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STAT4 IS A GENETIC RISK FACTOR FOR SYSTEMIC SCLEROSIS IN A CHINESE POPULATION

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Abstract

Systemic sclerosis (SSc) is an immune-mediated and complex genetic disease. An association of single-nucleotide polymorphisms (SNPs) in the STAT4 gene with SSc has been reported in European Caucasians, North Americans and Japanese. We undertook the current study to examine whether the STAT4 SNPs are also associated with susceptibility to SSc and SSc subsets in a Han Chinese population. A total of 453 Han Chinese patients with SSc and 534 healthy controls were examined in the study. The SNPs rs7574865, rs10168266 and rs3821236 of the STAT4 gene were examined with SNP TaqMan assays. The T-allele carriers of rs7574865 and rs10168266 were strongly associated with the presence of anti-topoisomerase I (ATA) and pulmonary fibrosis in SSc patients, as well as with diffuse cutaneous SSc (dcSSc). The presence of anti-centromere (ACA) and limited cutaneous SSc (lcSSc) did not show significant association with any of the examined SNPs. The results were consistent with previous reports in other ethnic populations in

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supporting the notion that polymorphisms of STAT4 may play an important role in susceptibility to SSc. It also revealed different genetic aspects of SSc subsets in a Han Chinese population.

Keywords

scleroderma systemic sclerosis; polymorphism; stat4; genetics; Chinese population

Systemic sclerosis (SSc) or scleroderma is a rare and complex genetic disease. It is characterized clinically by thickening and fibrosis of the skin and involvement of multiple internal organs. Based on the extent of skin involvement, SSc is classified into limited cutaneous (lcSSc) and diffuse cutaneous (dcSSc) scleroderma. The latter subset is characterized by more rapid progression of skin and visceral involvement, as well as poorer prognosis (1). In addition, SSc also can be subgrouped by autoantibody subsets. The most common of these autoantibodies are directed against DNA topoisomerase I (ATA), centromeric proteins (ACA) and RNA polymerases III (anti-RNAP3) (2, 3). Importantly, clinical and serological presentations of SSc vary in different ethnic populations. For instance, the patients of Choctaw Native Americans, African Americans and Han Chinese are more likely to develop dcSSc compared to subjects of European descent (4-6). Furthermore, Choctaw Native Americans, African Americans, Koreans, Thais and Han Chinese have relatively higher frequencies of ATA (4-8).

Although etiopathogenesis of SSc is still unclear, genetic factors play a crucial role in SSc susceptibility. In addition to previously reported HLA-region genes, recent genome-wide association studies (GWAS) of SSc identified multiple non-HLA-region SSc-associated genetic loci and gene polymorphisms in European and US populations (9). One of the strongest association signals fall within the STAT4 gene. STAT4 stands for signal transducer and activator of transcription 4, and is a transcription factor functioning in T-cell differentiation and signaling in response to stimulation from cytokines and growth factors (10-12).

However, genetic heterogeneity in different ethnic populations may significantly impact the complex trait of SSc. Chinese SSc patients have unique serological and clinical features with high frequency of ATA and pulmonary fibrosis but low in anti-RNAP3 (6). Associations between the STAT4 and SSc have not been reported in Chinese SSc. Recently, we established a SSc cohort of Han Chinese through multicenter SSc consortium in China under the International Network of Scleroderma Clinical Care and Research (InSCAR) (http://www.inscar-global.org). The goal of the present study is to investigate the STAT4 polymorphisms in association with potential risk to or protection from SSc in Han Chinese.

MATERIALS AND METHODS

Patient enrolment

SSc patients were recruited from a multicenter study including hospitals and outpatient clinics in Shanghai, Hebei province, Sichuan province, and Hunan province in China. All patients met the American College of Rheumatology (ACR) classification criteria for SSc (13), or had at least 3 out of 5 CREST features (Calcinosis, Raynaud's phenomenon,

Esophageal dysmotility, Sclerodactyly, and Telangiectasia) with sclerodactyly being mandatory (14). A total of 453 SSc patients and 534 gender-matched and unrelated controls were examined in the studies. None of the controls had autoimmune diseases.

