Variants of C-C Motif Chemokine 22 (CCL22) Are Associated with Susceptibility to Atopic Dermatitis: Case-Control Studies

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Abstract

Atopic dermatitis (AD) is a common inflammatory skin disease caused by multiple genetic and environmental factors. AD is characterized by the local infiltration of T helper type 2 (Th2) cells. Recent clinical studies have shown important roles of the Th2 chemokines, CCL22 and CCL17 in the pathogenesis of AD. To investigate whether polymorphisms of the CCL22 gene affect the susceptibility to AD, we conducted association studies and functional studies of the related variants. We first resequenced the CCL22 gene and found a total of 39 SNPs. We selected seven tag SNPs in the CCL22 gene, and conducted association studies using two independent Japanese populations (1st population, 916 cases and 1,032 controls; 2nd population, 1,034 cases and 1,004 controls). After the association results were combined by inverse variance method, we observed a significant association at rs4359426 (meta-analysis, combined $p = 9.6 \times 10^{-9}$; OR, 0.74; 95% CI, 0.65–0.85). Functional analysis revealed that the risk allele of rs4359426 contributed to higher expression levels of CCL22 mRNA. We further examined the allelic differences in the binding of nuclear proteins by electrophoretic mobility shift assay. The signal intensity of the DNA-protein complex derived from the G allele of rs223821, which was in absolute LD with rs4359426, was higher than that from the A allele. Although further functional analyses are needed, it is likely that related variants play a role in susceptibility to AD in a gain-of-function manner. Our findings provide a new insight into the etiology and pathogenesis of AD.

Introduction

Atopic dermatitis (AD) is a pruritic and chronically relapsing inflammatory skin disease involving disturbed skin barrier functions, cutaneous inflammatory hypersensitivity and defects in the antimicrobial immune defense with a strong genetic background [1]. Predominant infiltration of Th2 cells is a hallmark of acute atopic AD skin lesions [2]. Most patients with AD have peripheral blood eosinophilia and increased serum IgE levels, which are reflected in an increased frequency of peripheral blood skin-homing Th2 cells producing IL-4, IL-5 and IL-13 [1]. C-C motif chemokine 22 (CCL22) and CCL17 are high-affinity ligands for CC-chemokine receptor 4 (CCR4) and induce selective migration of Th2 cells [3]. CCL22 plays a crucial role in controlling the trafficking of Th2 cells into sites of allergic inflammation and is considered to be involved in the pathology of
AD [4]. Keratinocytes from patients with AD highly express thymic stromal lymphopoietin (TSLP), and CCL22 is produced by TSLP-treated dendritic cells [5]. CCL22 is upregulated in lesional atopic dermatitis skin compared with healthy skin [6], and keratinocytes in the epidermal layer of AD skin express CCL17 and CCL22 [7]. Serum levels of CCL22 in AD patients are significantly higher than those found in normal controls [8], and the levels correlate positively with disease severity in AD patients [9]. Strong positive correlations between the levels of CCL17, CCL22, and total IgE in serum of patients with AD and SCORing Atopic Dermatitis (SCORAD) have also been reported [10]. Another study reported that overproduction of IgE induced CCL22 secretion from basophils, which are essential for IgE-mediated chronic allergic dermatitis [11]. These findings prompt-

