Neurosci Bull December 1, 2013, 29(6): 725–730. http://www.neurosci.cn
DOI: 10.1007/s12264-013-1381-4

·Original Article·

Synergistic effect of *MTHFR* C677T and *F2* G20210A polymorphisms on ischemic stroke

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ABSTRACT

The predisposition to stroke might involve interactive effects among variants in several genes. We tested this hypothesis by examining the influence of polymorphisms in methylenetetrahydrofolate reductase (*MTHFR)* (C677T) and prothrombin (*F2*) (G20210A) as risk factors for stroke in Morocco. The polymerase chain reaction-restriction fragment length polymorphism methods were used to analyze DNA from 91 stroke patients and 182 controls. Association between the two polymorphisms and the risk of stroke was estimated by four-level models for the analysis of genetic interaction. Neither the *MTHFR* 677TT nor the *F2* 20210GA genotype showed any significant association compared to the *MTHFR* CC and *F2* GG genotypes, respectively. An interactive effect between the *MTHFR* 677TT and *F2* 20210GA polymorphisms showed an increased risk of stroke. The odds ratios, in univariate and multivariate analysis, for the combined polymorphisms were 4.99 (95% CI, 1.75–14.2, P = 0.001) and 5.29 (95% CI, 1.63–17.1, P = 0.005), respectively.

Keywords: genotyping; prothrombotic polymorphisms; risk factor; *MTHFR*; *F2*; stroke

INTRODUCTION

Evidence from epidemiological studies has shown that stroke is a multifactorial disease[1], which involves interactions between acquired and genetic risk factors[2]. Despite reports of pathological links between genetic factors and ischemic stroke in several candidate gene-association studies, results are still controversial[3]. Two explanations have been suggested for the discrepancy: whether stroke is actually the end phenotype, and whether the effect of candidate polymorphisms might be weak when analyzed individually. To reflect an inherited susceptibility, it is assumed that the pathogenic processes result from the combined action of a wide spectrum of pathogenic alleles[4]. As important candidate genes for prothrombotic risk, the roles of the prothrombin (*F2*) G20210A and methylenetetrahydrofolate reductase (*MTHFR*) C677T polymorphisms in stroke have been reported in association studies[5].

An *MTHFR* gene polymorphism at position 677, in which a cytidine/thymidine substitution occurs, is associated in the homozygous state with an increased level of homocysteine (tHcy), particularly when the plasma folic acid level is reduced[6,7]. The association studies between homozygous *MTHFR* 677T and stroke show controversial results ranging from no effect[8-10] to a mild-to-moderate effect[11-13]. Another prothrombotic polymorphism occurs in position 20210 of the 3'-untranslated region of *F2*[14] and results in mutation of G to A. This substitution is associated with increased prothrombin plasma levels and confers an excessive risk of venous thrombosis[14]. However, studies assessing these markers as isolated gene mutations have come to conflicting conclusions: some reports suggest an association between *F2* G20210A mutation and an increased risk of stroke[11,13] but others do not[8,15]. In

addition, the few genetic studies[5,16] that investigated the association of interactive effects of these two candidate polymorphisms with stroke events were performed in a Caucasian population. No data are available for the Moroccan, an African population.

In this case-control study, we assessed the potential association between polymorphisms of the *F2* and *MTHFR* genes and ischemic stroke in stroke patients compared with age-matched controls (matched 1:2 for gender), of the same geographic origin.

PARTICIPANTS AND METHODS

Study Participants

In this study, patients with ischemic stroke, aged from 17 to 86 years, consecutively admitted to the Neurology Unit of Ibn Rochd Hospital Center in Casablanca between May 26, 2008 and September 30, 2009, were included. There were 46 men and 45 women with a mean age of 48 years. An established diagnosis of ischemic stroke was defined as described in our previous study[11]. The control group consisted of 182 healthy Moroccan people of similar age with 94 men and 88 women, who were enrolled for studies of the prevalence of genetic polymorphisms in the Laboratory of Genetics at the Faculty of Medicine in Casablanca. Data on standard risk factors for stroke from patients and controls were assessed as previously described[10,15].

