Dose-Related Association of \textit{MTHFR} 677T Allele With Risk of Ischemic Stroke

Evidence From a Cumulative Meta-Analysis

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\textbf{Background}—Data are conflicting concerning ischemic stroke risk associated with a common polymorphism in the gene encoding 5,10-methylenetetrahydrofolate reductase (\textit{MTHFR} 677C\textrightarrow{}T), which predisposes to hyperhomocysteinemia in vivo.

\textbf{Methods}—We performed a systematic review and meta-analysis of published relevant literature. We included cohort, case-control, or cross-sectional studies reporting the frequencies of heterozygous (CT) and homozygous (TT) genotypes in (a) all stroke/TIA (overall group) and (b) imaging-proven ischemic stroke (best-phenotyped group).

\textbf{Results}—Among 14,870 subjects, the pooled estimated risk of stroke/TIA associated with the 677T allele increased in a dose-dependent manner (T allele pooled OR 1.17, 95%CI 1.09 to 1.26, TT genotype pooled OR 1.37, 95%CI 1.15 to 1.64). An almost-identical relationship was observed when the analysis was restricted to imaging-proven ischemic stroke (T allele pooled OR 1.18, 95%CI 1.09 to 1.29, TT genotype pooled OR 1.48, 95%CI 1.22 to 1.8).

\textbf{Conclusion}—A graded increase in ischemic stroke risk with increasing \textit{MTHFR} 677T allele dose was observed, suggesting an influence of this polymorphism as a genetic stroke risk factor and supporting other evidence indicating a causal relationship between elevated homocysteine and stroke. \textit{(Stroke. 2005;36:1581-1587.)}

\textbf{Key Words:} cerebrovascular disorders ■ folic acid ■ gene mutation ■ homocysteine

Ischemic stroke is a leading cause of death and disability in the developed world.\textsuperscript{1} Despite recent advances in acute stroke therapy, effective prevention is an important strategy to reduce the overall burden of stroke worldwide. Established causal risk factors such as hypertension and smoking are estimated to account for \textasciitilde{}50\% of vascular disease risk.\textsuperscript{2} Therefore the identification of novel markers of stroke risk is of key importance, both for risk prediction and potential modification to reduce future events.

One such candidate marker is plasma total homocyst(e)ine (tHcy) level. Following early reports of premature vascular events in individuals with homocystinuria, laboratory and clinical studies have indicated that elevated tHcy may promote atherosclerosis and cause endothelial dysfunction.\textsuperscript{3–5} While retrospective epidemiological studies have reported an association between tHcy and stroke, such studies are prone to bias, due to alterations in tHcy induced by stroke itself, or confounding factors such as differences in dietary vitamin status between cases and controls.\textsuperscript{6–7} In contrast, prospective epidemiological studies have not consistently demonstrated an association between elevated tHcy and stroke, and the only completed clinical trial of tHcy-lowering therapy to date did not demonstrate a reduction in stroke events in the intervention arm.\textsuperscript{8–11} Given this uncertainty, independent lines of evidence supporting the relationship between tHcy and stroke are needed.

A common polymorphism (677C\textrightarrow{}T) in the gene encoding the N5,N10-methylenetetrahydrofolate reductase (\textit{MTHFR}) enzyme, which converts dietary folate to its active cofactor in Hcy catabolism, has been studied as a candidate genetic risk factor for stroke.\textsuperscript{12} As T allele dose increases, this functional polymorphism causes a graded elevation in tHcy in the mild-moderate range, most pronounced in individuals with low dietary folate consumption. However, epidemiological studies examining the association between the 677C\textrightarrow{}T variant and stroke have reported conflicting results.\textsuperscript{13} Family studies have indicated a 2-fold increased stroke risk associated with stroke in a first-degree relative, likely mediated both by shared environment and polymorphisms in several genes involved in stroke pathophysiology.\textsuperscript{2} Given an \textasciitilde{}20\% increased risk for each of 5 or 6 polymorphisms, individual studies examining the \textit{MTHFR} 677C\textrightarrow{}T polymorphism are likely to have been underpowered to detect a relationship (Type II error). We performed an updated systematic review and cumulative meta-analysis of available data addressing this question. We aimed to analyze and quantify the stroke...
risk associated with the 677T allele, with a sufficient sample size to address these power limitations.

