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Functional variants of *TNFAIP3* are associated with systemic lupus erythematosus in a cohort of Chinese Han population

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ABSTRACT

Tumor necrosis factor-alpha-induced protein 3 (*TNFAIP3*) is a negative regulator of *NF-κB* activity. We previously reported that the paired tandem polymorphic dinucleotides TT > A (rs148314165, rs200820567 of *TNFAIP3*) conferred the risk for systemic lupus erythematosus (SLE) in European and Korean populations. We investigated the genetic association of the TT > A variants, as well as the functional coding variant rs2230926 in exon 3 of *TNFAIP3* in 1229 Chinese Han SLE patients and 1608 matched population controls. We further evaluated the role of these variants in regulating expression of the *TNFAIP3* gene and *NF-κB* signaling pathway in their peripheral blood mononuclear cells from Chinese SLE patients. The TT > A variants and the *TNFAIP3* exon 3 coding variant rs2230926 demonstrated significant associations in SLE ($P_{TT>A} = 8.96 \times 10^{-12}$, odds ratio [OR] = 2.07, 95% confidence interval [CI] = 1.68–2.55). SLE patients carrying the risk A allele showed reduced messenger RNA expression of the *TNFAIP3* gene and increased expression of *NF-κB1* in PBMCs. Conditional analyses revealed that the TT > A variants are likely to be causal variants in Chinese Han SLE patients. The TT > A variants associated with Chinese Han SLE and negatively regulate the expression of the *TNFAIP3* gene resulting in enhanced *NF-κB* activity.

1. Introduction

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease that encompasses a diverse array of clinical manifestations, including organ-specific autoantibody production, immune complex-mediated inflammation of target organs, and dysregulated activity of the interferon pathway [1–3]. SLE is a non-curative disease that poses a growing challenge for healthcare [4], affecting an increasing number of people in China with a prevalence of 0.03%. SLE patients are regularly treated with broad-spectrum immunosuppressive agents, yet the adverse effects of these agents contribute to the considerable morbidity and accumulating mortality of the disease [2,5–8]. Therefore, a better genetic understanding of the pathogenesis of SLE will significantly advance the prognosis, diagnosis, and therapy of this disease in the future [9,10].

Tumor necrosis factor alpha-induced protein 3 (*TNFAIP3*) encodes A20, a ubiquitin-editing enzyme critical for negatively regulating the *NF-κB* signaling pathway in cells [3,6,11]. Multiple genetic studies have shown a consistent association between the variants of *TNFAIP3* and susceptibility of SLE. The most commonly replicated association in both European and Korean populations is a ~100 kb risk haplotype that spans the *TNFAIP3* locus [5,12,13]. Through targeted resequencing of European and Korean SLE patients enriched for this risk haplotype, a pair of adjacent polymorphic dinucleotides (rs148314165, rs200820567, collectively referred to as TT > A) were identified and confirmed to have the most significant association among the variants carried on the risk haplotype [5,12]. In previous studies, we mechanistically characterized the functional TT > A polymorphic dinucleotide in regulating the expression of *TNFAIP3* in cell lines derived from European SLE patients [2,12,14]. The TT > A variants bind to a

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nuclear protein complex containing the *NF-κB* and SATB1 transcription factors, which enables the physical interaction of the TT > A variants with the *TNFAIP3* promoter through long-range DNA looping [12]. While accumulating evidence has shown that the TT > A variants are associated with SLE in European and Korean populations, the existence of these variants in Chinese Han patients with SLE, as well as their role in susceptibility, remain largely elusive.

