Homocysteine, small-vessel disease, and atherosclerosis

An MRI study of 825 stroke patients

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ABSTRACT

Objective: We evaluated the relationship between hyperhomocysteinemia and methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism and small-vessel disease (SVD) and atherosclerotic large-vessel disease (LVD) in stroke patients.

Methods: A total of 825 noncardioembolic ischemic stroke patients whose plasma concentrations of total homocysteine were measured and whose MTHFR C677T polymorphism status was identified were included in this retrospective study. MRI of the brain and magnetic resonance angiography of the intracranial and extracranial cerebral arteries had been performed. SVD and LVD were assessed by the Scheltens scale (the SVD score) and by the number of atherosclerotic steno-occlusive arteries (the LVD score), respectively.

Results: The TT genotype of the MTHFR C677T polymorphism was associated with hyperhomocysteinemia ($p < 0.001$), but not with SVD ($p = 0.182$) or LVD ($p = 0.988$) scores. Multiple logistic regression analysis showed that hyperhomocysteinemia was associated with SVD (odds ratio [OR] 1.04; 95% confidence interval [CI] 1.01–1.07; $p = 0.005$) and LVD (OR 1.02; 95% CI 1.00–1.05; $p = 0.041$) scores. Hyperhomocysteinemia was related to the LVD score of extracranial arteries ($p = 0.008$), but not to the LVD score of intracranial arteries ($p = 0.730$). In multiple logistic regression analysis, however, hyperhomocysteinemia was not related to the LVD score of extracranial arteries ($p = 0.255$).

Conclusions: Hyperhomocysteinemia was associated with SVD of the brain and LVD of cerebral arteries. The MTHFR C677T polymorphism was not related to SVD and LVD, although the TT genotype was an important determinant of hyperhomocysteinemia. *Neurology*® 2014;83:695–701

GLOSSARY

CI = confidence interval; **IQR** = interquartile range; **LVD** = large-vessel disease; **MRA** = magnetic resonance angiography; **MTHFR** = methylenetetrahydrofolate reductase; **OR** = odds ratio; **SVD** = small-vessel disease; **tHcy** = total homocysteine.

High plasma concentrations of total homocysteine (tHcy), also known as hyperhomocysteinemia, are associated with ischemic stroke risk.[1] Numerous studies have evaluated the mechanisms through which homocysteine is associated with stroke. In earlier studies, atherosclerosis was proposed to be the main mechanism of stroke.[2,3] Subsequently, however, hyperhomocysteinemia was more closely linked to occlusions of small arteries than to atherosclerosis of large arteries.[4,5] Other studies showed that hyperhomocysteinemia is not associated with any specific levels of cerebral arteries.[6] Limitations such as few participants and insufficient evaluations of both small and large arteries may have contributed to these inconsistencies.

Genetic susceptibility can affect homocysteine metabolism. Homocysteine is derived from the essential amino acid methionine. Homocysteine metabolism depends on methylenetetrahydrofolate reductase (MTHFR), an enzyme involved in the remethylation of homocysteine to methionine. Therefore, genetic polymorphisms of this enzyme contribute to the development of hyperhomocysteinemia.[7] The homozygous form of MTHFR C677T reduces MTHFR activity and increases tHcy plasma concentrations.[8] However, it is unclear whether this polymorphism is directly associated with cerebral vasculopathy, and no studies have addressed the

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differential influences of the MTHFR C677T polymorphism on the burden and location of small-vessel disease (SVD) and large-vessel disease (LVD).

We sought to investigate the role of homocysteine and the MTHFR C677T polymorphism in cerebral vasculopathy according to the size and location of the involved arteries in a large number of stroke patients who underwent MRI of the brain and magnetic resonance angiography (MRA) of intracranial and extracranial cerebral arteries.

