperature</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-3 pro-3</td>
<td>Promoter</td>
<td>613</td>
<td>55</td>
<td>F: TGGGTITTAGAAAGGCCACCAAGCAGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: GGCAATGGCTCAGGAGGAAGGAA</td>
</tr>
<tr>
<td>MCP-3 ex-1</td>
<td>Exon 1</td>
<td>640</td>
<td>57</td>
<td>F: AAGTGAGCCGGCTCAGCAGATT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: TGGAGAGCTGCTTTTCTTGGAA</td>
</tr>
<tr>
<td>MCP-3 ex-2</td>
<td>Exon 2</td>
<td>584</td>
<td>57</td>
<td>F: CAAAGACCCGGACTTGGGACG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: ATGCTCAGGGATGAGGAGGA</td>
</tr>
<tr>
<td>MCP-3 ex-3</td>
<td>Exon 3</td>
<td>534</td>
<td>58</td>
<td>F: AGACTTCAGTTCTTTTATCTTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: TCCACCACAAATCCATGGAAGA</td>
</tr>
<tr>
<td>MCP-3 int-1</td>
<td>Intron 1</td>
<td>629</td>
<td>55</td>
<td>F: AGCGAGITTTGGGATCGGTTGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: ACACATGACTCCCCGGGA</td>
</tr>
<tr>
<td>MCP-3 int-2</td>
<td>Intron 1-terminater</td>
<td>1235</td>
<td>56</td>
<td>F: TTTGGGATCGGGTGATCAAAAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R: AATCACTCCTGAGAAGGACAGGGT</td>
</tr>
</tbody>
</table>

Discussion

Our study showed an association between polymorphism of CCL7 promoter and Mf reactivity to asthma-inducing allergen, whereby a haplotype of -456 A/- was exclusive to low responders. Expression of CCL7 mRNA and its compared to high responders (p=0.081). Similar expression pattern was found on CCL7’s receptors with p-values of 0.063 for CCR1 and 0.073 for CCR3.

DNA samples were evaluated to identify CCL7 polymorphism. Our analysis indicated 4 areas in the CCL7 gene that showed nucleotide sequence variation (Figure 3): the promoter (-460 G/A, -459 A/G, -456 A/-), exon 1 (9 A/G, 65 G/C), intron 1 (135 T/C, 254 T/C, 420 T/C, 453 A/G), and intron 2 (1205 T/A). Among those, the genetic variation in the promoter region was associated with reactivity phenotype wherein 3 polymorphic sites were found, which produced 4 haplotypes. One haplotype, particularly at position -456, was evident in animals that had low reactivity to allergen. The number of haplotypes and individual groupings based on these haplotypes are presented in Table 3.
receptors appeared to be higher in low responders group although they were not found to be statistically significant.

Asthma studies involving Mf rarely take into consideration the genetic background of the model despite the fact that asthma is a polygenic disorder and that genetic polymorphism are commonly found in nonhuman primates more than in humans.\(^{(24,25)}\) We have recently characterized CCL7 mRNA of Indonesian Mf.\(^{(23)}\) To our knowledge, our study is the first to indicate that CCL7 polymorphism may determine Mf responsiveness to allergen and asthma. The grouping based on CCL7 promoter haplotype showed an interesting result as Haplotype I was found to be specific to monkeys in the low responder group. Research on association of SNPs in the gene encoding chemokine and asthma phenotype is a developing field. A study in the Korean population found CCL7 polymorphism in the promoter region although it was not associated with asthma severity.\(^{(17)}\) In contrast, polymorphism within the CCL7 promoter region was associated with atopic asthma in Indian population.\(^{(16)}\) The association between asthma response and genetic variation also occurs in other chemokine-related genes. Previous study reported a link between CCL2 polymorphism and the nature of asthma response in families with asthma in Tunisia.\(^{(26)}\)

