Original Article

Association of β-defensin Gene Copy number variations with Ankylosing Spondylitis in Chinese population: A case control study

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Abstract

Objectives: To explore the association of β-defensin gene copy number variations (CNVs) with Ankylosing Spondylitis (AS).

Methods: In this study, 405 unrelated Chinese Han patients with AS and 401 unrelated healthy controls were enrolled. The copy numbers of DEFB4 gene (2 fragments) were measured by AccuCopy™ methods. The association of DEFB4 gene CNVs with AS susceptibility was analyzed by chi-square and logistic regression models. Besides, *P* values, Odds Ratio, and 95% confidence intervals (CIs) were used to estimate the effects of risk.

Results: The range of DEFB4_1 CN was 0 to 7 and the range of DEFB4_2 CN was 1 to 8 both in patients and controls. *P* value of χ^2 trend test for the association of DEFB4_1 and DEFB4_2 with AS were 0.607 and 0.005,

respectively. The results of DEFB4_2, compared with the individual having median 3-copies, those carrying ≤2-copies [OR=0.68, 95%CI (0.46,0.99), *P*=0.049; adjusted OR=0.69, 95%CI(0.47,1.03), *P*=0.067.]; and those carrying ≥4-copies [OR=0.62, 95%CI(0.45, 0.86), *P*=0.004; adjusted OR=0.64, 95%CI(0.46,0.88), *P*=0.006] was significantly associated with decreasing risk of AS. Univariate analysis showed that both DEFB4_1 and DEFB4_2 were associated with BASDAI. After adjusted by age, sex and disease duration, the results changed little, which demonstrated that high-copies may be linked with decrease in the risk of disease severity [OR=0.71, 95%CI (0.56, 0.90), *P*=0.005; OR=0.75, 95%CI (0.60, 0.94), *P*=0.013, respectively].

Conclusions: The CNs of DEFB4 gene may be associated with AS and involved in disease progression.

Keyword: Ankylosing Spondylitis, β-defensin copy number variation, case control study.

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Introduction

Ankylosing Spondylitis (AS), a common inflammatory arthritis that main symptom was inflammatory spinal pain. The overall prevalence of AS were 0.25% in European[1] and 0.2%~0.54% in Chinese[2]. It was generally observed in young people, and slightly prevailed in men than women[3] having a ratio of 2:1[4]. Up to now, the pathophysiology of AS was unknown, but it has long been known that the susceptibility to AS was almost associated with complex genetic and environmental factors, Besides, genetic analysis had indicated that the heritability of the disease exceeded 90%[5]. In the past decade, we have already known that human leukocyte antigen (HLA)-B27 which have contributed 16%~50% of the total genetic risk for the disease and B27 subtypes were associated with AS[6]. Genome-wide association study (GWAS) demonstrated that several areas from non-HLA regions like 1p, 2p, 2q, 4q, 5q, 6p, 9q, 10q, 16q, 19q, and 21q were related to AS[7], but their contributions to the development of disease were little. Now, another gene variation-copy number variations (CNVs) have been studied, and discovered that those were associated with many human diseases.

Copy number variations (CNVs) were simple different numbers of the same DNA sequence across different individuals which included not only simple deletions and duplications but also more complex multi-allelic variations. It was just another form of variation, complying with the rules of population genetics like other variants. In recent years, many CNVs have been detected to be associated with several autoimmune diseases, including rheumatoid arthritis[8], psoriasis[9], ankylosing spondylitis[10] and systemic lupus erythematous[11].

The β-defensin gene clusters at chromosome 8p23.1 was one of the most copy numbers variable regions of human genome. The gene dose partly determined the level of β-defensin which might contribute to the different susceptibility to microbial infections and intensity of

inflammation[12]. DEFB4 gene was a member of β-defensin which coded the human β-defensin 2 (hBD2), a small antibiotic peptides that have antimicrobial, modulating and enhancing immune function. Many previous studies have reported that DEFB4 gene CNVs were related to psoriasis[9, 13](increase) and Crohn's disease[12, 14] (either increase or decrease). However, whether the DEFB4 gene CNVs were also associated with AS have not been reported until now. In this study, we would explore the association of DEFB4 gene CNVs with AS in Chinese population.

Patients and methods

Subject

This study included 405 unrelated Chinese Han patients with AS and 401 unrelated healthy controls who were matched by ethnicity. The mean age of the patients and control was 27.91±8.93 years and 26.89±7.12, respectively. The sex ratio of male: female was 5.12:1 in patients and 4.21:1 in control group. All patients were from the department of Rheumatology and Immunology, the First Affiliated Hospital of Anhui Medical University, and all patients were diagnosed definitively according to the modify New York diagnostic criteria[15]. Moreover, this study was approved by the Anhui Medical University Ethics Committee and each participant signed an informed consent form after receiving a vernal explanation of the study. All demographic data of patients and control were collected, and Clinical data of patients were also obtained.

