Interplay between 3′-UTR polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene and the risk of ischemic stroke

Jung Oh Kim1,2, Han Sung Park1,2, Chang Soo Ryu1,2, Jung-Won Shin3, Jinkwon Kim3, Seung Hun Oh3, Ok Joon Kim3 & Nam Keun Kim1,2

Stroke incidence is a multifactorial disease and especially hyperhomocysteinemia is associated with a higher risk of stroke. Previous studies have reported a folate metabolism disorder associated with the MTHFR gene. We investigated four single nucleotide polymorphisms in the MTHFR 3′-UTR [2572 C>A (rs4846049), 4869 C>G (rs1537514), 5488 C>T (rs3737967), and 6685 T>C (rs4846048)] to elucidate associations between ischemic stroke prevalence and prognosis. We examined 511 consecutive patients with ischemic stroke. Additionally, we selected 411 sex-/age-matched control subjects from patients presenting at our hospitals during the same period. The MTHFR 2572 C>A and 6685 T>C were significantly associated with ischemic stroke prevalence in the cardioembolism subgroup (MTHFR 2572CC vs. CA+AA: AOR, 2.145; 95% CI, 1.203–3.827; P=0.010; MTHFR 6685TT vs. CC: AOR, 10.146; 95% CI, 1.297–79.336; P=0.027). The gene-environment combined effect was significant, with MTHFR 2572CA+AA and folate levels ≤3.45 ng/mL correlating with ischemic stroke incidence. In addition, the total homocysteine (tHcy) levels in subjects with MTHFR 2572AA were elevated compared to tHcy levels in subjects with MTHFR 2572CC. Therefore, we suggest that MTHFR 2572 C>A and 6685 T>C are associated with ischemic stroke pathogenesis. The combined effects of the MTHFR 3′-UTR polymorphisms and tHcy/folate levels may contribute to stroke prevalence.

Stroke is the third most common cause of death in many developed countries, and approximately 80% of stroke cases are ischemic in origin1,2. In South Korea, stroke is the most frequent cause of death after cancer and is more frequent than heart disease3,4. Stroke is a complex multifactorial and polygenic disease arising from a variety of gene–gene and gene–environment interactions5,6. Multiple factors, including hypertension (HTN), diabetes mellitus (DM), smoking, hyperlipidemia, and hyperhomocysteinemia, are associated with a higher risk of stroke1,7,8. In particular, hyperhomocysteinemia is considered an independent, potentially modifiable risk factor for ischemic stroke, and has been previously reported in several studies involving different ethnic groups9-12.

The 5, 10-methylenetetrahydrofolate reductase (MTHFR) protein performs a central reaction in folate metabolism. It irreversibly catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the primary circulating form of folate13,14. In addition, MTHFR converts 5, 10-methylenetetrahydrofolate into 5-methyltetrahydrofolate and provides the methyl group for homocysteine (Hcy) in methionine synthesis. It has been reported that elevation of Hcy in the blood is associated with an increased risk for arteriosclerosis, myocardial infarction, venous thrombosis, stroke, and neural tube defects15,16. The effect of several polymorphic genes involved in folate metabolism, including MTHFR on ischemic stroke susceptibility and progression, has been reported. The MTHFR gene is critical for Hcy and folate metabolism, and polymorphic variants of the enzymes

1Institute for Clinical Research, CHA Bundang Medical Center, School of Medicine, CHA University, Seongnam, 463-712, South Korea. 2Department of Biomedical Science, College of Life Science, CHA University, Seongnam, South Korea. 3Department of Neurology, CHA Bundang Medical Center, School of Medicine, CHA University, Seongnam, 463-712, South Korea. Correspondence and requests for materials should be addressed to O.J.K. (email: okjun77@cha.ac.kr) or N.K.K. (email: nkkim@cha.ac.kr)
involved in Hcy and folate metabolism also play an important role in determining the susceptibility of an individual to disease. Lower MTHFR enzyme activity, which can increase total plasma homocysteine (tHcy) levels and decrease plasma folate levels, contributes to stroke development. Folate concentrations inversely correlate with tHcy levels. The role of hyperhomocysteinemia in vascular and thromboembolic disease has been extensively studied. Previous studies reported significant vascular disease in patients with markedly elevated tHcy levels. The tHcy is hypothesized to increase thrombotic risk by inducing endothelial injury in venous and arterial vasculatures. Abnormal folate concentrations have also been implicated in the development of diseases, such as cardiovascular diseases, neural tube defects, cleft lip and palate, late pregnancy complications, and neurodegenerative and psychiatric disorders. Recent studies have shown the clinical impacts of polymorphisms in the 3′-UTR of certain genes, which may potentially bind to specific microRNAs (miRNAs) in various diseases. However, variants in the MTHFR 3′-UTR have not been extensively examined.

In the present study, we selected four single nucleotide polymorphisms (SNPs) in the MTHFR 3′-UTR region: MTHFR 2572 C > A (rs4846049), 4869 C > G (rs1537514), 5488 C > T (rs3737967), and 6685 T > C (rs4846048). We then determined their associations with ischemic stroke prevalence and prognosis. The minor allele frequencies of the studied polymorphisms are higher than 5% in the Asian population, and little is known about their genetic associations with ischemic stroke. Therefore, we investigated whether MTHFR 3′-UTR polymorphisms correlate with ischemic stroke susceptibility in Korean subjects.