METHODS

Patients. This retrospective study analyzed hospitalized patients enrolled in our stroke registry. Patients were included in the study when (1) the final diagnosis was ischemic stroke or TIA, (2) MRI of the brain and MRA of both intracranial and extracranial cerebral arteries were performed, and (3) the plasma concentrations of tHcy and the MTHFR C677T polymorphism status were measured between November 2002 and February 2005. Patients with cardioembolic stroke were not considered in our analyses because the purpose of our study was to investigate the relationship between homocysteine and the MTHFR polymorphism and the impairment of cerebral arteries.

Standard protocol approvals, registrations, and patient consents. This study was approved by the institutional review board of the Asan Medical Center. Because of the retrospective and observational nature of this study, the need for written informed consent was waived.

Homocysteine and the MTHFR C677T polymorphism. The methods used to measure tHcy and the MTHFR polymorphism have been previously described.[9] Briefly, after an overnight fast, samples of venous blood were collected in EDTA-containing tubes, and plasma was promptly separated by centrifugation at 1,800 rpm for 5 minutes. tHcy was analyzed within 1 hour of blood collection using a specific immunoassay (ADVIA Centaur; Bayer Corporation Diagnostics Division, Leverkusen, Germany). The average total coefficient of variation for tHcy was 7.6% during the study period. Leukocytes were isolated with erythrocyte lysis solution and genomic DNA was extracted using Puregene DNA isolation kits (Gentra Systems, Inc., Minneapolis, MN). The MTHFR C677T polymorphism was amplified by real-time PCR with melting point analysis (LightCycler System; Roche Diagnostics, Mannheim, Germany).

Imaging analysis of SVD and LVD. The 1.5T MRI protocol for this study included fluid-attenuated inversion recovery imaging or T2-weighted imaging, diffusion-weighted imaging, 3D time-of-flight MRA covering the intracranial arteries, and 3D contrast-enhanced MRA covering both the intracranial and extracranial arteries. The protocol has been detailed previously.[10]

The Scheltens system was used to score the extent of SVD on fluid-attenuated inversion recovery imaging or T2-weighted imaging (the SVD score) because this scale reflects the volume of leukoaraiosis and lacunar infarcts and covers supratentorial and infratentorial lesions.[10,11] Atherosclerotic lesions on MRA were visually graded: 0, stenosis less than 50%; 1, stenosis of 50%–99%; and 2, occlusion.[12] Regarding atherosclerotic LVD, assessment of the location included bilateral intracranial arteries (middle cerebral, anterior cerebral, posterior cerebral, and basilar arteries and intracranial portions of internal carotid and vertebral arteries) and extracranial arteries (extracranial portions of vertebral and internal carotid arteries, common carotid artery, and innominate and subclavian arteries). A steno-occlusion in an intracranial artery was considered significant when findings of both intracranial 3D time-of-flight and contrast-enhanced MRAs were consistent. Hypoplasia of a vertebral artery was not considered to be steno-occlusive. The sum of the involved cerebral arteries was defined as the LVD score.[12] MRI and MRA were interpreted jointly by 2 investigators who were blinded to clinical data and laboratory tests.

Data analysis. For univariate analysis, the Pearson χ^2 test, Student t test, 1-way analysis of variance, Mann-Whitney U test, Kruskal-Wallis test, and Jonckheere-Terpstra test were used, as appropriate. For multivariate logistic regression analysis, the SVD and LVD scores were dichotomized by using the uppermost quartile. Variables with a p value <0.1 by univariate analysis were selected for entry into multiple logistic regression analysis. Statistical significance was defined as a 2-tailed p value of <0.05. Post hoc comparisons of the Kruskal-Wallis test were performed using multiple Mann-Whitney U tests, and significance was adjusted as a 2-tailed p value less than 0.05 divided by the number of comparisons. All statistical analyses were performed with SPSS version 21 (IBM, Armonk, NY).

RESULTS

During the study period, 1,898 patients were admitted to our stroke center. Of these patients, 295 with cardioembolic stroke and 264 with hemorrhagic stroke were not considered for this study. Of 1,339 remaining patients, 383 without data on tHcy and the MTHFR polymorphism and 131 without MRI or MRA results were excluded. Thus, 825 patients were included in the final analysis. Baseline characteristics were not different between included and excluded patients (table e-1 on the *Neurology*® Web site at Neurology.org). Of the 825 finally included patients, 508 (61.6%) were men. The median patient age was 64 years (mean 63.8 ± 12.0 years). The median plasma concentration of tHcy was 12.8 μmol/L (interquartile range [IQR] 10.7–15.7 μmol/L) (table 1).