In addition to the TT > A functional variants, the same risk haplotype carries a coding variant, rs2230926 in exon 3, resulting in a substitution of phenylalanine to cysteine in the TNFAIP3 protein [15–17]. While the molecular mechanism underlying which coding variant influences *NF-κB* signaling is unknown, an *in vitro* assay has shown that the risk allele G of the coding variant is associated with decreased *NF-κB* signaling activity in comparison to the non-risk T allele [15,18]. Interestingly, no significant association of the haplotype carrying the coding variant was observed in African American SLE patients [5]. However, the TT > A variants were not present on the African American haplotype [5,16]. These data suggest that the coding variant influences the function of A20, but it is unlikely that the predominant causal variant impacts the risk of SLE [5,18]. Of the two SLE-related functional variants at the *TNFAIP3* locus, the coding variant rs2230926 has been associated with SLE in Chinese Han population, while the TT > A variants have not been investigated [17,19,20]. Additionally, the linkage disequilibrium (LD) between the two functional variants in Chinese Han patients remains unclear.

In this study, we assessed the genetic association of the two functional variants (TT > A and rs2230926) at the *TNFAIP3* locus in Chinese Han SLE patients. In addition, we further evaluated if TT > A variants influence the expression levels of the *TNFAIP3* gene in peripheral blood mononuclear cells (PBMCs) from Chinese Han SLE patients.

2. Material and methods

2.1. Patients and sample collection

The cohort of Chinese participants in this study consisted of 1229 SLE patients and 1608 healthy adult controls. All participants were recruited between March 2005 and August 2017 from The First Hospital of Jilin University (Changchun, Jilin Province, China), and all individuals self-reported to be of Chinese Han descent. All SLE patients were diagnosed according to the American College of Rheumatology (ACR) criteria established in 1997 and were randomly enrolled. There were no gender and age restrictions in the SLE or healthy control groups (Supplementary Table 2). Peripheral blood was collected to isolate the PBMCs for gene expression analyses. The protocol was approved by the Ethics Committee of the First Hospital of Jilin University and written informed consent was obtained from all participants.

2.2. DNA isolation and single-nucleotide polymorphisms (SNP) genotyping

Genomic DNA was isolated from the PBMCs using a DNA extraction kit (Zhiang Biotech, Changchun, China), according to the manufacturer's instructions. Sequences of the PCR primers are listed in Supplementary Table 3. The genotype at rs7749323 for each genomic DNA sample with PCR amplified target regions was determined using the TaqMan SNP genotyping assay kit (ThermoFisher Scientific Inc., Waltham, MA, USA). The assay followed the protocol in the manual, and PCR reactions were conducted on an OpenArray™ real-time PCR instrument (Applied Biosystems™, Foster City, CA, USA).

2.3. RNA isolation and quantitative RT-PCR

Total RNA from PBMCs was isolated using TRIzol (Invitrogen Inc., Carlsbad, CA, USA), per the manufacturer's instructions. RNA concentrations were measured using the NanoDrop (ThermoFisher Scientific Inc.). A total of 500 ng of total RNA from each patient was reverse transcribed using iScript cDNA Synthesis Kits (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in a final volume of 25 ul/reaction. Quantitative RT-PCR assays were performed using the Power SYBR Green Master Mixture (Applied Biosystems, Foster City, CA, USA) to determine the mRNA expression of *TNFAIP3* and *NF-κB1*, with the *GAPDH* gene being used as the internal control. The quantitative PCR reaction contained 10ul of 2X SYBR Green Master Mixture, 30 nM Forward and reverse primer, and 40 ng cDNA in 20ul volume. The quantitative PCR conditions were as follows: 95 °C for 10 min, (95 °C for 15 s, and 60 °C for 1 min) for 40 cycles. The sequences of PCR primers are listed in Supplementary Table 4. Fold changes were calculated according to the ÄÄCT method.

2.4. Statistical analysis

The associations of the functional variants in Chinese Han SLE patients were performed using a logistic regression in Plink, version 1.09. None of the variants failed a Hardy Weinberg test for genotypes proportions in controls using a p-value threshold of 0.01. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using logistic regression model. The comparisons of the transcript levels of *TNFAIP3* and *NF-κB1* in PBMCs between genotypes were performed using the one-way analysis of variance (ANOVA). A Brown – Forsythe test was performed for the equality of group variance. *P*-values less than 0.0025 were considered statistically significant.

