Associations of *BDNF* Genotype and Promoter Methylation with Acute and Long-Term Stroke Outcomes in an East Asian Cohort

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Abstract

**Background:** Brain derived neurotrophic factor (BDNF) has been shown to play an important role in poststroke recovery. BDNF secretion is influenced by genetic and epigenetic profiles. This study aimed to investigate whether *BDNF* val66met polymorphism and promoter methylation status were associated with outcomes at two weeks and one year after stroke.

**Methods and Findings:** A total of 286 patients were evaluated at the time of admission and two weeks after stroke, and 222 (78%) were followed one year later in order to evaluate consequences of stroke at both acute and chronic stages. Stroke outcomes were dichotomised into good and poor by the modified Rankin Scale. Stroke severity (National Institutes of Health Stroke Scale), physical disability (Barthel Index), and cognitive function (Mini-Mental State Examination) were measured. Associations of *BDNF* genotype and methylation status on stroke outcomes and assessment scale scores were investigated using logistic regression, repeated measures ANOVA and partial correlation tests. *BDNF* val66met polymorphism was independently associated with poor outcome at 2 weeks and at 1 year, and with worsening physical disability and cognitive function over that period. Higher *BDNF* promoter methylation status was independently associated with worse outcomes at 1 year, and with the worsening of physical disability and cognitive function. No significant genotype-methylation interactions were found.

**Conclusions:** A role for BDNF in poststroke recovery was supported, and clinical utility of *BDNF* genetic and epigenetic profile as prognostic biomarkers and a target for drug development was suggested.

Citation: Kim J-M, Stewart R, Park M-S, Kang H-J, Kim S-W, et al. (2012) Associations of *BDNF* Genotype and Promoter Methylation with Acute and Long-Term Stroke Outcomes in an East Asian Cohort. PLoS ONE 7(12): e51280. doi:10.1371/journal.pone.0051280

Editor: Albert Jeltsch, Universita¨t Stuttgart, Germany

Received August 3, 2012; Accepted October 31, 2012; Published December 11, 2012

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Funding: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0087344). The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Stroke is frequently associated with significant disability and impaired quality of life. Adverse outcomes after stroke are believed to be determined by a series of biochemical, hemodynamic, and neurophysiologic changes. Neurotrophic factors play a central role in this process through their neuroplastic effects [1]. Brain-derived neurotrophic factor (BDNF), the most abundant neurotrophin within the brain, is important for poststroke recovery, since it promotes neuro- and angiogenesis in animals [2,3]. A single nucleotide polymorphism (SNP) producing a valine-to-methionine substitution at codon 66 (val66met) in the *BDNF* gene is associated with reduced activity-dependent secretion of BDNF [4], and *BDNF* val66met/met mice have been found to exhibit greater deficits in poststroke locomotor functions and reduced angiogenesis [5]. However, associations in humans between the met allele and stroke outcomes have been less conclusive [6–9]. *BDNF* expression is also regulated by epigenetic chromatin remodeling, including DNA methylation of cytosines in cytosine-guanine (CpG) dinucleotides. An increase in CpG methylation at promoter regions on the *BDNF* gene have been found to be correlated with decreased neuronal synthesis of BDNF [10] and with an increased risk of bipolar disorder [11]. Based on these findings, it could be postulated that *BDNF* promoter methylation status will be associated with stroke outcomes, although this hypothesis has not been tested yet. Using data from a longitudinal study of a post-stroke cohort, we examined the roles of *BDNF* val66met polymorphism and promoter methylation status on outcomes at two weeks and one year after the index stroke.

