Signal transducer and activator of transcription 4 gene polymorphisms associated with rheumatoid arthritis in Northwestern Chinese Han population

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A B S T R A C T

Aims: Signal transducer and activator of transcription 4 (STAT4) gene encode a transcriptional factor that transmits signals induced by several key cytokines which play important roles in the development of autoimmune diseases. Recently, several single nucleotide polymorphisms (SNPs) in STAT4 gene have been reported to be significantly associated with Rheumatoid arthritis (RA) in different ethnic populations. We undertook this study to investigate whether the association of STAT4 genetic polymorphisms with RA is present in Northwestern Chinese Han population.

Main methods: A case–control association study in individuals with RA (n=208) and healthy controls (n=312) was conducted. Four SNPs (rs7574865, rs8179673, rs10181656, rs11889341) in STAT4 gene were genotyped by using polymerase chain reaction followed by denaturing high-performance liquid chromatography (PCR-DHPLC) and DNA sequencing.

Key findings: The genotype and allele distributions of four polymorphisms were significantly different in individuals with RA compared to controls, with SNP rs7574865 T allele and T/T genotype showing the most significant association with susceptibility to RA (uncorrected P=1×10^-4, OR=1.645, 95% CI=1.272–2.129; uncorrected P=4.8×10^-5, OR=3.111, 95% CI=1.777–5.447, respectively). Stratification studies showed that STAT4 gene polymorphisms were significantly associated with anti-cyclic citrullinated peptide (anti-CCP) positive subgroup in Northwestern Chinese Han population.

Significance: These findings strongly suggest that STAT4 genetic polymorphisms are associated with RA in Northwestern Chinese Han population, and support the hypothesis of STAT4 gene polymorphisms increasing the risk for RA across major populations.

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Introduction

Rheumatoid arthritis (RA) is a common chronic inflammatory autoimmune disease characterized by significant disability and early mortality, which affects ~1% of the adult population worldwide (Silman and Pearson, 2002). It is generally accepted that RA is a complex disease, with suspected interrelated contributions from genetic, infectious, environmental and hormonal factors (Firestein, 2003). Twin and family studies suggest that genetic factors contribute up to 60% of disease susceptibility to RA (MacGregor et al., 2000). To date, human leukocyte antigen (HLA) class II molecules have been documented as the most powerful genetic factors of RA across all populations, but they account for no more than one-third of the total genetic susceptibility (Newton et al., 2004; Cornelis et al., 1998). Recently, several non-HLA genes have been identified to contribute to RA susceptibility (Bowes and Barton, 2008). However, subsequent studies in European and Asian populations have got conflicting results (Yamada and Yamamoto, 2007; Kyogoku et al., 2004; Ikari et al., 2006; Orozco et al., 2005), which suggest that the genetic backgrounds of different ethnic groups should be taken into account (Colhoun et al., 2003).

Signal transducer and activator of transcription 4 (STAT4) gene encode a transcription factor which resides in cytosol and transmits the intracellular signals induced by cytokines including interleukin-12 (IL-12), IL-23, IL-27 and type 1 interferons (IFNs). Upon cytokine signaling, this transcription factor becomes phosphorylated and translocates to the nucleus to play an essential downstream role in the differentiation and proliferation of IL-12-dependent T helper 1 (Th1) cells (Watford et al., 2004). STAT4 is also important in the development of IL-17-secreting Th cells (Th17) in response to IL-23 (Mathur et al., 2007). Since Th1 and Th17 lineages work as crucial
effectors role in chronic inflammatory disorders, STAT4 gene may play an important role in the pathogenesis of RA (McInnes and Schett, 2007; Thierfelder et al., 1996).

Currently, several single nucleotide polymorphisms (SNPs) in STAT4 gene have been reported to be significantly associated with RA and systemic lupus erythematosus (SLE) (Amos et al., 2008; Remmers et al., 2007). And subsequent association studies between STAT4 gene polymorphisms and RA in Caucasians and Asians have yielded consistent results (Lee et al., 2007; Palomino-Morales et al., 2008; Barton et al., 2008; Kobayashi et al., 2008; Orozco et al., 2008; Martinez et al., 2008; Daha et al., 2009; Suarez-Gestal et al., 2009), suggesting that STAT4 gene polymorphisms may contribute to RA susceptibility across ethnic barriers (Lee et al., 2010; Ji et al., 2010). However, the recent study between SNP rs11889341 in STAT4 gene and RA only found association of the heterozygous CT genotype with female RA group in Northwestern Chinese Han population, and there was no significant association between rs11889341 and the total patients (Li et al., 2009). A followed replication failure was also reported in African Americans (Kelley et al., 2010). The two inconsistent results make STAT4 gene now controversial. Thus, we undertook this study to investigate whether the association of STAT4 genetic polymorphisms with RA is present in Northwestern Chinese Han population.

