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Relationship of *CARD8* Gene Polymorphisms with Susceptibility to Ankylosing Spondylitis: A Case-Control Study

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Statistical Analysis C
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Background: The aim of this case-control study was to evaluate the correlation of caspase recruitment domain-containing protein 8 (*CARD8*) gene rs2043211 (exon) and rs7253718 (intron) polymorphisms with the susceptibility of ankylosing spondylitis (AS) in the Chinese Han population.

Material/Methods: *CARD8* polymorphisms were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in 118 AS patients and 122 healthy persons. Linkage disequilibrium (LD) and haplotype analysis were carried out using Haploview software. Distribution differences of genotypes, alleles and haplotypes between the case and control groups were tested by chi-square test. Relative risk of AS was expressed by odds ratios (ORs) and 95% confidence intervals (CIs). Logistic regression analysis was used to adjust the results of association by clinical parameters.

Results: For rs2043211, distribution of variant allele T was obviously different between AS patients and healthy controls (P=0.046). It indicated that T allele might increase the susceptibility of AS (OR=1.441, 95%CI=1.006–2.065). Adjusted by clinical characteristics, the significance of difference was slightly decreased (P=0.050, OR=1.439, 95%CI=0.999–2.072). Strong LD existed between rs2043211 and rs7253718 polymorphisms, and rs2043211T-rs7253718G haplotype was significantly associated with increased AS susceptibility (OR=1.787, 95%CI=1.165–2.740). In subgroup analysis, we found that the TT genotype and T allele of rs2043211 significantly increased the risk of AS in males (TT versus AA: OR=2.554, 95%CI=1.079–6.049; T versus A: OR=1.661, 95%CI=1.067–2.586), but not females.

Conclusions: *CARD8* polymorphisms are likely to be associated with the elevated susceptibility of AS. Present results should be confirmed in the future studies.

MeSH Keywords: Caspases • Haplotypes • Polymorphism, Genetic • Spondylitis, Ankylosing

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Background

Ankylosing spondylitis (AS) is a kind of serum negative spinal joint disease belonging to rheumatic disease category. Its main symptom is persistent inflammation in the sacroiliac joints and spinal attachment points. AS mainly involves the spine and sacroiliac joints, causing rigidity and fibrosis of the spine and different degrees of lesions to bone, muscle, lung, eye, and other organs [1]. A number of epidemiological studies have shown that the prevalence of AS is widely increasing worldwide with some differences among different races and regions [2–4]. Its incidence in European populations is about 0.24%, and Asian populations is about 0.17%, and African black populations had the lowest incidence rate in 2014 [2]. AS commonly affects younger people with incidence of high disability and even death, causing life stress for patients and families and causing huge economic burdens for families and society. However, so far, the pathogenesis of AS still remains unclear. According to previous studies, AS is a complex multiple-factor disorder and is influenced by a number of environmental and genetic factors. Recently, the role of genetic predisposition has received more attention in the etiology of AS [5].

Caspase recruitment domain-containing protein 8 (CARD8), a member of caspase recruitment domain (CARD) family, is encoded by the *CARD8* gene located on chromosome 19q13.33. CARD contains a conserved homology domain which can mediate the protein-protein interactions among key apoptotic signaling molecules [6]. In addition, CARD participates in the nuclear factor kappa-B (NF-κB) signaling pathway via mediating the interactions of components in the upstream of the pathway [6]. NF-κB signaling pathway plays an important role in the immune and inflammatory responses, and in apoptosis [7–9]. CARD8 is found to regulate the inflammatory reaction by inhibiting NF-κB signaling [10]. Moreover, *CARD8* polymorphisms are also associated with many inflammatory diseases [11–16]. However, few articles have investigated the association of *CARD8* and polymorphisms with AS pathogenesis.

