Significance of Factor V, Prothrombin, MTHFR, and PAI-1 Genotypes in Childhood Cerebral Thrombosis

Emel Ozyurek,* Gunay Balta,* Aydan Degerliyurt,† Hülya Parlak,* Sabiha Aysun,† and Aytemiz Gürgey*

*Institute of Child Health and Section of Pediatric Hematology and †Section of Pediatric Neurology, Department of Pediatrics, Faculty of Medicine, Hacettepe University, Ankara, Turkey

Summary: The aim of this study was to evaluate the significance of factor V (FV) G1691A, prothrombin G20210A, methylenetetrahydrofolate reductase (MTHFR) C677T, and plasminogen activator inhibitor-1 (PAI-1) 4G/5G genotypes in development of childhood cerebral thrombosis (CT). A total of 113 Turkish children with CT were studied and compared with the control group. The carrier frequency of the factor V G1691A mutation was found to be significantly higher in the patient group (17.7%) than controls (7.4%). The presence of this genotype was associated with a 2.7-fold increased risk of developing CT (95% confidence interval [CI], 1.0–7.0). The prevalence of prothrombin G20210A mutation in 110 patients (4.5%) was insignificantly higher than controls (2.3%) (odds ratio, 2.0; 95% CI, 0.4–10.7). A statistically significant increase in the frequency of homozygous MTHFR C677T genotype was observed in 62 patients (11.3%) compared to controls (4.3%), and this genotype was associated with 2.8-fold increased CT risk (95% CI, 1.0–8.0). The incidence of PAI-1 4G/4G genotype in 65 patients (21.5%) was slightly lower than that of controls (26.0%), but the differences did not reach statistical significance (odds ratio, 0.8; 95% CI, 0.4–1.5). The results of this study suggested that factor V G1691A and MTHFR C677T genotypes may be associated with an increased risk of developing CT in Turkish children.
Key Words: Cerebral thrombosis—Childhood—Genetic risk factors.

Increasing evidence of neuroradiological data indicates that cerebral thrombosis (CT) is one of the common causes of sudden-onset focal neurologic deficit in children. It often results in neurologic sequelae. Despite much investigation, little is known about etiopathogenesis of the disease. A variety of disorders such as congenital and acquired heart diseases, systemic vascular disorders, hematological diseases, coagulopathies, and trauma are known as predisposing factors for development of the disease in children (1). Recently, epidemiological studies have also associated certain genetic variations with a high risk for CT in childhood (2–13).

There are considerable and consistent data indicating that factor V (FV) G1691A mutation is the most common genetic risk factor associated with development of thrombophilic disorders including childhood CT (2–5,14), despite some conflicting reports that are also present in the literature (6–8,15). On the other hand, the role of prothrombin (PT) G20210A mutation in the development of thrombophilic disorders including childhood CT still remains controversial (2–8,15). Methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism has been studied intensively as a genetic risk factor in various vascular thrombotic disorders; however, no consistent conclusions have yet been made about the role of this genotype in development of childhood CT (4,8–11). Although the 4G/5G genotype in the promoter of the plasminogen activator inhibitor-

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Address correspondence to Aytemiz Gürgey, MD, Hacettepe University, Faculty of Medicine, Department of Pediatrics, Section of Pediatric Hematology, Slhhlye, 06100, Ankara, Turkey; e-mail: agurgey@hacettepe.edu.tr.

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1 (PAI-1) gene has also been studied extensively in many thrombophilic disorders, the significance of this genotype in the development of childhood CT still remains to be clarified (12,13).

The purpose of this study was to evaluate the significance of FV G1691A, PT G20210A, MTHFR C677T, and PAI-1 4G/5G genotypes in the development of CT in Turkish children. The importance of underlying disorders and other predisposing risk factors was also evaluated in the patients with data available.

