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IκBα promoter polymorphisms in patients with Behçet's disease

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Abstract. To investigate the role of *IκBα* promoter polymorphisms in the development of Behçet's disease, eighty-six patients with Behçet's disease and 120 healthy controls were enrolled in this study. The *IκBα* -881A/G, -826C/T, -550A/T, -519C/T, and -297C/T polymorphisms were measured by the method of polymerase chain reaction/ restriction fragment length polymorphism. This study demonstrated that the genotype frequencies of *IκBα* -826C/T and -826T/T were significantly higher in the patients with Behçet's disease than in the controls. Both in the dominant and in the recessive models, the patients with Behçet's disease have higher frequencies of the *IκBα* -826T containing genotype than the controls. The allele frequency of *IκBα* -826T was significantly increased in the patients with Behçet's disease. The frequencies of the *IκBα* -881A -826T -550A -519C -297C and *IκBα* -881A -826T -550A -519T -297C haplotypes were significantly higher in the patients with Behçet's disease than in the controls. In contrast, the haplotype frequency of *IκBα* -881A -826C -550A -519C -297C in the patients with Behçet's disease was significantly decreased. This study also revealed that the Behçet's disease patients with *IκBα* -826T/T have higher prevalence of skin lesions than those without *IκBα* -826T/T. In summary, the *IκBα* -826T allele, *IκBα* -881A -826T -550A -519C -297C and *IκBα* -881A -826T -550A -519T -297C haplotypes might be associated with susceptibility to Behçet's disease. The *IκBα* -826T/T genotype was related to the development of skin lesions in the patients with Behçet's disease.

Keywords: IκBα, NFkB inhibitor, polymorphisms, Behçet's disease

1. Introduction

Behçet's disease is a chronic inflammatory systemic autoimmune disease characterized primarily by recurrent oral ulcers, genital ulcers, ocular inflammation, skin lesions, and vasculitis. Although the etiology of Behçet's disease is still unknown, multiple genes and environmental factors are involved in the pathogenesis of this disease. HLA-B51 is strongly associated with susceptibility to Behçet's disease in different ethnic groups [1]. The positive rate of HLA-B51 in Behçet's disease is about 60% [2]. However, the contribution of this allele to the overall genetic susceptibility to Behçet's disease is only about 19% [3]. Therefore, non-HLA genes may also be related to the pathogenesis of this disease [4–13]. A whole-genome screening also revealed the association of several non-HLA susceptibility loci with Turkish patients with Behçet's disease [14].

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Many pro-inflammatory cytokines are involved in the inflammatory process of Behçet's disease [13,15–18]. NFκB is related to the transcription of these pro-inflammatory cytokines, immune response, and anti-apoptotic genes [19–22]. Therefore, NFκB plays an important role in inflammatory diseases and in the development of autoimmunity [21,23]. IκB is an inhibitor of NFκB, which binds with NFκB in the cytoplasm and influences the transcriptional activity of NFκB. Therefore, IκB may also play an important role in inflammatory immunological diseases.

The IκB family includes IκBα, IκBβ, IκBγ, IκBδ, IκBε, IκBζ, IκB-R, Bcl-3, p100, and p105 [24,25]. All these proteins are characterized by the presence of multiple ankyrin repeats. IκBα, a classic form of the IκB family, consists of six ankyrin repeats, and can be found in cytoplasm and nucleus [25].

Several polymorphisms in the promoter region of *IκBα* including -881A/G (rs 3138053), -826C/T (rs 2233406), -550A/T (rs 2233407), -519C/T (rs 2233408), and -297C/T (rs 2233409) have been identified [26]. Several transcription factor binding sites have been demonstrated in the promoter region of *IκBα* (TFsearch web). The *IκBα* promoter polymorphisms may affect IκBα expression and influence the regulation of inflammatory response. A mutation in the coding region of *IκBα* might result in the over-expression of IκBα, which was implicated in the development of lymphoma [27].

