Hong Kong J. Dermatol. Venereol. (2014) **22**, 5-11

Original Article

Activation parameters in Behçet's disease
白塞氏病的活躍參數

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Objective: Behçet's disease (BD) is a chronic, multi-system vasculitis of unknown aetiology. Specific criteria to assess the activity of the disease, which has a relapsing course, have not been found, thus evaluation of the disease activity is mainly based on the clinical findings. Therefore, there is a need for laboratory markers that can be correlated with the disease activity. In this report, we aimed to search for parameters that may be used as an activation marker in BD. ***Methods:*** Serum levels of acute phase reactants were evaluated in active and inactive Behçet's disease patients. Mutations of factor V Leiden (FVL), prothrombin (PT) and methylenetetrahydrofolate reductase (MTHFR) were analysed and compared according to systemic involvement in patients with BD. ***Results:*** Erythrocyte sedimentation rate (ESR) was found to be higher in the active group, whereas it was lower in the inactive group. There was a statistically significant difference between these data ($p=0.023$). There was no significant difference among other parameters between these two groups which included D-dimer, C-reactive protein, fibrinogen and procalcitonin levels. The FVL mutation was detected in 25% of the BD patients with vascular involvement compared with 0% of the BD patients without vascular involvement. The difference was statistically significant ($p<0.022$). However, the prevalence of the PT and MTHFR mutations was normal in patients with BD according to systemic involvement. ***Conclusion:*** Results of the present study suggested that FVL mutation might be an additional risk factor for the development of vascular involvement in BD and increased ESR level was associated with active BD.

目標：白塞氏病是一種成因不明的慢性多系統血管炎，病情多反覆發作，現行並沒有一套準繩可靠的標準來評估其疾病的活躍度，病情評估只能建基於其臨床病徵。因此，有需要去發掘可對應臨床病情的實驗室指標。在這份報告中，我們的目標是要發掘 出可作爲白塞氏病活躍指標的參數。
方法：我們分別對病情活躍與不活躍的白塞氏病患者進行了急性期反應物的血清水平評估；並同時分析及比較萊登第五因子、凝血酶原和甲基四氫葉酸還原酶的基因突變率在不同程度的白塞氏

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病全身受累患者間的異同。**結果**：紅細胞沉降率在白塞氏病活躍群組較病情不活躍群組爲高，其數據在統計學上有顯著的差異（p=0.023）。在以上兩群組間，其他的研究參數包括 D- 二聚體、C- 反應蛋白、纖維蛋白原及降鈣素原的血清水平則未發現有顯著差異。另外，在百分之二十五有血管受累的白塞氏病患者被發現帶有萊登第五因子基因的突變，無血管受累群組中則沒有發現，兩組的差異在統計學上是顯著的（p<0.022）。此外，凝血酶原和甲基四氫葉酸還原酶的基因突變盛行率在對比白塞氏病全身受累不同程度的患者間則沒有分別。**總結**：本研究結果顯示萊登第五因子基因突變可能是白塞氏病血管受累的新發現風險因素，另外紅細胞沉降率水平的增加亦與白塞氏病的病情活躍度有關聯。

Keywords: Activation, Behçet's disease, parameters

關鍵詞：活躍，白塞氏病，參數

Introduction

Behçet's disease (BD) is a rare inflammatory, lifelong disorder of unknown cause, characterised by recurrent oral and genital ulcerations and skin lesions.[1] It is a multi-system disease that may present with cutaneous, articular, neurological, intestinal, pulmonary, urogenital or vascular manifestations. Vascular injury and autoimmune responses are characteristics of BD.[2] Evaluation of the disease activity in BD is still a major problem. A number of clinical and laboratory variables have been studied in these patients and some of them have been found to be well-correlated with the disease activity.[3]

In this study, we evaluated the possible relationship of acute phase reactants including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), fibrinogen, D-dimer and procalcitonin in patients with active and inactive BD, and we analysed mutations including factor V Leiden (FVL), prothrombin (PT), methylenetetrahydrofolate reductase (MTHFR)-A, MTHFR-C according to systemic involvement in patients with BD.

