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Special Article

Children With Stroke: Polymorphism of the MTHFR Gene, Mild Hyperhomocysteinemia, and Vitamin Status

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ABSTRACT

The aim of this study was to investigate a possible association among the thermolabile polymorphism, nucleotide 677 cytosine to thymidine point mutation (677 C→T) of the methylenetetrahydrofolate reductase (MTHFR) gene, hyperhomocysteinemia, serum folate, vitamins B_{12} and B_6, and stroke in children. Allele and genotype frequencies for the 677 C→T polymorphism in 21 children with stroke and 28 healthy children of the same age were studied. No differences in allelic frequency were detected between the two populations. However, the prevalence of homozygous 677 C→T was doubled in the stroke population (28.6%) compared to the healthy group (14.3%). Total plasma homocysteine (tHcy) levels were significantly increased in children aged 2 months to 15 years with stroke compared to reference values. No association was observed between the homozygous genotype (T/T) and hyperhomocysteinemia, nor between the T/T genotype and low folate levels (below the 95th percentile) in this group of patients. Vitamin concentrations in patients were not significantly different from reference values. Significant negative correlations were found between tHcy and folate and between tHcy and cobalamin, but not between tHcy and B_6 concentrations. In summary, a higher prevalence of hyperhomocysteinemia and the 677 C→T polymorphism were observed in children with stroke, but were not always associated. The systematic study of both abnormalities in children with stroke is recommended, so that hyperhomocysteinemia of any genetic origin can be corrected with vitamin supplementation. Moreover, the 677 C→T genotype is a strong factor for predisposition to hyperhomocysteinemia and recurrent risk of stroke that might also be prevented with folate supplementation. (*J Child Neurol* 2000;15:295–298).

Prospective and retrospective epidemiologic studies have recognized hyperhomocysteinemia to be an independent risk factor for arterial vascular disease and venous thrombosis in adult and pediatric patients.[1-7] Plasma homocysteine is determined by genetic and acquired factors,[3] but their nature and interactions are poorly characterized. Homozygosity for a point mutation at nucleotide 677 (677 C→T) of the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene is the most common inherited cause of mild hyperhomocys-

teinemia[9] and has also been related to an increased risk of cardiovascular disease[10] and early-onset stroke.[11] This polymorphism causes an alanine to valine (Ala225Val) mutation that is associated with production of a thermolabile enzyme with 50% less MTHFR activity.[9] The consequence is impaired synthesis of 5-methyl-tetrahydrofolate, the major circulatory form of folate, which provides the methyl group that is indispensable for homocysteine remethylation. Plasma homocysteine concentrations are also determined by nutritional factors such as folate, vitamin B_{12}, and vitamin B_6, which are closely related to its metabolism.[12] Deficiency of one of these vitamins may impair intracellular homocysteine metabolism and cause hyperhomocysteinemia.

In previous studies, an association between hyperhomocysteinemia and stroke in children was found[7,13] but the relationship to genetic and nutritional factors could not be analyzed. The aim of the present study was to investigate a possible association among the 677 C→T polymorphism of the MTHFR gene, hyperhomocysteinemia, serum folate, vitamins B_{12} and B_6, and stroke in children.

Table 1.   Prevalence of the 677 C→T Polymorphism of the MTHFR Gene

MTHFR Genotype	No. of Children With Stroke (%) (n = 21)	No. of Healthy Children (%) (n = 28)
C/C	6 (28.6)	7 (25.0)
T/C	9 (42.8)	17 (60.7)
T/T	6 (28.6)	4 (14.3)
Allele frequency		
C	0.5	0.55
T	0.5	0.45

MTHFR = 5,10-methylenetetrahydrofolate reductase.

MATERIALS AND METHODS

Patients

Twenty-one pediatric cases (age range, 2 months to 18 years) with acute ischemic stroke admitted to the Neurology Department of the Hospital Sant Joan de Déu were studied. Stroke was defined as an acute focal neurologic deficit of more than 24 hours' duration with evidence of cerebral infarction on brain computed tomography (CT) scan or magnetic resonance imaging (MRI). Cases of hemiplegic migraine, central nervous system infection, head injury, and cerebral tumor were excluded. Blood for genetic and biochemical studies was collected at least 1 month after the stroke episode. Eleven of the 21 stroke patients were on antiepileptic treatment (carbamazepine, valproic acid, clonazepam, or clobazam). The hospital ethics committee approved the stroke study, and blood samples were obtained in accordance with the World Medical Assembly's Helsinki Declaration of 1964, as revised in 1996.

Control Group

Genetic studies involved 28 healthy, randomly selected children free of vascular disease, referred to the Genetic Service. Patients and the control group members were of the same age range and genetic background (Caucasian). Reference values for homocysteine and vitamin levels were established respectively in 195 and 100 apparently healthy children (based on history and analytic data) who underwent presurgical analysis for minor surgery.