CCL7 is one of the chemokines that plays a role in the pathophysiology of asthma. It has been reported that higher CCL7 expression was associated with an increase in the number of eosinophils in bronchial mucosa of individuals with asthma compared to healthy individuals.\(^{(17)}\) In a microarray study of asthmatic monkeys, CCL7 was the gene with the highest expression.\(^{(18)}\) Here, we performed a preliminary study with limited number of animals and found a trend of differential CCL7 expression between asthmatic monkeys of different reactivity to allergen. Although not statistically significant, the expression of CCL7 and its receptors showed a higher trend in the BALf of low responders compared to high responders. As the BALf samples were taken at 24 hours following the asthma episode induced by airway challenge, this result may indicate that the chemokine response could have occurred immediately in the high responders which caused the expression to have decreased by 24 hours. In contrast,
low reactivity responders may have exhibited a slower chemokine response. It has been previously reported that the expression of CCL7 and other chemokines increased within 4 hours and decreased by 8 h post A. suum inhalation and was back to near baseline by 24 hours.(18) Since there was no BALf sample that represented an earlier timepoint (i.e., less than 24 hours post-allergen challenge), it is unclear whether low and high reactivity responders react differently during the acute phase of allergen inhalation. Alternatively, the result may be indicative of a compensatory mechanism that occurs with the high reactivity responders in their effort to decrease airway hyper-responsiveness. In non-allergic mice, down regulation of CCL7 was found to reduce rhinovirus-induced airway hyper-reactivity.(27) Compensation is a relatively common mechanism during asthma response whereby in transgenic models of asthma, it has been reported that compensatory mechanisms such as those involving up regulation of G-protein or ion channels occur to attenuate airway responsiveness or smooth muscle contractility.(28,29)

The CCL7 protein is a ligand which binds to receptors such as CCR1, CCR2 and CCR3. The binding activates monocytes, lymphocytes, dendritic cells, NK cells, and granulocytes.(30) In line with CCL7 expression, we found that CCR1 and CCR3 gene expressions of the high responders also showed a trend of lower expression compared to low responders. CCR2 and its other ligand (i.e., CCL2), however, showed a significantly higher expression in low responders. (31) This finding may indicate a synergistic role between CCL7 and its receptors in the pathogenesis of asthma. The RNA expression analysis from Mf BALf in this study are also limited to the airway response after 24 hours antigen challenge, which may not be relevant to the asthmatic lung or reflect the chronic aspect of asthma.

This study also suggests the likelihood that polymorphisms in the CCL7 gene promoter region are affect for gene expression of CCL7. Genetic variation in the promoter region allows for difference in transcription factors and such difference affect gene expression in respiratory tract muscles.(32) Our findings require further investigation to validate the use of CCL7 as a genetic marker to determine Mf reactivity to asthma-inducing allergens as a refined way to select for animals in a more efficient manner.

Information of CCL7 promoter haplotype may be a useful tool to identify Mf of certain asthma profile. Pre-screening of animals using such information will allow for an efficient and more accurate selection of asthma model candidate, and eventually lead to less variation in animal response during the study. As of now, skin test using allergens remains as the tool for selection of hypersensitive Mf. This process involves injection of allergen in several sites on the skin performed under anaesthesia. The use of genetic marker to select Mf candidate for asthma study needs to be developed further as a refined approach to improve animal welfare. This follows the principles of 3Rs (reduction, refinement and replacement), which is an important aspect in every research that involves animals.(33,34)

### Table 3. Identified SNP in promoter regions and haplotypes.

<table>
<thead>
<tr>
<th>Haplotyp</th>
<th>Promoter Region</th>
<th>n</th>
<th>Phentype Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>A - G - A</td>
<td>4</td>
<td>Low responders</td>
</tr>
<tr>
<td>II</td>
<td>A - G - A</td>
<td>1</td>
<td>High responder</td>
</tr>
<tr>
<td>III</td>
<td>A - A - A</td>
<td>1</td>
<td>High responder</td>
</tr>
<tr>
<td>IV</td>
<td>G - A - A</td>
<td>2</td>
<td>High responders</td>
</tr>
<tr>
<td>Total samples</td>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Polymorphism in CCL7 promoter region was associated with hyper-responsiveness of Mf to A. suum, and is therefore a potential genetic marker to support an efficient selection of Mf candidates for asthma study.

### Conclusion

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