Correlation of Polymorphisms of Natural Resistance-Associated Macrophage Protein 1 (*NRAMP1*) Gene and Smoking with the Risk of Rheumatoid Arthritis in Chinese Han People

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Background: In this study we report on the possible connection between single nucleotide polymorphisms (SNPs) in natural resistance-associated macrophage protein 1 (*NRAMP1*) gene and the risk of rheumatoid arthritis (RA) in the Chinese Han population.

Material/Methods: A total of 248 participants consisting of 116 RA cases and 132 healthy individuals were recruited for the current study. Genotyping for *NRAMP1* gene polymorphisms was implemented using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The chi-square test was used to detect discrepancies in genotype and allele frequencies between the RA case group and the control group. Odds ratios (ORs) with 95% confidence intervals (CIs) was used to evaluate relative risk of RA. The results were adjusted by logistic regression analysis.

Results: The TT genotype and T allele in rs17221959 showed dramatically different distribution between RA cases and healthy controls. After adjustment, TT genotype (OR=0.338, 95%CI=0.278–1.214, *P*=0.028) and T allele (OR=0.608, 95%CI=0.298–0.956, *P*=0.005) showed close association with reduced risk of RA. For rs1059823, no obvious diversity was uncovered in either genotype or allele distribution between the 2 groups. Interaction analysis showed that smoking decreased the protective function of TT in rs17221959.

Conclusions: This study suggested that the TT genotype and T allele in rs17221959 decreased RA risk. Smoking could decrease the protective effect of TT.

MeSH Keywords: **Arthritis, Juvenile • Multidrug Resistance-Associated Proteins • Polymorphism, Genetic • Smoking**

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Background

Rheumatoid arthritis (RA) is a common autoimmune disease which effects the inflammation of synovial joints and multiple organ [1], which could cause disability, systemic complications, and other symptoms. There are various cell-types associated with the development of RA such as macrophages, T-cells, B-cells, chondrocytes, and so on [2]. As a result, the pathology of RA is multifarious and complex. Generally speaking, the progress of the autoimmune reaction can be caused by endogenous or environmental factors [1]. Genetic and environment factors may play an important role in immune system activities and productive inflammatory responses [3]. In recent years, various risk factors for the onset of RA have been determined by many research studies, for example, genetic factors [4] including *PADI4* [5] and *SLC22A4* [6], environmental factors [7] such as smoking [8], and life habits factors such as diet [9], coffee intake [10], and alcohol [11]. All of these factors have different protective or risk roles in RA [12], but the exact mechanisms remain unknown.

The natural resistance associated macrophage protein 1 (*NRAMP*1) gene, also known as solute carrier family 11-member a1 (*SLC11A1*) gene, is associated with macrophage activation pathways [13]. The *NRAMP1* gene has been reported to be correlated with autoimmune and infectious diseases [14,15]. NRAMP1 is also a member of the metal transporter protein family, with the function of transferring iron (Fe) ions across the phagosome membrane [16]. As we know, the development of RA is associated with macrophage cell-types, the function of which are maintained by having enough Fe ions [17]. Even the mild loss of Fe ions can do significant damage to the immune status and to infection control [18]. The association between *NRAMP1* polymorphisms and the risk of RA has seldom been studied, and when studied, the results have been inconsistent.

In our current study, we measured the potential connection of *NRAMP1* polymorphisms with RA susceptibility in Chinese Han people. In order to survey the correlation of *NRAMP1* polymorphisms and smoking with the risk of RA, a further interaction analysis was performed. Thus, our study results could provide more data and the molecular basis for further research on pathogenesis of RA.

Material and Methods

RA patients and controls

This study included 116 RA patients and 132 healthy controls. The patients with RA were recruited from the Department of Rheumatism of West China Hospital, Sichuan University between September 2014 and October 2015. The criteria used for patient's diagnosis of RA were the 1987 revised criteria of the American College of Rheumatology [19]. Patients with cancer, or abnormal liver or renal function tests were excluded from this study. The healthy controls were recruited randomly from West China Hospital, Sichuan University between September 2014 and October 2015. The healthy controls were checked by normal physical tests. Distribution of age and gender in the control group were well matched with those in the RA case group ($P>0.05$). Smoking status of all the participants was also included in the study data. All the RA patients and controls were from the Chinese Han population without blood relationship with each other.

