Brief Report

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PRKCH polymorphism is associated with rheumatoid arthritis in a Chinese population

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Summary **Genetic factors have been widely considered to have a substantial effect on the susceptibility to rheumatoid arthritis (RA). The purpose of this study was to determine whether the four newly discovered polymorphisms in a genome-wide association study (GWAS) meta-analysis confer susceptibility to RA in a Chinese Han population. We conducted a case-control study involving 359 RA cases and 873 age-and gender-matched controls and performed genotyping of four single nucleotide polymorphisms (SNPs), rs227163, rs726288, rs3783782 and rs2469434, using the dye terminator-based SNaPshot method. Consequently, we detected significant differences of genotype distribution of rs3783782 in *PRKCH* between RA and controls. The minor allele frequencies (MAFs) of rs3783782 were significantly higher in RA patients compared to control subjects. Moreover, the rs227163 in *TNFRSF9* had higher MAFs in male RA compared with male controls. In addition, the polymorphism of rs3783782 in *PRKCH* was significantly associated with RA susceptibility (OR = 1.67, 95% CI = 1.32-2.11, $p = 1.32 \times 10^{-5}$). After stratification by gender, the minor (A) allele was strongly associated with increased risk for RA in males (OR = 1.87, 95% CI = 1.34-2.60; $p = 1.62 \times 10^{-4}$) and in females (OR = 1.51, 95% CI = 1.08-2.10; $p = 0.014$). For rs227163, the minor (C) allele was found to be associated with RA risk only in males (OR = 1.34, 95% CI = 1.02-1.75; $p = 0.036$). These findings for the first time confirmed that rs3783782 in *PRKCH* was associated with RA susceptibility in a Chinese population, and rs227163 in *TNFRSF9* was associated with RA risk in Chinese males; these SNPs may serve as genetic markers for RA.**

Keywords: Rheumatoid arthritis, single nucleotide polymorphisms, *PRKCH*

1. Introduction

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases, characterized by progressive joint destruction, autoantibody formation, and synovitis, eventually leading to functional disability. Epidemiological data has estimated that RA affects approximately 0.5-1.0% of the world's population and 0.2-0.37% of the Chinese population (*1,2*). Particularly, the prevalence of RA is estimated to be 2-4% in siblings, 5-10% in same-sex dizygotic twins, and even 12-30% in monozygotic twins, supporting the critical role of genetic factors in RA susceptibility (*3,4*). Human leukocyte antigen (HLA) class II molecules are well-studied genetic factors closely associated with RA development (*5*). However, the contribution of the HLA is considered to only account for 30% of the total genetic factors of susceptibility (*6*). Thus, it is critical to identify novel biomarkers responsible for RA susceptibility.

A genome-wide association study (GWAS) meta-analysis discovered 42 novel non-HLA RA risk loci at a genome-wide level of significance. Interestingly, four risk single nucleotide polymorphisms (SNPs) were found to be significantly different between the European and Asian populations, including rs3783782

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in *PRKCH*, rs227163 in *TNFRSF9*, rs726288 in *SFTPD*, and rs2469434 in *CD226* (*7*). The *PRKCH* gene encodes protein kinase C η, a member of protein kinase C (PKC). Although *PRKCH* was identified in 1990, its specific functions in the pathogenesis of RA have not been elucidated. It has been reported that *PRKCH* may be a susceptibility gene for RA in the Japanese population, but not in the French Caucasian population, suggesting the genetic diversity of patients with RA (*8,9*).

Tumor Necrosis Factor α (TNF-α) is a prototypical pro-inflammatory cytokine, which is highly expressed in the synovitis of RA patients and targeting TNF-α by monoclonal antibodies proves to be an effective therapeutic approach for this disease. TNF receptor superfamily member 9 (*TNFRSF9*) is a key factor for communication signals between many cell types during development of multiple organs (*10*). Surfactant protein D gene (*SFTPD*) is mainly synthesized in alveolar type II cells of the lung. Studies have shown that *SFTPD* plays a diverse role in the innate immune system, and suppression of T-lymphocyte proliferation and cytokine production (*11*). The Cluster of Differentiation-226 (*CD226*) is expressed on immune cells such as T lymphocytes, monocytes and natural killer (NK) cells. There is some evidence regarding the potential role of *CD226* gene polymorphisms in autoimmune diseases (*12*).

Currently, few studies have explored the potential association between these SNPs (rs3783782 in *PRKCH*, rs227163 in *TNFRSF9*, rs726288 in *SFTPD*, and rs2469434 in *CD226*) and RA susceptibility in the Chinese population. In this study, we selected these four SNPs for RA association in a Chinese Han population.