Methods

Data Acquisition and Abstraction

To identify all publications examining stroke risk associated with the MTHFR 677C>T polymorphism we performed a computerized search of MEDLINE and Science Citation Index (1966–March 2004) using the terms “cerebrovascular accident,” “cerebrovascular disorders,” “homocysteine,” “homocystin,” “amine oxidoreductases,” “MTHFR,” “point mutation,” and “polymorphism.” We also reviewed citations from retrieved articles and previous meta-analyses.

To minimize heterogeneity and facilitate interpretation of our results, the following inclusion criteria were specified: (1) retrospective case-control or cross-sectional study, or prospective cohort study design; (2) exposure defined as stroke risk associated with MTHFR TT and CT genotypes compared with CC genotype expressed as risk ratio or odds ratio (OR) with 95% confidence intervals, or with sufficient data to allow calculation of these effect measures; (3) with the dual aims of maximizing power and the accuracy of phenotype characterization, we specified 2 definitions of disease outcome: 1. Overall group: outcome defined as new focal neurological syndrome of sudden onset consistent with stroke or TIA, with or without neuroimaging confirmation 2. Best-phenotyped group: Potential misclassification bias associated with inclusion of hemorrhagic stroke and non-vascular TIA/stroke mimic syndromes was addressed by definition of a “best-phenotyped” outcome, defined as a clinical syndrome consistent with recent ischemic stroke (TIA excluded), with neuroimaging confirmation

Exclusion criteria were as follows: (1) study design other than case-control or cohort (eg, single case reports, case series, reviews, family studies or cross-sectional studies without controls); (2) studies of stroke exclusively in children or adolescents below 18 years of age; (3) studies of stroke populations not representative of the general population (eg, sickle cell disease, systemic lupus erythematosi, renal dialysis patients); (4) outcome defined as other than clinical stroke or TIA (eg, carotid atherosclerosis or intima-media thickness, silent brain infarction, combined stroke and coronary disease); (5) MTHFR genotype frequencies or CI not provided and/or not calculable

In many studies OR and 95% confidence intervals were not provided directly, but could be calculated from the provided data using standard formula.14 In other studies data were provided in subgroups—in these strata were combined, and pooled values calculated from subgroup specific data. Data were extracted by a single investigator according to the above criteria, and reviewed by a single Stroke Neurologist.

Data Synthesis and Analysis

To examine for heterogeneity between studies, a Breslow-Day test was performed.15 In a meta-analysis, a fixed-effect estimate assumes that a single true measure of effect exists for the population, which is estimated with some imprecision by individual studies. For calculation of a single pooled risk estimate, a precision-weighted average of individual study risk estimates is generated. By contrast, a random-effects estimate assumes that the true measure of effect varies for different populations under study. It represents a precision-weighted average of individual study estimates, with additional adjustment for significant between-study variation.16 When the Breslow-Day test indicated significant (P<0.05) heterogeneity of the samples under study, random-effects models were used, adjusting for both within-study and between-study variability. When the Breslow-Day test did not indicate significant heterogeneity, a fixed effects model was used.

Visual funnel plot inspection and statistical testing (Begg rank correlation and Egger regression asymmetry tests) were performed to examine for publication bias. The funnel graph plots individual study effect measures against their precision, measured by standard error (SE). Publication bias due to exclusion of small studies with negative results leads to asymmetry of the base of the funnel shaped plot. The Begg test attempts to quantify this asymmetry, based on the principle that publication bias will induce a skewed relationship between standardized effect estimates and variance of included studies.17 The Egger test uses regression to evaluate the relationship between standardized effect estimates and precision (defined as 1/SE). With publication bias, smaller studies reporting greater effect sizes will disproportionately skew this relationship, causing the regression line intercept to significantly deviate from the origin.18