Our previous studies showed that *IκBα* -826T might be associated with the development of rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) in Taiwan [28,29]. However, the correlation between *IκBα* promoter polymorphisms and Behçet's disease is still unknown. The purpose of the present study is to investigate the association of *IκBα* promoter polymorphisms with the development of Behçet's disease.

2. Material and methods

Eighty-six patients with Behçet's disease (59 females and 27 males; mean $\pm$ SD $= 47.2 \pm 12.9$ years) and 120 unrelated healthy controls (84 females and 36 males; mean $\pm$ SD $= 44.6 \pm 10.1$ years) were enrolled in this study. All of the patients and controls are Taiwanese. This study has been approved by the IRB of Kaohsiung Medical University Hospital. The diagnosis of Behçet's disease was made according to the International Study Group criteria for diagnosis of Behçet's disease [30]. The linkage disequilibrium in the promoter of *IκBα* could not be found in Chinese (HapMap). Therefore, the *IκBα* -881A/G, -826C/T, -550A/T, -519C/T, and -297C/T polymorphisms were detected in this study.

To determine the *IκBα* -881A/G and -826C/T polymorphisms, a set of primers with the following sequences: 5'- GGTCCTTAAGGTCCAATCG-3' and 5'-GTTGTGGATACCTTGCACTA- 3' (underlined: mismatched nucleotide) were used. PCR was carried out under the following conditions: initial denaturation at 95°C for 3 min and 5 cycles of denaturation at 95° C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min, and then 35 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min. A final extension phase was also performed at 72°C for 7 min. The restriction enzymes, *Tsp*R I and *Bfa* I, were used to determine the *IκBα* -881A/G and *IκBα* -826C/T polymorphisms, respectively.

The primers 5'- GCTTTCACAACTTCTACCTG- 3' and 5'- AGAGTGGAAA TGATGGCTG- 3' were used to determine the *IκBα* -519C/T polymorphisms. The amplification conditions consisted of initial denaturation at 96°C for 3 min, followed by 5 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min, and 30 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min, and then a final extension phase at 72°C for 7 min. Then the PCR product was digested with *Mnl* I.

To determine the *IκBα* -550A/T polymorphisms, a nested PCR was performed with the PCR product for determining -519C/T polymorphisms and a set of new primers. The sequences of primers were 5'-TTGCTGCAAAGAGCCTGCT- 3' (underlined: mismatched nucleotide) and 5'- AGAGTGGAAATGAT GGCTG- 3'. The amplification conditions were as follows: initial denaturation at 96°C for 3 min, followed by 5 cycles of denaturation at 95°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min, and 30 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min, and then a final extension at 72°C for 7 min. Then the PCR product was digested with *Sfc* I.

The primers 5'- GAAAGGACCGGCAGTTGG- 3' and 5'- GTACTTCCCTG CAGCCTG- 3' were used to determine the polymorphisms of *IκBα* -297C/T. The PCR was performed under the following conditions: initial denaturation at 96°C for 3 min and 5 cycles of denaturation at 95°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min, followed by 30

Table 1
The genotype frequencies of $I\kappa B\alpha$ promoter polymorphisms in the patients with Behçet's disease and the controls

