RESEARCH ARTICLE

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

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Abstract

BACKGROUND: C-C motif Ligand 7 (*CCL7*) has been reported to be associated with asthma severity in humans. Cynomolgus 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.

METHODS: 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.

RESULTS: 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).

CONCLUSION: 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.

KEYWORDS: asthma, *CCL7*, allergy, *Ascaris suum*, nonhuman primate

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Introduction

Respiratory diseases including asthma are often complex polygenic disorders that manifest as the result of exposure of genetically susceptible individuals to airway reactive substances within their environments.(1) Asthma is a chronic disorder of the respiratory tract, in which a severe inflammation causes obstruction of the airflow, airway hyperresponsiveness to stimuli, bronchospasm and symptoms such as cough, wheezing, dyspnoea and chest tightness.(2) Asthma remains the most common respiratory disease both in developing and developed countries.(3,4) Study in families affected with asthma suggest several chromosome regions may contain potential candidate genes associated with the disease.(5,6)

The nonhuman primate (NHP) is genetically, anatomically and immunologically similar to humans and represents a valuable model system for human airway disease.(7-9) Studies have reported the use of *Mf* in research of asthma drugs (10,11), but details on the model's genetic background is limited. Even though *Mf* are spontaneously

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 hypersentivity in monkeys.

In this study, we identified mRNA expression and sequenced six regions of *CCL7* gene in 8 adult *Mf*s. 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). Ampilification 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 1. Primer sequences used for RealTime PCR.

Gene	Forward (5'-3')	Reverse (5'-3')	Species
CCL7	GAGCTACAGAAGGACCACCAGT	AAGTCCTGGACCCACTTCTG	Human [17]
CCR1	CTGGTTGGAAACATCCTGGT	GGAAGCGTGAACAGGAAGAG	Human [29]
CCR3	GTGTTCACTGTGGGCCTCTT	GTGACGAGGAAGAGCAGGTC	Human [28]
GAPDH	CGGATTTGGTCGTATTGG	TCAAAGGTGGAGGAGTGG	Human [34]

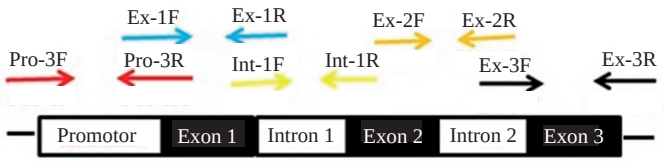

Figure 1. Primer position in analysis of *CCL7* SNPS in *Mf*. *Mf* covered the region of promoter (Pro-3F and Pro-3R), exon 1 (Ex-1F and Ex-1 R), exon 2 (Ex-2F and Ex-2 R), exon 3 (Ex-3 F and Ex-3 R), and intron 1 (Int-1F and Int-1 R). Primers are indicated as arrows and the target region are indicated as block .

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).(20) Analysis of sequencing results was carried out using Clustal W program of MEGA 5.2 (Pennsylvania State University, Pennsylvania, USA).(21) Analysis of haplotype was carried out using DnaSP program (Universitat de Barcelona, Barcelona, Spain).(22)

Results

The coding sequences of *Mf CCL7* was submitted to NCBI Genbank (MF062250).(23) The qRT-PCR results showed a trend of difference in the mRNA expression of *CCL7* and its receptors between low and high responders. Figure 2 shown gene expression of *CCL7*, *CCR1*, and *CCR3* of high responder group were down regulated compared to low responder with fold change value as 0.217, 0.228, and 0.115, respectively. Although not statitically significant, there was a trend of higher *CCL7* expression in low responders 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.

