Methylenetetrahydrofolate Reductase Polymorphisms and Homocysteine-Lowering Effect of Vitamin Therapy in Singaporean Stroke Patients

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Background and Purpose—Increased plasma total homocysteine (tHcy) levels are a risk factor for stroke and can be reduced with vitamin therapy. However, data on the tHcy-lowering effects of vitamins are limited largely to white populations. Thus, we aimed to determine in Singaporean patients with recent stroke: (1) the efficacy of vitamin therapy (folic acid, vitamin B12, and B6) on lowering tHcy, and (2) whether efficacy is modified by Methylenetetrahydrofolate reductase (MTHFR) gene polymorphism(s).

Methods—A total of 443 eligible patients were recruited after presenting with ischemic stroke within the past 7 months. Patients were randomized to receive either placebo or vitamins. Fasting blood samples collected at baseline and at 1 year were assayed for levels of plasma tHcy. Patients were genotyped for MTHFR C677T and A1298C polymorphisms.

Results—Mean baseline tHcy was similar in the 2 groups (placebo 13.7 μmol/L; vitamins 14.0 μmol/L; P=0.70). At 1 year, mean tHcy was 14.5 μmol/L in the placebo group compared with 10.7 μmol/L in the vitamin group (difference 3.8 μmol/L; 95% CI, 2.8 to 4.8 μmol/L; P<0.0001). MTHFR C677T genotype was an independent determinant of tHcy levels at baseline (P=0.005), but A1298C was not (P=0.08). Neither polymorphism significantly influenced the effect of vitamin therapy on tHcy at 1 year. The magnitude of the reduction in tHcy levels at 1 year with vitamin therapy was similar, irrespective of MTHFR genotypes.

Conclusions—Vitamin therapy reduces mean tHcy levels by 3.8 μmol/L in the Singaporean stroke population studied. MTHFR C677T but not A1298C is independently associated with tHcy levels at baseline, and neither impacts the tHcy-lowering effect of vitamins used in this study. (Stroke. 2006;37:456-460.)

Key Words: methylenetetrahydrofolate reductase ■ polymorphism ■ homocysteine ■ stroke
However, randomized trials of tHcy-lowering with vitamins have demonstrated conflicting results.6 The largest study to date is the Vitamin Intervention for Stroke Prevention (VISP) trial, a multicenter, double-blind, randomized controlled trial conducted in North America, that achieved only a 2.0-μmol/L reduction in tHcy in stroke patients of primarily white origin.6,8 Preliminary findings of white stroke patients enrolled into the VITATOPS trial in Perth, Australia, found a mean 3.7-μmol/L (95% CI, 2.7 to 4.7 μmol/L; P<0.001) reduction in tHcy after 6 months of vitamin therapy.10 The effect of vitamin therapy on tHcy lowering has not been extensively explored in Asian populations but is critically important because vitamin intake and response to vitamin therapy may vary across different ethnic groups and cultures. Furthermore, the prevalence of MTHFR polymorphism(s) and how these genetic variants may modify the effect of vitamins on tHcy lowering has not been examined in Asian stroke patients.

Therefore, the aims of this study were to determine: (1) the efficacy of the vitamin supplementation for 1 year on lowering tHcy levels, and (2) whether the efficacy of vitamin therapy in lowering tHcy is modified by MTHFR polymorphism(s) in Asian residents of Singapore with a recent ischemic stroke or transient ischemic attack (TIA).

Subjects and Methods

Study Population
This substudy of the VITATOPS trial was performed in Singapore General Hospital (SGH), Singapore. The study was designed and performed in observance of the local institutional guidelines. Written, informed consent was provided by all study participants. Between July 2000 and April 2004, we enrolled 443 consecutive consenting patients admitted to SGH after presenting with an ischemic stroke or TIA within 7 months of onset. All patients were investigated on demographic and vascular risk factor information and a thorough series of investigations for causes of stroke. Brain computerized tomography or MRI was used to confirm the clinical diagnosis of ischemic stroke. Patients not eligible for inclusion included those taking folic acid, vitamin B12, and B6, taking methotrexate for any reason, pregnant women, or women of child-bearing potential who were at risk of pregnancy, and patients with a limited life expectancy (<6 months). Eligible patients had venous blood specimens collected after an overnight fast to measure fasting plasma tHcy and for acquisition of genomic DNA.

