Association of *TNFAIP3* polymorphism with susceptibility to systemic lupus erythematosus in a Japanese population.

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Abstract

Recent genome-wide association studies demonstrated association of single nucleotide polymorphisms (SNPs) in the *TNFAIP3* region at 6q23 with systemic lupus erythematosus (SLE) in European-American populations. In this study, we investigated whether SNPs in the *TNFAIP3* region are associated with SLE also in a Japanese population. A case-control association study was performed on the SNPs rs13192841, rs2230926 and rs6922466 in 318 Japanese SLE patients and 444 healthy controls. Association of rs2230926 G allele with SLE was replicated in Japanese (allelic association *P*=0.033, odds ratio [OR] 1.47, recessive model *P*=0.023, OR 8.52). The association was preferentially observed in the SLE patients with nephritis. When the *TNFAIP3* mRNA levels of the HapMap samples were examined using GENEVAR database, presence of *TNFAIP3* rs2230926 G allele was associated with lower mRNA expression of *TNFAIP3* (*P*=0.013). These results indicated that *TNFAIP3* is a susceptibility gene to SLE both in the Caucasian and Asian populations.

Introduction

Nuclear factor-κB (NF-κB) is a transcription factor that regulates expression of various genes including pro-inflammatory cytokines. *TNFAIP3* (tumor necrosis factor alpha-induced protein 3) gene codes for A20, a ubiquitin-modifying protein required to inhibit NF-κB activation by several signaling pathways including TNF and Toll-like receptors [1]. *TNFAIP3* deficient mice exhibited multiple organ inflammation and damage involving kidneys and joints [2]. NF-κB induces expression of A20; thus, A20 plays a role in a negative feedback mechanism in NF-κB pathway.

The lupus phenotypes in Fcγ receptor IIb deficient mice were reduced by treatment with NF-κB inhibitors [3], suggesting a role of NF-κB activation in the pathogenesis of systemic lupus erythematosus (SLE). A recent study reported that A20 negatively regulates immunostimulatory effects of dendritic cells and attenuates antigen presentation [4]. Thus, reduced function of A20 may induce excessive activation of dendritic cells and leads to the loss of tolerance to self-antigens.

Since a genome-wide association study (GWAS) conducted by the Wellcome Trust Case Control Consortium reported association of *TNFAIP3* region single nucleotide polymorphisms (SNPs) with rheumatoid arthritis (RA) and Crohn's disease in 2007 [5], association of SNPs in the region surrounding *TNFAIP3* at 6q23 has been reported in several autoimmune diseases. Association of the *TNFAIP3* region has subsequently been replicated in RA [6, 7], and was also reported in SLE by two groups using GWAS [8, 9]. These observations implicated that *TNFAIP3* may be a common susceptibility gene to multiple autoimmune diseases.

Specifically, Musone et al. reported independent association of three SNPs in the *TNFAIP3* region, rs13192841, rs2230926 and rs6922466, with SLE in a European-American population [8]. The SNP rs2230926 is located in the coding region of *TNFAIP3* and introduces amino acid substitution of Phe to Cys at position 127 in the ovarian tumor domain, which was suggested to participate in the inhibitory function of A20 through deubiquitination of adaptor proteins [1]. Cys127 allele product was modestly less effective in inhibiting NF-κB activation by TNF than Phe127 [8].

Recently, an extensive meta-analysis and imputation study by Bates et al. [10] demonstrated that the strongest association was detected at an intron 2 SNP rs5029939, which was originally detected by Graham et al. [9], and was in tight linkage disequilibrium (LD) with rs2230926. They also showed that the carrier of the risk allele was at higher risk of developing nephritis and hematological manifestations [10]. SNPs rs13192841 and rs6922466 were mapped within 250kb upstream and downstream of the *TNFAIP3* region, respectively.

Recently, we demonstrated that *IRF5* [11], *STAT4* [12] and *BLK* [13] are associated with SLE not only in the Caucasians, but also in the Japanese. In this study, we carried out a case-control association study of the SNPs in the *TNFAIP3* region to examine whether *TNFAIP3* is involved in genetic predisposition to SLE also in Japanese.

Patients and Methods

Patients and controls

A case-control association study was conducted in 318 Japanese patients with SLE (19 males and 299 females, mean ± SD age 41.4 ± 13.5 years) and 444 healthy controls (219 males and 225 females, mean ± SD age 34.0 ± 9.9 years), recruited at the University of Tsukuba, Juntendo University and the University of Tokyo. All patients and healthy individuals were native Japanese living in the central part of the Japan. All patients met the criteria for SLE proposed by the American College of Rheumatology [14].