**Results**

**Genetic susceptibility of single markers in ischemic stroke subtypes and control subjects.** We investigated the MTHFR 2572 C > A, 4869 C > G, 5488 C > T, and 6685 T > C polymorphisms. Table 1 shows their genotype distributions in ischemic stroke patients and control subjects. The MTHFR 3′-UTR polymorphisms were not significantly correlated with ischemic stroke prevalence. To examine whether the effect of each polymorphism was confined to a specific subtype or related to generalized risks, we further separated the stroke group into three subgroups [large arterial disease (LAD), small-vessel disease (SVD), and cardioembolism (CE)] according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification. In subgroup analyses, LAD was significantly associated with the MTHFR 6685 T > C polymorphism [TT vs. TC: adjusted odds ratio (AOR), 0.517; 95% confidence interval (CI), 0.308–0.867; \( P = 0.012 \); TT vs. TC + CC: AOR, 0.543; 95% CI, 0.328–0.899; \( P = 0.018 \)]. The MTHFR 2572 C > A polymorphism (CC vs. CA: AOR, 2.098; 95% CI, 1.160–3.793; \( P = 0.014 \); CC vs. CA + AA: AOR, 2.145; 95% CI, 1.203–3.827; \( P = 0.010 \)) and 6685 T > C polymorphism (TT vs. CC: AOR, 10.146; 95% CI, 1.297–79.336; \( P = 0.027 \); TT + TC vs. CC: AOR, 10.504; 95% CI, 1.373–80.377; \( P = 0.024 \)) were significantly associated with the CE type (Table 1). In addition, we analyzed the association with/without patients of undetermined subtypes for ischemic stroke susceptibilities (Supplemental Table 1).

**Combined effects of MTHFR gene polymorphisms and clinical parameters on disease prevalence.** To determine additional clinical significance, we evaluated the combined effects of the gene environment. The MTHFR gene polymorphism elevated stroke prevalence in several conditions, including hypertension, diabetes mellitus, hyperlipidemia, smoking, high density lipoprotein–cholesterol levels, triglyceride levels, folate levels <3.45 nmol/mL, and tHcy levels ≥11.22 μmol/L (Table 2). The MTHFR 2572 CA + AA genotype was shown to have synergic effects with ischemic stroke prevalence. In particular, low folate levels were the most predictive, with MTHFR 2572 CA + AA and folate ≤3.45 nmol/L (AOR, 6.532; 95% CI, 2.592–16.46) shown to significantly increase ischemic stroke incidence. In addition, the MTHFR 4869 CG + GG genotype had a combinatorial effect with hypertension (AOR, 3.217; 95% CI, 1.653–6.372) and smoking (AOR, 5.067; 95% CI, 1.788–14.360), whereas the MTHFR 5488 CT + TT genotype was significant only in the smoking group (AOR, 2.740; 95% CI, 1.196–6.278). In addition, we performed stratified analyses for clinical factors including sex, age, hypertension, diabetes mellitus, hyperlipidemia, smoking status, folate levels, and homocysteine levels (Supplemental Table 2).

**Haplotype analysis with disease incidence.** The linkage disequilibrium (LD) of MTHFR polymorphisms at loci 2572 (rs4846049)/4869, (rs1537514)/5488, (rs3737967)/6685 (rs4846048) in ischemic stroke patients and control subjects is shown in the Supplemental Figure 1. There was a strong LD between loci 4869/5488 (\( D^′ = 0.944 \)), 2572/5488 (\( D^′ = 0.949 \)), and 2572/4869 (\( D^′ = 0.938 \)). To evaluate the combined effects of the MTHFR 3′-UTR SNP loci on ischemic stroke incidence, logistic regression for the combined genotypes and haplotypes was performed. The 2572CC/4869CC/5488CC/6685TTC (AOR, 0.095; 95% CI, 0.012–0.761; \( P = 0.027 \)), 2572CA/4869CC/5488CC/6685TT (AOR, 2.922; 95% CI, 1.342–6.361; \( P = 0.007 \)) 2572A/4869C-5488C-6685T (AOR, 0.062; 95% CI, 0.008–4.477; \( P = 0.001 \)), and 2572A-4869C-5488C-6685T (AOR, 2.239, 95% CI, 1.178–4.257, \( P = 0.013 \)) types contributed to ischemic stroke prevalence (Table 3). In the ischemic stroke subgroup, the MTHFR haplotypes were shown to have a significant association with CE subtype prevalence (Supplemental Table 3).

