Protein Z G79A and A-13G gene polymorphisms in Italian patients with Behçet's disease

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ABSTRACT

Objective. *To investigate potential associations between A-13G and G79A polymorphisms of the protein Z gene and venous thrombosis and other clinical manifestations in Italian patients with Behçet's disease (BD).*

Methods. *176 Italian patients who satisfied the International Study Group criteria for BD and 134 healthy age- and sex- matched blood donors were genotyped for A-13G and G79A polymorphisms of the protein Z gene by molecular methods. 113 and 112 of the 176 BD patients were also genotyped for factor V Leiden and prothrombin gene G20210A polymorphisms. Serological HLA class B51 typing was performed by a standard microlymphocytotoxicity technique. The patients were subgrouped according to the presence or absence of clinical manifestations.*

Results. *The distribution of allele and genotype frequencies of A-13G and G79A polymorphisms did not differ significantly between BD patients and healthy controls.*

The frequencies of carriage rates of protein Z G79A and A-13G polymorphisms in BD patients with and without DVT were similar. Similarly, no associations between thrombotic events and the protein Z gene polymorphisms studied were observed in BD patients carrying factor V Leiden or prothrombin gene G20210A mutations. No significant associations were observed between protein Z polymorphisms and the occurrence of specific clinical findings.

Conclusion. *No association between DVT and A-13G or G79A polymorphisms of the protein Z gene was found in Italian BD patients. Furthermore, these protein Z polymorphisms in BD do not seem to increase the risk of DVT due to factor V Leiden or prothrombin gene G20210A mutations.*

Introduction

Behçet disease (BD) is a multi-systemic inflammatory disorder, which preferentially affects oral and genital mucous membranes, skin and eyes (1-3). Vascular lesions, in particular subcutaneous thrombophlebitis and deep vein thrombosis (DVT), may also occur, being detected in 10-30% of patients with active disease (4, 5). Vasculitis is the pathological lesion underlying most clinical manifestations of BD, including venous thrombosis. However, thrombophilia may also play an important role in the pathogenesis of the thrombotic manifestations observed in BD (6).

Protein Z is a vitamin K-dependent plasma glycoprotein that acts as a cofactor for the protein Z-dependent protease inhibitor, which inhibits activated blood coagulation factor X (factor Xa) (7, 8). Several studies have reported low plasma protein Z levels in association with ischemic stroke (9-11). However, this issue remains controversial because other studies have found no association or mapped increased concentrations to an increased stroke risk (12-14). Low protein Z levels in human increase the risk of venous thrombosis due to factor V Leiden mutation or, to a lesser extent, due to prothrombin G20210A or hyperhomocysteinemia, suggesting a prothrombotic phenotype for protein Z deficiency (15, 16). Circulating protein Z levels were found to be decreased in patients with BD (17). Protein Z deficiency may have a role in the hypercoagulable/prothrombotic state of BD (18).

Intron F polymorphism G79A and promoter polymorphism A-13G of the protein Z gene have been found to influence protein Z plasma levels (19-22). In healthy carriers of the A allele of the intron F polymorphism protein Z plasma levels were lower than in

carriers of the G allele (19-22). G allele of the promoter polymorphism was also found to be associated with lower levels of protein Z in healthy subjects (21, 22). Therefore, genetically determined low levels of circulating protein Z could be implicated in promoting thrombophilia in BD.

The aim of our study was to determine whether or not these two protein Z gene polymorphisms were associated with an increased risk of venous thrombosis in Italian patients with BD. We also evaluated if the 2 protein Z gene polymorphisms studied could influence the prothrombotic tendency of BD patients carrying factor V Leiden and prothrombin gene G20210A mutations.

Materials and methods

Study population

Case patients were 176 consecutive patients with BD who were followed at 9 different Italian referral Centers over a 7-year period (1999-2005). All patients fulfilled the criteria developed by the International Study Group for BD (ISG) (23). The control group consisted of 134 sex and age matched healthy subjects who were unrelated volunteer blood donors. The median age of the controls was 34 years (range 19-44), 50% of whom were males. All study subjects were Caucasians who had been residing in Italy for at least one generation. No ethnic differences were present between patients and controls. The study was approved by the Ethics Committees of the participating centers and written informed consent was obtained from patients and controls before inclusion in the study.

