Polymorphisms in the *NLRP3* gene and risk of primary gouty arthritis

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Abstract. The aim of the present study was to investigate the association between genetic variants in 17 tagSNPs of the *NLRP3* gene and the susceptibility to primary gouty arthritis. A genotype-phenotype analysis of 480 primary gout and 480 control patients was performed. Samples from all the patients were collected from The Affiliated Hospital of Medical College (Qingdao, China). Seventeen tagSNPs of the *NLRP3* gene were amplified using polymerase chain reaction (PCR) and MassARRAY technology was used for single nucleotide polymorphism (SNP) genotyping. The genetic frequency of rs7512998 was significantly different between the gout and control patients (P<0.05), whereas no significant differences were identified for the remaining SNPs. The 17 SNPs conformed to the Hardy-Weinberg equilibrium (HWE) in the control group (P>0.05). The haplotype association among the 17 SNPs of the *NLRP3* gene indicated that no individual SNP was significantly associated with primary gouty arthritis. CTATCAGCGCCCAGTGC was the most common haplotype in the case and control groups, with a frequency of 0.224 and 0.243, respectively. However, the odds ratios (ORs) of the 8 haplotypes were not identified to be significantly associated with gouty arthritis (P>0.05 for all the 8 haplotypes). To the best of our knowledge, this is the first study to investigate the association between SNPs of the *NLRP3* gene and the risk of primary gouty arthritis, although no significant association was identified. Further clinical studies and functional analysis are required to explore the potential associations between *NLRP3* gene polymorphisms and the risk of primary gouty arthritis.

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Introduction

Primary gout affects 1-2% of adults (1-7) and is one of the most common types of metabolic disease; a group of diseases characterized by arthralgia, dysfunction, gouty nephropathy and uremia. Although primary gout is a polygenic hereditary disease, dietary habits and lifestyle changes, including increased alcohol and excessive meat and seafood consumption, may interact with the genetic factors to facilitate the pathogenesis and development of gout. Additionally, insulin resistance, abdominal obesity, dyslipidemia, arterial hypertension, diabetes and metabolic syndrome are strongly associated with hyperuricemia and gout (8,9). Previous studies have investigated the candidate genes that control the production and clearance of uric acid, that lead to hyperuricemia (2,10-12). However, epidemiological evidence has demonstrated that <10% of patients with hyperuricemia develop gout (13,14), indicating that genes that are not linked to the metabolism of uric acid may also contribute to the susceptibility for this disease.

Gout is one of the most common types of autoinflammatory arthritis and is characterized by an elevated serum urate (uric acid) level and the intra-articular deposition of monosodium urate (MSU) crystals. Uric acid and MSU are known to act as host-derived danger-associated molecular patterns by directly activating NALP3. The activation of NALP3 leads to the formation of the inflammasome; a protein platform that mediates the processing of intracellular interleukin (IL)-1β into its active form (15-17). An association between *NLRP3* gene (which encodes NALP3) polymorphisms and autoinflammatory diseases, including type 1 diabetes and inflammatory bowel disease, has been suggested in previous studies (18,19). Furthermore, multiple mutations in the *NLRP3* gene have been reported to be associated with familial periodic fever syndromes, including Muckle-Wells syndrome (MWS), familial cold-induced autoinflammatory syndrome (FCAS) and neonatal-onset multisystem inflammatory disease (NOMID) (20-24). It has been shown that serum IL-1β levels in these patients are markedly increased (20-24). In each of these diseases, treatment with the recombinant human IL-1 receptor antagonist (IL-1Ra) anakinra induced the recovery of patients, which indicates that the pathogenesis of these syndromes is

Table I. Demographic and clinical characteristics of the study population (mean ± SD).

