PLA1/A2 polymorphism of platelet glycoprotein receptors IIIa in Behçet's disease

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ABSTRACT

Objective. *To investigate potential associations between the PlA1/A2 polymorphism of the platelet glycoprotein receptor IIIA (GpIIIa) gene and venous thrombosis and other clinical manifestations in Italian patients with Behçet's disease (BD).*

Methods. *Two hundred consecutive Italian patients satisfying the International Study Group criteria for BD who were followed up for seven years and 241 healthy Italian age- and gender-matched blood donors were molecularly genotyped for the PlA1/A2 polymorphism of the platelet GpIIIa gene; 118 and 117 of the 200 BD patients were also respectively genotyped for factor V Leiden and prothrombin gene G20210A polymorphisms. A standard microlymphocytotoxicity technique was used to type serological HLA class B51. The patients were grouped on the basis of the presence or absence of clinical manifestations. The diagnoses of deep vein thrombosis (DVT) and superficial thrombophlebitis were initially made clinically, and then confirmed by means of ultrasonography or contrast venography. The distribution of the PlA1/A2 genotype was investigated, and odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated.*

Results. *The allele and genotype frequency of the PlA1/A2 polymorphism were not significantly different in the BD patients and controls, but the PlA2 allele was significantly more frequent in the BD patients with DVT than the controls (p=0.023; Pcorr=0.046; OR 2.0, 95% CI 1.1–3.7). There were no associations between thrombotic events and the PlA1/A2 polymorphism in the BD patients carrying factor V Leiden or prothrombin gene G20210A mutations. The PlA2 allele was significantly less frequent in the BD patients with genital ulcers than in those without (26.9% vs. 43.2%; p=0.022; P corr 0.044; OR 0.48, CI 0.27–0.88).*

Conclusions. *The PlA1/A2 polymorphism of the GpIIIa gene was associated with DVT in our Italian BD patients, but does not seem to increase the risk of DVT due to factor V Leiden or prothrombin gene G20210A mutations. There was a negative association between the A2 allele and genital ulcers.*

Introduction

Behçet's disease (BD) is a primary systemic vasculitis characterised by oral and genital ulcerations, various skin lesions, uveitis and, less frequently, vascular, central nervous system (CNS) and gastrointestinal involvement (1, 2). Thrombosis is common and has a reported frequency of 10–30% (3). Venous involvement is more frequent than arterial involvement, and most frequently manifests itself in the form of thrombophlebitis (4).

The pathogenesis of BD-related thrombosis is unknown, although endothelial dysfunction due to vasculitis and possibly thrombophilia may play an important role (2, 5). A number of studies have assessed the prevalence of thrombophilic factors in BD patients (6-10). Silingardi (11) found no association between factor V Leiden and G20210A mutations in the 3'-untranslated region of the prothrombin gene in Italian BD patients with DVT, and a meta-analysis revealed no differences in the prevalence of factor V Leiden, prothrombin G20210A or methylenetetrahydrofolate reductase mutation between BD patients and controls, although the former showed a statistically significant association between prothrombin G20210A and thrombotic events (12). An association between factor V Leiden

and thrombosis has been observed in a Turkish sub-analysis and a Middle Eastern population study (12, 13), which confirms that this association depends on ethnic background as it is not found in European patients.

Glycoprotein IIb/IIIa (GpIIb/IIIa) receptor is a platelet membrane glycoprotein that facilitates platelet aggregation by binding to fibrinogen and von Willebrand factor (vWF) (14). Various clinical data show that blocking Gp IIb/IIIa receptors helps to prevent major adverse cardiovascular (CV) events (15-17). Genetic variations in Gp IIb/IIIa receptors may be independent risk factors for coronary thrombosis (18, 19): a number of polymorphisms have been identified in the general population, the PlA1/A2 polymorphism of the platelet GpIIIa gene has been widely studied in CV disease, and the presence of the PlA2 allele has been associated with increased platelet aggregation *in vitro* and with ischaemic CV disease and vascular thrombosis in patients with giant cells arteritis (GCA) and the antiphospholipid syndrome (20-22).

The aim of this multicentre study was to evaluate for the first time the potential associations between PlA1/A2 polymorphism and venous thrombosis and other clinical manifestations in Italian patients with BD. We also investigated whether the PlA1/A2 polymorphism could influence the prothrombotic tendency of BD patients carrying factor V Leiden and prothrombin gene G20210A mutations.