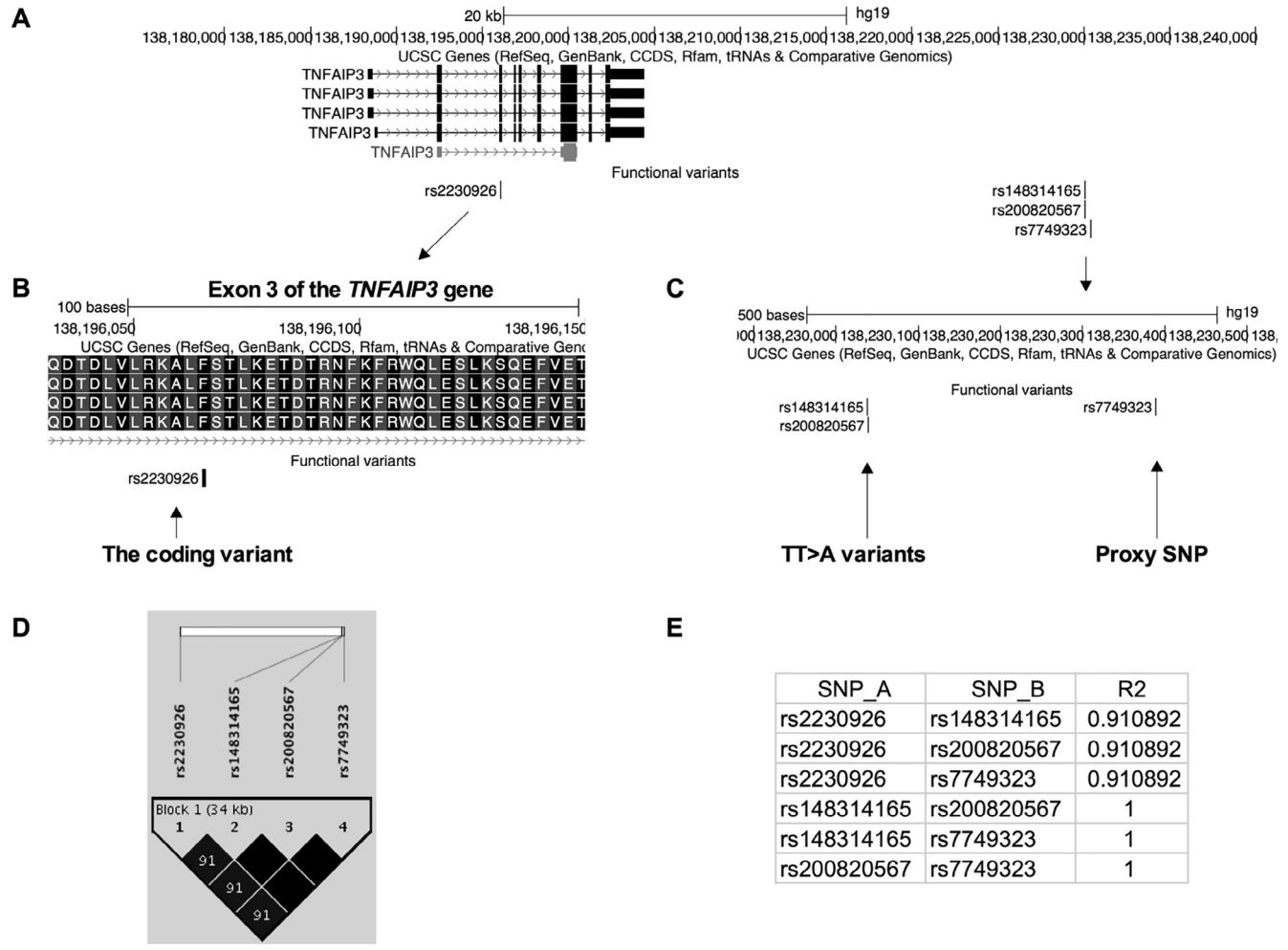
3. Results

3.1. rs7749323 is an excellent proxy SNP for genotyping the functional TT > A variants in Chinese Han ancestry