Characteristics	Gout patients (n=480)	Control patients (n=480)	P-value
Age (years)	52.48±13.12	60.57±9.45	<0.001[a]
Height (cm)	173.37±5.23	169.43±6.95	<0.001[a]
Weight (kg)	80.92±12.12	71.21±9.65	<0.001[a]
BMI (kg/m^2)	26.87±3.46	24.78±2.90	<0.001[a]
Circumference of waist (cm)	96.41± 8.89	91.60± 7.06	<0.001[a]
Circumference of hip (cm)	104.59±7.33	101.11±6.14	<0.001[a]
W/H ratio	0.92±0.52	0.91±0.51	<0.001[a]
Systolic blood pressure (mmHg)	136.75±19.84	132.48±16.49	<0.001[a]
Diastolic blood pressure (mmHg)	87.84±12.16	84.70±9.76	<0.001[a]
Blood glucose (mmol/l)	6.16±1.84	5.64±1.22	<0.001[a]
TG (mmol/l)	2.40±2.23	1.48±0.98	<0.001[a]
TC (mmol/l)	5.26±1.30	5.27±0.84	0.846
Urea nitrogen (mmol/l)	6.12±2.72	5.87±1.42	0.073
Creatinine (μmol/l)	91.45±33.53	94.17±15.93	0.110
Uric acid (μmol/l)	495.98±133.08	322.94±52.90	<0.001[a]

[a]Significant difference. SD, standard deviation; BMI, body mass index; W/H, waist/hip; TG, triglycerides; TC, total cholesterol.

strongly dependent on IL-1 (22). Gain-of-function mutations of the *NLRP3* gene were revealed in these patients, including missense mutations in MWS, chronic infantile neurological cutaneous and articular (CINCA) syndrome/NOMID and FCAS, which are located in exon 3 of the *NLRP3* gene (25). However, no previous studies have investigated the association of polymorphisms and haplotypes in the *NLRP3* gene with susceptibility to primary gout.

The aim of the present study was to investigate the association between genetic variants in 17 tagSNPs of the NLRP3 gene and the susceptibility to primary gouty arthritis, via the genotype-phenotype analysis of 480 primary gout and 480 control patients.

Materials and methods

Study population. A total of 480 primary gout and 480 control patients who were admitted to The Affiliated Hospital of Medical College, Qingdao University (Qingdao, China) were enrolled in this study. The gout patients were diagnosed by a clinical endocrinology physician, according to the criteria set by the American Rheumatism Association (26). Hyperuricemia was defined as uric acid levels >420 mmol/l in males and post-menopausal females, and >350 mmol/l in pre-menopausal females. Informed consent was obtained from all the patients. The clinical features of the patients were recorded. The criteria for inclusion in the control group were that patients did not have a family history of hyperuricemia and had no past medical history or potential indications of hypertension, coronary atherosclerotic heart disease, diabetes, hyperlipidemia, cancer, hepatic disease or renal disease.

The study protocol was in accordance with the Ethics Guidelines of the 1975 Declaration of Helsinki and was approved by The Affiliated Hospital of Medical College, Qingdao University.

Measurement of biochemical parameters. The plasma levels of blood glucose, creatinine, uric acid, total cholesterol (TC) and triglycerides (TG) in all the patients were measured using an automated multichannel chemistry analyzer (Toshiba 200FR; Toshiba Medical Systems, Tokyo, Japan).

Genotype analysis. Peripheral blood samples were obtained from the patients and DNA samples were preserved at -20˚C. Genomic DNA was isolated from the peripheral blood leukocytes and extracted according to the manufacturer's instructions (QIAamp DNA Blood Mini kit; Qiagen, Hiden, Germany). The ODs at 260 and 280 nm were determined using a UV spectrophotometer (DU-650; Beckman Coulter, Inc., Miami, FL, USA) to determine the DNA concentration.

Potentially functional SNPs of the *NLRP3* gene were selected from the HapMAP database (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap24_B36/). Seventeen tagSNPs were selected based on the standard (r^2, ≥0.08; MAF, >0.05). SNP genotyping was performed using the Sequenom MassARRAY iPLEX platform (Table II). The full length of the *NLRP3* gene is ~30 kbp, including 9 exons and 8 introns. A total of 12 pairs of primers were used for the polymerase chain reaction (PCR) and these were designed using the GeneTools and DNAMAN softwares, according to the manufacturer's instructions. PCR was carried out in a reaction volume of 20 μl, containing 50 ng of genomic DNA, 200 μM dNTP, 2.5 units of *Taq* DNA polymerase (Promega

Table II. Genotype distribution and allele frequencies of the *NLRP3* gene in the cases and control patients.