Materials and methods

Subjects

This study was approved by the Ethical Committee of Lanzhou University. After obtaining informed consent, 208 RA patients (68 males, 140 females) and 312 healthy controls (119 males, 193 females) were consecutively recruited from the First and the Second Hospital of Lanzhou University, respectively, the People’s Hospital of Gansu Province, the Traditional Chinese Medicine hospital of Gansu Province, and the affiliated Lanzhou Petrochemical hospital between July 2006 and November 2008. All patients who self-reported Han Chinese, have resided in Northwestern China for at least three generations and fulfilled the revised criteria for the classification of rheumatoid arthritis by American Rheumatism Association in 1987 (Arnett et al., 1988), and their families did not have any record of RA history. The controls were gender, age and ethnically-matched unrelated healthy people obtained from the checkup population in the above five hospitals (Table 1).

Autoantibody analysis

The status of rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) antibodies was available for about two-thirds of RA patients. Serum levels of RF were tested by laser nephelometry. Individuals with values ≥40 IU/ml were regarded as RF positive. Serum anti-CCP antibody levels were examined by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Immuno-noscan RA2, Generic Assays, Dahlewitz, Germany). A cut-off of 50 IU/ml was used as a criterion for anti-CCP antibody positivity.

Genotyping

Genomic DNA was extracted from EDTA-anticoagulated peripheral blood using standard methods (Blin and Stafford, 1976). All DNA samples were stored at −20 °C until analyzed. The four SNPs (rs7574865, rs8179673, rs10181656, rs11889341) in intron 3 of STAT4 gene were amplified by polymerase chain reaction (PCR) followed by denaturing high-performance liquid chromatography (DHPLC) on a Wave DNA Fragment Analysis System (Trans-gnomic Inc., San Jose, CA) as previously reported (Gross et al., 1999). The reference sequence (i.e. wild type sequence) of STAT4 gene was obtained from NCBI GenBank (NM_003151.2). The primer sequences for the four SNPs and the respectively melting temperature on PCR and DHPLC are listed in Supplementary table S1.

Genotyping was conducted by laboratory personnel who were blinded to subject status, and all of the samples were randomly selected to be genotyped again by a different author. To confirm the genotyping results by PCR-DHPLC, DNA samples were also randomly selected to be examined again by direct sequencing on an automated ABI PRISM 3730 Genetic Analyzer (Sheng Gong Ltd., Shanghai, China) (sequencing more than 10% of the total sample).

Statistical analysis

Hardy–Weinberg equilibrium was assessed by using χ² test. Comparisons of allele and genotype frequencies between RA patients and controls were performed by using 2 × 2 contingency tables with χ² analysis. Haplotype constructions were analyzed by using SHEsis software (Shi and He, 2005). Multiple testing was corrected by Bonferroni procedure (Benjamini et al., 2001). All P-values were two-tailed and P < 0.05 was considered to be statistically significant. All statistical analyses were performed with SPSS for Windows (version 16.0; SPSS Inc., Chicago, Illinois, USA).

Results

Distribution of the four polymorphisms in cases and controls

The four SNPs have been successfully genotyped in all subjects, and repeated DHPLC results and corresponding sequencing results were 100% concordant (Supplementary Figure S1). All SNPs were in Hardy–Weinberg equilibrium in both patients and controls, and were significantly associated with RA (P < 0.05) in Northwestern Chinese Han population, with rs7574865 exhibiting the strongest association (uncorrected P = 1×10⁻⁴, Odds Ratio (OR) = 1.645, 95% confidence interval (95% CI) = 1.272–2.129 for T vs. G; uncorrected P = 4.8×10⁻⁵, OR = 3.111, 95% CI = 1.777–5.447 for TT vs. GG). Furthermore, the associations remained significant except for rs11889341 after being corrected by Bonferroni procedure (Table 2).