Autoantibody tests

Patients' sera were tested for antinuclear antibodies (ANA) by indirect immunofluorescence using HEp-2 cells as antigen substrate (Antibodies, Davis, CA, USA). ATA was detected by passive immunodiffusion against calf thymus extracts (INOVA, Diagnostics). ACA was determined by indirect immunofluorescence using HEp-2 cells.

Genotyping

DNA samples were extracted from peripheral blood cells of patients and controls. Three single nucleotide polymorphisms (SNPs) of STAT4 (rs7574865 rs10168266, and rs3821236) were selected, and were purchased from the pre-designed SNP Assays of the Applies Biosystems (Foster City, CA, USA). The SNP TaqMan assays were performed to examine genotypes of each sample as described previously (7). The SDS2.4 software (Applied Biosystems) was used for allele determination. Genotyping data were examined with Hardy–Weinberg equilibrium.

Statistics

Exact *p*-values were obtained (Fisher's test) from 2×2 tables of allele counts and disease status. The *p* values less than 0.05 were considered statistically significant. Correction for multiple comparisons was performed according to Bonferroni's method. Specifically, a *p*-value less than 0.0167 was considered significant after adjustment for 3 comparisons of the examined SNPs. Of note, we considered each subset of SSc is an independent outcome for three SNPs.

RESULTS

SSc patients were 91.1% positive for ANA. There were 149 lcSSc (44%) and 190 dcSSc (56%), others were undefined. There were 361 patients examined for ATA with 172 positive (48%), 325 were examined for ACA with 42 positive (13%), 298 were examined for anti-RNAP3 with 7 positive (2.4%). In addition, there were 327 patients examined with chest X-ray and/or CT for diagnosis of pulmonary fibrosis, and 237 were positive (72.5%).

Homozygous GG genotype of rs7574865 were significantly decreased ($p=4.1\times10^{-4}$, OR=0.63), and T-allele carriers were significantly increased ($p=4.1\times10^{-4}$, OR=1.58) in SSc patients (Table I). Similarly, homozygous CC and T-allele carriers of rs10168266 were significantly decreased ($p=5.1\times10^{-3}$, OR=0.69) and increased ($p=5.1\times10^{-3}$ OR=1.44) in SSc patients, respectively (Table I).

The changes of rs7574865 and rs10168266 also were associated with ATA positive SSc or pulmonary fibrosis (PF) of SSc and dcSSc (Table II). Specifically, the T alleles of rs7574865 and rs10168266 were significantly increased, and represented risk alleles in these

three subsets of SSc, while the genotypes GG of rs7574865 and the CC of rs10168266 were associated with protection from these three subsets of SSc.

SNP rs3821236 did not show any association with SSc in general, but A-allele carriers were increased and homozygous GG genotype was decreased in pulmonary fibrosis of SSc (Table II). SSc patients with ACA or lcSSc did not show significant association ($p < 0.0167$) with any of the examined SNPs. Only a marginally significant *p* value (0.02) was observed in association between homozygous GG genotype or T-allele carriers of rs7574865 and lcSSc (Table II).

DISCUSSION

SSc is a complex genetic disease. Genetic studies of SSc have been reported in multiple ethnic populations. Different ethnic populations displayed some distinct clinical and serological features of SSc, which appeared also in association with specific genetic factors. Studies of a Han Chinese cohort herein demonstrated genetic association of the STAT4 gene with SSc susceptibility, which was consistent with the previous reports in other ethnic populations including Caucasians of North American, Spanish, and European ancestry (15), as well as Japanese (16). Moreover, specific genotypes of three SNPs (rs7574865, rs10168266 and rs3821236) of the STAT4 appeared in strong association with clinical and serological features including dcSSc subset, and SSc patients with ATA and pulmonary fibrosis, which are unique in Han Chinese SSc, in contrast to one SNP (rs7574865) which was reported in association with lcSSc and/or ACA in Caucasian Europeans and Japanese (15, 16).

Persistent and strong association between the STAT4 polymorphisms and SSc in multiple independent and different ethnic populations suggested an important role of STAT4 gene in pathogenesis of SSc. STAT4 is a transcription factor that transmits signals of type 1 interferon (INF), as well as interleukin (IL) -2, IL-12 and IL-23 in T cells and monocytes in regulation of innate and adaptive immunity (10-12). Three SNPs examined in the studies lay in intronic regions of the STAT4. Functional evidence of these SNPs has not been revealed. Future studies will be necessary to explore the possible functional correlations, such as with gene expression and splicing variants. Of note, STAT4-deficient mice displayed decreased levels of inflammatory cytokines by controlling T-cell activation and proliferation, and showed a protection against fibrosis induced by bleomycin (17), which supported an important role of STAT4 in potential pathogenesis of scleroderma.