$I\kappa B\alpha$ genotype	Behçet's $n = 86$ (%)	Controls $n = 120$ (%)	OR (95% CI)	p
-881 A/A	73 (84.9)	92 (76.7)	1	
A/G	12 (13.9)	26 (21.7)	0.6 (0.3–1.2)	NS
G/G	1 (1.2)	2 (1.7)	0.6 (0.1–7.1)	NS
-826 C/C	1 (1.2)	79 (65.8)	1	
C/T	13 (15.1)	35 (29.2)	29.3 (3.7–233.1)	< 0.001
T/T	72 (83.7)	6 (5.0)	948 (111.4–8065.2)	< 0.001
Dominant model				
C/C	1 (1.2)	79 (65.8)	1	
C/T + T/T	85 (98.8)	41 (34.2)	44.1 (5.9–328.3)	<0.001
Recessive model				
C/C + C/T	14 (16.3)	114 (95.0)	1	
T/T	72 (83.7)	6 (5.0)	97.7 (35.9–265.8)	< 0.001
-550 A/A	83 (96.5)	108 (90.0)	1	
A/T	3 (3.5)	11 (9.2)	0.4 (0.1–1.3)	NS
T/T	0 (0)	1 (0.8)	0.4 (0.04–4.9)	NS
-519 C/C	74 (86.0)	107 (89.2)	1	
T/C	11 (12.8)	12 (10.0)	1.3 (0.6–3.2)	NS
T/T	1 (1.2)	1 (0.8)	1.4 (0.1–23.5)	NS
-297 C/C	73 (84.9)	101 (84.2)	1	
C/T	12 (13.9)	17 (14.2)	1.0 (0.4–2.1)	NS
T/T	1 (1.2)	2 (1.7)	0.7 (0.1–7.8)	NS

NS: not significant.

cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 1 min, and then a final extension phase at 72°C for 7 min. The restriction enzyme *Hpy*8 I was used.

The polymorphisms of $I\kappa B\alpha$ -826C/T measured by the PCR/RFLP method were also confirmed by direct sequencing.

The chi-square test or the Fisher's exact test was used for statistical analysis. The p-value was corrected by the multiplication of the number of comparisons (*Pc*). The OR was calculated by the method of Woolf and by a modification of the method of Haldane. The estimated haplotype frequencies were determined by the EH program (Web Resources of Genetic Linkage Analysis).

3. Results

The distributions of the genotypes in the controls and the patients were compatible with the Hardy-Weinberg equilibrium. In comparison with $I\kappa B\alpha$ -826C/C, this study demonstrated that the genotype frequency of $I\kappa B\alpha$ -826C/T was significantly higher in the patients with Behçet's disease than in the controls (Table 1, $p < 0.001$, OR = 29.3, 95% CI = 3.7–233.1). A similar finding could also be observed in the genotype frequency of $I\kappa B\alpha$ -826T/T (Behçet's vs controls: $p < 0.001$, OR = 948, 95% CI = 111.4–8065.2). The genotype frequencies of $I\kappa B\alpha$ -826C/T polymorphisms, both in the dominant model (C/T + T/T vs C/C) and in the recessive model (T/T vs C/C + C/T), were significantly different between the patients with Behçet's disease and the controls. In the dominant model, the genotype frequency of $I\kappa B\alpha$ -826 C/T + T/T was significantly higher in the patients with Behçet's disease than in the controls ($p < 0.001$, OR = 44.1, 95% CI = 5.9–328.3). A similar finding could also be observed in the recessive model (T/T vs C/C + C/T: $p < 0.001$, OR = 97.7, 95% CI = 35.9–265.8).

We also found that the patients with Behçet's disease had a significantly higher allele frequency of $I\kappa B\alpha$ -826T than the controls (Table 2, $p < 0.001$, OR = 43.0, 95% CI = 23.2–79.8).

Direct sequencing was performed to verify the genotypes measured by the method of PCR/RFLP (Fig. 1). The results of PCR/RFLP were compatible with those of direct sequencing.

