**INTRODUCTION**

Atopic dermatitis (AD), also known as eczema, is the most common chronic inflammatory skin disease, adversely affecting 10%–20% of the pediatric population worldwide.\(^1\) The disorder, characterized by intense itching and recurrent eczematous lesions, seriously reduces pediatric patients’ quality of life and increases health care burden.\(^2,3\) Although the exact pathophysiology of AD remains unclear, genetic predisposition, immune dysregulation, and epidermal barrier dysfunction have been thought to be the critical factors in the pathogenesis and development of AD.\(^1,4,5\)

**TLR4** polymorphisms as potential predictors of atopic dermatitis in Chinese Han children

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**Abstract**

**Background**: Toll-like receptor 4 (TLR4) is considered to be involved in the pathogenesis and progression of atopic dermatitis (AD). In the present study, we evaluated the relationship between TLR4 gene polymorphisms and the susceptibility or severity of AD among Chinese Han children.  

**Methods**: A total of 132 AD patients and 100 healthy controls were enrolled in this study. Four single-nucleotide polymorphisms (rs19277914, rs11536891, rs7869402, and rs11536889) of the TLR4 gene were genotyped by multiplex PCR combined with next-generation sequencing.  

**Results**: Our results showed that a significantly reduced risk for AD was associated with C allele \((p = 0.008; \text{OR} = 0.41, \text{C vs. T})\), TC genotype \((p = 0.022; \text{OR} = 0.41, \text{TC vs. TT})\), and TC + CC genotype \((p = 0.010; \text{OR} = 0.39, \text{TC + CC vs. TT})\) of TLR4 rs11536891. The frequency of the haplotype GCCG (rs1927914–rs11536891–rs7869402–rs11536889) in AD patients was lower than that in the controls \((p = 0.010; \text{OR} = 0.38)\). Moreover, the results indicated that a higher risk of severe AD was related to the T allele \((p = 0.019; \text{OR} = 2.97, \text{T vs. C})\) and the TC genotype \((p = 0.021; \text{OR} = 3.34, \text{TC vs. CC})\) of TLR4 rs7869402. A risk haplotype of TLR4 (GTTG) was found in severe AD patients \((p = 0.010; \text{OR} = 5.26)\).

**Conclusions**: Our data suggested that TLR4 rs11536891 polymorphism was associated with the susceptibility to AD in Chinese Han children. And TLR4 rs7869402 might confer the severity of pediatric AD patients.

**KEYWORDS**  
atopic dermatitis, polymorphisms, susceptibility, Toll-like receptor 4
Toll-like receptors (TLRs), a family of transmembrane proteins, recognize pathogen-associated molecular patterns and then enable the specific innate and adaptive immune responses. It is well established that TLRs are expressed in a variety of immune/inflammatory cells and associated with different infectious and immune-mediated diseases. Among ten functional TLRs, TLR4 has been shown to be linked to atopic diseases including AD through mediating pro-inflammatory signals and immune intervention. Panzer et al. reported that the expression of TLR4 increased gradually from the basement layer to the upper stratum spinosum, including the granular layer in AD patients, while it was mainly located in the basement layer in normal skin. Moreover, the level of TLR4 expression in peripheral blood monocytes of patients with AD was higher than that of healthy subjects regardless of the severity of the disease. The results of an animal experiment explored that TLR4 could attenuate the hapten-induced skin inflammation through the inhibition of langerin-positive dendritic cells migration, suggesting that TLR4 might serve as a modulator of inflammatory and immune response during AD development.