SNP	Position	Genotype frequency						P-value	P-value for the HWE in controls	Alleles	MAFs	MAFs	
		Cases			Controls								
		1/1	1/2	2/2	1/1	1/2	2/2					Cases	Controls
rs4925648	247580568	319	146	14	320	127	25	0.112	0.81	C/T	0.14/T	0.182	0.188
rs7512998	247583221	387	87	5	393	79	2	<0.05	0.59	C/T	0.136/C	0.101	0.088
rs4925650	247584075	153	237	90	158	209	105	0.23	0.91	A/G	0.357/A	0.434	0.444
rs12137901	247584591	281	164	29	272	145	46	0.08	0.41	C/T	0.285/C	0.234	0.256
rs10754555	247584643	205	212	60	214	183	73	0.17	0.90	C/G	0.418/G	0.348	0.350
rs3806266	247587065	410	66	3	408	63	2	0.89	0.92	A/G	0.025/A	0.075	0.071
rs7525979	247587408	294	167	19	301	149	22	0.53	0.65	C/T	0.122/T	0.214	0.204
rs3806268	247587477	132	247	101	141	218	113	0.26	0.96	A/G	0.41/A	0.532	0.530
rs4925651	247587531	479	1	0	469	3	0	0.31	0.95	G/T	0.053/T	0.001	0.003
rs3738448	247593142	291	170	19	297	152	23	0.50	0.59	A/C	0.075/C	0.783	0.790
rs10925019	247595850	266	185	29	268	171	33	0.68	0.91	C/T	0.169/T	0.253	0.251
rs4612666	247599070	142	247	91	149	229	96	0.62	0.66	C/T	0.387/T	0.447	0.444
rs10754557	247599232	245	196	38	242	185	47	0.53	0.87	A/G	0.484/G	0.284	0.294
rs12143966	247601357	132	239	108	132	231	106	0.98	0.99	A/G	0.377/A	0.475	0.472
rs12239046	247604258	142	246	91	148	229	95	0.68	0.69	C/T	0.402/T	0.447	0.444
rs10159239	247607642	154	229	97	134	230	109	0.36	0.28	A/G	0.417/G	0.441	0.474
rs12565738	247609328	401	74	4	413	61	0	0.06	0.20	C/T	0.167/T	0.086	0.064

SNP, single nucleotide polymorphism; HWE, Hardy-Weinberg equilibrium; MAFs, minor allele frequencies.

Table III. Association between 8 haplotypes of the *NLRP3* gene and the risk of gouty arthritis.

Haplotype	Cases (freq)	Controls (freq)	χ^2	P-value	Odds ratio (95% CI)
CCGTGAGCGCCCAGTAT	44.96 (0.048)	36.93 (0.041)	0.5	0.475374	1.178 (0.751-1.846)
CTATCAACGCCCAGCGC	45.98 (0.049)	38.77 (0.043)	0.4	0.54526	1.146 (0.737-1.783)
CTATCAGCGCCCAACAC	83.23 (0.088)	74.51 (0.083)	0.2	0.651918	1.080 (0.774-1.507)
CTATCAGCGCCCAGTGC	211.20 (0.224)	216.97 (0.243)	0.8	0.375724	0.901 (0.715-1.135)
CTGCGGGCGCTTGACAC	43.71 (0.046)	47.99 (0.054)	0.5	0.497148	0.863 (0.564-1.320)
CTGTCGGTGACTAACAC	105.93 (0.112)	93.73 (0.105)	0.4	0.543856	1.098 (0.812-1.485)
CTGTCGGTGACTAGTGC	44.57 (0.047)	38.92 (0.044)	0.2	0.663469	1.104 (0.708-1.721)
TTGCGGGCGCTTGACAC	87.18 (0.092)	90.73 (0.101)	0.4	0.550771	0.908 (0.662-1.246)

CI, confidence interval; freq, frequency.