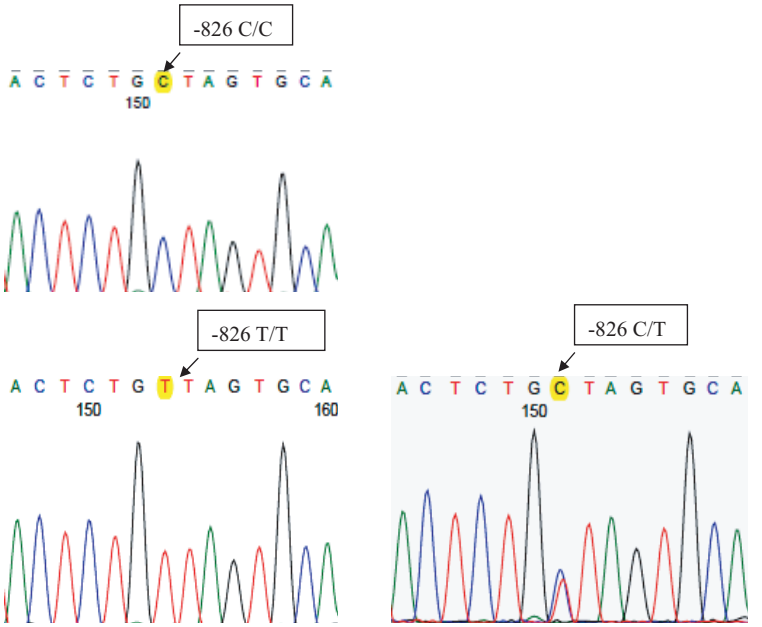
This study demonstrated that the estimated haplotype frequency of $I\kappa B\alpha$ -881A -826T -550A -519C -297C in the patients with Behçet's disease was significantly higher than that of the controls ($p < 0.001$, $Pc < 0.006$, OR = 34.14, 95% CI = 17.77–65.59). A similar finding could also be observed in the estimated haplotype frequency of $I\kappa B\alpha$ -881A -826T -550A -519T -297C (Behçet's disease vs controls: $p < 0.001$, $Pc < 0.006$,

Table 2
The allele frequencies of IκBα promoter polymorphisms in the patients with Behçet's disease and the controls

IκBα polymorphisms	Behçet's 2n = 172 (%)	Controls 2n = 240 (%)	OR (95% CI)	p
-881 A	158 (91.9)	210 (87.5)	1.6 (0.8–3.1)	NS
G	14 (8.1)	30 (12.5)		
-826 C	15 (8.7)	193 (80.4)		
T	157 (91.3)	47 (19.6)	43.0 (23.2–79.8)	< 0.001
-550 A	169 (98.3)	227 (94.6)	3.2 (0.9–11.5)	NS
T	3 (1.7)	13 (5.4)		
-519 C	159 (92.4)	226 (94.2)	0.8 (0.3–1.7)	NS
T	13 (7.6)	14 (5.8)		
-297 C	158 (91.9)	219 (91.3)	1.1 (0.5–2.2)	NS
T	14 (8.1)	21 (8.7)		

NS: not significant.

Sequences of *IκBα* -826 C/T polymorphisms:



RFLP results of *IκBα* -826 C/T polymorphisms:

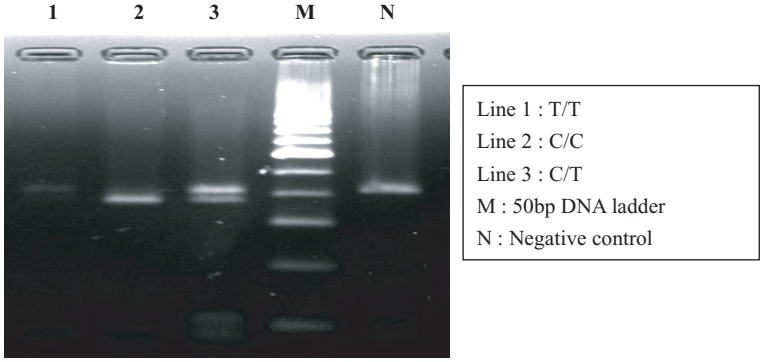


Fig. 1. The results of RFLP in IκBα -826C/T polymorphisms were confirmed by direct sequencing.

