Genetic and Plasma Markers of Venous Thromboembolism in Patients with High Grade Glioma

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ABSTRACT

Purpose: **Deep venous thrombosis/pulmonary embolism (DVT/PE) is a frequent complication in the course of cancer, particularly in brain tumors. We investigated genetic and plasma factors possibly associated with risk of DVT/PE in patients with high-grade glioma.**

Experimental Design: **In a case-control study, we studied polymorphisms of the genes coding for factor II (*G20210A*), factor V (*G1691A*), methylenetetrahydrofolate-reductase (*C677T*), tissue-type plasminogen activator (tPA; insertion/deletion), plasminogen activator inhibitor-1 (PAI-1; *4G/5G*), and vascular endothelial growth factor (VEGF; *C936T*). We also measured plasma levels of D-dimer, lipoprotein (lp) (a), homocysteine, VEGF, tPA, and PAI-1, comparing healthy control patients with patients with glioma or with patients with neurological nonneoplastic disease (multiple sclerosis).**

Results: **Genotype frequencies of polymorphisms analyzed were similar in patients with glioma and in healthy matched population. D-dimer, lp (a), homocysteine, VEGF, tPA, and PAI-1 plasma levels were significantly higher in patients with glioma than in healthy controls, whereas patients having neurological nonneoplastic disease had plasma values of these molecules not significantly different from healthy controls. VEGF, tPA, and PAI-1 were also found at high-plasma levels in patients carrying genotypes that, in healthy controls, were associated with "low-producing" phenotypes.**

Conclusions: **Genetic risk factors alone did not explain the high incidence of DVT/PE observed in patients with glioma. Higher plasma levels of molecules influencing the coagulation pathways indicate that the tumor itself might confer an increased risk of DVT/PE; thus, D-dimer, homocysteine, lp (a), VEGF, tPA, and PAI-1 look like good candidates to be evaluated as DVT/PE prognostic factors.**

INTRODUCTION

Deep venous thrombosis/pulmonary embolisms (DVT/PE) are common events in patients with brain tumors, affecting a proportion of patients ranging from 11 to 25% (1–3). Events are scattered through the follow-up; however, they involve a minority of patients, suggesting that prothrombotic factors released by the tumor itself may interact with genetic factors predisposing patients to DVT/PE. Because an effective prophylaxis might involve daily s.c. heparin lifelong, identification of a subset of patients at higher risk for DVT/PE could lead to a more tailored preventive treatment in selected individuals. We therefore undertook this study with the aim to identify putative genetic and plasma factors involved in DVT/PE and to differentiate patients with malignant glioma from healthy control patients.

Predisposition to sporadic DVT/PE has been associated with genetic polymorphisms located in the gene *F2* (coding for coagulation factor [FII]/prothrombin) and in the gene *F5* [coding for coagulation factor FV; Ref. 4]. FV activates the FII into thrombin, which regulates the maturation of fibrinogen into fibrin. Polymorphism *G1691A* of *F5* (also called Leiden mutation) confers resistance to inactivation of FV by protein C (4). Polymorphism *G20210A* of *F2* (also called prothrombin mutation) modifies the mRNA processing, causing an increase of mRNA and protein synthesis (4).

Plasma markers that could reveal activation of coagulation/fibrinolysis pathways are increased levels of D-dimer (5), lipoprotein (lp) (a) (6); homocysteine (7), tissue-type plasminogen activator (tPA), and plasminogen activator inhibitor-1 (PAI-1; Ref. 8).