Ethics approval was obtained from the Research Ethics Committees of West China Hospital, Sichuan University (IRB: 2014-126, dated December 12, 2012). Written informed consent was obtained from all the participants after they were informed with the details and purpose of the study. The sample collection process was carried out in accordance with the national ethics criteria for human genome research.

Genomic DNA extracted

Peripheral venous blood samples of 4 mL were obtained from each individual by standard venipuncture. DNA was extracted following the instructions of the manufacturer (Tiangen Biotech Co., Ltd., Beijing, China). The DNA samples were stored in the refrigerator at −20°C for future application.

Genotyping

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was carried out to identify the genotypes of the 2 polymorphisms. The region consisting of single nucleotide polymorphisms (SNPs) of *NRAMP1* was amplified using primers designed by Primer Premier 5 software according the general primer design principles (Table 1).

The PCR reaction system was 25 μL, which consisted of 4 μL DNA template, l μL forward primer, l μL reverse primer, 2.5 μL 10× Buffer, 2 μL MgC1$_2$, 2.5 μL dNTPs, 1 μL Taq enzyme, and 11 μL ddH$_2$O. The reaction system was mixed well.

The PCR conditions for rs17221959 were initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 7°C for 3 minutes. The PCR conditions for rs1059823 were initial denaturation at 95°C for 8 minutes followed by 40 cycles of 95°C for 20 seconds and 56°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. PCR products were preserved at −2°C for standby application.

Table 1. Primer sequences and restriction enzymes of polymorphisms for *NRAMP1* gene.

NRAMP1	Position	Primer	Restriction enzyme
rs17221959	Chr2 10879	F: 5'-CTTGTCCTGACCAGGCTCCT-3'	Nar I
		R: 5'-CATGGCTCCGACTGAGTGAG-3'	
rs1059823	Chr2 18093	F: 5'-CTGGACCAGGCTGGGCTGAC-3'	Msp I
		R: 5'-CCACCACTCCCCTATGAGGTG-3'	

Restriction enzymes digestion was finished in 20 µL reaction system, including 1.5 µL restriction enzyme, 8 µL PCR products, 1.5 µL 10× Buffer, and 9 µL ddH$_2$O. The digested products were separated by electrophoresis on 1.5% TBE agarose gel to confirm the size of amplicons.

Statistical analysis

The direct count method was implemented to calculate the frequencies of genotype and allele in the RA case group and the control group. Hardy-Weinberg equilibrium (HWE) was estimated using the chi-square test. Discrepancies in the frequencies of the genotype and allele of *NRAMP1* gene polymorphisms between the RA case group and the control group were compared by the chi-square test. Effects of genotypes and alleles on RA were evaluated by odds ratios (ORs) with 95% confidence intervals (CIs). Moreover, the results were adjusted using the logistic regression analysis, and the confounding factors included age, gender, smoking, family history, and comorbidities. When $P<0.05$, differences were considered to be statistically significant. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) 18.0 for Windows (Chicago, IL, USA).

Results

Demographic and clinical characteristics

In our study, there were 116 patients with RA, including 42 females and 74 males with an age range of 52±7.6 years. The control group included 51 females and 81 males with the age range of 53.8±8.2 years. The RA case group and the control group were matched in age and gender ($P=0.552$ and $P=0.693$, respectively). Smoking status was divided into 2 groups: smoking and non-smoking. The smoking group included 88 RA patients and 89 control participants; the non-smoking group included 28 RA patients and 43 control participants. In the RA case group, 75.86% of RA patients were smokers; the percentage in the control group was 67.42%. There was no significant different between the 2 groups ($P=0.142$). There were 32 RA cases who had hypertension, whereas, there were only 12 control participants diagnosed with hypertension ($P<0.001$).