Table 3
The estimated haplotype frequencies of *IκBα* promoter polymorphisms in the patients with Behçet's disease and the controls

IκBα haplotype	Behçet's disease	Controls	OR(95% CI)	p	*Pc*
-881A -826C -550A -519C -297C	0.063	0.753	0.02 (0.01–0.04)	< 0.001	< 0.006
-881A -826C -550A -519C -297T	0.006	0.01	–	–	–
-881A -826C -550A -519T -297C	0.002	0.01	–	–	–
-881A -826C -550T -519C -297C	0.000	0.015	–	–	–
-881A -826T -550A -519C -297C	0.779	0.054	34.14 (17.77–65.59)	< 0.001	< 0.006
-881A -826T -550A -519C -297T	0.000	0.004	–	–	–
-881A -826T -550A -519T -297C	0.057	0.000	30.48 (3.93–236.52)	< 0.001	< 0.006
-881A -826T -550T -519C -297C	0.006	0.008	–	–	–
-881G -826T -550A -519C -297C	0.013	0.045	0.22 (0.05–1.00)	NS	NS
-881G -826T -550A -519C -297T	0.047	0.045	1.02 (0.42–2.52)	NS	NS
-881G -826T -550A -519T -297T	0.000	0.022	0.11 (0.01–0.93)	NS	NS

−: The$p$ value was not calculated due to the fact that the number of cases is too small. *Pc*: corrected p value.

Table 4
Associations between the *IκBα* -826T/T genotype and clinical manifestations of Behçet's disease

		IκBα -826T/T		p	OR (95% CI)
		+,$n = 72$ (%)	−, $n = 14$ (%)		
Uveitis	+	13 (18.1)	6 (42.9)	NS	
	−	59 (81.9)	8 (57.1)		
Cutaneous	+	16 (22.2)	2 (14.3)	NS	
vasculitis	−	56 (77.8)	12 (85.7)		
Peripheral	+	10 (13.9)	2 (14.3)	NS	
Neuropathy	−	62 (86.1)	12 (85.7)		
Skin lesion	+	60 (83.3)	5 (35.7)	0.001	9.0 (2.6–31.6)
	−	12 (16.7)	9 (64.3)		

Skin lesions include erythema nodosum, psudofolliculitis, papulopustular lesion or acneiform nodule.

OR = 30.48, 95% CI = 3.93–236.52). In contrast, the estimated haplotype frequency of *IκBα* -881A -826C -550A -519C -297C was significantly decreased in the patients with Behçet's disease when compared with that of the controls ($p < 0.001$, $Pc < 0.006$, OR = 0.02, 95% CI = 0.01–0.04).

The associations of the *IκBα* -826T/T genotype with the clinical manifestations of Behçet's disease were shown in Table 4. The patients with Behçet's disease, carrying the *IκBα* -826T/T genotype, have higher prevalence of skin lesions including erythema nodosum, pseudofolliculitis, papulopustular lesion or acneiform nodules than the patients without *IκBα* -826T/T.

4. Discussion

This study demonstrated that the *IκBα* -826T allele, *IκBα* -881A -826T -550A -519C -297C haplotype and *IκBα* -881A -826T -550A -519T -297C haplotype might be related to susceptibility to Behçet's disease in Taiwan.

NFkB activation may modulate the expression of anti-apoptotic genes leading to apoptosis resistance in T-cell subsets of Behçet's disease, and plays an important role in the pathogenesis of Behçet's disease [31]. The anti-apoptogenic effect of NFkB is caused by the expression of anti-apoptogenic molecules including Bcl-xL, XIAP, IAP, and TRAFs [32]. IκB inhibits the transcription function of NFκB. Different IκB molecules preferentially inhibit the distinct NFkB/Rel protein dimmer [24]. The central portions of IκB molecules contain several ankyrin repeats. The ankyrin repeats bind to the Rel homology domain of NFkB/Rel, which causes NFkB to remain in the cytoplasm by masking the nuclear localization sequence of NFkB. Nuclear import of IκBα is also found [33,34]. When IκBα is expressed in the nucleus, it can inhibit the interaction of NFkB with DNA and promote the export of NFkB from the nucleus to the cytoplasm [35]. The C-terminal domain of IκB may block DNA binding by NFkB and dissociate DNA-bound NFkB dimmers [36].