The "gold standard" for measuring and evaluating diseased activity in AS was the Bath AS Disease Activity Index (BASDAI), which contained 6 questions about fatigue, spinal pain, peripheral arthritis, enthesitis, and morning stiffness (range 0 to 10)[16]. In this study, patients were considered as having active AS if BASDAI ≥ 4[17].

AccuCopy™ method for determination DEFB4 copy number

The copy numbers of DEFB4 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[18]. The forward and reverse primers of target segments were provided in **Table 1 and t**he genetic structure of DEFB4 CNV were presented in **Figure 1.** The PCR reaction was prepared in 20μl for each sample, containing 1×Multiplex PCR Master Mix (Qiagen, Valencia, CA, USA), 0.05μM each primer, 1×Competitive DNA Mix (Genesky Biotechnologies) and ~10ng genomic DNA. The PCR program followed as bellow: 95ºC 10min; 11 cycles x (94ºC 20s, 65ºC-0.5ºC /cycle 40s, 72 ºC 1min30s); 24cycles x (94ºC 20s, 59ºC 30s, 72 ºC 1.5min); 60ºC 60 min; 4 ºC forever.

PCR products were diluted 20-fold before being run by capillary electrophoresis using ABI (Carlsbad, CA, USA) 3730XL genetic analyzer. Raw data were analyzed by GeneMapper4.0 (ABI) and the height and area data for all specific peaks were exported into a Microsoft Excel file. The sample/competitive (S/C) peak ratio was calculated for all four target segments and four reference segments. The S/C ratio for each target fragment was first normalized based on four reference segments, respectively. The four normalized S/C ratios were further normalized to the median value in all samples for each reference segment, respectively, and then averaged. If one of the four normalized S/C ratios deviated >25% from the average of the other two, then it was excluded for further analysis. The copy number of each target segment was determined by the average S/C ratio times two, given that the copy numbers of four reference segments were two in the diploid genome.

Statistics Analysis

Data were assembled according to the case control study design. Patients were divided into different group according to their copy numbers. The χ^2 test and Wilcoxon rank sum test were used to assess the differences in the distributions of demographic characteristics and the CNV-DEFB4 genotypes between patients and control. Association between the CNV-DEFB4 genotypes and AS risk was estimated by adopting an unconditional logistic regression model and χ^2 trend test. Association between the DEFB4 copy number and BASDAI risk was estimated using an unconditional logistic regression model with or without adjustments of age, sex and disease duration. We calculated two-sided 95% confidence intervals (CIs) based on Wald's tests. Statistical power was calculated by PASS (NCSS, USA). The significance level was set at α=0.05, however, after multiple testing corrections by adopted Bonferroni methods, then, P<0.025 was considered to be statistical significant. All statistical analyses were performed on SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL).

Results

Demographics of the study sample

A total of 806 DNA samples (405 AS and 401 health control) were tested. The mean age of the patients and controls were 27.91±8.93 years and 26.89±7.12, respectively. The males in patients and controls were 338 and 324, respectively. There were no statistical significant differences between the two groups with respect to age (t=1.77, P=0.076) and sex (χ^2=1.13, P=0.288). The range of disease duration was 0 to 29 years and the median disease duration was 2.08 years, and 64% patients were HLA-B27 positive.

Frequency distribution of DEFB4 gene copy number

We examined two fragments (DEFB4_1 and DEFB4_2) of DEFB4 gene, which locate at human chromosome 8, and detected that a high overlap occurred in the region of those two

fragments. Results showed that the range of DEFB4_1 CN was 0 to 7 and the range of DEFB4_2 CN was 1 to 8. The median of DEFB4_1 and DEFN4_2 were 3 copies, the majority had four copies of DEFB4_1 in patients (31.31%) and control group (34.34%), and the majority had three copies of DEFB4_2 in patients (35.99%) and control group (27.14%), whereas 1, 7 and 8 were uncommon. Frequency distribution of DEFB4 gene copy numbers were showed in **Table 2.**