Polymorphisms. The MTHFR C677T polymorphism status was associated with a higher plasma concentration of tHcy: a median (IQR) tHcy plasma concentration for the CC genotype was 12.2 μmol/L (10.2–14.6 μmol/L); the CT genotype, 12.7 μmol/L (10.7–15.9 μmol/L); and the TT genotype, 14.5 μmol/L (11.3–17.4 μmol/L) ($p < 0.001$). However, this polymorphism status was not directly related to SVD ($p = 0.182$) or LVD ($p = 0.988$) scores. When stratified by the location of involved arteries, the MTHFR C677T polymorphism was not associated with LVD in intracranial ($p = 0.211$) or extracranial ($p = 0.364$) arteries.

Homocysteine and SVD. SVD was significantly associated with older age, male sex, hypertension, diabetes mellitus, smoking, and higher plasma concentrations of tHcy (table 2). Binary logistic regression analysis indicated that tHcy was related to SVD

Table 1 Patient characteristics

Variables	n = 825
Age, y	64.0 (56.0–72.0)
Sex, male	508 (61.6)
Hypertension	623 (75.5)
Diabetes mellitus	275 (33.3)
Hypercholesterolemia	241 (29.2)
Smoking	267 (32.4)
Alcohol consumption	24 (2.9)
NIH Stroke Scale score on admission	4.0 (2.0–7.0)
Days from symptom onset to admission	1.0 (0–2.0)
tHcy concentration, μmol/L	12.8 (10.7–15.7)
MTHFR C677T polymorphism	
CC genotype	271 (32.8)
CT genotype	390 (47.3)
TT genotype	164 (19.9)
SVD score	10.0 (4.0–17.5)
LVD score	2.0 (0–4.0)
LVD score, intracranial artery	2.0 (0–3.0)
LVD score, extracranial artery	0 (0–2.0)

Abbreviations: LVD = large-vessel disease; MTHFR = methylenetetrahydrofolate reductase; SVD = small-vessel disease; tHcy = total homocysteine.
Data are given as a number (column %) or median value (interquartile range).

(odds ratio [OR] 1.04; 95% confidence interval [CI] 1.01–1.06; $p = 0.002$). We also observed a positive correlation between the quartiles of tHcy and the SVD score (p for trend = 0.001) (figure 1A). We further compared our stroke patients with 56 stroke-free, age-matched (median 64.0 years), and sex-matched controls (male, 61%) recruited from a community welfare center. The median plasma concentration of tHcy in the controls was 11.1 μmol/L (IQR 8.6–15.6 μmol/L), which was lower than that of stroke patients ($p = 0.014$). We found that patients in the third ($p = 0.002$) and fourth ($p = 0.001$) quartiles of the SVD score had higher plasma concentrations of tHcy than controls (figure e-1A). After adjustment for age, sex, hypertension, diabetes mellitus, smoking, and alcohol consumption, tHcy was associated with the SVD score (adjusted OR 1.04; 95% CI 1.01–1.07; $p = 0.005$) (table 3).