In addition to the originally recruited controls, genotype data of 934 Japanese healthy controls were retrieved from the "JSNP550typed" dataset in the JSNP DATABASE (http://snp.ims.u-tokyo.ac.jp/jsnp_gtype_access.html) and analyzed [15].

This study was reviewed and approved by the research ethics committees of University of Tsukuba, Juntendo University and the University of Tokyo. Informed consent was provided by all donors.

DNA samples

Genomic DNA was extracted from peripheral blood leucocytes using QIAamp blood kit (QIAGEN, Hilden, Germany) and amplified using the GenomiPhi DNA Amplification Kit (Amersham Biosciences, Piscataway, NJ, USA), following the manufacturer's instructions. Whole genome amplification products were used for genotyping.

Genotyping

Genotyping of SNPs was carried out using the TaqMan genotyping assay (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. The TaqMan probes used in this study were as follows: Assay ID: C___1575524_10 (rs13192841), C___7701116_10 (rs2230926), and C__29431844_10 (rs6922466). Amplification was performed in a 10μl reaction volume containing 5μl of TaqMan Genotyping Master Mix and 0.125ul of TaqMan genotyping assay mix using 7300

Real-Time PCR System (Applied Biosystems). Thermal cycling conditions consisted of initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Genotyping of 934 controls in the "JSNP550typed" dataset was performed using the Illumina HumanHap550 BeadChip (Illumina, Inc., San Diego, CA), as indicated in the JSNP DATABASE.

Association of *TNFAIP3* SNP with *TNFAIP3* mRNA expression

Association of *TNFAIP3* rs2230926 genotype with *TNFAIP3* mRNA expression was examined using the mRNA data from lymphoblastoid cell lines derived from 44 JPT (the Japanese in Tokyo, Japan) and 45 CHB (the Han Chinese in Beijing, China) HapMap individuals available from the database of the Gene Expression Variation (GENEVAR) project at the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/humgen/genevar/), as previously described [11]. Genotypes of rs2230926 in HapMap samples were available from HapMap database (http://www.hapmap.org/index.html.en). Because there was no subject with G/G genotype, mRNA levels of *TNFAIP3* were compared between rs2230926 G/T (n=24) and T/T (n=65) subjects.

Statistical analysis

Association analyses were conducted by chi-square test using 2 x 2 contingency tables. When 1 or more of the variables in the contingency tables was 5 or less, Fisher's exact test was employed. Because this study intended to examine whether the association of *TNFAIP3* is replicated in Japanese, correction for multiple testing was not applied and the uncorrected *P* values are shown.

Based on the allele frequencies in Japanese, the sample size of this study (318 patients and 444 controls) had the power of 80% to detect association when the genotype relative risk is 1.55 (rs13192841), 1.65 (rs2230926) and 1.45 (rs6922466), respectively [16].

To examine LD between the SNPs in the *TNFAIP3* region, LD parameters (D' and r^2) were calculated using Haploview version 4.0 software (Broad Institute of MIT and Harvard, Cambridge, MA), based on data from the 444 healthy Japanese controls. Difference in the mRNA expression between the rs2230926 G/T and T/T subjects in the HapMap subjects was analyzed by unpaired Student's t-test.

Results and Discussion

To examine whether *TNFAIP3* is associated with SLE in a Japanese population, an association study of rs13192841, rs2230926 and rs6922466 was conducted in 318 Japanese patients with SLE and 444 healthy controls. As observed in Caucasians [8], LD was modest among the examined three SNPs in 444 healthy Japanese controls (r^2: 0.00-0.11, *D'*: 0.04-0.40). On the other hand, the intron 2 SNP rs5029939, which showed the strongest association in the study of Bates et al. [10], was in strong LD with rs2230926 also in Japanese (HapMap JPT: r^2=0.86, *D'*=1.0). None of the control genotypes were significantly deviated from Hardy-Weinberg equilibrium.

The association of rs2230926 with SLE was replicated in the Japanese, while evidence for association of rs13192841 and rs6922466 was not detected (Table 1). To confirm our findings, the patients' genotypes were also compared with the genotypes of 934 Japanese controls available from JSNP DATABASE. As shown in Table 1, rs2230926 G allele frequency was significantly increased in SLE patients compared with JSNP controls. On the other hand, association of rs13192841 and rs6922466 was not detected, even when the JSNP controls were used for comparison.

Because the minor allele frequency of rs6920220, previously shown to be associated both with RA and SLE in European-American populations [6, 7, 9], was only 0.006 in the HapMap JPT samples, association was not examined for this SNP.