Both patients and controls gave informed consent for DNA analysis before entering the study, which was approved by the Ethics Committee of our institution.

Genotyping Analysis

DNA was isolated using the salt method[17] from 5–10 mL venous blood samples collected in tubes containing disodium EDTA and kept at 4°C until use. A slightly modified method[18] was used to identify the *F2* G20210A polymorphism. PCR produced an amplicon of 486 bp, and this was followed by *Hind*III digestion for mutation detection. For *MTHFR* C677T, an adapted amplification method[6] produced an amplicon of 198 bp containing the mutation, which was identified by *Hin*fI digestion.

Statistical Analysis

Statistical comparison between the two groups was performed based on Fisher's exact test for dichotomous variables, and on Student's *t*-test for continuous variables. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for each prothrombotic polymorphism. Further analyses such as four-level models, using the data from the two-by-four table (table not shown) of cases and controls in each of the four possible combinations of the two genetic factors, were undertaken to assess interactions between these two polymorphisms as stroke risk factors. A logistic regression model was designed to include age, sex, alcohol intake, smoking, and a history of hypertension or diabetes mellitus as controlling variables, and the adjusted ORs with 95% CIs were estimated. The Statistical Package for the Social Sciences (version 17; SPSS, Chicago, IL) was used for analyses.

RESULTS

Clinical Characteristics of Participants

The clinical characteristics of cases and controls are shown in Table 1. Some major predisposing factors were more common in the cases than in the controls. Among these conventional risk factors, hypertension, age >50 years, diabetes mellitus, smoking, and alcohol abuse are independent risk factors for cerebrovascular events in patients.

Prevalence of *MTHFR* and *F2* Polymorphisms

The genotype prevalence of *MTHFR* C677T and *F2* G20210A along with the combined homozygote and heterozygote frequencies for the two groups are shown in Table 2. No homozygous AA for the *F2* G20210A polymorphism was found in stroke patients and controls. There were no significant differences between the stroke patients and control group, although the patients showed an elevated frequency of the *MTHFR* TT genotype. Similarly, no statistical difference was found for the heterozygous *F2* 20210GA polymorphism genotype (Table 2). When the combined *MTHFR* C677T and *F2* G20210A polymorphisms were compared with the reference genotype CCGG in univariate and multivariate analysis, an interactive effect of *MTHFR* 677TT and *F2* 20210GA (TTGA) was found (Table 3) with a 4.99- to 5.29-fold risk compared to 1.79-fold for *MTHFR* 677TT alone, or to 2.36-fold (not significantly different) for *F2* 20210GA. This risk effect disappeared

Table 1. Clinical characteristics of cases and controls

Characteristics	Cases *n* = 91 (%)	Controls *n* = 182 (%)	*P* value[#]
Age (years, mean ± SD)	49 ± 15.28	46.25 ± 11.44	0.095
Sex (male)	46 (50.5)	94 (51.6)	0.864
>50 years old	37 (40.7)	49 (26.9)	0.022
Hypertension	46 (50.5)	18 (9.9)	<0.001
Diabetes mellitus	16 (17.6)	16 (8.8)	0.037
Current smoking	20 (22)	13 (7.1)	0.001
Alcohol intake	14 (15.4)	4 (2.2)	<0.001

[#]*P* value obtained using Fisher's exact test.

Table 2. Prevalence of prothrombotic risk factor polymorphisms in cases and controls

Polymorphism	Cases (%)	Controls (%)	Crude OR	CI (95%)	*P*	Adjusted OR	CI (95%)	*P**
MTHFR								
677TT *vs* CC	17.2	10.4	1.79	0.71–4.53	0.213	1.71	0.59–4.98	0.322
677CT *vs* CC	40.7	44.4	0.86	0.50–1.47	0.580	0.84	0.43–1.62	0.579
TT+CT *vs* CC	47.3	47.8	0.97	0.59–1.62	0.932	0.95	0.59–1.76	0.880
F2								
20210GA *vs* GG	12.1	5.5	2.36	0.96–5.79	0.060	2.45	0.86–7.24	0.106

**P* value obtained using logistic regression model adjusted for age, sex, smoking, alcohol intake, history of hypertension, and history of diabetes.