Materials and methods

Study population

The case patients were 200 consecutive BD patients who attended nine Italian referral centres for a period of seven years (1999-2007). All of the patients fulfilled the criteria developed by the International Study Group for BD (ISGB) (23). The control group consisted of 241 healthy, age- and gender-matched, unrelated volunteer blood donors (50% males) with a median age of 34 years (range 19-44). All of the study subjects were Caucasians who had been residing in Italy for at least one generation, and there were no ethnic differences between the patients and controls.

The diagnoses of deep vein thrombosis (DVT) and superficial thrombophlebitis were initially made clinically, and then confirmed by means of ultrasonography or contrast venography.

The study was approved by the Ethics Committees of the participating centres, and written informed consent was obtained from all of the subjects before study entry.

HLA class I typing

A standard microlymphocytotoxicity technique was used to type serological HLA class I in peripheral blood lymphocytes. One hundred and sixty of the 200 patients were typed for the HLA-B51 allele. The control group consisted of 228 Italian healthy subjects.

DNA extraction and genotyping

DNA was isolated from venous blood samples by means of standard three-step phenol/chloroform proteinase K extraction, and stored at 4°C until further analysis. The primers were designed on the basis of the sequence of Tanaka *et al*. (24). A 247 bp fragment from exon 2 of the Gp IIIa gene was amplified with 1 unit of AmpliTaq (Perkin Elmer, Weiterstadt, Germany), 0.2 mmol/L deoxynucleotides, 20 pmol downstream primer GP-3 5'- CTGCAGGAGGTA GAGAGTCGCCATAG, 20 pmol upstream primer GP-4 5'- GTGCAATC CTCTGGGGACTGACTTG and 1.5 mmol/L magnesium chloride in a final volume of 25 μL using a Perkin Elmer 9600 Thermal Cycler (Perkin Elmer, Foster City, USA). The PCR conditions were 35 cycles for 20 s at 94°C, 20 s at 68°C, and 20 s at 72°C. PCR efficiency was checked on 2% agarose gel for 20 min at 120 V. Fifteen microlitres of the amplified product were digested overnight with 5 units of BsmI (MBI Fermantas, Vilnius, Lithuania) in a final volume of 20 μL at 37°C. The fragments were separated on 2.5% agarose gel for 60 min at 80 V.

Of the 200 BD patients, 118 and 117, respectively, were genotyped for factor V Leiden and prothrombin gene G20210A polymorphisms by means of PCR and previously described allele-specific restriction enzyme techniques (11).

Statistical analysis

The data were statistically analysed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA; version 14.0, 2006). The frequencies of the alleles and genotypes in the patients and controls were compared using the chi-square test. Odds ratios (ORs) were calculated together with their 95% confidence intervals (95% CI). Corrected *p*-values (*P*corr) were calculated by multiplying the p value by the number of compared alleles or genotypes. The cases and controls were tested for conformity to Hardy-Weinberg equilibrium using a 2x2 chi-square test between observed and expected numbers. The statistical power of association was calculated Power for Association With Errors (PAWE) software (http://linkage.rockefeller.edu/pawe/pawe.cgithe)

Results

Table I shows the demographic and clinical characteristics of the 200 Italian BD patients. Thirty-six patients had DVT: 34 (17%) had DVT of the legs and two patients isolated intracardiac thrombosis (one with associated Budd-Chiari syndrome and extensive inferior vena cava and leg vein thromboses). There were no arterial lesions. The demographic and clinical characteristics of the patients with and without DVT were not significantly different. In an Italian population-based study the frequency of DVT in the two 10-year age groups (20–29 years and 30–39 years) corresponding to the age at disease onset of BD patients ranges between 1–2% (25).

The genotype frequencies of the control and case populations were in Hardy-Weinberg equilibrium. Table II shows that the allele and genotype frequencies of the PlA1/A2 polymorphism were not significantly different between the two groups, and there was no difference in the number of carriers of the PlA2 allele (A2/A2+A1/A2). The power of the allelic tests for the PlA1/A2 polymorphism was 40%.