**Differences of blood coagulation factors according to MTHFR polymorphisms.** Analyses of variance were used to show differences in blood coagulant factors (fibrinogen, antithrombin, platelet, activated partial thromboplastin time, and prothrombin time), folate, and tHcy levels according to genotype (Table 4). The tHcy levels in subjects with the MTHFR 2572A AA polymorphism were elevated compared to the tHcy levels in subjects with the MTHFR 2572CC (\( P = 0.011 \)). Additionally, the MTHFR 5488 and 6685 mutant genotypes had higher tHcy levels than that of the wild-type genotypes (MTHFR 5488, \( P = 0.020 \); MTHFR 6685, \( P = 0.005 \)). Supplemental Table 4 shows the combination models with MTHFR 677 C > T, which measured the Hcy levels. The MTHFR 677-2572 combination group (677CC-2572AA: 12.14 ± 5.56; \( P = 0.010 \)) and MTHFR 677-6685 combination group (677CC-6685CC: 14.61 ± 6.53; \( P < 0.0001 \)) were significantly different compare to each wild type genotype.
| Genotypes | Controls (n = 411) | Case (n = 511) | AOR (95% CI)* | P† | LAD (n = 205) | AOR (95% CI)* | P‖ | SVD (n = 149) | AOR (95% CI)* | P‖ | CE (n = 55) | AOR (95% CI)* | P‖ |
|-----------|------------------|----------------|--------------|-----|---------------|--------------|-----|--------------|--------------|-----|-------------|--------------|-----|
| **MTHFR 2572 C>A** | | | | | | | | | | | | | |
| CC | 280 (68.1) | 333 (65.2) | 1.000 (reference) | 137 (66.8) | 1.000 (reference) | 97 (65.1) | 1.000 (reference) | 28 (50.9) | 1.000 (reference) | | | | |
| CA | 122 (29.7) | 163 (31.9) | 1.216 (0.90–1.638) | 0.199 | 64 (31.2) | 1.126 (0.767–1.653) | 0.544 | 47 (31.5) | 1.222 (0.794–1.880) | 0.363 | 25 (45.5) | 2.098 (1.160–3.793) | 0.014 |
| AA | 9 (2.2) | 15 (2.9) | 1.575 (0.653–3.800) | 0.312 | 4 (2.0) | 0.932 (0.262–3.315) | 0.913 | 5 (3.4) | 1.718 (0.528–5.586) | 0.369 | 2 (3.6) | 2.432 (0.463–12.767) | 0.294 |
| Dominant (CC vs. CA + AA) | | | | | | | | | | | | | |
| Recessive (CC + CA vs. AA) | | | | | | | | | | | | | |
| HWE-P | 0.308 | 0.352 | | | | | | | | | | | |
| **MTHFR 4869 C>G** | | | | | | | | | | | | | |
| CC | 368 (89.5) | 457 (89.4) | 1.000 (reference) | 179 (87.3) | 1.000 (reference) | 134 (89.9) | 1.000 (reference) | 50 (90.9) | 1.000 (reference) | | | | |
| CG | 43 (10.5) | 54 (10.6) | 1.075 (0.689–1.676) | 0.750 | 26 (12.7) | 1.379 (0.797–2.388) | 0.251 | 15 (10.1) | 0.980 (0.503–1.909) | 0.952 | 5 (9.1) | 0.822 (0.304–2.220) | 0.699 |
| GG | 0 (0.0) | 0 (0.0) | N/A | 0 (0.0) | N/A | 0 (0.0) | N/A | 0 (0.0) | N/A | | | | |
| Dominant (CC vs. CG + GG) | | | | | | | | | | | | | |
| Recessive (CC+C vs. GG) | N/A | N/A | N/A | N/A | N/A | N/A | N/A | | | | | | |
| HWE-P | 0.263 | 0.208 | | | | | | | | | | | |
| **MTHFR 5488 C>T** | | | | | | | | | | | | | |
| CC | 351 (85.4) | 450 (88.1) | 1.000 (reference) | 177 (86.3) | 1.000 (reference) | 131 (87.9) | 1.000 (reference) | 49 (89.1) | 1.000 (reference) | | | | |
| CT | 59 (14.4) | 57 (11.2) | 0.837 (0.556–1.258) | 0.391 | 28 (13.7) | 1.134 (0.681–1.890) | 0.629 | 16 (10.7) | 0.740 (0.396–1.385) | 0.347 | 6 (10.9) | 0.754 (0.304–1.871) | 0.543 |
| TT | 1 (0.2) | 4 (0.8) | 2.738 (0.296–25.290) | 0.375 | 0 (0.0) | N/A | 0.998 | 2 (1.3) | 4.486 (0.391–51.468) | 0.228 | 0 (0.0) | N/A | 0.998 |
| Dominant (CC vs. CT + TT) | | | | | | | | | | | | | |
| Recessive (CC+CT vs. TT) | 2.857 (0.307–26.552) | 0.356 | N/A | 0.998 | 4.679 (0.402–54.450) | 0.218 | N/A | N/A | 0.998 | | | | |
| HWE-P | 0.364 | 0.151 | | | | | | | | | | | |
| **MTHFR 6685 T>C** | | | | | | | | | | | | | |
| TT | 326 (79.3) | 426 (83.4) | 1.000 (reference) | 180 (87.8) | 1.000 (reference) | 122 (81.9) | 1.000 (reference) | 40 (72.7) | 1.000 (reference) | | | | |
| TC | 83 (20.2) | 78 (15.3) | 0.795 (0.556–1.137) | 0.209 | 23 (11.2) | 0.517 (0.308–0.867) | 0.012 | 25 (16.8) | 0.921 (0.551–1.540) | 0.753 | 13 (23.6) | 1.297 (0.655–2.567) | 0.456 |
| CC | 2 (0.5) | 7 (1.4) | 2.902 (0.553–15.240) | 0.208 | 2 (1.0) | 1.418 (0.174–11.537) | 0.744 | 2 (1.3) | 2.775 (0.326–23.649) | 0.351 | 2 (3.6) | 10.146 (1.297–79.336) | 0.027 |
| Dominant (TT vs. TC + CC) | 0.846 (0.597–1.200) | 0.349 | 0.543 (0.328–0.899) | 0.018 | 0.966 (0.585–1.595) | 0.892 | 1.481 (0.773–2.837) | 0.237 | | | | | |
| Recessive (TT+TC vs. CC) | 3.199 (0.613–16.682) | 0.168 | 1.693 (0.209–13.693) | 0.622 | 2.842 (0.349–23.145) | 0.329 | 10.504 (1.373–80.377) | 0.024 | | | | | |
| HWE-P | 0.175 | 0.123 | | | | | | | | | | | |