Methods

Study overview and participants

This analysis was carried out as a component of a larger parent study, which seeks to investigate neurologic and psychiatric
morbidity in stroke survivors using a naturalistic prospective design which has been previously described in detail [12]. Participants were consecutively recruited from all patients with recent ischemic stroke hospitalized within the Department of Neurology of Chonnam National University Hospital, Gwangju, South Korea. Stroke severity was assessed in all hospitalized patients at the time of admission (prior to treatment, usually within 6 hours of stroke onset) and treatment was carried out by the research neurologists based on the guidelines for the management of stroke [13] over the study period. About 2 weeks poststroke, all patients were approached regarding study participation. Inclusion criteria were: i) confirmed ischemic stroke by brain magnetic resonance imaging (MRI), or computed tomography (CT) if MRI was contraindicated; ii) ability to complete the necessary investigations and questionnaires; and iii) capacity to understand the objective of the study and provide informed consent. Exclusion criteria were: i) severe physical illnesses which were life-threatening or interfering with the recovery from stroke; ii) communication difficulties due to dysphasia or dysarthria precluding informed consent and questionnaire completion; iii) any of the following comorbid neuro-psychiatric conditions: dementia, Parkinson’s disease, brain tumor, epilepsy, psychoses, alcohol and substance dependence; iv) severe physical illnesses limiting movement prior to stroke; and v) Mini-Mental State Examination (MMSE) [14] score of <16. The recruitment period was from 2006 to 2010 and attempts were made to follow up all participants after one year. Overall, assessments were made at the time of admission, 2 weeks and 1 year after the stroke to investigate, in the context of the wider study, a broad range of consequences of stroke at both acute and chronic stages. All participants gave written informed consent and the study was approved by the Chonnam National University Hospital Institutional Review Board.

Of 423 patients eligible and consenting to participate in the study, 286 (68%) agreed to provide blood samples and formed the sample for this analysis. There were no significant differences between participants and non-participants with respect to any demographic and clinical characteristics (all p-values ≥0.1). At 1 year, 222 (78%) were followed up (MMSE was available in 201). Those present or not at 1 year did not significantly differ at baseline with respect to any demographic and clinical characteristic (all p-values≥0.1). The mean (SD) time from admission to assessment points were 12.3 (3.0) days and to 1 year were 13.2 (3.6) months.

Evaluations for stroke severity

Global disability was evaluated by the modified Rankin Scale (mRS) [15], the scores of which range from 0–6 with higher scores indicating more severe disability. Stroke severity was measured using the National Institutes of Health Stroke Scale (NIHSS; scores ranging 0–42 with higher scores indicating more severe pathology) [16], physical disability was measured with the Barthel Index (BI; scores ranging 0–100 with lower scores indicating more severe disability) [17], and cognitive function was evaluated by the MMSE (scores ranging 0–30 with lower scores indicating lower cognitive function). Scores on the mRS, NIHSS, and BI were obtained at admission, at 2 week and at 1 year assessment points, while the MMSE scores were available at the 2 week and at 1 year evaluation points.

BDNF genotyping and DNA methylation analysis

Blood samples were obtained in a subsample who agreed to this. DNA was extracted from venous blood, and genotyping and DNA methylation analysis were conducted using standard procedures. For genotyping, polymerase chain reaction (PCR) and the PCR-based restriction fragment length polymorphism (RFLP) assays were performed. The primer sequences used were the forward primer 5’-ACTCTGGAGCGTGATGAG-3’ and the reverse primer 5’-ACTACTGAGCATGACCCTGGAG-3’. The amplification conditions were pre-denaturation at 95 °C for 5 minutes, followed by 40 cycles consisting of denaturation at 95 °C for 30 seconds, 62 °C for 30 seconds and 72 °C for 30 seconds, and post-elongation at 72 °C for 5 minutes, with a final maintenance step at 4 °C. The PCR products were digested at 37 °C with the corresponding restriction enzyme (NcoI), and gel electrophoresis was used to detect the 169G (val, 99 and 72 bp fragments) and 196A (met, 171 bp fragment) alleles. The BDNF promoter region for analyzing methylation status is presented in Figure 1. These data have been deposited in GenBank (accession number: BankIt1568919 BDNF JX848620). A CpG-rich region of the promoter between −694 and −577, relative to the transcriptional start, including seven CpG sites were analyzed as in other studies [18,19]. Genomic DNA (1 µg) was extracted from leukocytes using QIAPrep DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer’s suggested protocol, and was bisulfite-treated using the EpiTech Bisulfite Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. A 118 bp fragment of BDNF promoter was amplified by PCR from bisulfite-treated DNA using the forward and reverse primers designated in Figure 1. PCR conditions were 95 °C for 15 minutes, followed by 45 cycles of 95 °C for 15 seconds, 57 °C for 30 seconds, and 72 °C for 15 seconds, with a final extension of 5 minutes at 72 °C. PCR products were sequenced using the PSQ 96M Pyrosequencing system (Biotage) according to the manufacturer’s protocol with the following sequencing primers designated in Figure 1. The methylation percentage at each CpG region was quantified using the Pyro Q-CpG software, version 1.0.9 (Biotage). The genotype was categorized as ‘val/val’, ‘val/met’, and ‘met/met’. The individual methylation percentages at seven CpG sites of a promoter region and their average values were estimated.

