Genetic susceptibility to Systemic Lupus Erythematosus is associated with CTLA-4 Gene Polymorphisms in the Chinese Population

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1 Introduction

Systemic lupus erythematosus (SLE) is a chronic multisystem autoimmune disease and is considered to be caused by complex interactions between genetic risk, environmental and hormonal factors that result in an immune dysregulation and autoantibody production ensued (Edberg et al., 2008; Kyttaris et al., 2006; Sawalha et al., 2008). Epidemiological studies reported that SLE is more common in Asians (46.7/100 000) than in Caucasians (20.7/100 000), and ethnicity also influences the age of onset and severity of its manifestations (Lau et al., 2006; Rus et al., 2002).

The precise aetiopathogenesis of SLE is still unclear; however, the search for genes and molecular interactions that influence disease has promoted our understanding of pathogenesis and genetic contributions to autoimmunity in SLE (Sekigawa et al., 2004). Genetic association studies and recent advances in the field of single nucleotide polymorphisms (SNPs) have been highly successful in identifying several loci associated with disease susceptibility (Gregersen & Olsson, 2009; Harley et al., 2006).

The CTLA-4 molecule has a suppressive effect on T-cell activation and might contribute to maintain immune tolerance by blocking CD28-dependent T cell activation through interactions with its ligand CD80/86 on antigen presenting cells (Harley et al., 2006). The CTLA-4/B7 complex can compete with the CD28/B7 complex and convey an inhibitory influence to the T cell affecting T cell development, cytokine production and immune reactions (Gregersen & Olsson, 2009).

CTLA-4 would therefore, be an important negative regulator of T-cell responses, and its dysregulation has the potential to affect the pathogenesis of SLE by altered activation of T cells to self-antigens (Carreno B.M. & Collins M., 2002; Walunas et al., 1996). The CTLA-4 gene is located within the risk region on chromosome 2q33, and several polymorphisms have been reported in this gene. However, only few of them have been studied for association with SLE susceptibility, of which, two are located within the promoter region: a T/C change at position –1722 and an A/G transition at position -1661 (Hudson et al., 2002; Lee et al., 2005; Ueda et al., 2003). The former could alter transcription factor binding sites, whereas the latter may alter the potential response element for myocyte enhancer factor 2 (MEF2)( Ling et al., 1999). Hence, allelic variations of these two sites might lead to a differential susceptibility to SLE resulting from unbalanced or inefficient immune responses.

Although CTLA-4 polymorphism has been shown to be associated with a number of autoimmune diseases, including SLE, Graves' disease, multiple sclerosis and type 1 diabetes, however, the associations have not been always replicated in different populations (Kristiansen et al., 2000; Lee et al., 2005; Ueda et al., 2003). Recent studies showed that the CTLA-4 polymorphism plays an important role in SLE in some populations, which has not been confirmed in Chinese. Using a case-control study design, we have investigated the role of CTLA-4 polymorphism at positions –1661 and –1722 on SLE susceptibility in our Chinese SLE population in central China's Hubei Province.

2 Patients and Methods

2.1 Study Population

A total of 148 patients (17 males and 131 females) meeting the 1997 revised criteria of the American College of Rheumatology (ACR) for SLE (Hochberg, 1997) were recruited from Renmin and Zhongnan Hospitals of Wuhan University, Wuhan, China. Controls were 170 healthy volunteer with no history of autoimmune disease, collected for a case–control study. All patients and controls were Han Chinese re-
siding in the central part of China. The study was approved by the hospitals’ Ethics Committee of Wuhan University, and all subjects were consented to participate in the study.

2.2 DNA Extraction and Genotyping

DNA from patients and controls was extracted from peripheral blood with DNA flash kit 2.0 (HaiGene Biotechnology Co.Ltd., Gentra Systems Corp.) according to the standard protocol from the manufacturer. The Polymorphisms at positions −1661 and −1722 were analyzed by PCR–RFLP (polymerase chain reaction–restriction fragments length polymorphism), using the specific oligonucleotide primers (Sangon, Shanghai, China), 5’ CTAAGAGCATCCGGCTTGACCT 3’ and 5’TTGGTGTGATGCACAGAAGCC TTTT 3’. PCR amplification conditions were carried out as follows: initial denaturation at 94 °C for 5 minutes, then thirty cycles at 94°C (15 s), 60°C (30 s), 72°C (45 s), and one final extension at 72°C for 5 min. The products of the PCR were digested with BbvI or MseI at 37°C for 4 h, and then were analyzed by 2% agarose gel electrophoresis stained with ethidium bromide. After resolving, the −1722 T/C polymorphism was determined by detecting a 486 bp digested fragment (T allele) or two fragments of 270 and 216 bp (C allele) (Figure 1). The -1661A/G polymorphism was determined by detecting a 486 bp fragment (G allele) or two fragments of 347 and 139 bp (A allele) (Figure 2).