2. Materials and Methods

2.1. *Ethical approval*

This study was approved by the Ethics Committee of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital. Informed consent was obtained from all individual participants included in the study. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

2.2. *Subjects*

A total of 1,232 Chinese subjects, including 359 patients with RA (201 men and 158 women; mean age 51.3 ± 13.07 years) were recruited from the Sichuan Provincial People's Hospital. 873 age-and-gender-matched healthy controls (481 men and 392 women; mean age 51.2 ± 15.81 years) were recruited from the physical examination center. All patients were diagnosed with RA according to the American College of Rheumatology criteria (*2*). Demographic data and laboratory testing were obtained by reviewing hospital records of the hospital.

2.3. *Genomic DNA extraction*

Genomic DNA from all participants was isolated from peripheral blood leukocytes using a Gentra Puregene Blood DNA kit (Minneapolis, MN, USA) according to the manufacture's protocol. The concentration of DNA samples was measured by NanoDrop 2000 (Thermo Scientific, USA).

2.4. *Genotyping*

The DNA sequences containing the target SNPs were amplified by polymerase chain reaction (PCR) with designed primers (Table 1). PCR amplification was performed on each sample in a 20-μL reaction volume containing 50 ng of genomic DNA and 2×Taq Master Mix. After PCR amplification, the concentration of each primer was defined. Then, the products of the four SNPs were added together in the same concentration for multiplex reaction. SNP genotyping was performed using the dye terminator-based SNaPshot method according to the protocol (Applied Biosystems, USA). The SNaPShot primers included the 20-60bp upstream sequence or reverse complement of the downstream sequence of each SNP position (Table 1). All probe Primers were synthesized by Sangon Biotech (Shanghai, China). The SNPs analysis was performed on the ABI 3130XL genetic analyzer (Applied Biosystems, USA). The genotypes of the SNPs were determined using Genemapper software 5.0 (Applied Biosystems, USA).

2.5. *Statistical analysis*

All of the statistical analyses were performed using the software Statistical Product and Service Solutions (SPSS) version 17.0 (Prentice Hall International, Chicago, USA). The p values of the SNPs were calculated using an additive model. Hardy-Weinberg equilibrium was tested for each allele using the χ^2 test. The unadjusted odds ratios of the alleles and genotypes were estimated by the χ^2 test. Odds ratios (ORs) and 95 % confidence intervals (95 % CIs) were calculated. $P < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. *Clinical characteristics in RA patients and controls*

Between 10 February 2017 and 30 May 2018, 359 patients with RA and 873 sex-and-age-matched controls were enrolled. All subjects were Han Chinese. In RA

Table 1. Primers used in this study

Primer Name	Sequence	Tm (℃)
rs726288-F	GGTTCCCTCTGGGACTTTTC	54
rs726288-R	AGAGCAGCAGGAATCCAAAA	50
rs726288-SNapSHOT-primer	AGGCAAATGTGCACCACACTCCCAGCCTGC	
rs3783782-F	CCAAGAACCTCATGCCGTAT	52
rs3783782-R	CCTGAGGTCAGGAGTTCGAG	56
rs3783782-SNapSHOT-primer	TTTTAAGACAGAGTCTCGCTCTGTTGCCCAGGCTGGAGTG	
rs2469434-F	GGCTCCACCAGATTAACCAA	52
rs2469434-R	ACCACAGCAATCGTCAACAG	52
rs2469434-SNapSHOT-primer	AAAAAGTTCTAGAGGCCTGGACTTGCAATTGGTGTCTGAAGGGCAGGGTT	
rs227163-F	CCTGGAAAGTCATCCAGGTC	54
rs227163-R	CCTCTCTTTCACCACCCACA	54
rs227163-SNapSHOT-primer	ACGTTTTTTCTAGGGAATTGGTCATTTTGTCTGAGTTTTCAAATGTATTGGCAAAAACC	

Tm, DNA melting temperature.

Table 2. Characteristics of the RA cases and controls

Characteristics	Total RA cohort (n = 359)	Healthy Controls (n = 873)	p value
Male, n (%)	201 (56)	481 (55)	0.08
Age (years), mean (SD)	51.3 (13.07)	51.2 (15.81)	0.97
BMI (kg/m^2), mean (SD)	21.6 (3.16)	20.4 (4.32)	0.53
Age of onset (years), mean (SD)	45.2 (13.84)	-	
Disease duration (years), median (p25-p75)	3.0 (1.0-9.0)	-	

RA, rheumatoid arthritis; BMI, Body Mass Index; p25-p75, 25th to75th percentile.

group, the mean age of all the RA patients was 51.3 years old. In healthy controls, the mean age was 51.2 years old. The characteristics of the study participants are shown in Table 2. The sex ratio, mean age and body mass index (BMI) of the RA patients were not statistically different from those of controls. Not all SNPs were successfully genotyped in every individual. The results of the genotyping experiments are given in Table 3.