D-dimer is a product of fibrin degradation; increased D-dimer plasma levels reveal the activation of fibrinolysis. Lp (a) binds to fibrin occupying the plasminogen binding site (9); inhibits tissue factor pathway inhibitor (10), thus removing a block of coagulation cascade; induces PAI-1; and inhibits tPA (11), leading to fibrinolysis inhibition. Homocysteine, which derives from the methionine metabolism, is also a thrombogenic factor; plasma levels higher than normal have been associated with vascular diseases (12). The clotting role of homocysteine could be explained by its capacity to induce FV (13) and tissue factor (14), which binds to FVII, initiating the extrinsic way of coagulation cascade. These procoagulant mechanisms could also be supported by homocysteine inhibition of thrombomodulin (15, 16), the activator of protein C. Finally, homocysteine inhibits tPA binding to endothelial cell, leading to down-regulation of fibrinolysis (17). Homocysteine plasma level is regulated by the activity of the enzyme methylenetetrahydrofolate reductase (MTHFR), which catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a cosubstrate for homocysteine remethylation to methyonine. The gene coding for MTHFR has a biallelic polymorphism, *C677T*; individuals carrying *T/T* genotype have a thermolabile enzyme with reduced activity, leading to higher plasma levels of homocys-

teine (18). Despite the clear effect on thrombotic risk conferred by high levels of homocysteine, the carriage of *T/T* genotype of *MTHFR* polymorphism is not widely accepted as risk factor for DVT/PE (19).

In patients with brain malignancy, additional risk factors for thrombotic complications arise from the close relationship between tumor and activation of blood coagulation. Tumor cells produce tissue factor, which initiates the coagulation cascade. Tumor and tumor-associated endothelial cells are able to produce and release the following factors that influence the activation of coagulation cascade: vascular endothelial growth factor (VEGF), PAI-1, and tPA (20, 21). Allelic variants of the genes coding for VEGF (*C936T*; Ref. 22), for PAI-1 (*4G/5G*; Ref. 23), and for tPA (insertion/deletion [I/D]; Ref. 24) have been associated with different plasma levels or activity of these proteins. Genotypes associated with high production of VEGF, tPA, and PAI-1 could regulate the balance between fibrin production and degradation, influencing the risk of DVT/PE. A correlation between risk of DVT/PE and an allele of VEGF, tPA, and PAI-1 genetic polymorphisms is not yet well defined.

To identify risk factors predisposing patients with high-grade glioma to DVT/PE, we analyzed, by case control study, the genetic polymorphisms of *F2*, *F5*, *MTHFR*, *VEGF*, *PAI-1*, and *PLAT* (the gene coding for tPA) and the plasma levels of D-dimer, lp (a), homocysteine, VEGF, PAI-1, and tPA in patients and in healthy controls. We then correlated genotypes with phenotypes (plasma levels) in both patients and healthy controls, as follows: *MTHFR* genotypes with homocysteine plasma levels and *VEGF*, *PAI-1*, and *PLAT* genotypes with plasma levels of molecules coded by these genes.

PATIENTS AND METHODS

Patients and Controls. Our cohort of patients with glioma includes 250 patients, 75 with anaplastic astrocytoma and 175 with glioblastoma multiforme. For simplicity, all patients with these high grade gliomas (WHO classification grade III and IV) will be grouped with the acronym AA/GBM (anaplastic astrocytoma/glioblastoma multiforme). Patients were enrolled over a period of 4 years.

Ninety patients affected by multiple sclerosis were enrolled consecutively over a period of one year and contributed to our study with a venous peripheral blood donation. Plasma values of these patients have been used as a pathological non-neoplastic neurological control group.

Healthy control group (*n* = 270) includes healthy blood donors and healthy volunteer staff personnel of National Neurological Institute C. Besta.

All individuals included in this study had the same ethnicity (Italian Caucasian). Patients and healthy controls gave informed consent to donate anonymous biological samples for research use. Peripheral blood samples were taken by venipuncture in patients with AA/GBM a median of 4 months (range 1–40 months) after surgery and, in patients with multiple sclerosis, during remission phase of the disease. Patients with glioma were under treatment with 1,3-bis(2-chloroethyl)-1-nitrosourea and cisplatin every 6 weeks. Very few patients (*n* = 20) received radiotherapy at the time of blood draw; all of the others had been treated by external beam radiotherapy (60 Gy). Pa-

tients with multiple sclerosis were under treatment with IFN-β or azathioprine. Healthy control samples were taken during blood donation or during routine control of health status of the staff of our institute.

