BRIEF REPORT

Copy Number Variations of Interleukin-17F, Interleukin-21, and Interleukin-22 Are Associated With Systemic Lupus Erythematosus

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Objective. Systemic lupus erythematosus (SLE) represents the classic prototype of systemic autoimmune disease. The identification of the Th17 cell subset has provided new understanding regarding the underlying mechanisms of autoimmunity. Copy number variations (CNVs) have been discovered to have phenotypic consequences and are associated with various types of diseases. We undertook this study to explore a possible association between CNVs of Th17 cell–related genes and the risk of SLE.

Methods. We extracted genomic DNA and RNA from 938 SLE patients and 1,017 healthy controls. We examined CNVs of Th17 cell–related genes, including retinoic acid receptor–related orphan nuclear receptor γt, STAT-3, interleukin-6 (IL-6), transforming growth factor β, tumor necrosis factor α, IL-17A, IL-17F, IL-21, IL-22, IL-23A, CCL20, and CCR6, and levels of messenger RNA (mRNA) for IL-17F, IL-21, and IL-22.

Results. Genotype and allele frequencies for copy number amplifications of IL-17F, IL-21, and IL-22 were found to be significantly higher in SLE patients than in healthy controls. CNVs of IL-17F, IL-21, and IL-22 had no synergistic contribution to SLE. The mRNA expression of IL-17F, IL-21, and IL-22 in the samples with >2 copies of DNA was significantly higher than that in those with 2 copies of DNA.

Conclusion. Our findings indicate that CNVs of IL-17F, IL-21, and IL-22 are associated with the risk of SLE.

Th cells, also called autoreactive effector CD4+ T cells, have been associated with the pathogenesis of several autoimmune disorders. In earlier studies, Th1 cells were implicated as the causal agents in the pathogenesis of autoimmunity (1). Recently, the identification of the subset of Th17 cells, which are potent producers of interleukin-17A (IL-17A) and IL-17F, has provided a new understanding of the underlying mechanisms of autoimmunity (2). Because Th17 cells are associated with the production of mediators of inflammation, much attention has been given to their potentially pathogenic role in autoimmune disease. The involvement of Th17 cells in rheumatoid arthritis (RA) has been explored (3). Furthermore, there is increasing evidence both in humans (4,5) and in mouse models (6) suggesting that IL-17–producing cells play a role in the progression of systemic lupus erythematosus (SLE).

SLE represents the classic prototype of systemic autoimmune disease in which loss of immune tolerance to self antigens leads to activation and expansion of autoreactive lymphocytes, uncontrolled production of several autoantibodies, and release of mediators of inflammation that ultimately damage multiple organs. It is generally assumed that the derangement of the im-
mune system in both human and murine SLE takes place as a result of dysregulation of immune T cell tolerance. The etiology of SLE remains unclear, although both genetic and environmental factors are involved (7). Two recent genome-wide association studies based on single-nucleotide polymorphisms have confirmed some previously associated foci and revealed new genetic regions related to SLE (8). However, the role of copy number variations (CNVs) in the genetic contribution to SLE still awaits extensive investigation.

CNVs have been defined by the presence of variable copies of genomic regions in different individuals. Several methodologies, such as the most commonly used array-based comparative genomic hybridization, have been used for genome-wide CNV detection and genotyping. CNVs have been discovered to have phenotypic consequences and associate with various types of diseases including mental disorders, RA, and others (9). So far, no genome-wide CNV studies have been performed for SLE. Examples of CNV loci associated with SLE include C4, Fcγ receptor type IIIb (FcγRIIIb), Toll-like receptor 7 (TLR-7), and CCL3-like 1. Our previous study also identified the association between CNVs of H3 histamine receptor and SLE (10). In the present study, we focused on Th17 cell–related genes, aiming to explore the possible association between the CNVs of these genes and the risk of SLE.

