

C677T and A1298C methylenetetrahydrofolate reductase (MTHFR) polymorphisms as factors involved in ischemic stroke

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Abstract

Background: Ischemic stroke is a major health problem. Data regarding the possible association between ischemic stroke and the polymorphism of methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C are still conflictual. **Aim:** The study tried to assess the association of the two MTHFR polymorphisms with ischemic stroke in a series of patients from a unique hospital center. **Materials and Methods:** The study comprised a total of 127 patients (67 with non-cardioembolic ischemic stroke diagnosed by computed tomography or magnetic resonance imaging) and 60 control cases. The method we used was reverse hybridization performed on peripheral blood for C677T and A1298C polymorphisms. In all patients a careful clinical examination, laboratory analyses of cholesterol, glucose amount and triglycerides, as well as their medical history were available. **Results:** The mean age of stroke patients was 68.73 years, and 55.2% were males. Gene analysis for C677T disclosed the presence of TT genotype in more control subjects than in stroke series (15% and 7.46% respectively). Also, the overall T allele (CT+TT cases) was present in 71.6% of control cases, as compared with 44.7% stroke patients. 1298C allele was almost equally distributed among the two series. No statistically significant correlations of the two genotypes with infarct localization and dimensions and with other potential risk factors (hypertension, lipids, diabetes mellitus) were observed. **Conclusions:** The two MTHFR polymorphisms, C677T and A1298C, seemed not related to the onset of ischemic stroke in our study. However, they could be rather involved in hemorrhagic stroke, as seen in our control patients. Further evaluation on larger series is mandatory since homocysteine activity (related to MTHFR activity) could be easily influenced by folate or cobalamin derivatives.

Keywords: ischemic stroke, methylenetetrahydrofolate reductase, gene polymorphisms, C677T, A1298C, pathogenesis.

Introduction

Ischemic stroke represents almost 90% of cerebrovascular diseases, being the third cause of mortality after cardiac diseases and cancer, but the leading cause of morbidity with almost four millions annual stroke survivors [1]. Several risk factors for developing ischemic stroke are described, including diabetes mellitus, hypertension and age [2]. Apart that, a complex of genetic and environmental factors is described as being involved in its determinism [3, 4].

Among biological factors, plasma homocysteine levels are considered a major risk factor for vascular diseases, including stroke [5]. Several studies suggest a positive and dose-dependent association between the serum concentration of total homocysteine and the risk of stroke, which is independent of other vascular risk factors [6]. Mutations in genes of the homocysteine metabolic pathway may confer an increased risk for ischemic stroke because of elevated plasma homocysteine levels. A common polymorphism (C677T) in the gene encoding 5,10-methylenetetrahydrofolate reductase (MTHFR), a critical enzyme in homocysteine metabolism, has been reported by several studies to be associated with both elevated plasma homocysteine

levels and increased stroke risk [7–9]. Individuals with the MTHFR 677 TT genotype have been shown to have only 30% of in vitro MTHFR enzyme activity compared with the wild type, whereas those with the heterozygous CT genotype have been found to have 60% of wild-type MTHFR enzyme activity [7]. Many studies addressed MTHFR polymorphisms related to stroke, cancer, birth defects, recurrent abortion, and cardiovascular disorders [10–16].

Methylenetetrahydrofolate reductase is a key enzyme in the folate metabolic pathway. It catalyses the conversion of 5,10-methylenetetrahydrofolate (5,10-methylene-THF) to 5-methyltetrahydrofolate (5-methyl-THF). It could be used as a predictive marker for ischemic stroke in patients with associated type 2 diabetes [17]. MTHFR C677T polymorphism effects seem to be great in regions with low dietary folate consumption [18]. However, some recent studies failed to detect a direct association between any single polymorphisms of homocysteine metabolic pathway genes and ischemic stroke [19]. Except C677T, a second common mutation within the gene coding for MTHFR, A1298C, results in significantly reduced catalytic activity of the enzyme, even though only fewer studies focused on this polymorphism [20–26]. The two

polymorphisms presumably act in an additive manner, increasing the risk for ischemic stroke [27].