Three NFkB binding sites, which are required for induction of gene expression, have been demonstrated in the promoter of *IκBα* [37]. A putative binding

site for transcription factors ROR alpha 1 and ROR alpha 2 is in the position of *IκBα* -881 (TFsearch web). The *IκBα* -826C/T polymorphisms are near a putative binding site of transcription factor GATA-2 (TFsearch web). Another putative binding site for transcription factor C/EBP is in the position of *IκBα* -519 (TFsearch web). Therefore, the polymorphisms in the *IκBα* promoter may affect the binding of transcriptions, and then influence the expression of *IκBα*.

This study demonstrated that the *IκBα* -826T allele might be related to susceptibility to Behçet's disease. We also found that the *IκBα* -881A -826T -550A -519C -297C and *IκBα* -881A -826T -550A -519T -297C haplotypes played significant roles in the development of Behçet's disease. Our previous study showed that the *IκBα* -826T -550A -519C haplotype and *IκBα* -881A -826T -550A -519C -297C haplotype were associated with susceptibility to other autoimmune diseases in Taiwan [28,29,38]. These haplotypes may be related to the promoter activity of *IκBα* and the production of IκBα, and then influence the function of NFkB, which plays an important role in the inflammatory and immune responses. The promoter activities of various *IκBα* promoter polymorphisms were detected with a luciferase reporter assay. It showed that individuals with *IκBα* -826T allele had a lower promoter activity than those with *IκBα* -826C (unpublished data). Although the sample size of this study is limited, the power for the *IκBα* -826C/T polymorphisms is more than 95%. The numbers of patients and controls may suffice for this study.

Mutations in the *IκBα* are associated with other autoimmune diseases. An 8 bp insertion in the promoter region of *IκBα* (*IκBα* -708 ins 8) prevented the development of primary progressive multiple sclerosis [39]. The *IκBα* polymorphisms may also be associated with Crohn's disease or autoimmune diabetes mellitus [40,41]. The single nucleotide polymorphisms in the 3′-UTR were significantly increased in these patients. Mutations of *IκBα* may also be associated with the development of other inflammatory diseases and malignancies [42,43]. Mozzato-Chamay showed that the *IκBα* -881G/ -826T haplotype protected the development of scarring tracoma, an inflammatory disease, in Gambia [26]. The *IκBα* -881G -826T -297T haplotype was significantly associated with sarcoidosis in the UK and the Netherlands, and the *IκBα* -826T allele was related to the stage of sarcoidosis [44]. *IκBα*-deficient mice died of a wasting disease that was attributed to over-expression of TNFα [45]. Klement showed that *IκBα* deficiency also resulted in a sustained NFkB response and severe widespread dermatitis in mice [46].

Table 5
Associations of *IκBα* polymorphisms with various autoimmune diseases

Diseases	Associated *IκBα*	Population	Ref.
Multiple sclerosis (primary progressive type)	decreased -708ins8	German	[39]
LADA	increased AA genotype (rs696 in 3'-UTR)	Czech	[41]
Crohn's	increased AA genotype (rs696 in 3'-UTR)	German	[40]
PsA	no	Canadian	[47]
RA	increased -826T (rs 2233406)	Taiwanese	[28]
Behcet's	increased -826T (rs 2233406)	Taiwanese	present study

LADA: latent autoimmune diabetes in adult; PsA: psoriatic arthritis. RA: rheumatoid arthritis; SLE: systemic lupus erythematosus.

This study also demonstrated that the *IκBα* -826T/T genotype was associated with the development of skin lesions in the patients with Behçet's disease.

The polymorphisms of *IκBα* have been demonstrated to be associated with some autoimmune diseases. The associations of these polymorphisms with various autoimmune diseases were summarized in Table 5.

In conclusion, the *IκBα* -826T allele, *IκBα* -881A -826T -550A -519C -297C haplotype, and *IκBα* -881A -826T -550A -519T -297C haplotype may be related to susceptibility to Behçet's disease. In contrast, the *IκBα* -881A -826C -550A -519C -297C haplotype may prevent the development of Behçet's disease in Taiwan. Moreover, the *IκBα* -826T/T genotype is associated with skin lesions in patients with Behçet's disease.

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