PATIENTS AND METHODS

Study populations. The present study included 2 cohorts. The first cohort was from Shenzhen Hospital, Peking University and represented the population of Southern China. This cohort included 532 SLE patients (47 male and 485 female; median age 28 years, range 12–56 years) and 576 healthy controls (52 male and 524 female; median age 29 years, range 16–49 years). The replication cohort was from Huashan Hospital, Fudan University and represented the population of Northern China. This cohort included 406 SLE patients (39 male and 367 female; median age 29 years, range 13–54 years) and 441 healthy controls (43 male and 398 female; median age 30 years, range 14–51 years). Characteristics of the study subjects are shown in Supplementary Table 1, available on the Arthritis & Rheumatism Web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131. All patients fulfilled the American College of Rheumatology criteria for SLE (11). The study was approved by the ethics committees of Shenzhen Hospital, Peking University and Huashan Hospital, Fudan University. All subjects provided written informed consent. The investigations were conducted according to the Declaration of Helsinki principles.

Quantification of copy numbers. SYBR Green–based quantitative polymerase chain reaction (PCR) was performed using the Bio-Rad Chromo4 real-time PCR system. Average copy numbers of RNase P in normal candidates (copy numbers = 2) were used as control (12). The copy numbers were calculated using the comparative C_{T} method. Cutoff values of 0.25, 0.75, 1.25, 1.75, and 2.25 were used to define the copy numbers as 0, 1, 2, 3, and 4, respectively. The primers for initial quantification are listed in Supplementary Table 2, available on the Arthritis & Rheumatism Web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131. The primers for validation of IL-17F, IL-21, and IL-22 are listed in Supplementary Table 3, available on the Arthritis & Rheumatism Web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

Southern blots. Genomic DNA was digested with restriction endonucleases and separated with agarose gel. The gel was washed and treated sequentially with 0.25M HCl, denaturation solution (1.5M NaCl/0.5M NaOH), and neutralization solution (1.5M NaCl/0.5M Tris, pH 7.0). The DNA was transferred to a nitrocellulose membrane using the Whatman 3MM filter paper wick method and crosslinked with ultraviolet irradiation. Southern blotting was performed by prehybridization at 37°C for 2 hours, hybridization with a radioactive probe at 65°C overnight, and washing sequentially with 2× saline–sodium citrate (SSC)/0.1% sodium dodecyl sulfate (SDS), 1× SSC/0.1% SDS, and 0.1× SSC/0.1% SDS. The bands of interest were observed by direct exposure on x-ray film.

Cell sorting and quantitative reverse transcription–PCR. Peripheral blood cells were collected and red blood cells were lysed with hypotonic buffer. For each staining reaction, 10⁶ white blood cells were stained in a 100 µl system with an optimized amount of phycoerythrin-conjugated anti-CD3ε (BD PharMingen). The cells were incubated at 4°C in the dark for 1 hour, washed twice with phosphate buffered saline, and subjected to cell sorting with a BD FACSAria II cell sorter (BD Biosciences). Total RNA was isolated from the sorted T cell population using the AxyPrep Blood Total RNA MiniPrep Kit (BD Biosciences). First-strand complementary DNA (cDNA) was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Fermentas China). Quantitative PCR was performed using the Chromo4 real-time PCR system. The relative messenger RNA (mRNA) levels of target genes were calculated using the comparative C_{T} method with GAPDH as the internal control. Data from 3 independent experiments were analyzed using Student’s t-test. The primers were as follows: for IL-17F, forward 5’-ATG-ACA-GTG-AAG-ACC-CTG-CAT-3’ and reverse 5’-TTG-GGG-ATT-TTC-GCA-GCT-GC-3’; for IL-21, forward 5’-CAT-GGA-GAT-TGT-CAT-CTG-TC-3’ and reverse 5’-CAG-AAA-TCG-GAC-CAA-GTC-AT-3’; for IL-22, forward 5’-TTC-CAG-CAG-CCC-TAT-ATC-ACC-3’ and reverse 5’-GCT-CAC-TCA-TAC-TGA-CTC-G-3’; for GAPDH, forward 5’-CAG-CCT-CAA-GAT-CAT-CAG-CA-3’ and reverse 5’-TGT-GGT-CAT-GAG-TCC-TTC-CA-3’.