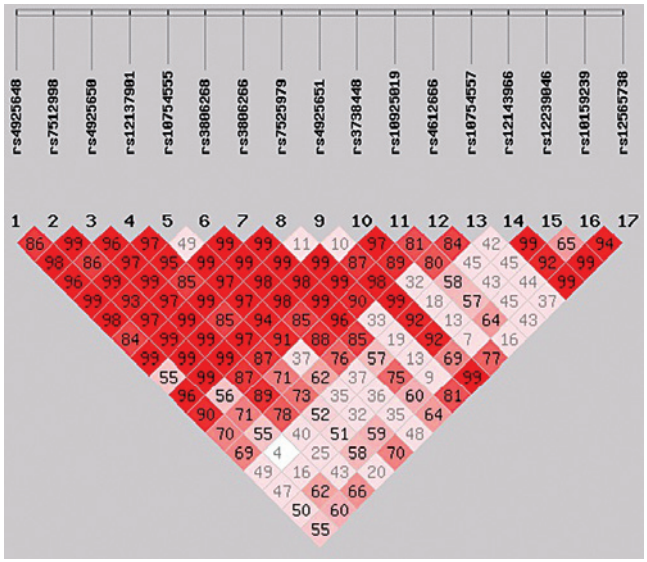

Figure 1. Linkage disequilibrium (LD) pattern among 17 single nucleotide polymorphisms using Haploview analysis.

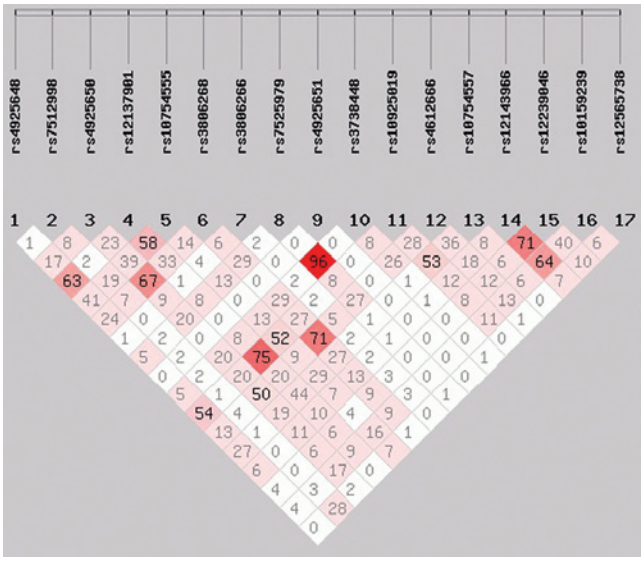

Figure 2. The r^2 measure of linkage disequilibrium (LD) among 17 single nucleotide polymorphisms using Haploview analysis.

Corporation, Madison, WI, USA) and 200 μM of primers. The conditions of the PCR were as follows: 94˚C for 2 min, 35 cycles of 94˚C for 30 sec, an annealing temperature reduced to 64˚C for 30 sec and 72˚C for 1 min. The PCR products were analyzed using electrophoresis on 1.0% agarose gel. For quality control, genotyping was performed without knowledge of the case/control status of the subjects and 5% of the total number of case and control patients were selected at random and re-genotyped by different investigators; the reproducibility was 100%.

Statistical analysis. Stata 8.0 (StataCorp, College Station, TX, USA) was used to perform statistical analysis. Continuous variables are expressed as the mean ± standard deviation (SD), while categorical variables are expressed as frequencies and percentages. The χ^2 test and Student's t-test were used to compare clinical characteristics between the two groups. The χ^2 test was also used to determine the Hardy-Weinberg equilibrium (HWE) in the control patients. Differences between the non-continuous variables, genotype distribution and allele frequency were assessed using the χ^2 test. Haploview 4.2 was used to calculate linkage disequilibrium blocks and haplotype association risk (27). The odds ratio (OR) and 95% confidence interval (CI) were evaluated using binary logistic regression analysis. Comparisons were two-sided and P<0.05 was was considered to indicate a statistically significant difference.

Results

Subject characteristics. The clinical characteristics of the subjects are provided in Table I. The gout patients had significantly higher blood uric acid, triglyceride and blood glucose levels, body mass index and waist-hip ratio compared with the control patients (P<0.01). No significant difference was observed in serum cholesterol, urea nitrogen and creatinine levels between the two groups (Table I).

HWE and linkage disequilibrium (LD)/haplotype analysis. The genetic frequency of rs7512998 was significantly different between the gout and control patients (P<0.05; Table II),

whereas no significant differences were observed for the remaining SNPs. Seventeen SNPs conformed to the HWE in the control patients (P>0.05; Table II). The minor allele frequencies of SNPs containing rs4925651, rs3806266 and rs3738448 were notably low (<10%).