Table 1. Comparison of genotype frequencies and AOR of MTHFR gene polymorphisms between the ischemic stroke, subtypes, and control subjects. Abbreviation; AOR, adjusted odds ratio; MTHFR, methylenetetrahydrofolate reductase; CI, confidence interval; LAD, large artery disease; SVD, small vessel disease; CE, cardioembolism; HWE, Hardy-Weinberg equilibrium. *The P-value calculated by multiple logistic regression on the basis of risk factors such as age, gender, hypertension, hyperlipidemia, and diabetes mellitus.
Table 2. Ischemic stroke incidence by interactions with hypertension, diabetes mellitus, hyperlipidemia, smoking, HDL-cholesterol, folate, and homocysteine. Adjusted by age, sex, hypertension, diabetes mellitus, hyperlipidemia, and smoking. Abbreviations are defined in Table 1. *HDL-cholesterol has a different standard value for males and females, with 40 for males and 50 for females. †3.16 nmol/L is based on the bottom 15% of the homocysteine levels in patients and controls. ‡16.36 μmol/L is based on the top 15% of folate levels in patients and controls. $16.36 μmol/L is based on the top 15% of the homocysteine levels in patients and controls.

**Discussion**

Hcy is a well-known thrombotic factor in vascular diseases including coronary artery disease, heart disease, arteriosclerosis, myocardial infarction, venous thrombosis, chronic kidney disease, and ischemic stroke. There is increasing evidence that Hcy may affect the coagulation system and the resistance of the endothelium to thrombosis. Moreover, Hcy may interfere with the vasodilator and antithrombotic functions of nitric oxide. Notably, vascular complications reported in patients with homocystinuria are related to thrombosis rather than atherosclerosis, and a relationship between tHcy levels and the incidence of thrombotic events has recently been reported in patients with systemic lupus erythematosus.

Previous studies have identified the MTHFR gene as being associated with ischemic stroke prevalence. Numerous studies reported that MTHFR 677 C>T was associated with increased stroke risk, likely because the MTHFR 677 T allele decreased MTHFR gene activity. Other studies reported that the methylation pattern of CpG island regions in the MTHFR gene had decreased MTHFR activity, causing an abnormality for tHcy and MTHFR gene activity. **During a mean follow-up period of 7 years, 98 patients (17.5%) died. We investigated whether the MTHFR 3'-UTR genotypes were associated with long-term overall survival (OS) after ischemic stroke using Kaplan-Meier analyses. However, we did not find significant associations with individual MTHFR 3'-UTR genotypes (Supplemental Figure 2).**
Table 1. ‡The false discovery rate-adjusted P-value was calculated by multiple logistic regression on the basis of risk factors such as age, gender, hypertension, hyperlipidemia, and diabetes mellitus. Abbreviations are defined in method.

| Gene       | Controls freq. (n = 411) | Case freq. (n = 511) | AOR (95% CI) | \( P^\dagger \)  | \( P^\ddagger \) |
|------------|--------------------------|----------------------|--------------|-----------------|-----------------|
| MTHFR      |                          |                      |              |                 |                 |
| CC/CC/CT/TT| 0.652                    | 0.644                | 1.000 (reference) |    |                 |
| CC/CC/CC/TT| 0.029                    | 0.002                | 0.095 (0.012–0.761) | 0.027 | 0.149           |
| CC/CC/CT/TT| 0.000                    | 0.002                | 0.393 (0.021–7.323) | 0.531 | 0.680           |
| CA/CC/CC/TT| 0.022                    | 0.065                | 2.922 (1.342–6.361) | 0.007 | 0.077           |
| CA/CC/CT/TC| 0.141                    | 0.145                | 1.106 (0.745–1.642) | 0.618 | 0.680           |
| CA/CC/CT/TT| 0.024                    | 0.010                | 0.475 (0.157–1.438) | 0.188 | 0.528           |
| CA/CC/CT/TC| 0.010                    | 0.002                | 0.309 (0.034–2.844) | 0.299 | 0.581           |
| CA/CG/CT/TT| 0.085                    | 0.092                | 1.218 (0.746–1.989) | 0.431 | 0.677           |
| AA/CC/CC/TT| 0.005                    | 0.002                | 0.524 (0.044–6.192) | 0.608 | 0.680           |
| AA/CC/CC/CC| 0.005                   | 0.014                | 3.004 (0.575–15.704) | 0.192 | 0.528           |
| AA/CC/CT/TC| 0.005                    | 0.002                | 0.628 (0.056–7.100) | 0.707 | 0.707           |
| AA/CC/CT/TC| 0.007                    | 0.002                | 0.295 (0.027–3.213) | 0.317 | 0.581           |

Table 3. Combined genotype and allele frequencies of the MTHFR 3′-UTR polymorphisms for ischemic stroke patients and control subjects. †The P-value was calculated by multiple logistic regression on the basis of risk factors such as age, gender, hypertension, hyperlipidemia, and diabetes mellitus. Abbreviations are defined in Table 1. ‡The false discovery rate-adjusted P-value for multiple hypothesis testing using the Benjamini-Hochberg method.