Intervention
Patients were assigned randomly through the VITATOPS study website to receive a combination of 2.5 mg folic acid, 0.5 mg vitamin B12, and 25 mg B6, or the placebo, as a single tablet, once daily, using a vitamin therapy.10 The effect of vitamin therapy on tHcy lowering has not been extensively explored in Asian populations but is critically important because vitamin intake and response to vitamin therapy may vary across different ethnic groups and cultures. Furthermore, the prevalence of MTHFR polymorphism(s) and how these genetic variants may modify the effect of vitamins on tHcy lowering has not been examined in Asian stroke patients.

Follow-Up
Follow-up appointments were scheduled 1 year after randomization. At follow-up, data were collected on compliance with study treatment and nonstudy vitamins, and a repeat venous blood specimen was collected after an overnight fast for the measurement of tHcy.

Blood Collection and Laboratory Techniques
Twenty-milliliter fasting venous blood samples were collected into EDTA anticoagulant before randomization and at 1 year. Samples were immediately placed on ice and after transport to the laboratory, were centrifuged at 4000 rpm for 10 minutes. Plasma and cells were separated and stored at −80°C until analysis. Plasma tHcy concentration was determined by fluorescent polarization immunoassay method (Abbott Laboratories).

DNA was isolated from blood cells using commercially available QIAsamp DNA Blood Mini kit (Qiagen GmbH) according to the manufacturer protocol. The regions containing the 2 MTHFR polymorphisms were amplified separately according to established methods using standard polymerase chain reaction–restriction fragment length polymorphism with HindIII and MboII restriction enzymes (New England BioLabs) to determine the C677T11 and A1298Cgenotypes, respectively. Quality control for the DNA analyses was maintained by the use of both positive and negative controls in each set of analyzed samples, assayed in duplicate, and results were confirmed independently by 2 laboratory personnel.

Statistical Analysis
For the purposes of the present analyses, only a third-party statistician (Q.Y.) not involved in any aspects of the design or day-to-day running of the VITATOPS trial was unblinded to treatment allocation.

Means and proportions were calculated for baseline demographics and the prevalence of vascular risk factors in the placebo and vitamin treatment groups. The significance of any differences between the 2 groups was examined with Student’s t test for means or χ2 test for proportions.

The primary analysis was a comparison of mean tHcy concentrations at 1 year between the placebo and vitamin treatment groups based on the intention-to-treat principle. The significance of difference in tHcy concentrations between the 2 treatment groups was examined using Student t test. The association between MTHFR polymorphisms and tHcy at baseline and 1 year after randomization were examined using separate multivariable regression models with and without adjustment for age, gender, baseline tHcy levels, randomization time, and conventional vascular risk factors (hypertension, diabetes, hypercholesterolemia, smoking, and history of previous vascular events).

Statistical analysis was performed using the SAS software version 8.2 (SAS Institute). Significance was determined by a 2-sided P value <0.05.

Results
A total of 443 patients were recruited into the Singapore VITATOPS substudy. A total of 221 patients were randomly assigned to the placebo and 222 to receive the vitamins. A total of 107 patients (54 from the placebo group and 53 from the vitamin group) did not undergo repeat blood collection at 1 year, leaving 167 patients randomized to the placebo and 169 patients randomized to the vitamin therapy for inclusion in our analysis.