### Table 1. Frequencies of polymorphisms of the CCL22 gene.

| SNP*       | Location   | Amino acid | MAF; | NCBI† |
|------------|------------|------------|------|-------|
| 1 -3075G/A | 5′-flanking region | -         | 0.125 | rs223884 |
| 2 -2938G/A | 5′-flanking region | -         | 0.208 | rs223885 |
| 3 -2903T/A | 5′-flanking region | -         | 0.333 | rs223886 |
| 4 -2668G/T | 5′-flanking region | -         | 0.458 | rs34569362 |
| 5 -2550G/C | 5′-flanking region | -         | 0.458 | rs76295899 |
| 6 -2511G/T | 5′-flanking region | -         | 0.458 | rs4784799 |
| 7 -2191G/C | 5′-flanking region | -         | 0.042 | rs76720124 |
| 8 -1795G/A | 5′-flanking region | -         | 0.458 | rs34885482 |
| 9 -1775G/T | 5′-flanking region | -         | 0.083 | rs72784894 |
| 10 -1618C/T | 5′-flanking region | -         | 0.458 | rs77239447 |
| 11 -1515G/T | 5′-flanking region | -         | 0.333 | rs223887 |
| 12 -1338A/G | 5′-flanking region | -         | 0.208 | rs182668 |
| 13 -961G/A | 5′-flanking region | -         | 0.208 | rs223888 |
| 14 -740A/G | 5′-flanking region | -         | 0.083 | rs3760077 |
| 15 -488T/C | 5′-flanking region | -         | 0.333 | rs223889 |
| 16 -215WT/DelG | 5′-flanking region | -         | 0.333 | rs314179 |
| 17 SC/A | exon 1 | Ala2Asp | 0.125 | rs4359426 |
| 18 88C/A | intron 1 | -         | 0.458 | rs2074543 |
| 19 493T/C | intron 1 | -         | 0.458 | rs72784897 |
| 20 559G/A | intron 1 | -         | 0.333 | rs223816 |
| 21 902C/T | intron 1 | -         | 0.333 | rs223817 |
| 22 2030G/C | intron 2 | -         | 0.208 | rs223818 |
| 23 2134T/C | intron 2 | -         | 0.208 | rs223819 |
| 24 2198T/C | intron 2 | -         | 0.208 | rs223820 |
| 25 2314G/A | intron 2 | -         | 0.292 | rs598366 |
| 26 2936A/G | intron 2 | -         | 0.125 | rs170359 |
| 27 3062A/G | intron 2 | -         | 0.458 | rs73557194 |
| 28 3766T/A | intron 2 | -         | 0.042 | |
| 29 3970G/A | intron 2 | -         | 0.125 | rs223823 |
| 30 4064WT/InsAAAAC | intron 2 | -         | 0.125 | rs72030112 |
| 31 522T/C | 3′ UTR | -         | 0.125 | rs170360 |
| 32 5978WT/DelT | 3′ UTR | -         | 0.125 | rs75450696 |
| 33 5979C/G | 3′ UTR | -         | 0.375 | rs5718204 |
| 34 6089T/C | 3′ UTR | -         | 0.125 | rs223823 |
| 35 6621A/G | 3′ UTR | -         | 0.458 | rs121565 |
| 36 6910G/A | 3′ UTR | -         | 0.417 | rs668559 |
| 37 7858C/T | 3′-flanking region | -         | 0.458 | rs3859048 |
| 38 7883G/A | 3′-flanking region | -         | 0.458 | rs72301 |
| 39 8021G/A | 3′-flanking region | -         | 0.042 | rs1165093 |

*Numbering according to the genomic sequence of CCL22 (AC003665). Position 1 is the A of the initiation codon.
1Minor allele frequencies (MAF) in the screening population (N = 12).
1NCBI, number from the dbSNP of NCBI (http://www.ncbi.nlm.nih.gov/SNP/).
1SNPs were genotyped in this study.
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Figure 1. Pairwise linkage disequilibrium between 34 SNPs. LD was measured by D’/LOD (upper) and r² (lower) estimated using the Haploview 4.2 program (http://www.broad.mit.edu/mpg/haploview/). Boxed variants were genotyped in this study.

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ed us to conduct an association and functional study to test whether genetic variations of CCL22 contribute to AD susceptibility.

Several association studies using genetic variants of genes CCL17 and CCR4 in the CCR4 pathway have been conducted to discover genetic components in the pathogenesis of atopic dermatitis [12,13]. A promoter polymorphism of CCL17, -431C>T, increases the promoter activity and the 431T allele influences higher serum levels of CCL17 [12], but genetic variants in the CCL17 gene are not associated with susceptibility to AD. A recent study also reported that C1014T polymorphism in the CCR4 gene was not associated with AD [13]. However, those studies were performed with small sample sizes and without replication studies. Genetic study of the CCL22 gene has not been conducted.

In this study, we focused on the CCL22 gene, resequenced the gene regions including all exons and introns, and carried out linkage disequilibrium mapping. We performed an association study using two independent populations and functional analyses of the related variants.