Demographic and clinical covariates

Those characteristics potentially associated with stroke outcomes were considered as covariates in this analysis. Age, gender, year of education, previous history of stroke, and comorbidity with heart disease, hypertension and diabetes were recorded according to information obtained from the participant or their caregiver, or by medical records, as appropriate. Stroke location by hemisphere was divided into left, right, and bilateral using the brain MRI or CT imaging. Blood pressure, and serum glucose and total cholesterol levels were measured. Depressive symptoms were evaluated by the Hamilton Depression Rating Scale (HAM-D) [20], scores ranging 0–52 with higher scores indicating more severe pathology.

Statistical analyses

Patients were dichotomized into two groups at each evaluation point by applying the mRS score cut-off 1 (no significant disability)/2 (slight disability). Demographic and clinical characteristics, scores on NIHSS and BI at admission, and HAM-D scores at 2 weeks were compared according to the mRS score cut-off at admission using t-tests or χ² tests as appropriate. To compare the BDNF genotype distribution and methylation percentages between patients with good outcome (mRS grade≤1) and poor outcome (mRS grade≥2) at 2 weeks and 1 year, pairwise and multiple logistic regression analyses were conducted adjusting for those demographic and clinical characteristics at admission which were associated with the mRS outcome (p-value<0.1). The interactive effects of BDNF genotype and methylation percentages on the
outcomes were investigated using multivariable logistic regression models with the same adjustment. To investigate the associations with scores on stroke assessment scales (NIHSS, BI, and MMSE) across the evaluation points, repeated measures ANOVA tests for BDNF genotype and partial correlation tests for BDNF methylation percentages were conducted adjusting for those demographic and clinical characteristics at admission which were associated with the mRS grades (p-value < 0.1). Additional analyses were carried out to compare those demographic and clinical characteristics at admission which were associated with the mRS grades (p-value < 0.1) by BDNF genotype and tertiles of CpG average methylation percentage, and to investigate the associations between BDNF genotype and promoter methylation percentages. Statistical analyses were carried out using SPSS 13.0 software.

Results

Demographic and clinical characteristics

Overall distributions of sample characteristics are summarized in the first column of Table 1. These characteristics are compared