3.2. *Hardy-Weinberg equilibrium test*

There were no significant deviations from Hardy–Weinberg equilibrium test (HWE) at any of the four SNPs. The p values for the tests of HWE were 0.106 *vs*. 0.906 for rs3783782, 0.769 *vs*. 0.689 for rs726288, 0.689 *vs*. 0.188 for rs2469434, and 0.425 *vs*. 0.704 for rs227163, in cases and controls, respectively.

3.3. *Genotype and allele frequencies of the four SNPs in RA patients and controls*

The genotypes distribution *and allele* frequencies of the four SNPs are shown in Table 3 and Table 4, respectively. RA is a chronic inflammatory disease manifested by joint synovial tissue inflammation, and activation of T cells is involved in the synovial inflammatory response, tissue damage and bone invasion (*13-15*). Previous studies have shown that the protein kinase C (PKC) family plays an important role in T lymphocyte activation and autoimmune disorders (*16*). Therefore, *PRKCH*, as a member of the PCK family, is speculated to be associated with the pathophysiologic mechanism of RA (*17*). Indeed, Takata *et al*. reported *PRKCH* as a susceptibility gene for RA in a Japanese population. They found that multiple SNPs of *PRKCH* may influence susceptibility to this disease (*8*). Nevertheless, another study by Teixeira *et al*. confirmed that these susceptibility alleles of *PRKCH* were not associated with RA in a French Caucasian population (*9*). These contrary findings suggest the variation of *PRKCH* across different ethnic populations and identification of these variants in other populations is necessary. In our study, we observed a significant difference of genotype distribution of *PRKCH* rs3783782 between RA patients and control subjects ($p = 3.09 \times 10^{-5}$). The minor allele frequencies (MAFs) of rs3783782 were significantly higher in RA patients compared to control subjects both in males (18.1% in RA *vs*. 10.6% in control, $p = 1.62 \times 10^{-4}$) and females (22.1% in RA *vs*. 15.8% in control, p = 0.014).

Controlling the excessive production of pro-inflammatory cytokines such as tumor necrosis factor (TNF) represents a remarkable therapeutic approach for RA treatment (*18*). TNFRSF9 is a 30 kDa membrane glycoprotein which belongs to the tumor necrosis factor receptor (TNFR) family. TNFRSF9 is widely expressed

Table 3. Genotype frequencies of the four SNPs in RA patients and controls

SNPs	Gene	Gender	RA, *n* (%)	Control, *n* (%)	*p* value
rs726288	*SFTPD*	Male	200	475	0.056
		TT	7 (3.50)	19 (4.0)	
		CT	29 (14.50)	155 (32.63)	
		CC	164 (82.00)	301 (63.37)	
		Female	157	387	0.54
		TT	10 (6.37)	20 (5.17)	
		CT	64 (40.76)	143 (36.95)	
		CC	83 (52.87)	224 (57.88)	
		All			0.41
rs3783782	*PRKCH*	Male	196	478	3.99×10^{-4}
		AA	5 (2.55)	8 (1.67)	
		AG	61 (31.12)	85 (17.78)	
		GG	130 (66.33)	385 (80.54)	
		Female	154	386	0.035
		AA	4 (2.60)	6 (1.55)	
		AG	60 (38.96)	110 (28.50)	
		GG	90 (58.44)	270 (69.95)	
		All			3.09×10^{-5}
rs2469434	*CD226*	Male	199	455	0.34
		CC	28 (14.07)	46 (10.11)	
		CT	93 (46.73)	222 (48.79)	
		TT	78 (39.20)	187 (41.10)	
		Female	157	384	0.85
		CC	17 (10.83)	48 (12.50)	
		CT	75 (47.77)	177 (46.09)	
		TT	65 (41.40)	159 (41.41)	
		All			0.77
rs227163	*TNFRSF9*	Male	199	454	0.09
		CC	14 (7.04)	25 (5.50)	
		CT	80 (40.20)	148 (32.60)	
		TT	105 (52.76)	281 (61.89)	
		Female	150	366	0.98
		CC	9 (6.0)	20 (5.46)	
		CT	55 (36.67)	128 (34.97)	
		TT	91 (60.67)	218 (59.56)	
		All			0.21

RA, Rheumatoid Arthritis; MAF, minor allele frequency; SNPs, single nucleotide polymorphisms; OR, odds ratio.