The PlA2 allele was significantly more frequent in the BD patients with DVT than in the controls (p=0.023; Pcorr=0.046; OR 2.0, 95% CI 1.1–3.7), as was the carriage rate (p=0.044;

Table I. Demographic and clinical characteristics of 200 Italian patients with Behçet's disease.*

	Total BD (n=200)	BD without DVT (n= 164)	BD with DVT (n=36)
Males	104 (52.0)	86 (52.4)	18 (50.0)
Females	96 (48.0)	78 (47.6)	18 (50.0)
Age at disease onset, years (mean ± SD)	30 ± 12	30 ± 11	31 ± 15
Disease duration, years (mean ± SD)	11 ± 8	11 ± 8	11 ± 9
Oral ulcer	198 (99.0)	162 (98.8)	36/36 (100)
Cutaneous lesions	164 (82.0)	134 (81.7)	30 (83.3)
Papulopustular lesions	108 (54.0)	87 (53.0)	21 (58.3)
Erythema nodosum	81 (40.5)	65 (39.6)	16 (44.4)
Genital ulcer	119 (59.5)	104 (63.4)	15 (41.7)
Epididymitis	14 (7.0)	11 (6.7)	3 (8.3)
Eye lesions	112 (56.0)	87 (53.0)	25 (69.4)
Anterior uveitis	63 (31.5)	50 (30.5)	13 (36.1)
Posterior uveitis/retinal vasculitis	87 (43.5)	69 (42.1)	18 (50.0)
Arthritis	83 (41.5)	66 (40.2)	17 (47.2)
Central nervous system involvement	33 (16.6)	28 (17.1)	5 (13.9)
Subcutaneous thrombophlebitis	20 (10.0)	15 (9.1)	5 (13.9)
Positive pathergy test**	44/104 (42.3)	31/81 (38.2)	13/23 (56.5)
HLA-B51***	107/160 (66.9)	88/133 (66.2)	19/27 (70.4)
Factor V Leiden mutation§	9/118 (7.6)	8/91 (8.8)	1/27 (3.7)
Prothrombin gene G20210A mutation†	8/117 (6.8)	6/90 (6.7)	2/27 (7.4)

*Numbers (%) unless otherwise stated. BD: Behçet's disease; DVT: deep vein thrombosis.
**104 patients underwent the pathergy test. ***160 patients underwent HLA typing.
§118 patients underwent factor V Leiden mutation investigation. †117 patients underwent prothrombin gene G20210A mutation investigation.

Table II. Frequencies of alleles, genotypes and carriage of the PlA1/A2 polymorphism in patients with Behçet's disease and controls.*

	Behçet's disease n=200	Controls n=241	*p*-value	OR (95% CI)
Alelles				
A2	74/100 (18.5)	68/482 (14.1)	0.081	1.4 (1.0–2.0)
A1	327/400 (81.5)	414/482 (85.9)		
Genotypes				
A2/A2	6 /200 (3.0)	8/241 (3.3)		
A2/A1	61/200 (30.5)	52/241 (21.6)	NS	
A1/A1	133/200 (66.5)	181/241 (75.1)		
Carriage rate				
A2/A2 + A2/A1	67/200 (33.5)	60/241 (24.9)	0.057	1.5 (1.0–2.3)
A1/A1	133/200 (66.5)	181/241 (75.1)		
A1/A1 + A2/A1	194/200 (97.0)	233/241 (96.7)	NS	0.9 (0.3–2.6)
A2/A2	6/200 (3.0)	8/241 (3.3)		

*Number/total number examined (%).
OR: odds ratio; 95% CI: 95% confidence interval; NS: not significant.

*P*corr=0.088; OR 2.2, 95% CI 1.1-4.5) (Table III). However, the significance was lost after correcting the *p*-values. The power of the allelic tests for the PlA1/A2 polymorphism was 53%.

The possible associations between the PlA1/A2 polymorphism and the clinical manifestations of BD (see Table I) were evaluated by comparing the patients with and without specific manifestations. The PlA2 allele was significantly less frequent in the BD patients with genital ulcers than in those without (26.9% *vs*. 43.2%; *p*=0.022; *P*corr 0.044; OR 0.48, 95% CI 0.27–0.88). The power of the allelic tests for the PlA1/A2 polymorphism in these patients was 46%.