Polymorphism Genotyping. Genomic DNA was extracted from 200 μl of whole blood with commercial kit (Euroclone, Devon, United Kingdom). PCR amplifications were performed using *Taq*DNA polymerase (Roche Diagnostic GmbH, Mannheim, Germany) with standard procedures and specific primers that amplified the following polymorphisms: *F2 G20210A* (forward, 5′-ACACATGTGTTCGCCTGAA-3′; backward for G allele, 5′-GCACTGGGAGCATTGAAGCTC-3′; and backward for A allele, 5′-CCCAGAGAGCTGCCCAT-GAATTTCACTGGGAGCATTGAAGATT-3′); *F5 G1691A* (forward, 5′-TGCCCAGTGCTTAACAAGACCA-3′; backward for G allele, 5′-ACAAAATACCTGTATTCCTC-3′; and backward for A allele, 5′-GAATTTCTGAAAGGTTACTTCA-ATTACAAAATACCTGTATTCATT-3′); *MTHFR C677T* (18); *VEGF C936T* (22); *PAI-1 −675 4G/5G* (25); and *PLAT* intron 8 *I/D* (26).

Plasma Dosages. Peripheral blood samples were taken by venipuncture in vacutainer tubes containing Na-citrate or EDTA as anticoagulant. Citrate blood samples were used for the determination of D-dimer, lp (a), tPA, and PAI-1. EDTA blood samples were used for the determination of homocysteine and VEGF. The vacutainers used for the determination of homocysteine, D-dimer, lp (a), tPA, and PAI-1 were immediately placed at 0°C to 4°C and centrifuged at 900 × *g* within one hour. Vacutainers used for the determination of VEGF were immediately centrifuged.

D-dimer, lp (a), VEGF, tPA, and PAI-1 values were determined by commercial enzyme immunoassay kits [D-dimer (AGEN Biomedical, Ltd., Acacia Ridge, Australia); lp (a) and tPA (Byk-Sangtec Diagnostica, Dietzenbach, Germany); VEGF (Pierce Endogen, Rockford, IL); and PAI-1 (Hyphen BioMed, Andrésy, France)]. Homocysteine values were determined by fluorescent polarized immunoassay method using AXSYM System Instrument (Abbot Diagnostic Division, Oslo, Norway).

Statistical Analyses. The Hardy-Weinberg equilibrium was verified for all tested populations. Genotype frequencies were compared by 3×2 χ^2 test. Plasma values were compared using Mann-Whitney test (SAS software).

RESULTS

We analyzed the genotypes distribution of polymorphisms associated with DVT/PE risk in a AA/GBM cohort of patients and in age- and sex-matched healthy controls. The genotype frequencies of the *F2 G20210A* polymorphism and the *F5 G1691A* polymorphism were not significantly different in AA/GBM patients compared with healthy controls (*F2*: *A/G* 4% in healthy controls and 5% in AA/GBM; *G/G* 96% in healthy controls and 95% in AA/GBM; *F5*: *A/G* 4% in healthy controls and 1% in AA/GBM; *G/G* 96% in healthy controls and 99% in AA/GBM; χ^2 values 0.145 for *F2* and 3.55 for *F5*; *P* not significant). Similarly, the genotype distributions of polymorphisms *MTHFR C677T*, *VEGF C936T*, *PLAT I/D*, and *PAI-1 4G/5G* were similar between healthy control patients and AA/GBM patients (*MTHFR*: *C/C* 32%, *C/T* 42%, *T/T* 26% in

Table 1 Plasma levels of D-dimer, lp[a] (a), homocysteine, VEGF, tPA, and PAI-1 in healthy controls, in pathological controls (patients with multiple sclerosis), and in patients with glioma (AA/GBM)

In columns are indicated also *P* of each comparison with healthy controls.