The goal of our study was to assess the presence of the two genetic polymorphisms of MTHFR in a series of ischemic stroke patients, compared to other subjects presenting with hemorrhagic stroke or other neurological diseases as control series.

Materials and Methods

The study was conducted with the approval of the Ethic Committee of the institutions involved and written informed consent was obtained from all subjects or their relatives (for comatose patients) for the inclusion in the study. A total of 127 patients were recruited, comprising 67 cases with ischemic stroke and 60 control subjects which were age and sex matched to the stroke patients. The inclusion criteria for stroke patients were the presence of an ischemic stroke at the present hospital admission, regardless a previous event of the same category. The neurological deficits were confirmed in all cases by computerized tomography (CT), or magnetic resonance imaging (MRI) scan. Transcranial Doppler ultrasound, CT angiography or MR angiography were performed in selected cases as ancillary techniques. The control cases comprised patients with brain hemorrhage, neurodegenerative diseases, epileptic seizures, brain tumors, or other neurological conditions and no ischemic stroke at present or in their medical history. Cases with ischemic stroke of cardioembolic origin (i.e., with atrial fibrillation) were also discarded from both series, since an associated thrombotic mechanism in the cranial arteries could not be excluded in association to atrial thrombosis with subsequent embolic phenomena. Patients with atrial fibrillation but no cerebral ischemic lesions were, however, included in the control series.

In each patient, one mL of whole blood was harvested in EDTA-coated vials and stored at -80°C .

MTHFR gene polymorphisms (C677T and A1298C) were studied by reverse hybridization. Isolated DNA samples were screened for two MTHFR gene polymorphism (C677T and A1298C) using the strip assay GenoType MTHFR test (Hain Lifescience, Nehren, Germany). The test is based on DNA STRIP technology and permits the combined molecular genetic characterization of C677T and A1298C mutation of the human methylenetetrahydrofolate reductase (MTHFR) gene. The whole procedure consists in: DNA isolation, multiplex amplification with biotinylated primers and a reverse hybridization. Genomic DNA was isolated from the whole blood and DNA isolation was performed using the Wizard® Genomic DNA Purification kit (Promega, Madison, USA) according to the manufacturer's recommendations. PCR Amplification with biotinylated primer was performed using a Verity® 96-Well Thermal Cycler (Applied Biosystems, Carlsbad, USA) and a cycling program consisting of pre-polymerase chain reaction (PCR) at 95°C for 15 minutes, followed by 10 cycles at 95°C for 30 seconds; 58°C for 2 minutes; 20 cycles at 95°C for 25 seconds, 53°C for 40 seconds, 70°C for 40 seconds, and a final extension of 8 minutes

at 72°C . Amplification products were checked by 3% agarose gel electrophoresis. The amplicons has a length of 64bp (C677T) and 83bp (A1298C) respectively as seen in Figure 1.

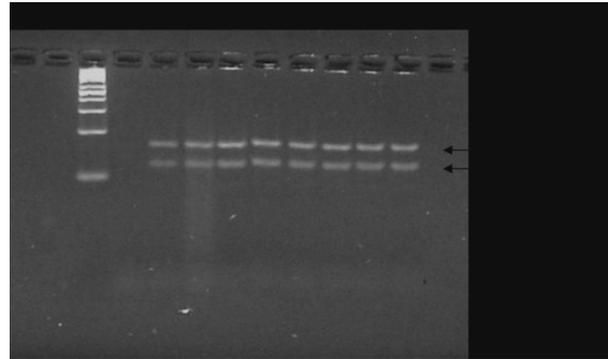


Figure 1 – Verification of the PCR products. The PCR amplification products were analyzed using ethidium-bromide-stained 3% on agarose gel. Lane 1: Negative control with no template; Lanes 2–9: Probes. DNA Ladder 100 bp.

The MTHFR C677T/A1298C polymorphism was detected in the amplified products using reverse hybridization to specific mutant and wild oligonucleotide probes by a colorimetric microwell plate method.