The HLA-B51 allele was significantly more frequent in the BD patients than the healthy controls (66.9% *vs*. 21.9%; *p*=0.0001; OR 7.2, 95% CI 4.6–11.3).

We also investigated possible associations with the PlA1/A2 polymorphism by stratifying the BD patients on the basis of their HLA-B51 status. Although limited by the small number of patients, no significant associations were observed in either group (data not shown).

In a previous study including some of these 200 patients, we did not find any association between venous thrombosis and factor V Leiden or G20210A mutations in the 3'-untranslated region of the prothrombin gene (11). Factor V Leiden and prothrombin gene G20210A mutations were carried by respectively 9/118 (7.6%) and 8/117 patients (6.8%) (Table I). The only factor V Leiden-positive patient with venous thrombosis did not carry the PlA2 allele, but it was present in heterozygous form in three of the eight factor Leiden-positive patients without venous thrombosis. The allele was present in one of the two patients with prothrombin gene G20210A mutations and venous thrombosis, and in three of the six patients with prothrombin gene G20210A mutations without venous thrombosis. None of these associations was statistically significant.

Discussion

Platelet GpIIb/IIIa is a membrane receptor that facilitates platelet aggregation and thrombus formation on the injured vessel wall by binding to fibrinogen and vWF (14, 15). Platelets represent a link between inflammation, thrombosis, and atherogenesis (15). Thrombotic occlusions at the sites of vascular inflammatory lesions are frequent in BD patients, and may be facilitated by the presence of underlying atherosclerotic lesions (26).

The PlA1/A2 polymorphism of the GpIIIa gene, which is caused by a T-to-C nucleotide substitution at position 1565, leads to the substitution of proline by leucine at position 33 of the mature GpIIIa receptor. A1 is the more common allele, and A2 the presumed variant (18). Approximately 25% of people of Northern European ancestry are PlA2 positive, whereas only 2% are homozygous for PlA2 (18). Various lines of evidence suggest that the PlA2 allele of the PlA1/A2 polymorphism may be an independent risk factor for

Table III. Frequencies of alleles, genotypes and carriage of the PlA1/A2 polymorphism in Behçet's disease patients with and without deep vein thrombosis and controls.*

Carriage rate	BD with DVT (36) A	BD without DVT (164) B	Controls (n=241) C	A *vs*. C *p*-value OR (95% CI)	B *vs*. C *p*-value OR (95% CI)	A *vs*. B *p*-value OR (95% CI)
PlA1/A2 polymorphisms Alleles						
	18/72 (25.0)	55/328 (16.8)	68/482 (14.1)	0.023	0.310	0.128
	54/72 (75.0)	273/328 (83.2)	414/482 (85.9)	(*P*corr 0.046) 2.0 (1.1–3.7)	1.2 (0.8–1.8)	1.7 (0.9–3.0)
Genotypes						
A2/A2	3/36 (8.3)	3/164 (1.8)	8/241 (3.3)	0.079	0.127	0.094
A2/A1	12/36 (33.3)	49/164 (29.9)	52/241 (21.6)	(*P*corr 1.58)		
A1/A1	21/36 (58.3)	112/164 (68.3)	181/241 (75.1)			
Carriage rate						
A2/A2 + A2/A1	15/36 (41.7)	52/164 (31.7)	60/241 (24.9)	0.044	0.142	0.329
A1/A1	21/36 (58.3)	112/164 (68.3)	181/241 (75.1)	(*P*corr 0.088) 2.2 (1.1–4.5)	1.4 (0.9–2.2)	1.5 (0.7–3.2)
A2/A2	3/36 (8.3)	3/164 (1.8)	8/241 (3.3)	0.160	0.536	0.073
A1/A2 + A1	33/36 (91.7)	161/164 (98,2)	233/241 (96.7)	(*P*corr 0.320) 2.7 (0.7–10.5)	0.5 (0.1–2-1)	4.9 (0.9–25.2)

*Number/total number examined (%). BD: Behçet's disease; DVT: deep vein thrombosis; OR: odds ratio; 95% CI: 95% confidence interval.

acute myocardial infarction (AMI), coronary artery disease (19, 20), restenosis after stent placement, and stroke caused by large-vessel disease (27, 28). The association between ischaemic CV disease and A1/A2 heterozygosity is controversial, but A2/A2 homozygosity is associated with a 3-4 times increased risk of ischaemic CV disease, particularly in young men (28). The role of this polymorphism has also been evaluated in patients with autoimmune diseases, and the A2 allele has been linked to an increased risk of arterial thrombosis in patients with antiphospholipid syndrome, regardlesss of whether it is primary or secondary to systemic lupus erythematosus (29).