Statistical analysis. Allele and genotype distributions were compared by chi-square test or Fisher’s exact test. Odds ratios (ORs) and 95% confidence intervals were calculated according to Woolf’s method, using SPSS 10.0 software for Windows. P values less than 0.05 were considered significant.
<table>
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*P* values were calculated by comparison of patients versus controls in each population. IL-17F = interleukin-17F; OR = odds ratio; 95% CI = 95% confidence interval.
ASSOCIATION BETWEEN CNVS OF TH17 CELL–RELATED GENES AND RISK OF SLE.

We examined the CNVs of Th17 cell–related genes including Th17 lineage–specific transcription factors (retinoic acid receptor–related orphan nuclear receptor γ [RORγt] and STAT-3), Th17 cell–related cytokines (IL-6, transforming growth factor β [TGFβ], tumor necrosis factor α [TNFα], IL-17A, IL-17F, IL-21, IL-22, and IL-23A), the Th17 cell–related chemokine (CCL20), and the Th17 cell–related receptor (CCR6). Primers for these genes are listed in Supplementary Table 2, available on the Arthritis & Rheumatism Web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131. Actual data from the quantitative PCR are shown in Supplementary Table 4, available on the Arthritis & Rheumatism Web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

Genotype and allele frequencies for copy number amplifications of IL-17F, IL-21, and IL-22 were found to be significantly higher in SLE patients than in healthy controls (Table 1), suggesting that CNVs of these 3 genes were associated with the risk of SLE (ORs of 3.67, 12.24, and 6.48, respectively). CNVs of the other genes showed no significant difference between SLE patients and controls (data not shown). Southern blots from representative samples were used to directly demonstrate the nature and extent of the CNV at the genomic

RESULTS

Association between CNVs of Th17 cell–related genes and risk of SLE. We examined the CNVs of Th17 cell–related genes including Th17 lineage–specific transcription factors (retinoic acid receptor–related orphan nuclear receptor γ [RORγt] and STAT-3), Th17 cell–related cytokines (IL-6, transforming growth factor β [TGFβ], tumor necrosis factor α [TNFα], IL-17A, IL-17F, IL-21, IL-22, and IL-23A), the Th17 cell–related chemokine (CCL20), and the Th17 cell–related receptor (CCR6). Primers for these genes are listed in Supplementary Table 2, available on the Arthritis & Rheumatism Web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131. Actual data from the quantitative PCR are shown in Supplementary Table 4, available on the Arthritis & Rheumatism Web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

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Figure 1. Southern blots of interleukin-17F (IL-17F), IL-21, and IL-22 from representative samples. A, Fifteen micrograms of DNA was digested with endonuclease enzymes as indicated. B–D, Shown are DNA agarose gel images (left) and Southern blots (right) of IL-17F (B), IL-21 (C), and IL-22 (D) from representative samples with 2 or 4 copies of DNA.

Figure 2. Messenger RNA levels of interleukin-17F (IL-17F), IL-21, and IL-22 in blood samples from patients with systemic lupus erythematosus (SLE). Total RNA was isolated from sorted T cells and then reverse transcribed. SYBR Green–based quantitative polymerase chain reaction was used to measure the relative mRNA levels of IL-17F, IL-21, and IL-22. The average mRNA level in the SLE samples with 2 copies of DNA was set to 1. Data from 3 independent experiments were analyzed by Student’s t-test. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the minimum and maximum values. * = P < 0.05 versus samples with 2 copies of DNA.
level (Figure 1). The initial quantification of IL-17F, IL-21, and IL-22 was further validated with 2 additional sets of primers, and similar results were obtained (see Supplementary Table 5, available on the Arthritis & Rheumatism Web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

**Combinational analysis of the allele frequencies of IL-17F, IL-21, and IL-22 in SLE patients.** We did not perform linkage analysis for the IL-17F, IL-21, and IL-22 genes, since they are located on chromosomes 6, 4, and 12, respectively. However, it is reasonable to assume that CNVs of these genes may simultaneously exist in certain patients and synergistically contribute to the risk of SLE. If so, higher frequencies of simultaneous amplification of these genes should be observed in SLE patients than in controls. We performed combinational analysis of these genes (see Supplementary Table 6, available on the Arthritis & Rheumatism Web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131). The results showed that frequencies of simultaneous amplification versus single-gene amplification were not significantly higher in SLE patients than in controls ($P = 0.803$ for IL-17F and IL-21, $P = 0.276$ for IL-17F and IL-22, $P = 0.754$ for IL-21 and IL-22), suggesting that these genes had no synergistic contribution to SLE.

