Association of *TLR7* gene copy number variations with ankylosing spondylitis in a Chinese population: a case control study

M. Wang[1,2], S. Xu[3], X. Zhang[1,2], M. Chen[1,2], R. Han[1,2], X. Hu[1,2], Y. Yuan[1,2], Y. Ma[1,2], J. Yang[1,2], M. Wu[1,2], R. Liu[1,2], G. Jiang[1,2], F. Pan[1,2,4]

[1]Department of Epidemiology and Biostatistics, School of Public Health, Anhui Medical University, Anhui, China; [2]The Key Laboratory of Major Autoimmune Diseases, Anhui Province, China; [3]Department of Rheumatism and Immunity, the First Affiliated Hospital of Anhui Medical University, Anhui, China; [4]Menzies Research Institute Tasmania, University of Tasmania, Hobart, Australia.

Abstract

Objective

To explore the association of TLR7 gene copy number variations (CNVs) with the susceptibility of ankylosing spondylitis (AS).

Method

The case control study was performed in 649 Chinese Han patients with AS and 628 healthy controls. The copy numbers of TLR7 gene (2 fragments) were measured by AccuCopy™ methods. Chi-square and logistic regression models were performed to investigate the association of TLR7 gene CNVs with AS. Odds ratio (ORs) and 95% confidence intervals (CIs) was calculated to estimate AS risk and the Bonferroni correction was applied owing to multiple testing.

Result

The logistic regression analysis showed that one copy was significantly associated with AS susceptibility after Bonferroni correction (for the TLR7_1 fragment: OR=1.458, 95%CI(1.098,1.936), p=0.009; for the TLR7_2 fragment: OR=1.451, 95%CI (1.093,1.927), p=0.010), and this association still exists after adjustment of age and sex (for the TLR7_1 fragment: adjusted OR=2.066, 95%CI (1.318,3.238), p=0.002; for the TLR7_2 fragment: adjusted OR=2.061, 95%CI (1.315,3.230), p=0.002). However, logistic regression analysis stratified by gender showed a higher OR in males (for the TLR7_1 fragment: OR(95%CI)=7.987(3.756,16.983); for the TLR7_2 fragment: OR(95%CI)=7.947(3.738,16.897)) than in females (for the TLR7_1 fragment: OR(95%CI)=0.204(0.080,0.524); for the TLR7_2 fragment: OR(95%CI)=0.204(0.080,0.524)).

Conclusion

We conclude that the lower copy number (=1) of TLR7 gene confers a risk factor for AS susceptibility in males but a protective factor in females.

Key words

ankylosing spondylitis, copy number variations, *TLR7*, case-control

*Mengmeng Wang, MD**
*Shengqian Xu, PhD**
Xu Zhang, MD
Mengya Chen, MD
Renfang Han, MD
Xingxing Hu, MD
Yaping Yuan, MD
Yubo Ma, MD
Jiajia Yang, MD
Meng Wu, MD
Rui Liu, PhD
Guangming Jiang, PhD
Faming Pan, PhD

**These authors contributed equally to this work and are co-first authors.*

Please address correspondence to:
Prof. Faming Pan,
Department of Epidemiology and Biostatistics,
School of Public Health,
Anhui Medical University,
81 Meishan Road, Hefei,
Anhui 230032, China.
E-mail: famingpan@ahmu.edu.cn

Received on September 23, 2017; accepted in revised form on January 29, 2018.

Funding: this study was supported by grants from the National Natural Science Foundation of China (30771849, 81273169 and 81773514) and the Natural Science Fund of Education Department of Anhui province (KJ2014A127).

Competing interests: none declared.

Introduction

Ankylosing spondylitis (AS), the prototypic seronegative arthropathy, mainly invades the axial and sacroiliac joints (1). At present, there are no exact pathogenesis, perfect diagnosis and effective treatment strategies targeted at AS. Given these factors, it is essential to explore the underlying mechanisms of AS (2). For past several decades, it has been suggested that immunologic factors and genetic predispositions play vital roles in the development and progression of AS (3). Substantial evidence revealed that AS had strong genetic predisposition and the heritability of the disease exceeded 90% (4, 5). Human leukocyte antigen (HLA)-B27 has been verified to be associated with AS, which only contributed 16%~50% of the genetic susceptibility to the disease (6). Single nucleotide polymorphisms (SNPs) in non-HLA regions have been studied frequently to identify positive locus associated with disease susceptibility, but their contributions to the development of disease were little (7, 8). Recently, the most prominent form of genomic variation known as copy number variation (CNV), which refers to different numbers of the same DNA sequence due to deletions, duplications and insertions, plays a vital role in medical genetics and contributes to human genetic diversity (7). For example, it is known that CNVs have been verified to be associated with many autoimmune diseases including rheumatoid arthritis (RA), psoriasis and systemic lupus erythematous (SLE) (9-11). Furthermore, Yu and Hou *et al*. reported that complement component C4 and FAS gene copy numbers are associated with susceptibility to Behçet disease (12, 13).

