Short Communication

Copy number variations of HLA-DRB5 is associated with systemic lupus erythematosus risk in Chinese Han population

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Systemic lupus erythematosus (SLE) is a polygenic, systemic, autoimmune disease. Copy number variants (CNVS) have been discovered to be associated with a number of complex disorders. We undertook the current study to explore the potential associations between genomic CNVS and SLE in Chinese Han population. In the discovery stage, seven SLE patients were examined with the high-density comparative genomic hybridization microarrays in the screening test for SLE associated CNVS. Then, in the validation stage, 135 SLE patients and 219 matched healthy subjects were investigated for the CNVS of gene HLA-DRB5 by AccuCopy™ technology. Quantitative polymerase chain reaction was carried out to determine the copy number (CN) and mRNA level of HLA-DRB5 in SLE patients. Although the mRNA level of HLA-DRB5 between the CN deletion group and the CN normal group in SLE patients was not statistically positive (P = 0.46), our results still showed more CN of HLA-DRB5 in SLE patients than in healthy controls (P = 3.98 × 10⁻⁵). Odds ratio for CN deletion was 0.38 (95% confidence interval (CI), 0.23–0.61, P = 7.79 × 10⁻⁵) and for CN duplication was 1.89 (95% CI, 0.56–7.66, P = 0.37), respectively. These findings indicated that CNVS of HLA-DRB5 was associated with the risk of SLE, and CN deletion appeared to be protective for SLE.

Keywords copy number variations; systemic lupus erythematosus; human leukocyte antigen

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Introduction

Systemic lupus erythematosus (SLE) is a polygenic autoimmune disease, which is characterized by the development of many autoantibodies and the deposition of antibody–antigen immune complexes in many kinds of organs. The resulting clinical features range from skin rashes to organ-threatening glomerulonephritis. Although the critical etiology of SLE still remains unknown, a number of previous studies have confirmed the role of both the genetic risks and environmental factors [1]. Till now, several genome-wide association studies (GWAS) have been performed in SLE patients and have confirmed some previously associated loci and revealed new genetic regions related to SLE [2–4].

Structural variation is recognized as a rich source of genetic heterogeneity in the human genome, and copy number variations (CNVS) are also included. Increasing studies of CNVS across the genome have highlighted its role in the etiology of genetic disease susceptibility. Thus far, variation in gene copy number (CN) has been associated with a number of complex inflammatory and infectious disorders, including SLE. For instance, an association between high CN at the beta-defensin and psoriasis, and CCL3L1 CN replication and the susceptibility to HIV-1 infection were previously reported [5,6]. Meanwhile, examples of CNVS loci associated with SLE include deficiencies in the complement factor 4 gene (C4A/C4B) [7], low Fcγ receptor type III b (FCGR3B) CN [8], duplication in chemokine (C-C motif) ligand 3-like 1 (CCL3L1) [9], and increased amplification of histamine H4 receptor (HRH4) [10].

Here, we undertook a comparative genomic hybridization (CGH) microarray study to explore more specific CNVS that potentially contribute to genetic susceptibility to SLE disease and then validated our findings in a case–control study to confirm the association.

Materials and Methods

Patients and controls
A total of 354 subjects were enrolled. One hundred and thirty-five cases [mean (SD) 38.7 (13.9) years; 87.4%
females] with SLE and 219 ethnically and geographically matched healthy blood donors [mean (SD) 68.4 (6.6) years, 49.8% females]. Detailed clinical and serological information about SLE patients were shown in Table 1. All patients were recruited from a multicenter study including hospitals and outpatient clinics in Shanghai and Jiangsu province in China, and met the American College of Rheumatology classification criteria for SLE. The ethical approvals of the studies were obtained from each Institutional Review Board.