**Correlation between mRNA levels and DNA copy numbers.** Next, we investigated whether the mRNA levels of IL-17F, IL-21, and IL-22 were positively correlated with their copy numbers. The samples from the SLE patients were divided into 2 groups: copy numbers = 2 and copy numbers >2. Fifty representative blood samples in each group were selected and subjected to cell sorting to define the T cell population. As shown in Figure 2, the mRNA expression of IL-17F, IL-21, and IL-22 in the sorted T cells with >2 copies of DNA was significantly higher than that in those with 2 copies of DNA, suggesting that CNVs have phenotypic consequences.

**DISCUSSION**

Th17 cells are associated with the production of mediators of inflammation; thus, they have potentially pathogenic roles in autoimmune disease. It has been shown that IL-17–producing cells play a role in progression of SLE both in humans (4,5) and in mouse models (6). Th17 cells produce IL-6, TNFα, IL-21, IL-22, IL-1β, and granulocyte–macrophage colony-stimulating factor in addition to IL-17A and IL-17F (2). In our study, we examined the CNVs of Th17 cell–related genes including Th17 lineage–specific transcription factors (RORγt and STAT-3), Th17 cell–related cytokines (IL-6, TGFβ, TNFα, IL-17A, IL-17F, IL-21, IL-22, and IL-23A), the Th17 cell–related chemokine (CCL20), and the Th17 cell–related receptor (CCR6). Copy number amplifications of IL-17F, IL-21, and IL-22 were found to be associated with SLE.

CNVs have been clearly shown to have the potential to directly or indirectly influence a healthy individual’s susceptibility to disease, for example, by varying the gene dosage of certain disease-causing genes (9). Since no genome-wide CNV studies have yet been performed in SLE, our study extends the previously reported examples of CNV loci associated with SLE, which include C4, FcYRIIIB, TLR-7, CCL3-like 1, and H4 histamine receptor.

It is possible that CNVs of IL-17F, IL-21, and IL-22 may synergistically contribute to the risk of SLE. However, in our study, combinational analysis did not reveal such a phenomenon. This indicates that CNVs of these genes separately contribute to the risk of SLE. It is thought that phenotypic effects of CNVs are brought about by changes in expression levels (13). In the present study, increased copy numbers of IL-17F, IL-21, and IL-22 correlated with elevated mRNA levels. Expression differences with CNV genotypes were also seen in normal subjects, not just in SLE patients. In general, up-regulation of Th17 cell–produced cytokines may result in stronger autoimmune responses. Supporting this notion is the fact that IL-17 expression was increased at the site of inflammation in patients with RA, psoriasis, multiple sclerosis, and uveitis (14). Another Th17 cell–produced cytokine, IL-21, a member of the IL-2 family of cytokines, can amplify the Th17 cell response in an autocrine manner and even substitute for IL-6 in the stimulation of Th17 cell differentiation (15).

In conclusion, the results of this study suggest that genetic variations in IL-17F, IL-21, and IL-22 copy numbers contribute to the risk of SLE. The implication of IL-17F, IL-21, and IL-22 in SLE risk might help to identify new targets for therapy in this disease. In addition, information about CNVs of these genes may help to define populations with high susceptibility to SLE.

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AUTHOR CONTRIBUTIONS
All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Wei Zhang had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Yu, Guan, Ye, Wan, W. Zhang.
Acquisition of data. Peng, Shao, Yue, J. Zhang, Yang.

REFERENCES
5. Wong CK, Ho CY, Li EK, Lam CW. Elevation of proinflammatory cytokine (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) concentrations in patients with systemic lupus erythematosus. Lupus 2000;9:589–93.