The human TLR4 receptor gene is located on the chromosome (9q32-33). TLR4 single-nucleotide polymorphisms (SNPs) have been indicated to be involved in the pathogenesis of various inflammatory and immune-related disorders such as rheumatoid arthritis, asthma, and ulcerative colitis. Nevertheless, the results of the genetic researches on the effect of TLR4 SNPs in systemic vasculitis were inconsistent. To our knowledge, previous studies on the relationship between TLR4 SNPs and AD have been only performed in Italian children, Russians, Ukrainian children, and German population. Up to now, there have been no studies on the linkage between TLR4 gene polymorphisms and AD in Chinese population. Therefore, the present study is carried out in this direction to explore the association of TLR4 gene SNPs with the susceptibility and severity of AD in Chinese Han children, so as to estimate the population-specific predictive effect of TLR4 genetic variation on AD.

2 | MATERIALS AND METHODS

2.1 | Subjects

In this study, the children with AD visiting the dermatology clinic of the Children’s Hospital of Zhejiang University School of Medicine were recruited from February 2021 to August 2021. The inclusion criteria were defined as follows: patients newly diagnosed with AD based on the AD diagnostic criteria of Hanifin and Rajka; less than 18 years old; all subjects and their parents agreed to participate in the study. Children with other skin diseases or any other systemic inflammatory and autoimmune disorders or a history of dermatological medication were excluded. The severity of AD was evaluated according to the Severity Scoring of Atopic Dermatitis (SCORAD) index. In order to analyze the correlation between TLR4 gene SNPs and the AD severity, the cases were divided into two subgroups, and named as mild-to-moderate subgroup (index: 0–50) and severe subgroup (index >50).

During the same period, 100 unrelated healthy controls with no history of atopic or any other autoimmune diseases were selected from the children who underwent routine physical examination in the same hospital. All cases and controls enrolled in this study were of Chinese Han ethnicity. Their demographic and clinical information including gender and age were collected and described in Table 1. The study was adopted by the Institutional Ethics Committee of the Children’s Hospital of Zhejiang University School of Medicine in accordance with the principles of the Declaration of Helsinki.

### 2.2 | Genomic DNA extraction and genotype assessment

Peripheral blood samples from each subject were collected in ethylenediaminetetraacetic acid (EDTA) vials (1 ml). Two hundred microlitres of peripheral blood was taken from each sample for DNA extraction and genotype assessment. Genomic DNA was extracted using the Biospin Genomic DNA Purification Kit (BIOER Technology; #BSC0651). Genomic DNA concentration and purity were determined by Nanodrop1000c spectrophotometer (Thermo Scientific). The integrity of DNA was then detected by agarose gel electrophoresis (1%). The qualified DNA samples were stored at −20°C until the genotype analysis and then at −80°C for long-term storage.

Four SNPs in TLR4 gene were selected including rs1927914, rs11536891, rs7869402, and rs11536889. Genotyping was evaluated by multiplex PCR combined with next-generation sequencing on Illumina X-10, a high-throughput genotyping platform of Shanghai BioWing Applied Biotechnology Company (http://www.biowing.com.cn/). The primers for TLR4 rs19...
27914 were 5'–GTTGATGGAGTCTACAAGAGTTTG–3' (forward) and 5'–TTGTAAAGCTTTTAGGACAGTGTC–3' (reverse). The primers for TLR4 rs11536891 were 5'–TCAAAACTGGAAAATATGACCACAG–3' (forward) and 5'–ACACACACTTTCAAAATAC–3' (reverse). The primers for TLR4 rs7869402 were 5'–TGGGATCCCTCCCCTGTAGC–3' (forward) and 5'–AGGAGCATGCCCCACAGG–3' (reverse). The primers for TLR4 rs11536889 were 5'–CTTTAGGGAGACACAGATGGCTG–3' (forward) and 5'–GAACATTCTTTTCTGGGAACCTTC–3' (reverse).