Material and Methods

Patients

We studied 47 patients with BD (23 males, 24 females). All patients fulfilled the criteria for diagnosis of BD according to the International Study Group for BD.[4] All procedures followed the tenets of the Declaration of Helsinki. All patients underwent a complete ophthalmological and systemic examination in order to define the ophthalmic and systemic involvement, and disease activity. Active and inactive periods of Behçet's patients were clinically determined. Serum samples were drawn during clinically active (active BD; one or more clinical manifestations present; 12 patients with BD) and inactive disease (inactive BD; no clinical sign present; 35 patients with BD). At the time of clinical assessment, patients were included in the active group if they had at least one of the following clinical findings: oral ulcers, genital ulceration, active uveitis, recent arthritis, papulopustular or pseudo-follicular cutaneous lesions, and neurological involvement. None of the patients recevied corticosteroid or immunosuppressive therapy during the study period and all of them recevied colchicine therapy. Written informed consent was obtained from all participants.

Methods

Erythrocyte sedimentation rate was determined according to the Westergren method using anticoagulant-containing whole blood. Venous blood was collected in vacutainers without additive, allowed to clot for 30 minutes at room temperature and centrifuged at 3000 g for five minutes to obtain the serum. Serum aliquots were stored at -80°C until biochemical analysis. Haemolysed samples were excluded. Serum CRP

levels were determined by the nephelometric method (Beckman Array 360 Protein System, Beckman Coulter, Fullerton, California, USA). Fibrinogen levels were determined using high-sensitivity standard reagent IL (Instrumentation Laboratory (IL), Milano, Italy). Procalcitonin was measured on a Kryptor system (BRAHMS USA, Annapolis, MD) by a homogenous time-resolved amplified cryptate emission immunometric fluorescent assay. D-dimers (split products of cross-linked fibrin) were measured by micro-plate enzyme immunoassay using the respective monoclonal antibodies (Innogenetics, NW Zwijndrecht, Belgium).

The FVL, PT, MTHFR-A, and MTHFR-C gene mutations were tested by rapid PCR amplification of genomic DNA extracted from the patients' peripheral blood cells. Venous blood samples were collected in plastic tubes containing 0.1 mol/L trisodium citrate. Plasma was separated by centrifugation at 2000 g for 20 minutes at room temperature and stored at -70°C until analysis for activated protein C resistance. The activated protein C resistance of the samples was measured using a Trombolyzer Compact XR automated system (Behnk Electronic, Norderstedt, Germany). Extracted DNA was used for the detection of FVL, PT (G20210A), MTHFR-A, and MTHFR-C mutations (light cycler factor V Leiden mutation detection kit, light cycler prothrombin G20210A mutation detection kit; light cycler MTHFR A1298C mutation detection kit and light cycler MTHFR C677T mutation detection kit, Roche Molecular Biochemicals, Mannheim, Germany). These systems perform rapid polymerase chain reaction (PCR) by melting curve analysis by monitoring the fluorescence.

Statistical analysis

All statistical analyses were performed using SPSS version 11.5 for Windows computer software (SPSS, Chicago, Ill., USA). The Pearson chi-squared and Fisher exact chi-squared tests were used to compare acute phase reactants in active and inactive groups. Gene mutations according to systemic involvement including ocular, vascular and articular were analysed by the Pearson chi-squared and Fisher exact chi-squared tests. P value <0.05 were considered statistically significant.

Results

The subjects were between 18 to 65 years of age (mean age 37.6±12.5 years). The activity of acute phase reactants is shown in Table 1.

Comparison of the levels of D-dimers, CRP, fibrinogen and procalcitonin in active and inactive groups did not reveal any significant difference ($p>0.05$). These results are summarised in Table 2. The ESR level was higher in active BD patients (58.3%, 7/12) compared with that in inactive patients (22.9%, 8/35). The statistical analysis showed a significant difference between the active and inactive patient groups ($p=0.023$). The higher ESR level was detected in seven active (53.3%) and eight inactive (46.7%) patients with BD. Normal ESR levels were detected in 27 inactive patients with BD (84.4%) and five active patients with BD (15.6%). Descriptive statistics and *p* values are summarised in Table 2.