Results

Search Results

The computerized search found 787 publications. Of these, 756 did not satisfy the inclusion and exclusion criteria. Nine studies exclusively on childhood stroke cases were excluded. Four studies reported a small number of cases <18 years among a larger adult sample and were included.36,50,52,54 In 5 instances, results from previously-reported cases were included in later analyses—to avoid potential duplication of reporting we included the larger publication from each group.8

Thirty-one studies met inclusion criteria in the overall group (outcome defined as clinical stroke or TIA, with or without neuroimaging confirmation).26–56 Only 2 studies reported crude ORs for ischemic stroke associated with TT and CT genotypes.40,42 Crude ORs were recalculated in the remaining studies because (a) no OR provided in original publication (14 studies)† (b) adjusted but not crude OR provided (5 studies)41,43,45,53,54 (c) risk was calculated only for TT genotype compared with a combined CT+CC group (6 studies)26,30,31,33,43,46 (d) subgroups with imaging-proven intracerebral hemorrhage included so ORs specific for IS calculated (4 studies).37,48,52,56 One study36 tested OR separately for white and black ethnicities—these were included separately, making a total of 32 study samples in the overall analysis (Table).

Pooled OR of Stroke Associated With 677T Allele in Overall Group

For the overall group, 32 studies with 6110 stroke/TIA cases (mean age range 33.3 to 78.8 years;56.9% male) and 8760 controls (mean age range 32.2 to 74.3 years;52.2% male) were included. All studies followed a retrospective case-control design. Nine studies measured tHcy and 5 measured folate levels. All used HinfI restriction digestion with gel electrophoresis for detection of 677C>T. No between-study heterogeneity was detected for the analysis of risk associated with CT genotype in this group (P=0.33). The pooled OR estimate for stroke/TIA associated with CT genotype was 1.17 (95% CI 1.09, 1.26, P<0.001). Significant between-study heterogeneity was detected for the analysis of risk associated with TT genotype in the overall group (Breslow-Day P<0.001). The random-effects pooled OR estimate for stroke/TIA associated with TT genotype was 1.37 (95% CI 1.15, 1.64, P<0.001; Figure 1).

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*References 6, 27, 30, 33, 44, 55, 62–65
†References 27–29, 32, 34–36, 38, 39, 49–51, 55
We examined the influence of individual studies on between-study heterogeneity by sequential exclusion of those with the greatest variation from the majority. 26–28,36,37,44 Following exclusion of the 6 studies with widest variability, statistical testing for between-study heterogeneity remained significant (P = 0.01). However, the pooled stroke OR associated with TT genotype calculated by both fixed and random effects models was equivalent (OR 1.28, 95% CI 1.01–1.61). The influence of study variation on the pooled stroke OR associated with CT and TT genotypes of MTHFR, compared with the CC genotype, in the overall and best-phenotyped groups.
Pooled OR of Stroke Associated With 677T Allele in Best-Phenotyped Group

Twenty-two studies fulfilled criteria for inclusion in the best-phenotyped group (outcome defined as neuroimaging-confirmed ischemic stroke). Reasons for exclusion of the remaining 10 studies from the overall group were (a) TIA included (5 studies)29–33 (b) clinically-defined stroke without neuroimaging (3 studies)26–28 (c) both TIA and absent neuroimaging (2 studies).34,35 These 22 studies included 4740 ischemic stroke cases (mean age range 33.3 to 78.8 years; 55.5% male) and 7486 controls (mean age range 32.2 to 72.9 years; 52.6% male). Six reported tHcy levels and 4 reported folate levels. No heterogeneity was detected for the analysis of risk associated with CT genotype (Breslow-Day $P=0.12$).

The fixed-effect pooled OR estimate for neuroimaging-confirmed ischemic stroke associated with CT genotype was 1.18 (95% CI 1.09, 1.29, $P=0.001$). For the analysis of risk associated with TT genotype, significant between-study heterogeneity was detected (Breslow-Day $P=0.006$). The random-effects pooled OR estimate of neuroimaging-confirmed ischemic stroke associated with TT genotype was 1.48 (95% CI 1.22, 1.8, $P<0.001$).

