Association of Toll-like receptor 2 polymorphisms with gout

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Abstract. Gout is the most common autoinflammatory arthritis characterized by elevated serum urate and recurrent attacks of intra-articular crystal deposition of monosodium urate (MSU) in tissues. The pathogenesis of gout has not been fully determined, although certain genetic factors are involved in the development of gout. Accumulated data suggested that MSU crystal-induced inflammation is a paradigm of innate immunity. As Toll-like receptors (TLRs) are the underlying mechanisms of the innate immune response, the present study aimed to investigate whether *TLR2* polymorphisms are associated with gout. Two single-nucleotide polymorphisms (Arg677Trp and Arg753Gln, rs5743708) in *TLR2* were genotyped by polymerase chain reaction-restriction fragment length polymorphism and the -196 to -174 del polymorphism was investigated using the allele-specific polymerase chain reaction in 431 individuals (215 patients with gout and 216 healthy controls). *TLR2* Arg677Trp and Arg753Gln genotyping indicated that all the positive samples were of the wild-type genotype. No significant differences in genotype (χ^2=1.686, P=0.430) and allele (χ^2=1.430, P=0.232) frequencies of the -196 to -174 del polymorphism between the patients with gout and the control groups was observed. Our results suggested that the *TLR2* Arg677Trp, Arg753Gln and the -196 to -174 del polymorphisms were not associated with susceptibility to primary gouty arthritis.

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Introduction

Gout is an inflammatory and immune disease caused by monosodium urate (MSU) deposition in the synovial membrane of the joints, synovial cyst, cartilage and other tissues. Gout is characterized as acute or chronic arthritis, gouty stone, joint deformity, chronic interstitial nephritis and uric acid urolithiasis. In the past, gout was referred to as the 'disease of the wealthy' due to its association with the quality of life. Epidemiological evidence suggests that gout affects ~3.9% of adults and 12.6% of the elderly (aged >80 years) in the United States (1).

The pathogenesis of gout remains unclear. The fact that <10% of patients with hyperuricosuria (2) develop gout indicates that genetic factors may contribute to the genesis and development of gout. A previous study (3) reported that the recognition of naked MSU crystals by specific toll-like receptors (TLRs), such as TLR2 (4), is a major factor in determining the inflammatory potential of MSU crystal deposits and the course of gouty arthritis. Polymorphisms of *TLR2* deteriorate the function of proteins associated with certain diseases, for example, the *TLR2* Arg753Gln polymorphism is associated with an increased risk of infective endocarditis (5), vitiligo (6) and atopic derma (7). Although the results of Hussein *et al* (8) indicated that the genotype and allele frequencies of *TLR2* Arg753Gln were not associated with asthma or allergic rhinitis, a significant correlation with disease severity was observed. Brown *et al* (9) reported that this mutation may abrogate TLR2 signaling in response to human cytomegalovirus. Furthermore, the -174 to -196 del of *TLR2* gene polymorphisms may confer an increased susceptibility to breast cancer development (10), gastric cancer (11) and prostate cancer (12). Therefore, the present study aimed to investigate the frequencies of *TLR2* gene polymorphisms (Arg677Trp, Arg753Gln and the -196 to -174 del) in patients with gout and explore the association between the polymorphisms and the susceptibility to gout in a Han Chinese population.

Table I. Clinical characteristics of the GA and control groups (means ± SD).

Characteristics	GA group (n=215)	Control group (n=216)	P-value
Age, year	54.59±13.23	52.64±10.58	0.091[b]
Gender, male, %	93.49	92.59	0.715[b]
Median disease duration, years	5	-	-
UA, μmol/l	477.70±106.64	319.59±58.03	<0.001[a]
GLU, mmol/l	6.02±1.27	5.57±1.34	0.001[a]
TG, mmol/l	2.14±1.41	1.60±0.75	0.000[a]
TC, mmol/l	5.05±1.08	4.91±0.85	0.150[b]

Data were compared by the Student's t-test. [a]P<0.05 and [b]P>0.05 compared to the control group. GA, gouty arthritis; SD, standard deviation; UA, serum uric acid; GLU, serum glucose; TC, total cholesterol; TC, triglycerides.

Materials and methods

Subjects. Unrelated patients (n=215) diagnosed with primary gouty arthritis (GA) were recruited from the Affiliated Hospital of North Sichuan Medical College (Nanchong, China) and the Affiliated Hospital of Medical College, Qingdao University (Qingdao, China) between January, 2008 and December, 2012. The clinical diagnosis of gout was established by the revised American College of Rheumatology classification criteria (13). The control group included 216 healthy subjects confirmed by medical examination at the Affiliated Hospital of North Sichuan Medical University during the corresponding period. All the subjects were of Han Chinese descent. The clinical data and measurements, including serum uric acid (UA), serum glucose (GLU), total cholesterol (TC) and triglycerides (TG), were assessed at the Department of Clinical Laboratory, the Affiliated Hospital of North Sichuan Medical University. The study was approved by the Ethics Committee and informed consent was obtained from all the participants.