### 2.3 Statistical analysis

The software of Statistical Package for Social Science (SPSS) version 22.0 for Windows (IBM) was used to perform the statistical analysis. Continuous variables were expressed as mean ± standard deviation, and t test or variance analysis was used for comparison between groups. The categorical variables were expressed as percentage or ratio. Fisher’s exact test or Pearson’s chi-square test was used for comparison between groups. Genotypic frequencies of all subjects were checked for Hardy-Weinberg equilibrium (HWE) before analysis. HWE, linkage-disequilibrium, and haplotypes were performed by using Haploview version 4.2 program (D' > 0.5 and r^2 > 0.33 means strong linkage disequilibrium). All p values were bilateral, and p < 0.05 was the threshold for statistical significance. The correlation between TLR4 polymorphisms and susceptibility or severity of AD, was assessed under different genetic models (codominant, dominant, recessive). The unconditional logistic regression analysis was used to calculate the odds ratio (OR) and 95% confidence intervals (CIs) of the different groups or subgroups.

### 3 RESULTS

#### 3.1 General characteristics

This case-control study enrolled 132 AD patients and 100 healthy controls. As presented in Table 1, there were 73 males and 59 females in the case group, with a mean age of 2.6 ± 2.1 years. The control children with a mean age of 4.9 ± 1.9 years, consisted of 66 males and 34 females. No significant difference was found between the cases and controls in gender and mean age. Among these cases, 99 (75.00%) were confirmed as mild-to-moderate and 33 (25.00%) were diagnosed as severe in severity. There was no significant difference in gender and average age between the mild-to-moderate subgroup and the severe subgroup.

### TABLE 2 Allele frequencies of four TLR4 gene SNPs in AD cases and the controls

| SNPs     | Allele | MAF (A/B) | HWE-p (AD) | OR (95% CI) | p value |
|----------|--------|-----------|------------|-------------|---------|
| rs1927914| G/A    | 0.38      | 0.58       | 1.29 (0.89–1.87) | 0.183   |
| rs11536891| C/T    | 0.05      | 1.00       | 0.41 (0.20–0.81) | 0.008   |
| rs7869402| T/C    | 0.07      | 1.00       | 0.95 (0.45–1.99) | 0.889   |
| rs11536889| C/G   | 0.25      | 0.35       | 1.06 (0.69–1.64) | 0.780   |

Note: A: minor alleles; B: major alleles; Abbreviations: AD, atopic dermatitis; CI, confidence interval; HWE-p, p-value of Hardy-Weinberg equilibrium; MAF, minor allele frequency; OR, odds ratio; SNP, single nucleotide polymorphism; TLR4, Toll-like receptor 4.

*Statistically significant (p < 0.05).

#### TABLE 3 Genotype analysis of TLR4 rs11536891 in codominant, dominant, and recessive models in AD cases and the controls

| Genotype model | AD n = 132 [n (%)] | Control n = 100 [n (%)] | OR (95% CI) | p value |
|---------------|--------------------|--------------------------|-------------|---------|
| Codominant    |                    |                          |             |         |
| TT            | 118 (89.39)        | 76 (76.77)               | 1.00        |         |
| TC            | 14 (10.61)         | 22 (22.22)               | 0.41 (0.20–0.85) | 0.022   |
| CC            | 0 (0)              | 1 (1.01)                 | -           |         |
| Dominant      |                    |                          |             |         |
| TT            | 118 (89.39)        | 76 (76.77)               | 1.00        |         |
| TC + CC       | 14 (10.61)         | 23 (23.23)               | 0.39 (0.19–0.81) | 0.010   |
| Recessive     |                    |                          |             |         |
| TT + TC       | 132 (100.00)       | 98 (98.99)               | 1.00        |         |
| CC            | 0 (0)              | 1 (1.01)                 | -           | 0.190   |

Abbreviations: AD, atopic dermatitis; CI, confidence interval; OR, odds ratio; TLR4, Toll-like receptor 4.

*Statistically significant (p < 0.05).
The successful genotyping rate of the four SNPs was 99.57%–100%. The distribution of all SNP genotypes in the case group, the control group, and each subgroup conformed to the HWE test (p > 0.05).