The CNVs genotypes and AS risk

As it was shown in table 2, the median of DEFB4_1 and DEFN4_2 were 3 copies, we then divided the samples into lower (two or fewer), central (three) and higher(four or more), **Fig 2 and 3,** the results of χ^2 test of DEFB4_1 and DEFB4_2 were 1.89 and 8.68, respectively, and *P* value were 0.388 and 0.013, respectively. Multiple testing corrections of DEFB4_2, ≥ 4-copies compared with 3-copies had statistical significance (*P*=0.004), ≤ 2-copies compared with 3-copies was marginal statistical significant (*P*=0.049). The statistical power of DEFB4_1 and DEFB4_2 were 0.22 and 0.75, respectively. For the DEFB4_2 gene, compared with the individuals whose had 3-copies, the individuals whose carried ≤ 2-copies were significantly associated with decreasing risk of AS [OR=0.68, 95%CI (0.46, 0.99), *P*=0.049], but adjusted by age and sex, there were no significant difference (adjusted OR=0.69, 95%CI(0.47, 1.03), *P*=0.067); and ≥ 4-copies conferred a 0.62-fold decrease risk of AS compared with 3-copies[OR=0.62, 95%CI(0.45, 0.86), *P*=0.004), adjusted OR=0.64, 95%CI(0.46, 0.88), *P*=0.006]. χ^2 trend test used to explore the association of DEFB4-CNV with AS, which showed that *P* value of DEFN4_1 was 0.607, and DEFB4_2 was 0.005. (**Table 3**) .

Association of CNVs with BASDAI risk

As shown in table 3, we found that the CNVs of DEFB4 gene were associated with AS susceptibility. We assumed that whether the CNVs would influence the severity of AS, we further studied the association of CNVs and BASDAI using an unconditional logistic regression model with or without adjustments of age, sex and disease duration. Univariate analysis showed that both DEFB4_1 and DEFB4_2 were associated with BASDAI, high-copies were related to decreasing risk of the disease severity [OR=0.71, 95%CI (0.56, 0.90), *P*=0.005; OR=0.75, 95%CI (0.60,0.94), *P*=0.013, respectively]. After adjusted by age, gender and disease duration, we found that CNVs of DEFB4 gene were associated with BASDAI, moreover, it was worthy to note that it was independent of age, sex and disease duration. (**Table 4**)

Discussion

Defensin genes have been the focus of the present susceptibility study as the functional consequences of such mutation appeared to be associated with reduced expression and presumably reduced activity of the resulting peptides. β-defensin was coded by defensin gene - a small cationic peptides with antimicrobial activity, which was shown to be an important player in the innate immune system[19-21]. Recently, more study reported that great genetic heterogeneity could be observed in human β-defensin gene locus and may be a result of selection[22], Furthermore, many previous studies have reported that DEFB4 gene CNVs were associated with psoriasis[9, 13] and Crohn's disease[12, 14]. However, it had not found relevant study about AS, so we designed this study to explore the association of DEFB4 gene CNVs with AS susceptibility. The copy numbers of DEFB4 gene were range from 1 to 8 in our study, which is similar with the previous study[23].

AS was a complicated autoimmune disease, and the susceptibility to AS was almost associated with complex genetic and environmental factors. Recently, a GWAS had found five deletion-type CNV regions, in 1q32.2, 2q31.2, 6p21.32, 13q13.1 and 16p13.3, were related to an increased risk of AS[24]. There existed a correlation between copy number and β-defensin gene expression at the mRNA level[25]. It was only when higher copy numbers of β-defensin were present, transcription was significant up-regulated by TNF-α, which resulted in better anti-microbial activity in *vitro*[26]. Previous study showed that a low level of β-defensin caused by low CN may lead to ineffective defense against microorganisms in skin or mucosa and then triggered a chronic inflammatory state[27].

In our study, the fragment of DEFB4_1, it was not statistical significant with susceptibility of AS, which may be as a result of the statistical power was lower. But in regard to the fragment of DEFB4_2, we found that higher CN (≥4) may conferred a 0.62-fold decreasing risk of AS compared with 3-copies. And the results were also showed that lower CN(≤2) had marginal statistical significance compared with 3-copies, but after adjusted by age and sex and multiple testing corrections, it had no significance. These two fragments of DEFB4 gene had different significance with susceptibility of AS, which may be owing to the different of the two test region, in fact. Combined with previous studies, we drawn a conclusion that the CN of DEFB4 gene may play a different role in different autoimmune diseases.

Considering the association of DEFB4-CNV with susceptibility of AS may be affected by SNPs, we had queried relevant literatures and referred to the previous studies, which had found that the distribution of alleles at the -2,173 SNP in the DEFB4 gene did not agree with the Hardy-Weinberg equilibrium, and the existence of a DEFB4 paralogous gene with a 99.4% sequence homology could explain why a larger cohort of subjects does not agree with the

Hardy–Weinberg equilibrium for previously identified SNPs by direct sequencing of the gene[28]. In view of that, the results suggested that the association of DEFB4 gene and susceptibility of disease may be due to CNV rather than SNPs.