To investigate the haplotype association among the 17 SNPs of the *NLRP3 gene*, the r^2 measure of LD was estimated and demonstrated that only one polymorphism, rs7525979, had a high LD (r^2>0.9; Figs. 1 and 2). No individual SNP was demonstrated to be significantly associated with primary gouty arthritis.

Among 134 haplotypes in the 17 SNPs, 8 haplotypes of the *NLRP3* gene accounted for 98% of the subjects and were included in the analysis (Table III). The haplotype CTATCAGCGCCCAGTGC was the most common among the case and control groups, with a frequency of 0.224 and 0.243, respectively. However, the ORs of the 8 haplotypes were not identified to be significantly associated with gouty arthritis (P>0.05 for all the 8 haplotypes).

Polymorphisms of the NLRP3 gene and primary gouty arthritis. Conditional logistic regression analysis demonstrated that the 17 SNPs were not significantly different between the groups and no significant ORs for the risk of gouty arthritis were noted (data not shown).

Discussion

To the best of our knowledge, the present study is the first investigation on the potential association of the risk of gouty arthritis with polymorphisms and haplotypes in a presumptive promoter region of the *NLRP3* gene. However, this study did not demonstrate a significant association between SNPs of the *NLRP3* gene and gouty arthritis. Female gout patients were not included in the present study, since the incidence of gouty arthritis in females is lower compared with that in males.

A previous genome-wide association study demonstrated that the NLRP3 inflammasome gene is associated with a number of autoimmune diseases, including familial cold urticaria (28) and MWS (29), and numerous inflammatory diseases (30), including Crohn's disease (31), obesity-induced inflammation and insulin-resistant FCAS (32). The activation of NLRP3 inflammasomes results in an inflammatory response driven by the secretion of IL-1β, a pro-fibrotic cytokine, which is essential in the pathogenesis of inflammation-induced pulmonary fibrosis (33). Furthermore, *NLRP3* inflammasomes induce the exposure of MHC-II on macrophage/antigen-presenting cell surfaces for a rapid non-self antigen presentation (34) and are important in the maturation and activation of dendritic cells (35,36). Thus, the activation of *NLRP3* drives local inflammation and the acquired immune response. More than 30 different SNPs located within exon 3 of the *NLRP3* gene that encode the nucleotide binding site domain and boundary regions have been identified (32). However, results of the present study have shown that mutations of the *NLRP3* gene were not associated with the risk of gouty arthritis. There are several possible explanations for this result: The full-length of the *NLRP3* gene is ~30 kbp, including 9 exons and 8 introns; in the present study, only 17 SNPs were selected from 388 SNPs of the *NLRP3* gene, which may not account for all the potentially susceptible SNPs. Furthermore, the sample size in this study was small and the statistical power to determine the authentic susceptible SNP loci in low allele frequencies was low. Therefore, further studies are required to confirm the results of the present study.

There were a number of limitations in the present study. Firstly, we focused solely on genetic variants and only 17 SNPs were selected among 388 SNPs of the *NLRP3* gene. Therefore, more functional studies on the effects of genetic variants on transcription factor binding activity and changes at the protein level are required. Secondly, although the association between polymorphisms in the *NLRP3* gene and the risk of gouty arthritis was investigated, the potential association between additional risk factors and gouty arthritis were not assessed. Therefore, a more detailed data analysis may allow the association between additional risk factors and genetic variants to be elucidated. Thirdly, the sample size of the present study was small and all the subjects were of the same ethnicity; thus, our results may not be representative of the various ethnicities in China and other countries. The experiments performed in this study should be repeated in other independent populations using larger sample sizes. Moreover, the gout phenotypes were assessed using the medical history of the patients. The data for several phenotypic disease characteristics, including the duration of gout history, disease location and whether the patient had metabolic syndrome, insulin resistance, cardiovascular disease or chronic syndrome, were not available. Therefore, further studies should consider the detailed clinical histories of the patients.

To the best of our knowledge, this is the first study to investigate the association between SNPs of the *NLRP3* gene and the risk of primary gouty arthritis; however, no association was detected. Clinical studies and functional analysis are required to investigate the mechanisms underlying the association between *NLRP3* gene polymorphisms and/or mutations and the risk of primary gouty arthritis.

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