Table 1 shows the distribution of baseline characteristics among ischemic stroke patients according to treatment allocation. The ethnic distribution of the study subjects appeared to be broadly representative of the Singaporean population with ≈86% Chinese, 7% Malays, and 7% Indians, and the risk factor profile was typical of ischemic stroke patients in Singapore.

Patients were randomized into the study within an average of 83 days after index ischemic stroke event. The baseline clinical and laboratory characteristics of patients who did not undergo repeat blood collection at 1 year did not differ significantly from the remaining study population (data not shown).

Mean baseline plasma tHcy levels were not statistically different in the 2 treatment groups with tHcy of 13.7 μmol/L (SD 4.4) in patients randomized to the placebo and 14.0 μmol/L (SD 5.2) in patients randomized to the vitamins (P=0.70; Table 2). At 1 year, mean tHcy levels were 14.5 μmol/L (SD
Table 1 demonstrates a significant association between the *MTHFR* C677T polymorphism and mean baseline tHcy levels. The 677TT genotype was associated with the highest mean tHcy (16.7 μmol/L [SD 7.8]), compared with 677CT (14.1 μmol/L [SD 4.9]) and 677CC (13.4 μmol/L [SD 4.2]; P=0.005). The *MTHFR* A1298C was not significantly associated with mean baseline tHcy (P=0.08). At 1 year, the interaction between vitamin treatment efficacy and the *MTHFR* genotypes on plasma tHcy difference (1 year from baseline) was not significant with (C677T P for interaction=0.93; A1298C P for interaction=0.32) and without (data not shown) adjustment for age, gender, baseline tHcy levels, randomization time, and conventional vascular risk factors (hypertension, diabetes, hypercholesterolemia, smoking, and history of previous vascular events).

### Discussion

The principal findings of this substudy of the VITATOPS trial are: (1) vitamin therapy compared with the placebo significantly reduces mean tHcy concentrations at 1 year by 3.8 μmol/L (P<0.0001) in the United States and Canada, which coincided with the conduct of the trail. The 3.8-μmol/L mean reduction in the tHcy levels at 1 year in our study population approximates the 4.0-μmol/L reduction projected in the VITATOPS trial and is comparable to the 2.0-μmol/L reduction observed in the VISP trial, which randomized 3680 stroke survivors to receive high-dose (folic acid 2.5 mg; vitamin B12 0.4 mg; B6 25 mg) or low-dose (folic acid 20 μg; vitamin B12 6.0 μg; B6 200 μg) vitamins. The smaller-than-expected treatment effect of high-dose vitamins seen in VISP is most likely attributable to the implementation on January 1, 1998, of fortification of cereal grain flour products with folic acid (0.4 to 1.4 mg/lb) in the United States and Canada.
A systematic review of individual patient data from 30 prospective and retrospective studies involving a total of 5073 coronary events and 1113 stroke events indicated that after adjusting for confounding factors caused by known vascular risk factors and correction for regression dilution caused by random variation in tHcy measurements, a 25% lower usual tHcy concentration in the blood (~3 μmol/L) was associated with an 11% (OR, 0.89; 95% CI, 0.83 to 0.96) lower risk of a coronary event, and a 19% (OR, 0.81; 95% CI, 0.77 to 0.85) lower risk of stroke by 24% (95% CI, 15% to 33%) and ischemic heart disease by 16% (95% CI, 11% to 20%).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Placebo (n=167)</th>
<th>Vitamins (n=169)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD) Mean Difference</td>
<td>Mean (SD) Mean Difference</td>
</tr>
<tr>
<td>CC (n=201)</td>
<td>13.4 (4.2) +0.7</td>
<td>10.2 (2.9) -3.2</td>
</tr>
<tr>
<td>CT (n=112)</td>
<td>14.1 (4.9) +1.0</td>
<td>11.4 (5.5) -2.7</td>
</tr>
<tr>
<td>TT (n=23)</td>
<td>16.7 (7.8) -1.7</td>
<td>12.0 (3.4) -4.7</td>
</tr>
</tbody>
</table>