Table 3. Odds ratios (ORs) of interactive effects between *MTHFR* C677T and *F2* G20210A polymorphisms on stroke using the four-level models

Genotypes	*F2*											
	GG						GA					
MTHFR	Crude OR	CI (95%)	*P*	Adjusted ORs	CI (95%)	*P***	Crude OR	CI (95%)	*P*	Adjusted OR	CI (95%)	*P***
CC	1	Reference groups					4.82	0.42–54.7	0.216	5.72	0.34–94.5	0.223
CT	0.93	0.52–1.68	0.472	0.93	0.45–1.94	0.866	2.18	0.84–5.64	0.084	2.23	0.73–6.78	0.156
TT	2.45	0.87–6.88	0.075	2.47	0.75–8.16	0.052	4.99	1.75–14.2	0.001	5.29	1.63–17.1	0.005

***P* value between combined genotype risk factors “TTGA”, “CTGG”,“CTGA”, and “CCGA” of the two polymorphisms compared with the reference genotype (CCGG) obtained using logistic regression model adjusted for age, sex, smoking, alcohol intake, history of hypertension, and history of diabetes.

when *MTHFR* 677CT was combined with *F2* 20210GA (CTGA) as well as *MTHFR* 677CC with 20210GA (CCGA). Our results showed that 22% of the cases carried at least the combination of both risk factor polymorphisms (TTGA),

against 9.3% in controls (P = 0.001 and P = 0.005 after adjustment for conventional risk factors).

Interaction Effects of Candidate Genotypes on Stroke

Using four-level model analysis, the significant interaction effects with or without covariates after multiple tests were confirmed when the homozygote *MTHFR* TT and heterozygote *F2* GA co-existed, with *P*-values at 0.001 and 0.005. The risk for stroke was higher in carriers of the *MTHFR* TT and *F2* GA risk alleles (TTGA) than in those without risk alleles (CCGG). The combination of both polymorphisms as risk allele variants revealed that the presence of *MTHFR* T (CT and TT) in the absence of *F2* A (GG), showed no or the lowest rising risk for stroke. Besides, there was no significant risk for stroke occurrence when *F2* was present without the *MTHFR* risk allele. The ORs and *P*-values of the logistic regression analysis on the interactive genetic models for each polymorphism, with and without covariates, are summarized in Table 3.

DISCUSSION

It is recognized that stroke is a multifactorial disease, the susceptibility to which is increased by the combined presence of genetic and environmental factors. However, individually, genetic factors appear to have modest effects. In this study, we assessed the polygenic nature of stroke by analyzing the association of two genetic risk factors involved mainly in the prothrombotic pathway and estimated their interaction with major stroke risk factors. Similar to our previous reports[10,15] and others, *F2*[8,19-21] or *MTHFR* polymorphism[9,22-24] had no association with stroke. Nevertheless, some reports demonstrated an association between *F2*[25-27] or *MTHFR*[8, 21] and an increased risk of stroke. These discrepancies may be due to the sample size, specific selection criteria, and variable prevalence of these genetic or other risk factors in different ethnic populations.

As reported by Kelly *et al.*[28] and Kim *et al.*[29], our study supported the hypothesis that genetic factors have an interactive effect. In fact, when comparing the combined *MTHFR* 677TT and *F2* 20210GA with the reference genotypes CC and GG, an interactive effect was found. It is notable that, in parametric analyses to assess the strength of association between stroke and both the *MTHFR* and *F2* gene polymorphisms, carriers of the risk alleles had a five-fold increase in the risk of stroke over participants without the risk alleles. Our results are in concordance with the study by Pezzini *et al.*[5] who reported a gene dose effect on the risk of cerebral ischemia and an interactive effect of genetic factors, especially that the increased risk of stroke was more pronounced in participants with more than one genetic marker.