This study has several limitations. First, the mechanisms by which 3′-UTR polymorphisms in the MTHFR gene affect stroke development remain unclear. Second, the controls in our study were not completely healthy serum folate levels that were associated with ischemic stroke occurrence. Moreover, we have shown an association with ischemic stroke risk based on computational epigenetic profiling of CpG islands in the MTHFR gene. However, epigenetic regulation of the MTHFR gene could occur via another mechanism that included RNA interference with miRNA binding. In addition, a previous study showed differential mRNA expression levels of the MTHFR gene according to 3′-UTR polymorphisms.

Therefore, understanding the genesis of ischemic events due to decreased MTHFR activity might explain why 3′-UTR polymorphisms could affect ischemic events, stroke occurrence, and patient prognosis. Polymorphisms in the 3′-UTR region could affect mRNA stability and translation, which may significantly impact gene expression by abolishing, weakening, or creating miRNA binding sites. Currently, there are not sufficient data to indicate that miRNA binding activity is modulated depending on MTHFR 3′-UTR polymorphisms. One study reported that miR-149 binding activity was affected by the MTHFR 3′-UTR polymorphisms. In addition, we have shown an association with ischemic stroke occurrence and progression of ischemic stroke because their expression is altered in some genotypes.

In conclusion, we investigated the relationship of the MTHFR 2572 C > A and 6685 T > C polymorphisms with ischemic stroke incidence and progression. The MTHFR 2572 C > A and 6685 T > C polymorphisms were shown to increase the risk of embolisms of cardiac origin and ischemic stroke occurrence, and the prevalence of large-artery-origin ischemic stroke had a decreased odds ratio (OR) for the MTHFR 6685 T > C polymorphism. Moreover, the combination of MTHFR 2572 CA + AA genotypes and serum folate levels or tHcy levels were synergic for ischemic stroke susceptibility, whereas other MTHFR 3′-UTR polymorphisms were not associated with serum folate and tHcy for ischemic stroke risk. Therefore, we hypothesized that the MTHFR 3′-UTR regulates one-carbon metabolism through miRNA binding. We found that these genotypes and haplotypes positively correlated with the occurrence and unfavorable prognosis of stroke, according to vascular disease risk factors, including hypertension, diabetes mellitus, HDL-C levels, tHcy levels, and folate levels.

This study has several limitations. First, the mechanisms by which 3′-UTR polymorphisms in the MTHFR gene affect stroke development remain unclear. Second, the controls in our study were not completely healthy.
| Genotypes | Folate (nmol/L) | tHcy (μmol/L) | Fibrinogen (mg/dL) | Antithrombin (%) | PLT (10^3/μL) | aPTT (sec) | PT (sec) |
|-----------|----------------|---------------|-------------------|-----------------|---------------|-----------|---------|
|           | Mean ± SD (N)  | CV, %         | Mean ± SD (N)     | CV, %           | Mean ± SD (N) | CV, %     | Mean ± SD (N) | CV, % |
| CC        | 7.56 ± 5.60 (608) | 74.1         | 10.89 ± 5.33 (612) | 48.9            | 418.07 ± 128.28 (407) | 30.7      | 94.80 ± 29.46 (408) | 31.1 |
|           | 246.33 ± 76.57 (612) | 31.1         | 30.89 ± 6.47 (536) | 20.9            | 11.75 ± 0.80 (536) | 6.8       |
| CA        | 8.05 ± 5.93 (281)  | 73.7         | 9.94 ± 3.77 (281)  | 37.9            | 420.94 ± 126.74 (190) | 30.1      | 93.34 ± 16.82 (191) | 18.0 |
|           | 239.85 ± 64.13 (279) | 26.7         | 32.95 ± 19.32 (237) | 58.6            | 11.85 ± 1.19 (237) | 10.0      |
| AA        | 6.76 ± 4.00 (24)   | 59.2         | 11.98 ± 5.50 (24)  | 45.8            | 433.22 ± 154.08 (18) | 35.6      | 91.71 ± 17.60 (17)  | 19.2 |
|           | 265.13 ± 211.05 (23) | 79.6         | 34.11 ± 6.02 (19)  | 17.6            | 11.60 ± 0.83 (19) | 7.2       |
| P         | 0.358           | 0.011*       | 0.870             | 0.242*          | 0.055*        | 0.240     |
| Callele   | 7.65 ± 5.66 (1497) | 74.0         | 10.71 ± 5.09 (1505) | 47.5            | 418.61 ± 127.87 (1004) | 30.5      | 94.22 ± 27.50 (1503) | 29.1 |
|           | 245.13 ± 74.42 (1309) | 30.4         | 31.27 ± 10.11 (1309) | 32.3            | 11.77 ± 0.88 (1309) | 7.5       |
| Allele    | 7.86 ± 5.70 (329)  | 72.5         | 10.23 ± 4.11 (329)  | 40.2            | 422.89 ± 130.77 (226) | 30.9      | 93.09 ± 16.87 (235) | 18.1 |
|           | 243.43 ± 98.26 (325) | 40.4         | 33.11 ± 18.06 (275) | 54.5            | 11.82 ± 1.14 (275) | 9.6       |
| P         | 0.551           | 0.114        | 0.651             | 0.453           | 0.725         | 0.019*    | 0.410     |