3.2 | Gene polymorphisms in AD patients and healthy children

The allele frequencies of four TLR4 SNPs in AD cases and controls were shown in Table 2. The minor allele frequency (MAF) of TLR4 rs11536891 was significantly different between AD patients and the healthy children (p = 0.008). Compared with T allele, the C allele of rs11536891 in TLR4 gene was associated with a reduced risk of AD (OR = 0.41, 95% CI: 0.20–0.81).

The genotype frequencies of four TLR4 SNPs in AD cohort and healthy controls were further analyzed in three genetic models including codominant, dominant, and recessive models. As shown in Table 3, a significant relevance was found between TLR4 rs11536891 and AD susceptibility in both codominant and dominant model. The risk of AD in children with TC genotype was lower than that in children with TT genotype (OR = 0.41, 95% CI: 0.20–0.85, p = 0.022). Additionally, the frequency of rs11536891 TC + CC genotype was significantly decreased as compared to that in TT genotype (OR = 0.39, 95% CI: 0.19–0.81, p = 0.010).

Nevertheless, the allele and genotype frequencies of the other three SNPs (rs1927914, rs7869402, and rs11536889) displayed no significant differences between AD patients and the controls.

Moreover, we estimated the linkage disequilibrium and haplotype construction. The four polymorphic sites of TLR4 gene were in a state of linkage disequilibrium with one another in all participants (All D’ > 0.5 and r² > 0.33). As shown in Table 4, among the five haplotypes, the frequency of GCCG composed of rs1927914, rs11536891, rs7869402, and rs11536889 in TLR4 gene (TLR4/GCCG) was significantly lower in AD patients than in controls (OR = 0.38, 95% CI: 0.18–0.79, p = 0.010, GCCG vs. ATCG).

3.3 | Gene polymorphisms in mild-to-moderate cases and severe cases

As shown in Table 5, the MAF of TLR4 rs7869402 was significantly different between mild-to-moderate cases and severe cases (p = 0.019). The T allele of rs7869402 was associated with a higher risk of severe AD, compared with C allele (OR = 2.97, 95% CI: 1.15–7.66).

As shown in Table 6, TLR4 rs7869402 was significantly related to the severity of AD in genotype analysis (p = 0.021). The risk of severe AD in patients with TC genotype was significantly higher than that in patients with CC genotype (OR = 3.34, 95% CI: 1.22–9.14).

For allele and genotype frequency of the other three TLR4 SNPs (rs1927914, rs11536891, and rs11536889), no significant differences were observed between severe subjects and mild-to-moderate subjects.

All four polymorphic sites of TLR4 gene were in linkage disequilibrium with one another in AD patients (All D’ > 0.5 and r² > 0.33). As shown in Table 7, among the five haplotypes, the frequency of GTTG formed by rs1927914, rs11536891, rs7869402, and rs11536889 in TLR4 gene (TLR4/GTTG) was significantly higher in patients with severe AD (OR = 5.26, 95% CI: 1.51–16.67, p = 0.010, GTTG vs. ATCG).

4 | DISCUSSION

Genetic variation, immune disorder, and epidermal barrier dysfunction have been considered to be involved in the pathogenesis of AD. TLR4 is the main receptor of lipopolysaccharide, which participates in the perception of pathogens and triggers a pathogen-specific immune response. Moreover, TLR4 is expressed on the surface of a multiple kinds of skin cells and implicated in the occurrence of several inflammatory skin diseases. Both animal and clinical researches to date have established that TLR4, as an amplifier of inflammatory response, plays an important role in the initiation and progression of AD. Additionally, previous studies have demonstrated that some

| Haplotypes         | AD n = 132 [n (%)] | Control n = 100 [n (%)] | OR (95% CI)          | p value |
|--------------------|--------------------|--------------------------|----------------------|---------|
| (rs1927914, rs11536891, rs7869402, rs11536889) |                     |                          |                      |         |
| ATCG               | 100 (78.13)        | 65 (33.26)               | 1.00                 |        |
| GTCG               | 66 (25.21)         | 51 (25.84)               | 0.89 (0.54–1.45)     | 0.641   |
| ATCC               | 62 (23.54)         | 45 (22.74)               | 0.96 (0.58–1.59)     | 0.879   |
| GCCG               | 14 (5.30)          | 24 (12.16)               | 0.38 (0.18–0.79)     | 0.010*  |
| GTTG               | 18 (6.74)          | 10 (5.24)                | 1.11 (0.46–2.70)     | 0.810   |

