Concise report

Higher \textit{DEFB4} genomic copy number in SLE and ANCA-associated small vasculitis

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Abstract

\textbf{Objective.} Evidence shows that defensins are involved in the pathogenesis of SLE and ANCA-associated small vasculitis (AASV). The copy number variation of \textit{DEFB4} has been proposed to be susceptible to inflammatory disorders. This study aims to investigate whether the \textit{DEFB4} genomic copy number variations associate with the susceptibility to these two autoimmune diseases.

\textbf{Methods.} A total of 1178 Chinese people were enrolled, including Panel 1 comprising 240 SLE patients and 275 matched controls, Panel 2 comprising 303 SLE patients and 248 matched controls and Panel 3 with 112 AASV patients. The \textit{DEFB4} copy number was typed by a paralogue ratio test (PRT), and all the subjects in Panel 1 were also typed using the restriction enzyme digest variant ratio (REDVR) for validation.

\textbf{Results.} The results from PRT and REDVR were highly concordant ($R = 0.911$, $P = 3.85 \times 10^{-199}$) and allowed copy numbers to be assigned into integer classes with high confidence. Comparison of mean \textit{DEFB4} copy number revealed a small increase in cases with SLE both in Panel 1 ($P = 0.063$) and Panel 2 ($P = 0.017$). When pooling Panels 1 and 2 together, the association was reinforced ($P = 0.002$) in SLE. Such association was also observed in AASV ($P = 0.009$).

\textbf{Conclusion.} We found that a higher \textit{DEFB4} gene copy number was associated with both SLE and AASV.

\textbf{Key words:} \textit{DEFB4}, gene copy number, SLE, AASV.

Introduction

Defensins are highly conserved cationic peptides with a vast spectrum of anti-microbial activity. Several immunomodulatory functions, including chemotactic effects and induction of pro-inflammatory cytokines, have been identified. Thus defensins constitute an important part of the first defence against micro-organisms and connect the innate and adaptive immune system [1]. In SLE, it was reported that active SLE can be distinguished by a remarkably homogeneous gene expression pattern with overexpression of defensin-related and IFN-induced genes [2, 3], and accumulating data evidenced that defensins were involved in SLE pathogenesis [4–6]. For example, up-regulated mRNA and protein expression were observed in SLE patients, and increased anti-defensin antibodies were found in the sera of patients with SLE, but they decreased after therapy with CSs [3, 5, 6]. More recently, it was reported that such elevated values of human neutrophil peptide (HNP; \textit{z}-defensin 1–3) and human \textit{b}-defensin 2 (\textit{hBD2}; \textit{DEFB4}) could be observed in both SLE and granulomatosis with polyangiitis (Wegener’s) [6, 7]. Therefore, further studies on defensins in autoimmune diseases are needed, which may lead to a better understanding of disease mechanisms at the junction of the innate and adaptive immune response.

The copy number variation in \textit{b}-defensin genes on human chromosome 8 has been proposed to underlie susceptibility to inflammatory disorders, such as psoriasis [8], chronic obstructive pulmonary disease [9], IBD [10, 11], HIV infection and severe acute pancreatitis. There is a correlation between copy number and \textit{b}-defensin gene expression at the mRNA level [12]. Only when higher copy numbers of \textit{b}-defensin genes were present was transcription significantly up-regulated by...
AAAGAT-3, which resulted in better anti-microbial activity in vitro [9]. In this study we investigated whether genomic copy number variations of the β-defensin gene cluster associate with susceptibility to two prototype autoimmune diseases, SLE and ANCA-associated small vasculitis (AASV).

Methods

Patients and controls

All patients were recruited from Peking University First Hospital from 1997 to 2010. A total of 1178 subjects were enrolled. Panel 1 comprised 240 cases [mean (s.d.) 32.6 (12.1) years; 82.7% females] with SLE and 275 ethnically and geographically matched healthy blood donors [33.6 (11.3) years, 75.8% females]. Panel 2 comprised 303 SLE patients [31.8 (11.1) years, 84.7% females] and 248 controls [36.1 (10.5) years, 63.7% females]. SLE patients were diagnosed according to the revised and updated criteria established by the ACR [13]. Panel 3 included 112 subjects [56.4 (15.1) years, 43.1% females] with AASV, who were ANCA seropositive and had biopsy-proven necrotizing glomerulonephritis [14]. The genetic study was approved by the medical ethics committee of Peking University and informed written consent was obtained from all participants.