The most prominent feature observed in the Han Chinese cohort appeared to be the association between the T alleles of rs7574865 and rs10168266 and pulmonary fibrosis in SSc patients, which achieved the strongest *p*-values in the studies. Moreover, the SSc patients carrying T alleles of rs7574865 and rs10168266 also were more likely to have ATA in circulation, and presented with dcSSc. It is in contrast to previous reports that homozygous TT of rs7574865 was associated with lcSSc in Caucasians of Spanish and Dutch SSc (15), and with ACA positive SSc in Japanese population (16). Reasons of these discrepancies are still unclear. One possible explanation may be the differences in the number of SSc subsets studied in different ethnic populations. For instance, in reported

Caucasians of Spanish and Dutch studies, the numbers of dcSSc patients were 90 (27%) and 30 (23%), respectively, and the majority of the patients were lcSSc patients [242 (73%) and 101 (77%), respectively] (15). In reported Japanese studies of STAT4, a total of 88 (31%) ATA *versus* 87 (31%) ACA positive SSc patients were examined (16). In contrast, the SSc patients of Han Chinese examined in our studies were 190 (56%) dcSSc and 172 (48%) ATA positive. On the other hand, the numbers of lcSSc and ACA positive Chinese SSc patients were 149 (44%) and 42 (13%), respectively. Limited numbers in specific subsets of SSc may affect the statistic power. Therefore, a larger sample size of SSc subsets may be needed to verify their associations with alleles of rs7574865. In addition, it is also important to consider genetic heterogeneity between Han Chinese and other ethnic populations that may significantly impact the complex trait of SSc.

In summary, this is the first report of genetic studies of STAT4 in Han Chinese SSc. The results were consistent with previous reports in other ethnic populations in supporting the notion that polymorphisms of STAT4 may play an important role in susceptibility to SSc. It also revealed different genetic aspects of SSc subsets, which may need a larger number of SSc patients for verification.

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Table I

Association of two SNPs of STAT4 with SSc of Han Chinese.

		Control	SSc		
rs7574865		**N (%)**	**N (%)**	***p***	**OR (95%CI)**
genotype	GG	265 (50)	174 (38)	**4.1×10^{-4}**	0.63(0.49-0.82)
	TT	55 (10)	62 (14)	0.1	1.38(0.92-2.07)
	GT	214 (40)	217 (48)	**0.0135**	1.37(1.06-1.78)
allele carrier	G	479 (90)	391 (86)	0.1	0.72(0.48-1.09)
	T	269 (50)	279 (62)	**4.1×10^{-4}**	1.58(1.22-2.05)
total examined		534	453		
rs10168266					
genotype	CC	251 (48)	173 (39)	**0.0051**	0.69(0.53-0.9)
	TT	58 (11)	68 (15)	0.051	1.45(0.98-2.15)
	CT	218 (41)	206 (46)	0.14	1.21(0.93-1.5)
allele carrier	C	469 (89)	379 (85)	0.051	0.69(0.47-1.02)
	T	276(52)	274 (61)	**0.0051**	1.44(1.11-1.88)
total examined		527	447		

A p-value of multiple comparisons less than 0.0167 was considered significant after adjustment for 3 comparisons (rs7574865, rs10168266 and rs3821236, note: rs3821236 did not show any association). N: number; OR: odds ratio, CI: confidence interval

Table II

Association of two SNPs of STAT4 with clinical and serological features of SSc in Han Chinese.