	Healthy controls		Multiple sclerosis			AA/GBM		
	Mean ± SD	(*n*)	Mean ± SD	(*n*)	*P*	Mean ± SD	(*n*)	*P*
D-dimer ng/ml	29.7 ± 6.52	(74)	73.22 ± 60.94	(11)	0.384	109.25 ± 248.14	(72)	0.012
lp (a) mg/dL	23.69 ± 25.3	(92)	20.70 ± 26.94	(45)	0.336	38.63 ± 37.78	(79)	0.002
Homocysteine μmol/liter	10.21 ± 3.82	(160)	9.90 ± 2.9	(27)	0.979	16.53 ± 15.6	(50)	<0.0001
VEGF pg/ml	60.95 ± 70.89	(29)	n.a.	n.a.	n.a.	103.43 ± 75.5	(31)	0.018
tPA ng/ml	4.15 ± 2.72	(133)	4.91 ± 3.81	(58)	0.425	6.83 ± 3.4	(106)	<0.0001
PAI-1 ng/ml	14.08 ± 14.98	(43)	14.37 ± 11.78	(40)	0.134	24.31 ± 15.94	(83)	<0.0001

[a] lp, lipoprotein; VEGF, vascular endothelial growth factor; tPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; AA/GBM, anaplastic astrocytoma/glioblastoma multiforme.

healthy controls; *C/C* 27%, *C/T* 51%, *T/T* 22% in AA/GBM; *VEGF*: *C/C* 74%, *C/T* 24%, *T/T* 2% in healthy controls; *C/C* 75%, *C/T* 23%, *T/T* 2% in AA/GBM; *PLAT*: *D/D* 26%, *I/D* 43%, *I/I* 31% in healthy controls; *D/D* 26%, *I/D* 47%, *I/I* 28% in AA/GBM; *PAI-1*: *4G/4G* 22%, *4G/5G* 57%, *5G/5G* 20% in healthy controls; *4G/4G* 16%, *4G/5G* 55%, *5G/5G* 29% in AA/GBM; χ^2 values were 2.24 for *MTHFR*, 0.41 for *VEGF*, 2.59 for *PLAT*, and 4.68 for *PAI-1*; *P* not significant).

We then measured plasma levels of factors possibly associated with DVT/PE risk in the whole AA/GBM cohort and in age- and sex-matched healthy controls. Inflammatory status up-regulates plasma pro-coagulant factors and inhibits fibrinolytic activity (27). As inflammatory non-neoplastic control, we measured plasma factors also in a group of patients affected by an autoimmune inflammatory disease of the central nervous system (multiple sclerosis). As shown in Table 1, D-dimer, lp (a), homocysteine, VEGF, tPA, and PAI-1 plasma levels were significantly higher in AA/GBM patients than in healthy controls, whereas plasma levels of these proteins measured in multiple sclerosis patients were not significantly different from those of healthy controls.

We observed a wide range of variability of plasma values of the molecules measured in the normal population and, furthermore, in the patient groups. One possible explanation of plasma level variability is the association between a particular polymorphic genotype and a high-producing phenotype.

Individuals having *MTHFR TT* genotype had homocysteine mean levels higher than individuals carrying *C+* genotypes (*C/C* or *C/T*) both in healthy controls (*T/T*: 12.88 ± 4.93 μmol/liter; *C+*: 10.60 ± 2.60 μmol/liter; *P* = 0.015) and in AA/GBM patients (*T/T*: 31.74 ± 32.26 μmol/liter; *C+*: 13.56 ± 7.86 μmol/liter; *P* = 0.034; Fig. 1).

Mean plasma levels of VEGF were higher in *C/C* (76.59 ± 87.43 pg/ml) compared with *C/T* (44.20 ± 44.81 pg/ml) healthy controls, although this difference did not reach statistical significance (*C/C versus C/T* in controls, *P* = 0.275), probably because normal VEGF plasma values varied over a wide range (0–86.5 pg/ml) and could be occasionally higher than 150 pg/ml. The association of *C/T* genotype with a low-producing phenotype was not evident in the AA/GBM patient group; mean levels of VEGF were 112.49 ± 70.50 pg/ml in *C/C* patients and 93.76 ± 81.83 in *C/T* patients (*C/C versus C/T* in patients, *P* = 0.429; Fig. 2).