DNA extraction

Peripheral blood was collected from all subjects. Genomic DNA was isolated from whole blood using a modified salt extraction technique, concentrated by ethanol precipitation, resuspended in Tris-EDTA (TE) buffer and stored at −30°C until use. DNA concentration and quality [including optical density (OD) 260/OD 280 and OD 260/OD 230 measurements] were determined by a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Paralogue ratio test

A paralogue ratio test (PRT) was performed using the HSPD21 system, as previously reported [10], which specifically amplified chr8 (DEFB) in conjunction with restriction products from chr21, with products differing by 8bp. Primers were HSPD21F (5′-GAGGTCACTGTGATC AAAGAT-3′) and FAM-labelled HSPD21R (5′-AACACTCA AAAGAT-3′). Genomic DNA of 10–20 ng was amplified in a final volume of 20 μl with 0.5 mM forward primer and 0.5 mM FAM-labelled reverse primer, in a buffer containing final concentrations of 12 μM dH2O, 10× PCR buffer 2 μl, 2.5 mM dNTP 1.6 μl, 25 mM MgCl2 1.2 μl and 1 U Taq polymerase (Takara, Dalian, China).

Participants were amplified at 94°C for 5 min followed by 22 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 30 s; followed by a single chase phase of 57°C for 1 min, then 72°C for 20 min to reduce levels of ssDNA products. In all cases, PRT measurements were carried out on mixed plates containing randomly interspersed case, control and reference DNA samples, as well as no DNA blanks. Electrophoresis was analysed on an ABI 3130xl Genetic Analyzer using GeneScan software (Applied Biosystems).

Restriction enzyme digest variant ratio

All the patients in Panel 1 were typed using both PRT-based methods and the restriction enzyme digest variant ratio (REDVR). REDVR was done using reported primers (HSPD5.8F 5′-CCAGATGACCGAGGTCC-3′ and HEX-labelled primer HSPD5.8R 5′-TTTTAAGTTCA CGAATTACGC-3′) to amplify two products, one from the variable copy near DEF4 and one from an unrelated reference copy on chromosome 5 [8]. Genomic DNA of 10–20 ng was amplified in a final volume of 20 μl, with 0.5 mM forward primer and 0.5 mM HEX-labelled reverse primer, in a Roche FastStart High Fidelity System (Roche Cat No. 0355340001) and with the same conditions described above, except using 30 cycles. Test and reference amplicons are distinguished by restriction digestion (HaeIII, New England Biolabs) and electrophoresis.

Statistical analysis

A comparison of the median copy number of DEF4 between patients with SLE or AASV and controls was performed using the Mann–Whitney U-test. Logistic regression was used to model the effect of copy number of DEF4 on the disease incidence when gender and age were included as covariates. Tests of association with disease status were also carried out using χ² tests on 3×2 contingency tables, classifying samples into low (three or fewer), central (n=4) or high (five or more) copy number categories. All hypothesis tests in this study were two tailed. If necessary, meta-analysis was conducted with the Mantel–Haenzel method. Statistical analyses were performed using SPSS16.0 for Windows (SPSS, Inc., Chicago, IL, USA). A value of P <0.05 was considered statistically significant. For multiple replications, uncorrected P-values were reported.

Results

DEF4 genomic copy number was analysed in 1178 Chinese individuals, and varied between two and seven per diploid genome in the current Chinese cohort. The results from PRT and REDVR were highly concordant (R=0.911, P=3.85×10⁻¹⁹⁵) and allowed copy number to be assigned into integer classes with high confidence. Comparison of mean DEF4 copy number revealed a small increase in cases with SLE both in Panel 1 (mean 3.96 vs 3.79, P=0.063) and Panel 2 (3.99 vs 3.80, P=0.017). When pooling Panels 1 and 2 together, the association was reinforced (3.98 vs 3.79, P=0.002) in SLE. Such an association was also observed in AASV (4.05 vs 3.79, P=0.009) compared with pooled control subjects. In the multivariate analysis, when controlling for gender and age by logistic regression analysis, DEF4 genomic copy number was also found to be associated with susceptibility to SLE [P=0.002, odds ratio (OR) 1.23, 95% CI 1.08, 1.39] and AASV (P=0.010, OR 1.49, 95% CI 1.10, 2.01).

