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The plasminogen activator inhibitor (PAI)-1 promoter 4G/4G genotype is not associated with ischemic stroke in a population of German children

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Abstract: *Objectives*: To investigate the relationship between an insertion/deletion (4G/5G) polymorphism of the plasminogen activator inhibitor (PAI)-1 gene and childhood patients with a past history of ischemic stroke. *Methods*: The PAI-1 4G/4G genotype and the coinheritance with lipoprotein (Lp) (a) levels, the factor V (FV) G1691A mutation, the prothrombin (PT) G20210A variant, and the methylenetetrahydrofolate reductase (MTHFR) T677T genotype were studied in 198 Caucasian children with stroke and 951 controls (same age, sex and ethnical distribution). In a randomly selected subgroup of patients/controls ($n=60$) PAI-1 activities have been investigated. *Results*: The distribution of the 4G/5G genotypes was no different in childhood stroke patients and controls, with a 4G allele frequency of 55.8% in patients compared with 53.8% in control subjects ($P=0.49$). The 4G/4G genotype compared with the remaining genotypes was present in 43 cases and 167 (17.6% vs. 21.7%; OR/CI: 1.30/0.89–1.98; $P=0.3$). PAI-1 activity was significantly elevated ($P<0.001$) in the patient group. *Conclusions*: Data presented here suggest that the 4G/4G genotype is not a major risk factor in the aetiology of childhood ischemic stroke.

Ulrike Nowak-Göttl[1], Ronald Sträter[1], Andrea Kosch[1], Arnold von Eckardstein[2], Rosemarie Schobess[3], Petra Luigs[1], Petra Nabel[1], Heinrich Vielhaber[4], Karin Kurnik[5], Ralf Junker[2] for the Childhood Stroke Study Group*

[1]Paediatric Haematology and Oncology, University Hospital Münster, [2]Institute of Clinical Chemistry and Laboratory Medicine and Institute of Arteriosclerosis Research, University of Münster, [3]Paediatric Haematology and Oncology, University Hospital Halle/Saale, [4]Department of Paediatrics, Lachnerstrasse Children's Hospital, [5]Department of Paediatrics, University Hospital Munich, Germany

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Correspondence: Prof. Dr. U. Nowak-Göttl, Department of Paediatric Haematology and Oncology, Westfälische Wilhelms-Universität Münster, Albert Schweitzer-Str. 33, D-48149 Münster, Germany
Tel: +49–251/8347783
Fax: +49–251/8347828
e-mail: leagottl@uni-muenster.de

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Within the past decade, various genetic defects of proteins regulating blood coagulation, particularly those affecting the physiological anticoagulant or fibrinolytic systems, have been well established as risk factors of thromboembolic events in adults (1). In the developed countries cardiovascular disease, i.e. myocardial infarction or stroke, are the leading causes of death in adults, influenced by several factors such as age, hypertension, smoking, diabetes and dyslipidemia (2, 3). In childhood patients, however, ischemic cerebrovascular accidents are very rare, with an estimated incidence of about 1 per 100,000 per year. Non-genetic risk factors of arterial cerebrovascular accidents in children and adolescents include congenital heart malformations, vascular abnormalities, endothelial damage, infectious diseases, as well as some rare inborn errors of metabolism (4–6). Since stroke is a thrombotic process in at least 80% of cases, many studies in adults have focused on hemostatic markers and increasingly also on prothrombotic gene polymorphisms with respect to stroke types, age at onset, severity and outcome (7–14). Very recently, we have shown that prothrombotic risk factors which in adults are mainly associated with venous thromboembolism, i.e. the factor V (FV) G1691A

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mutation and the 20210A allele within the 3′-untranslated region of the prothrombin (PT) gene, are significant risk factors in childhood spontaneous stroke (15–19). In addition, the methylenetetrahydrofolate reductase (MTHFR) T677T genotype which causes a thermolabile variant of this enzyme and appears to facilitate the manifestation of hyperhomocysteinemia, especially in individuals with undernutrition with folic acid, has been discussed as a genetic risk factor for vascular disease and stroke in this selected group of very young stroke patients (15). Finally, as in adults, elevated lipoprotein (Lp) (a) has been identified as a genetically determined risk factor for spontaneous stroke in childhood patients beyond the neonatal period (12, 15). Besides the established prothrombotic risk factors mentioned above, a decreased fibrinolytic activity due to increased levels of plasminogen activator inhibitor (PAI)-1 has been shown in patients suffering from deep venous thrombosis and in symptomatic childhood carriers of the FV G1691A gene mutation (20, 21). In adult stroke patients high circulating levels of tissue plasminogen activator and PAI-1 have been described (22–26), and in addition some studies suggest that elevated PAI-1 levels are associated with the 4G/4G genotype of the recently described deletion/insertion 4G/5G polymorphism in the PAI-1 gene (27, 28).

Therefore the present multicenter case-control study has been undertaken to unravel the relationship between the insertion/deletion (4G/5G) polymorphism in the promoter region of the plasminogen activator inhibitor (PAI)-1 gene, alone or coinherited with risk factors of spontaneous ischemic stroke in children.