	ATA (+)			PF (+)			lcSSc			dcSSc		
rs7574865												
alleles	***N* (%)**	***p***	**OR (95%CI)**	**N (%)**	***p***	**OR (95%CI)**	**N (%)**	***p***	**OR (95%CI)**	**N (%)**	***p***	**OR (95%CI)**
GG	61 (35)	**0.0012**	0.56 (0.38-0.81)	81 (35)	**1.2×10^{-4}**	0.54 (0.39-0.75)	58 (39)	**0.02**	0.65 (0.44-0.95)	67 (35)	**6.4×10^{-4}**	0.55 (0.39-0.79)
TT	29 (17)	**0.021**	1.77 (1.05-2.95)	38 (16)	**0.02**	1.69 (1.06-2.7)	22 (15)	0.13	1.51 (0.86-2.64)	23 (12)	0.49	1.2 (0.69-2.07)
GT	82 (48)	0.079	1.36 (0.95-1.95)	115 (49)	**0.018**	1.45 (1.05-1.99)	69 (46)	0.17	1.29 (0.88-1.89)	100 (53)	**0.0027**	1.66 (1.17-2.35)
G	143 (83)	**0.021**	0.57 (0.34-0.95)	196 (84)	**0.02**	0.59 (0.37-0.95)	127 (85)	0.13	0.66 (0.38-1.17)	167 (88)	0.49	0.83 (0.48-1.45)
T	111 (65)	**0.0012**	1.79 (1.24-2.6)	153 (65)	**1.2×10^{-4}**	1.86 (1.34-2.59)	91 (61)	**0.02**	1.55 (1.05-2.28)	123 (65)	**6.4×10^{-4}**	1.81 (1.27-2.59)
total	172			234			149			190		
rsl0168266												
CC	53 (32)	**3.1×10^{-4}**	0.51 (0.35-0.75)	80 (35)	**7.7×10^{-4}**	0.58 (0.41-0.81)	59 (39)	0.08	0.72 (0.49-1.06)	64 (34)	**0.0015**	0.57 (0.4-0.82)
TT	31 (18)	**0.011**	1.84 (1.11-3.04)	43 (18)	**0.0049**	1.84 (1.17-2.89)	25 (17)	0.06	1.63 (0.95-2.79)	25 (13)	0.386	1.25 (0.73-2.12)
CT	83 (50)	0.058	1.4 (0.97-2.01)	109 (47)	0.15	1.26 (0.91-1.73)	65 (44)	0.62	1.1 (0.75-1.61)	98 (52)	**0.009**	1.56 (1.1-2.21)
C	136 (81)	**0.011**	0.54 (0.33-0.9)	189 (82)	**0.0049**	0.54 (0.35-0.85)	124 (83)	0.06	0.61 (0.36-1.05)	162 (87)	0.386	0.8 (0.47-1.37)
T	114 (68)	**3.1×10^{-4}**	1.96 (1.33-2.88)	152 (66)	**7.7×10^{-4}**	1.73 (1.24-2.41)	90 (60)	0.08	1.39 (0.94-2.04)	123 (66)	**0.0015**	1.75 (1.22-2.51)
total	167			232			149			187		
rs3821236												
AA	40 (24)	0.43	1.18 (0.76-1.82)	54 (23)	0.472	1.15 (0.78-1.68)	35 (24)	0.48	1.17 (0.74-1.84)	36 (19)	0.6	0.89 (0.57-1.39)
GG	40 (24)	**0.019**	0.62 (0.41-0.94)	57 (25)	**0.015**	0.65 (0.45-0.93)	40 (27)	0.14	0.74 (0.48-1.13)	56 (30)	0.36	0.85 (0.58-1.23)
AG	88 (52)	0.128	1.31 (0.91-1.88)	121 (52)	0.99	1.3 (0.94-1.79)	73 (49)	0.43	1.16 (0.79-1.7)	96 (51)	0.2	1.24 (0.88-1.76)

	ATA (+)			PF (+)			lcSSc			dcSSc		
rs7574865												
alleles	***N* (%)**	***p***	**OR (95%CI)**	**N (%)**	***p***	**OR (95%CI)**	**N (%)**	***p***	**OR (95%CI)**	**N (%)**	***p***	**OR (95%CI)**
A	128 (76)	**0.0194**	1.6 (1.06-2.44)	175 (75)	**0.015**	1.54 (1.07-2.22)	108 (73)	0.14	1.35 (0.89-2.07)	132 (70)	0.36	1.18 (0.81-1.72)
G	128 (76)	0.43	0.85 (0.55-1.31)	178 (77)	0.47	0.87 (0.59-1.29)	113 (76)	0.48	0.86 (0.54-1.35)	152 (81)	0.6	1.12 (0.72-1.74)
total	168			232			148			188		

A p-value of multiple comparisons less than 0.0167 was considered significant. PF: pulmonary fibrosis