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The association of the BLK gene with SLE was replicated in Chinese Han

Zheng Zhang · Kun-Ju Zhu · Qiang Xu · Xue-Jun Zhang · Liang-Dan Sun · Hou-Feng Zheng · Jan-Wen Han · Cheng Quan · Sheng-Quan Zhang · Li-Qiong Cai · Sheng-Xin Xu · Xian-Bo Zuo · Hui Cheng · Sen Yang

Abstract Systemic lupus erythematosus (SLE) is an autoimmune disease influenced by genetic and environmental factors. Recently, single nucleotide polymorphisms (SNPs) in the region of B lymphoid tyrosine kinase (BLK) have been shown to be associated with SLE in Caucasian population. In this paper, we genotyped SNP rs2248932 in 1,396 SLE patients of Chinese Han and 4,362 ethnically matched control subjects by using the Sequenom MassArray system. We confirmed that SNP rs2248932 in BLK gene was significantly associated with SLE ($P = 1.41 \times 10^{-8}$ for the allele frequency, Odds ratio [OR] = 0.74, 95% confidence interval (CI): 0.66–0.82).The association of BLK in Chinese SLE patients was consistent with a dominant model ($P = 8.88 \times 10^{-9}$, OR = 0.69, 95% CI: 0.61–0.79). In contrast to the Caucasian, this risk allele was the major allele in the Chinese Han; the risk allele frequency was higher in Chinese Han than in Caucasian. We did not find the association between this SNP and any subphenotype of SLE. The SNP rs2248932 was correlated to the expression of BLK mRNA. We conclude that the association of the BLK region with SLE was replicated in Chinese Han population living in mainland.

Keywords Systemic lupus erythematosus · Association · Chinese · BLK

Z. Zhang · K.-J. Zhu · Q. Xu · X.-J. Zhang · L.-D. Sun · H.-F. Zheng · J.-W. Han · C. Quan · S.-Q. Zhang · L.-Q. Cai · S.-X. Xu · X.-B. Zuo · H. Cheng · S. Yang (✉)
Department of Dermatology, Institute of Dermatology,
The First Affiliated Hospital, Anhui Medical University,
81 Meishan Road, 230032 Hefei, Anhui,
People's Republic of China
e-mail: ayzxj@vip.sina.com

Z. Zhang · K.-J. Zhu · Q. Xu · X.-J. Zhang · L.-D. Sun · H.-F. Zheng · J.-W. Han · C. Quan · S.-X. Xu · X.-B. Zuo · H. Cheng · S. Yang
The MOE Key Laboratory of Dermatology,
Hefei, Anhui, People's Republic of China

Introduction

Systemic lupus erythematosus (SLE) (OMIM 152700) is an autoimmune disease characterized by a striking preponderance in female, multisystem involvement, and autoantibodies directed primarily against nuclear antigens. Pathogenic mechanisms have been partly elucidated and defects in immune complex clearance, B-cell tolerance, and T-cell function have all been described [1]. SLE predominantly affects women (at a 9:1 ratio), particularly during childbearing years [2, 5]. Prevalence of the disease and severity of its manifestations vary among different populations. It affects a higher proportion of people of non European descent [2, 4, 19]. The prevalence of SLE ranges from 31 to 70 cases per 100,000 persons among Chinese [21].

Recent work on genome-wide association (GWA) studies has discovered more than ten new genes associated with SLE [3, 7, 8, 11, 18]. Hom et al. [11] first reported association of BLK with disease risk in SLE. The SNP rs13277113, located in the promoter region of BLK, was found to be related with reduced expression of BLK and increased expression of C8orf13 (OR = 1.39 $P = 1 \times 10^{-10}$). The association of BLK with SLE was confirmed by Harley et al. on SNP rs2248932 (OR = 1.22, $P = 7 \times 10^{-10}$), association on SNP rs13277113 was not directly examined [9]. SNP rs2248932 was located in the first intron of BLK, 43 kb downstream of rs13277113. These two SNPs were also replicated in Hong Kong Chinese [21].

They have moderate LD to each other ($r^2 = 0.57$ in CHB), but the association of rs2248932 in Hong Kong Chinese was not very significant (OR = 0.84 on the minor allele, $P = 0.034$). Here, we tested the association of rs2248932 in larger samples to replicate the association of this SNP between SLE and Chinese Han population. Our results revealed both similarities and differences compared to the findings from population of European origin, and Hong Kong Chinese living in mainland of China.