Table 4. Altered plasma folate and homocysteine levels, and blood coagulation factors according to MTHFR genotypes. Abbreviations: tHcy, total plasma homocysteine; PLT, platelet; aPTT, activated partial thromboplastin time; PT, prothrombin time. Other abbreviations are defined in Table 1. *One-way analysis of variance test. †Independent two-sample t-test.
Table 5. Baseline characteristics of control and stroke patients

Abbreviations: MetS, metabolic syndrome; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBS, fasting blood sugar; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; T. chol, total cholesterol; aPTT, activated partial thromboplastin time; tHcy, total plasma homocysteine; BUN, blood urea nitrogen. Other abbreviations are defined in Table 1. *P-values were calculated by two-sided t-test for continuous variables and chi-square test for categorical variables.

provide the first evidence for 3’-UTR variants in MTHFR as potential biomarkers of stroke prevention and prognosis, a prospective study using a larger cohort of patients is warranted to validate these findings.

Methods

Ethics statement. All study protocols of participants were reviewed and approved by The Institutional Review Board of CHA Bundang Medical Center and followed the recommendations of the Declaration of Helsinki. Study subjects were recruited from the South Korean provinces of Seoul and Gyeonggi-do between 2000 and 2008. The Institutional Review Board of CHA Bundang Medical Center approved this genetic study in June 2000 and informed consent was obtained from the study participants.

Study population. We included 511 consecutive patients with ischemic stroke referred from the Department of Neurology at CHA Bundang Medical Center, CHA University. Ischemic stroke was defined as a stroke (a clinical syndrome characterized by rapidly developing clinical symptoms and signs of focal or global loss of brain...
function) with evidence of cerebral infarction in clinically relevant areas of the brain according to brain imaging using magnetic resonance imaging (MRI). The date and cause of death were identified using death certificates from the Korean National Statistical Office. Patients who were alive on Dec 31, 2012 were censored at that point. The death statistics of the Korean National Statistical Office have been previously reported to be reliable.

Based on clinical manifestations and neuroimaging data, two neurologists classified all ischemic strokes into four causative subtypes using the TOAST criteria as follows: (1) large-artery disease (LAD), significant (≥50%) stenosis of a relevant cerebral artery confirmed by cerebral angiography; (2) small-vessel disease (SVD), an infarction lesion <15 mm in diameter, and classic lacunar syndrome without evidence of a cerebral cortical dysfunction or potentially detectable cardiac sources for embolism; (3) cardioembolism (CE), presumably due to an embolus arising in the heart, as detected by cardiac evaluation; and (4) undetermined pathogenesis, in which the cause of stroke could not be determined or patients with two or more potential causes. The frequencies of the stroke subtypes were 40% LAD (n = 205), 29% SVD (n = 149), 11% CE (n = 55), and 20% undetermined pathogenesis (n = 102). These proportions are similar to previously reported values for the Korean population.

We selected 411 sex- and age-matched (±5 years) control subjects from patients presenting at our hospitals during the same period for health examinations, including biochemical testing, electrocardiogram analyses, and brain MRI. Control subjects did not have a recent history of cerebrovascular disease or myocardial infarction. Exclusion criteria were the same as those used in the patient group. The demographic and laboratory data of patients with ischemic stroke, subtype patients [LAD, SVD, and CE] and control subjects are summarized in Table 5. In our sample, 43.1% and 42.1% of stroke patients and control subjects were male, respectively. The mean ages of stroke patients and the control population were 62.96 ± 10.90 years and 62.82 ± 10.61 years, respectively. There were few significant differences between the two groups. Ischemic stroke patients were significantly more likely to have hypertension, fibrinogen, increased tHcy levels, and decreased folate levels (P < 0.05).

Estimation of tHcy and folate levels. Within 48 hours of stroke onset, we collected plasma samples to measure tHcy and folate levels. Twelve hours after the patient's previous meal, we collected whole blood in a tube containing anticoagulant. Tubes were centrifuged for 15 minutes at 1000 × g to separate the plasma. The tHcy concentrations were measured using a fluorescent polarizing immunooassay with the IMx system (Abbott Laboratories, Chicago, IL, USA), and folate concentrations were measured using a radioimmunoassay kit (ACS 180; Bayer, Tarrytown, NY, USA). The concordance of quality control samples was 100%.