2.3 Statistical Analyses

We tested for Hardy-Weinberg equilibrium (HWE) among cases and controls. Allelic and genotypic frequencies were calculated by direct counting. The chi-square test with Yates correction and Fisher exact test were used to compare genotypes and alleles frequencies. Statistical significance was defined as P<0.05. The odds ratio (OR) was calculated to measure the strength of the association observed.

Figure 1: PCR restriction fragment length polymorphism results of −1722 T to C substitution in CTLA-4 promoter region. 1. CC Genotype; 2. TT Genotype; 3.TC Genotype.
3 Results and Discussion

The genotypic frequencies for the two sites tested were not found to be deviated from those predicted from Hardy-Weinberg equilibrium in both SLE patients and controls. Genotype and allele frequencies of the −1722 T/C and 1661 A/G polymorphisms are shown in Table 1 and Table 2. As observed, the genotypes at position −1722 were strongly associated with SLE. The frequency of the T allele on the −1722 SNP was significantly increased in SLE patients: 57.8% versus 40.6% in controls (P < 0.001, OR = 2.002). While the detected C allele frequency in the controls was significantly elevated in comparison with that in the SLE patients (59.4% versus 42.2%). The frequencies of T/T homozygotes and T/C heterozygotes were also significantly higher in patients than in controls (28.4% vs 17.1%, P = 0.016, OR = 1.926; 58.8% vs 47.1%, P = 0.037, OR = 1.605). Conversely, the frequencies of C/C homozygotes was considerably higher in controls than in patients (35.8% vs 12.8%, P < 0.01, OR = 0.263). We observed no significant difference in the distribution of the alleles and genotypes for the -1661 site between patients and healthy subjects.

Notwithstanding the convincing evidence that CTLA-4 polymorphism plays an important role in susceptibility to SLE, contradictory result has been reported among different populations (Lee et al., 2005). Several studies have observed a significant association of SLE with CTLA-4 gene polymorphisms (Ahmed et al., 2001; Fernandez-Blanco et al., 2004; Hudson et al., 2002; Lee et al., 2005; Ulker et al., 2009). While on the contrary, other studies showed a lack of association with that genetic variation (Aguilar et al., 2003; Liu et al., 2001; Parks et al., 2004). Reasons for the variability in associations are still ambiguous. Thus, investigating the frequencies and distribution of variants CTLA-4 genes across populations are essential for understanding disease association and discovery of population differences,
<table>
<thead>
<tr>
<th>promoter -1722</th>
<th>SLE n=148</th>
<th>Controls n=170</th>
<th>$\chi^2$</th>
<th>P-value</th>
<th>OR (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genotype frequency</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TT</td>
<td>42(28.4%)</td>
<td>29(17.1%)</td>
<td>5.846</td>
<td>0.016</td>
<td>1.926(1.127-3.293)</td>
</tr>
<tr>
<td>TC</td>
<td>87(58.8%)</td>
<td>80(47.1%)</td>
<td>4.362</td>
<td>0.037</td>
<td>1.605(1.028-2.503)</td>
</tr>
<tr>
<td>CC</td>
<td>19(12.8%)</td>
<td>61(35.8%)</td>
<td>22.315</td>
<td>&lt; 0.001</td>
<td>0.263(0.148-0.468)</td>
</tr>
<tr>
<td><strong>Allele frequency</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>171(57.8%)</td>
<td>138(40.6%)</td>
<td>18.701</td>
<td>&lt; 0.001</td>
<td>2.002(1.459-2.747)</td>
</tr>
<tr>
<td>C</td>
<td>125(42.2%)</td>
<td>202(59.4%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Phenotype</strong></td>
<td></td>
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<tr>
<td>T</td>
<td>129(87.2%)</td>
<td>109(64.1%)</td>
<td>6.182</td>
<td>0.013</td>
<td>1.574(1.100-2.253)</td>
</tr>
<tr>
<td>C</td>
<td>106(71.6%)</td>
<td>141(82.9%)</td>
<td></td>
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</tr>
</tbody>
</table>

**Table 1:** Genotypic distribution and allelic frequencies of -1722 CTLA-4 polymorphisms in Chinese SLE patients and healthy controls.