Patients, materials and methods

Ethics

The present study is part of a prospective multicenter study and was performed in accordance with the ethical standards laid down in a relevant version of the 1964 Declaration of Helsinki and approved by the medical ethics committee at the Westfälische Wilhelms-University, Münster, Germany.

Patients

All infants and children aged from 6 months to 16 yr with first onset of spontaneous ischemic stroke admitted to one of the participating study centres were consecutively included in the study. From October 1995 to October 1999, 198 Caucasian patients (median age at first thrombotic onset 4.9 yr, range 6 months to 16 yr (male, $n=93$) with spontaneous ischemic stroke were recruited from different geographic areas of Germany as previously described (15).

Preterm infants and term neonates <6 months of age, and childhood stroke patients with known underlying diseases, i.e. congenital or acquired heart diseases, cerebral vascular abnormalities, endothelial damage, infectious diseases, adiposity, renal diseases, hypertension, collagen tissue diseases and metabolic disorders, were not enrolled in the present data analysis.

At acute onset of spontaneous ischemic stroke the majority of patients presented with hemiparesis ($n=143$), seizures ($n=28$) or ataxia ($n=27$). The corresponding brain lesions with territorial infarction were predominantly found in the left medial artery ($n=119$), right medial artery ($n=52$) or vertebro-basilar system ($n=27$).

As previously reported, the diagnoses of ischemic strokes were confirmed by an external neuroradiologist upon the results of computed tomography, magnetic resonance imaging or angiography according to criteria previously published by Ringelstein *et al.* (29).

Control population

951 Caucasian controls [same age, sex and ethnical distribution; potential bone marrow donors (315), elective surgery (636); median (range) age 5 yr (6 months to 16 yr: male, $n=446$)] from the same geographic areas as the patients were investigated with parental consent.

Genotyping

For genetic analysis we obtained venous blood in EDTA-treated sample tubes (Sarstedt, Nümbrecht, Germany), from which cells were separated by centrifugation at 3000 *g* for 15 min. The buffy coat layer was then removed and stored at $-70\,^{\circ}C$ pending DNA extraction by a spin column procedure (Qiagen, Hilden, Germany). The PAI-1 4G/5G genotype, the G1691A polymorphism in the FV gene, the G20210A polymorphism in the prothrombin gene, and the C677T polymorphism in the MTHFR gene were determined in controls and patients with ischemic stroke by the polymerase chain reaction as described earlier (30–33).

Measurement of Lp(a) and PAI-1 activity

With informed parental consent, 6–9 months after the acute stroke event fasting blood samples were collected by peripheral venipuncture into plastic tubes containing 1/10 by volume of 3.8% trisodium citrate (Sarstedt, Nümbrecht, Germany), and placed immediately on melting ice. Platelet-poor plasma was prepared by centrifugation at 3000 *g* for 20 min

Table 1. Overall frequency distribution of the PAI-1 4G/5G genotypes in Caucasian childhood stroke patients and controls. Odds ratios and 95% confidence intervals

PAI-1 genotype	Patients ($n=198$)	Controls ($n=951$)	Odds ratio (95%–CI)
4G/4G	65 (32.8%)	275 (28.9%)	1.20 (0.86–1.7)
4G/5G	91 (46.0%)	473 (49.7%)	0.86 (0.63–1.2)
5G/5G	42 (21.2%)	203 (21.4%)	0.99 (0.68–1.44)

at 4 °C, aliquoted in polystyrene tubes, stored at −70 °C and thawed immediately before the assay procedure. As described earlier Lp(a) were quantified by ELISA (COALIZA Lp(a); Chromogenix, Mølndal, Sweden. The critical cut-off level for Lp(a) concentrations was defined at 30 mg/dl, previously identified by us as the risk threshold value for venous thrombosis in childhood (34).

In a randomly selected group of patients ($n=60$) and controls ($n=60$) fasting PAI-1 activities were additionally investigated 6–9 months after the acute stroke onset: PAI-1 activity was measured with Coatest PAI-1 (Chromogenix, Mølndal, Sweden) (21).

Statistics

Odds ratios (OR) and 95% confidence intervals (CI) were calculated with respect to the distribution of the PAI-1 genotypes, i.e. 4G/4G, 4G/5G and 5G/5G each compared with the remaining groups, in patients and controls. In addition, the 4G allele frequency in patients and controls was compared by χ^2-analysis. Calculations of medians, ranges and nonparametric statistics (Mann–Whitney test, Spearman rank correlation) were performed with the Stat View 5.0 program. Furthermore, analysis of variance was performed to investigate PAI-1 activity and PAI-1 genotypes. The significance level was set at 0.05. In addition, all non-descriptive statistical analyses were performed using the MedCalc software package (MedCalc, Mariakerke, Belgium).