Homocysteine and LVD. LVD was significantly associated with older age, hypertension, diabetes mellitus, and higher plasma concentrations of tHcy (table 2). Binary logistic regression analysis indicated that tHcy was related to LVD (OR 1.03; 95% CI 1.00–1.05; $p = 0.031$). We observed a positive correlation between the quartiles of tHcy and the LVD score (p for trend = 0.031) (figure 1B). We also found that patients in the second ($p = 0.010$), third ($p = 0.017$), and fourth ($p = 0.003$) quartiles of the LVD score had higher plasma concentrations of tHcy than age- and sex-matched normal controls (figure e-1B). Further analysis showed that tHcy plasma concentration was related to the LVD score of extracranial arteries (OR 1.03; 95% CI 1.01–1.06; $p = 0.008$), but not to the LVD score of intracranial arteries (OR 1.00; 95% CI 0.98–1.03; $p = 0.730$) (table e-2; figure 1, C and D). Patients with LVD in the intracranial ($p = 0.020$), extracranial ($p = 0.005$), and both intracranial and extracranial ($p = 0.003$) arteries had higher plasma concentrations of tHcy than controls (figure e-1C). After adjustment for age, hypertension, diabetes mellitus, and NIH Stroke Scale on admission, tHcy plasma concentration was associated with the LVD score (adjusted OR 1.02; 95% CI 1.00–1.05; $p = 0.041$) (table 3). However, regarding arterial location, multiple logistic regression analysis did not show that tHcy was related to the LVD score of extracranial arteries ($p = 0.255$) (table e-3).

DISCUSSION In the current study, we analyzed the relationships among hyperhomocysteinemia, the MTHFR C677T polymorphism, SVD of the brain, and LVD of cerebral arteries in 825 patients with noncardioembolic ischemic stroke. Patients with hyperhomocysteinemia had a higher risk of SVD than those without hyperhomocysteinemia. Hyperhomocysteinemia was also significantly related to LVD, but not to its location. The TT genotype of the MTHFR C677T polymorphism was significantly associated with hyperhomocysteinemia, but this genotype was not directly related to the SVD or LVD scores.

Small cerebral arteries are susceptible to homocysteine-induced endothelial dysfunction,[13] and clinical studies have shown an association between hyperhomocysteinemia and SVD.[4,14–16] A major mechanism of hyperhomocysteinemia-induced vasculopathy is endothelial dysfunction, which is related to a decreased bioavailability of endothelium-derived nitric oxide and subsequently inhibited vasodilation and increased thrombosis.[17] Hyperhomocysteinemia also promotes arteriolar hypertrophy and alteration of vascular mechanics, which may result in occlusions of small arteries.[18] Most of the previous studies that showed associations between hyperhomocysteinemia and SVD investigated the presence of SVD rather than the extent of SVD. In this study, we found that higher plasma concentrations of tHcy were associated with higher burdens of SVD.

The mechanism of homocysteine-induced atherosclerosis is poorly understood but complex pathways of inflammation have been suggested.[19] Abnormalities in the coagulation system involving increases in clotting

Table 2 Factors associated with SVD and LVD

	SVD[a]			LVD[b]		
	No (n = 619)	Yes (n = 206)	p	No (n = 524)	Yes (n = 301)	p
Age, y	63.0 (54–69)	70.0 (63–77)	<0.001	64.0 (54.0–72.0)	65.0 (59.5–73.0)	<0.001
Sex, male	395 (63.8)	113 (54.9)	0.022	323 (61.6)	185 (61.5)	0.959
Hypertension	444 (71.7)	179 (86.9)	<0.001	373 (71.2)	250 (83.1)	<0.001
Diabetes mellitus	220 (35.5)	55 (26.7)	0.020	146 (27.9)	129 (42.9)	<0.001
Hypercholesterolemia	177 (28.6)	64 (31.1)	0.499	155 (29.6)	86 (28.6)	0.759
Smoking	213 (34.4)	54 (26.2)	0.030	174 (33.2)	93 (30.9)	0.495
Alcohol consumption	22 (3.6)	2 (1.0)	0.075	18 (3.4)	6 (2.0)	0.241
NIH Stroke Scale score on admission	4.0 (2.0–7.0)	4.0 (2.0–7.0)	0.641	4.0 (2.0–7.0)	4.0 (2.0–7.0)	0.060
Days from symptom onset to admission	1.0 (0–2.0)	1.0 (0–2.0)	0.122	1.0 (0–2.0)	1.0 (0–2.0)	0.266
Creatinine, mg/dL	0.9 (0.7–1.0)	0.8 (0.7–1.0)	0.651	0.9 (0.7–1.0)	0.8 (0.7–1.0)	0.318
tHcy concentration, μmol/L	12.7 (10.5–15.4)	13.2 (11.1–16.7)	0.002	12.6 (10.5–15.5)	13.3 (10.9–16.1)	0.031
MTHFR C677T polymorphism			0.182			0.988
CC genotype	202 (32.6)	69 (33.5)		172 (32.8)	99 (32.9)	
CT genotype	285 (46.0)	105 (51.0)		247 (47.1)	143 (47.5)	
TT genotype	132 (21.3)	32 (15.5)		105 (20.0)	59 (19.6)	