Materials and methods

Subjects

Most of our SLE patients and healthy controls were from middle area of China. All subjects were of self-reported Chinese Han, including 1,396 cases (1,279 women and 117 men) with the mean age of 34.79 years. SLE in patients was diagnosed according to the criteria of the American College of Rheumatology (ACR) [10]. The clinical diagnosis of all subjects was confirmed by at least two dermatologists. Additional clinical information was collected from the subjects through a full clinical checkup. Controls were healthy individuals without SLE, autoimmune and systemic disorders, and family history of SLE (including first-, second- and third-degree relatives), including 4,362 controls (2,094 women and 2,268 men) with a mean age of 34.44 years.

Genotyping

Genomic DNA was extracted using Flexi Gene DNA kits (QIAGEN, Germany). All cases and controls were genotyped for SNP using the Sequenom MassArray system (Sequenom iPLEX assay) at The MOE Key Laboratory of Dermatology, Hefei, Anhui, China according to manufacturer's instructions. Approximately 15 ng of genomic DNA was used to genotype each sample. Locus-specific polymerase chain reaction (PCR) and detection primers were designed using the MassARRAY Assay Design 3.0 software (Sequenom). The sample DNAs were amplified by multiplex PCR reaction and the PCR products were then used for locus-specific single-base extension reaction. The resulting products were desalted and transferred to a 384-element SpectroCHIP array. Allele detection was performed using MALDI-TOF MS. The mass spectrograms were analyzed by the MassARRAY TYPER software (Sequenom). To evaluate the quality of the genotype data, 117 samples (64 cases and 53 controls) in this study were also genotyped by Illumina Human610-Quad BeadChips, which were also included in our previous GWAS [8]. The concordance rate between the genotypes from two platforms was 99.15%. In addition, we have a repeat sample as

the array-inside quality control and a repeat sample between the SpectroCHIP as CHIP-between quality control in each 384-element SpectroCHIP array.

Statistical analysis

The SNP was analyzed for an association with the disease by means of comparison of the minor allele frequency in patients and controls using PLINK [16], and it was tested for significant deviation from Hardy–Weinberg equilibrium in controls and passed the test with P values >0.05. In addition to the allelic test of association, the additive genetic model was calculated (dominant, additive or negative).

Association with subphenotype was analyzed by comparing cases with a certain subphenotype with controls, cases without the subphenotype as controls. We also performed case-only analyses to examine the risk that is conferred by this SNP on subtypes of SLE. P value, OR, and 95% CI were determined for each subphenotype.

Real-Time PCR analysis the mRNA

To analyze the mRNA of the BLK, we isolated the PBMC from the fresh blood of the controls and cases. Then we extracted total cellular RNA by using Trizol purchased from Invitrogen. After quantification, 1 μg of total cellular RNA was used to conduct reverse transcription with a Promega RT kit (A3800) and an oligo (dT) primer. The PCR was completed in a 50-μl reaction system containing 200 nM of primers (sequences were described in Table 1), 120 nM of TaqMan probe, and TaqMan Universal PCR Master Mix from Roche (NJ, USA). Samples were amplified in the Applied Biosystem 7500 Real-Time PCR System for 40 cycles with the following conditions: denaturation for 15 s at 95°C, and annealing and extension for 40 s at 60°C. The IL-15Rα was detected by PCR as follows: denaturation for 40 s at 95°C, annealing for 40 s at 55°C, and extension for 40 s at 72°C for 32 cycles. All primers and probes were purchased from Takara Company (DaLian, China).