There were 10 RA patients confirmed with diabetes, and 14 control participants diagnosed with diabetes. In addition, 16 RA patients had coronary artery disease, and 10 control participants exhibited coronary artery disease. The distributions of diabetes and coronary artery diseases were similar between the 2 groups ($P>0.05$ for both groups). In addition, the presence of family history of RA was significantly higher in the RA group than in the control group (45 RA patients versus 12 control participants, $P<0.001$) (Table 2).

Distributions of genotype and allele and the risk of RA

In the current study, the RA case group and the control group could represent the general populations with which the genotype frequencies of the 2 SNPs were in agreement with HWE. The distributions of genotypes and alleles in rs17221959 were markedly different. Frequencies of genotype CC, CT, and TT were 68.10%, 27.59%, and 4.31% in RA cases, and 53.03%, 34.85%, and 12.12% in control participants, respectively (Table 3), while the difference for the genotype TT had statistical significance ($P=0.012$). TT genotype might decrease the risk of RA with OR=0.277 (95%CI=0.096–0.795). A similar result was also found for the T allele ($P=0.003$). T alleles for rs17221959 play a protective role in the risk of RA (OR=0.527, 95%CI=0.344–0.807). After adjustment using logistic regression analysis, TT genotype (OR=0.338, 95%CI=0.278–1.214, $P=0.028$) and T allele (OR=0.608, 95%CI=0.298–0.956, $P=0.005$) also showed close association with reduced risk of RA. Similar distributions ($P>0.05$) in the genotypes GG, GA, and AA, and the alleles G and A for rs1059823 were also observed.

Interaction analysis for genotype distributions and smoking

Frequencies of genotype CC, CT, and TT for "never smoked" participants were 17.24%, 5.17%, 1.24% in the RA case group and 13.64%, 11.36%, and 7.58% in the control group (Table 4). Compared with the control group, TT genotype decreased the risk of RA with OR=0.180 (95%CI=0.035–0.934). For the smoking participants, distribution of CC, CT, and TT did not show significant effects for the risk of RA. Comparing the smoking and non-smoking groups, smoking did increase the risk of RA.

Table 2. Demographic and clinical characteristics of the patients and controls.

Characteristic	Case (n=116)		Control (n=132)		P Value
Gender (Female/Male)	42/74		51/81		0.693
Age (mean ±SD) (years)	52.4±7.6		53.8±8.2		0.552
Smoking status (%)					
Current or ever	88	(75.86)	89	(67.42)	0.142
Never	28	(24.14)	43	(32.58)	
Hypertension (%)					
Yes	32	(27.59)	12	(9.09%)	<0.001
No	84	(72.41)	120	(90.91%)	
Diabetes (%)					
Yes	10	(8.62)	14	(10.61)	0.598
No	106	(91.38)	118	(89.39)	
Coronary artery disease (%)					
Yes	16	(13.79)	10	(7.58)	0.111
No	100	(86.21)	122	(92.42)	
Family history of RA (%)					
Yes	45	(38.79)	12	(9.09%)	<0.001
No	71	(61.21)	120	(90.91%)	

Table 3. Counts and frequencies of genotype and allele for *NRAMP1* polymorphisms in case and control groups.

Genotype/ allele	Case (n=116)		Control (n=132)		Rough		Adjustment		P_{HWE}
	n	%	n	%	*P*	OR (95%CI)	*P*	OR (95%CI)	
rs17221959									0.0612
CC	79	68.10	70	53.03	–	–	–	–	
CT	32	27.59	46	34.85	0.086	0.616 (0.354–1.073)	0.102	0.728 (0.278–1.214)	
TT	5	4.31	16	12.12	0.012	0.277 (0.096–0.795)	0.028	0.338 (0.069–0.912)	
C	190	81.90	186	70.45	–	–	–	–	
T	42	18.10	78	29.55	0.003	0.527 (0.344–0.807)	0.005	0.608 (0.298–0.956)	
rs1059823									0.1105
AA	64	55.17	66	50.00	–	–	–	–	
AG	39	33.62	49	37.12	0.476	0.821 (0.477–1.413)	0.552	0.901 (0.396–1.521)	
GG	13	11.21	17	12.88	0.560	0.789 (0.354–1.755)	0.631	0.882 (0.465–1.923)	
A	167	71.98	181	68.56	–	–	–	–	
G	65	28.02	83	31.44	0.406	0.849 (0.577–1.250)	0.555	0.918 (0.428–1.4670	

HWE – Hardy-Weinberg equilibrium; The values of *P* and OR were adjusted by clinical characteristics of subjects.