Stratification analysis of rs7574865 in subgroups according to the presence of RF and anti-CCP antibodies

Since SNP rs7574865 exhibited the strongest association with RA in the present study, we analyzed the allele and genotype frequencies of rs7574865 among RA patients stratified by autoantibody status (Table 3). The stratification study showed that rs7574865 T allele was significantly associated with RF-positive and anti-CCP-positive subgroups (uncorrected P = 0.037, OR = 1.446, 95% CI = 1.021–2.047)

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Table 1: Characteristics of RA patients and controls.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>RA patients</th>
<th>Controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number</td>
<td>208</td>
<td>312</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>140 (67.31)</td>
<td>193 (61.86)</td>
<td>0.205&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Male</td>
<td>68 (32.69)</td>
<td>119 (38.14)</td>
<td></td>
</tr>
<tr>
<td>Age, mean ± SD years</td>
<td>46.74 ± 16.44</td>
<td>47.04 ± 13.37</td>
<td>&gt;0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Age of onset, years</td>
<td>40.7 ± 11.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of disease, years</td>
<td>10.1 ± 9.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of disease, years</td>
<td>10.1 ± 9.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> P value calculated by Pearson chi-square test (All frequency > 0.05) or Fisher’s exact test (Any frequency < 0.05).

<sup>b</sup> P value calculated by student t test.

<sup>c</sup> Clinical data were not available for some cases.
intervals (95% CIs) were calculated in the indicated patient subgroups in comparison to controls.

The most common haplotype (CGTC) which was formed by the major allele of each SNP showed a protective effect to all RA patients. While the second common haplotype (TTCG), formed by the minor allele of rs7574865 and the major alleles of the others (uncorrected OR=0.471, 95% CI=0.354–0.626). While the second common haplotype (TTCG), formed by the minor allele of each SNP, only showed a moderate risk effect for all the patients (uncorrected P=0.009, OR=1.394, 95% CI=1.001–1.942).

Linkage analysis of the four SNPs in STAT4 gene in controls showed strong associations of rs7574865 T allele and TT genotype with RF-positive subgroup (uncorrected P=0.001, OR=2.510, 95% CI=1.219–5.169 for RF-positive; P=0.001, OR=3.478, 95%CI=1.611–7.508 for anti-CCP-positive) but also with RF-negative and anti-CCP-negative subgroups (uncorrected P=0.038, OR=2.424, 95% CI=1.033–5.689 for RF-negative; uncorrected P=0.024, OR=2.462, 95% CI=1.108–5.467 for anti-CCP-negative). However, after being corrected by Bonferroni procedure, we only found significant associations of rs7574865 T allele and TT genotype with anti-CCP-positive subgroup (corrected P=0.012 and 0.004, respectively), and a marginal association of rs7574865 TT genotype with RF-positive subgroup (corrected P=0.044, Table 3).

### Discussion

In the present study, we investigated the association of a candidate gene STAT4 with RA in a Chinese population. The four examined SNPs in intron 3 of STAT4 gene were associated with 1.3–1.6-fold increased susceptibility to Northwestern Chinese Han RA patients. These findings strongly support the hypothesis that STAT4 gene polymorphisms are also associated with RA in Northwestern Chinese Han population, and are similar to the results collected from major populations worldwide (Remmers et al., 2007; Lee et al., 2007; Palomino-Morales et al., 2008; Barton et al., 2008; Kobayashi et al., 2008; Orozco et al., 2008; Zervou et al., 2008; Martinez et al., 2008; Daha et al., 2009; Suarez-Gestal et al., 2009). Being a complex disease, clinical heterogeneity of RA may come from many factors such as the absence or presence of RF and anti-CCP antibodies. Our stratification study after Bonferroni correction showed that rs7574865 T allele and TT genotype were still significantly associated with RA susceptibility (corrected P=1.4×10^{-6} and 1.5×10^{-5}, respectively).

### Table 2

Genotype/allele frequencies of the four SNPs in STAT4 gene in RA patients and controls.