Genotyping. DNA was extracted using the G-DEX blood extraction kit (iNTRON Biotechnology, Inc., Seongnam, Republic of Korea). The four best-studied SNPs in the MTHFR gene were determined by a documentary search, which included four 3′-UTR SNPS (2572 C > A, rs4846049; 4869 C > G, rs1537514; 5488 C > T, rs3737967; and 6685 T > C, rs4846048). All SNP sequences were obtained from the HapMap database (http://www.hapmap.org). The MTHFR 2572 C > A and 4869 C > G polymorphisms were analyzed by the polymerase chain reaction-restriction fragment length polymorphism method. Real-time polymerase chain reaction (PCR) was used to analyze the MTHFR 5488 C > T and 6685 T > C polymorphisms. For each polymorphism, 30% of the PCR assay samples were randomly selected and repeated, and followed by DNA sequencing, to validate the RFLP findings. Sequencing was performed using an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The concordance of quality control samples was 100%.

Statistical analyses. To analyze baseline characteristics, we used chi-square tests for categorical data and Student’s t-tests or analyses of variance were used for continuous data. We estimated associations of MTHFR gene polymorphisms with ischemic stroke incidence using adjusted ratios (AORs) and 95% confidence intervals (CIs) from multivariate logistic regression analyses. Adjustments were performed for sex, age, HTN, DM, hyperlipidemia, and smoking, because they are well-established risk factors for ischemic stroke. To evaluate the impact of MTHFR gene polymorphisms on all-cause mortality, we conducted hazard ratios (HRs), and 95% CIs from Kaplan-Meier survival analyses. Analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA) and Medcalc version 12.7.1.0 (Medcalc Software, Mariakerke, Belgium). Haplotypes for multiple loci were estimated using the expectation-maximization algorithm with SNPAlyze (Version 5.1; DYNACOM Co. Ltd., Yokohama, Japan).

References
1. The World Health Report 2002: Reducing risks, promoting healthy life. World Health Organization web site. http://www.who.int/whr/2002/en/. Accessed October 4, 2003.
2. Goldstein, L. B. et al. Primary prevention of ischemic stroke: a statement for healthcare professionals from the Stroke Council of the American Heart Association. Stroke 32, 280–299 (2001).
3. The Korea National Statistical Office Report 2009: Change in leading causes of death (1999–2009). Korea National Statistical Office web site. http://www.kosis.kr/ups3/service/ch_file_down.jsp?PCODE=YD&FILE_NAME=ups3/upload/101/YD/VD0005.xls&SEQ=8. Accessed June 26, 2011.
4. Cho, Y. et al. Predisposing roles of paraoxonase-1 genetic variants in Korean ischemic stroke patients. Genes Genom 37, 579–586 (2015).
5. Kluijtmans, L. A. et al. Genetic and nutritional factors contributing to hyperhomocysteinemia in young adults. Blood 101, 2483–2488 (2003).
6. Gellekink, H., den Heijer, M., Heil, S. G. & Blom, H. J. Genetic determinants of plasma total homocysteine. Semin Vasc Med 5, 98–109 (2005).
7. Iso, H. et al. Serum triglycerides and risk of coronary heart disease among Japanese men and women. Am J Epidemiol 153, 490–499 (2001).
8. Kitamura, A. et al. High density lipoprotein cholesterol and premature coronary heart disease in urban Japanese men. Circulation 89, 2533–2539 (1994).
9. Han, L. et al. Homocysteine, ischemic stroke, and coronary heart disease in hypertensive patients: a population-based, prospective cohort study. Stroke 46, 1777–1786 (2015).

10. Kelly, P. J. et al. Homocysteine, MTHFR 677C→T polymorphism, and risk of ischemic stroke: results of a meta-analysis. Neurology 27, 529–536 (2002).

11. Jeon, S. B. et al. Homocysteine, small-vessel disease, and atherosclerosis: an MRI study of 825 stroke patients. Neurology 83, 695–701 (2014).

12. Wei, L. K. et al. Clinical relevance of MTHFR, eNOS, ACE, and ApoE gene polymorphisms and serum vitamin profile among Malay patients with ischemic stroke. J Stroke Cerebrovasc Dis 24, 2017–2025 (2015).

13. Sharp, L. & Little, J. Polymorphisms in genes involved in folate metabolism and colorectal neoplasia: a HuGE review. Am J Epidemiol 159, 423–443 (2004).

14. Fowler, B. The folate cycle and disease in humans. Kidney Int Suppl 78, S221–229 (2001).

15. Toyoda, K. Recurrent small-artery disease in hyperhomocysteinaemia: widowers' stroke syndrome? Intern Med 43, 869–872 (2004).

16. Van der Put, N. M. et al. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet 62, 1044–1051 (1998).

17. Blom, H. J. & Smulders, Y. Overview of homocysteine and folate metabolism. With special references to cardiovascular disease and neural tube defects. J Inherit Metab Dis 34, 75–81 (2011).

18. Ho, V., Massey, T. E. & King, W. D. Effects of methionine synthase and methylenetetrahydrofolate reductase gene polymorphisms on markers of one-carbon metabolism. Genes Nutr 8, 571–580 (2013).

19. Kim, O. J. et al. Influence of combined methionine synthase (MTR 2756A>G) and methylenetetrahydrofolate reductase (MTHFR 677C>T) polymorphisms to plasma homocysteine levels in Korean patients with ischemic stroke. Yonsei Med J 48, 201–209 (2007).

20. Brevis, A. et al. Plasma concentration of folate as a biomarker for the intake of fruit and vegetables: the Hordaland Homocysteine Study. Am J Clin Nutr 81, 434–439 (2005).