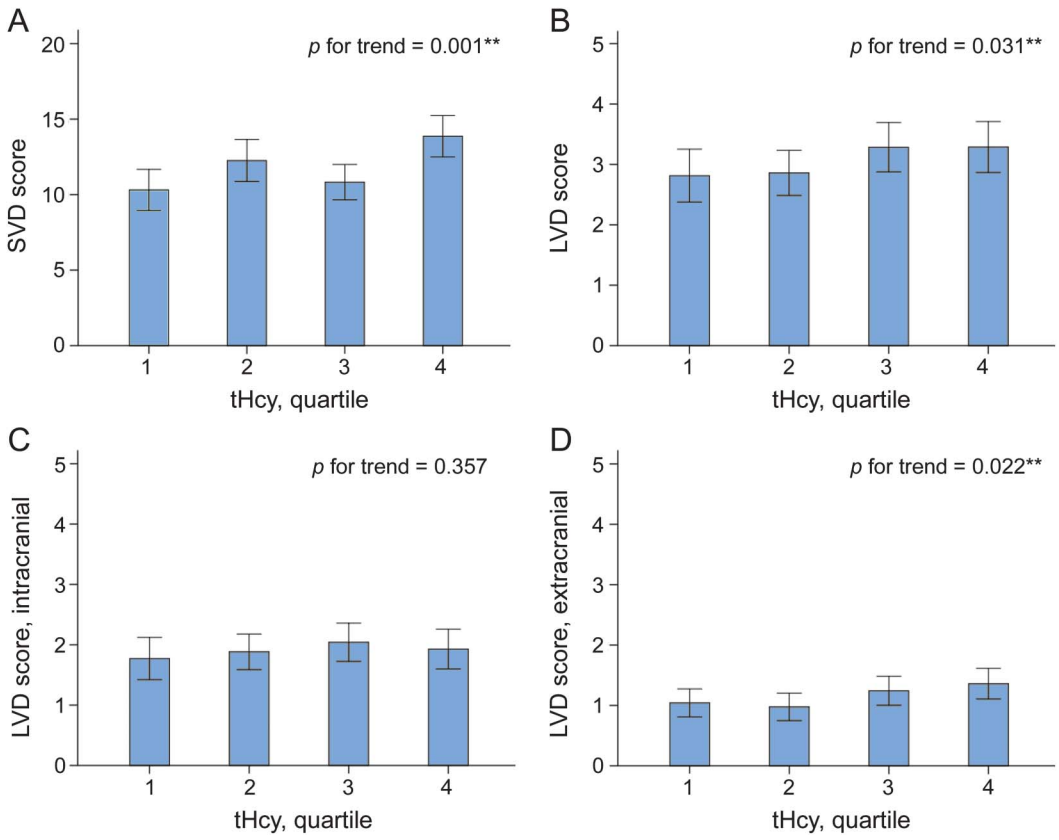
Abbreviations: LVD = large-vessel disease; MTHFR = methylenetetrahydrofolate reductase; SVD = small-vessel disease; tHcy = total homocysteine.
Data are given as a number (column %) or median value (interquartile range).
[a] The uppermost quartiles of the SVD scores (≥18).
[b] The uppermost quartiles of the LVD scores (≥4).