The hybridization of amplicons products includes the following steps: chemical denaturation of the amplification products, hybridization of a single-stranded, biotin-labeled amplicons to membrane-bound probes, stringent washing, addition of streptavidine/alkaline phosphatase conjugate and a mediated staining reaction. The single-stranded amplicon binds specifically to the analog probes during hybridization, while non-specifically bound amplicons are removed in subsequent washing steps.

During the conjugate reaction, the specifically bound amplicon is marked with the enzyme alkaline phosphatase and is then made visible in a colorimetric detection reaction. In this way, a specific banding pattern develops on the DNA STRIP as seen in Figure 2.

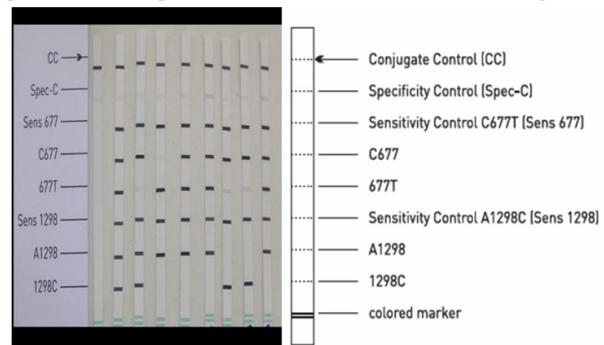


Figure 2 – Staining pattern of hybridized DNA for MTHFR genotyping for representative patients' samples with the corresponding genotypes indicated. Lane 1: Negative control; Lane 2: Normal positive control; Lane 3: 677CC/1298AC; Lane 4: 677TT/1298AA; Lane 5: 677CT/1298AA; Lane 6: 677CT/1298AA; Lane 7: 677CC/1298CC; Lane 8: 677CC/1298CC; Lane 9: 677CT/1298AA.

Statistic analysis of data was performed using Excel software running under Windows. Logistic regression

and two sample Student *t*-test for continuous data were used with $p < 0.05$ considered as statistically significant.

Results

The stroke series comprised 67 patients (37 males, 30 females; 49–87-year-old, mean 68.73, standard error 1.414). The 60 control patients included 35 males and 25 females, with a mean age of 71.26 years, standard error 1.413). Table 1 shows the distributions of clinical characteristics in patients with stroke and control subjects. In the stroke series, higher values of hypertension and cholesterol were obvious, while triglycerides, cardiac ischemic disease and diabetes mellitus were much more frequent in the control series.

Table 1 – The clinical characteristics of patients included in the two series taken into the study

	Stroke (%)	Control (%)
Male (%)	37 (55.22)	35 (58.33)
Age [years]	68.73±11.57	71.26±10.94
Hypertension	57 (85)	43 (71.66)
Diabetes mellitus	15 (22.38)	34 (56.66)
Hypercholesterolemia	21 (41.79)	9 (15)
Hypertriglyceridemia	9 (13.43)	17 (28.33)
Cardiac ischemic disease	18 (26.86)	25 (41.6)
Atrial fibrillation	0	22 (36.66)

In this study, CT allele was positive in 30 stroke patients (44.7%) but in 43 subjects from the control series (71.6%). An almost double percentage of TT homozygosity was also present in the control series as compared with the stroke patients. Data are summarized in Figure 3. The 1298C polymorphism was present in almost equal proportions in the two series (55.2% and 58.3% respectively). The data are summarized in Figure 4. In sum, no relationship was seen for one of the two polymorphisms in an individual or additive manner with the ischemic stroke presence or extent. No relationship of the two genotypes with the presence of hypertension, increased cholesterol, glucose or triglycerides, atrial fibrillation or cardiac ischemic disease association was found to be statistically significant ($p > 0.05$).