<table>
<thead>
<tr>
<th>promoter -1661</th>
<th>SLE n=148</th>
<th>Controls n=170</th>
<th>$\chi^2$</th>
<th>P-value</th>
<th>OR (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genotype frequency</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>AA</td>
<td>59(39.9 %)</td>
<td>63(37.1%)</td>
<td>0.263</td>
<td>0.608</td>
<td>1.126(0.716-1.771)</td>
</tr>
<tr>
<td>AG</td>
<td>67(45.2%)</td>
<td>76(44.7%)</td>
<td>0.010</td>
<td>0.920</td>
<td>1.132(0.730-1.755)</td>
</tr>
<tr>
<td>GG</td>
<td>22(14.9%)</td>
<td>31(18.2%)</td>
<td>0.647</td>
<td>0.421</td>
<td>0.783(0.431-1.423)</td>
</tr>
<tr>
<td><strong>Allele frequency</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>185(62.5%)</td>
<td>202(59.4%)</td>
<td>0.633</td>
<td>0.426</td>
<td>1.139(0.827-1.568)</td>
</tr>
<tr>
<td>G</td>
<td>111(37.5%)</td>
<td>138(40.6%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Phenotype</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>126(85.1%)</td>
<td>139(81.8%)</td>
<td>0.207</td>
<td>0.649</td>
<td>1.090(0.752-1.578)</td>
</tr>
<tr>
<td>G</td>
<td>89(60.1%)</td>
<td>107(62.9%)</td>
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</tbody>
</table>

**Abbreviations:**

CTLA-4 = cytotoxic T-lymphocyte antigen 4;
SLE = systemic lupus erythematosus;
OR = odds ratios;
95% CI = 95% confidence interval

**Table 2** Genotypic distribution and allelic frequencies of –1661 CTLA-4 polymorphisms in Chinese SLE patients and healthy controls.
especially for Chinese as they have a much higher SLE prevalence than the Europeans (Lau et al., 2006; Rus et al., 2002). The interval encompassing the CTLA-4 locus on chromosome 2q33 has been reported to show linkage to SLE in two genome-wide association studies (GWAS) studies (Cantor et al., 2004; Graham et al., 2006). A very recent GWAS has confirmed that variation in the CTLA-4 gene has been associated with a genetic risk of SLE (Budarf et al., 2011). Although, this polymorphism may has not yet reached stringent thresholds of lupus GWAS performed in some Chinese cohorts, however, the association has been observed in multiple independent studies in various ethnic populations including Asian (Ahmed et al., 2001; Barreto et al., 2004; Budarf et al., 2011; Cantor & Collins, 2004; Fernandez-Blanco et al., 2004; Graham et al., 2006; Hudson et al., 2002; Lee et al., 2005; Ulker et al., 2009), and there is strong biological evidence sufficient to conclude that CTLA-4 polymorphism confers susceptibility to SLE through its crucial functions in T cell activation/regulation (Budarf et al., 2011; Graham et al., 2006; Lindqvist et al., 2000; Liu et al., 2003; Ueda et al., 2003; Wong et al., 2005).

Recently, a GWAS study confirmed a SLE susceptibility locus at chromosome 2q32.3 in Chinese population that is near the region 2q33 which encodes the genes for CTLA-4 (Han et al., 2009). As polymorphisms that are near each other have a tendency be inherited together, something worth further pursuit in future studies. In Asian people, positive associations of the CTLA-4 polymorphism with SLE were reported in Korean (Hudson et al., 2002) and Japanese (Ahmed et al., 2001), while no such association was found in Malaysian population (Kek-Heng et al., 2010). Significantly, a meta-analysis by Lee et al. found a close association between SLE and exon 1 at +49 of CTLA-4 gene, especially in Asians (Lee et al., 2005). Liu, MF et al did not observe an association of CTLA-4 polymorphism with SLE in Taiwanese, but suggested that it is possible that this polymorphism could affect some specific clinical features (Liu et al., 2001). This discrepancy with the findings of the current study may be partially attributed to the variations among Chinese living in different geographical regions (Xu et al., 2009).

The results of this study confirm the involvement of CTLA-4 polymorphisms at the promoter –1722 on SLE susceptibility in the Chinese population. Our findings are in agreement with the results of other ethnic groups demonstrating the influence of this polymorphism in the susceptibility to SLE. On the contrary, the genotypic frequencies for the –1661 site were not found to be significantly different between patients and controls. Other studies on –1661 polymorphism in Korean with SLE also could not find a positive correlation (Hudson et al., 2002). In addition, no overall associations were seen between this polymorphism and SLE in African-Americans (Parks et al., 2004). Being inexplicit, despite the short distance between the two locations, only –1722 promoter-region polymorphisms was associated with SLE, while the other –1661 is not. This possibly due to some functional differences between the two sites on regulatory properties of this promoter might affect basal promoter activity and gene expression. Polymorphisms in promoter regions may affect the gene expression quantitatively or qualitatively by altering transcription factor binding sites or other controlling domains (Shastry, 2002).