Genomic DNA preparation. Whole blood samples (2 ml) were collected from each subject by the standard venipuncture method (5). Genomic DNA was extracted from whole blood samples using the TIANamp Blood DNA kit (Tiangen Biotech, Co., Ltd., Beijing, China) following the manufacturer's instructions.

Analysis of the TLR2 polymorphisms. The single-nucleotide polymorphisms, Arg677Trp and Arg753Gln in *TLR2* were genotyped by digestion with the restriction enzyme, *Aci*I (Fermentas, Burlington, Canada), following polymerase chain reaction (PCR) amplification. The procedure was as follows: an initial denaturation step at 94˚C for 5 min, amplification was performed by 38 cycles at 94˚C for 30 sec, 58˚C for 30 sec and 72˚C for 30 sec, followed by a final elongation cycle at 72˚C for 5 min and then digested with *Aci*I at 37˚C for 15 min. The products were electrophoresed on 3% agarose gel. Polymorphisms at *TLR2*-196 to -174 del were investigated using the PCR method following the procedures described by de Oliveira *et al* (11). Products were examined by electrophoresis in 1.5% agarose gel.

Statistical analysis. Statistical analyses were performed using SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA). A Student's t-test was performed to compare the clinical parameters between patients with gout and the healthy control subjects. Hardy-Weinberg equilibrium was assessed using the χ^2 test for each *TLR2* polymorphism. Significance of association was determined by χ^2 or Fisher's exact test. The P-value, odds ratios (OR) and 95% confidence intervals (CI) were calculated. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical parameters. A total of 431 subjects were enrolled in this study, including 215 patients with primary gout (200 men and 15 women) and 216 healthy controls (200 men and 16 women). The two groups were matched for age and gender. The UA levels of the GA group were significantly higher compared to that of the control group (P<0.05). Compared to the control group, the serum levels of GLU (P<0.05), TG (P<0.05) and TC (P>0.05) were increased in the GA group (Table I).

In the GA group, 20% of patients (43/215) exhibited high serum GLU levels (fasting glucose concentration was >6.11 mmol/l) and >30% of patients had hyperlipemia (fasting plasma TG concentration was >1.7 mmol/l, TC level was >5.72 mmol/l or both exceeded the normal value).

TLR2 gene polymorphisms. We genotyped three polymorphisms of the *TLR2* gene, Arg677Trp, Arg753Gln and -196 to -174 del (Table II). The positive rate of polymorphisms in the samples was 96.52% (207/215 in the GA group and 209/216 in the control group). The *Aci*I restriction enzyme identified the CCGC sequence, which existed in the wild-type sequence of Arg677Trp and Arg753Gln polymorphisms of *TLR2*. When the wild-type of the two polymorphisms was digested by *Aci*I, there were three bands of 38, 75 and 227 bp. Two bands (75 and 265 bp in Arg753Trp, 38 and 302 bp in Arg677Gln) were presented when the mutation of one polymorphism of *TLR2* had occurred. The amplified DNA of -196 to -174 del was 286 bp for the insertion allele and 264 bp for the deletion allele.

Table II. Primer sequences, restriction enzymes and fragment sizes for the toll-like receptor 2 gene.

Gene polymorphisms	Primers	Enzyme ˚C/time	Fragment, bp	Refs.
TLR2 R677W *TLR2* R753Q	F: 5'-GCCTACTGGGTGGAGAACCT-3' R: 5'-GGCCACTCCAGGTAGGTCTT-3'	*Aci*I 37˚C, 15 min	Total: 341 Wild-type: 38,75,227 R677W: 75,265 R753Q: 38,302	(14)
-196 to -174 del	F: 5'-CACGGAGGCAGCGAGAAA-3' R: 5'-CTGGGCCGTGCAAAGAAG-3'	-	ins/ins: 286 ins/del: 286,264 del/del: 264	(11)

F, forward; R, reverse.

Table III. Genotype frequencies of toll-like receptor 2 gene polymorphisms in the GA and control groups.

Gene polymorphisms	Genotype frequencies	GA group, n=215 (%)	Control group, n=216 (%)	Pearson χ^2	P-value	OR (95% CI)
TLR2 R677W						
	CC	207 (96.28)	209 (96.76)			
	TC	0	0			
	TT	0	0			
TLR2 R753Q						
	GG	207 (100)	209 (100)			
	AG	0	0			
	AA	0	0			
-196 to -174 del	ins/ins	27 (13.04)	24 (11.48)	1.686	0.430	
	ins/del	96 (46.38)	87 (41.63)			
	del/del	84 (40.58)	98 (46.89)			
	ins	150 (36.23)	135 (32.30)	1.430	0.232	1.191 (0.894-1.587)
	del	264 (63.77)	283 (67.70)			

GA, gouty arthritis; TLR2, toll-like receptor 2; OR, odds ratio; 95% CI, confidence interval; ins, insertion; del, deletion.