Importantly, a GWAS exploring CNVs with AS risk had been conducted in Korea (14). The result revealed that nine CNV regions were associated with AS, which included five susceptible regions (1q32.2, 2q31.2, 6p21.32, 13q13.1 and 16p13.3) and four protective regions (1p34.2, 11q22.1, 14q24.2 and 22q11.1) in AS. Notably, our previous studies also found that the copy numbers of DEFB4 gene might be associated with AS and involved in disease progression (15), and that a lower copy number of FCGR3A and FCGR3B genes was significantly associated with increasing risk of AS (16). These results made contributions to the pathogenesis of AS and motivated us to further research other gene CNVs.

Toll-like receptors (TLRs), which play an important role in innate immune response and inflammatory or autoimmune diseases, are members of the family of pattern recognition receptors (PRRs) together with RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) (17, 18). *TLR7* gene, a member of Toll-like receptors (TLRs), which is located at Xp22.3-p22.2, and has two introns and three exons (19). It mainly recognises a variety of small antiviral compounds and the viral single-stranded RNA, causing a series of signal transduction and inducing the release of cytokines such as TNF-α, IL-1, IL-6, IL-12 and IFN-α (20). These cytokines play a vital role in the development of autoimmune diseases and thus *TLR7* has been verified to be associated with inflammatory or autoimmune diseases. Many previous studies have reported that *TLR7* gene CNVs were related to the risk of behcet disease and systemic lupus erythematosus (21, 22). As an immune-related disease, AS shares some genetic overlaps and pathogenic mechanisms with SLE (23). Thus we hypothesised that *TLR7* gene CNVs might be associated with AS susceptibility. In this study, we would explore the relationships of *TLR7* gene CNVs with AS susceptibility in Chinese Han population.

Materials and methods

Study subjects

This study recruited 649 unrelated Chinese Han patients with AS and 628 healthy controls who were matched by ethnicity. The AS patients and the healthy controls were drawn from the Department of Rheumatology and Immunology, physical examination center, respectively, both in the First Affiliated Hospital of Anhui Medical University. All patients were diagnosed according to the 1984 modified New York criteria (24) and controls were free of autoimmune disease or such family history. Moreover, the study was approved by

Table I. The information of two fragments and connection primes for *TLR7* gene.

probe	Chromesome	Location (ref37Database)[a]	Amplification length (sample, competitive)[b]	Primer binding region 1	Primer binding region 2
TLR7_1	X	12903790-12903950	189(+0,-2)	CATTTGACAGAAATTCCTGGAGGT	TTTGACCCCAGTGGAATAGGTACA
TLR7_2	X	12906638-12906825	216(+0,-2)	GGAAAAGGCTCTGTGGGAGTT	CCAGGCCTCTCCTTGGTAAAC

[a] GRCH37 reference primary assembly; [b] sample: sample DNA, competitive: competitive DNA.

the ethics committees of Anhui Medical University, and informed consents were obtained from all subjects. The study was performed in accordance with the Declaration of Helsinki.

Copy number (CN) estimation

First, the DNA extraction was conducted. Peripheral blood was collected from all subjects in vacuum blood tubes contained EDTA-K2. Genomic DNA was isolated from 2-mL whole blood using a QIAGEN kit according to the manufactures instructions (QIAGEN, Hilden, Germany) and was stored at -20ºC before CNV genotypes detection. Then we designed two sets of probes to examine the copy number of *TLR7* gene, and both of the probes have two primers (details see Table 1). Probes information was referred to the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway). The copy numbers of *TLR7* gene (2 fragments) were measured by AccuCopy™ method (Genesky Biotechnologies Inc, Shanghai, China). The basic molecular principle of competitive PCR amplification for AccuCopy™ was illustrated by Du *et al*. and the study showed that AccuCopy™ was a reliable method for multiple CNV genotyping (25). Furthermore, we detected two fragments of the *TLR7* gene because the *TLR7*gene sequence is long and the specific sequence (the first and last exons) of the *TLR7* gene is amplified to represent the other sequences. The primers of target segments were provided in Table I.

