

Copy number variation of beta-defensin genes in Behçet's disease

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ABSTRACT

Objectives. Behçet's disease (BD) may be triggered by infectious agents in genetically susceptible persons. Human β -defensin 2 is an inducible antimicrobial peptide, the level of which can be influenced by copy number (CN) of the *DEFB4*. We investigated the relationship between copy number variation (CNV) of *DEFB4* and BD.

Methods. One hundred and ninety-seven patients with BD and 197 healthy controls were enrolled. After measuring CN of *DEFB4* with a paralogue ratio test, the CNV was compared between patients and controls. CNV was also analysed in comparison with the clinical manifestations of BD.

Results. The CN of *DEFB4* was unimodally distributed among the study subjects with mean CN of 4.57 and standard deviation of 1.28. BD samples had numerically lower CN than controls, but the difference was not statistically significant (4.49 ± 1.21 vs. 4.65 ± 1.36 , $p=0.245$). Regarding the relationship between CN of *DEFB4* and clinical manifestations, there was no difference of CNV depending on the clinical manifestations.

Conclusion. We found no significant difference in CNV of *DEFB4* between patients with BD and controls. Our results suggest that CNV of *DEFB4* may not contribute to the pathogenesis of BD.

Introduction

Behçet's disease (BD) is a rare, chronic, inflammatory disease of unknown etiology, characterised by recurrent oral and genital ulcers, ocular and cutaneous lesions, as well as visceral organ involvement (1). Epidemiologic studies suggest that the interaction between genetic and environmental factors plays a crucial role in the disease development (1-2). As for many other immune dysregulatory diseases, infectious agents are thought to trigger the pathology in

genetically susceptible patients such as HLA-B51 carriers (2).

β -defensins are small antibiotic peptides that have direct antimicrobial, immune-enhancing and modulating and cytokine-like activity in inflammatory signalling pathways. They are part of the innate immune system and are encoded by *DEFB* genes in three main gene clusters: two on chromosome 20 and one on chromosome 8p23.1 (3).

Genetic alterations include qualitative changes and quantitative changes which include change of chromosome number and CNV of a gene (4). CNV is the most common type of quantitative variation among different individuals. Several autoimmune diseases have been found to be associated with CNV of a specific gene: these include systemic lupus erythematosus with complement factor 4 (C4) gene, and Wegener's granulomatosis and microscopic polyangiitis with FCGR3B (5). The β -defensin genes on 8p23.1 with the exception of *DEFB1* but including *DEFB4*, *SPAG11*, *DEFB103*, *DEFB104*, *DEFB105*, *DEFB106*, and *DEFB107* are on a large repeat unit of which the copy number is highly polymorphic and varies between 2 and 12 copies (6). The gene dose partly determines β -defensin level, and the variation of β -defensin level might contribute to different susceptibility to microbial infections and intensity of inflammation (7).

Previous case control studies showed an association of psoriasis with increased copy number (CN) of *DEFB4* (6), and Crohn's disease with either increased or decreased CN of *DEFB4* (7-8). In genetically susceptible patients, a minor infection may not be controlled in individuals with decreased β -defensin level and may lead to Behçet's skin and mucosa manifestation. Therefore we investigated whether there is a relationship between CN variation (CNV) of *DEFB4* and susceptibility to BD in Koreans.

Competing interests: none declared.

Patients and methods

Patients and controls

A total of 197 consecutive Korean patients with BD were enrolled from the Rheumatology Clinic of Seoul National University Hospital between March 2003 and February 2009. The diagnosis of BD was mainly based on the diagnostic criteria of international study group (ISG) for BD (9). Fifteen patients did not fulfill the ISG criteria but fulfilled the possible category of diagnostic criteria from the Behçet Syndrome Research Committee of Japan (10). The healthy controls were composed of the same number of Korean healthy blood donors. Mean age of the patients and controls were comparable (43.5 ± 10.3 vs. 43.4 ± 10.2 years, $p=0.993$) as was the percentage of males (49.5% vs. 47.4% , $p=0.702$). The institutional review board of Seoul National University Hospital approved the study and informed consent was obtained from all the patients and controls.

Parologue ratio test

To measure the CN of β -defensin gene, we applied the parologue ratio test (PRT) of Armour *et al.* with minor modifications (11). PRT is a novel comparative multiplex polymerase chain reaction (PCR) method in which CN can be accurately measured by amplifying a dispersed repeated sequence (*HSPD8*) near a target gene (*DEFB4*) simultaneously with a similar reference gene on another chromosome (*HSPDP3* in chromosome 5) using a single pair of PCR primers, one of which is fluorescently labelled. By comparing the amount of PCR products from the copy-variable parologue with the reference gene, CNV can be accurately measured (12).