A number of single nucleotide polymorphisms (SNPs) have been identified in *CARD8*. rs2043211 is located on the exon region of *CARD8* gene, and it can introduce a stop codon at codon 10 (Cys10Stop) and produce a truncated CARD8 protein. rs7253718 is a novel mutation in the intron region of CARD8 with the low frequency minor allele. rs2043211 has been found to be significantly associated with AS risk in southern Sweden populations [17]. Polymorphism distribution is different in various populations. Therefore, the present study aimed to assess the influences of *CARD8* rs2043211 and rs7253718 SNPs on the susceptibility of individuals to AS in a Chinese Han population.

Material and Methods

Case and control groups

All of the participants were recruited from the Yongchuan Hospital of Chongqing Medical University during from September 2017 to December 2018. There were 118 newly diagnosed AS patients, including 82 males and 36 females aged 17 to 71 years old with an average age of 36.15±13.09 years. AS patients were diagnosed by 2 clinical doctors following the previously published standards [18]. Patients with joint diseases, or other systemic diseases during the perinatal period were excluded from the case study group.

There were 122 healthy individuals who had a normal medical examination enrolled as the control group including 78 males and 44 females. Their age range was from 19 to 75 years old with the average age of 38.40±15.13 years. Individuals with histories of chronic and immune diseases during the perinatal period were excluded. The controls were frequency-matched with AS patients in age and gender.

This study complied with Helsinki declaration and was approved by the Ethics Committee of the Yongchuan Hospital of Chongqing Medical University. The research participants were all from the Chinese Han population and they had no blood relationship between each other. They all knew about the research process and signed the written informed consent. Afterwards, the professionally trained investigators recorded the relevant information of all participants and collected blood samples according to the national ethics criteria of human genome research.

DNA extraction

We collected 3 mL of venous blood from each participant, who had fasted for 12 hours, and we put the blood sample into an anticoagulative tube with ethylene diamine tetra acetic acid dipotassium salt (EDTA-2K). The whole blood genomic DNA of samples were extracted using Beijing TIANGEN biochemical blood genome DNA extraction kit according to the manufacturer's instructions and then stored in –20°C for further applications.

Genotyping method of *CARD8* SNPs

Genebank database of the NCBI was used to find the complete sequence of *CARD8* gene. Then we designed polymerase chain reaction (PCR) primer sequences using Primer Premier 5.0 software and synthesized in Shanghai Sangon Biotech Co., Ltd. (Table 1).

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Table 1. Primer sequences of *CARD8* gene rs2043211 and rs7253718 polymorphisms.

SNP		Primer sequence	Primer length (bp)
rs2043211	For.	5'-ACCCTGTGTTTCTGAGACCCTTTG-3'	108 bp
	Rev.	5'-GATAGTTGACACTCAGGAACAGCACG-3'	
rs7253718	For.	5'-CCCTTCACTGTCTTGCTC-3'	218 bp
	Rev.	5'-CTGTATTGGGTCATTCTTG-3'	

The PCR reaction system used a volume of 25 μL mixture including 2.0 μL DNA template, 0.8 μL forward primer, 0.8 μL reverse primer, 2.5 μL 10×loading buffer, 1.5 μL $MgC1_2$, 2.0 μL dNTPs, 0.5 μL Taq DNA enzyme, and 14.9 μL ddH_2O. Amplification conditions of PCR were 95°C pre-denaturation for 5 minutes; followed by 45 cycles of 95°C degeneration for 30 seconds, 62°C annealing for 30 seconds, 72°C extension for 30 seconds, and finally 72°C extension for 10 minutes. PCR products were tested by 1% agarose gel electrophoresis (AGE).

The enzyme reaction system used a volume of 20 μL, including 3.0 μL restriction enzyme (*Alw*I for rs2043211 and *Hind*III for rs7253718), 10.0 μL PCR products, 2.0 μL 10x buffer solution, and 5.0 μL double distilled water. Then the mixture was digested in a water bath at 37°C overnight. The enzyme-digested products were separated by 3% AGE.