Mutation Detection

Genomic DNA was prepared from peripheral blood leukocytes using a standard phenol extraction and ethanol precipitation method.[14] Primers for polymerase chain reaction (PCR) amplification were as described by Frosst and colleagues.[9] Template DNA (100 ng) was amplified by using 23 µl of PCR Supermix (Life Technologies, Rockville, MD) and 10 pmol of each primer in a final volume of 25 µl. The PCR conditions were as follows: 30 cycles of denaturation at 94°C for 40 sec, a single annealing and extension step at 56°C for 30 sec, and a final elongation step of 5 min at 72°C. The PCR products were digested with *Hinf I* (New England Biolabs, Beverly, MA) and analyzed by nondenaturing polyacrylamide gel electrophoresis and ethidium bromide staining.

Total Plasma Homocysteine

Fasting total plasma homocysteine (tHcy) (the sum of all homocysteine forms that generate this amino acid by reduction) was determined by high performance liquid chromatography (HPLC), with fluorescence detection of the 7-fluorobenzo-2oxa-1,3-diazole-4-sulfonate (SBDF) derivatives.[15] Total plasma homocysteine was independent of sex and increased significantly with age in the general pediatric population. Therefore, patients were distributed into three age groups for comparison to the reference values for similar ages (2 months to 10 years, 11 to 15 years, and 16 to 18 years). The details of the study, methods, and results of the healthy pediatric population are published elsewhere.[15] Hyperhomocysteinemia was defined as tHcy values above the 95th percentile of the distribution within the pediatric population.[7]

Vitamins

Serum folate and cobalamin were determined by radioimmunoassay (Gamma LKB Wallac 1272, Cambridge, UK), and by HPLC (Perkin Elmer Integral 4000, Beaconsfield, UK) with fluorescence detection (LC 240, Chromsystem Kit, Martinsried, Germany) for vitamin B_6. Patients' serum vitamin concentrations were compared to our reference values, which are independent of age and sex for the pediatric population.

Statistical Methods

The Hardy-Weinberg equilibrium test was applied to allelic frequencies. Statistical analyses were performed using the statistical package SPSS version 6.1.2. The chi-squared test was used to analyze allele and genotype differences between the control and stroke populations, and the association of genotype to hyperhomocysteinemia. The relationship between homocysteine and vitamin concentrations was tested using the Pearson correlation coefficient. Nonparametric Mann-Whitney U-test was applied to compare tHcy and vitamin values of patients to those of reference values, with a 95% confidence interval.

RESULTS

No differences in the allele frequency of the thermolabile 677 C→T polymorphism of the MTHFR gene were detected between the two populations, both being in Hardy-Weinberg equilibrium (Table 1). However, the prevalence of a homozygous 677 C→T genotype (T/T) in the stroke population (28.6%) was double that of the healthy control group (14.3%), although statistical significance could not be attained due to the small sample size.

Plasma total homocysteine was significantly higher in children with stroke (aged 2 months to 15 years) compared to reference values for similar ages (Table 2). Moreover, 15 (71.4%) of 21 stroke patients showed mild hyperhomocysteinemia, defined as values above the 95th percentile of the reference pediatric population. No association was observed between T/T genotype and hyperhomocysteinemia, nor between T/T genotype and low folate levels (folate level < 25th percentile) in this group of patients. Only 8 of 16 patients with hyperhomocysteinemia were on antiepileptic treatment.

Table 2.   Plasma Total-Homocysteine and Vitamin Concentrations in Children With Stroke and Reference Populations

Factor	Children With Stroke			Reference Population		
	Median (range)		n	Median (range)		n
Total-Homocysteine (μmol/L)						
2 months–10 years	9.0	(4.9–11.4)*	8	5.8	(3.7–8.0)	106
11–15 years	10.5	(8.6–31.2)†	11	6.6	(5.1–9.3)	58
16–18 years	10.8	(6.1–15.5)‡	12	8.1	(5.7–10.8)	31
Vitamins						
Folate (nmol/L)	16.1	(3.9–9.1)‡	21	13.0	(6.1–26.2)	100
Vitamin B_{12} (pmol/L)	530	(229–714)‡	21	415	(234–731)	100
Vitamin B_6 (nmol/L)	39.3	(13.8–56.8)‡	21	48.7	(13.8–75.4)	100

Comparison to reference value:
*$P < .0023$.
†$P < .0001$.
‡Not significant.

Vitamin concentrations for patients were not significantly different from the reference values (Table 2). Significantly negative correlations were found between tHcy and folate ($r = -0.5752$; $P < 0.01$) and between tHcy and cobalamin ($r = -0.6330$; $P < 0.01$), but not between tHcy and B_6 concentrations.