factors, tissue factor expression, and platelet aggregation and dysfunction of fibrinogen and thrombin have also been proposed.[20] Several investigators have suggested an association between hyperhomocysteinemia and atherosclerosis.[3,21,22] However, most of these studies assessed the extent of atherosclerosis by measuring plaque area or intima-media thickness in carotid arteries with duplex sonography. Estimating atherosclerosis in the extracranial portions of the carotid arteries is insufficient for assessing atherosclerosis because atherosclerosis can develop anywhere throughout the cerebral arteries. MRA can estimate atherosclerosis in both intracranial and extracranial arteries. However, few studies have used MRA for assessing the association between hyperhomocysteinemia and atherosclerosis of cerebral arteries. A recent MRA study found no association between hyperhomocysteinemia and LVD in both extracranial and intracranial arteries.[23] However, the authors did not evaluate the burden of LVD, just the presence of LVD. A small retrospective study found that hyperhomocysteinemia was related to moderate to severe atherosclerosis,[24] but it did not evaluate the arterial location. In the present study, we found that higher concentrations of tHcy were associated with higher burdens of LVD, especially in the extracranial cerebral arteries.

Our study showed that the MTHFR C677T polymorphism was significantly associated with hyperhomocysteinemia but this polymorphism was not directly related to SVD and LVD. The MTHFR C677T polymorphism is a common genetic cause of hyperhomocysteinemia.[25] Several studies have shown a relationship between the TT genotype and the development of ischemic stroke,[14,26] whereas others have failed to show such associations.[27,28] These discrepancies may be due to small study populations and methodologic limitations in assessing arteriopathy. In the present study, using MRI and MRA, we could not find an association between the MTHFR polymorphism and SVD or between the MTHFR polymorphism and LVD, despite including a large number of patients and evaluating both intracranial and extracranial cerebral arteries. It remains possible, however, that our sample size is not large enough to reveal the association between MTHFR polymorphism and vasculopathy. We also could not find a locational preference of SVD and LVD according to genotype. However, these findings do not mean that homocysteine is not a risk factor for cerebrovascular diseases. Nongenetic factors or confounders may have important roles in the pathogenesis of homocysteine-driven vasculopathy.

There have been expectations that the ingestion of B vitamins (folate, vitamin B_6, and vitamin B_{12}) may decrease the incidence of stroke because B vitamins can lower plasma concentrations of homocysteine.[25] A population-based study revealed that stroke mortality in the United States and Canada declined following folic acid fortification of grain products.[29] A meta-analysis of

Figure 1 MRI and magnetic resonance angiography findings according to homocysteine



Mean small-vessel disease (SVD) (A) and large-vessel disease (LVD) (B) scores according to the quartile of the total homocysteine (tHcy) plasma concentration. Mean LVD scores in the intracranial (C) and extracranial (D) arteries are shown. The numbers in the boxes are *p* values. $^{**}p < 0.05$. Error bars indicate the 95% confidence interval of each value.

retrospective studies also showed the positive effect of folate supplementation in prevention of stroke.[30] However, clinical trials to lower tHcy with B vitamins have produced equivocal results. The Heart Outcomes Prevention Evaluation trial failed to show a beneficial effect of B vitamin therapy on the composite of death from cardiovascular causes, but it showed a significant decrease in the incidence of stroke in subgroup analysis.[31] In contrast, the Vitamin Intervention for Stroke Prevention trial and the Vitamins to Prevent Stroke trial did not reveal the beneficial effect of B vitamin supplementation on recurrent stroke in patients with previous stroke and

Table 3 Factors associated with SVD and LVD on multiple logistic regression analysis

	SVD[a]		LVD[b]	
	AOR (95% CI)	*p*	AOR (95% CI)	*p*
Age, y	1.07 (1.05–1.08)	<0.001	1.02 (1.01–1.04)	0.001
Sex, male	1.11 (0.74–1.65)	0.617		
Hypertension	2.41 (1.51–3.86)	<0.001	1.66 (1.14–2.40)	0.008
Diabetes mellitus	0.65 (0.45–0.94)	0.022	1.94 (1.43–2.64)	<0.001
Smoking	0.94 (0.61–1.43)	0.759		
Alcohol consumption	0.36 (0.08–1.60)	0.177		
NIH Stroke Scale on admission			1.03 (1.00–1.06)	0.104
tHcy concentration, μmol/L	1.03 (1.00–1.06)	0.035	1.02 (1.00–1.05)	0.041

Abbreviations: AOR = adjusted odds ratio; CI = confidence interval; LVD = large-vessel disease; SVD = small-vessel disease; tHcy = total homocysteine.
[a] The uppermost quartiles of the SVD scores (≥18).
[b] The uppermost quartiles of the LVD scores (≥4).