Statistical analysis

PASW Statistics 18.0 software was used to analyze the data. Significant level was set to 0.05. Linkage disequilibrium (LD) and its correlation coefficient (D') between *CARD8* rs2043211 and rs7253718 SNPs were assessed by Haploview software (Figure 1). The χ^2 test was used to check whether the genotype distributions matched Hardy-Weinberg equilibrium (HWE) in the control group. Comparison of genotypes, alleles, and haplotypes in *CARD8* polymorphisms between the 2 groups were also tested by chi-square test. Odd ratios (ORs) and 95% confidence intervals (CIs) were used to evaluate the effect of *CARD8* polymorphisms to AS susceptibility. Meanwhile, the results were adjusted by clinical features of participants using logistic regression analysis.

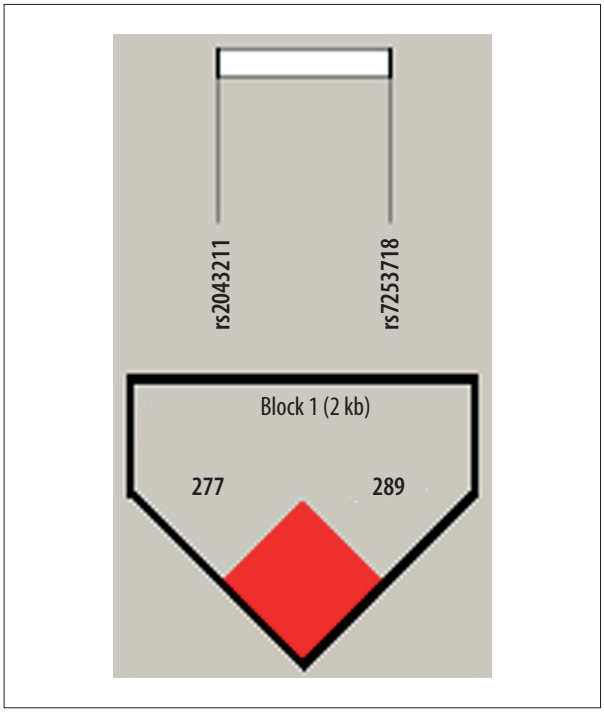

Figure 1. Linkage disequilibrium (LD) status of *CARD8* rs2043211 and rs7253718 polymorphisms, D'=1.0, r^2=0.258.

Results

The basic characteristics of study participants

The basic information of study participants in the case and control groups was investigated and recorded by a trained doctor, the detailed results are listed in Table 2. The mean age of AS patients and the controls was respectively 36.15±13.09 years and 38.40±15.13 years. The number of males and females in the case group was 82 males and 36 females, the number in the control group was 78 males and 44 females. There was no significant difference between the 2 groups in age or gender (P>0.05). Body mass index (BMI) of the AS patients and the controls was similar (23.30±2.45 kg/m^2 and 23.31±2.46 kg/m^2, respectively, P=0.978). In this study population, smoking, drinking, and exercise habit were not the influence factors of AS occurrence (P>0.05). But people with family history of AS easily suffered from AS, compared with people without family history (P=0.006). Nearly 85% of AS patients were positive-HLA-B27 and the ratio was 13.93% in the controls, so HLA-B27 was the biomarker of AS (P<0.001). The mean duration of AS was 5.36±2.31 years.

HWE test

Genotype distributions of the 2 SNPs (rs2043211 and rs7253718) in the control group respectively met the HWE

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Table 2. Basic characteristics of participants.

Basic features	Cases n=118(%)	Controls n=122(%)	*P*	χ^2/t
Age (year)	36.15±13.09	38.40±15.13	0.220	–1.523
Gender (male/female)	82/36	78/44	0.361	0.834
Body mass index (kg/m^2)	23.30±2.45	23.31±2.46	0.978	–0.042
Smoking (%)	34 (28.81)	26 (21.31)	0.180	1.801
Drinking (%)	26 (22.03)	22 (18.03)	0.439	0.600
Family history (%)	17 (3.39)	5 (0.82)	0.006	7.655
Exercise habit (%)	34 (28.81)	43 (35.25)	0.286	1.139
HLA-B27 (positive/%)	98 (83.05)	17 (13.93)	<0.001	114.817
Duration (year)	5.36±2.31			

Table 3. Frequency comparisons of genotypes and alleles in *CARD8* gene polymorphisms.