### Results

#### Polymorphisms of the CCL22 gene and LD mapping

We identified a total of 39 polymorphisms (Table 1). We next performed linkage disequilibrium (LD) mapping and calculated

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**Table 2. Clinical characteristics of the subjects.**

| Source                          | Case                  | Control                |
|---------------------------------|-----------------------|------------------------|
| **1st population**             |                       |                        |
| Source                          | The University of Tokyo | Control volunteers     |
| Keio University                 |                       |                        |
| Kyushu University               |                       |                        |
| Takao Hospital                  |                       |                        |
| Number of samples               | 916                   | 1,032                  |
| Ethnicity                       | Japanese              | Japanese               |
| Female                          | 43.6%                 | 33.0%                  |
| Age (mean ± sd)                 | 30.1±9.5              | 48.5±13.7              |
| **2nd population**             |                       |                        |
| Source                          | BioBank Japan          | University of Tsukuba  |
| Number of samples               | 1,034                 | 1,004                  |
| Ethnicity                       | Japanese              | Japanese               |
| Female                          | 43.8%                 | 54.4%                  |
| Age (mean ± sd)                 | 30.8±12.7             | 50.0±9.2               |

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**Table 3. Genotype counts and case-control association test results of seven tag SNPs.**

| db SNP ID | Allele | Case 1/2 | 1/1 | 2/2 | N 1/1 | 1/2 | 2/2 | N | Case | Control | Frequency of allele 2 | P value | OR (95%CI) |
|-----------|--------|----------|-----|-----|-------|-----|-----|---|------|---------|----------------------|---------|-----------|
| 1st population |        |          |     |     |       |     |     |   |      |         |                      |         |           |
| rs223889   | T/C    | 321      | 435 | 151 | 907   | 360 | 502 | 161| 1023 | 0.406   | 0.403               | 0.82    | -         |
| rs4359426  | C/A    | 706      | 191 | 12  | 909   | 736 | 269 | 16 | 1021 | 0.118   | 0.147               | 0.0072  | 0.77(0.64–0.93)     |
| rs2074543  | G/C    | 386      | 404 | 113 | 903   | 447 | 469 | 110| 1026 | 0.349   | 0.336               | 0.39    | -         |
| rs223818   | A/G    | 563      | 311 | 39  | 913   | 596 | 369 | 56 | 1021 | 0.213   | 0.236               | 0.093   | -         |
| rs121565   | A/G    | 294      | 439 | 173 | 906   | 325 | 509 | 195| 1029 | 0.433   | 0.437               | 0.0072  | 0.77(0.64–0.93)     |
| rs658559   | G/A    | 333      | 434 | 134 | 901   | 374 | 491 | 162| 1027 | 0.390   | 0.397               | 0.65    | -         |
| rs3859048  | C/T    | 399      | 410 | 103 | 912   | 466 | 448 | 108| 1022 | 0.338   | 0.325               | 0.40    | -         |
| 2nd population |        |          |     |     |       |     |     |   |      |         |                      |         |           |
| rs223889   | T/C    | 639      | 497 | 163 | 1029 | 364 | 485 | 150| 999  | 0.400   | 0.393               | 0.65    | -         |
| rs4359426  | C/A    | 815      | 202 | 12  | 1029 | 722 | 249 | 22 | 999  | 0.110   | 0.148               | 0.00037 | 0.71(0.59–0.86)     |
| rs2074543  | G/C    | 404      | 484 | 133 | 1021 | 418 | 459 | 120| 997  | 0.367   | 0.351               | 0.26    | -         |
| rs223818   | A/G    | 647      | 331 | 42  | 1020 | 585 | 351 | 57 | 999  | 0.203   | 0.234               | 0.019   | 0.84(0.72–0.97)     |
| rs121565   | A/G    | 317      | 530 | 179 | 1026 | 317 | 500 | 180| 997  | 0.433   | 0.431               | 0.92    | -         |
| rs658559   | G/A    | 389      | 486 | 154 | 1029 | 363 | 479 | 148| 990  | 0.386   | 0.391               | 0.71    | -         |
| rs3859048  | C/T    | 425      | 484 | 117 | 1026 | 441 | 446 | 113| 1000 | 0.350   | 0.336               | 0.35    | -         |
| Combined   |        |          |     |     |       |     |     |   |      |         |                      |         |           |
| rs223889   | T/C    | 690      | 932 | 314 | 1936 | 724 | 987 | 311| 2022 | 0.403   | 0.398               | 0.63    | -         |
| rs4359426  | C/A    | 1521     | 393 | 24  | 1938 | 1458| 518 | 38 | 2014 | 0.114   | 0.147               | 0.0000096| 0.74(0.65–0.85)    |
| rs2074543  | G/C    | 790      | 888 | 246 | 1924 | 865 | 928 | 230| 2023 | 0.359   | 0.343               | 0.16    | -         |
| rs223818   | A/G    | 1210     | 642 | 81  | 1933 | 1181| 720 | 113| 2014 | 0.208   | 0.235               | 0.0044  | 0.86(0.77–0.95)     |
| rs121565   | A/G    | 611      | 969 | 352 | 1932 | 642 | 1009| 375| 2026 | 0.433   | 0.434               | 0.93    | -         |
| rs658559   | G/A    | 722      | 920 | 288 | 1930 | 737 | 970 | 310| 2017 | 0.388   | 0.394               | 0.56    | -         |
| rs3859048  | C/T    | 824      | 894 | 220 | 1938 | 907 | 894 | 221| 2022 | 0.344   | 0.330               | 0.21    | -         |