PATIENTS AND METHODS

Patients

The subjects of this study were 113 consecutive, unrelated Turkish children with CT (100 with ischemic stroke and 13 with sinovenous thrombosis) who were referred for consultation to Hacettepe University Children's Hospital, Section of Pediatric Hematology, in Ankara, between October 1995 and February 2002. The patients consist of 37 girls and 76 boys aged from newborn to 160 months (mean, 50 months). A standardized questionnaire was completed regarding the past thrombotic episodes and family history of thrombosis for all patients. The CT was diagnosed by computed tomography, magnetic resonance imaging, or magnetic resonance angiography. Ischemic stroke was treated with aspirin (1 mg/kg/day) or low molecular weight heparin (LMWH) for at least 3 months, and sinovenous thrombosis with unfractionated heparin for 10 days followed by LMWH for at least 3 months. The levels of protein C (PC), protein S (PS), and antithrombin III (ATIII), and antiphospholipid antibodies (APA) were detected by the standard assays.

The control groups also recruited between the years 1995 and 2002 have been described in our previous reports (16–19). The subjects of these groups met the inclusion criteria as being randomly selected, unrelated, apparently healthy individuals without family history or any evidence of present thrombosis who were either blood bank donors or healthy brothers or sisters of the patients visiting our hospital for mild illnesses (common cold, etc.). Informed consent was obtained from all participating individuals and/or their parents.

Genotyping

All 113 patients were analyzed for FV G1691A, 110 for PT G20210A, 62 for MTHFR C677T, and 65 for PAI-1 4G/5G genotypes by using the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) methods. Genomic DNA was isolated from peripheral blood by using standard methods. FV G1691A genotype was identified by PCR amplification of genomic DNA using the primer pairs described previously (20). Digestion of 267-base-pair (bp) PCR products with the enzyme *Mnl*1 yielded 163-, 67-, 37-bp DNA fragments in normal, and 200- and 67-bp in mutant alleles. PT G20210A genotype was studied by using the primer pairs described previously (21). *Hind*III digestion of the 345-bp PCR product produced 322- and 23-bp DNA fragments in the mutant allele whereas the 345-bp DNA fragment remained undigested in the normal allele. MTHFR C677T genotype was detected by amplification of the 198-bp DNA region by the primer pairs defined before (22). *Hinf*I digestion generated 175- and 23-bp DNA fragments in the mutant, an undigested 198-bp fragment in the normal allele. PAI-1 gene promoter 4G/5G genotype was analyzed by using the previously described primers flanking the polymorphism (23). Digestion of the 163-bp PCR products with *Bse*L1 created 107- and 56-bp DNA fragments in the presence of the 4G, and 74-, 56-, and 34-bp fragments in the presence of the 5G allele.

Statistical Analysis

χ^2 analysis on the SPSS statistical package (version 10) was used to estimate the statistical significance of differences between the frequencies of the genotypes observed in the patient group with those in the control groups. Homozygosity and heterozygosity for A alleles of both FV G1691A and PT G20210A, homozygosity for T allele of MTHFR C677T, and homozygosity for the 4G allele of PAI-1 4G/5G genotypes were taken as risk-elevating genotypes and absence of these genotypes as reference category in the statistical analysis. The odds ratios (OR) were calculated and are given with 95% confidence intervals (CI). Statistical significance of other parameters was also tested by χ^2 analysis.

RESULTS

Clinical analysis of 113 patients with CT revealed that recurrent thrombosis was present in only one patient, and family history of thrombosis in 10 patients (9%). Underlying disorders present in 51 patients of the total (45.1%) were as

follows: 24 infection (21.1% of total), 6 perinatal anoxia (5.3%), 6 congenital heart disease (5.3%), 6 malignancy (5.3%), 3 connective tissue diseases (2.7%), 3 trauma (2.7%), 2 moya moya disease (1.8%), and 1 homocystinuria (0.9%). No patient had patent foramen ovale, catheter-related thrombosis, or history of immobilization.

Predisposing risk factors for the patients with available data are presented along with underlying disorders in Table 1. Among the 65 patients, 5 (1 congenital) were deficient for PC (8%). Protein S deficiency was detected in 7 (1 congenital) of 66 patients (10.6%). Two of these patients had both PC and PS deficiencies. One of the 65 patients had ATIII deficiency (1.5%). This patient was also deficient for PS. Of 41 patients, 13 were positive for APA (32%). Two of 31 patients with available data had slightly higher homocysteine levels than normal.

The distributions of FV G1691A, PT G20210A, MTHFR C677T, and PAI-1 4G/5G genotypes in children with CT and control groups are given in Table 2. Some patients were not successfully genotyped for all genes tested, thus explaining the variations in the total number of patients in the table. The distributions of all of the genotypes in the patient group were in Hardy-Weinberg equilibrium.