Genotype/ allele	Cases n=118 (%)	Controls n=122 (%)	P_{HWE}	χ^2	*P*	OR (95% CI)	*P**	OR* (95% CI)
rs2043211			0.467					
AA	25 (21.19)	41 (33.61)		–	–	1.00		
TA	62 (52.54)	56 (45.90)		3.651	0.056	1.816 (0.982–3.358)	0.085	1.727 (0.927–3.220)
TT	31 (26.27)	25 (20.49)		3.727	0.054	2.034 (0.985–4.197)	0.057	2.041 (0.978–4.261)
A	112 (47.46)	138 (56.56)		–	–	1.00		
T	124 (52.54)	106 (43.44)		3.980	0.046	1.441 (1.006–2.065)	0.050	1.439 (0.999–2.072)
rs7253718			0.990					
GG	67 (56.78)	74 (60.65)		–	–	1.00		
AG	45 (38.14)	42 (34.43)		0.381	0.537	1.183 (0.693–2.020)	0.626	1.144 (0.666–1.966)
AA	6 (5.08)	6 (4.92)		0.027	0.869	1.104 (0.340–3.590)	0.692	1.279 (0.378–4.328)
G	179 (75.85)	190 (77.87)		–	–	1.00		
A	57 (24.15)	54 (22.13)		0.276	0.600	1.120 (0.733–1.713)	0.565	1.135 (0.738–1.744)

HWE – Hardy-Weinberg equilibrium; * *P* and OR values were adjusted by clinical parameters of participants. OR – odds ratio; CI – confidence interval.

test (Table 3, *P*>0.05), the results suggested that our study participants had similar genetic backgrounds and were recruited from the same one Mendelian population.

Genotype distributions of *CARD8* polymorphisms in case and control groups

TA and TT genotype frequencies of rs2043211 SNP were higher in the case group than that the control group, but the differences were non-significant (Table 3, *P*>0.05) and the differences were still non-significant after adjusted by clinical features (*P*>0.05). While the variant allele of rs2043211 T had an obviously higher frequency in the cases (*P*=0.046) and adjusted by clinical features of participants, the significance difference was slightly decreased (*P*=0.050), T allele might positively associate with the susceptibility of AS (OR=1.441, 95%CI=1.006–2.065; adjusted OR=1.439, 95%CI=0.999–2.072). However, no distinct difference existed in the rs7253718 AG and AA genotype frequencies between AS patients and healthy controls (*P*>0.05). Similar result was observed in the rs7253718 A allele. Thus,

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Table 4. Analyses of LD and haplotypes in *CARD8* rs2043211 and rs7253718 polymorphisms.

Haplotypes	Cases 2n=236 (%)	Controls 2n=244 (%)	χ^2	*P*	OR (95%CI)
rs2043211A-rs7253718G	55 (23.31)	84 (34.43)	–	–	1.00
rs2043211A-rs7253718A	57 (24.15)	54 (22.13)	3.465	0.063	1.612 (0.974–2.669)
rs2043211T-rs7253718G	124 (52.54)	106 (43.44)	7.137	0.008	1.787 (1.165–2.740)

Table 5. The genotype and allele of *CARD8* polymorphisms.

Genotype/allele		Age (year) ≤40	Age (year) >40	χ^2	*P*	OR (95% CI)
rs2043211	AA	18 (22.22)	7 (18.92)	–	–	1.00
	TA	41 (50.62)	21 (56.76)	0.281	0.596	0.759 (0.274–2.104)
	TT	22 (27.16)	9 (24.32)	0.007	0.932	0.951 (0.296–3.056)
	A	77 (47.53)	35 (47.30)			1.00
	T	85 (52.47)	39 (52.70)	0.001	0.973	0.991 (0.571–1.718)
rs7253718	GG	48 (59.26)	19 (51.35)			1.00
	AG	29 (35.80)	16 (43.24)	0.649	0.420	0.717 (0.319–1.611)
	AA	4 (4.94)	2 (5.41)	0.067	0.796	0.792 (0.134–4.688)
	G	125 (77.16)	54 (72.97)			1.00
	A	37 (22.84)	20 (27.03)	0.486	0.486	0.799 (0.425–1.502)

we suggested that rs7253718 SNP lack of association with AS susceptibility.