BLK Primers

Forward 5′AGAGGATGCCTGCTGGATTTC
Reverse 5′TGGAATTCATGCGCTCAATG

Results

Association of SLE with SNP rs2248932

We genotyped rs2248932 in BLK in our samples and found highly significant association of this SNP ($P = 1.41 \times 10^{-8}$,

Table 1 Distribution of alleles and genotypes for the rs2248932 C/T polymorphism in systemic lupus erythematosus patients and in controls

	Controls ($n = 4{,}362$)	Case ($n = 1{,}396$)	OR (95% CI)	P value
Allele				
T	6,495 (74.4%)	2,227 (79.8%)		
C	2,229 (25.6%)	565 (20.2%)	0.74 (0.66–0.82)	*1.41×10^{-8}
Genotype				
TT	2,412 (55.3%)	894 (64.0%)	Reference	
CT	1,671 (38.3)	439 (31.5%)	0.71(0.62–0.81)	
CC	279 (6.4%)	63 (4.5%)	0.61(0.46–0.81)	#4.64×10^{-8}
Recessive model				
C/C (vs. C/T + T/T)	**279 (6.4%)**	**63 (4.5%)**	**0.69 (0.52–0.91)**	**0.010**
Dominant model				
C/C + C/T (vs. T/T)	1,950 (44.1%)	502 (36%)	0.69 (0.61–0.79)	8.88×10^{-9}

Bold values indicate the result of dominate model

OR Odds ratio, *95% CI* 95% confidence intervals

* Patients vs controls using a 2×2 contingency table

\# Patients vs controls using a 3×2 contingency table

OR = 0.74, [95% CI: 0.66–0.82]) with SLE. Full genotype and allele data were shown in Table 1. Risk allele T was the major allele in Chinese Han population. In genotypic analysis, the association of rs2248932 (allele C) was compatible with a dominant model ($P = 8.88 \times 10^{-9}$).

To clarify the relationship of two SNPs rs2248932 and rs13277113, we extracted genotype data from our previous GWAS, including 1,047 cases and 1,205 controls [8].They are all from the same geographic setting. These two SNPs have moderate LD ($r^2 = 0.53$). By logistic regression controlling, the effect of rs2248932, the P value of rs13277113 did not become significant. ($P = 1.06 \times 10^{-3}$, but the P value of the SNP in GWAS stage of the study was 3.07×10^{-7}).

Subphenotype-control analysis

The counts and percentages for each of the 11 ACR classification criteria for SLE were listed in Table 2. Most SLE patients were associated with high level of ANA (97%); only about 6% patients associated with serositis and neurologic disorder.

The high heterogeneity of SLE manifestations may give clues on different pathogenesis of the disease. Here we analyzed rs2248932 in BLK in Chinese Han SLE patients. We performed analyses stratified according to the 11 ACR criteria. Table 3 illustrated a spectrum of subphenotypes of SLE and the analysis of subphenotype stratification. We observed that the P values of arthritis, anti-dsDNA autoantibodies, hematologic disorder, malar rash, and ANA were higher in SLE (+) patients. However, case-only analysis in our study did not produce any significant difference.

Table 2 Count and frequency of clinical characteristics in SLE

Subphenotypes	SLE (+) (%)	SLE (−) (%)
Anti-nuclear autoABs	1,348 (97)	48 (3)
Arthritis	871 (62)	525 (38)
Photosensitivity	752 (54)	644 (46)
Sm-autoantibodies	496 (36)	900 (64)
Anti-dsDNA autoantibodies	759 (54)	637 (46)
Hematologic disorder	1,017 (73)	379 (27)
Malar rash	870 (62)	526 (38)
Oral ulcers	414 (30)	982 (70)
Serositis	89 (6)	1,307 (94)
Renal disorder	690 (49)	706 (51)
Neurologic disorder	80 (6)	1,316 (94)
Discoid rash	204 (15)	1,192 (85)

Real-time PCR analysis

In order to detect whether the variant of the SNP rs2248932 was associated with the BLK expression, we determined the relative mRNA expression of this gene by quantitative real-time RT-PCR on total RNA purified from human PBMCs. The results in Fig. 1 showed the risk C allele of rs2248932 was associated with the lower levels of messenger RNA expression of BLK, Homozygotes for the C allele had an expression level that was approximately 30% of that of homozygotes for the T allele, and C/T heterozygotes had intermediate levels.

