Copy number variations of Interleukin-12B and T-bet are associated with systemic lupus erythematosus

Bo Yu1,2,3,*, Yong Shao1,2,3,*, Xupeng Yue1,3, Jie Zhang1,2, Ming Guan4, Jun Wan1,3,5 and Wei Zhang1,5

Abstract

Objectives. Th1 cells have been implicated as the causal agents in the pathogenesis of autoimmunity. SLE represents the classical prototype of systemic autoimmune disease. Copy number variations (CNVs) have been discovered to have phenotypic consequences and associate with various types of diseases. The current study aims to explore a possible association between CNVs of Th1 cell-related genes and the risk of SLE.

Methods. Genomic DNA and RNA from 532 SLE patients and 576 healthy controls were extracted. CNVs of Th1 cell-related genes (T-bet, Stat4, IL-12A, IL-12B, IFN-g, IP-10 and CXCR3) as well as Th2 and Treg cell-related genes (c-Mef, GATA3, Foxp3, IL-6 and TGF-β) were examined, and mRNA levels of IL-12B and T-bet were examined.

Results. Frequencies of IL-12B and T-bet CNVs in SLE patients were significantly higher than those in healthy controls. CNVs of IL-12B and T-bet had no synergistic contribution to SLE. The mRNA levels of IL-12B and T-bet in the samples with more than two copies of DNA were significantly higher than those with two copies of DNA.

Conclusions. CNVs of IL-12B and T-bet are associated with the risk of SLE.

Key words: Systemic lupus erythematosus, Interleukin-12B, T-bet, Copy number variations.

Introduction

Autoreactive effector CD4+ T cells, also called Th cells, have been associated with the pathogenesis of several autoimmune disorders. The traditional model suggests that in tissue-specific autoimmune diseases Th1 cells are the pro-inflammatory effectors of autoimmunity, whereas Th2 cells function to antagonize this effect [1, 2]. Th1 cells were implicated as the causal agents in the pathogenesis of autoimmunity in earlier studies [3]. Recently, the identification of the Th17 subset, which is a potent producer of IL-17A and IL-17F, has provided a new understanding as to the underlying mechanisms of autoimmunity [4]. The role of Th1 in autoimmune disease, however, cannot be discounted. Th17 cells appear to drive pathology in some models and Th1 cells do so in others, even for the same autoimmune disease, and it is unclear whether SLE is a Th1 and/or Th17-mediated disease at present [5].

SLE represents the classical 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 inflammatory mediators that ultimately damage multiple organs. It is generally assumed that the derangement of the immune system in this disorder takes place from a dysregulation of immune T-cell tolerance in both human and murine SLE. The aetiology of SLE remains unclear, although both genetic and environmental factors are involved [6, 7]. Two recent genome-wide association studies (GWASs) based...
on single nucleotide polymorphisms (SNPs) have confirmed some previously associated foci and revealed new genetic regions related to SLE [8, 9]. However, the role of copy number variations (CNVs) in the genetic contribution to SLE still awaits extensive investigation.

CNVs were 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 (aCGH), were utilized 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 [10]. CNVs could be causative variants in disease susceptibility and severity through different mechanisms. Extra copies of a gene may contribute to overexpression of proteins, whereas deletion of a gene may lead to deficient consequences. Furthermore, CNVs might have functional impact through direct structural disrupting/alteration of certain genes. So far, no genome-wide CNV studies were performed for SLE. However, the CNVs in genetic susceptibility and severity of SLE have been investigated in the past few years [11]. Examples of CNV loci associated with SLE include complement component 4 (C4), Fc-gamma receptor 3B (FCGR3B), Toll-like receptor 7 (TLR7), chemokine (C-C motif) ligand 3-like 1 (CCL3L1). Our previous study also identified the association between CNVs of histamine H4 receptor and SLE [12]. In this study, we focused on Th1 cell-related genes, aiming to explore the possible association between the CNVs of these genes and the risk of SLE.

Materials and methods

Study populations

The present study included 532 SLE patients (47 males and 485 females; median age 28 years, range 12–56) and 576 healthy controls (52 males and 524 females; median age 29 years, range 16–49). All patients fulfilled the ACR criteria for SLE [13]. Patient characteristics were shown in supplementary table 1, available as supplementary data at Rheumatology Online. The study was approved by the ethical committee of the Shenzhen Hospital, Peking University. The individuals gave their written informed consent. The investigations were conducted according to the Declaration of Helsinki principles.