Haplotype analysis of *CARD8* rs2043211 and rs7253718 polymorphisms in AS patients

Strong LD was discovered between *CARD8* rs2043211 and rs7253718 SNPs (D'=1.0) and 3 haplotypes were identified, namely rs2043211A-rs7253718G, rs2043211A-rs7253718A, and rs2043211T-rs7253718G (Table 4). Distributions of rs2043211T-rs7253718G haplotype had obvious difference between case and control groups (*P*=0.008), which indicated that it was significantly associated with the increased risk of AS (OR=1.787, 95%CI=1.165–2.740). However, the frequencies of rs2043211A-rs7253718A haplotype had no significant association with AS susceptibility.

The association analysis of *CARD8* polymorphisms with age in AS patients

We all known that AS easily affect younger people, so we explored whether *CARD8* polymorphisms were associated with age of AS patients in this study. The results are shown in Table 5. Unfortunately, we did not find a significant distribution difference of genotypes or alleles of rs2043211 or rs7253718 between AS patients with age ≤40 years and >40 years old groups (*P*>0.05).

Subgroup analysis of the association between *CARD8* polymorphisms and AS risk based on gender

In this study, we also conducted the subgroup analysis based on gender due to the high incidence in males and the results are listed in Table 6. We found that TT genotype and T allele frequencies of rs2043211 were significantly higher in male patients than that in healthy males (*P*<0.05), so they were associated with the increased risk of AS in males (TT versus AA: OR=2.554, 95%CI=1.079–6.049; T versus A: OR=1.661, 95%CI=1.067–2.586). but rs2043211 was not correlated with the occurrence risk of AS in females (*P*>0.05). There was no significant association between rs7253718 and AS risk in males or females (*P*>0.05).

Discussion

AS is a common chronic inflammatory rheumatic disease and mainly affects the axial skeleton and peripheral joints. It has

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Table 6. The subgroup analysis of the association between CARD8 polymorphisms and AS risk based on gender.

Genotype/ allele	Male				Female			
	Cases (n=82)	Controls (n=78)	*P*	OR (95% CI)	Cases (n=36)	Controls (n=44)	*P*	OR (95% CI)
rs2043211								
AA	19 (23.17)	28 (35.90)			6 (16.67)	13 (29.54)		
TA	37 (45.12)	35 (44.87)	0.241	1.558 (0.741–3.277)	25 (69.44)	21 (47.73)	0.095	2.579 (0.835–7.969)
TT	26 (31.71)	15 (19.23)	0.031	2.554 (1.079–6.049)	5 (13.89)	10 (22.73)	0.914	1.083 (0.255–4.596)
A	75 (45.73)	91 (58.33)			37 (51.39)	47 (53.41)		
T	89 (54.27)	65 (41.67)	0.024	1.661 (1.067–2.586)	35 (48.61)	41 (46.59)	0.799	1.084 (0.581–2.023)
rs7253718								
GG	49 (59.76)	51 (65.38)			18 (50.00)	23 (52.27)		
AG	28 (34.15)	23 (29.49)	0.493	1.267 (0.644–2.493)	17 (47.22)	19 (43.18)	0.770	1.143 (0.465–2.810)
AA	5 (6.09)	4 (5.13)	0.706	1.301 (0.330–5.130)	1 (2.78)	2 (4.55)	0.721	0.639 (0.054–7.617)
G	126 (76.83)	125 (80.13)			53 (73.61)	65 (73.86)		
A	38 (23.17)	31 (19.87)	0.473	1.216 (0.712–2.076)	19 (26.39)	23 (26.14)	0.971	1.013 (0.499–2.056)