Another independent systematic review of 20 prospective studies (involving 3820 participants) of tHcy and disease risk concluded that lowering tHcy by 3 μmol/L from current levels would reduce the risk of stroke by 24% (95% CI, 15% to 33%) and ischemic heart disease by 16% (95% CI, 11% to 20%). Data obtained from 3680 study subjects in the VISP study also found a strong and graded association between baseline tHcy levels and outcomes. A 3 μmol/L lower tHcy level was associated with a 10% lower risk of stroke (P=0.05), a 26% lower risk of coronary events (P<0.001), and a 16% lower risk of death (P=0.001) in the low-dose vitamin group and a nonsignificantly lower risk in the high-dose group by 2% for stroke, 7% for coronary events, and 7% for death.

Based on these data, if elevated tHcy is indeed causally related to cardiovascular diseases, the 3.8-μmol/L reduction in plasma tHcy levels achieved with the VITATOPS trial vitamin therapy in our study population should reduce the risk of subsequent vascular events by ≥15%. This is important because the planned study is powered to detect with a type I error of 5% and type II error of 20%, assuming an average follow-up of 2 years.

In agreement with other reports, we found an association between the MTHFR C677T polymorphism and baseline tHcy levels (P=0.005). However, unlike previous studies, we did not find an association between the A1298C genotype and baseline tHcy levels, nor did we demonstrate a significant association between the C677T or A1298C genotypes and the effect of the vitamins on tHcy lowering. This contrasts with previous studies conducted primarily in white populations that demonstrated a larger reduction in tHcy levels in subjects with the 677TT genotype compared with other C677T genotypes. This may be explained by ethnic differences, but our study may also have lacked power to demonstrate a significant effect of genotype on treatment effect. No studies to date have examined the association between the A1298C genotype and response to the vitamin therapy.

The strengths of our study are that it was randomized, double blinded, and placebo controlled, thus minimizing systematic bias and error in treatment allocation and outcome evaluation. Plasma tHcy measurements were undertaken after the patient had been fasting overnight and were measured by a single technique in a single laboratory. The statistical analysis included a multiple regression analysis of factors independently associated with plasma tHcy concentrations, and we adjusted for these factors when examining the influence of vitamin treatment and the MTHFR genotype on tHcy.

The limitations of our study are that our findings may only be applicable to ischemic stroke and TIA patients in Singapore and not to other individuals in other parts of the world where nutritional status and supplementation may differ. Although folic acid supplementation is not mandatory in Singapore, it is available over the counter. During the course of the blinded trial follow-up, very few patients have admitted to consuming nontrial vitamin preparations. Moreover, there was no significant difference in data on the baseline tHcy levels in the entire study population according to time of randomization by quartiles (data not shown), thus there is no evidence in the sample evaluated that folic acid supplementation is increasing, suggesting that a similar scenario is likely to occur in our population. The study population is made up of 3 major ethnic groups, mainly the Chinese (76%), Malays (14%), and Indians (7%), with other minorities making up the rest of the 3%. We are interested to determine whether any differences in the tHcy levels, genotypes, and response to treatment exist between the 3 ethnic groups because the dietary intake and genetic makeup may differ. However, our study, because of the limited patients available for the Malays and Indians, is underpowered to determine this association. The study also necessarily relied on patients surviving 1 year...
after randomization and returning for follow-up, but survival bias or loss to follow-up is unlikely to influence the results because the number of patients who died during the treatment period or failed to return for review was modest, and their baseline characteristics were similar to those who returned for review.

In conclusion, our results indicate that in Singaporean ischemic stroke patients, the vitamin therapy (folic acid, vitamin B12, and B6) effectively reduces mean tHcy levels by 3.8 μmol/L after 1 year of treatment. The efficacy of treatment was not modified by the MTHFR genotype, although the C677T genotype is an independent determinant of tHcy at baseline.

Acknowledgments
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References
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