The genetic association between TLR-1, -2, -4, and -6 gene polymorphisms and rheumatoid arthritis (RA) susceptibility in a Chinese Han population

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Abstract
Background: The toll-like receptor (TLR) genes were shown to be involved in the pathogenesis of RA. We aimed to investigate the genetic associations between the TLR-1, -2, -4, and -6 genes polymorphisms and RA susceptibility in a Chinese Han population.

Methods: Six polymorphisms (TLR-1 (rs5743610, rs5743618), -2 (rs5743708), -4 (rs4986790, rs4986791), and -6 (rs5743810)) in TLRs genes were genotyped in 360 patients with RA and 560 matched healthy controls by using direct sequencing method. The ORs and 95% CIs were evaluated using a standard logistic regression analysis.

Results: No significant association between the allelic, dominant and recessive models of TLR-1 rs5743618, TLR-2 rs5743708, TLR-4 rs4986790 and rs4986791, and TLR-6 rs5743810 polymorphisms and RA risk was observed (p>0.05). However, significant associations were detected between the allelic, dominant and recessive models of TLR-1 rs5743618 and RA risk (allelic: OR[95%CI]= 2.21 [1.73, 2.81], p<0.0001; dominant: OR[95%CI]= 2.33 [1.75, 3.09], p<0.0001; recessive models: OR[95%CI]= 3.70 [1.85, 7.41], p=0.0002). In addition, the TLR6 rs5743810 was found to be associated with the RF - and anti-CCP - antibody in RA group (RF: OR[95%CI]= 2.29 [1.42, 3.69], p=0.0007; anti-CCP: OR[95%CI]= 2.33 [1.39, 3.89], p=0.001).

Conclusions: The allelic, dominant, and recessive models of TLR1 rs5743618 might be associated with RA susceptibility. And the TLR6 rs5743810 might be associated with RF and anti-CCP antibody in RA in Chinese Han population.

Background
RA is a chronic, aggressive and systemic autoimmune disease [1]. The incidence of RA varies by region and race, and the prevalence of RA in China is about 0.3–0.5% [2]. The number of females is approximately three times that of males [2]. Epidemiological studies have found that the incidence of RA is characterized by familial aggregation and co-twin disease, and the heritability of RA in identical twins is 53–65% [3–4]. However, the etiology and pathogenesis of RA are hardly known yet [5].

TLRs are the specific type I transmembrane receptors and pathogenic pattern recognition receptors in the natural immune system, and play an important role in inflammation, cell signal transduction and
apoptosis [6-7]. It has been found that TLRs, especially TLR2 and TLR4, on the cell surface play a key role in the development of RA [8]. Studies showed that the expressions of TLR-2, -3, -4 and –7 in synovial tissues, synovial fibroblasts, peripheral blood monocytes and CD14+ macrophages in synovial fluid of RA patients significantly increased [10-11]. In addition, according to Spitzer et al., TLRs could interact and modulate with each other. TLR1 and TLR4 can form heterodimer complexes. And their extracellular segments can bind to TLR4 and block the connection between TLR4 and ligand, thus blocking the signal transduction of TLR4 [12]. Furthermore, Bulut et al. [13] suggested that the synergistic effect of TLR2 and TLR6 may be involved in the identification of all TLR2 ligands. Further experiments also confirmed that the fused TLR2 and TLR6 could inhibit the activation of the ligands of TLR2 and TLR4 [12].

One of the possible mechanisms driving TLRs dysfunction in RA is genetic variants in genes encoding these receptors. Recently, increasing number of studies have been carried out on the TLRs gene polymorphisms and the susceptibility of RA. Most of studies have focused on the genetic association between TLR2 and TLR4 genes polymorphisms and RA susceptibility. Two well-known missense mutations "Asp299Gly (rs4986790)" and "Thr399Ile (rs4986791)" in TLR4 gene have been shown to be correlated with RA risk. However, such associations were not shown in all the investigated populations [14-16]. As regards TLR2 Arg753Gln (rs5743708), it has been shown not to be the risk factor for arthritis in a Spain population [17]. As for TLR1 and TLR6, no correlation of TLR1 Arg80Thr (rs5743610), Ser602Ile (rs5743618), and TLR6 Ser249Pro (rs5743810) polymorphisms in RA was observed [18].

However, evaluation of the associations between the TLRs, especially the TLR1, TLR2 and TLR6 gene polymorphisms and RA susceptibility in Chinese Han population has been very limited. Thus, by utilizing a case-control method we aim to investigate whether polymorphisms of the TLR-1, -2, -4, and –6 genes polymorphisms contribute to the development of RA in a Chinese Han population.