The diagnosis of deep vein thrombosis (DVT) and subcutaneous thrombophlebitis was based on clinical data and confirmed by ultrasonography or contrast venography.

HLA class I typing

Serological HLA class I typing was performed by a standard microlymphocytotoxicity technique, using peripheral blood lymphocytes. Out of the 175 Italian patients with BD, 162 were typed for HLA-B51 allele. The control group was made up of 228 Italian healthy subjects.

DNA extraction and genotyping

DNA was extracted from peripheral blood leukocytes using phenol/chloroform method, according to standard procedures.

The detection of the protein Z A-13G polymorphism and G79A polymorphism was performed using a based PCR-RFLP method using the PCR primers described by Lichy *et al*. (19).

For analysis of the protein Z A-13G polymorphism in the promoter region, a 272-bp fragment was amplified by polymerase chain reaction (PCR) using the following primers:

5'-GGGTCCTCTGAGCCTTCACCGTTCATTT-3' and 5'-CAGGCACAACAGACAGGTAAGCCAGATG-3'. Polymerase chain reaction was carried out in a P.E. 9600 (Perkin Elmer Cetus) T.C. in 25 μl reaction volume containing 100 ng template DNA, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, 200 μM each of dATP, dCTP, dGTP, dTTP (Amersham Pharmacia Biotech), 2.5 mM MgCl2, 0.5 μM each primer and 1 U Taq DNA Polymerase (Perkin Elmer).

Following an initial denaturation step (2 min at 95°C), samples were subjected to 35 cycles of 95°C for 20 sec, 64°C for 20 sec, 72°C for 20 sec with a final extension time of 3 min at 72°C.

After incubation for 2 hour at 37°C with *Hin*fI, an isoschizomere of *Hha*I (MBI Fermentas), the G allele yielded 2 DNA fragments of 157 and 115 bp on a 2% agarose gel after ethidium bromide staining. The A allele was not digestible. 168 of 176 BD patients were genotyped for protein Z A-13G polymorphism.

The G79A polymorphism of intron F of the PZ gene was analyzed by amplification of a 320-bp sequence using the primers 5'-TAACACCATAGACAGAGTCCGATATTCGC-3' and 5'-ATGAACTCGGCATTAGAACATGGTTGGAA-3'. Polymerase chain reaction was performed with the same condition of the A-13G polymorphism except that annealing temperature was 66°C. Digestion for 2 hour at 37°C with an isoschizomere of *Hpa*I, *Bst*HPI (MBI Fermentas), yielded 2 products of 221 and 99 bp in length in the presence of the A allele, whereas the G allele was not digestible. 161 of 176 BD patients were genotyped for protein Z G79A polymorphism.

113 and 112 of the 176 BD patients were genotyped by PCR and allele-specific restriction enzyme techniques for factor V Leiden and prothrombin gene G20210A polymorphisms. The techniques used to determine these two polymorphisms are described in a previous manuscript published by our group (6).

Statistical analysis

Statistical analysis was done using SPSS statistical package (SPSS Inc., Chicago, IL, USA). The frequencies of the alleles and genotypes among the case patients and controls were compared by chi-square test. Odds ratios (OR) were calculated together with their 95% confidence intervals (95% CI). For multiple comparisons, *p*-values were corrected (*p* corr) for the numbers of comparisons made, and results were considered significant when *p* corr values were less than 0.05. We performed power calculation for an unmatched case-controls study and estimated relative risk using Power and Sample Size calculation version 2.1.31 software.

Results

The demographic and clinical characteristics of the 176 Italian patients with BD are reported in Table I. We observed 46 (26.1%) patients with vascular lesions. The 2 most common lesions were subcutaneous thrombophlebitis (17/176, 9.7%) and DVT of the legs (32/176, 18.2%). Two patients had isolated intracardiac thrombosis and 1 patient had associated Budd-Chiari syndrome and extensive inferior vena cava and leg vein thromboses. Therefore, a total of 34 patients had DVT. Arterial lesions were not present. There were no significant differences between patients with and without DVT regarding demographic and clinical features.