Discussion

In this study, we explored the possible correlation of *NRAMP1* polymorphisms with RA risk in Chinese Han people.

The distribution of genotypes and alleles for rs17221959 and rs1059823 was compared between the RA case group and the control group; significant deviations were found for TT and T distribution of the polymorphism rs17221959 between the RA

Table 4. Interaction analysis for the polymorphism of *NRAMP1* and different smoking status.

Smoking status	Genotype	Case (n=116)		Control (n=132)		OR (95%CI)
		n	%	n	%	
Never	CC	20	17.24	18	13.64	–
	CT	6	5.17	15	11.36	0.360 (0.115–1.127)
	TT	2	1.72	10	7.58	0.180 (0.035–0.934)
Smoking	CC	59	50.86	52	39.39	1.021 (0.488–2.136)
	CT	22	18.97	31	23.48	0.639 (0.276–1.478)
	TT	3	2.59	6	4.55	0.450 (0.098–2.068)

case group and the control group. A protective function was found for TT genotype and T allele for the risk of RA. But no evident dissimilarity was detected for rs1059823 between the 2 groups. Further interaction analysis for rs17221959 of *NRAMP1* gene for smoking status was performed; the results suggested that smoking might decrease the protective function of the TT genotype for the risk of RA.

This is the first study of a potential connection between *NRAMP1* polymorphisms and RA susceptibility in the Chinese Han population. A study of the association of *NRAMP1* gene and RA for a Korean population was reported by Yong et al. [20], and significant correlation was found between 3 polymorphisms (823C/T, D543N, and 1729+55del4) of *NRAMP1* and the risk of RA. The association of 823C/T (17221959) and RA is consistent with our results. Our results were also supported by a study by Yen et al. for a population in the Taiwan Province in China [15]. A study investigating the possible function of *NRAMP1* gene polymorphisms in susceptibility and clinical outcome of RA for a Spanish population was performed by Rodriguez et al. [21], and found effects on RA with radiological severity by promoter polymorphisms *NRAMP1* gene. Rs1059823 has been reported to be associated with tuberculosis in East India [22], type 1 diabetes of European ancestry population [23], and chronic obstructive pulmonary disease of a Korean population [24].

RA is one of the chronic inflammatory diseases which could be caused by auto-reactive immune responses. NRAMP1 plays a major role in modulating vesicle trafficking of macrophages [25]. RA onset is reported to be associated with tuberculosis (TB) [26]. The *NRAMP1* gene a number of effects on the pathways of macrophage activation. These effects may not only increase resistance to infections but also cause susceptibility

to autoimmune diseases. There are many research studies supporting the association of TB and *NRAMP1* gene polymorphisms. A study performed by Fernández-Mestre et al. provided support for 3'UTR variants linked to TB occurrence [27]. Polymorphic genes have been related to TB in a Chinese population, and were also identified by a study of Wu et al. [25]. The immune traits of *NRAMP1* gene have been identified in an animal model [28].

RA is a complex disease, and there are many factors that influences the risk of RA. As a risk factor of most diseases, smoking has also been studied in association with the development of RA in Northern European and American populations [29–31]. There are more than 4000 toxic substances involved in smoking, such as nicotine, carcinogens, organic compounds, solvents, gas substances, and others [32], most of which do harm to the body. The association of smoking with other factors, such as polymorphisms of genes, has been analyzed in many studies.

Conclusions

A correlation of *NRAMP1* gene polymorphisms and the risk of RA were evaluated in our study, which provided evidence for future research direction on the pathology of RA. However, with a limited sample size, our results cannot rule out the effects of other factors on RA. More studies with larger sample sizes and various populations should be performed to validate our study results. Our study findings have set a direction for further studies of RA in genetic and functional areas. We should note that studies with different sample populations and sample sizes will be useful for further studies of the pathology of RA, which could provide a molecular basis for the study of RA.

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