Among 113 children with CT, 2 were homozygous (1.8%) and 18 were heterozygous (15.9%) for the FV G1691A mutation. Overall carrier frequency of the mutation (17.7%) was significantly higher in this group, compared to control group (7.4%), and the presence of this mutation showed an association with a 2.7-fold increased risk of developing CT in Turkish children (95% CI, 1.0–7.0) (Table 2). The frequency of risk elevating the A allele was 9.7% in the patient group and 3.7% in the control group, and the differences between the frequencies were also statistically significant (OR, 3.2; 95% CI, 1.3–8.0). The carrier frequency (17%) and positive association remained the same when 100 patients with ischemic stroke were used as an independent group in the statistical analysis (OR, 2.6; 95% CI, 1.0–6.8). The remaining 13 patients with sinovenous thrombosis had even higher carrier frequency (23.1%), but this study fails to assess its significance because the number of patients in this group was too small to obtain reliable results from the statistical analysis.

The distributions of underlying disorders and other predisposing risk factors among the patients with and without FV G1691A mutation are also presented in Table 1. The frequency of underlying disorder within the patients carrying the mutation was found lower than those patients

TABLE 1. Distribution of Underlying Disorders and Other Predisposing Risk Factors Among the Patients With and Without FV G1691A Mutation*

FV G1691A Mutation	Type of Cerebral Thrombosis	Underlying Disorder			Protein C Deficiency			Protein S Deficiency			ATIII Deficiency			APA Positivity		
		N	Total	%	N	Total	%	N	Total	%	N	Total	%	N	Total	%
Hom&Het	Ischemic stroke	6	17	35	2	12	17	3	11	27	0	9		0	3	
	Sinovenous thr.	1	3	33	0	2		0	2		0	2		0	1	
	Sum	7	20	35	2	14	14	3	13	23	0	11		0	4	
Normal	Ischemic stroke	37	83	45	3	43	7	4	45	9	1	46	2	11	34	32
	Sinovenous thr.	7	10	70	0	8		0	8		0	8		2	3	66
	Sum	44	93	47	3	51	6	4	53	8	1	54	2	13	37	35
	Total	51	113	45	5	65	8	7	66	11	1	65	2	13	41	32

*The patients with available data were documented.

ATIII = antithrombin III; APA = antiphospholipid antibody; Hom = homozygote (A/A); Het = heterozygote (A/G); Normal = normal (G/G) genotypes; N = the number of patients positive for the given phenotype; Sum = sum of the patients with ischemic stroke and sinovenous thrombosis for the given phenotype within each group of FV G1691A mutation; Total = total number of patients studied for the given phenotype.

TABLE 2. Distribution of Genetic Risk Factors in Children With Cerebral Thrombosis and Controls

	Patients			Controls*				
Genotypes	Total	N	(%)	Total	N	(%)	OR	95% CI
FV G1691A	113			81				
Hom (A/A)		2	(1.8)		0	(0.0)	2.7†	1.0–7.0*
Het (G/A)		18	(15.9)		6	(7.4)		
Normal (G/G)		93	(82.3)		75	(92.6)		
				Ref. 16				
PT G20210A	110			87				
Het (G/A)		5	(4.5)		2	(2.3)	2.0	0.4–10.7
Normal (G/G)		105	(95.5)		85	(97.7)		
				Ref.17				
MTHFR C677T	62			185				
Hom (T/T)		7	(11.3)		8	(4.3)	2.8	1.0–8.0
Het (C/T)		22	(35.5)		87	(47.0)		
Normal (C/C)		33	(53.2)		90	(48.6)		
				Ref.18				
PAI-1	65			281				
4G/4G		14	(21.5)		73	(26.0)	0.8	0.4–1.5
4G/5G		31	(47.7)		112	(40.0)		
5G/5G		20	(30.8)		96	(34.0)		
				Ref.19				

*The control group data of our laboratory were also reported in the references given for each genotype.

†Both homozygous and heterozygous genotypes for FV G1691A mutation were taken as risk-elevating genotypes in the statistical analysis.