Discussion

In this study we verified SNP rs2248932 in BLK associated with SLE. We also found some differences between Euro-

Table 3 Association of rs2248932 with SLE analyzed by subphenotype stratification

Subphenotype	Comparison	OR(95% CI) P value
Neurologic disorder	SLE (+) versus Control	0.59, (0.42–0.84), 2.72×10^{-3}
	SLE (−) versus Control	0.75, (0.68–0.84), 1.39×10^{-7}
	SLE (+) versus SLE (−)	0.70, (0.45–1.10), 0.1198
Arthritis	SLE (+) versus Control	0.75, (0.66–0.85)7, 0.18×10^{-6}
	SLE (−) versus Control	0.72, (0.61–0.84), 3.47×10^{-5}
	SLE (+) versus SLE (−)	1.05, (0.87–1.28), 0.6142
Photosensitivity	SLE (+) versus Control	0.80, (0.70–0.91), 6.84×10^{-4}
	SLE (−) versus Control	0.68, (0.58–0.78), 1.07×10^{-7}
	SLE (+) versus SLE (−)	1.20, (0.9872–1.441), 0.06762
Anti-sm antibody	SLE (+) versus Control	0.74, (0.66–0.83), 7.56×10^{-7}
	SLE (−) versus Control	0.74, (0.66–0.83), 2.51×10^{-7}
	SLE (+) versus SLE (−)	0.97, (0.74–1.28), 0.8244
Anti-dsDNA autoantibodies	SLE (+) versus Control	0.74, (0.66–0.83), 2.21×10^{-7}
	SLE (−) versus Control	0.75, (0.66–0.84), 1.54×10^{-6}
	SLE (+) versus SLE (−)	0.96, (0.74–1.24), 0.7471
Hematologic disorder	SLE (+) versus Control	0.77, (0.68–0.86), 8.07×10^{-6}
	SLE (−) versus Control	0.67, (0.56–0.81), 1.81×10^{-5}
	SLE (+) versus SLE (−)	1.16, (0.94–1.45), 0.1727
Malar rash	SLE (+) versus Control	0.75, (0.66–0.85), 7.15×10^{-6}
	SLE (−) versus Control	0.72, (0.62–0.84), 3.54×10^{-5}
	SLE (+) versus SLE (−)	1.05, (0.86–1.270), 0.6543
Oral ulcers	SLE (+) versus Control	0.76, (0.64–0.90), 1.76×10^{-3}
	SLE (−) versus Control	0.72, (0.65–0.82), 2.25×10^{-7}
	SLE (+) versus SLE (−)	1.05, (0.86–1.29), 0.6341
Serositis	SLE (+) versus Control	0.77, (0.57–1.04), 9.10×10^{-2}
	SLE (−) versus Control	0.74, (0.66–0.82), 2.00×10^{-8}
	SLE (+) versus SLE (−)	1.05, (0.72–1.53), 0.7862
Renal disorder	SLE (+) versus Control	0.76, (0.66–0.87), 9.13×10^{-5}
	SLE (−) versus Control	0.72, (0.62–0.82), 1.92×10^{-6}
	SLE (+) versus SLE (−)	1.07, (0.89–1.29), 0.4953
ANA	SLE (+) versus Control	0.73, (0.66–0.81), 6.76×10^{-9}
	SLE (−) versus Control	0.97, (0.70–1.35), 0.8613
	SLE (+) versus SLE (−)	0.69, (0.40–1.20), 0.18
Discoid rash	SLE (+) versus Control	0.74, (0.59–0.93), 9.76×10^{-3}
	SLE (−) versus Control	0.74, (0.66–0.83), 8.42×10^{-8}
	SLE (+) versus SLE (−)	1.00, (0.77–1.30), 0.9887

SLE (+), patients positive for a certain phenotype

SLE (−), patients negative for a certain phenotype

pean and Asian populations, even differences between east and middle area of China.

Risk allele T was the major allele in the Chinese Han population; its allele frequency was 74.4% compared with 39.3% in Caucasian. The minor allele frequency (MAF) of SNP rs2248932 in Chinese Han populations living in mainland and Hong Kong were 25.6 and 26.5% in controls, 23.2 and 20.2% in cases, respectively. Although the *P* value of association with SLE were 1.41×10^{-8} in patients living in mainland and 0.034 in patients living in HongKong, they remained broadly consistent with each other, but our result was more significant. We speculated that there were several