Quantification of copy numbers

Quantitative PCR was performed through BioRad Chromo4 real-time PCR system (Heracles, CA, USA). Average copy numbers of RNase P in normal candidates (copy numbers = 2) were used as control [14]. The copy numbers were calculated by using the comparative C(T) method. Cut-off 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 are listed in supplementary table 2, available as supplementary data at Rheumatology Online.

Quantitative RT–PCR

Total RNA was isolated from whole blood by using AxyPrepTM Blood Total RNA MiniPrep Kit (Axygen, Union City, CA, USA) according to the manufacturer’s instruction. First-strand cDNA was synthesized with ReverTraTM First Stand cDNA Synthesis Kit (Fermentas China, Shenzhen, Guangdong, China). Quantitative PCR was performed through BioRad Chromo4 real-time PCR system. The relative mRNA levels of target genes is presented by using the comparative C(T) method with GAPDH as the internal control. Data from three independent experiments were analysed by Student’s t-test and \( P < 0.05 \) was considered statistically significant. The primers are: IL-12B, forward: 5’ CAG CAG TTG GTC ATC TCT TGG and reverse: 5’ GGT CCA GGT GAT ACC ATC TTC T; T-bet, forward: 5’ GGT TGC GGA GAC ATG CTG A and reverse: 5’ GTA GGC GTA GGC TCC AAG G; GAPDH, forward: 5’ CAG CCT CAA GAT CAT CAG CA and reverse: 5’ TGT GAG CAT GAG TCC TTC CA.

Statistical analysis

Statistical analysis comparing the allele and genotype distributions was performed using chi-squared test or Fisher exact test. Odds ratios (ORs) and 95% CIs were calculated according to Woolf’s method, using the SPSS 10.0 software for Windows (IBM, Chicago, IL, USA). \( P < 0.05 \) were considered to be statistically significant. Student’s t-test was used to compare the mRNA levels in the SLE samples. The average mRNA level in the SLE samples with two copies of DNA was set as 1. Data from three independent experiments were analysed. The non-normal distribution data were presented as 50% median (25 and 75% median) and range (maximum and minimum). \( P < 0.05 \) was considered to be statistically significant.

Results

The association between CNVs of Th1 cell-related genes and the risk of SLE

We have previously identified the association between CNVs of histamine H4 receptor and SLE [12]. Using the same method, we examined the CNVs of Th1, Th2 and Treg cell-related genes including Th1 lineage-specific transcription factors (T-bet and Stat4), Th1 cell-related cytokines (IL-12A, IL-12B and IFN-\( \gamma \)), Th1 cell-related chemokine (IP-10) and receptor (CXCR3), Th2 lineage-specific transcription factors (c-Mef and GATA3), Treg lineage-specific transcription factors (Foxy3) and Treg cell-related cytokines (IL-6 and TGIF-\( \beta \)). Primers for these genes are listed in supplementary table 2, available as supplementary data at Rheumatology Online. Frequencies of IL-12B and T-bet CNVs in SLE patients were significantly higher than those in healthy controls (Table 1, genotype and allele frequencies), suggesting that CNVs of these genes are associated with the risk of SLE (OR = 5.51 and 7.39, respectively). CNVs of the other genes showed no statistical difference between SLE patients and controls (data not shown).
Combinational analysis of the allele frequencies of IL-12B and T-bet in SLE patients

We did not perform linkage analysis for IL-12B and T-bet since they are located on chromosome 5 and 17, respectively. However, it is reasonable to assume that CNVs of the two genes may simultaneously exist in certain patients and synergistically contribute to the risk of SLE. If so, high frequencies of simultaneous amplification of these genes should be observed in some SLE patients. We then performed combinational analysis of the allele frequencies of these genes in SLE patients (supplementary table 3, available as supplementary data at Rheumatology Online). Frequencies of simultaneous amplification were not significantly higher than that of single-gene amplification (P = 0.415), suggesting that these genes had no synergistic contribution to SLE.

The correlation between mRNA levels and DNA copy numbers

Next, we checked whether the mRNA levels of IL-12B and T-bet were positively correlated with their copy numbers. The samples from the SLE patients were divided into two groups: copy numbers = 2 and copy numbers > 2. As shown in Fig. 1, expressions of IL-12B and T-bet in the samples with > 2 copies of DNA were significantly higher than those with two copies of DNA, suggesting that CNVs have phenotypic consequences.