The TT > A variants represent a deletion of T followed by a T to A transversion at chromosome 6 positions 138,272,732–138,271,733, 42 kb downstream of the *TNFAIP3* gene. The SNP rs7749323, which is located 349 bp downstream of the TT > A variants, has been used as a perfect proxy SNP to identify the TT > A variants in people of European and Korean descent [5,12,14]. To evaluate the existence of the TT > A variants and to ensure that the rs7749323 is in complete LD with the TT > A variants, we resequenced the 800 bp DNA fragment (centered on the TT > A variants) in 100 healthy controls and 100 Chinese SLE patients. As shown in Fig. 1 and Supplementary Table 1, the TT > A variants are in complete LD with the proxy SNP rs7749323 with an r^2 value of 1. The minor allele frequencies of the TT > A variants were 0.05 in the healthy controls and 0.11 in the Chinese Han SLE patients in the sequenced samples. These data demonstrate the existence of the TT > A variants in Chinese Han individuals and suggest that rs7749323 is a perfect proxy SNP of the TT > A variants.

3.2. Coding variant rs2230926 and the distal variant rs7749323 are associated with SLE in the Chinese Han population

The demographics of the 1229 SLE patients and 1608 matched healthy controls are shown in Supplementary Table 2. There were no significant differences in the mean age or gender distribution between the SLE cases and healthy control subjects. We genotyped the coding variant rs2230926 and the proxy SNP rs7749323 in the SLE cases and matched healthy controls. As shown in Table 1, single-marker association analyses using logistic regression with additive model suggested that both variants demonstrated significant associations in Chinese Han patients with SLE (rs2230926, $P = 1.60 \times 10^{-11}$, OR = 1.91 and rs7749323 [in complete LD with TT > A], $P = 8.96 \times 10^{-12}$, OR = 2.07). We also assessed the associations using logistic regression with dominant and recessive models (Supplementary Table 5). Interestingly, we observed a stronger association between the TT > A



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SNP_A	SNP_B	R2
rs2230926	rs148314165	0.910892
rs2230926	rs200820567	0.910892
rs2230926	rs7749323	0.910892
rs148314165	rs200820567	1
rs148314165	rs7749323	1
rs200820567	rs7749323	1

Fig. 1. Relative locations of functional variants of the *TNFAIP3* gene in the chromosome 6. A. Relative locations of the *TNFAIP3* gene, exon 3 variant rs2230926, TT > A variants, and the proxy SNP rs7749323 for TT > A variants. B and C. Zoom in view of functional variants as indicated in the figure. D. LD structure analyses of the four variants. E. List of r^2 between variants.

variants and the susceptibility of SLE in our cohort.

3.3. Conditional analyses suggest a causative role of the TT > A variants in Chinese Han SLE patients

The coding variant rs2230926 has demonstrated significant associations with the susceptibility of SLE in the European and Korean populations [5]. Conditioning on the TT > A variants or its proxy SNP rs7749323 ($r^2 = 1.0$), for the Chinese Han population, reduced the association signal to the baseline ($P = 0.6475$), as shown in Table 2. However, conditioning on the coding variant, rs2230926, revealed the residual association in the TT > A variants and the proxy SNP

Table 2
Conditional association analysis for the exon 3 variant rs2230926 and the TT > A variants in Chinese Han SLE.

Chr	Variant	Pos (hg38)	A1	A2	$P_{con\_rs2230926}$	$P_{con\_TT>A}$
6	rs2230926	137874929	T	G	N/A	0.6475
6	rs148314165	137908901	GT	G	0.01349	N/A
6	rs200820567	137908903	T	A	0.01349	N/A
6	rs7749323	137909252	G	A	0.01349	N/A

Chr., chromosome. Pos., chromosome position.
A1, major allele. A2, minor allele. $P_{con\_rs2230926}$, Conditional association analysis for SNP rs2230926. $P_{TT>A}$, Conditional association analysis for SNP rs2230926.

Table 1
Functional genetic variants at the *TNFAIP3* locus associated with SLE in Chinese Han.