We next tested whether rs2230926 in *TNFAIP3* was associated with the development of nephritis. Frequency of rs2230926 G allele was significantly increased in SLE with nephritis (12.2%) as compared with healthy controls (7.5%, *P*=0.010, OR 1.70, 95%CI 1.13-2.56), while significant difference was not observed between SLE without nephritis (8.6%) and healthy controls (Table 2). This finding is in agreement with Bates et al., who demonstrated preferential association of *TNFAIP3* with SLE patients with nephritis in Caucasians [10]. Bates et al. also reported association of *TNFAIP3* with hematological manifestations [10]. We did not carry out this analysis, however, because more than 90% of our patients fulfilled ACR classification criteria for hematologic disorder, especially lymphopenia (1500/μl).

A previous study suggested that the product of the risk allele, rs2230926 G (Cys127), may be modestly less efficient in inhibiting TNF-induced NF-κB activity [8]. To gain more insight into the functional significance of rs2230926, association of *TNFAIP3* mRNA levels with genotype was evaluated using the HapMap JPT and CHB subjects. Data on the mRNA levels in B cell lines from HapMap samples were obtained from GENEVAR database (http://www.sanger.ac.uk/humgen/genevar/). As shown in Figure 1, the levels of *TNFAIP3* mRNA was decreased in carriers of the rs2230926 G allele (G/T subjects) as compared with non-carriers (T/T) (P=0.013). This finding is in agreement with a previous study which demonstrated that *TNFAIP3* intronic SNP rs5029930, which is strong LD with rs2230926 G, was associated with lower expression of *TNFAIP3* mRNA expression in peripheral mononuclear cells [17]. Thus, our findings suggested that the molecular mechanisms of association of *TNFAIP3* haplotype containing rs2230926 G and rs5029939 G may involve both the functional change caused by the amino acid substitution at the protein level and the reduced expression level of A20 protein.

A GWAS of SLE in a Chinese Han population was very recently reported for the first time in non-Caucasian populations [18]. In the study, association of *TNFAIP3* rs2230926, but not rs13192841 and rs6922466, with SLE was demonstrated, which was consistent with our results in Japanese. Thus, taken together with previous observations, our study established *TNFAIP3* as a susceptibility gene to SLE common to Caucasian and Asian populations. Of particular interest, both the GWAS in Chinese [18] and a large-scale study in Caucasians [19] identified association of TNFAIP3 interacting protein 1 (*TNIP1*), which binds to A20 and is thought to inhibit NF-κB signaling. These findings underscore the crucial role of NF-κB regulation pathway in the genetic background of SLE in multiple populations.

Conclusions

The association of rs2230926 in the coding region of *TNFAIP3* with SLE was replicated in a Japanese population. Our finding demonstrated that *TNFAIP3* is a

susceptibility gene to SLE both in the Asian and Caucasian populations.

Acknowledgements

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Table. 1 Association of the *TNFAIP3* region SNPs with SLE in a Japanese population.

	Genotype n (%)			Allele n (%)	vs. controls (n=444)				vs. JSNP controls (n=934)			
					Allelic association		Recessive model[#]		Allelic association		Recessive model[#]	
					P	OR(95%CI)	*P*	OR(95%CI)	*P*	OR(95%CI)	*P*	OR(95%CI)
rs13192841	A/A	G/A	G/G	A								
SLE	5(1.6)	64(20.1)	249(78.3)	74(11.6)	0.43	1.14(0.82-1.58)	0.29	2.35(0.58-9.51)	0.45	1.12(0.84-1.48)	0.36	1.64(0.55-4.88)
controls	3(0.7)	86(19.4)	355(80.0)	92(10.4)		reference		reference				
JSNP controls*	9(1.0)	179(19.2)	745(79.8)	197(10.6)						reference		reference
rs2230926	G/G	T/G	T/T	G								
SLE	6(1.9)	56(17.6)	256(80.5)	68(10.7)	0.033	1.47(1.03-2.09)	0.023	8.52(1.45-50.1)	6.2X10^{-4}	1.17(1.26-2.33)	0.049	2.97(1.00-8.82)
controls	1(0.2)	65(14.6)	378(85.1)	67(7.5)		reference		reference				
JSNP controls*	6(0.6)	110(11.8)	818(87.6)	122(6.5)						reference		reference
rs6922466	G/G	A/G	A/A	G								
SLE	12(3.8)	86(27.0)	220(69.2)	110(17.3)	0.80	0.97(0.74-1.26)	0.97	0.99(0.46-2.09)	0.20	0.86(0.68-1.09)	0.40	0.76(0.40-1.45)
controls	17(3.8)	124(27.9)	303(68.2)	158(17.8)		reference		reference				
JSNP controls*	46(4.9)	274(29.3)	614(65.7)	366(19.6)						reference		reference

OR: odds ratio, 95%CI: confidence interval.