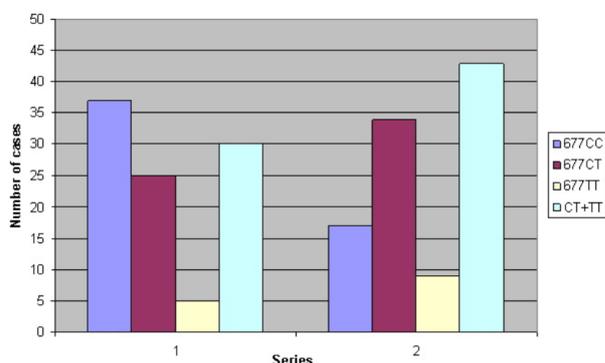


Figure 3 – Graph illustrating the distribution of C677T polymorphism of MTHFR gene. An obviously higher percentage of cases of the control series exhibit the T polymorphism; this is also reflected in the total number of T alleles in the two series, which is higher in the control series. 1: Stroke series; 2: Control series.

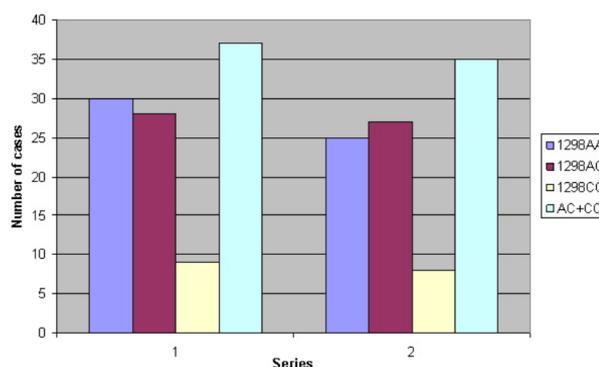


Figure 4 – Graph with the A1298C polymorphism of MTHFR gene in the two series. The number of cases is approximately equal for each polymorphism. 1: Stroke series; 2: Control series.

Discussion

In our study of C677T polymorphism, we found that T allele was mostly present in the control series (CT plus TT 71.6%), when compared with stroke patients (only 44.7%). TT homozygosity was also almost double in frequency in the control (15%) vs. stroke (7.46%) series. This is different from other recent papers dealing with the same subject [28]. A possible explanation could be the presence in this control series of patients with hemorrhagic stroke, whose pathogenic mechanisms could also be influenced by the presence of C677T allele in the vascular bed fragilization with consecutive blood extravasation.

Overall MTHFR C677T polymorphism effects related to ischemic stroke are extremely variable according to multiple published reports. It appears to be related to ischemic brain events mostly in Asian patients [9, 29–32]. On the other hand, with rare exceptions [33] several papers failed to show a direct relationship of C677T polymorphism on the apparition of ischemic stroke or to the dimensions of the infarcted area in Caucasian populations [34–38]. Its influence as a certain risk factor seems to be more obvious in young subjects [39]. Even some Asian series of patients did not show a significant relationship of C677T polymorphism with ischemic stroke [40].

For the second genetic polymorphism, in our study, the only minor differences of A1298C between the two series probably reflect an even lower impact of this genetic modification (than of C677T) in the onset and evolution of an ischemic brain event.

Of the other risk factors identified, arterial hypertension was present in approximately equal proportions in the two series, as were increased values of triglycerides or cholesterol. Unlike that, diabetes was present in a much higher percentage in control subjects than in those with atherothrombotic ischemic stroke.

A potential weakness in our study was the lack of homocystein determination. This was first caused by the fact that it is not currently available as a procedure in our laboratories, and second, because we intended to see mostly the simple association of the two genotypes with the apparition of cerebral ischemic stroke and not to also assess the potential link between genotype and thrombosis, that could be the raised homocystein levels.

The presence in the control series of subjects with hemorrhagic stroke is also a potential confusing element, since in these patients the presence of 677T and 1298C alleles could also play a role, possibly by tissue changes in the arterial wall structure, with consecutive blood extravasation.

☒ Conclusions

The etiology and pathogenesis of ischemic stroke is complex and several risk factors could be involved. Among these, genetic polymorphisms in MTHFR gene did not appear to be significant in our study. Interactions of environmental and genetic factors on larger series are needed in order to assess the real contribution of each element in this devastating disease.

Acknowledgments

The study was supported by a grant from the Ministry of Education, PNII 42–168/2008.

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Received: October 1st, 2011

Accepted: December 15th, 2011