Note: The haplotype frequencies under 0.05 was not included in the haplotype analysis.
Abbreviations: AD, atopic dermatitis; CI, confidence interval; OR, odds ratio; TLR4, Toll-like receptor 4.
*Statistically significant (p < 0.05).
TLR4 genetic variants are associated with the susceptibility to several atopic and autoimmune diseases, such as asthma, Henoch-Schönlein purpura, and rheumatoid arthritis.\textsuperscript{10,26,27} As far as we know, however, only a few studies were carried out to investigate the predictive role of TLR4 genetic risk in AD. For instance, a study performed in Italian children detected the prevalence of TLR4 \textsuperscript{-D299G (14.9%)} in the patients with AD.\textsuperscript{20} Another genotyping study in Russia showed a strong association between AD and TLR4 (Asp299Gly).\textsuperscript{21} Besides, the meta-analysis study further presented a correlation between the polymorphism of TLR4 rs4986790 and a high risk of AD in the Caucasian population.\textsuperscript{22} 

In Chinese Han children, our results revealed an association between TLR4 polymorphisms and the susceptibility to AD. In contrast with T allele and TT genotype, children with C allele and TC genotype of TLR4 rs11536891 have a lower risk of AD. There was a significantly decreased risk of having AD disease in children with haplotype of TLR4/GCCG, which were composed of SNP rs1927914, rs11536891, rs7869402, and rs11536889. Considering the differences in TLR4 gene polymorphism in different ethnic populations, the other four SNPs instead of TLR4 Asp299Gly (rs4986790) were selected according to the procedural screening program under the specific conditions, as well as the reports from the TLR4 SNP-related studies.\textsuperscript{12,27–29} So far, very few studies have focused on the relevance of these four TLR4 SNPs with AD, even though they have been confirmed to play roles in the pathogenesis of the atopic and inflammatory diseases.\textsuperscript{10,12,26,27} Thus, our results could provide baseline information for analyzing the etiologic effect and the risk predictive ability of TLR4 gene polymorphisms on AD.

In addition to the etiology of AD, studies on prediction of the severity of AD in patients remains challenging. Several inflammation-associated gene polymorphisms are linked with the severity of AD, which could be used to predict potential severity.\textsuperscript{30,31} Our results showed the association of C allele and CC genotype of TLR4

### TABLE 5 Allele frequencies of four TLR4 SNPs in patients with mild-to-moderate and severe AD

| SNPs      | Allele | MAF     | HWE-p | OR (95% CI)       | p value |
|-----------|--------|---------|-------|-------------------|---------|
|           | A/B    |         |       | Mild-to-moderate  | Severe  |
| rs1927914 | G/A    | 0.35    | 0.19  | 1.52 (0.87–2.68)  | 0.143   |
| rs11536891| C/T    | 0.05    | 1.00  | 0.58 (0.19–1.80)  | 0.341   |
| rs7869402 | T/C    | 0.05    | 1.00  | 2.97 (1.15–7.66)  | 0.019*  |
| rs11536889| C/G    | 0.23    | 0.78  | 0.75 (0.40–1.40)  | 0.364   |

Note: A: minor alleles; B: major alleles.

Abbreviations: AD, atopic dermatitis; CI, confidence interval; HWE-p, p-value of Hardy-Weinberg equilibrium; MAF, minor allele frequency; OR, odds ratio; SNP, single nucleotide polymorphism; TLR4, Toll-like receptor 4.

*Statistically significant (p < 0.05).