To examine the effect of individual studies on heterogeneity, we sequentially excluded those with widest variability from the majority of studies in this group. Following exclusion of the 5 studies with widest variability [36–39,44], no residual heterogeneity was detected. Among the remaining 17 studies with proven ischemic stroke, the fixed-effects pooled OR was 1.32 (95% CI, 1.17, 1.49, $P<0.001$).

Analysis for Publication Bias

Among the overall group, funnel plot visual inspection suggested minor asymmetry among smaller, less precise studies (Figure 2) but did not suggest important over-representation of smaller “positive” studies which may have biased the analysis. This was supported by statistical testing (Begg $P=0.12$, Egger $P=0.18$), which failed to detect significant bias.

MTHFR 677TT Risk and Continent of Origin

High nutritional folate intake modifies the effect of the MTHFR T allele (resulting in a smaller increase in tHcy levels) and may influence the risk of stroke associated with the polymorphism. Folate intake is likely to differ across continents, as vitamin supplement intake is greater and cereal grain fortification programs exist in North America, but not most European or Asian countries. TT genotype is also more frequent in Asian compared with white individuals, and least common in those of African ethnic origin.19 As insufficient folate data were available to directly examine the interaction between genotype and folate status, we stratified studies in the overall group by continent as an indirect surrogate of folate status (Figure 3). To maximize statistical power for all subgroup analyses, we further included 5 studies57–61 which calculated stroke ORs for TT homozygotes compared only to [CT+CC] genotypes.

The pooled OR estimate associated with TT genotype was higher in Asia (OR 1.37; 95%CI 1.05, 1.79, $P=0.02$) than in Europe (OR 1.17; 95%CI 1.02, 1.34, $P=0.03$). As only 2 North American studies met inclusion criteria, these provided insufficient power to allow a precise risk estimate although a similar trend was observed (OR 1.56; 95%CI 0.9, 2.75, $P=0.1$).

MTHFR 677TT Risk and Ethnic-Regional Groups

Carrier rates of the 677T polymorphism differ across white, Asian, and black ethnic groups. However, ethnic subgroups may in turn exhibit different carrier rates or environmental susceptibility to disease-specific polymorphisms, which may in part explain ethnic and regional differences in stroke risk.20 To examine this question, we calculated pooled stroke risk estimates among Asian and European studies categorized by 6 regions broadly corresponding to areas of ethnic similarity (China, Japan, South Korea, Northwestern Europe, Central Europe, Italy). Within these regions, no heterogeneity between studies was detected. The pooled risk of stroke associated with TT genotype was significantly elevated among Japanese and Italian studies, but not other regions (Figure 3).

MTHFR 677TT Risk and Stroke Mechanism

It is unclear whether elevated tHcy predisposes to stroke of particular pathophysiological mechanisms. Different studies have reported associations between tHcy and both large- and small-vessel disease, intra-atrial thrombus in atrial fibrilla-
tion, and craniocervical arterial dissection. To investigate for a relationship between MTHFR TT genotype and stroke mechanism, we examined included studies stratified by stroke subtype. Thirteen studies provided data on underlying stroke mechanism,§ and subtype-specific TT genotype frequencies were available in only 5 of these.33,44,45,49,56 Within this small sample, no association was found between TT genotype and large-artery, small-artery, and cardioembolic stroke.

**Discussion**

The main finding of this analysis of almost 15,000 subjects is that the MTHFR 677C—T polymorphism was associated with increased risk of stroke in a graded, dose-dependent fashion. The pooled estimate in TT homozygotes was twice that observed in heterozygotes, suggesting an additive influence of the T allele on stroke risk. The risk estimates were essentially unchanged after exclusion of TIA, or those without brain imaging confirmation. The analysis suggests that it is highly unlikely that the findings may be due to chance (Type 1 error) or bias favoring publication of ‘positive’ studies. These findings support the concept of this gene variant as a susceptibility polymorphism for sporadic ischemic stroke, particularly when viewed in the context of a recent meta-analysis which found that TT genotype was associated with coronary artery disease in 23,920 subjects.21

Although the magnitude of the increased stroke risk for an individual is relatively modest compared with established vascular risk factors, these findings may have important public health implications, as 30% to 50% of most populations are CT heterozygotes and 3% to 15% are TT homozygotes.19 Despite its frequency, further research is required to investigate whether routine screening for the presence of the polymorphism may improve prediction of vascular risk.