Materials
Study subjects
The experimental protocol was evaluated and approved by the Ethical Committee of The First
Affiliated Hospital of Soochow University (China) (EC/19021, 12/10/2019). Written informed consent for genetics analysis was obtained from all individuals. 360 patients who fulfill the American College of Rheumatology 1987 criteria for RA were enrolled in the present study. And 560 healthy controls that matched with gender, age and ethnicity were also included. Detailed information of patients and controls were concluded in Table 1. All subjects were of Chinese Han origin.

Table 1
Clinical Characteristics of RA patients and healthy controls

| Clinical Characteristics | Case (Mean ± SD) | Control (Mean ± SD) | p     |
|-------------------------|------------------|---------------------|-------|
| Sex ratio (Female/Male) | 3.61 (282/78)    | 3.48 (435/125)      | 0.17  |
| Age (years)             | 43.1 ± 10.4      | 39.8 ± 12.6         | 0.43  |
| Median of evolution (months) | 102 ± 73.53 | -                   |       |
| Early onset before 40 years | 189 (52.4%) | -                   |       |
| Bone erosions n (%)     | 129 (35.7%)      | -                   |       |
| Shared epitope          | 131 (36.5%)      | -                   |       |
| Active RA (DAS28 ≥ 5.1) | 221 (61.4%)      | -                   |       |
| ESR (mm/h)              | 31.5 ± 12.1      | 4.6 ± 1.8           | < 0.001 |
| CRP (mg/l)              | 27.3 ± 11.6      | 4.7 ± 1.4           | < 0.001 |
| IgA IU/ml               | 79.3 ± 20.5      | 75.1 ± 14.4         | 0.79  |
| IgG IU/ml               | 114.2 ± 17.9     | 6.3 ± 3.0           | < 0.001 |
| IgM IU/ml               | 157.9 ± 22.4     | 7.7 ± 4.8           | < 0.001 |
| RF +, %                 | 266 (77.4%)      | 0 (0%)              | < 0.001 |
| CCP +, %                | 254 (70.5%)      | 0 (0%)              | < 0.001 |

Abbreviation: SD: Standard Deviation; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; RF: rheumatoid factor; Disease activity score 28 (DAS28): a score for evaluation of RA activity by assessing the state of 28 joints; anti-CCP: anti-cyclic citrullinated peptide

Genotyping
Genomic DNA was extracted by standard phenol chloroform method. Genotyping detection was performed on 6 polymorphisms of TLR-1 (rs5743610, rs5743618), TLR-2 (rs5743708), TLR-4 (rs4986790, rs4986791), and TLR-6 (rs5743810) genes by direct sequencing.

Statistical analysis
The SPSS 20.0 were applied for statistical analysis of the data. The Hardy-Weinberg equilibrium (HWE) of the SNPs in control group was tested with $\chi^2$. The chi-squared test was used for the difference of genotype and allele frequency between control and RA groups. The corresponding odd ratios (ORs) and 95% confidence intervals (CIs) of RA risk of individuals with genetic models including allelic, dominant and recessive models of selected SNPs were calculated using logistic regression analysis. In addition, the genetic association between the alleles of selected SNPs and autoantibody status (rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP)) were also performed. The p values were adjusted ($P_{adj}$) by Bonferroni correction in multiple comparisons. Statistical significance
was set at \( p < 0.05 \). The calculation power was analyzed by using the G*Power software.

Results

A total of 360 patients with RA and 560 healthy controls were included in the present study. The genotype distribution was in HWE in the control group for all the SNPs (\( p > 0.05 \))(Table 2). Moreover, our study obtained > 74.5% power at the 5% significant level (2-tailed)(Table 2).