The association of the I allele with high-tPA plasma levels was significant in healthy controls (*D/D* mean levels, 4.32 ± 1.54 ng/ml, *versus I+* mean levels, 6.85 ± 3.46 ng/ml, *P* = 0.020) but not in AA/GBM cohort (*D/D* mean levels, 7.12 ± 3.91 ng/ml, *versus I+* mean levels, 7.17 ± 4.00 ng/ml, *P* = 0.861); patients carrying *D/D* genotype had high-tPA plasma levels as well as those carrying the other genotypes (Fig. 3).

Mean plasma levels of PAI-1 were higher in healthy controls carrying the *4G* allele (15.64 ± 15.90 ng/ml) compared with *5G/5G* healthy individuals (8.9 ± 10.45 ng/ml), although this difference was not significant (*4G+* versus *5G/5G* in healthy controls, *P* = 0.201; normal range 4–43 ng/ml, occasionally up to 50 ng/ml). In AA/GBM patient, individuals carrying *5G/5G* genotype had PAI-1 plasma levels (24.16 ± 16.40) as high as those of *4G+* group (29.42 ± 15.81; *4G+* versus *5G/5G* in AA/GBM patients, *P* = 0.443; Fig. 4).
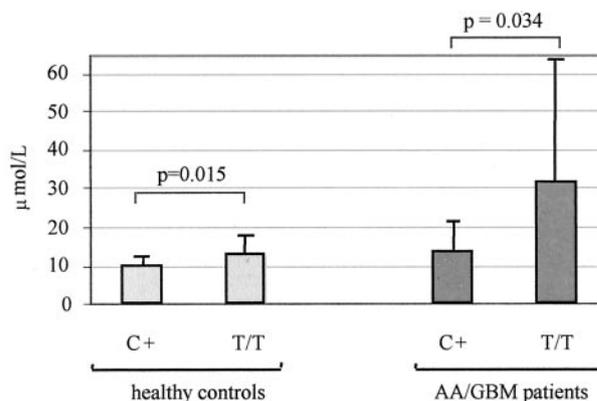
Fig. 1 Plasma levels of homocysteine in healthy controls and patients carrying different *MTHFR C677T* genotypes. *C/C* and *C/T* individuals had similar mean values of homocysteine, and thus they have been included in a unique group (*C+*). Mean values were as follows: in healthy controls, *C+* (*n* = 48) 10.60 ± 2.60 μmol/liter and *T/T* (*n* = 21) 12.88 ± 4.93 μmol/liter, and in anaplastic astrocytoma/glioblastoma multiforme (AA/GBM) patients, *C+* (*n* = 40) 13.56 ± 7.86 μmol/liter and *T/T* (*n* = 8) 31.74 ± 32.26 μmol/liter. Comparisons between *C+* and *T/T* individuals showed significant differences in both healthy controls and AA/GBM groups.
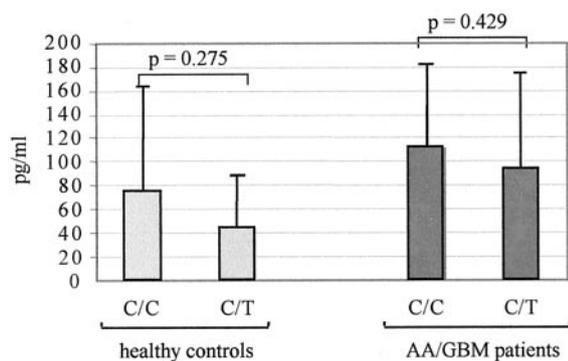
Fig. 2 Plasma levels of vascular endothelial growth factor (VEGF) in healthy controls and patients carrying different *VEGF C936T* genotypes. Numbers of *TT* individuals were too low, and thus they were not included in the analyses. Mean values were as follows: in healthy controls, *C/C* ($n = 16$) 112.49 $\pm$ 70.50 pg/ml and *C/T* ($n = 15$) 93.76 $\pm$ 81.83 pg/ml, and in anaplastic astrocytoma/glioblastoma multiforme (AA/GBM) patients, *C/C* ($n = 15$) 76.59 $\pm$ 87.43 pg/ml and *C/T* ($n = 14$) 44.20 $\pm$ 44.81 pg/ml. Comparisons between *C+* and *T/T* individuals showed no significant differences in either healthy controls or AA/GBM groups.
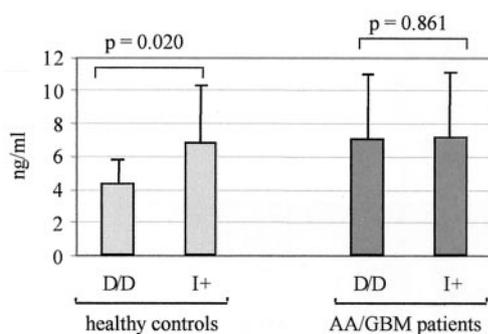