Our data on major cardiovascular risk factors (hypertension, diabetes, alcohol, and smoking) showed that these standard risks were not comparable between cases and controls. However, it is recognized that neither polymorphism operates *via* these risk factors in the occurrence of ischemic stroke[30]. We performed logistic regression analysis with adjustment for these standard risk factors and found an interaction with genetic factors in the occurrence of ischemic stroke in our population. Evaluation of the cumulative effect of numerous polymorphisms in association with other cardiovascular risk factors should focus on an appropriate biological interaction between modifiable exposure and genotype, as reported in previous studies conducted on the occurrence of myocardial infarction in young adults[31,32] and in ischemic stroke patients[5,16,24]. However, as previously reported[3], our findings suggest that the probability that a genetic predisposition to a complex phenotype such as stroke is mediated by a strong influence of one or two genes must be weak. Mechanistic studies may allow the elucidation of the pathogenic processes by which these risk alleles contribute to the occurrence of stroke. The 20210G-A variant in the 3-prime untranslated region of the *F2* gene is associated with elevated plasma prothrombin levels and increased thrombin formation[33], leading to a pro-coagulant state by altering mRNA stability. The *MTHFR* 677C-T mutation, resulting in an Ala222-to-Val (A222V) substitution, is correlated with reduced enzymatic activity and leads to significantly elevated plasma tHcy levels[6]. Thus, in a meta-analysis of association between serum tHcy concentration and cardiovascular disease, Wald *et al.*[34] showed that the increase of 5 μmol/L tHcy level linked to 677C-T mutation was associated with an increased risk of stroke. Morocco is a country with a mediterranean diet (high consumption of vegetables and fruit) that has cardioprotective effects. Samman *et al.*[35] reported reduced tHcy concentrations and significantly decreased cardiovascular events and total deaths in patients adapted to this diet. We suggested

that the high folate intake in the Moroccan diet might nullify the potential effect of *MTHFR* polymorphism on the risk of stroke events in this population. Moreover, dietary protection has been reported in neurotoxicity and other neurological disorders[36].

However, neither prothrombin nor homocysteine measurement has been performed to assess their correlation with stroke in our population. It would therefore be of interest to adjust for this biochemical factor to determine whether the entire effect of *MTHFR* or *F2* polymorphisms is mediated *via* homocysteine or prothrombin, respectively. On the other hand, our findings are limited by the small sample size and the lack of a replicate population. External validation is required despite this gene-gene interaction found in our analysis. The other limitation of our study lies in using the four-level model to assess the interactive effects of the candidate genotypes on stroke; because of the large confidence intervals, this needs to be confirmed in studies with a larger number of participants. The finding that the combination of both risk genotypes reached statistical significance does not mean that there is a biological interaction. We suggest that this statistical interaction should be taken with caution.

In conclusion, the present study was initiated to investigate the combined interaction between *F2* G20210A and *MTHFR* C677T polymorphisms that previously failed to show individual association with ischemic stroke in Morocco[10,15]. We found that these polymorphisms were not risk factors for ischemic stroke when tested individually. However, when combining both polymorphisms, we found an interaction effect on the occurrence of stroke. This suggests that the effects of genetic interactions linked to the prothrombin pathway modulate the risk of stroke. Further validations are necessary in other populations, as well as in a Moroccan population with a greater sample size. As emphasized previously[37], assessment of independent data from a similar study design is required to obtain further insight on the scientific importance of our findings. Therefore, the physiological function and molecular mechanisms of the involvement of these prothrombotic risk polymorphisms in stroke need to be investigated to clarify the association. The development of preventative and treatment strategies requires more knowledge of this gene-gene interaction. Regarding the result of this study, we suggest that screening of these two polymorphisms individuually as genetic test in stroke is not advisable, at least in the Moroccan population.

ACKNOWLEDGMENTS

We thank the participants and the members of the Neurology Unit for their help with data collection. We are also grateful to Belgium Technical Cooperation in the Democratic Republic of Congo for support.

Received date: 2012-10-27; Accepted date: 2013-04-01

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