| Genes  | SNPs            | Case  | Control   | OR[95%CI] (Allelic model) | OR[95%CI] (Dominant model) | OR[95%CI] (Recessive model) | HWE in controls | power |
|--------|-----------------|-------|-----------|--------------------------|---------------------------|-----------------------------|-----------------|-------|
| TLR1   | Arg80Thr (rs5743610) | 178/136 /46 | 270/234 /47 | 1.08 [0.88, 1.32], 0.47  | -                         | -                           | 0.18            | > 0.05 | 74.5 |
| TLR1   | Ser602Ile (rs5743618) | 207/126 /27 | 425/123 /12 | 2.21 [1.73, 2.81], < 0.0001 | < 0.001                   | < 0.01                     | 0.0012          | > 0.05 |
| TLR2   | Arg753Gln (rs5743708) | 256/96/8 | 433/122/5  | 1.38 [1.05, 1.81], 0.02  | 0.12                      | 0.18                       | -               | > 0.05 |
| TLR4   | Asp299Gly (rs4986790) | 360/0/0 | 560/0/0   | -                         | -                         | -                          | -               | > 0.05 |
| TLR4   | Thr399Ile (rs4986791) | 360/0/0 | 560/0/0   | -                         | -                         | -                          | -               | > 0.05 |
| TLR6   | Ser249Pro (rs5743810) | 222/116 /22 | 363/181 /16 | 1.22 [0.97, 1.53], 0.10  | -                         | -                          | 0.12            | > 0.05 |

\( ^a \) \( p \) value were calculated using Fisher’s exact test.

\( ^b \) The Bonferroni’s correction was carried out to adjust the \( p \) value.

Abbreviation: **"-": not significant; SNP, single nucleotide polymorphism; OR, odds ratio; 95% CI, 95% confidence intervals; -, not calculated; RA, Rheumatoid arthritis; \( p_{adj} \), \( p \) adjusted.

The association between TLRs genes polymorphisms and RA risk

Significant associations were detected between the recessive model of the TLR1 rs5743610 (OR[95%CI] = 1.60 [1.04, 2.46], \( p = 0.03 \)), TLR6 rs5743810 (OR[95%CI] = 2.21 [1.15, 4.27], \( p = 0.02 \)) and RA risk. However, the significant associations disappeared after the Bonferroni correction (rs5743610: \( p_{adj} = 0.18 \); rs5743810: \( p_{adj} = 0.12 \)) (Table 2).

Significant difference was found between the distributions of the allelic and dominant models of the TLR2 rs5743708 (allelic model: OR[95%CI] = 1.38 [1.05, 1.81], \( p = 0.02 \); dominant model: OR[95%CI] = 1.39 [1.02, 1.87], \( p = 0.03 \)) in RA and control groups. However, these significant associations did not
exist after the Bonferroni correction (p > 0.05) (Table 2).

Additionally, the frequencies of the allelic (OR[95%CI] = 2.21 [1.73, 2.81], p < 0.0001), dominant (OR[95%CI] = 2.33 [1.75, 3.09], p < 0.0001), and recessive models (OR[95%CI] = 3.70 [1.85, 7.41], p = 0.0002) of TLR1 rs5743618 were significantly different in RA group from that in the control group, even after the Bonferroni correction (p < 0.05).

Furthermore, no TLR4 rs4986790 and rs4986791 mutation was detected in both RA patients and control subjects (Table 2).

The association between risk allele in TLRs genes polymorphisms and clinical characteristics

Correlation between the TLR-1, -2, -4, and -6 risk alleles and the status of RF and anti-CCP in patients with RA was evaluated by regression analysis.

As shown in Table 3, 360 patients with RF and anti-CCP information available were divided into two groups (RF status: RF-positive (RF+) RA group and RF-negative (RF-) RA group; anti-CCP status: anti-CCP-positive (anti-CCP+) RA group and anti-CCP-negative (anti-CCP-) RA group). Results revealed that no significant association was detected between TLR1 rs5743610, rs5743618, TLR2 rs5743708, TLR4 rs4986790, rs4986791 and RF status, as well as anti-CCP status (p > 0.05). Moreover, the distribution of the TLR6 rs5743810 in RF+ (OR[95%CI] = 2.29 [1.42, 3.69], p = 0.0007) and anti-CCP+ (OR[95%CI] = 2.33 [1.39, 3.89], p = 0.001) RA groups were significantly different from those in RF− and anti-CCP− RA groups. And these significant associations existed even after the Bonferroni’s correction (p < 0.05) (Table 3).
Table 3
TLR1,2,4,6 gene alleles frequencies and autoantibody levels in patients with rheumatoid arthritis