TIA.[32,33] Other clinical trials also found that B vitamin treatment did not prevent stroke. However, main outcomes of such clinical trials might be null because benefit of B vitamins in patients with normal renal function was cancelled out by harm in those with impaired renal function. In patients with renal dysfunction, asymmetric dimethylarginine, a nitric oxide antagonist, may accumulate from folic acid metabolism.[34] Administration of cyanide from cyanocobalamin may also lead to endothelial dysfunction by consuming nitrous oxide.[35] Thus, it seems likely that methylcobalamin should be used instead of cyanocobalamin in patients with renal impairment.[35] B vitamin doses might also not be enough, because metabolic B_{12} deficiency is prevalent in elderly patients.[35] In addition, the main outcomes of these trials consisted of stroke with sudden neurologic deficits. SVD may cause an insidious cognitive decline that may be difficult to recognize as stroke in clinical trials. Short follow-up durations (<5 years) may also be insufficient to observe anti-atherosclerosis effects of homocysteine-lowering treatment because atherosclerosis is a slow process that takes 30–40 years from initial development to a clinical event.[36] Therefore, results of previous B vitamin trials may have underestimated the benefits of the homocysteine-lowering strategy. Nevertheless, a recent meta-analysis of 14 randomized controlled trials revealed that B vitamin supplementation for homocysteine reduction significantly reduced stroke events.[37]

In addition to the inherent limitations of a retrospective design and a selection bias, this study has other limitations. First, regarding SVD of the brain, we did not differentiate leukoaraiosis from lacunar infarcts. Both types of radiologic abnormalities are included in the category of SVD but they may differ in pathogenesis.[38] However, it is often difficult to distinguish lacunar infarcts from leukoaraiosis. Therefore, we semiquantitatively analyzed them altogether based on a validated grading system. A previous study showed that plasma concentrations of tHcy were associated with both silent brain infarcts and leukoaraiosis.[39] Second, mild atherosclerotic changes may have been missed or atherosclerotic changes may have been exaggerated on MRA. Although MRA can reveal stenoocclusions in both intracranial and extracranial arteries, this imaging modality has limited positive predictive values for 50%–99% stenosis, despite high negative predictive values.[40] Third, we regarded stenoocclusive lesions on MRA as atherosclerotic LVD. However, an MRA lesion presumed to be atherosclerotic may have another pathology, such as dissection, vasculitis, or moyamoya disease. Fourth, we only had a single measurement of tHcy and did not measure plasma concentrations of B vitamins and other biomarkers that may interact with tHcy. Fifth, it is possible that hyperhomocysteinemia was the result, not the cause, of stroke. The fact that the MTHFR polymorphism was associated with hyperhomocysteinemia, but not vasculopathy, supports this possibility. Finally, other polymorphisms than the MTHFR C677T polymorphism were not measured. Thus, our findings need to be interpreted cautiously.

Despite these limitations, our findings are based on a large number of patients and detailed radiologic analysis of the brain and cerebral arteries and provide a better understanding of previously inconsistent results on the effects of hyperhomocysteinemia on the pathogenesis of stroke. Future studies evaluating the benefits of homocysteine-lowering therapy may need to include the extent of SVD of the brain and LVD of cerebral arteries.

AUTHOR CONTRIBUTIONS

Dr. Jeon: drafting/revising the manuscript, study concept or design, analysis and interpretation of data, and acquisition of data. Dr. Kang: drafting/revising the manuscript and acquisition of data. Dr. Kim: drafting/revising the manuscript and acquisition of data. Dr. Kwon: drafting/revising the manuscript, study concept or design, analysis of interpretation of data, and acquisition of data.

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DISCLOSURE

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