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Genotype/Allele</th>
<th>Genotype/Allele frequency</th>
<th>P value*</th>
<th>OR (95% CI)</th>
<th>P corr*</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs11889341</td>
<td>CC</td>
<td>133 (42.6)</td>
<td>1</td>
<td>1.147</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>145 (46.5)</td>
<td>0.083</td>
<td>1.128</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>34 (10.9)</td>
<td>0.168</td>
<td>1.128</td>
<td>1</td>
</tr>
<tr>
<td>rs7574865</td>
<td>CC</td>
<td>133 (42.6)</td>
<td>1</td>
<td>1.147</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>151 (48.4)</td>
<td>0.044</td>
<td>1.471</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>17 (5.4)</td>
<td>0.011</td>
<td>1.559</td>
<td>1</td>
</tr>
<tr>
<td>rs8179673</td>
<td>TT</td>
<td>136 (43.6)</td>
<td>1</td>
<td>1.147</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>138 (44.2)</td>
<td>0.024</td>
<td>1.147</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>140 (44.9)</td>
<td>0.042</td>
<td>1.147</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>410 (65.7)</td>
<td>1</td>
<td>1.147</td>
<td>1</td>
</tr>
<tr>
<td>rs10181656</td>
<td>CC</td>
<td>151 (48.4)</td>
<td>0.044</td>
<td>1.472</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>144 (46.2)</td>
<td>0.012</td>
<td>1.472</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>27 (8.7)</td>
<td>0.019</td>
<td>1.472</td>
<td>1</td>
</tr>
</tbody>
</table>

Values are number (%); MAFL = minor allele frequency; OR, odds ratio; 95% CI = 95% confidence interval; P corr (corrected P) calculated by Bonferroni procedure.

* P-values calculated by Pearson chi-square test.

### Table 3

Genotype frequencies of rs7574865 in RA patients, stratified by rheumatoid factor and anti-CCP autoantibody status.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Genotype frequency</th>
<th>T vs. G</th>
<th>TT vs. GG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>OR (95% CI)</td>
<td>P corr</td>
</tr>
<tr>
<td>Control</td>
<td>144 (46.2)</td>
<td>1.146 (1.021–2.047)</td>
<td>0.148</td>
</tr>
<tr>
<td></td>
<td>141 (45.2)</td>
<td>1.146 (1.021–2.047)</td>
<td>0.148</td>
</tr>
<tr>
<td></td>
<td>27 (8.7)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>RF+</td>
<td>34 (39.1)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>37 (42.5)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>16 (18.4)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>RF−</td>
<td>22 (36.7)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>28 (46.7)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10 (16.7)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Anti-CCP+</td>
<td>23 (32.4)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>33 (46.5)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>15 (21.1)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Anti-CCP−</td>
<td>26 (38.2)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>30 (44.1)</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Values are number (%); P-values calculated by Pearson chi-square test; P corr (corrected P) calculated by Bonferroni procedure; The odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated in the indicated patient subgroups in comparison to controls.

* Clinical data were not available for some cases.
immunity (Kaplan, 2005). Transgenic STAT4 knockout mice have been reported to have impaired Th1 differentiation, interferon-γ production, and cell-mediated immunity and are very susceptible to intracellular infections (Watford et al., 2004), but they have less severe disease in proteoglycan-induced arthritis (Finnegan et al., 2002). And collagen-induced arthritis in mice could be ameliorated by antisense oligonucleotides directed at STAT4 (Hildner et al., 2007; Klinman et al., 2005; Shirota et al., 2004). All these suggest that STAT4 may play pivotal roles in both initiation and maintenance of inflammatory process and may be a potential therapeutic target for RA.

Until now, two alternatively spliced isoforms have been described for STAT4, STAT4α and STAT4β, which lacks 44 amino acids at the C terminus of the full-length STAT4α is not as efficient as STAT4α in directly inducing IFN-γ gene expression activated by IL-12 in Th1 cells (Hoey et al., 2003). A recent study has documented that STAT4 mRNA was over-expressed in osteoblasts with a risk haplotype formed by rs10181656 and rs7582694 (the two strongest SLE-associated SNPs) compared with osteoblasts with non-risk haplotypes (Sigurdsson et al., 2008). The expression level of STAT4 in Peripheral Blood Mononuclear Cell (PBMC) was also reported to be correlated with the risk allele of STAT4 rs7574865 (Abelson et al., 2009).

Since the four susceptibility SNPs are located within the third intron of STAT4 gene, they probably have an influence on the level of STAT4 transcription and splice variation (Korman et al., 2008). However, the precisely functional roles of these risk SNPs remain to be elucidated.

**Conclusion**

In brief, our results confirm a genetic association of STAT4 with RA in Northwestern Chinese Han population, which strongly support the hypothesis that STAT4 may be a common RA susceptibility marker across different ethnic groups. Further functional studies on polymorphisms of STAT4 gene would lead to a greater understanding of RA and make it possible to develop novel therapies.

**Supplementary materials related to this article can be found online at doi:10.1016/j.jifs.2011.05.012.**

**Conflict of interest statement**

The authors declare that there are no conflicts of interest.

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