DISCUSSION

Cerebrovascular diseases in children are a heterogeneous group of disorders caused by the influence of multiple genetic-susceptibility loci and nongenetic factors. Dissecting the contribution of single factors is the starting point for understanding the etiology and pathophysiology of such complex traits. Hyperhomocysteinemia has been implicated as one risk factor for vascular diseases. In a previous study,[7] plasma tHcy concentrations were analyzed in a group of 68 children with stroke, and an association between mild hyperhomocysteinemia and stroke was found, with an odds ratio of 10.3 (confidence interval, 4.6 to 23.2). To further analyze genetic and nutritional factors relating hyperhomocysteinemia with stroke, patients were recruited for study during their clinical and biochemical follow-up. Only 21 of 68 earlier patients could be studied again, which probably introduced a selection bias in the original population. Some of the original group of patients had died as a consequence of stroke, and others were from distant areas of the country and not available for study.

One candidate gene for susceptibility to vascular diseases is the MTHFR gene,[9] since mutations that reduce the function of this enzyme may cause hyperhomocysteinemia. Several studies have analyzed the contribution of the thermolabile mutation 677 C→T to vascular diseases, with controversial results.[16] An increased prevalence of the T/T genotype has been demonstrated in vascular disease patients relative to controls in studies from the Netherlands,[10] Ireland,[17] Italy,[11,18] and Japan.[19,20] However, other large epidemiologic studies have failed to detect significant differences.[21,22] The different conclusions may be due to factors related to the selection of patients, such as age and clinical inclusion criteria,[11] and to the variable prevalence of the 677 C→T polymorphism in different geographic areas.[23]

This study has focused on analyzing the association between homozygosity for the 677 C→T thermolabile muta-tion and stroke in pediatric patients. No previous studies have been done on stroke patients below 18 years of age. Clinical inclusion criteria were well-defined, in order to minimize clinical heterogeneity. In addition, a wide variation of the 677 C→T allele among different populations has been reported,[18,23,24] and an increase in the frequency of T/T individuals has been documented in this country in people born after 1982,[25] probably due to early folate treatment for all pregnant women to prevent neural-tube defects. Therefore, the pediatric stroke population (mean age of 9 years) was compared to a healthy control group of the same age and genetic background to ensure homogeneity of the two populations.

The results presented in this article showed twice the expected prevalence of homozygosity for the thermolabile 677 C→T polymorphism in children with stroke. The sample could have been biased by the death of some patients, and the difference was not large enough to achieve statistical significance. Nevertheless, these results are in agreement with those found by Soriente, who reported a 1.7-fold increase in the prevalence of thermolabile 677 C→T polymorphism for early-onset stroke (mean age, 38 years) relative to control population.[11] Reference ranges for plasma tHcy in children and adolescents from different geographic areas are very similar.[15,26-28] Most studies have concluded that tHcy values increase with age but are independent of sex, although Osganian and colleagues[27] observed slight differences between boys and girls in a pediatric population of 3524 patients ranging in age from 13 to 14 years. In the present study, mild hyperhomocysteinemia, or tHcy values above the 95th percentile, was observed in 71% of stroke patients. This is a marked increase from that previously reported by the authors, in which only 36% of stroke cases had hyperhomocysteinemia. The differences between the two studies could be explained by selection bias.

No association between the T/T genotype and hyperhomocysteinemia was found. Folate levels, which were normal in 20 of 21 of the patients, might have modified the tHcy levels, since the association between the mutant genotype and hyperhomocysteinemia is especially noteworthy when folate status is low.[29]

Children offer a good population for genetic association studies, since they are free from most acquired determinants of hyperhomocysteinemia that are common in adults, such

as tobacco, coffee, and alcohol use, impaired renal function, and reduced vitamin levels.[8] Treatment with antiepileptic drugs is one of the few acquired factors contributing to hyperhomocysteinemia in children, owing to its antifolate action.[7] Only 8 of 16 patients with hyperhomocysteinemia in this study were on antiepileptic treatment, so this factor would have only partially contributed to the increased tHcy concentrations that were found. Vitamin levels were within the normal range in most of the stroke patients. However, the negative correlation observed between vitamin levels and tHcy concentrations in these patients, as well as in a healthy population,[27] support the key role of these vitamins in maintenance of normal plasma tHcy concentrations.

Finally, mild hyperhomocysteinemia in children may increase with age due to lifestyle factors.[30] Since hyperhomocysteinemia is associated with an increased risk of recurrent stroke[20] without a threshold value,[8] it is important to detect in children to allow correction with folate supplementation.[31] It is probably not the genotype per se that confers an increased risk for stroke, but rather the interaction of this genotype with less than optimal levels of dietary folate that raises plasma tHcy levels.[23]

In summary, hyperhomocysteinemia and the 677 C→T polymorphism were more prevalent but not uniformly present in children with stroke. It is therefore suggested that systematic study of both abnormalities in children with stroke be performed, because hyperhomocysteinemia, whatever its genetic origin, should be corrected with vitamin supplementation. Moreover, the 677 C→T genotype is a strong predisposing factor for hyperhomocysteinemia, and folate supplementation might also prevent recurrent risk of stroke.

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