a high degree of genetic predisposition and is strongly associated with histocompatibility antigen HLA-B27 (human leukocyte antigen-B27) [19,20]. AS usually occurs in young adults, especially in young men. AS is a progressive disease with inconspicuous symptoms in the early stage including low back pain, tendon or ligament bone attachment point inflammation, and other symptoms. Then symptoms of early morning stiffness, limited lumbar movement in all directions, reduced thoracic activity, and other clinical signs appear. In the later stage, it may appear as ankylosis of the spinal column and even loss of mobility [21,22]. So far, its exact etiology and pathogenesis is not clear; studies have shown that the occurrence of AS may be related to genetic background, chronic infection, autoimmune disorders, endocrine disorders, and so on [23–26]. A recent study suggests that AS is the result of the interaction of environmental and genetic factors [27]; environmental factors may be the causes of the disease, and genetic susceptibility the basis for AS pathogenesis.

CARD8 is involved in innate immunity which involves the inhibition of NF-κB activation [28]. Besides, it acts as a negative regulator for the NLRP3 (nucleotide binding oligomerization domain-like receptor pyrin domain containing 3) inflammasome [29]. Knockdown of *CARD8* gene will upregulate the secretion of IL-1β (interleukin 1, beta) which is synthesized by activated macrophages as an important mediator for inflammatory response [29]. Ko et al. revealed that the loss of the *CARD8* gene could increase the onset of inflammatory diseases [30]. *CARD8* rs2043211 polymorphism is a truncated mutation and might alter the protein function in inflammasome-mediated processes and the suppression of NF-κB [31], and rs7253718 is a novel mutation in the intron region of *CARD8* with the substitution of A/G base. A previous study found that a genetic variant of *CARD8* contributed to the severity of rheumatoid arthritis (RA) [10]. As an inflammatory disease, AS may be implicated by the *CARD8* gene. However, the relationship of the *CARD8* gene and AS has been rarely reported until now.

In our current study, we explored the genetic association of *CARD8* rs2043211 and rs7253718 polymorphism with AS risk in a Chinese Han population. Family history of AS was the independent risk factor of AS and HLA-B27 was a biomarker of AS. For polymorphism, the variant allele T of rs2043211 was more frequent in AS patients than in the controls, revealing that T allele might contribute to the risk of AS development. This result was in line with the study by Kastbom et al. which found that the A allele of rs2043211 significantly decreased the susceptibility of individuals to AS [17]. In addition, its A allele heralded a decreased trend for the risk of systemic juvenile idiopathic arthritis [14]. Other studies showed that rs2043211 polymorphism had no significant association with the risk of RA [15,16]. Nevertheless, genotype and allele frequencies of rs7253718 polymorphism had no statistically significant difference between AS patients and healthy controls. This study explored the role of *CARD8* rs2043211 and rs7253718 SNPs in AS development as risk-based study in a Chinese Han population.

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As a complex disease, AS is not only affected by a single SNP, but multiple factors also attributed to the combined effects of AS. Thus, we assessed the LD between rs2043211 and rs7253718 SNPs, so as to explore the implication of the interaction between these 2 SNPs in AS patients. High LD existed between rs2043211 and rs7253718 SNPs and T-G haplotype conferred on 1.787 times increased risk of AS occurrence. Additionally, we also investigated the genotypes and alleles distribution of *CARD8* polymorphisms between the different age groups in AS patients and the results showed the influence of *CARD8* polymorphisms on AS risk was not associated with age.

Meanwhile, some limitations in the present study should be noted. The relatively small sample size in this study could reduce the statistical power. The study was limited to Chinese Han population and so cannot represent the whole population. Various environmental factors involved in AS development were not considered in this study. Therefore, further studies should be carried out to confirm our results with large sample size and various population; and the mechanism of *CARD8* polymorphisms in AS development also needs to be revealed in the future.

Conclusions

CARD8 rs2043211 polymorphism may contribute to the risk of AS development in the Chinese Han population, but not the novel SNP rs7253718. Moreover, the interaction of *CARD8* rs2043211 and rs7253718 polymorphisms appears to play an important role in AS development.

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