| Genes | SNPs            | RF+ (freq.) | RF− (freq.) | p^a, OR[95%CI] | Padj | Anti-CCP+ (freq.) | Anti-CCP− (freq.) | p^a, OR[95%CI] | Padj |
|-------|-----------------|-------------|-------------|----------------|------|-------------------|-------------------|----------------|------|
| TLR1  | Arg80Thr (rs5743610) | 0.11        | 0.13        | 0.83 [0.48, 1.44], 0.5 | -    | 0.25              | 0.21              | 1.36 [0.86, 2.14], 0.19 | -    |
| TLR1  | Ser602Ile (rs5743618) | 0.33        | 0.29        | 1.39 [0.86, 2.25], 0.18 | -    | 0.34              | 0.32              | 1.19 [0.74, 1.92], 0.47 | -    |
| TLR2  | Arg753Gln (rs5743708) | 0.33        | 0.29        | 1.39 [0.86, 2.25], 0.18 | -    | 0.34              | 0.32              | 1.19 [0.74, 1.92], 0.47 | -    |
| TLR4  | Asp299Gly (rs4986790) | 0.00        | 0.00        | -               | -    | -                 | -                 | -               | -    |
| TLR4  | Thr399Ile (rs4986791) | 0.00        | 0.00        | -               | -    | -                 | -                 | -               | -    |
| TLR6  | Ser249Pr (rs5743810) | 0.32        | 0.22        | 2.29 [1.42, 3.69], 0.0007 | 0.0042 | 0.41              | 0.33              | 2.33 [1.39, 3.89], 0.001 | 0.006 |

^a p value were calculated using Fisher’s exact test.

Abbreviation: SNP, single nucleotide polymorphism; OR, odds ratio; 95% CI, 95% confidence intervals; -, not calculated; RF: rheumatoid factor; anti-CCP: anti-cyclic citrullinated peptide

Discussion

TLRs play an important role in both innate and adaptive immune responses [19]. In innate immunity, TLRs directly trigger the intracellular bactericidal mechanism or induce the production of immune inflammatory factors based on the recognition of pathogenic microorganisms to expand the non-specific defense [20]. In adaptive immune response, TLRs can induce dendritic cells to mature, activate them to secrete cytokines and chemokines, and induce their expression of costimulatory molecules [21]. In addition, TLRs could activate naive B cells, affect the intensity and quality of the memory T cell response, and initiate the CD8^+^T cell response against soluble protein antigen [22]. Thus, TLRs are widely involved in various pathways of specific immune response and in the occurrence and development of chronic immune inflammatory diseases. Furthermore, increased expression of TLRs was found in peripheral blood mononuclear cells, synovial tissues, synovial fluid, synovial macrophages and synovial fibroblasts in RA patients. And, TLR-2, -3, -4, -5, -7, -8 and -9 were also demonstrated to involve in the development of RA [23–24].

As a member of an important pattern recognition receptor, TLR2 can not only bind with exogenous ligands such as lipoproteins, peptidoglycan, lipophosphoric acid, lipophosphoric mannose, glycosyl, yeast
polysaccharides, and glycolipids, but also recognize endogenous danger associated molecular patterns (DAMPs) such as hyaluronic acid (HA), heat shock proteins (HSP), and high-mobility group protein 1 (HMGB1) [25]. Studies have shown that HSP72 can be actively or passively released to the outside of the cell, and can bind to TLR2 on the surface of immune cells to participate in immune response [26]. Additionally, TLR2 is involved in the recognition of HMGB1. HMGB1 is highly expressed in active RA, which can be used as an important indicator to evaluate the disease activity of RA [27]. There was a positive correlation between the expression of HMGB1 and TLR2 in active RA patients [28]. Furthermore, Li et al. showed that the expression of TLR2 in peripheral blood mononuclear cells increased in active RA patients, and the expression of TLR2 was closely related to disease activity indicators such as disease activity score (DAS), serum c-reactive protein, and blood sedimentation [29]. Blocking TLR2 can prevent the spontaneous release of cytokines in vitro synovial graft cultures of RA, suggesting that targeting TLR2 may play an important role in the treatment of RA [30]. However, little is known about the relation of TLR2 gene polymorphisms and the susceptibility of RA. In the present study, we found no association between the TLR2 rs5743708 and RA risk, as well as the RF and anti-CCP status, which was similar with the results reported by Sa’nchez et al. in a Spanish population [17]. Although, no significant association was found between TLR2 rs5743708 and RA in the present study, we could not rule out that the impact of TLR2 gene in the pathogenesis of RA. This SNP may be linked to other SNPs, which could regulate the expression or function of TLR. Further studies with larger number of subjects are needed to investigate the genetic association between TLR2 rs5743708 and RA risk.