DNA extraction
Peripheral blood was collected from all subjects. Genomic DNA was isolated from whole blood using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) and stored at −20°C until use. DNA concentration and quality (including optical density 260/280 and 260/230 measurements) were determined by a Nanodrop Lite spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

Genome-wide CNVS analysis
Seven SLE patients were examined by Agilent SurePrint G3 Human CGH Microarrays (1 × 1 M) following the manufacturer’s protocol (Agilent, Santa Clara, USA). Commercial genomic DNA (Promega, Madison, USA) was used as the internal control. Briefly, genomic DNA of each subject was treated with restriction digestion, and then was labeled with ULS-Cy5 (for patients) and ULS-Cy3 (for sex-matched controls). The labeled products of one patient and control were mixed and hybridized to the array for 40 h at 60°C. Then, the array was washed and scanned on an Agilent Microarray Scanner. The data were extracted by Agilent Feature Extraction 10.7.3.1 and analyzed by Agilent Workbench 7.0. ADM-2 was used as statistical algorithm with P-value threshold of 0.05. CN duplications or deletions of at least five consecutive oligomers on the array were selected for further analysis. The genome-wide common CNVS in human populations were adopted from the public data via Database of Genomic Variants (http://projects.tcag.ca/variation/) [11] and our private Chinese CNV data [12], which were applied to distinguish the common CNVS from the novel CNVS. Genes present in these common aberrations regions (refer to as CNV regions) were identified using human genome browser at UCSC [13].

AccuCopy™ technology for CNVS detection
HLA-DRB5 CNVS were validated with the AccuCopy assay [a multiple competitive real-time polymerase chain reaction

Table 1. The clinical and laboratory data at the time of inpatient for 135 SLE patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>All patients (n = 135)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (female)</td>
<td>118 (87.4%)</td>
</tr>
<tr>
<td>Age (years ± SD)</td>
<td>38.7 ± 13.9</td>
</tr>
<tr>
<td>SLEDAIa (mean ± SD)</td>
<td>20.29 ± 9.55</td>
</tr>
<tr>
<td>Immunological indicators (g/l)</td>
<td></td>
</tr>
<tr>
<td>Serum C3 (g/l)</td>
<td>0.63 ± 0.26</td>
</tr>
<tr>
<td>Serum C4 (g/l)</td>
<td>0.13 ± 0.07</td>
</tr>
<tr>
<td>ANAs (%)</td>
<td>121 (89.6%)</td>
</tr>
<tr>
<td>Anti-dsDNA antibodies (%)</td>
<td>69 (51.1%)</td>
</tr>
<tr>
<td>Anti-Sm (%)</td>
<td>63 (46.7%)</td>
</tr>
<tr>
<td>Anti-SSA (%)</td>
<td>100 (74.1%)</td>
</tr>
<tr>
<td>Anti-SSB (%)</td>
<td>43 (31.8%)</td>
</tr>
</tbody>
</table>

aSLEDAI, SLE disease activity index.

Table 2. Whole genome CNV profiles for each seven SLE patients

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>CNV type</th>
<th>Number</th>
<th>Length (SD), kb</th>
<th>Gain/loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL11-0004F</td>
<td>cCNV</td>
<td>57</td>
<td>101.846 (290.5158)</td>
<td>21/36</td>
</tr>
<tr>
<td></td>
<td>nCNV</td>
<td>3</td>
<td>51.469 (70.70524)</td>
<td>0/3</td>
</tr>
<tr>
<td>SL11-0008F</td>
<td>cCNV</td>
<td>57</td>
<td>68.961 (153.8646)</td>
<td>22/35</td>
</tr>
<tr>
<td></td>
<td>nCNV</td>
<td>4</td>
<td>24.124 (24.30854)</td>
<td>2/2</td>
</tr>
<tr>
<td>SL11-0011F</td>
<td>cCNV</td>
<td>55</td>
<td>60.924 (120.427)</td>
<td>20/35</td>
</tr>
<tr>
<td></td>
<td>nCNV</td>
<td>3</td>
<td>10.481 (4.294353)</td>
<td>2/1</td>
</tr>
<tr>
<td>SL11-0021F</td>
<td>cCNV</td>
<td>59</td>
<td>110.111 (319.930)</td>
<td>24/35</td>
</tr>
<tr>
<td></td>
<td>nCNV</td>
<td>4</td>
<td>18.084 (17.87053)</td>
<td>1/3</td>
</tr>
<tr>
<td>SL11-0025F</td>
<td>cCNV</td>
<td>31</td>
<td>142.451 (418.9751)</td>
<td>10/21</td>
</tr>
<tr>
<td></td>
<td>nCNV</td>
<td>9</td>
<td>24.476 (30.16832)</td>
<td>6/3</td>
</tr>
<tr>
<td>SL11-0047F</td>
<td>cCNV</td>
<td>50</td>
<td>87.447 (251.0645)</td>
<td>18/32</td>
</tr>
<tr>
<td></td>
<td>nCNV</td>
<td>9</td>
<td>13.108 (13.01129)</td>
<td>3/6</td>
</tr>
<tr>
<td>SL11-0002M</td>
<td>cCNV</td>
<td>49</td>
<td>108.738 (329.4251)</td>
<td>22/27</td>
</tr>
<tr>
<td></td>
<td>nCNV</td>
<td>4</td>
<td>37.035 (37.83676)</td>
<td>1/3</td>
</tr>
</tbody>
</table>