In brief, PCR was performed with genomic DNA from patients and HSPD5.8 forward primer (CCAGATGAGAC-CAGTGTCC) and FAM or HEX labelled HSPD5.8 reverse primer (TTTAAAGT-TCAGCAATTACAGC). Sample DNA was amplified using polymerase chain reaction (PCR) (Peltier Thermal Cycler, PTC-200, Bio-Rad Laboratories Inc., California, USA). PCR products were then cut with *HaeIII* (Takara Bio Inc., Shiga, Japan) after purification. The en-

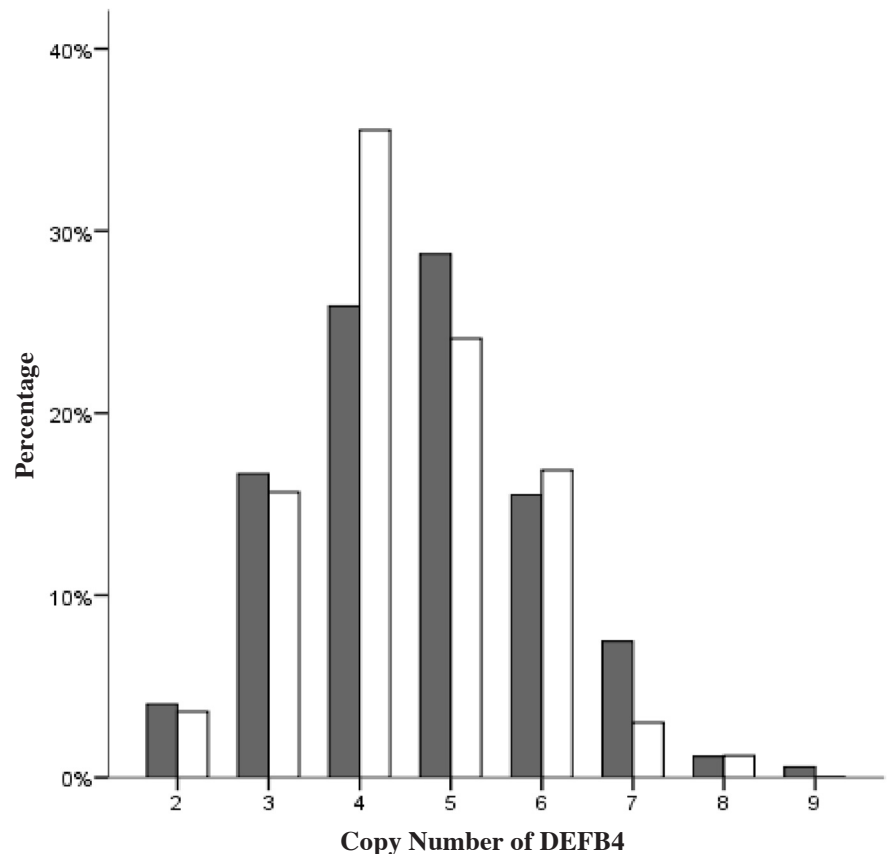


Fig. 1. Distribution of *DEFB4* copy number in healthy controls and patients with Behçet's disease. There was no significant difference in the distributions of copy number variation between healthy controls and patients with Behçet's disease ($p=0.257$).

zyme-cut products were then separated by capillary electrophoresis on an ABI 3130 Genetic Analyzer. Fluorescent intensity was measured with GeneScanTM-500 and the data was analysed with ABI Data Collection software v3.0 and GeneMapper 4.0.

All experimental procedures were done with FAM- and HEX-labelled primers. When the difference of both was less than 15% of their mean, those values were accepted for further analysis.

Statistical analysis

Data was presented as integer numbers and frequencies for categorical variables, and mean $\pm$ standard deviation for continuous variables. For comparison between groups, unpaired Student's *t*-test or 1-way analysis of variance were performed. Statistical tests were performed using SPSS version 17 (SPSS Inc., Chicago, Illinois).

Results

A total of 394 blood samples (197 BD

and 197 healthy controls) were tested. Twenty-two BD samples and 3 healthy control samples failed to produce results. Among the remaining samples, 9 BD sampled and 20 healthy control samples showed more than 15% difference between FAM- and HEX-labelled products. Therefore, 166 BD samples and 174 healthy control samples were able to enter into the final analysis.

The CN of *DEFB4* was unimodally distributed among the study subjects with mean CN of 4.57 and standard deviation of 1.28. The median CN was 4 with interquartile (IQ) of 4 and 5. The modal CN in healthy controls was 5 [IQ: 4-5], but 4 in patients with BD [IQ: 4-5]. However, there was no significant difference in the mean CN of *DEFB4* between healthy control and BD groups (4.65 ± 1.36 vs. 4.49 ± 1.21 , $p=0.245$) (Fig. 1, Table I).

Among patients with BD, we investigated the relationship between CN of *DEFB4* and clinical manifestations. There was no significant difference

Table I. Copy number variation (CNV) of *DEFB4* in Korean DNA samples.

	All	Healthy controls	Patients with BD	<i>p</i> -value*
Sample number	394	197	197	
Successful samples	369	194	175	
Samples with acceptable results	340	174	166	
CNV				
Mean $\pm$ SD	4.57 $\pm$ 1.28	4.65 $\pm$ 1.36	4.49 $\pm$ 1.21	0.245
Median [I.Q]	4 [4–5]	5 [4–5]	4 [4–5]	

BD: Behçet's disease; SD: standard deviation; IQ: interquartile. Of 394 samples, 340 (86.3%) samples were valid for analysis. **p*-value for CNV between healthy control and patients with BD.