Chr	Variant	Pos (hg38)	A1	A2	MAF		OR (95% CI)	P
					Cases	Controls		
6	rs2230926	137874929	T	G	0.10291	0.05597	1.912 (1.567–2.332)	1.60E−10
6	rs148314165	137908901	GT	G	0.09764	0.04975	2.072 (1.681–2.554)	8.96E−12
6	rs200820567	137908903	T	A	0.09764	0.04975	2.072 (1.681–2.554)	8.96E−12
6	rs7749323	137909252	G	A	0.09764	0.04975	2.072 (1.681–2.554)	8.96E−12

Chr., chromosome. Pos., chromosome position.
A1, major allele. A2, minor allele. MAF, minor allele frequency. The odds ratio (OR) was calculated with respect to the minor allele. P values were Adjusted for sex and age.

($P = 0.01349$) (Table 2). Furthermore, we performed a haplotype analysis and revealed a risk haplotype (haplotype 2) carrying both risk alleles of these variants is significantly associated with SLE in Chinese Han. Interestingly, a haplotype carrying only the risk allele of the rs2230926 coding variant exhibited no significant association. Because the frequency of the haplotype 3 was significantly lower than the haplotype 2, further association study is required. These results suggested that the TT > A variants are likely to be causal variants for SLE in our studied cohort of Chinese Han patients.

3.4. Risk allele of the TT > A variants is correlated with reduced expression of TNFAIP3 mRNA and increased expression NF-κB1 mRNA

The risk allele of the TT > A polymorphic dinucleotide reduces messenger RNA (mRNA) expression of *TNFAIP3* in European SLE patients [5,11,12]. Therefore, we assessed the role of the TT > A variants in regulating the expression of the *TNFAIP3* gene in Chinese Han patients with SLE. We isolated PBMCs from 64 SLE patients with different genotypes at the TT > A variants (47 TT/TT, 15 TT/A, and 2 A/A) and determined the transcript levels of the *TNFAIP3* gene using RT-PCR assays. In line with other published studies [2,12,14], individuals carrying the risk A allele displayed a significant reduction in the levels of *TNFAIP3* mRNA expression (Fig. 2A). The *TNFAIP3* gene encodes protein A20, which is a negative regulator of *NF-κB* signaling. Therefore, we further assessed the expression of *NF-κB1* mRNA in the PBMC samples with different genotypes at the TT > A variants. As expected, PBMCs from individuals carrying the risk A allele had a significant increase in *NF-κB1* mRNA expression, compared to that of the G allele carriers (Fig. 2B). Because the coding variant is in strong LD with the TT > A variants, we also assessed correlation between the rs2230926 variant and mRNA expression of *TNFAIP3* and *NF-κB1*. A similar correlation was also observed (Supplementary Fig. 1).

4. Discussion

The precise pathogenesis of SLE remains to be elucidated. Genome-wide association studies and candidate gene scans have identified multiple genetic variants at the *TNFAIP3* locus that are consistently associated with SLE in various populations [5,12,13,21,22]. Two well-documented SLE-associated functional variants, the TT > A variants and the coding variant rs2230926, were shown to influence the expression of the *TNFAIP3* gene and enzymatic activity of the encoded-protein A20. Both variants were associated with reduced inhibition of *NF-κB* signaling [3,5,6,18,23].

It has been reported that the two variants are in LD with an r^2 values between 0.8 and 1.0 in different populations [24,25]. The coding variant rs2230926 has demonstrated significant associations with SLE in European, African, and Asian populations, including the Chinese Han population [5,16,19,20]. TT > A variants were also shown to influence the risk for SLE in both European and Korean populations [26]. However, a previous large-scale genotyping study in African individuals suggested that the TT > A variants are only present in African American samples with an overall allele frequency of 0.53% (n = 2,252), which is likely due to European admixture [5,11]. To evaluate whether the TT > A variants are present in the Chinese Han chromosome, we re-sequenced ~800 bp covering the TT > A region of 100 SLE patients and 100 population-matched healthy controls. The results showed that the TT > A variants existed in the studied Chinese Han population with an allele frequency of 4% in the controls and 9% in the SLE cases. Additionally, the sequencing data also demonstrated that the proxy SNP rs7749323 of the TT > A variants in both European and Korean populations is also a perfect proxy SNP for the TT > A variants in Chinese Han individuals.