BASDAI score was usually used to measure and evaluate disease activity in AS, in our study, patients were considered as active AS if BASDAI ≥ 4[17]. Analysis by logistic regression model, higher CNV of DEFB4 gene may decrease the risk of disease severity with or without adjusted by age, sex and disease duration. The conventional therapy for AS were non-steroidal anti-inflammatory drugs (NSAIDs) and disease modifying anti-rheumatic drugs (DMARDs), such as sulfasalazine or methotrexate, in our study, the patients who were also treatment with these conventional drugs, taking into the therapy may affect the progress of AS, we had added this variable into analysis, and results showed that effect of DEFB4-CNV has not change, so we think the effect of DEFB4-CNV on BASDAI was independent of therapy. But further study is strongly needed to demonstrate how to regulated disease activity.

To our knowledge, this was the first study to independently analyze the relationship between the copy number variations of DEFB4 gene and susceptibility to AS, which have already demonstrated that higher CN of DEFB4 gene were significant associated with decreasing AS and BASDAI risk, which suggested that higher CN of β-defensin gene may be play an protective role in the development of AS. Therefore, we will continue to explore how it influence the onset of AS.

Acknowledgment

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Conflict of interest

None.

Reference

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Figure legends

Figure 1 the genetic structure of DEFB4 CNV and the position of the primer sets.

Figure 2 the frequency distribution of CN in DEFB4_2.

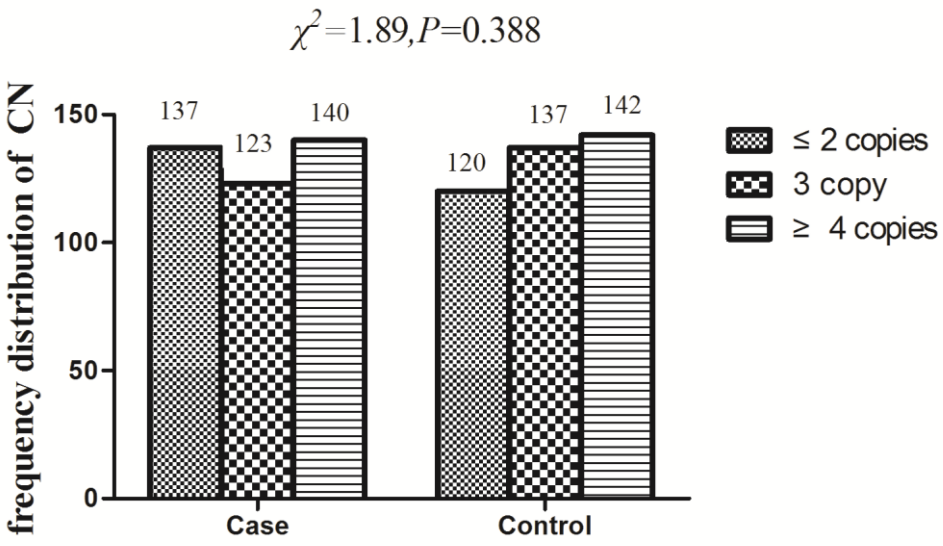

Figure 3 the frequency distribution of CN in DEFB4_2.

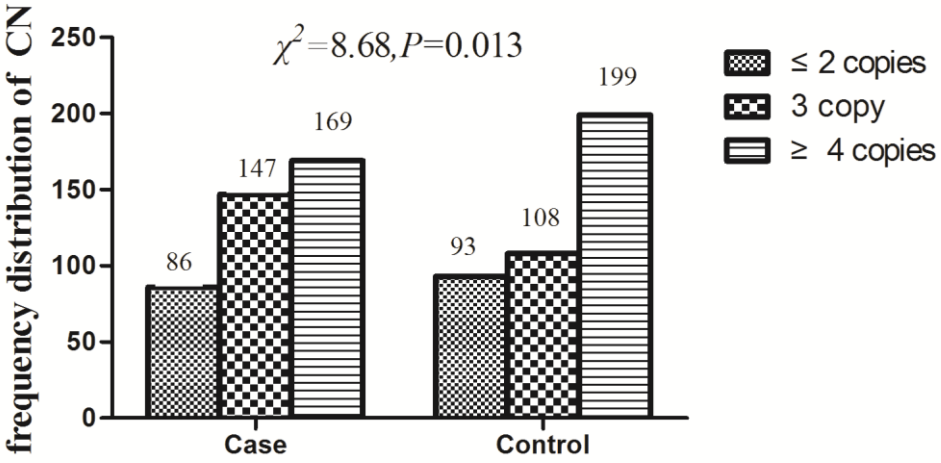

Table 1 the information of two fragment and connection primes for DEFB4 gene.