| Table 1. Baseline characteristics by modified Rankin Scale (mRS) grades at admission. |
|---------------------------------------------------------------|
| All patients (N = 286) | mRS=1 (N = 98) | mRS=2 (N = 188) | p-value* |
|------------------------|---------------|------------------|---------|
| Age, mean (SD) years   | 64.5 (9.5)    | 63.1 (9.4)       | 65.2 (9.5) | 0.087 |
| Gender, N (%) male     | 169 (59.1)    | 63 (64.3)        | 106 (56.4) | 0.197 |
| Education, mean (SD) year | 8.5 (5.0)   | 8.9 (4.9)        | 8.3 (5.1) | 0.319 |
| Previous stroke, N (%) | 28 (9.8)      | 8 (8.2)          | 20 (10.6) | 0.504 |
| Heart disease, N (%)   | 27 (9.4)      | 13 (13.3)        | 14 (7.4) | 0.110 |
| Hypertension, N (%)    | 141 (49.3)    | 44 (44.9)        | 97 (51.6) | 0.282 |
| Diabetes, N (%)        | 84 (29.4)     | 30 (30.6)        | 54 (28.7) | 0.739 |
| Stroke hemisphere, N (%) |            |                  |         |
| Left                  | 130 (45.5)    | 49 (50.0)        | 81 (43.1) | 0.495 |
| Right                 | 141 (49.3)    | 45 (45.9)        | 96 (51.1) |         |
| Systolic blood pressure, mean (SD) mmHg | 138.5 (21.0) | 138.0 (25.6) | 138.8 (18.2) | 0.801 |
| Diastolic blood pressure, mean (SD) mmHg | 84.2 (15.1) | 82.2 (16.2) | 85.2 (14.5) | 0.111 |
| Glucose, mean (SD) mg/dL | 148.4 (69.2) | 150.3 (77.4) | 147.4 (64.6) | 0.736 |
| Total cholesterol, mean (SD) mg/dL | 188.7 (38.9) | 191.2 (38.5) | 187.4 (39.2) | 0.444 |
| National Institutes of Health Stroke Scale, mean (SD) score | 3.4 (1.2) | 1.1 (0.8) | 4.6 (3.3) | <0.001 |
| Barthel Index, mean (SD) score | 80.1 (23.0) | 97.6 (5.8) | 71.0 (23.3) | <0.001 |
| Hamilton Depression Rating Scale at 2 week, mean (SD) score | 7.9 (6.3) | 6.9 (6.1) | 8.4 (6.3) | 0.047 |

*p-value using t-tests or χ² tests, as appropriate.

Figure 1. BDNF promoter regions for analyzing methylation status. Of the CpG-rich region of BDNF promoter, the portion analyzed by bisulfite pyrosequencing is shown. The CpGs are in underlined and bold font and numbered. Forward and backward primers and sequencer are designated. Numbering of the gene sequence is relative to the transcriptional start site.

doi:10.1371/journal.pone.0051280.g001
Table 2. Confounding factors at baseline by BDNF genotype and tertiles of CpG average methylation percentage.

| BDNF genotype | val/val (N = 66) | val/met (N = 157) | met/met (N = 63) | p-value* |
|---------------|-----------------|------------------|-----------------|----------|
| Age           | 64.4 (10.1)     | 64.3 (9.4)       | 64.9 (9.2)      | 0.912    |
| National Institutes of Health Stroke Scale | 3.2 (3.4) | 3.5 (3.0) | 3.4 (3.3) | 0.801 |
| Barthel Index | 81.0 (23.2)     | 79.2 (22.5)      | 81.4 (24.0)     | 0.777    |
| Hamilton Depression Rating Scale | 7.3 (5.9) | 8.0 (6.5) | 8.2 (6.0) | 0.674 |

| Tertiles of BDNF promoter CpG average methylation percentage |
|---------------------------------------------------------------|
| Low (30–43%) | Middle (44–48%) | High (49–70%) | p-value* |
|---------------|-----------------|---------------|----------|
| 63.4 (8.9)   | 65.2 (9.9)      | 64.8 (9.7)    | 0.386    |
| 3.3 (2.5)    | 3.8 (3.6)       | 3.2 (3.2)     | 0.362    |
| 79.5 (20.3)  | 78.7 (25.1)     | 82.1 (23.3)   | 0.565    |
| 8.0 (6.4)    | 7.1 (5.9)       | 8.7 (6.6)     | 0.234    |

*p-value derived from ANOVA.
Data are mean (SD).
doi:10.1371/journal.pone.0051280.t002

Table 3. BDNF genotype and promoter methylation percentages by stroke outcome status at 2 week and at 1 year.