TLR4 plays an important role in mediating immune response. It can recognize endogenous ligands including HSPs, fragments of hyaluronic acid, and fibronectin, which are released by cells undergoing stress, damage, or necrotic death, and are abundantly present in inflamed synovial joints of patients with RA [31]. Ren et al. showed that TLR4 expression on the surface of CD14+ monocytes in peripheral blood of RA patients was up-regulated and positively correlated with serum IL-18 level, while negatively correlated with DAS28, suggesting that TLR4 may indirectly participate in the pathogenesis of RA [32]. TLR4 rs4986790 and rs4986791 causing change in amino acid were
identified to reduce the response of TLR4 to stimulation with LPS and probably endogenous ligands [33]. Lines of studies have reported the association between TLR4 rs4986790 and rs4986791 and RA in multiple populations. However, the results were inconsistent in different ethnicity groups. Radstake et al. found that TLR4 rs4986790 was associated with RA susceptibility, but not with RA disease activity and prognosis in a Dutch population [15]. However, no association was found between the TLR4 rs4986790 and rs4986791 and RA in other Caucasian populations including French [18], Finn [34], Spanish [17], and British [16]. The results of the relationship of TLR4 rs4986790 and rs4986791 and RA in Asian populations were more complex. In the present study, both the TLR4 rs4986790 and rs4986791 were found to be not polymorphimic, which was similar with the previous results reported by Zheng et al. [14], Yuan et al. [35], and Kang et al. [36]. In addition, no significant association between the TLR4 rs4986790 and rs4986791 polymorphisms and RA risk was identified by meta-analysis [37–38]. Thus, we may conclude that the TLR4 rs4986790 and rs4986791 did not contribute to the pathogenesis of RA. These results need to be confirmed in larger number of case-control studies.

TLR1 and TLR6 are TLR2 co-receptors that increasing the number of ligands and inducing different transduction pathways [39–40]. Studies have revealed that TLR1 rs5743610 and rs5743618 were associated with complicated skin and skin structure infections (cSSSIs) [41] and Crohn's disease [42]. In addition, the TLR6 rs5743810 was shown to be associated with a reduced risk of childhood asthma [43] and an increased risk of invasive aspergillosis [44]. To our knowledge, this is the first study of the genetic association between the allelic, dominant and recessive models of TLR1 rs5743610, rs5743618, and TLR6 rs5743810 and RA susceptibility was conducted. Although, no evidence of which was detected in our study, we could not draw the conclusion that TLR1 rs5743610, rs5743618, and TLR6 rs5743810 were not associated with RA susceptibility for the relatively small sample size.

Notable, significant associations were observed between the TLR6 rs5743810, but not the TLR1 rs5743610, rs5743618 and RA status, as well as anti-CCP status, which was different form the results reported by Jaen et al. [18]. This inconsistent may due to the different genetic background in the two populations. For the function of this SNP is still hardly known. We hypothesized that TLR6 rs5743810
might be associated with disease severity by increasing autoantibody production. To confirm this result, functional study is necessary in the future.

Conclusions
Our results suggested that the allelic, dominant, and recessive models of TLR1 rs5743618 might be associated with the pathogenesis of RA. And the TLR6 rs5743810 might be associated with clinical characteristics (RF and anti-CCP antibody) in RA in Chinese Han population. Further studies of the TLR1 rs5743618 and TLR6 rs5743810 in ethnically well-defined populations as well as functional studies of this variation are warranted to evaluate their role in the pathogenesis of RA.

Abbreviations
RA: rheumatoid arthritis; TLR: toll-like receptor; SNP: single nucleotide polymorphism; EC: Ethical Committee; HWE: Hardy-Weinberg equilibrium; ORs: odd ratios; CIs: confidence intervals; RF: rheumatoid factor; anti-CCP: anti-cyclic citrullinated peptide; DAMPs: danger associated molecular patterns; HA: hyaluronic acid; HSP: heat shock proteins; HMGB1: high-mobility group protein 1; DAS: disease activity score; cSSSIs: complicated skin and skin structure infection;

Declarations

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors’ contributions
QYF and PYQ designed the experiments and drafted the manuscript. CBQ and SXW collected the samples and carried out the genotyping. SXW and PYQ contributed to the statistical analysis. QYF are project leader and planned the study. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.
Consent for publication

Consents to publish have obtained from all subjects or their guardians.

Ethics approval and consent to participate

The study was approved by the Ethical Committee of The First Affiliated Hospital of Soochow University (China) (EC/19021, 12/10/2019). Written informed consents for genetic analysis were obtained from all subjects or their guardians.

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