Further classification of samples into low (three or fewer), central (n=4) or high (five or more) copy number categories showed an excess of samples with five or more copies among SLE (27.9%, P=0.005) and AASV (27.7%,...
Patients compared with controls (19.9%) (Fig. 1 and supplementary data, available at Rheumatology Online). To determine whether DEFb4 copy number had a stronger association with specific clinical manifestations of SLE and AASV, we compared its mean CN in cases stratified by the presence or absence of autoantibodies (anti-dsDNA, anti-Sm and anti-RNP antibodies in SLE; ANCA specificities in AASV), organ involvement and disease activity index. We observed that the presence of anti-RNP antibody was associated with higher DEFb4 copy number (4.19 vs 3.78, \( P = 0.008 \)).

Discussion

To our knowledge, our study is the first to investigate the association of \( \beta \)-defensin genomic copy number with SLE and AASV. We found higher DEFb4 gene copy number associated with both SLE and AASV, which supported the role of the defensin system in autoimmunity. However, such an increase in copy number was modest (increase by 0.2 in SLE and 0.3 in AASV), with ORs of 1.2 for SLE and 1.5 for AASV, which may be the reason why we did not observe that it contributes greatly to disease development in our current cohort. The precise mechanisms of its association with the presence of anti-RNP antibody are still unknown. Even so, what we found was consistent with recent reports considering associations between DEFb4 gene copy number and Crohn’s disease. Aldhous et al. [10] reported that they failed to replicate the associations of \( \beta \)-defensin copy number with Crohn’s disease. However, they also observed a borderline significant difference (\( P = 0.032 \)) with higher \( \beta \)-defensin copy number in UK cases than that in matched controls [10], and in the recent report from the Welcome Trust Case-Control Consortium (WTCCC) on genome-wide copy number variation (CNV) associations in eight common diseases, although higher \( \beta \)-defensin copy numbers were observed in RA and Crohn’s disease, such an association became non-significant after correction for multiple testing [15]. The same non-significant disease associations in whole-genome level were also reported with regard to CCL3L1 and FCGR3A/B [15], which have recently been verified to be associated with SLE and replicated in large case-control studies from different populations [16]. All in all, it reinforced the notion that CNVs were more complex than single nucleotide polymorphisms (SNPs) in disease association studies, and common variants may exert modest genetic effect in determining different genetic risk. It was of great significance to type the copy number precisely and to adopt proper statistical analysis [17-19].

Environment, especially infections, may be involved in the pathogenesis of SLE and AASV. Thus recent experimental and clinical studies have placed new emphasis on the role of the innate immune system in SLE. Among many of the remarkably varying gene expression patterns in SLE, overexpression of defensin-related genes was one of the most prominent pathways. hBD2 (DEFb4) was a highly inducible, anti-microbial peptide, which may have an important role in the innate immune response. It was reported that elevated hBD2 could be detected in sera from SLE and AASV patients; copy number was found to affect expression of human DEFb4. Thus what we found may give a plausible explanation for this; that is, DEFb4 gene CNV may affect autoimmune disease susceptibility by a dosage effect. A more recent study observed that great genetic heterogeneity could be observed in the human \( \beta \)-defensin gene locus and may be a result of selection [20]. Hence, further, more widespread associations may be needed to determine whether such genetic heterogeneity accounts for some of the geographical or ethnic variations in various populations. Further studies analysing the role of defensin in autoimmune diseases are needed, as this may lead to a better understanding of disease mechanisms with possible implications for future treatment strategies.

**Rheumatology key messages**

- Higher DEFb4 gene copy number is associated with both SLE and AASV.
- DEFb4 gene CNV may affect autoimmune disease susceptibility by a dosage effect.
- Further studies analysing the role of defensin in autoimmune diseases are needed.

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Supplementary data
Supplementary data are available at Rheumatology Online.

References