Table II. *DEFB4* copy number according to clinical characteristics.

	Characteristics		<i>p</i> -value
	Absent (patient no.)	Present (patient no.)	
Oral ulcer	n.a.	n.a.	n.a.
Genital ulcer	4.72 $\pm$ 1.16 (43)	4.40 $\pm$ 1.22 (122)	0.137
Erythema nodosum	4.28 $\pm$ 1.17 (69)	4.64 $\pm$ 1.21 (97)	0.055
Acne	4.50 $\pm$ 1.31 (90)	4.50 $\pm$ 1.05 (74)	1.000
Uveitis	4.63 $\pm$ 1.21 (99)	4.31 $\pm$ 1.16 (65)	0.095
Retinitis	4.51 $\pm$ 1.19 (158)	4.33 $\pm$ 1.37 (6)	0.729
Saline skin test	4.43 $\pm$ 1.31 (67)	4.51 $\pm$ 1.11(79)	0.713
Vascular Behçet	4.47 $\pm$ 1.18 (143)	4.71 $\pm$ 1.31(21)	0.381
Intestinal ulcer	4.47 $\pm$ 1.21 (158)	4.75 $\pm$ 1.04 (8)	0.530
Deep vein thrombosis	4.46 $\pm$ 1.19 (140)	4.71 $\pm$ 1.23 (24)	0.357
Thrombophlebitis	4.50 $\pm$ 1.19 (162)	4.50 $\pm$ 2.12 (2)	1.000
NeuroBehçet	4.49 $\pm$ 1.20 (154)	4.60 $\pm$ 1.17 (10)	0.786
Arthritis	4.41 $\pm$ 1.17 (74)	4.55 $\pm$ 1.24 (92)	0.430

in the CNV depending on the clinical manifestations (Table II).

Discussion

β -defensins are small cationic peptides that form an important part of the innate immune system (3). Both over- and under-expression might lead to immune-dysregulatory phenomenon and the CNV of β -defensin was associated with immunologic diseases such as Crohn's disease (7–8) and psoriasis (6). Since BD is a representative autoimmune disease which can be triggered by infection, it can be associated with CNV of β -defensin. There have been some clinical observations suggesting increased β -defensin gene dose in BD. Individuals with myelodysplastic syndromes were found to have a trisomy 8 in their bone marrow. Some of these patients developed mucocutaneous ulcerations, resembling one of the main clinical features of BD. Furthermore, patients with BD who have bone marrow failure showed a higher frequency of cytogenetic abnormalities with trisomy 8 (13). These suggest that

trisomy 8 induces over-expression of some genes in chromosome 8, such as *DEFB4*, as a result of increased gene dose. Since β -defensin plays an important role in immune activation at epithelial surfaces and has cytokine like properties, overexpression may lead to inflammation of skin, causing mucocutaneous ulceration. In psoriasis, an inflammatory skin disease, CN of *DEFB4* CN was increased compared with healthy controls (6).

On the other hand, a low level of β -defensin caused by low CN may lead to ineffective defense against microorganisms in skin or mucosa and trigger a chronic inflammatory state. Since one of the pathogenic mechanisms for BD is believed to be an autoimmune process triggered by infectious agents, patients with lower CN of *DEFB4* could be more susceptible. In accordance, there is a report suggesting lower CN of *DEFB4* in Crohn's disease (7) which shows some similarities to BD in the respect of refractory intestinal ulcers, recurrent oral ulcers, uveitis, inflammatory skin lesions and intermittent arthritis.

There is one study investigating the relationship between CN of β -defensin gene and BD. Becker *et al.* quantified CNs of the α - and β -defensin in patients with constitutional trisomy 8 and with Behçet's syndrome, and showed that *DEFA1A3* CN among the patients with Behçet's syndrome was lower than controls, while *DEFB4* and *DEFB104* were not higher (14). However, the small number of BD patients (n=14) in that study warranted further studies with bigger sample size.

Despite these functional considerations and a relatively large number of patients, we could not observe a significant difference of CN of β -defensin gene in BD. In addition to the genuine absence of an effect of β -defensin CNV on BD risk, these observations could have several other possible explanations. First, sample size may still not be enough to show a statistically significant difference. Second, the effect of CNV of *DEFB4* may be different between different populations (15). However, the distribution of β -defensin gene CNV is similar between our group and previously studied results, so that population differences are unlikely to be a source of negative results (12) Third, CN may not be accurately captured with the current measurement method. However, the validity of PRT has been already established against multiplex amplifiable probe hybridisation (MAPH) and multiplex ligation-dependent probe amplification (MLPA) which are considered as gold-standard methods for measuring CN (11). In addition, we investigated only β -*DEFB4* in this study. Therefore, it is still possible that CNV of other defensin genes might be related to development of BD.

In conclusion, we could not find any difference in CN of *DEFB4* between patients with BD and healthy controls, suggesting that CN of β -defensin gene may not be a critical factor in the development of BD.

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