cCNV, common CNV; nCNV, novel CNV.
Twenty microliters PCR reaction was prepared for each sample; containing 1 x AccuCopy™ PCR Master Mix, 1 x Fluorescence Primer Mix, 1 x Competitive DNA mix, and ~10 ng sample DNA. The PCR program was described as follows: 95°C for 10 min; 11 cycles of 94°C for 20 s, 65°C–0.5°C/cycle for 40 s, 72°C for 1.5 min; 24 cycles of 94°C for 20 s, 59°C for 30 s, 72°C for 1.5 min; 60°C for 60 min; 4°C to the end. PCR products were diluted 20-fold before loaded on ABI 3730XL sequencer (Life technologies, Carlsbad, USA). Raw data were analyzed by GeneMapper4.0 and height/area data for all specific peaks were exported into an excel file. The sample/competitive (S/C) peak ratio was calculated for all target fragments and three reference genes, and the S/C ratio for each target fragment was first normalized to three reference genes, respectively. The three normalized S/C ratios were further normalized to the median value in all samples for each reference gene, respectively, and then averaged. If one of the three normalized S/C ratios deviated more than 25% from the average of the other two, it was excluded from further analysis. The CN of each target fragment was determined by the average S/C ratio multiplied by two. The primers used to amplify target CNVS regions in AccuCopy™ technology were shown in Supplementary Table S1. In order to verify the reliability of AccuCopy assay, PCR was performed to validate the HLA-DRB5 CN. The primers are as shown in Figure 1. CGH results for CNVS of the HLA-DRB5 regions in seven SLE patients. Black dots represent normal hybridization signal, green dots represent decreased hybridization signal, and red dots indicate increased hybridization signal.
follows: forward primers (5′–3′), ATGACAGGAAGAGA TTTCAACTAACA; reverse primers (5′–3′), GGGCTTGTAGT GCTTCGCTG. REF-F8E01 was used as positive control. Forward primers (5′–3′): CCATGGGGATGCTTAGGAC; reverse primers (5′–3′): CACAACCATCCTAACCCGATG. The PCR was performed in 20 μl mixture including 1 × GC buffer I (Takara Bio, Tokyo, Japan), 2.5 mM Mg²⁺, 0.2 mM dNTP, 0.2 μM of each primer, 1 U HotStarTaq polymerase (Qiagen), and 1 μl template DNA. The cycling program was 95°C for 15 min; 8 cycles of 94°C for 15 s, 66°C for 40 s, 72°C for 1 min; 22 cycles of 94°C for 15 s, 60°C for 30 s, 72°C for 1 min; 72°C for 2 min. The PCR products were visualized using electrophoresis on 1% agarose gels stained with ethidium bromide (Supplementary Fig. S1).

Measurement of HLA-DRB5 mRNA transcripts

Another 15 SLE patients were also enrolled, and the clinical information was shown in Supplementary Table S2. Total RNA was extracted from the peripheral blood by using Trizol Reagent (Sigma, St Louis, USA) and reverse-transcription (Takara Bio) was performed. Real-time PCR [A 10 μl mixture was prepared for each reaction and included 5 μl 2 × SYBR Green Taq polymerase (Takara Bio), 0.2 μl 50 × Rox Reference DyeII, 0.2 μl 10 μM of each primer, 3.4 μl ddH₂O, and 1 μl template DNA. The cycling program was 95°C for 30 s; 38 cycles of 95°C for 5 s, 62°C for 30 s.] was performed using SYBR Green chemistry [forward primers (5′–3′): AGTGACACTGAT CGTCCCGTTGA AGAAATGAC] on an ABI 7500 machine (Life technologies) and the well-established relative quantification 2⁻ΔΔCt method, GAPDH was used as an endogenous control.