| BDNF | At 2 weeks post-stroke | At 1 year post-stroke |
|------|-------------------------|-----------------------|
|      | Good outcome (N = 163)  | Poor outcome (N = 123) | OR (95% CI) | p-value* | Good outcome (N = 146) | Poor outcome (N = 76) | OR (95% CI) | p-value* |
| Genotype, N (%) |                      |                       |             |          |                      |                       |             |          |
| val/val          | 45 (27.6)             | 21 (17.1)             | Ref         |          | 39 (26.7)            | 12 (15.8)             | Ref         |          |
| val/met          | 86 (52.8)             | 71 (57.7)             | 1.97 (0.98–3.97) | 0.018    | 80 (54.8)            | 41 (53.9)             | 1.65 (0.76–3.59) | 0.038    |
| met/met          | 32 (19.6)             | 31 (25.2)             | 2.75 (1.20–6.31) |          | 27 (18.5)            | 23 (30.3)             | 3.14 (1.29–7.68) |          |
| Methylation percentages, mean (SD) |
| CpG 1          | 13.7 (3.5)            | 13.8 (3.5)            | 1.00 (0.93–1.08) | 0.988    | 13.6 (3.5)           | 14.2 (3.9)            | 1.06 (0.98–1.15) | 0.179    |
| CpG 2          | 97.8 (7.0)            | 96.6 (10.0)           | 0.98 (0.95–1.01) | 0.253    | 96.3 (9.7)           | 98.5 (5.7)            | 1.04 (0.99–1.09) | 0.068    |
| CpG 3          | 72.3 (6.3)            | 72.5 (6.4)            | 1.00 (0.96–1.05) | 0.881    | 72.4 (6.1)           | 72.3 (7.0)            | 1.00 (0.96–1.05) | 0.928    |
| CpG 4          | 30.8 (7.5)            | 29.4 (6.8)            | 0.99 (0.93–1.00) | 0.062    | 28.9 (6.5)           | 32.3 (7.5)            | 1.09 (1.04–1.15) | 0.001    |
| CpG 5          | 55.6 (12.2)           | 54.6 (10.1)           | 1.00 (0.97–1.01) | 0.392    | 55.9 (12.5)          | 54.5 (10.9)           | 0.99 (0.96–1.01) | 0.350    |
| CpG 6          | 19.5 (16.4)           | 23.0 (18.2)           | 1.00 (1.00–1.04) | 0.033    | 19.7 (15.7)          | 23.6 (19.7)           | 1.02 (0.99–1.03) | 0.079    |
| CpG 7          | 29.5 (21.9)           | 30.4 (19.2)           | 1.01 (0.99–1.02) | 0.468    | 27.1 (20.2)          | 35.4 (21.3)           | 1.02 (1.01–1.04) | 0.005    |
| CpG average    | 45.6 (6.1)            | 45.7 (6.6)            | 1.01 (0.97–1.06) | 0.660    | 44.9 (6.0)           | 47.3 (5.5)            | 1.08 (1.03–1.04) | 0.003    |

*p-value using logistic regression likelihood ratio tests adjustment for age and baseline scores on National Institutes of Health Stroke Scale, Barthel Index, and Hamilton Depression Rating Scale.
doi:10.1371/journal.pone.0051280.t003
Table 4. Multiple regression analyses of BDNF genotype and promoter methylation on poor stroke outcomes at 2 weeks and at 1 year.

| BDNF | At 2 weeks post-stroke | At 1 year post-stroke |
|------|------------------------|-----------------------|
|      | OR (95% CI) | p-value | OR (95% CI) | p-value |
| Genotype |                |          |                |          |
| val/val | Ref | Ref | Ref | Ref |
| val/met | 1.87 (0.92–3.78) | 0.027 | 1.53 (0.69–3.37) | 0.048 |
| met/met | 2.58 (1.12–5.95) | 0.048 | 2.53 (1.01–6.38) | 0.048 |

Methylation site

| CpG average | NA | 0.96 (0.86–1.07) | 0.414 |

| CpG 4 | NA | 1.09 (1.02–1.17) | 0.011 |
| CpG 6 | 1.01 (0.99–1.03) | 0.051 | NA |
| CpG 7 | NA | 1.02 (0.99–1.05) | 0.092 |

* p-value using logistic regression likelihood ratio tests adjustment for age and baseline scores on National Institutes of Health Stroke Scale, Barthel Index, and Hamilton Depression Rating scale.