The significant increase in the C allele observed in the controls is assumed to play a protective role against SLE in Chinese. On the other hand, The T/T and T/C genotypes of –1722 T/C polymorphisms were associated with higher risk to have SLE. Hudson et al studied CTLA-4 Polymorphisms at positions T/C –1722 in Korean population and found that T allele was more frequent in SLE patients while C allele was decreased in the controls and suggested that the C allele could contribute protectively to SLE in the Korean population. In contrast, Fernandez-Blanco et al found that the C allele of the –1722 T/C SNP was associated with SLE susceptibility in the Spanish SLE patients. However, another study did not find any significant association between the genetic polymorphisms at the –1722 T/C SNP of the CTLA-4 gene and Spanish SLE patients (Aguilar et al., 2003). Although there were significant associa-
tions in two of the three studies described above. However, a published meta-analysis study did not un-
veil a significantly increased odds ratio for this polymorphism (Lee et al., 2005). Takeuchi et al. (2005)
also reported a slight increase in the allele frequency of –1722 C in Japanese patients with SLE compared
to the controls, but the difference was not statistically significant.

Because there are relatively few studies, it is difficult to explain the contradictories at the promoter
–1722 polymorphism in SLE, but they may be due to a different genetic background and a possible role
for racial and ethnic influences in the pattern of haplotypes on the CTLA-4 locus between various popula-
tions in SLE predisposition (Lau et al., 2006; Rus et al., 2002). Variations in associations among popula-
tions maybe also related to difference in patient characteristics or the distribution of other risk factors that
interact with CTLA-4 concerning SLE. Moreover, genetic evidence suggests that genetic heterogeneity, a
common phenomenon in complex diseases, are responsible for a considerable portion of this variability.
We believe that there is not sufficient research on CTLA-4 polymorphisms to identify and demonstrate
the role of these important genetic variants that confer susceptibility to SLE susceptibility among differ-
ent populations.

Whereas some polymorphisms on the CTLA-4 gene have been analyzed in various populations for
investigating an association with SLE, only one of these, the CTLA-4 A/G polymorphism at position +49
in exon 1 has been widely studied, mainly in European populations (Lee et al., 2005). Accordingly, we
expect that the results of our study to reinforce the interest of focusing on analyzing the role of CTLA-4
polymorphism in increased susceptibility to SLE. Furthermore, an elevated level of soluble CTLA-4 in
sera has been described in SLE, with a positive and significant correlation between plasma sCTLA-4
concentration and SLE activity (Liu et al., 2003; Wong et al., 2005). Besides, the therapeutic use of
CTLA4Ig which is a soluble fusion protein that interferes with T cell activation by inhibiting the
B7/CD28 costimulatory interaction, appears to delay or extenuate disease development in experimental
models of lupus (Dall’Era & Davis, 2004). In this way, the results observed should provide new postu-
lates for the immunological role of this co-stimulatory molecule in the pathogenesis of SLE and should
facilitate recent advances in the exploration of therapeutic agents targeting T-cell activation in this dis-
ease (Liu et al., 2003).

Other polymorphisms have also been described in the promoter region (-658 and-318), the CT60
(A/G) and at the 3-end of the gene as well as the microsatellite (AT)(n) in the 3'-untranslated region (3'-
UTR) of the CTLA-4 gene. Nevertheless, the reported results have been inconsistent across different eth-
nic populations [27,39]; (Johnson et al., 2001; Torres et al., 2004). Hence, further explorations of these
polymorphisms are needed in order to more fully examine SLE associations with CTLA-4 locus in Chi-
nese population. Allelic and genotypic frequencies may vary between the populations; therefore, disease
association studies and interpreting their results offer a possible route to understanding the influence of
these genetic variants on disease aetiology and potentially to the development of new treatments.

4 Conclusions

CTLA-4 polymorphism at positions –1722 was significantly associated with SLE and may be a risk fac-
tor for SLE susceptibility in Chinese. Our results concur with the majority of those published supporting
the important influence of CTLA-4 polymorphisms in the susceptibility to SLE. Nevertheless, further
study in terms of the functional analysis of polymorphisms on the CTLA-4 gene needs to be done, and
larger population studies in different ethnic groups should be performed in the future.
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References