Results

PAI-1 4G/4G genotypes

The overall distribution of the 4G/5G genotypes (Table 1) was no different in childhood stroke patients and controls with a 4G allele frequency of 55.8% in patients (221 of 396) compared with 53.8% in the control group ($P=0.49$). Twenty-two out of all patients with the 4G/4G genotype suffered from further prothrombotic risk factors, i.e. the FV G1691A gene mutation ($n=6$), the MTHFR T677T genotype ($n=5$), and increased Lp (a) ($n=8$). Furthermore, combinations between the 4G/4G genotype with two further risk factors, i.e. the FV F1691A and PT G20210A, FV G1691A and MTHFR T677T, and PT G20210A along with MTHFR T677T were found in 1 patient each but not in the control group.

PAI-1 activities

In the randomly selected group of patients and controls median/range PAI-1 activity (11.8/2–34 AU/ml) determined clearly beyond the acute stroke onset was significantly elevated compared with the control subjects (6.1/0–17 AU/ml; $P<0.0001$). In Table 2 the distribution of the 4G/5G genotypes and the corresponding median (range) PAI-1 activity is shown. Analysis of variance revealed no statistical significant result.

Discussion

The present multicenter case-control study was performed to investigate the relationship between the insertion/deletion (4G/5G) polymorphism in the promoter region of the PAI-1 gene and a past history of spontaneous ischemic stroke in childhood. In addition, since some authors claim an association of elevated PAI-1 and stroke patients (24, 26), in a randomly selected subgroup of patients PAI-1 activities were measured.

Data presented here demonstrate that neither the PAI-1 4G/4G genotype alone nor co-inheritance with further prothrombotic risk factors, i.e. FV G1691A, PT G20210A, MTHFR T677T or increased Lp(a) levels, is associated significantly with an increased risk of spontaneous ischemic stroke in early childhood and adolescence. This is in accordance with previously published data that arterial and venous thrombosis is not associated with the 4G/5G polymorphism in a large cohort of US men, and in young patients with minor stroke (35, 36).

Table 2. Subgroup analysis of PAI-1 genotypes and PAI-1 activity (median and range values) in patients and controls

PAI-1 genotype	PAI-1 activity (AU/ml)	
	Patients	Controls
4G/4G	11.2 (2.1–34.0)	8.7 (1–17)
4G/5G	12.8 (4.9–25.4)	5.0 (0–8.0)
5G/5G	12.1 (2.0–26.0)	6.7 (0–14.8)

This 4G/5G promoter polymorphism showed differential transcriptional responses to IL-1 in HepG2 cells with a higher rate of PAI-1 synthesis in cells containing the 4G/4G genotype (37). In 1991 Dawson *et al.* (27) suggested that the 4G site binds an enhancer, whereas the 5G allele binds both an enhancer and a suppressor leading to higher rates of transcription with the 4G/4G genotype. In addition, it has been demonstrated that the 4G/5G promoter site was triglyceride-dependent with the highest PAI-1 levels in subjects with the PAI-1 4G/4G genotype and elevated triglyceride concentrations, respectively (38, 39). However, although PAI-1 levels in homozygous 4G carriers have been reported to be approximately 25% higher than in 5G/5G subjects (40–42), the insulin-resistant state has been described to be more important for PAI-1 plasma levels than genetic traits (43).

Numerous studies on coronary heart disease (CHD) have produced a mixture of publications with controversial results. Eriksson *et al.* (28) suggested in a small-scale study in young males the allele-specific increase in basal transcription of the PAI-1 gene to be associated with myocardial infarction, Mansfield *et al.* (37) identified the 4G/4G genotype as risk factor for CHD in subjects with non-insulin-dependent diabetes mellitus, Margaglione *et al.* (44) described the association of the 4G allele with a family history of CHD, whereas Doggen *et al.*, (45) Ossei-Gerning *et al.* (41) and Junker *et al.* (46) did not confirm the link between the PAI-1 4G allele and CHD or the onset of early myocardial infarction in young men, respectively. Very recently, Gardemann *et al.* (47) suggested that the 4G/4G genotype of the PAI-1 gene polymorphism is associated with the extent of CHD in patients at high cardiovascular risk, i.e. in individuals with high body mass index, hypertension or smoking. The discrepancies, however, reported in the studies mentioned above, might be explained by different sample sizes, individual environmental factors or the extent of additional non-hemostatic risk factors, recently underlined by Gardemann *et al.* (47).

Similar to findings of Catto *et al.* (22) in 558 elderly Caucasian stroke patients, PAI-1 activities were significantly elevated 6–9 months after the acute stroke onset in the randomly selected subgroup of childhood patients compared with control subjects. However, since blood samples for PAI-1 activity were collected beyond the acute stroke onset, increased PAI-1 activities due to the acute phase of stroke onset could be ruled out, suggesting an ongoing and prolonged fibrinolytic shut down in childhood patients suffering from ischemic stroke.

In conclusion, although spontaneous stroke in childhood as one rare entity of cardiovascular disease is different from adult ischemic stroke with respect to non-hemostatic and prothrombotic risk profiles, (2, 3, 6, 15) in this multicenter case-control study we suggest that similar to adult patients the 4G allele of the PAI-1 4G/5G promoter polymorphism is not a major risk factor of spontaneous ischemic stroke in childhood and early adolescence.

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