Statistical analysis

Both patients and controls were divided into two groups with one copy and two copies. The χ^2 test was used to assess the differences in the distributions of demographic characteristics and the *TLR7* copy numbers between patients and control. Association between the *TLR7* copy numbers and AS risk was determined by fitting an unconditional logistic regression model. Furthermore, the logistic regression models adjusted for sex and age were used to calculate the ORs of one copy number and two copy numbers in disease susceptibility. Stratification analysis by sex was performed to explore the correlation of *TLR7* copy numbers and AS risk between different genders. A *p*-value <0.05 was considered to be statistically significant and Bonferroni correction for multiple comparisons was applied appropriately when *p*-value for a truly significant result was calculated as 0.05/2. All statistical analyses were performed in SPSS v. 16.0 for Windows (SPSS Inc., Chicago, IL).

Results

Demographics and clinical characteristics of the study sample

A total of 1277 DNA samples (649 AS and 628 health controls) were tested. The mean age of the patients and controls was 28.33±8.93 and 27.83±7.58 years, respectively. The males/females in patients and controls were 541/108 and 512/108, respectively. The age of the healthy controls and the AS patients was comparable (t=1.06, *p*=0.29) and so was the sex ratio (χ^2=0.13, *p*=0.71). With respect to AS patients, 64% patients were HLA-B27 positive.

Frequency distribution of TLR7 gene copy number

For each sample, the copy number in two fragments (*TLR7_1* and *TLR7_2*) of *TLR7* gene triplicates was examined and the raw data were shown in Figure 1. There was a high overlap in the region of the two fragments, that is to say the two fragments were highly homologous. The copy number of *TLR7_1* and *TLR7_2* ranged from 1 to 2 per diploid genome. Frequency distribution of *TLR7* gene copy numbers were showed in Table II.

The CNV genotypes and AS risk

As shown in Table II, the copy numbers of both *TLR7_1* and *TLR7_2* was one

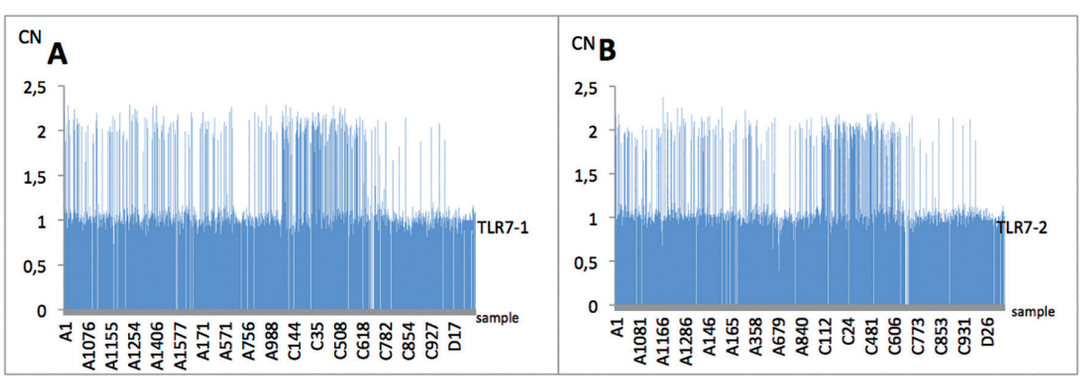

Fig. 1. The raw data of CN in *TLR7*_1 (A) and *TLR7*_2 (B). The Y-axis means copy number of *TLR7* gene of each sample, and X-axis means all of the enrolled cases including ankylosing spondylitis (AS) patients and healthy controls.
CN: copy number

Table II. The frequency distribution of two TLR7 gene fragment copy number.

Copy number	TLR7_1		TLR7_2	
	Case, n(%)	Control, n(%)	Case, n(%)	Control, n(%)
1	522 (83.1%)	456 (77.2%)	523 (83.1%)	459 (77.1%)
2	106 (16.9%)	135 (22.8%)	106 (16.9%)	135 (22.9%)
Total	628 (100.0%)	591 (100.0%)	629 (100.0%)	594 (100.0%)

Table III. The association between TLR7 gene CNV and AS risk.

	Case	Control	χ^2	Crude OR(95%CI)	p^*	Adjusted OR(95%CI)#	p_{adj}*
TLR7_1							
1 copy	522	456	6.827	1.458 (1.098,1.936)	0.009	2.066 (1.318,3.238)	0.002
2 copies	106	135		Ref			
TLR7_2							
1 copy	523	459	6.665	1.451 (1.093,1.927)	0.010	2.061 (1.315,3.230)	0.002
2 copies	106	135		Ref			

Adjusted in a logistic regression model that include age.
*p-value remained statistically significant after Bonferroni correction.
(p-values <0.025 were considered statistically significant).