Table 1. Acute phase reactants in patients with Behçet's disease

Acute phase reactants		N	%
D-dimer	Normal	44	93.6
	Elevated	3	6.4
ESR	Normal	32	68.1
	Elevated	15	31.9
CRP	Normal	23	56.1
	Elevated	18	43.9
Fibrinogen	Normal	33	75.0
	Elevated	11	25.0
Procalcitonin	Normal	32	100.0
Activity	Inactive	35	74.5
	Active	12	25.5

ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein

Table 2. Acute phase reactants according to disease activity

		Inactive BD		Active BD		p value
		N	%	N	%	
D-dimer	Normal	34	97.1	10	83.3	0.156
	Elevated	1	2.9	2	16.7	
ESR	Normal	27	77.1	5	41.7	**0.023**
	Elevated	8	22.9	7	58.3	
CRP	Normal	20	64.5	3	30.0	0.056
	Elevated	11	35.5	7	70.0	
Fibrinogen	Normal	27	81.8	6	54.5	0.070
	Elevated	6	18.2	5	45.5	
Procalcitonin	Normal	25	100.0	7	100.0	

BD: Behçet's disease; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein

The frequency of gene mutations in patients with BD is described in Table 3. The systemic manifestations including ocular, articular and vascular in each BD patient in relation to the FVL, PT and MTHFR gene mutations are summarised in Tables 4, 5 and 6 respectively. Only the FVL mutation was higher in BD patients with vascular involvement. The difference was statistically significant (p=0.022). The FVL gene mutation was detected in four BD patients (25%) with vascular involvement. No FVL gene mutation was seen in the BD patients without vascular involvement. Comparison of the frequency of PT and MTHFR mutations according to systemic involvement did not show any statistically significant difference (p>0.05).

Discussion

Behçet's disease is a multi-systemic disease characterised by oral and genital ulcerations and uveitis, but many additional manifestations of the disease such as neurological, articular and vascular are well known.[1,2] A number of clinical and laboratory variables have been studied in these patients and some of them have been found to be well correlated with disease activity.[3] Muftuoglu et al observed that ESR and CRP levels were usually moderately elevated but did not correlate well with disease activity.[5] Valesini et al observed high levels of ESR correlated with eye lesions, hypopyon, erythema nodosum and

Table 3. The frequency of gene mutations in patients with Behçet's disease

		N	%
PT gene mutation	None	36	90.0
	Heterozygous	4	10.0
FVL gene mutation	None	35	89.7
	Heterozygous	3	7.7
	Homozygous	1	2.6
MTHFR-A gene mutation	None	14	35.0
	Heterozygous	21	52.5
	Homozygous	5	12.5
MTHFR-C gene mutation	None	13	31.7
	Heterozygous	26	63.4
	Homozygous	2	4.9
Ocular involvement	Absence	25	61.0
	Presence	16	39.0
Vascular involvement	Absence	25	61.0
	Presence	16	39.0
Articular involvement	Absence	26	63.4
	Presence	15	36.6

PT: prothrombin; FVL: factor V Leiden; MTHFR: methylenetetrahydrofolate reductase

Table 4. The frequency of gene mutations in patients with ocular Behçet's disease

		Ocular involvement				
		Absence		Presence		
		N	%	N	%	p value
PT	None	22	91.7	14	87.5	0.667
	Mutation	2	8.3	2	12.5	
FVL	None	21	91.3	14	87.5	0.70
	Mutation	2	8.7	2	12.5	
MTHFR-A	None	10	40.0	4	26.7	0.392
	Mutation	15	60.0	11	73.3	
MTHFR-C	None	8	32.0	5	31.3	0.96
	Mutation	17	68.0	11	68.8	

PT: prothrombin; FVL: factor V Leiden; MTHFR: methylenetetrahydrofolate reductase

Table 5. The frequency of gene mutations in patients with angio-Behçet's disease

		Vascular				
		Absence		Presence		
		N	%	N	%	p value
PT	None	21	87.5	15	93.8	0.638
	Mutation	3	12.5	1	6.3	
FVL	None	23	100.0	12	75.0	**0.022**
	Mutation	0	0.0	4	25.0	
MTHFR-A	None	10	40.0	4	26.7	0.392
	Mutation	15	60.0	11	73.3	
MTHFR-C	None	8	32.0	5	31.3	0.96
	Mutation	17	68.0	11	68.8	

PT: prothrombin; FVL: factor V Leiden; MTHFR: methylenetetrahydrofolate reductase

Table 6. The frequency of gene mutations in patients with articular Behçet's disease