Table 4. MAFs of the four SNPs in RA patients and controls

SNPs (MAF)	Gene	Gender	RA, *n* (%)	Control, *n* (%)	OR (95% CI)	*p* value
rs726288 (T)	*SFTPD*	Male	43 (10.8)	193 (20.3)	1.129 (0.85-1.50)	0.399
		Female	84 (26.8)	183 (23.6)	1.179 (0.87-1.59)	0.28
		All	127 (17.9)	376 (21.8)	1.15 (0.94-1.42)	0.181
rs3783782 (A)	*PRKCH*	Male	71 (18.1)	101 (10.6)	1.87 (1.34-2.60)	1.62×10^{-4}
		Female	68 (22.1)	122 (15.8)	1.51 (1.08-2.10)	0.014
		All	139 (19.9)	223 (12.9)	1.67 (1.32-2.11)	1.32×10^{-5}
rs2469434 (C)	*CD226*	Male	149 (37.4)	314 (34.5)	1.135 (0.89-1.45)	0.308
		Female	109 (34.7)	273 (35.5)	0.964 (0.73-1.27)	0.79
		All	258 (36.2)	587 (35.0)	1.056 (0.88-1.27)	0.558
rs227163 (C)	*TNFRSF9*	Male	108 (27.1)	198 (21.8)	1.34 (1.02-1.75)	0.036
		Female	73 (24.3)	168 (23.0)	1.03 (0.76-1.42)	0.83
		All	181 (25.9)	366 (22.3)	1.20 (0.97-1.47)	0.087

SNPs, single nucleotide polymorphisms; MAF, minor allele frequency; RA, rheumatoid arthritis; OR, odds ratio.

in a variety of immune cells, including activated T/B, NK, and NKT cells (*10*). Previous studies have found that SNPs of *TNFRSF9* are associated with autoimmune disorders, such as psoriatic arthritis in Europeans by GWAS, and RA in African Americans (*19,20*). In our study, we did not find a significant difference in genotype distribution of *TNFRSF9* rs227163 between RA patients and controls ($p = 0.21$), but we found that rs227163 appeared to have higher MAFs in male RA compared male controls (27.1% in male RA *vs*. 21.8% in male controls, $p = 0.036$). However, we failed to detect any differences between RA and control subjects with respect to rs726288 and rs2469434 genotype distribution and allele frequencies.

3.4. *Polymorphisms of the four SNPs and risk of RA*

Furthermore, we evaluated the effects of rs3783782 in *PRKCH* and rs227163 in *TNFRSF9* for RA risk, as shown in Table 4. As a result, we found rs3783782 polymorphism was significantly associated with RA susceptibility (OR = 1.67, 95% CI = 1.32-2.11, $p = 1.32 \times 10^{-5}$). After stratification by gender, the minor (A) allele was strongly associated with increased risk for RA in males (OR = 1.87, 95% CI = 1.34-2.60; $p = 1.62 \times 10^{-4}$) and in females (OR = 1.51, 95% CI = 1.08-2.10; $p = 0.014$). For rs227163, the minor (C) allele was found to be associated with RA risk only in males (OR = 1.34, 95% CI = 1.02-1.75; $p = 0.036$). Unfortunately, we failed to find any obvious association of rs227163 polymorphism with female RA (OR = 1.03, 95% CI = 0.76-1.42; $p = 0.83$).

Previous studies have shown that *SFTPD* and *CD226* is critically involved in the innate immune system by suppressing T cell proliferation and cytokine production (*11,12*). Accumulating evidence also supports the role of *SFTPD* polymorphisms in a diversity of human diseases, such as chronic obstructive pulmonary disease, obesity, and RA (*21-23*). However, we found no association of *SFTPD* (rs726288) and *CD226* (rs2469434) with RA in a Chinese Han population, probably due to the SNP loci and genetic differences in different populations.

There are some limitations in this study. First, the study sample size is relatively small, and more participants need to be involved to confirm our findings. Second, the physiological and pathophysiological functions of SNPs of *PRKCH* and TNFRSF9 in RA have not yet been investigated.

In conclusion, these findings for the first time confirmed that rs3783782 in *PRKCH* was associated with RA susceptibility in a Chinese population, and rs227163 in *TNFRSF9* was associated with RA risk in Chinese males. These SNPs may serve as genetic markers for RA. Further functional investigations are needed to elucidate the precise role of *PRKCH* and *TNFRSF9* in RA pathogenesis.

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