While performing an association study in 1229 Chinese Han SLE cases and 1608 healthy controls, we observed significant associations of the coding variant rs2230926 in exon 3 of the *TNFAIP3* gene and the TT > A polymorphic dinucleotide with SLE susceptibility in the Chinese Han population. We also assessed associations of these *TNFAIP3* variants in Lupus nephritis (LN) in 208 SLE patients having clinical data on LN status (102 LN and 106 SLE without LN). We observed significant associations of both functional variants in LN as well as SLE without LN individuals (Supplementary Table 6). Conditioning on the TT > A variants diminished association signals of other variants. However, further genetic study in different populations and/or functional studies using animal model are required to dissect the causal variant. A haplotype analysis revealed a single risk haplotype carrying risk alleles of both functional variants exhibited significant association with SLE. Interestingly, a haplotype with a frequency of 0.006 carrying the risk allele of the rs2230926 variant and non-risk allele of the TT > A variants showed no significant association with SLE in our cohort (Table 3). Considering the sample size of the study and low haplotype frequency, further association studies in large cohort of SLE are critically needed. Additionally, new statistical methods are emerging and that might also gain our understanding of role of these variants in influencing risk for SLE [27].

The *TNFAIP3* gene encodes the ubiquitin-modifying enzyme A20, a

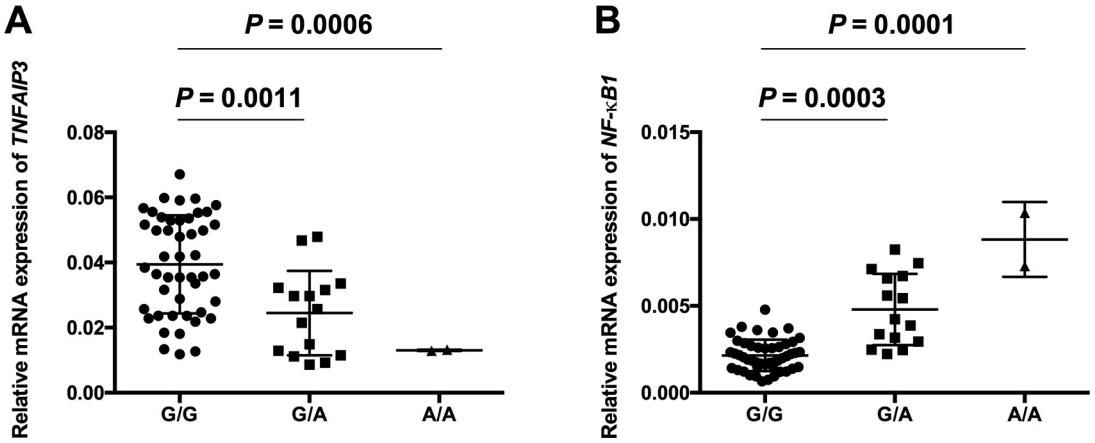

Fig. 2. The TT > A variants correlated with reduced mRNA expression of the *TNFAIP3* gene and increased mRNA expression of *NF-κB1*. The x-axis displays the three different genotypes for SNP rs7749323 corresponding to homozygote non-risk, heterozygote risk, and homozygote risk of the variant. The y-axis indicates the relative expression levels of *TNFAIP3* mRNA. Each dot represents the expression level of *TNFAIP3* (A) or *NF-κB1* (B) for one individual. All samples were stratified according to their genotype at the proxy SNP rs7749323 variant. *P*-values were calculated using the one-way analysis of variance (ANOVA) ($P_{TNFAIP3} = 0.0006$, $P_{NF\text{-}\kappa B1} = 0.0001$) or unpaired *t*-test when the comparison was made between G/G group and G/A group ($P_{TNFAIP3} = 0.0011$, $P_{NF\text{-}\kappa B1} = 0.0003$). n = 47 for G/G; n = 15 for G/A; n = 2 for A/A.