*P* values of the two populations were calculated by logistic regression analysis under an additive model. The combined *P* values were calculated using the inverse variance method. OR, odds ratio; CI, confidence interval; -, not significant.

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Association of CCL22 SNPs with susceptibility to atopic dermatitis

We recruited 916 cases and 1,032 control subjects for the 1st population and 1,034 cases and 1,004 control subjects for the 2nd population, respectively (Table 2). We genotyped seven tag SNPs and all genotype frequencies are shown in Table 3. The rs4359426 (A2D) SNP was associated with AD under an additive genotype model by logistic regression analysis in the first population ($P=0.0072$; OR, 0.77; 95% CI, 0.64–0.93) (Table 3). In a replication study, rs4359426 was also associated with AD in the second population ($P=0.00037$; OR, 0.71; 95% CI, 0.59–0.86) (Table 3). The direction of association of the SNP was similar in both of the populations. We combined the results using inverse variance method, and observed a significant association at rs4359426 (meta-analysis, $P=0.0000096$; OR, 0.74; 95% CI, 0.63–0.85) (Table 3). We next performed further mapping analyses using two genetic variants, rs170360 and rs223823. The two SNPs were selected from among SNPs that were in strong LD ($r^2>0.87$) with rs4359426 (Figure 2). Among the three variants, the strongest association was observed at rs4359426 (Table 4).

Contribution of 5′UTR rs4359426 SNP to mRNA expression levels of CCL22

Next, using allele-specific transcript quantification (ASTQ), we evaluated whether the related variants could affect the mRNA expression level in EBV-transformed lymphoblastoid cells. As rs4359426 was located at the 16th nucleotide from the 5′ end of the CCL22 gene (NM_002990.3), we were not able to design primers of the SNP for ASTQ analysis. We therefore designed PCR primers to encompass a SNP in the 3′-UTR of CCL22 (rs170360) that was in strong LD with rs4359426 (Figure 3). We isolated total RNA from 24 cell lines that were heterozygous with rs170360, and genomic DNA was used as a control for equal biallelic representation. Predicted haplotype frequencies are shown in Figure 3B. The ratio of PCR products was approximately 1.6 for cDNAs and 1.0 for genomic DNA from 21 subjects who were heterozygous for rs4359426 (Figure 3C, left panel); however, such

Table 4. Genotype counts and case-control association test results for SNPs rs4359426, rs170360 and rs223823.