Table IV: The association analysis by gender between TLR7 gene CNV and AS risk.

	Case	Control	Crude OR (95%CI)	p^*	Adjusted OR (95%CI)#	p_{adj}*
Male						
TLR7_1						
1 copy	516	428	7.987 (3.756,16.983)	<0.001	8.036 (3.774,17.107)	<0.001
2 copies	8	53	Ref			
TLR7_2						
1 copy	517	431	7.947 (3.738,16.897)	<0.001	8.019 (3.767,17.072)	<0.001
2 copies	8	53	Ref			
Female						
TLR7_1						
1 copy	6	24	0.204 (0.080,0.524)	0.001	0.221 (0.085,0.572)	0.002
2 copies	98	80	Ref			
TLR7_2						
1 copy	6	24	0.204 (0.080,0.524)	0.001	0.221 (0.085,0.572)	0.002
2 copies	98	80	Ref			

Adjusted in a logistic regression model that include age.
*p-value remained statistically significant after Bonferroni correction.
(p-values <0.025 were considered statistically significant).

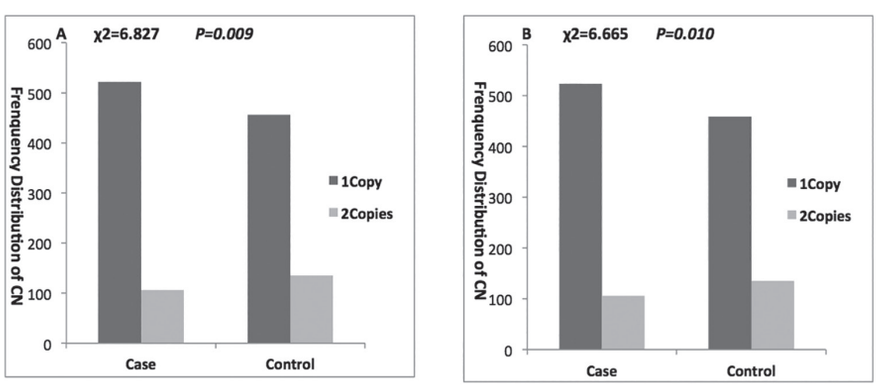

Fig. 2. The frequency distribution of CN between AS cases and controls in *TLR7*_1 (A) and *TLR7*_2 (B). The CN were divided into two categories, p-values above the histogram represent the results of chi-squared tests of *TLR7*_1 and *TLR7*_2, respectively. CN: copy number.

copy or two copies. Thus, we divided the samples into one copy group and two copies group. Figure 2 revealed that the frequencies of *TLR7_1* and *TLR7_2* gene CNVs were both significantly different between the cases and controls after Bonferroni correction (χ^2=6.827, p=0.009 for the *TLR7_1*; χ^2=6.665, p=0.010 for the *TLR7_2*).

As shown in Table III, the results of logistic regression model revealed that one copy number was significantly associated with AS disease susceptibility after adjusting for sex and age (For *TLR7-1*, compared with two copies, adjusted OR=2.066, 95%CI=(1.318,3.238), p_{adj}=0.002; For *TLR7-2*, compared with 2 copies, adjusted OR=2.061, 95%CI=(1.315,3.230), p_{adj} =0.002). Overall, the lower copy number had the higher risk for AS. However, after stratification analysis by gender, the results were controversial between males and females (see Table IV). Comparing to two copies, one copy confers increased risk to AS in males (For *TLR7_1*, adjusted OR(95%CI)= 8.036(3.774,17.107), P_{adj}<0.001; For *TLR7_2*, adjusted OR(95%CI)= 8.019(3.767,17.072), P_{adj}<0.001) but decreased risk in females (For *TLR7_1* and *TLR7_2*, adjusted OR(95%CI)= 0.221(0.085,0.572), P_{adj}=0.002). All the above results remained significant after Bonferroni correction.

To determine whether *TLR7* copy number associated with specific clinical manifestations of AS, we compared the difference in cases between the CNVs and the erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), HLA-B27, and Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), respectively, but we could not find any association between the clinical manifestations and *TLR7* CNVs (data not shown).

Discussion

To the best of our knowledge, this is the first study to explore the relationship between *TLR7* CNVs and AS susceptibility in the Chinese Han population. In the present study, CNV of two *TLR7* gene fragments (*TLR7*_1 and *TLR7*_2) was detected in the Chinese

Han population and the effects of this CNV on AS were investigated.