N = the number of patients positive for the given genotype; Total = total number of patients studied for the given genotype; Hom = homozigote; Het = heterozigote for the rare alleles of the genotypes.

with no mutation; 7 of 20 patients with the mutation (35%) and 44 of 93 patients without the mutation (47%) had underlying disorder (OR, 1.7; 95% CI, 0.6–4.6) (Table 1). Additionally, as is presented in Table 3, the frequency of the mutation among the patients with underlying disorder was also lower than that of the patients without it; 7 of 51 patients (14%) with underlying disorder and 13 of 62 patients (21%) without carried the mutation; however, the differences between the frequencies were not statistically significant (OR, 1.7; 95% CI, 0.6–4.6).

As also indicated in Table 1, the frequencies of PC and PS deficiencies were higher among the patients with the mutation (14% and 23%, respectively) than the patients without (6% and 8%, respectively), and ATIII deficiency and APA positivity were not detected in the small number of patients with the mutation whereas they were represented in patients without the mutation, with 2% and 35%, respectively.

The carrier frequency of the PT G20210A mutation given in Table 2 did not show statistically significant difference between 110 patients with CT (4.5%) and the control group (2.3%) (OR, 2.0; 95% CI, 0.4–10.7). This observation did not differ significantly when the 97 patients with ischemic stroke (4.1%) were analyzed as a separate group (OR, 1.8; 95% CI, 0.3–10.2). The carrier frequency of the mutation was found to be relatively high in the 13 patients with sinovenous thrombosis (7.7%). No homozygosity was detected for the mutation in the study.

TABLE 3. Distribution of FV G1691A Mutation and MTHFR C677T Genotype Among Patients With and Without Underlying Disorder

	FV G1691A Mutation						MTHFR C677T Genotype					
Underlying	Hom&Het			Normal			Hom			Het&Normal		
Disorder	N	Total	%	N	Total	%	N	Total	%	N	Total	%
Present	7	51	14	44	51	86	3	24	13	21	24	88
ND	13	62	21	49	62	79	4	38	11	34	38	90
Total	20	113	18	93	113	82	7	62	11	55	62	89

Hom = homozygote for the rare allele (A/A for FV; T/T for MTHFR); Het = heterozygote (G/A for FV; C/T for MTHFR); Normal = normal (G/G for FV; C/C for MTHFR) genotypes; N = the number of patients positive for the given genotype; Total = total number of patients studied for the given genotype; ND = not detected.

Of 62 patients with CT, 7 were homozygous (11.3%) and 22 were heterozygous (35.5%) for the rare T allele of the MTHFR C677T polymorphism (Table 2). The difference in the frequencies of the homozygous genotype between the patients and the controls (4.3%) was statistically significant, and the presence of this genotype in the homozygous state was associated with a 2.8-fold increased risk of developing CT in Turkish children (95% CI, 1.0–8.0). However, the allele frequency of the risk-elevating T genotype was 29% and 28% in patient and control groups, respectively (OR, 1.0; 95% CI, 0.7–1.7). As given in Table 3, three of 24 patients with underlying disorder (13%) and 4 of 38 patients without (10.5%) were homozygous for the T allele of MTHFR C677T genotype (OR, 0.8; 95% CI, 0.62–4.0). Separate analysis of 56 patients with ischemic stroke revealed even higher prevalence of homozygous MTHFR genotype (12.5%) than that of the control group (OR, 3.2; 95% CI, 1.0–9.0). No homozygosity for the T allele was detected in the subgroup of patients with sinovenous thrombosis.

Table 2 shows that 14 of 65 children with CT had PAI-1 gene 4G/4G, 31 4G/5G, and 20 5G/5G genotypes; a statistically nonsignificant decrease in the frequency of 4G/4G genotype was observed in the patient group (21.5%) compared to the control group (26%) (OR, 0.8; 95% CI, 0.4–1.5). The prevalence of 4G/4G genotype in the group of 57 patients with ischemic stroke was also similar (22.8%) and statistically nonsignificant (OR, 0.8; 95% CI, 0.4–1.7). One of 8 patients with sinovenous thrombosis had the 4G/4G genotype (12.5%).