| Allele    | db SNP ID | 1/2 | 1/1 | 1/2 | 2/2 | N | 1/1 | 1/2 | 2/2 | N | Case | Control | Frequency of allele 2 | P value | OR (95%CI) |
|-----------|-----------|-----|-----|-----|-----|---|-----|-----|-----|---|------|---------|----------------------------------|---------|------------|
| 1st population | rs4359426 | C/A | 706 | 191 | 12 | 909 | 736 | 269 | 16 | 1021 | 0.118 | 0.147 | 0.0072 | 0.77(0.64–0.93) |
| rs170360  | T/C      | 695 | 199 | 12 | 906 | 734 | 269 | 20 | 1023 | 0.123 | 0.151 | 0.011  | 0.78(0.65–0.95) |
| rs223823  | T/C      | 728 | 170 | 11 | 909 | 765 | 252 | 10 | 1027 | 0.106 | 0.132 | 0.0093 | 0.77(0.63–0.94) |
| 2nd population | rs4359426 | C/A | 815 | 202 | 12 | 1029 | 722 | 249 | 22 | 993 | 0.110 | 0.148 | 0.00037 | 0.71(0.59–0.86) |
| rs170360  | T/C      | 792 | 220 | 19 | 1031 | 728 | 238 | 26 | 992 | 0.125 | 0.146 | 0.055  | 0.84(0.70–1.00) |
| rs223823  | T/C      | 823 | 189 | 8  | 1020 | 780 | 193 | 19 | 992 | 0.100 | 0.116 | 0.11   | 0.85(0.70–1.04) |
| Combined  | rs4359426 | C/A | 1521 | 393 | 24 | 1938 | 1458 | 518 | 38 | 2014 | 0.118 | 0.147 | 0.0000096 | 0.74(0.65–0.85) |
| rs170360  | T/C      | 1487 | 419 | 31 | 1937 | 1462 | 507 | 46 | 2015 | 0.123 | 0.151 | 0.0017 | 0.81(0.72–0.93) |
| rs223823  | T/C      | 1551 | 359 | 19 | 1929 | 1545 | 445 | 29 | 2019 | 0.106 | 0.132 | 0.0030 | 0.81(0.70–0.93) |

$P$ values of the two populations were calculated by logistic regression analysis under an additive model. The combined $P$ values were calculated using the inverse variance method. OR, odds ratio; CI, confidence interval.

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differences were not observed in cells from three subjects who were homozygous for the C allele at rs4359426 (Figure 3C, right panel). These results implied an effect of rs4359426 and/or variants in strong LD with rs4359426 on mRNA expression levels of CCL22.

rs4359426 and rs170360 are in absolute LD in the HapMap Caucasian populations. We further examined the expression patterns of rs4359426 and rs170360 using Genevar 3.0.2 dataset, and confirmed that the expression patterns were similar to our findings (data not shown).

Transcription factor binding to the rs223821 SNP

As rs4359426 was in absolute LD with rs170359, rs223821 and rs72030112 (n = 94) (Figure 2), we further examined the allelic differences of these three SNPs in the binding of nuclear proteins by electrophoretic mobility shift assay (EMSA). We could not find any specific binding of nuclear factor(s) to oligonucleotides containing rs170359 and rs72030112. However, we observed that the signal intensity of the DNA-protein complex derived from the G allele of rs223821 was higher than that from the A allele in the presence of THP-1 nuclear extract stimulated with LPS (1 μg/ml) (Figure 4). We confirmed that the complex was diminished by an excess amount of a non-labeled allele-specific competitor probe (Figure 4). This result suggested that an unidentified nuclear factor(s) interacted with the genomic region at intron 2 of CCL22 and the SNP might have an allele-specific effect on expression through varying affinity for a transcription factor.