We have previously evaluated the role of this polymorphism in patients with GCA and found an association between PlA2/A2 homozygosity and anterior ischaemic optic neuritis, which suggests that thrombosis may play a role in causing the cranial ischaemic complications of GCA (21).

In the present study, we decided to study for the first time the PlA1/A2 polymorphism in a large group of Italian BD patients to test the hypothesis that it may be associated with thrombotic events. We found no difference between the BD patients with and without DVT, but the A2 allele was more frequent in the patients with DVT than in the healthy controls. Although this finding requires further confirmation, it is potentially important because the increased platelet aggregation induced by the A2 allele may contribute to the development of thrombotic events, if though it has been shown that the increased platelet aggregation that contributes to vascular dysfunction and thrombosis in BD patients is associated with reduced nitric oxide (NO) production and that it is present without agonists in spontaneous platelet aggregation (SPA) (30, 31).

A number of studies have evaluated the role of thrombophilic factors in BD patients with conflicting results (32-37). Some have found that BD patients with thrombotic manifestations more frequently carry factor V Leiden and prothrombin GA20210 mutations (10, 11); however, a meta-analysis did not reveal any difference in the prevalence of factor V Leiden, prothrombin G20210A or methylenetetrahydrofolate reductase mutation between BD patients and controls, although there was a statistically significant association between prothrombin G20210A and thrombotic events in the patients with BD (12). Furthermore, it has been suggested that protein C or protein S deficiency, impaired fibrinolytic activity, platelet hyperactivity, increased Lp(a) lipoprotein, and the presence of antiphospholipid antibody might play a role in the hypercoagulability of BD (6-9, 36-38).

We investigated whether the PlA1/A2 polymorphism influenced the prothrombotic tendency of our patients carrying factor V Leiden or prothrombin gene G20210A mutations, but found no association between the PlA2 allele and DVT in either group. However, these analyses were limited by the small number of patients with DVT and these mutations.

Vascular lesions are fairly common in BD patients (5), with estimates ranging from 10% to 30% of cases (4). Blood vessels of all sizes may be affected by BD (39), but arterial involvement is less frequent than venous involvement, which is found in approximately 85% of cases of BD-related vasculopathy (40). Superficial thrombophlebitis and DVT are the most common vascular lesions, with DVT particularly affecting the veins of the lower limbs and to a lesser extent the superior and inferior vena cava and the upper limbs (39, 40). The frequency of DVT in our consecutive series of 200 Italian patients with BD was 17%, which is similar to its 16% prevalence in Turkish patients (40).

Arterial lesions are characterised by both thrombosis and aneurysms, and are a leading cause of mortality (2), but none of our patients had arterial lesions. The pathogenesis of thrombosis in patients with BD is not clear, but vasculitis seems to play a key role (2, 5). However, other factors must also be involved because not all BD patients develop thrombosis (41-43). One of these is thrombophilia, and various abnormalities in endothelial cell function have also been described (26, 44, 45), which may be secondary to vasculitis and may predispose to vascular lesions.

Venous lesions seem to be mainly due to vessel inflammation as it has been shown that combined therapy with anticoagulants and immunosuppressants is not superior to immunosuppressants alone in decreasing the frequency of recurrent DVT in BD patients (46).

Finally, we found that the A2 allele was significantly less frequent in our BD patients with genital ulcers than in those without, which suggests that the PlA1/A2 polymorphism may protect against the development of genital ulcers. However, the mechanisms by which it does so are still unknown, and further studies are necessary to replicate this finding and clarify this point. No difference was found between the BD patients as a whole and the healthy controls.

In conclusion, we found that the PlA1/A2 polymorphism of the GpIIIa gene is associated with DVT in BD patients. This finding is potentially important because of the association between PlA2 and increased platelet aggregation, but requires confirmation in other populations. Furthermore, the polymorphism seems to have a protective effect on the development of BD-related genital ulcers.

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