DISCUSSION

Clinical data in this study revealed that approximately half of the patients with CT had underlying disorder. Although the presence of underlying disorder as a risk factor were frequently reported in the literature, it may be noteworthy in this study that infection was the most common underlying disorder found in Turkish children with CT, yet vascular and cardiac disorders in other populations were reported (7,24). The high prevalence of PC and PS deficiencies observed in this study (8% and 11%, respectively) may verify the general notion of association of thrombosis with acquired PC and PS deficiencies (7,24). Despite the presence of a few consistent reports, the prevalence of acquired ATIII deficiency in this study was relatively low (1.5%) compared to the prevalences of 7% to 33% given in a number of reports in the literature (6–8,24). The presence of APA has been increasingly reported in children with cerebral ischemia and is regarded as one of the most common acquired prothrombotic states (3,7,24). However, taken the small number of patients into consideration, increased prevalence of APA (32%) observed in this study may only provide preliminary data on the association of this factor with development of CT in Turkish children.

The results of the present study suggested that the FV G1691A mutation is a genetic risk factor for development of CT in Turkish children. Ignoring a few conflicting reports, this result is consistent with the general agreement on this mutation being a significant hereditary thrombophilia contributing to hypercoagulability in

thrombosis including childhood CT (2–5,14). Actually, given the metanalysis study including 37 reported studies, it is important here to emphasize that CT was the most common disorder associated with the FV G1691A mutation (25). In our previous study, CT was also the largest group (59.4%) among the 32 thrombotic children with FV G1691A mutation (26).

The lower frequencies of underlying disorder among patients with the FV G1691A mutation (Table 1), and the FV G1691A mutation among patients with underlying disorder (Table 3), may suggest that FV G1691A mutation is an independent risk factor for development of CT in Turkish children, and that this mutation does not necessarily need the presence of underlying disorder to trigger thrombosis. This finding is also supported by the results of the previous studies on children with idiopathic ischemic stroke (2,4).

In the literature, there is no agreement on the effect of PT G20210A mutation in development of childhood CT. In some studies, the patient group had significantly higher prevalences of PT G20210A mutation than the controls (2,4), in others, however, the frequency of this mutation in children with CT was found not to be different significantly than that of controls (3,5–8). The results of this study were consistent with the latter observations indicating that the presence of PT G20210A was not associated with the risk of developing CT in children.

This study suggested that the homozygous risk-elevating T allele of MTHFR C677T genotype may be associated with an increased risk of developing CT including ischemic stroke in Turkish children. Consistent results were reported for the idiopathic group with childhood ischemic stroke although contradictory reports were also present for both idiopathic and nonidiopathic ischemic stroke in children (4,8,10,11). The discrepancy between these studies may be the result of other genetic, environmental, and nutritional factors. On the other hand, similar distribution of homozygous MTHFR C677T genotype among the patients with and without underlying disorder may suggest that coexistence of underlying disorder with this genotype was not a prerequisite for development of CT in Turkish children.

In this study, no association was found between the PAI-1 gene 4G/5G genotype and the risk of developing CT including ischemic stroke in children. Similar results were also obtained in our previous study on a total of 80 patients with CT including both children and adults (19). Our findings were also consistent with the results of the presently available two other distinct studies indicating that this genotype alone did not contribute to the development of the disorder in children (12,13).

The FV G1691A mutation was suggested as a risk factor for development of sinovenous thrombosis in a study on German children (14), but not in a study on Argentinean and Canadian children (7,15). In this study, the frequencies of the FV G1691A and the PT G20210A mutations were markedly higher in children with sinovenous thrombosis than controls. Unfortunately, our study population was too small to appropriately assess the association of these mutations and risk of sinovenous thrombosis in children. Therefore, the result on sinovenous thrombosis would only provide preliminary data for future studies.

In summary, this study revealed that about half of the Turkish children with CT had underlying disorder and the most common one was infection. This study also suggested that the FV G1691A mutation and the homozygous MTHFR C677T polymorphism may be associated with an increased risk of developing CT including ischemic stroke in these children. The coexistence of underlying disorders was not a prerequisite for these genotypes to contribute to the development of CT. Neither PT G20210A mutation nor PAI-1 gene 4G/5G genotype proved to be significant predictors of CT risk in children. In conclusion, the investigation of FV G1691A mutation and MTHFR C677T genotype in children with CT may help better manage patients with the disease.

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