The samples showed three bands of Arg677Trp and Arg753Gln polymorphisms, indicating that the GA and control groups were of the wild-type genotype. Our results suggested that there were no significant associations between the Arg677Trp and Arg753Gln polymorphisms of *TLR2* with gout risk. However, different genotypes of the *TLR2* ins/del polymorphism were observed using the Hardy-Weinberg equilibrium. In the control group, the genotype frequencies were 11.48% for ins/ins, 41.63% for ins/del and 46.89% for del/del (χ^2=0.484, P=0.487); the allele frequency was 32.30% for the ins allele and 67.70% for the del allele. In the GA group, the genotype frequencies were 13.04% for ins/ins, 46.38% for ins/del and 40.58% for del/del; the allele frequency was 36.23% for the ins allele and 63.77% for the del allele. There were no significant differences in genotype (χ^2=1.686, P=0.430) and allele (χ^2=1.430, P=0.232) frequencies of the -196 to -174 del polymorphism between the GA and the control groups. The del allele was not associated with the risk of gout (OR=1.191, 95% CI: 0.894-1.587). The genotype and allele frequencies of TLR gene polymorphisms in the control and GA groups are presented in Table III.

Discussion

Gout is a disorder caused by the precipitation or deposition of MSU in tissues and organs. The pathogenesis of gout has not yet been determined; however, 1-2% of primary gout is induced by the defect of purine metabolic enzyme. Studies have reported (15) that inflammation and immunity, particularly innate immunity, is associated with the generation and development of gout.

Genetic variants of innate immune receptors may be associated with the risk of disturbances. The polymorphisms of *TLR2* may affect host defense, disease progression and are linked to certain disease susceptibilities. By contrast, the *TLR2* +597CC genotype exhibited protective effects against colorectal cancer decreasing the risk by 5-fold (16) in a Portuguese population. The *TLR2* Arg753Gln gene

polymorphism was associated with the increased levels of specific IgE in patients with allergic diseases (17). Thus, this mutation renders TLR2 signaling incompetent by impairing its tyrosine phosphorylation, dimerization with TLR6, and recruitment of the myeloid differentiation primary response gene 88 (MyD88) and MyD88-adapter-like proteins (18).

However, the correlation between gout and *TLR2* gene polymorphisms remains unclear. The present study investigated whether *TLR2* polymorphisms (Arg753Gln, Arg677Trp and -196 to -174 del) affect the development of gout in a Han Chinese population. Gel electrophoresis results presented three bands in all the samples indicating no mutations of the *TLR2* gene polymorphisms, Arg753Gln and Arg677Trp, in the GA and healthy control groups. Our results confirmed that the two polymorphisms of *TLR2* did not exist in subjects who exhibited the wild-type allele for Arg753Gln and Arg677Trp. These results are consistent with previous studies in India (19,20) and China (21,22).

The -196 to -174 del polymorphism in the *TLR2* gene causes a 22-bp nucleotide deletion, which alters the promoter activity of TLR2. The *TLR2* del/del genotype was reported to decrease the transactivation of the promoters. In a North Indian population, the variant allele of *TLR2* (ins/del + del/del) was found to increase the risk of bladder cancer (23). Additionally, the -196 to -174 del/del genotype of *TLR2* may increase the risk of late-onset Alzheimer's disease (24). In contrast to the results of de Oliverira *et al* (11), Zeng *et al* (25) found that the variant allele of *TLR2* (ins/del + del/del) significantly decreased the risk of gastric cancer in a Chinese population. By contrast, Hishida *et al* reported no association between the risk of gastric cancer and the *TLR2* -196 to -174 del polymorphism in a Japanese population (26). Our findings indicate that there were no significant differences in genotype and allele frequencies of the -196 to -174 del polymorphism between the GA and control groups. The del allele was not associated with the risk of gout in the Han Chinese subjects.

This study has reported that the serum levels of UA, GLU, TG and TC were higher in the GA group compared to those in the healthy control group. The fasting glucose concentration in 20% of patients with gout was abnormal and >30% of patients exhibited hyperlipemia. Results of the present study suggest that gout is a type of metabolic disease, which is accompanied by metabolic dysfunction. Diet control is required for patients with gout.

In conclusion, the *TLR2* polymorphisms, Arg753Gln, Arg677Trp and -196 to -174 del, were not significantly associated with the risk of primary GA in a Han Chinese population. Accumulated data have indicated that TLR2 plays a pivotal role in the pathogenesis of gout (27,28). TLR2 has a cytoplasmic Toll/interleukin(IL)-1 receptor (IL-1R) domain, which participated in the activation of downstream signaling pathways, including the activation of MyD88, interleukin-1 receptor-associated kinase, tumor necrosis factor receptor-associated factor, IκB kinase and nuclear factor-κB, and ultimately induced the expression of proinflammatory messenger RNA (mRNA), such as the mRNA of IL-1β, transforming growth factor (TGF)α and TGFβ1 (29). When chondrocytes are exposed to MSU crystals, this signaling pathway may be activated (30). Therefore, TLR2 not only recognizes naked MSU crystals, but also influences the release of inflammatory cytokines, such as IL-1β, which is the core factor inducing the symptoms and signs of GA (31). Further studies focusing on other SNPs of *TLR2* are required to determine whether there is an association between gout and *TLR2* gene polymorphisms.

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