Fig. 3 Plasma levels of tissue type plasminogen activator (tPA) in healthy controls and patients carrying different *PLAT* insertion/deletion (*I/D*) genotypes. *I/D* and *I/I* individuals had similar mean values of tPA and thus they have been grouped (*I+*). Mean values were as follows: in healthy controls, *D/D* ($n = 10$) 4.32 $\pm$ 1.54 ng/ml and *I+* ($n = 31$) 6.85 $\pm$ 3.46 ng/ml, and in anaplastic astrocytoma/glioblastoma multiforme (AA/GBM) patients, *D/D* ($n = 15$) 7.17 $\pm$ 4.00 ng/ml and *I+* ($n = 41$) 7.26 $\pm$ 4.01 ng/ml. Comparisons between *D/D* and *I+* individuals showed significant difference in healthy controls, but not in AA/GBM group.

DISCUSSION

The events of DVT/PE, particularly frequent and often fatal in patients with glioma (1–3), prompted us to search markers of DVT/PE risk. We reasoned that patients with glioma had a risk of DVT/PE, and we first focused our study in the identification of markers of DVT/PE risk that characterized the whole AA/GBM cohort of patients. Apart from risk factors such as age (>60 years), tumor size, grade of tumor malignancy, surgery, and chemotherapy (1, 28), other events seem to expose patients with brain tumors to DVT/PE risk. We analyzed both genetic and plasma factors possibly associated with DVT/PE risk in a group of AA/GBM patients, with the aim to identify putative differences with a control population as a prerequisite for a

follow-up study in which these altered parameters should be monitored and related to DVT/PE events in the course of the disease. Alleles associated with risk of DVT/PE, *F2 20210A* and *F5 1691A*, and polymorphisms of genes coding for proteins involved in coagulation pathways (*MTHFR, VEGF, PLAT,* and *PAI-1*) were not differently distributed in AA/GBM patients compared with healthy controls. Thus, these genetic risk factors alone did not explain the high frequency of DVT/PE occurrence in patients with glioma.

Increased plasma levels of D-dimer, lp (a), homocysteine, VEGF, tPA, and PAI-1 characterized the AA/GBM cohort when compared with healthy controls. All these molecules are involved in the coagulation cascade; although D-dimer is a marker of fibrinolytic activity, all of the other molecules are able to modulate the coagulation process, potentially leading to or revealing a prothrombotic status. Increased levels of VEGF, PAI-1, and tPA could be caused by the production and release of these molecules by the tumor cells (20). In fact, we found these molecules at high levels even in patients carrying a genotype associated with a low-producing phenotype; the presence of the tumor could overcome the genetic background. On the contrary, homocysteine levels correlated with the genotype carriage, being higher in individuals carrying *MTHFR TT* genotypes, both in patients and healthy controls. Patients, anyway, had homocysteine values significantly higher than healthy controls; homocysteine levels are strongly influenced by environmental factors, particularly diet and chemotherapy (29–33), and thus the higher values observed in patients compared with controls could partly be attributable to the tumor itself but possibly also to the lifestyle and drug treatment of patients with tumor. Plasma lp (a) shows that wide quantitative variation among individuals and diet or drugs could only slightly modulate lp (a) plasma levels (34–37). Lp (a) could play a crucial role in modulating the coagulation status of patients with tumor; it may influence coagulation cascade, and thus regulation of lp (a) levels in patients with glioma deserves further investigation.