		Articular involvement				
		Absence		Presence		
		N	%	N	%	p value
PT	None	23	92.0	13	86.7	0.622
	Mutation	2	8.0	2	13.3	
FVL	None	21	87.5	14	93.3	0.559
	Mutation	3	12.5	1	6.7	
MTHFR-A	None	8	32.0	6	40.0	0.608
	Mutation	17	68.0	9	60.0	
MTHFR-C	None	8	30.8	5	33.3	0.865
	Mutation	18	69.2	10	66.7	

PT: prothrombin; FVL: factor V Leiden; MTHFR: methylenetetrahydrofolate reductase

the presence of HLA-B51.[6] We found that a higher ESR correlated with the activity of BD. However, we did not find a statistically significant difference between active and inactive BD in the D-dimer, CRP, fibrinogen and procalcitonin levels.

Vascular involvement is found in about 25% of patients with BD.[7] In recent years, the prevalence and role of the thromboembolic manifestations have been investigated. Although the precise pathogenic mechanism underlying the thrombotic tendency in BD is not completely understood, some homeostatic abnormalities including endothelial dysfunction, plasma hypercoagulability, and deficiency of several factors involved in the anticoagulation pathway have been shown to induce thrombotic events. A genetic factor V mutation, consisting of substitution of glutamine for arginine at position 506, the FVL, renders it resistant to proteolytic inactivation of activated protein C and thus predisposes the patient to thrombosis. This mutation increases the risk of thromboembolic disease by 5- to 10-fold in the heterozygous state and about 50- to 100-fold in the homozygous state. An association of BD and venous thrombosis with FVL has been suggested in adults.[8] In another study, it was found that among patients with BD who had ocular involvement, the prevalence of FVL was higher.[9] Four of our patients with the FVL mutation had vascular involvement. The prevalence of the FVL mutation in our angio-BD patients was significantly different from that in BD-patients without vascular involvement. We showed that 10.03% of the patients with BD had the FVL gene polymorphism and this mutation was a risk factor for angio-BD.

In some studies, an association between the PT gene mutation and thrombosis in BD has been shown. The G20210A mutation in the 3'-untranslated region of the thrombin gene may contribute to higher PT levels. A consequence of a higher translation efficiency or higher stability of the transcribed mRNA accounts for the high plasma PT levels. It has been observed that the PT G20210A polymorphism is associated with a three- to fourfold increased risk of venous thrombosis.[8] We demonstrated a high prevalence of the PT gene mutation in patients with BD. There is considerable variation in the prevalence of this polymorphism in normal populations: it has been reported to range from 0% to 6.5%. We found that 10% of the patients with BD had PT gene polymorphism and this mutation was not a risk factor for systemic involvement. This finding is also consistent with the literature.[10-12]

Recent studies have demonstrated that hyperhomocysteinaemia is a risk factor for deep atherothrombosis and vein thrombosis in the general population. Homocysteine is a sulphur-containing amino acid that is formed by demethylation of methionine, and hyperhomocystinaemia is caused by a combination of inherited and environmental factors. Some research suggests that the enzyme 5,10-MTHFR, which translates homocysteine into methionine by re-methylation, may be one of the main factors that regulates plasma homocysteine levels.[10] Homozygosity for the C677T mutation of the MTHFR gene is associated with reduced activity and increased thermo-lability of the enzyme. This mutation is considered to be the most common genetic cause of elevated homocysteine levels. Individuals who are homozygous for the MTHFR C677T mutation often have elevated plasma homocysteine. Further, homozygosity for this mutation has been implicated as a risk factor for venous thrombosis; however, not all studies support this finding.[11] The rates of mutation for the MTHFR A1298C and MTHFR C677T mutation in our patients were 65% and 68.3%, respectively. The distribution of MTHFR genotypes was similar in the active and inacitve groups ($p>0.05$), and analysis showed that MTHFR mutation was not a risk factor for angio-BD. This finding is consistent with the literature.[10,13,14]

Our findings suggest a possible use of ESR level as a marker in discriminating BD patients with active and inactive disease. Also, it suggested that FVL mutation might be an additional risk factor

for the development of vascular involvement. Further studies in larger patient series are needed to determine the prevalence of these mutations in BD and whether they constitute a major risk factor for the development of thrombosis and other systemic manifestations of the disease.

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