21. McCully, K. S. Vascular pathology of homocysteinaemia: implications for the pathogenesis of arteriosclerosis. Am J Pathol 56, 111–128 (1969).

22. Joachim, E. et al. The methylenetetrahydrofolate reductase polymorphism (MTHFR c.677C>T) and elevated plasma homocysteine levels in a U.S. pediatric population with incident thromboembolism. Thromb Res 132, 170–174 (2013).

23. Park, S. Y. et al. Different impact of hyperhomocysteinemia on cerebral small vessel ischemia and cervico-cerebral atherosclerosis in non-stroke individuals. Thromb Res 131, e12–e16 (2013).

24. Nazzí, F. H., Sameer, A. S. & Ganaie, B. A. Folate: Metabolism, genes, polymorphisms and the associated diseases. Gene 533, 11–20 (2014).

25. Iwasaki, T., Tanaka, K., Kawano, M., Itonaga, I. & Tsumura, H. Tumor-suppressive microRNA-let-7a inhibits cell proliferation via targeting of E2F2 in osteosarcoma cells. Int J Oncol 46, 1543–1550 (2015).

26. Kong, Y. et al. Polymorphism of the OLIRI 3′ UTR potential microRNA binding site and risk of Alzheimer's disease: a meta-analysis. Genet Mol Res 13, 10162–10172 (2014).

27. Kim, H. J. et al. miRNA-105 and -128 function as rheostats modulating MMP-2 activities by downregulation of TIMP-2 and upregulation of MT1-MMP. Genes Genom 38, 217–223 (2016).

28. Nygard, O. et al. Plasma homocysteine levels and mortality in patients with coronary artery disease. N Engl J Med 337, 230–236 (1997).

29. Cui, R. et al. Serum total homocysteine concentrations and risk of mortality from stroke and coronary heart disease in Japanese: The JACC study. Atherosclerosis 198, 412–418 (2008).

30. Efthymiou, C. et al. Homocysteine levels and MTHFR polymorphisms in young patients with acute myocardial infarction: a case control study. Hellenic J Cardiol 53, 189–194 (2012).

31. Soltanpour, M. S. et al. Methylenetetrahydrofolate reductase C677T mutation and risk of retinal vein thrombosis. J Res Med Sci 18, 487–491 (2013).

32. Jamison, R. L. et al. Effect of homocysteine lowering on mortality and vascular disease in advanced chronic kidney disease and end-stage renal disease: a randomized controlled trial. JAMA 298, 1163–1170 (2007).

33. Holmes, M. V. et al. Effect modification by population dietary folate on the association between MTHFR genotype, homocysteine, and stroke risk: a meta-analysis of genetic studies and randomised trials. Lancet 378, 584–594 (2011).

34. Casas, J. P. et al. Effect of inhibitors of the renin-angiotensin system and other antihypertensive drugs on renal outcomes: systematic review and meta-analysis. Lancet 366, 2026–2033 (2005).

35. Gotzsche, P. C. et al. Effect of genetic variants associated with plasma homocysteine levels on stroke risk. Stroke 45, 1920–1924 (2014).

36. Wei, L. K. et al. A potential epigenetic marker mediating serum folate and vitamin B12 levels contributes to the risk of ischemic stroke. Biomed Res Int 2015, 167976 (2015).

37. Wei, L. K. et al. Methylenetetrahydrofolate reductase CpG islands: Epigenotyping. J Clin Lab Anal 30, 333–344 (2016).

38. Wu, C. et al. The human MTHFR 486A/G polymorphism increases coronary heart disease risk through modifying miRNA binding. Nutr Metab Cardiovasc Dis 23, 693–698 (2013).

39. Jeon, Y. J. et al. Genetic variants in 3′-UTRs of methylenetetrahydrofolate reductase (MTHFR) predict colorectal cancer susceptibility in Koreans. Sci Rep 5, 11006 (2015).

40. Kim, H. C., Choi, D. P., Ahn, S. V., Nam, C. M. & Suh, I. Six-year survival and causes of death among stroke patients in Korea. Neuroepidemiology 32, 94–100 (2009).

41. Adams, H. P. Jr et al. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter trial: TOAST. Trial of Org 10172 in Acute Stroke Treatment. Stroke 24, 35–41 (1993).

42. Lee, B. C. & Roh, J. K. International experience in stroke registries: Korean Stroke Registry. Am J Prev Med 31, S243–S245 (2006).

Acknowledgements

This study was partly supported by a grant of the Korea Healthcare Technology R&D Project (H115C1972010015 and H116C1559), Ministry for Health, Welfare & Family Affairs, Republic of Korea and partially supported by the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2015R1D1A1A09057432 and 2016R1D1A1B03930141).

Author Contributions

Conceived and designed the experiments: J.O.K., N.K.K. Performed the experiments: J.O.K., C.S.R. Analyzed the data: J.O.K., H.S.P., J.K., S.H.O., O.J.K., N.K.K. Contributed reagents/materials/analysis tools: J.W.S., J.K., S.H.O., O.J.K., N.K.K. Wrote the paper: J.O.K., N.K.K. Other: Article editing: J.O.K., O.J.K., N.K.K.
Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-12668-x.

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017