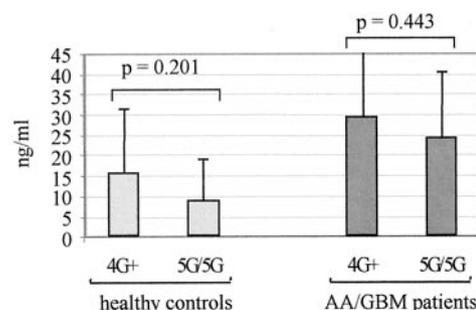The results of our study exclude the existence of major

Fig. 4 Plasma levels of plasminogen activator inhibitor 1 (PAI-1) in healthy controls and patients carrying different *PAI1* 4G/5G genotypes. *4G/4G* and *4G/5G* individuals had similar mean values of PAI-1, and thus they have been grouped (*4G+*). Mean values were as follows: in healthy controls, *4G+* ($n = 33$) 15.64 $\pm$ 15.90 ng/ml and *5G/5G* ($n = 10$) 8.9 $\pm$ 10.45 ng/ml, and in anaplastic astrocytoma/glioblastoma multiforme (AA/GBM) patients, *4G+* ($n = 40$) 29.42 $\pm$ 15.81 ng/ml and *5G/5G* ($n = 10$) 24.16 $\pm$ 16.40 ng/ml. Comparisons between *4G+* and *5G/5G* individuals showed no significant differences in either healthy controls or AA/GBM group.

differences in the genetic polymorphisms concerning the analyzed molecules in a malignant glioma patient population, strongly suggesting the overwhelming role of the release of these factors (possibly within the tumor) in obtaining plasma concentrations higher than in controls. In patients with glioma, the molecules that we investigated could act in concert and interact modifying the coagulation pathways. Glioma cells express VEGF, tPA, and PAI-1 during the tumor growth (20). The increase of VEGF, which in glioblastoma multiforme is sustained also by amplification of epidermal growth factor receptor (38), induces tPA and thus maturation of plasminogen into plasmin (39); in parallel, the inhibitor of tPA (PAI-1) is induced in concert with tPA (21); up-regulation of PAI-1 leads to a prothrombotic status by inhibition of plasminogen activation and, consequently, by reduction of fibrinolytic activity. The balance between PAI-1 and tPA activity could determine the pro- or the antithrombotic status. This balance could be influenced by lp (a), which reduce the secretion of tPA (11) and enhance the production of PAI-1 (40). Lp (a) binds to fibrin acting as a plasminogen competitor and further down-regulating fibrinolysis (9). Moreover, lp (a) down-regulates tissue factor pathway inhibitor (10), whereas homocysteine up-regulates tissue factor (14); both mechanisms activate the extrinsic way of coagulation. Homocysteine could also induces FV (13) and sustain its activation by inhibiting thrombomodulin (15, 16).

Because we found high levels of D-dimer, lp (a), homocysteine, VEGF, tPA, and PAI-1 in patients with glioma, we can suppose that, in these patients, the coagulation status may be modified by the tumor. A longitudinal study is in progress, with the aim to assess whether elevated plasma levels of these molecules are to some extent related to an increased frequency of DVT/PE events and tumor growth or to a relapse during the follow-up of patients treated by surgery followed by chemoradiotherapy.

ACKNOWLEDGMENTS

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