The allele and genotype frequencies of protein Z gene G79A and protein Z gene A-13G polymorphisms in BD patients and in the control group are shown in Table II. The distributions did not differ significantly between BD patients and healthy controls.

Table I. Demographic and clinical features of 176 Italian patients with Behçet's disease*.

	Total BD (n=176)	BD without DVT (n=142)	BD with DVT (n=34)
Males	95 (54.0)	78 (54.9)	17 (50.0)
Females	81 (46.0)	64 (45.1)	17 (50.0)
Age at disease onset, mean ± SD, years	29 ± 12	29 ± 11	31 ± 16
Disease duration, mean ± SD, years	11 ± 8	10 ± 8	11 ± 10
Oral ulcer	174 (98.9)	140 (98.6)	34/34 (100)
Cutaneous lesions	147 (83.5)	118 (83.1)	29 (85.3)
Papulopustular lesions	97 (55.1)	77 (54.2)	20 (58.8)
Erythema nodosum	69 (39.2)	54 (38.0)	15 (44.1)
Genital ulcer	107 (60.8)	92 (64.8)	15 (44.1)
Epididymitis	12 (6.8)	9 (6.3)	3 (8.8)
Eyes lesions	102 (58.0)	79 (55.6)	23 (67.6)
Anterior uveitis	59 (33.5)	47 (33.1)	12 (35.3)
Posterior uveitis/retinal vasculitis	77 (43.8)	61 (43.0)	16 (47.1)
Arthritis	74 (42.0)	57 (40.1)	17 (50.0)
Central nervous system involvement	28 (15.9)	25 (17.6)	3 (8.8)
Subcutaneous thrombophlebitis	17 (9.7)	12 (8.5)	5 (14.7)
Positive pathergy test**	39/91 (42.9)	27/69 (39.1)	12/22 (54.5)
HLA-B51***	92/141 (65.2)	74/115 (64.3)	18/26 (69.2)
Factor V Leiden mutation§	9/113 (8.0)	8/87 (5.6)	1/26 (3.8)
Prothrombin gene G20210A mutation†	8/112 (7.1)	6/86 (7.0)	2/26 (7.7)

*Data presented as number (%) unless otherwise stated. BD: Behçet's disease; DVT: deep vein thrombosis; **Pathergy test was performed in 91 patients (69 without DVT and 22 with DVT); ***HLA typing was performed in 141 patients (115 without DVT and 26 with DVT); §Factor V Leiden mutation was performed in 113 patients (87 without DVT and 26 with DVT); †Prothrombin gene G20210A mutation was performed in 112 patients (86 without DVT and 26 with DVT).

Table II. Allele and genotype frequencies of protein Z G79A and protein Z A-13G polymorphisms in BD patients and controls*.

Variable	Behçet's disease (n=176)	Controls (n=134)	*p*	Odds ratio (95% CI)
Protein Z G79A†				
Alleles				
A	65/322 (20.2)	50/268 (18.7)	NS	1.1 (0.7-1.7)
G	257/322 (79.8)	218/268 (81.3)		
Genotypes				
AA	5/161 (3.1)	3/134 (2.2)		
AG	55/161 (34.2)	44/134 (32.8)	NS	
GG	101/161 (62.7)	87/134 (64.9)		
Protein Z A-13G§				
Alleles				
G	38/336 (11.3)	20/268 (7.5)	NS	1.6 (0.9-2.8)
A	298/336 (88.7)	248/268 (92.5)		
Genotypes				
G/G	0 /168 (0)	0/134 (0)		
A/G	38/168 (22.6)	20/134 (14.9)	NS	
A/A	130/168 (77.4)	114/134 (85.1)		

*Values are the number/total number examined (%). 95% CI: 95% confidence interval; NS: not significant; †Protein Z G79A polymorphism was performed in 161 of 176 patients with Behçet's disease; §Protein Z A-13G polymorphism was performed in 168 of 176 patients with Behçet's disease.

Given the sample size (161 patients with BD and 134 controls) and the allele frequencies of the polymorphism examined, we can conclude with 80% certainty that there is a genetic relative risk of 1.7 for BD in carriers of the intron F G79A (rs3024734) polymorphism of the protein Z gene.