TLR7, an X-linked gene, belongs to the family of pattern-recognition receptors which play a pivotal role in innate immune system (22, 26). It has been reported that *TLR7* may recognise endogenous nucleic acid ligands included in immune complexes and intensify or lead to autoimmunity in pathological conditions (27-29). Importantly, a possible role of interferon family molecules (interferon-γ) in AS has been verified (30). Furthermore, previous studies demonstrated that *TLR7* are closely related to the production of interferon family molecules including interferon-γ and interferon -α (31-33). This may explain a role of *TLR7* in the pathogenesis of AS. Then previous study has revealed that *TLR7* gene expression level was significantly higher in patients with psoriasis than in healthy controls (34). It was reported that phenotypic effect of CNVs could be the change of the expression levels (35). Consequently, *TLR7* gene CNVs may affect the susceptibility to Psoriasis. Psoriasis is not only an immune-related disease but also an extra-articular manifestation of spondyloarthritides which include AS (1). This can provide potential evidence about the relationship between *TLR7* gene CNVs and AS. With respect to CNVs, it has been taken for granted that CNVs in *TLR7* are correlated with the modulation of the autoimmune response to nuclear material (36). Relevant studies have shown that CNVs of *TLR7* had an association with childhood onset SLE, RA, and Graves' disease (22, 26). AS is an autoimmune and arthritis disease, and it shares several common aspects of pathogenesis with other autoimmune diseases such as SLE and RA, so genetic overlaps might exist between AS and other autoimmune diseases (23). This may also explain our result that TLR7 gene CNVs were significantly associated with AS.

Our results showed that both the two fragments of *TLR7* CNV were associated with genetic susceptibility to AS, and a harmful effect of lower CN on the development of AS was observed in Chinese population. For *TLR7_1* and *TLR7_2*, one copy might confer a 1.458-fold and 1.451-fold increasing risk of AS, relative to two copies. After adjusted by sex and age, it remained significant between AS susceptibility and *TLR7* CNVs. Interestingly, we found that the effect of *TLR7* CNV on AS was greater and the conclusion was contradictory when the analysis was just conducted in males or females. That is to say, we detected a harmful effect of lower CN on AS susceptibility in males but protective effect in females. Regarding to males, we found that one copy might confer a 7.987-fold and 7.947-fold increasing risk of AS compared with two copies in *TLR7_1 and TLR7_2*. With respect to females, lower CN (=1) might confer a 0.204-fold decreasing risk of AS compared with normal copy (=2) both in *TLR7_1* and *TLR7_2*. This gender-related difference in *TLR7* function has been studied previously due to the role of X-linked gene. For example, SLE predominantly affects women and this may be explained as follows. As mentioned above, *TLR7* was closely related to the secretion of IFN-α, which play an important role in developing SLE and healthy women produce more IFN-αthan men (37). Recently, an X-linked gene named the interleukin 1 receptor-associated kinase 1 (*IRAK1*) was included as a risk factor for SLE (38). These explanations undoubtedly supported that *X-linked* gene confers different risk between females and males, which was consistent with the gender-related difference in our study. However our results showed that one copy in *TLR7* gene had the harmful effect for AS in males but protective effect in females. In other words, both males having one copy and females having two copies were related with increased risk of AS. Since the *TLR7* gene is located on chromosome X, one copy of *TLR7* should be the normal in males and two copies of *TLR7* should be the normal in females. Then according to the stratification analysis by gender, the 93.9% of males carried one copy of *TLR7* while two copies were detected in 85.5% of females. This result may contribute to the well-known conclusion that the incidence of AS is much higher in males. The opposite effect of *TLR7* between different genders could not be explained explicitly at present, it may be due to the protective effect of IFN-α in females or the higher exposure to certain environmental factors in males. In future, we should examine the level of *TLR7* expression and its downstream signaling pathways especially the production of IFN-α to explore the mechanism of gender-related difference. Therefore, future studies with larger sample size, especially of the female population, will be required to confirm the importance of *TLR7* CNV as a genetic marker of AS. Our study was performed in Han Chinese and whether the same correlation can be replicated in other ethnic populations is also subject of further studies.

Conclusions

In conclusion, this study for the first time suggests that CNVs in *TLR7* are associated with AS and that the lower copy number presents a risk effect for male AS and protective effect for female in AS.

Acknowledgments

We thank all AS patients and healthy controls for providing the plasma and information necessary for this study.

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