probe	Gene fragment	Chromosome	Location (ref37 Database)[a]	Amplification length (sample,competitive)[b]	Primer binding region 1	Primer binding region 2
DEFB4-1	DEFB4A	chr8	7752565-7752729	193(+0,-2)	TGCCCTATTTAATTGAACCAAGCAT	CAACCATAGTCTCAGCAGTTGTAGCA
	DEFB4B	chr8	7273864-7274028			
DEFB4-2	DEFB4A	chr8	7272415-7272618	232(+0,-2)	TGTTTTTGGTGGTATAGGCGATCC	ATGTCGCACGTCTCTGATGAGG
	DEFB4B	chr8	7753997-7754200			

[a]: GRCH37 reference primary assembly

[b]:sample: sample DNA, competitive: competitive DNA.

Table 2 the frequency distribution of two DEFB4 gene fragment copy number

Copy number	DEFB4_1		DEFB4_2	
	Case, n(%)	Control, n(%)	Case, n(%)	Control, n(%)
1	3 (0.73)	3 (0.75)	23 (5.56)	24 (6.03)
2	20 (4.58)	28 (7.02)	63 (15.22)	69 (17.34)
3	114 (28.40)	89 (22.31)	146 (35.99)	108 (27.14)
4	123 (31.31)	137 (34.34)	103 (26.09)	102 (25.63)
5	103 (25.24)	102 (25.56)	43 (11.59)	73 (18.34)
6	31 (8.01)	30 (7.52)	15 (3.62)	13 (3.27)
≥7	0(0.00)	2(0.50)	6(1.93)	9(2.26)
Total	400((100.00)	399(100.00)	399(100.00)	398(100.00)

Table 3 the association between DFB4 gene CNV and AS risk

	Case	Control	Crude OR(95%CI)	*P*	P^b	Adjusted OR(95%CI)[a]	*P*	P^b
DEFB4_1								
3 copies	123	137	Ref					
⩽2 copies	137	120	1.27(0.90,1.79)	0.173	>0.025	1.26(0.89,1.78)	0.195	>0.025
⩾4 copies	140	142	1.09(0.78,1.54)	0.586	>0.025	1.08(0.77,1.52)	0.654	>0.025
Trend test *P*-value			0.607			0.606		
DEFB4_2								
3 copies	147	108	Ref					
⩽2 copies	86	93	0.68(0.46,0.99)	0.049	>0.025	0.69(0.47,1.03)	0.067	>0.025
⩾4 copies	169	199	0.62(0.45,0.86)	0.004	<0.025	0.64(0.46,0.88)	0.006	<0.025
Trend test *P*-value			0.005			0.005		

[a] Adjusted in a logistic regression model that include age, sex.

[b]: Multiple testing corrections: $P<0.025$ has statistical significance

Table 4 the association between DEFB4 gene CNV and BASDAI

	Model 1			Model 2			Model 3		
	β	OR, 95%CI	*P*	β	OR, 95%CI	*P*	β	OR, 95%CI	*P*
DEFB4_1									
DEFB4	-0.35	0.71(0.56,0.90)	0.005	-0.36	0.70(0.55,0.90)	0.004	-0.36	0.70(0.55,0.90)	0.004
age	0.04	1.04(1.01,1.07)	0.003	0.05	1.05(1.02,1.08)	0.002	-	-	-
sex	-0.51	0.60(0.32,1.15)	0.124	-0.41	0.66(0.34,1.31)	0.234	-0.42	0.66(0.33,1.33)	0.242
disease duration	0.08	1.08(1.03,1.14)	0.001	-	-	-	0.10	1.10(1.05,1.16)	<0.01
DEFB4_2									
DEGB4	-0.28	0.75(0.60,0.94)	0.013	-0.31	0.74(0.58,0.93)	0.009	-0.32	0.72(0.58,0.92)	0.008
age	0.04	1.04(1.01,1.07)	0.003	0.04	1.04(1.01,1.07)	0.004	-	-	-
sex	-0.51	0.60(0.32,1.15)	0.124	-0.59	0.55(0.28,1.09)	0.082	-0.61	0.55(0.27,1.07)	0.077
disease duration	0.08	1.08(1.03,1.14)	0.001	-	-	-	0.09	1.09(1.03,1.15)	0.001

Model 1: Univariate analysis of binary logistic, Model 2: adjusted by age and sex, Model 3: adjusted by sex and Duration of disease.