Discussion

CCL22 plays an important role in the recruitment of Th2 cells into the inflammatory lesions of Th2-related diseases such as AD [14]. A recent study reported upregulation of CCL17, CCL18 and CCL22 expression in patients with AD, and suggested that the disease-specific chemokines might recruit specific memory T-cell subsets into the skin [15]. The plasma levels of CCL22 are
Genetic studies reveal underlying cellular pathways, and in some cases, point to new therapeutic approaches. A recent study using a humanized model of asthma showed a critical role for DC-derived CCL17 and CCL22 in attracting Th2 cells and inducing airway inflammation [16]. In the study, administration of a CCR4-blocking antibody abolished airway eosinophilia, goblet cell hyperplasia, IgE synthesis and bronchial hyperreactivity [16]. IL-13 is an important mediator of Th2 immune responses, and there may be IL-13-positive cells in AD skin lesions [17]. A recent study has shown that IL-13 induces a significant increase in the expression of CCL22 in human keratinocytes, and blocking of CCL22 in IL-13-stimulated cells results in 70–90% inhibition in migration of CD4+CCR4+ T cells [18]. These findings suggest that targeting the CCL22/CCR4 pathway might be therapeutically efficacious as a new treatment for atopic dermatitis.

The involvement of CCL22 has been reported in several immune-mediated diseases. A recent study has shown by immunohistochemistry that CCL22 is not expressed in normal skin and is markedly expressed in the lesions of atopic dermatitis, allergic contact dermatitis, and psoriasis vulgaris [19]. Another report has shown that CCL22 is present within the synovial membrane in rheumatoid arthritis and osteoarthritis patients and in high amounts in the synovial fluid of patients with rheumatoid arthritis and psoriatic arthritis [20]. To examine whether the functional SNPs found in this study are associated with these diseases will be needed for understanding of the interconnectivity of the molecular mechanisms underlying distinct diseases.

In summary, we found a significant association between susceptibility to AD and polymorphisms affecting CCL22 expression in Japanese populations. Our findings strongly support the important role of CCL22 in AD. Although the effect of the non-synonymous SNP on protein function remains unclear, it is likely that related variants play a role in susceptibility to AD in a gain-of-function manner. Further functional analyses and replication studies in other populations are needed; however, our findings provide insights into the pathophysiology of AD.

Materials and Methods

Subjects
A total of 1,950 case subjects with AD were recruited from several hospitals as described [21]. Case subjects in a second population were obtained from the BioBank Japan [22]. All case subjects were diagnosed according to the criteria of Hanifin and Rajka [23]. A total of 1,032 control volunteers in the first set who had no history of AD were recruited by detailed physicians’ interviews. For the second set, a total of 1,004 controls who had never been diagnosed with AD were recruited during their annual health checkup in the University of Tsukuba (Table 2). All individuals were Japanese and gave written informed consent to participate in the study. This research project was approved by the ethics committees at the Institute of Medical Science, the University of Tokyo and the RIKEN Yokohama Institute.

Resequencing of the CCL22 gene and genotyping
We first resequenced the CCL22 region to identify genetic variations using DNA from 12 subjects with AD. We surveyed the gene from 3 kb of the 5’ flanking region to a 1 kb continuous 3’ flanking region of the last exon on the basis of genomic sequences from the NCBI database (NC_000016.9). The PCR product was reacted with BigDye Terminator v3.1 (Applied Biosystems), and sequences were assembled and polymorphisms identified using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, MI).

Genotyping of the seven SNPs in CCL22 was performed by the TaqMan™ allele-specific amplification (TaqMan-ASA) method (Applied Biosystems) and multiplex-PCR based Invader assay (Third Wave Technologies).

Allele-specific transcript quantification (ASTQ)
We conducted allelic expression analyses by TaqMan assay using SNP genotyping probes as described [24]. EBV-transformed lymphoblastoid cells were obtained from the Health Science Research Resources Bank of Japan. Genomic DNA was used as a
control for equal biallelic representation. The allelic ratio for each cDNA and genomic DNA was measured. Corrected \( P \) values of less than 0.05 were judged to be significant. The expression patterns of SNPs were obtained from Genevar (GENe Expression VARiation) 3.0.2 (Wellcome Trust Sanger Institute). We examined the influence of amino acid substitution on the structure using SIFT software (http://sift.jcvi.org/) and PolyPhen-2 (polymorphism phenotyping v2) (http://genetics.bwh.harvard.edu/pph2/).

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Author Contributions

Conceived and designed the experiments: TH MT. Performed the experiments: TH KT STanaka HM TSasaki TE MA HE STakeuchi MF EN YN MK. Wrote the paper: MT. Supervised the study: NK YN.

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