Table 3
Haplotype analysis revealed a single risk haplotype associated with SLE in Chinese Han.

	rs2230926	rs148314165	rs200820567	rs7749323	Freq			Chi Square	P value
					All	Case	Controls		
Haplotype 1	T	GT	T	G	0.924	0.897	0.944	43.705	3.82E−11
Haplotype 2	G	G-	A	A	0.070	0.097	0.050	48.168	3.91E−12
Haplotype 3	G	GT	T	G	0.006	0.006	0.006	0.063	0.802

Freq., frequencies of haplotypes.

potent anti-inflammatory molecule that suppresses *NF-κB* signaling activity [6]. Genetic association and functional genomic studies have linked polymorphisms and mutations in the *TNFAIP3* gene to SLE susceptibility [5,8,10,22]. We previously studied the correlation of the TT > A variants with expression of the *TNFAIP3* gene and characterized the molecular mechanisms underlying which the TT > A variants may influence the expression of *TNFAIP3 in vitro* [12,14]. Since the TT > A variants are present in the Chinese Han SLE patients, we further assessed the role of the risk variants in regulating expression of the *TNFAIP3* gene using PBMC from the patients in our cohort. In line with previously published studies [2,12,14], the risk allele of the variants significantly reduced mRNA expression of the *TNFAIP3* gene, which enhanced *NF-κB* signaling. We further assessed the role of rs2230926 coding variant in regulating the mRNA expression of *TNFAIP3* and *NF-κB1*. Because the two variants are in strong LD, the result showed rs2230926 variant also influences gene expression of *TNFAIP3* and *NF-κB1* (Supplementary Fig. 1). To further validate the role of the variants in regulating *TNFAIP3* expression in lymphoblastoid cell lines, we assessed gene expression data and rs7749323 (a proxy variant for the TT > A variant) genotypes in Asian HapMap and the 1000 Genomes Project, respectively [28]. In line with other published studies [5], we observed that the normalized expression level of *TNFAIP3* was significantly associated with rs7749323 (Bonferroni-corrected $P = 0.0029$). Additionally, previous bioinformatic analysis and functional studies showed that the risk allele of rs2230926 results in a Phe127 – Cys127 change of the A20 protein that was less effective at inhibiting TNF-induced NF-κB activity [18]. Because the rs2230926 and the rs7749323 variants were in strong LD, further investigations are needed to uncover the role that TT > A variants in regulating *TNFAIP3* expression in various types of immune cells.

In this study, we demonstrated significant genetic associations of two reported functional variants with Chinese Han SLE and further investigated roles of the variants in modulating mRNA expression of *TNFAIP3* and *NF-κB1* in PBMCs from SLE patients. These data further suggested a role of genetic variants in *TNFAIP3* gene in influencing risk for SLE through a NF-kappa B-mediated signaling pathway.

5. Conclusions

In summary, we demonstrated that the TT > A variants and exon 3 coding rs2230926 variant of the *TNFAIP3* gene are present in our cohort of Chinese Han SLE patients. These minor alleles of the variants are significantly associated with the risk for SLE. The functional evaluation of *TNFAIP3* expression in the PBMCs from these Chinese Han patients further suggested a causative role of the TT > A variants in the pathogenesis of SLE.

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Author contributions

M.X,W. G., L. W., X. L., D. Y., X. S., Q. W. performed the genomic DNA isolation and SNP genotyping; M. X. and W. G. performed the functional studies; M. X. W. G, and J. Y. participated in the clinical samples and data collection; S. W. and X. L. wrote the manuscript, and all authors participated in the proofreading.

Competing financial interests

All authors listed in the manuscript declare no potential conflicts of interest.

In the last five years, we do not receive any special funding to support this work. All samples are collected from a non-profit research institute of the First Hospital of Jilin University. All authors are employed by the First Hospital of Jilin University or the Jilin University. All authors do not have potential conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2018.11.008.

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