### TABLE 6 Genotype analysis of TLR4 rs7869402 in patients with mild-to-moderate and severe AD

| Genotype | Mild-to-moderate, n (%) | Severe, n (%) | OR (95% CI) | p value |
|----------|-------------------------|---------------|-------------|---------|
| C/C      | 89 (89.90)              | 24 (72.73)    | 1.00        |         |
| T/C      | 10 (10.10)              | 9 (27.27)     | 3.34 (1.22–9.14) | 0.021*  |

Abbreviations: AD, atopic dermatitis; CI, 95% confidence interval; OR, odds ratio; TLR4, Toll-like receptor 4.

*Statistically significant (p < 0.05).

### TABLE 7 Haplotype frequencies of TLR4 gene in patients with mild-to-moderate and severe AD

| Haplotypes (rs1927914, rs11536891, rs7869402, rs11536889) | Mild-to-moderate n = 99 [n (%)] | Severe n = 33 [n (%)] | OR (95% CI) | p value |
|----------------------------------------------------------|---------------------------------|-----------------------|-------------|---------|
| ATCG                                                     | 83 (42.25)                      | 17 (25.75)            | 1.00        |         |
| GTCG                                                     | 49 (24.92)                      | 17 (26.18)            | 1.89 (0.83–4.17) | 0.130   |
| ATCC                                                     | 44 (22.40)                      | 18 (26.86)            | 2.04 (0.93–4.55) | 0.074   |
| GCCG                                                     | 9 (4.55)                        | 5 (7.58)              | 2.86 (0.78–10.00) | 0.121   |
| GTTG                                                     | 10 (5.05)                       | 8 (11.70)             | 5.26 (1.51–16.67) | 0.010*  |

Note: The haplotype frequencies under 0.05 was not included in the haplotype analysis.

Abbreviations: AD, atopic dermatitis; CI, 95% confidence interval; OR, odds ratio; TLR4, Toll-like receptor 4.

*Statistically significant (p < 0.05).
rs7869402 with severe AD. Meanwhile, the present study provided evidence that TLR4/GTTG might be the risk factor for severe AD patients. Molecular mechanism correlation studies indicated that TLR4-associated signaling pathways including janus kinase-signal transducer and activator of transcription (JAK-STAT), tumor necrosis factor (TNF), and mammalian target of rapamycin (mTOR) pathways, play certain roles in the development of atopic dermatitis.32–34 That might be the potential reason why TLR4 polymorphisms could be associated with the severity of AD, and the relevant mechanisms need to be explored in further investigation.

However, there are some shortcomings in the present study. The main limitation is that the sample size is still not enough to draw a more powerful conclusion, although the participants in this study have been proven to be representative in evaluating the compliance with HWE balance. In future, the multicenter studies with a larger sample size are needed to further investigate the relationship between genetic variation in TLR4 and AD. Moreover, for ethical considerations, there is no in-depth examination on the expression of TLR4 in skin tissues of subjects, especially that in the skin of healthy controls. In addition, there were limited data on AD patients’ characteristics such as serum immunoglobulin E, complement 3, complement 4, and cytokines (interferon-γ, interleukin-4, interleukin-10). This information would be helpful to analyze the severity and molecular mechanism of AD.

In conclusion, the present study revealed that the TC genotype of TLR4 rs11536891 and the TLR4/GCCG haplotype confer the decreased susceptibility to AD in Chinese Han children. TLR4 rs7869402 TC genotype and the TLR4/GTTG haplotype might be used to predict a high risk of severe AD in Chinese pediatric population.

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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS
Jianrong Shi and Lin He designed the research, completed the experimental part of the study, collected the laboratory parameters and patients’ information, performed the statistical analysis, drew the figures, and drafted the manuscript. Ran Tao, Huiwen Zheng, Wei Li, Shuanghuang Huang, and Yunling Li completed the experimental part of the study and collected the laboratory parameters. Shiqiang Shang supervised the entire study and provided academic guidance throughout the study process.

DATA AVAILABILITY STATEMENT
The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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