* Genotype data of healthy Japanese individuals obtained from JSNP DATABASE.

[#] Recessive model for the minor allele.

Table 2. Association of *TNFAIP3* rs2230926 with presence or absence of nephritis.

rs2230926	Genotype			Allele	Allelic association		Recessive model*		Dominant model*	
	G/G	T/G	T/T	G	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)
SLE										
with nephritis (n=168)	4(2.4)	33(19.6)	131(78.0)	41(12.2)	0.010	1.70(1.13-2.56)	0.022	10.8(1.85-63.2)	0.035	1.62(1.03-2.53)
without nephritis (n=145)	2(1.4)	21(14.5)	122(84.1)	25(8.6)	0.55	1.16(0.72-1.87)	0.15	6.20(0.75-51.1)	0.77	1.08(0.64-1.81)
Controls (n=444)	1(0.2)	65(14.6)	378(85.1)	67(7.5)		reference		reference		reference

OR: odds ratio, 95%CI: confidence interval. Parentheses in the genotype and allele frequency columns indicate percentages.

*Recessive and dominant models for the G allele.

Figure Legend

Figure 1. Association of *TNFAIP3* rs2230926 with mRNA levels of *TNFAIP3*. Because there was no subject with G/G genotype, *TNFAIP3* mRNA levels were compared between rs2230926 G/T (n=24, mean ± SD: 12.0 ± 0.43) and T/T (n=65, mean ± SD: 12.2 ± 0.33) subjects by unpaired Student's t-test. The mRNA levels were obtained from the mRNA expression profiling data in the B cell lines from the HapMap JPT and CHB subjects (GENEVAR database).
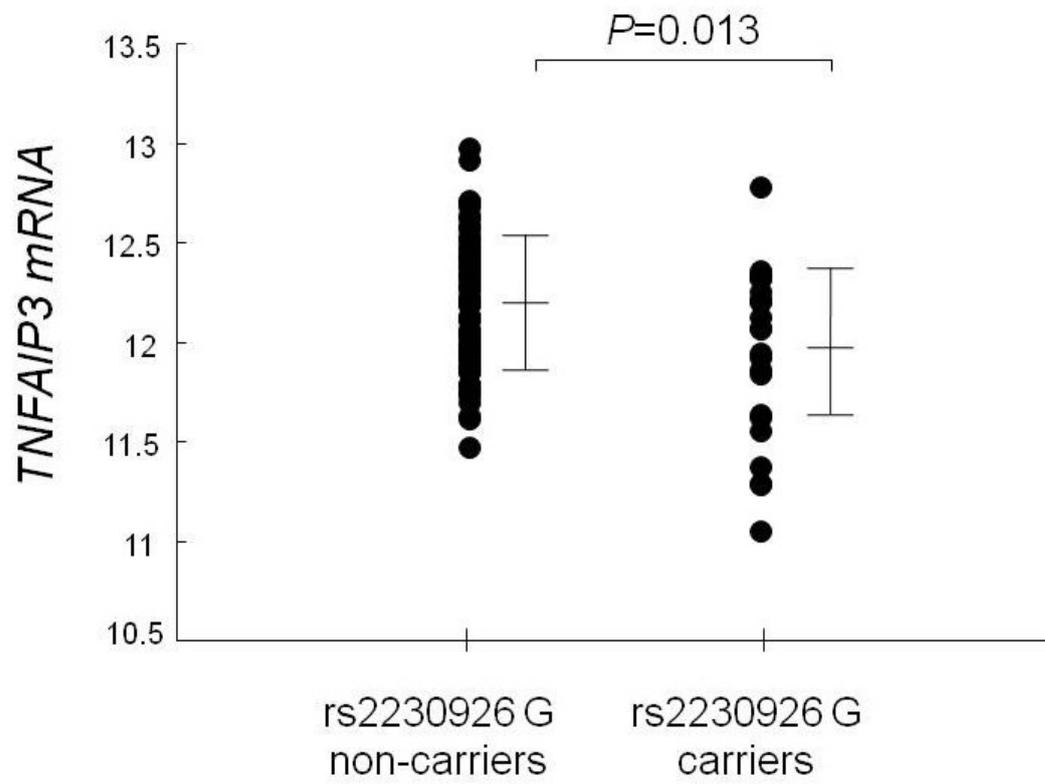
Figure 1