Discussion

In earlier studies, Th1 cells were implicated as the causal agents in the pathogenesis of autoimmunity as these cells possessed the ability to transfer disease and elevated levels of IFN-γ were detected in vivo in areas of inflammation. In our study, we examined the CNVs of Th1

**Table 1** Genotype and allele frequencies for copy number amplifications of IL-12B and T-bet in SLE cases and controls

<table>
<thead>
<tr>
<th>Genotype frequencies</th>
<th>Allele frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-amplification/ non-amplification (copy number = 2)</td>
<td>Non-amplification/ Amplification (copy number = 3)</td>
</tr>
<tr>
<td>g.588E-10</td>
<td>5.51 (3.02, 10.08)</td>
</tr>
<tr>
<td>3 1001</td>
<td>63</td>
</tr>
<tr>
<td>1 1139</td>
<td>13</td>
</tr>
<tr>
<td>0 1018</td>
<td>46</td>
</tr>
<tr>
<td>4 8.70E-08</td>
<td></td>
</tr>
</tbody>
</table>

For IL-12B, the OR for amplification/amplification (copy number = 4) was 5.51 (95% CI: 3.02, 10.08) compared to non-amplification/amplification (copy number = 2) in cases. For T-bet, the OR for amplification/amplification (copy number = 4) was 7.39 (95% CI: 3.32, 16.44) compared to non-amplification/amplification (copy number = 2) in cases.

**Fig. 1** mRNA levels of IL-12B and T-bet in the blood samples of SLE patients. Total RNA was isolated from blood samples, and then reverse transcribed. SYBR-green-based quantitative PCR was used to measure the relative mRNA levels of IL-12B and T-bet. The average mRNA level in the SLE samples with two copies of DNA was set as 1. Data from three independent experiments were analysed by Student’s t-test. The non-normal distribution data were presented as 50% median (25 and 75% median) and range (maximum and minimum). *P < 0.05.
cell-related genes (T-bet, Stat4, IL-12A, IL-12B, IFN-γ, IP-10 and CXCR3) as well as Th2 and Treg cell-related genes (c-Mef, GATA3, Foxp3, IL-6 and TOG-Fβ) and copy number amplifications of IL-12B and T-bet, which are both Th1 cell-related genes, were found to be associated with SLE. However, studies in several autoimmune disease models suggested a more complex story. Mice genetically deficient in IFN-γ were not only not protected but also exhibited enhanced susceptibility in many models of autoimmunity. The identification of Th17 cells, a CD4+ T-cell subset that produces IL-17, has helped to shed some light on this apparent paradox. These cells, like Th1 cells, have the capacity to cause T-cell-mediated inflammation and autoimmune disease. In the future, it would be conceivably necessary to examine the CNVs of Th17 cell-related genes.

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 [10]. Since no genome-wide CNVs studies were performed for SLE so far, our study extends the examples of CNVs loci associated with SLE including C4, FCGR3B, TLR7, CCL3L1 and H4 receptor and SLE. It is possible that CNVs of IL-12B and T-bet may synergistically contribute to the risk of SLE. However, in our study, combinational analysis did not reveal such phenomenon. This tells us that CNVs of these genes separately contribute to the risk of SLE. It is expected that phenotypic effects of CNVs are supposedly brought about by changes in expression levels [15]. In our case, increased copy numbers of IL-12B and T-bet correlated with the elevated mRNA levels. In general, up-regulation of Th1-produced cytokine may result in stronger autoimmune responses. It is assumed that the derangement of the immune system in this disorder takes place from a dysregulation of immune T-cell tolerance in both human and murine SLE. In the experimental autoimmune uveitis (EAU) model, cells polarized to the Th1 phenotype are able to transfer EAU and experimental autoimmune encephalomyelitis (EAE) with comparable severity to Th1-deficient mice [16, 17]. Mice lacking IL-12B were protected in a variety of autoimmune models [3, 18]. T-bet-deficient mice that lack a Th1 response are resistant to EAE [19, 20]. In conclusion, the results of this study suggest that genetic variations in IL-12B and T-bet copy numbers contribute to the risk of SLE. The implication of IL-12B and T-bet in SLE might help to identify or apply new candidates for therapeutic targeting of SLE. Also, information about CNVs of these genes may help to define the population with high susceptibility to SLE.

Acknowledgements
We thank Shenzhen Biomedical Research Support Platform for the technical help.

Funding: The study was supported by the Research Grants of Shenzhen Science and Technology Project.

Disclosure statement: The authors have declared no conflicts of interest.

Supplementary data
Supplementary data are available at Rheumatology Online.

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