Given the sample size (168 patients with BD and 134 controls) and allele frequencies of the polymorphism examined, we can conclude with 80% certainty that there is a genetic relative risk of 2.4 for BD in carriers of the A-13G (rs2273971) promoter polymorphism of the protein Z gene.

The comparisons of the frequencies of carriage rates of protein Z gene G79A and protein Z gene A-13G polymorphisms in controls and in BD patients with and without DVT are shown in Table III. No significant differences were found.

The possible associations between protein Z gene G79A and protein Z gene A-13G polymorphisms, on the one hand, and BD clinical manifestations defined in Table I, on the other hand, were evaluated comparing patients with and without specific manifestations. Only a weak association was found between A-13G G allele and anterior uveitis. However, after correction for the number of comparisons tested, this association was no longer significant.

The HLA-B51 allele frequency was significantly higher in BD patients compared with healthy controls (67.3% *vs.* 21.9%; p=0.0001, OR 7.3, 95% CI 4.6-11.5). We also investigated possible associations of the 2 polymorphisms studied with BD stratifying on HLA-B51. Although this analysis was limited by the low number of patients studied, no significant associations were observed in HLA-B51-positive or HLA-B51-negative patients (data not shown).

In a previous study published by our group that includes part of these 176 patients we did not find any association between venous thrombosis and the factor V Leiden mutation or G20210A mutation in the 3'-untranslated region of the prothrombin gene (6). Nine of 113 (8.0%) patients carried factor V Leiden and 8 of 112 (7.1%) prothrombin gene G20210A mutations, respectively (Table I).

Allele A of G79A polymorphism and allele G of A-13G polymorphism were not present in the only factor V Leiden-positive patient with venous thrombosis, while allele A was present in heterozygous form in 4 of the 8 factor Leiden-positive patients without venous thrombosis and allele G in the heterozygous form in 2 of these 8 patients.

Allele A of G79A polymorphism and allele G of A-13G polymorphism were present in one (heterozygous form) and in none, respectively, of the 2 patients with prothrombin gene G20210A mutation and venous thrombosis, while

Table III. Comparisons of carrier rates of protein Z G79A and protein Z A-13G polymorphisms in controls and Behçet's disease patients with and without deep vein thrombosis*.

Carriage rate	BD with DVT A	BD without DVT B	Controls (n=134) C	A *vs.* C *p*	B *vs.* C *p*	A *vs.* B *p*
Protein Z G79A†						
A	13/33 (39.4)	47/128 (36.7)	47/134 (35.1)	NS	NS	NS
G	20/33 (60.6)	81/128 (63.3)	87/134 (64.9)			
Protein Z A-13G§						
G	8/31 (25.8)	30/137 (21.9)	20/134 (14.9)	NS	NS	NS
A	23/31 (74.2)	107/137 (78.1)	114/134 (85.1)			

*Values are the number/total number examined (%). BD: Behçet's disease; DVT: deep vein thrombosis' †Protein Z G79A polymorphism was performed in 161 patients (33 with DVT and 128 without DVT); §Protein Z A-13G polymorphism was performed in 168 patients (31 with DVT and 137 without DVT).

allele A was present in the heterozygous form in 1 of the 6 patients with prothrombin gene G20210A mutation but without venous thrombosis and allele G in none of these 8 patients.
None of these associations was statistically significant.