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

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

Primer	Region	Amplicon	Annealing Temperature	Primer Sequence
MCP-3 pro-3	Promoter	613	55	F: TGGGTTTTAGAAAGCCACCAGG
				R: GGGCAATGTGCTTCAAGGAGAA
MCP-3 ex-1	Exon 1	640	57	F: AAGTGCACCGGCTCAGCAGATT
				R: TGCAGAGCTGCTGTTTCTGGAA
MCP-3 ex-2	Exon 2	584	57	F: CAAAGACACCGGACTTGGGACG
				R: ATGCCTCAGGGATGGAGGAGGA
MCP-3 ex-3	Exon 3	534	58	F: AGACTTCAGTTCTTTTATCCTGG
				R: TCCACCAAAATCCATGGAAGA
MCP-3 int-1	Intron 1	629	55	F: AGCAGAGTTTGGGATCGGGTGA
				R: ACACATTACAGCTTCCGGGGA
MCP-3 int-2	Intron 1-terminater	1235	56	F: TTTGGGATCGGGTGATCAAAAA
				R: AATCACTCTGAGAAAGGACAGGGT

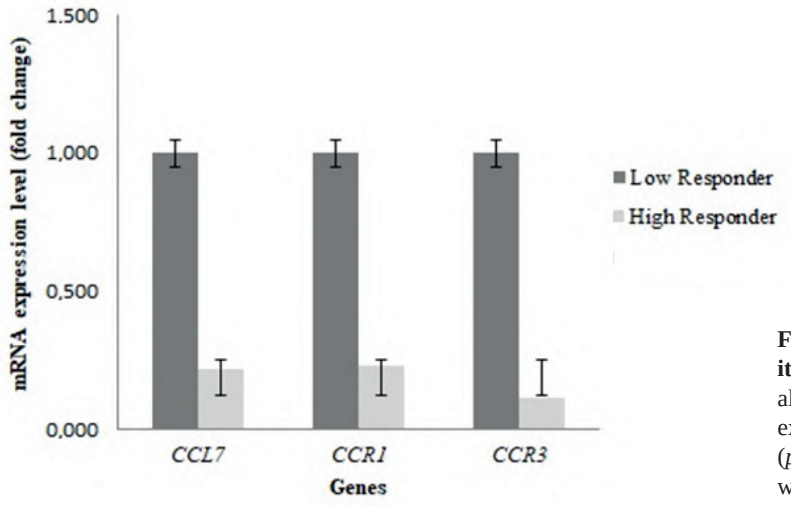

Figure 2. Level of mRNA expression for *CCL7* and its receptors. No significant difference was observed although high responder *Mf* showed a trend of lower expression compared to low responder *Mf* for *CCL7* (p=0.08), CCR1 (p=0.06), and CCR3 (p=0.07). Values were means, error bars = SD.

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,

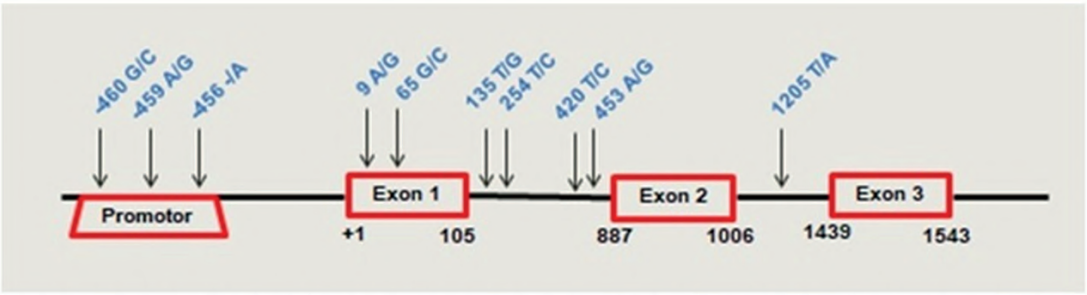

Figure 3. Gene map and SNPs position in *CCL7 Mf*. Coding exons are marked by red blocks and first base of translation site was denoted as nucleotide +1. Nucleotide position of the genes was indicated with the number and the arrow was indicated position of SNPs. There are SNPs in Promoter (-460 G/A, -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).

Table 3. Identified SNP in promoter regions and haplotypes.

Haplotype	Promoter Region			*n*	Phenotype Remarks
	-460	-459	-456		
I	A	G	A	4	*Low responders*
II	A	G	-	1	*High responder*
III	A	A	-	1	*High responder*
IV	G	A	-	2	*High responders*
Total samples				**8**	

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 base line 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)

Conclusion

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.

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