Statistical analysis

The distributions of CN between patients and controls after CN assignment according to the pre-defined threshold were compared by using χ² test for the trend in proportions with R project. Logistic regression models were constructed to determine the odds ratio (OR) and 95% confidence intervals (CIs) with the adjustment of gender using SPSS (version 17.0, SPSS Inc., 2008, Chicago, USA). Thresholds for deletions and duplications were empirically set at below 0.59 and above 1.77, respectively, in the above CNVS validation assays according to the manufacture’s instruction. All samples were tested in duplicates.

Results

CNV discovery in SLE patients

CGH microarrays were utilized to detect CNVS genome widely in six female and one male SLE patients. The clinical information about these seven SLE patients was shown in Supplementary Table S3. There were 394 CNVS (average 56.3, SD = 7.8) found in 7 individuals including 152 gains and 242 losses, 358 common and 36 novel CNVS in the whole genome (Table 2). The results showed aberrant CN at 6p21.32 in the majority of SLE patients. In particular, aberrant CN of a 42 kb fragment at the region containing the HLA-DRB5 showed in five of the seven SLE patients. The CGH results for CNV of the DRB5 region were shown in Fig. 1.

Association between HLA-DRB5 CNVS and susceptibility of SLE

Among our samples, all of the 354 individuals (135 SLE patients and 219 controls) met the criterion that CNV genotyping rate was >80%. The CNVS distributions were shown in Fig. 2. The χ² test for trend showed more CN of HLA-DRB5 in SLE patients than in healthy controls (P = 3.98 × 10⁻⁵). χ² test showed OR for CN deletion was 0.38 (95% CI, 0.23–0.61, P = 7.79 × 10⁻⁵) and for CN duplication was 1.89 (95% CI, 0.56–7.66, P = 0.37), respectively. Logistic regression analysis indicated significant association between CNVS and SLE after gender adjustment (OR = 1.64, 95% CI, 1.33–2.01, P < 0.0001) (Table 3). This evidence indicated that CN deletion of HLA-DRB5 appeared to be protective for SLE.

Correlation between HLA-DRB5 CN and mRNA level in SLE patients

We then investigated whether the mRNA level of HLA-DRB5 was positively correlated with its CN. Thus, both DNA and RNA samples from another 15 SLE patients were collected. The CN of HLA-DRB5 was detected by 

Figure 2. CN density of HLA-DRB5 in SLE cases (red) and controls (black) in the AccuCopy assay. X-axis represents CN status, Y-axis represents density values of CN distribution.
AccuCopy™, and the mRNA level was measured with the quantitative PCR. As shown in Fig. 3, the mRNA expression of HLA-DRB5 between the CN deletion group and the CN normal group was not significantly different ($P = 0.46$).

**Discussion**

To date, CNVS of genes has been well validated to be associated with several common diseases. As a complex disease with heterogeneous clinical phenotype, multifactorial etiologies such as genetic and environmental factors have been implicated in SLE. So far, increasing evidence has demonstrated the importance of CNVS in SLE [7–10,16]. Our results further proofed CNVS as a high-risk factor for SLE disease susceptibility.

Human leukocyte antigen (HLA) class II molecules are involved in different stages of immunological processes. This group of molecules could potentially account for several disease pathogenesis through influencing the presentation of antigens that share molecular mimicry or altered expression levels of these HLA. Here, we found that CNVS of HLA-DRB5 was associated with SLE disease susceptibility. A recent study has revealed the involvement of HLA-DRB5 CNVS in the development of Keloid disease through minor puncture wounds in Caucasians [17]. For the first time, we discovered that HLA-DRB5 CNVS was genetically associated with SLE in Han Chinese. As a fact, previous studies have reported that HLA-DRB5 locus is almost exclusively carried by HLA-DRB1*15 and HLA-DRB1*16 haplotypes in northern European populations [18], but as we know that the linkage disequilibrium (LD) patterns differ between populations, for instance, Louthrenoo et al. [19] found this LD in northern Thai population, while Odani et al. [20] did not find this phenomenon in Japanese population. Hence, further investigation may be still necessary to identify whether HLA-DRB5 haplotypes exist in Chinese Han population.

In the current study, we also tried to explore the functional consequences of CNVS at HLA-DRB5 through analysis of variations in the expression of HLA-DRB5 mRNA level. As shown above, our data were suggestive, but not conclusive, that HLA-DRB5 CN deletion was correlated with lower mRNA level, although it is not statistically significant, which may due to the small sample size used.

**Supplementary Data**

Supplementary data are available at *ABBS* online.

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**References**