Discussion

We observed a 26.1% frequency of vascular lesions in our consecutive series of Italian patients with BD. This frequency is similar to that reported by Koç *et al*. in a series of Turkish patients (4). These authors reported a prevalence of vascular involvement in BD of 27.7%. Superficial thrombophlebitis and DVT are the most common vascular lesions observed in BD. Arterial lesions are less frequently reported and they were not present in our series of Italian patients. The preferential involvement of the venous vessels may partially explain the absence of increased atherosclerosis in this vasculitis (24). We compared the demographic and clinical characteristics of the patients with and without DVT. Differently from previous studies (4, 25) that reported a higher frequency of males, pathergy positivity, and eye lesions in patients with vascular involvement, no significant differences were found in our patients.
Protein-Z is a vitamin K-dependent glycoprotein synthesized in the liver. In the presence of Ca^{+2} and phospholipids, protein Z forms a complex with activated coagulation factor X and serves as a co-factor for the rapid inhibition of factor Xa by protein Z-dependent protease inhibitor (PZI), enhancing PZI activity more than 1000 fold (7, 8). Normal protein Z levels are necessary for proper factor Xa inhibition and recent studies have suggested an association between protein Z deficiency and thrombosis. Low plasma protein Z levels have been associated with an increased risk for ischemic stroke, acute coronary events, arterial and venous thrombosis, pregnancy-related complications and retinal vessel occlusion (9-11, 26-32). Furthermore, the combination of protein Z deficiency with other thrombophilic factors may increase the thrombotic risk. Decreased circulating protein Z levels increase the prothrombotic tendency of Factor V Leiden and are correlated with an increased risk for arterial thrombosis in patients with the antiphospholipid antibody syndrome (15, 16, 33). Plasma protein Z levels are significantly reduced in patients with BD (17). There is evidence of universal activation of haemostatic system in BD (18). Alterations of protein Z concentrations could thus contribute to the hypercoagulable/prothrombotic state of BD.
Vasculitis plays a key role in the pathogenesis of thrombosis in BD. However, other factors must also be involved, since thrombosis does not invariably develop in all BD patients. Thrombophilia may be involved in the pathogenesis of thrombotic events in BD. Factor V Leiden mutation and the prothrombin G20210A mutation are the most common causes of inherited thrombophilia. These two mutations have been investigated in BD patients with conflicting results regarding possible associations with thrombotic events (6, 34-41). Allelic heterogeneity between different ethnic groups and inadequacy of sample size due to the small number of patients with thrombosis enrolled may explain the conflicting findings. A recent meta-analysis demonstrated an association of factor V Leiden and prothrombin mutation with thrombosis in BD (42). When studies from Turkey were excluded from the meta-analysis, only the prothrombin G20210A mutation was associated with thrombosis. We have also studied these two polymorphisms in Italian patients with BD (6). Prothrombin mutation was significantly associated with more severe ocular disease (posterior uveitis/retinal vasculitis), but we did not find any association between venous thrombosis and factor V Leiden and prothrombin gene G20210A mutations.
A strong genetic control of plasma protein Z levels has been reported (19-22). Both A-13G and G79A polymorphisms of the protein Z gene are independent determinants of protein Z concentrations in the blood. The A allele of the G79A polymorphism and the G allele of the A-13G polymorphism have been associated with lower circulating levels of protein Z.
We decided to study these two polymorphisms of the protein Z gene in BD patients to test the hypothesis of a possible association between these two polymorphisms and thrombotic events in BD patients.
We did not find any association between the A-13G and G79A polymorphisms of the protein Z gene and venous thrombosis in Italian patients with BD. Although no association with these

polymorphisms was found, we can not exclude that protein Z levels were decreased in our patients with BD, especially in those with venous thrombosis. Unfortunately in the present study, plasma protein Z levels could not be assayed in patients because either plasma were not available or patients were treated with oral anticoagulants.

Low protein Z levels in human increase the risk of venous thrombosis due to factor V Leiden or, to a lesser extent, due to prothrombin G20210A mutations.

Therefore, we evaluated if the protein Z mutations studied could influence the prothrombotic tendency of our patients with BD carrying factor V Leiden and prothrombin gene G20210A mutations. No association between the alleles associated with low protein Z levels with DVT was found in the patients with factor V Leiden or prothrombin gene G20210A mutations.

However, these analyses were limited by the low number of patients with DVT and factor V Leiden or prothrombin G20210A mutations studied.

In our study, we also evaluated whether A-13G and G79A polymorphisms of the protein Z gene were associated with specific clinical findings comparing patients with and without certain manifestations. However, no associations were found.

Conclusion

In conclusion, we did not find any association between venous thrombosis and the A-13G or the G79A polymorphisms of the protein Z gene in Italian BD patients. These results do not support the hypothesis that a genetically determined regulation of protein Z levels may predispose to thrombotic events in BD. Furthermore, these protein Z polymorphisms in BD do not seem to increase the risk of DVT due to factor V Leiden or prothrombin gene G20210A mutations.

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