reasons. First, the MAF of SNP rs2248932 was different in the two groups of cases. As we have known, SLE has very variable clinical subphenotypes; the high heterogeneity of manifestations may give clues on different pathogenesis and genetic basis of the disease [12]. The reason for difference may be due to that cases of HongKong have different components of subphenotypes from ours. When comparing subphenotypes and prevalence around China in different geographic groups, apparent differences between Chinese Han living in mainland and Hong Kong [14, 21] were found. Second, studies use different methods of ascertainment, cases from different medical records or distinct
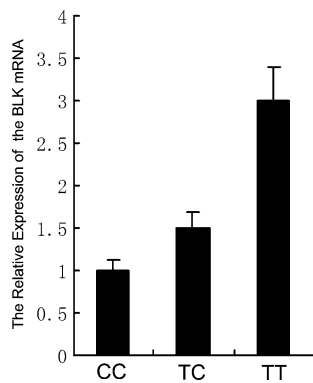
Fig. 1 Correlation of Variant in BLK gene with Expression Level in PBMC Relative mRNA expression of the BLK, as determined by quantitative real-time RT-PCR on total RNA purified from human PB-MCs of healthy controls and cases. Data represent mean ± SD. We analyzed 18 individuals with TT, 15 with TC, and 5 with CC. BLK expression correlated with genotypes of rs2248932. TT versus CC, $P = 0.0004$ (Student's t test)

environmental factors might also lead to the difference. In conclusion, genetic associations often vary with different population groups, even in the same ethnicity of Chinese Han, and hence future SLE genetic studies will require strong worldwide collaborations.

We use our previous GWAS of SLE to clarify the relationship of SNPs (rs2248932 and rs13277113). Risk allele T of rs13277113 showed an increased frequency in cases compared to controls, but after exclusion of rs2248932 the trend was not significant. This result seems too inconsistent with previous report [20], thus reinforcing the role of BLK in SLE and supporting the robustness of the study.

In Chinese, the association of SNP(rs2248932 allele C) was compatible with a dominant model, and the recessive model of the risk allele C did not reach statistical significance ($P = 0.010$). Compared with T/T, the genotype of homozygote C/C ($OR_{Hom} = 0.61$) had relatively similar OR with the heterozygote C/T ($OR_{Het} = 0.71$). This result may provide more information for following study of BLK.

BLK is a src family tyrosine kinase involved in signal transduction downstream of B-cell receptor. Expression of BLK is highly restricted to the B-cell lineage [6]. The function of BLK in human was not clear. It has been hypothesized that BLK is one of the tyrosine kinases that transduces signals downstream of the B-cell receptor. Hom et al. [11] reported that altered protein levels of BLK might influence tolerance mechanisms in B cells, predisposing persons to systemic autoimmunity, and it has a redundant role in mice (given the lack of a phenotype in blk-deficient mice).

There were several successes in the study of genotype-phenotype associations in SLE and other autoimmune diseases. For example, PTPN22 has been shown to be associated with anti-cyclic citrullinated peptide (anti-CCP) [15]

and rheumatoid factor (RF) [13] autoantibody positive RA. PDCD1 has been shown to be associated with lupus nephritis and anti-phospholipid antibodies [18]; STAT gene has been shown to be associated with nephritis and with the production of autoantibodies to double-stranded DNA [17].

However, Stratified analysis on genotype-phenotype associations of rs2248932 in BLK with SLE in our study did not show significant differences for this locus with any subphenotypes, which suggested that BLK might not be associated with a certain phenotype of SLE. The possible explanation for BLK gene's lack of association with any subphenotype of SLE might be that it generally influenced the tolerance mechanisms in B cells, but not for a specific phenotype of disease. The limitation of our research is the difficulty in obtaining accurate phenotype data; some of the phenotypes are related to disease activity, and may fluctuate naturally or as a result of treatment.

Some studies have confirmed that BLK was associated with SLE, and the risk allele A of SNP rs13277113 was associated with the lower expression of BLK [11]. Our results showed that the risk C allele of rs2248932 was associated with the lower levels of messenger RNA expression of BLK, which suggested that SNP rs2248932 influenced the expression of the BLK. BLK mediates tolerance in B cells and the altered protein levels of BLK may result in the SLE. Meanwhile, we should further explore how the BLK acts in the processing of SLE development.

In summary, our study evaluated SNP rs2248932 from BLK and further defined its role in SLE risk. Its association with SLE was confirmed in Chinese Han population. Exploring the relationship of genetic background of different populations could help us improve our overall understanding the pathogenesis of this disease. Of course, further studies are needed to confirm or dissect the genuine functional variants and to understand how these polymorphisms affect SLE.

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