As few studies provided information on plasma folate, we stratified our analysis by continent as an indirect surrogate of population folate status. We considered this a reasonable approach because both folate levels and the prevalence of vitamin supplementation intake have been reported to be significantly higher in North America compared with Europe and other continents.22,23 We found that the association between TT genotype and stroke was consistent within both European and Asian studies. A similar trend among North American studies did not reach statistical significance, possibly reflecting a reduced effect of the polymorphism in this more folate-replete population, although it is difficult to make a precise judgment because of small sample size.

Racial-ethnic differences in distribution of the C677—T polymorphism are well-described.19,20,25 To further refine racial-ethnic heterogeneity, we explored for hidden population stratification within continents by categorizing studies according to regions with broadly-similar historical population migration characteristics. While this approach limited power for some analyses, it revealed significant associations between TT genotype and stroke among individuals from Italy and Japan, when compared with other regions. Further study is required to investigate whether this finding is due to population differences in T allele frequency, folate status, or other genetic or environmental influences.

Our findings confirm and extend those of a recent analysis which employed a Mendelian randomization approach to investigate the consistency between the observed and expected stroke risk associated with TT genotype calculated from prior data on its effect on tHcy.23 In contrast, our analysis demonstrates the additive influence of T allele dose, places greater emphasis on the accuracy of ischemic stroke phenotyping, and examines the consistency of the association across ethnic groups.

As with all meta-analyses, our analysis has limitations that must be considered when interpreting the findings. First, we reported crude ORs to avoid potential inaccuracies resulting from combining adjusted ORs from studies that had adjusted for different variables. To validly examine the influence of confounding on the relationship between 677T allele and stroke risk, we recommend that future analyses should be performed using large pooled data sets of individual patient data. Second, as no prospective studies have addressed our question, all included studies followed a retrospective case-control design. Thus, while unlikely, we cannot exclude the possibility of undetected bias (eg, survival bias). Finally, insufficient data were available for direct analysis of the influence of TT genotype on either stroke subtype or on stroke risk for different categories of folate status.

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§References 27, 30, 33, 41, 44, 45, 46, 49, 51, 54, 56, 57, 60
Our findings provide evidence in support of a causal role for tHcy in the etiology of ischemic stroke due to atherosclerosis and other mechanisms, as it is plausible that the 677C→T variant exerts its influence other than by impaired tHcy metabolism. In the absence to date of definitive benefits of tHcy-lowering therapy for prevention of vascular events, evidence supporting the “homocysteine hypothesis” has mainly rested on observational data from experimental studies, clinical studies of patients with homocystinuria, and epidemiological studies demonstrating higher tHcy among individuals with vascular disease. While conclusive clinical trial data are required, studies indicating an association between the 677C→T polymorphism and vascular outcomes further suggest a role of tHcy in the pathogenesis of vascular disease.

Our findings also support the contention that population-wide folic acid grain fortification programs, already introduced in the United States and Canada for the prevention of neural tube defects, may also provide benefit in preventing stroke. Dietary folic acid intake inhibits the influence of the MTHFR 677C→T substitution on plasma tHcy, by facilitating tHcy remethylation to methionine. The Framingham investigators and others have demonstrated reductions in plasma tHcy following population folic acid grain fortification programs. The greatest reductions were observed in individuals with highest pretreatment tHcy, in whom tHcy has been reported to confer greatest risk of vascular disease. A recent meta-analysis of over 15,000 individuals without vascular disease reported a mean tHcy difference between CC and TT homozygotes of 1.93 μmol/L, with a significant tHcy reduction of 0.05 μmol/L for each 1 nmol/L increase in serum folate. It is possible that folate supplementation may have most benefit among T allele homozygotes or heterozygotes, or in populations where the prevalence of the TT genotype is high. Analysis of genetic subgroups within large clinical trials of tHcy-lowering therapy will be required to address this hypothesis.

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