Table 5. Multivariate analyses examining the interactive effects of BDNF val66met polymorphism and promoter methylation on poor stroke outcomes at 2 weeks and at 1 year.

| BDNF val66met polymorphism x CpG average | At 2 weeks (N=286) | At 1 year (N=222) |
|------------------------------------------|-------------------|-------------------|
|                                            | Wald | OR (95% CI) | Wald | OR (95% CI) |
| val66met polymorphism x CpG 1            | 2.55 | 0.90 (0.79–1.02) | 0.43 | 0.96 (0.84–1.09) |
| val66met polymorphism x CpG 2            | 0.02 | 1.01 (0.94–1.08) | 0.15 | 0.97 (0.85–1.11) |
| val66met polymorphism x CpG 3            | 0.18 | 0.98 (0.91–1.07) | 2.42 | 0.91 (0.81–1.03) |
| val66met polymorphism x CpG 4            | 0.04 | 1.01 (0.94–1.08) | 0.01 | 0.99 (0.92–1.08) |
| val66met polymorphism x CpG 5            | 0.83 | 0.96 (0.89–1.05) | 0.01 | 1.00 (0.96–1.05) |
| val66met polymorphism x CpG 6            | 0.20 | 0.99 (0.97–1.09) | 0.43 | 1.01 (0.98–1.04) |
| val66met polymorphism x CpG 7            | 0.02 | 1.01 (0.98–1.02) | 0.21 | 1.01 (0.98–1.03) |
| val66met polymorphism x CpG average      | 0.49 | 0.98 (0.91–1.05) | 0.21 | 1.02 (0.94–1.10) |

All data are adjusted for age and baseline scores on National Institutes of Health Stroke Scale, Barthel Index, and Hamilton Depression Rating scale.

doi:10.1371/journal.pone.0051280.t005
Discussion

Principal findings in this longitudinal study of a post-stroke cohort were that the BDNF val66met polymorphism was independently associated with acute and long-term poor outcomes, and with worsening of physical disability and cognitive function. Higher BDNF promoter methylation status was independently associated with long-term but not with acute outcome, and was significantly associated with the worsening of physical disability and cognitive function particularly over one year. No significant effects on stroke severity measured by the NIHSS were observed. No significant genotype-methylation interactions were found.

BDNF is the most abundant neurotrophin and regulates neuronal plasticity within the brain [1]. The crucial role of BDNF in stroke recovery has been repeatedly suggested in animal studies [23], and BDNF administration improves sensory motor function improvement is associated with BDNF upregulation [23], and BDNF administration improves sensory motor recovery [3,24,25], whereas BDNF blockade prevents recovery [5,26]. However, this hypothesis has been controversial in human research, in that no significant increase in blood BDNF levels were found after stroke [27], and associations between the BDNF met allele and ischemic stroke outcomes were not found to be significant [6,7], although significant associations have been found in aneurismal subarachnoid haemorrhage [8,9]. To our knowledge, our study is the first to report significant associations between the met allele and outcomes over 1 year after ischemic stroke. Ethnic differences in the risk allele frequency may underlie the positive findings. Our sample had higher BDNF met allele (49%) compared to reports from Western populations (18–21%) [6,8,9], although allele frequencies were similar to reports from other Asian populations [28]. In addition, the BDNF met allele frequency of this stroke patients were similar to that from a population based study of a Korean elderly (47%) [29]. These may have increased the statistical power to detect associations, and also raise the question of public health relevance in East Asian populations in terms of the increased genetic vulnerability to poor outcomes after stroke.

CpG methylation status at promoter regions on the BDNF gene may influence stroke outcomes, since it also regulates BDNF release [10], and so we took the opportunity to test the hypothesis within a defined post-stroke cohort. To our knowledge, ours is the first report of BDNF methylation status with respect to stroke outcomes. As postulated, higher methylation percentages were independently associated with outcomes particularly at 1 year after stroke. These are consistent with recent findings that BDNF methylation is associated with memory consolidation [30] and exercise related neuronal plasticity [31] in rats, although these were not stroke models.

It is noteworthy that measures of higher BDNF methylation status were more strongly associated with longterm stroke outcomes, while the BDNF met allele was associated with both acute and longterm outcomes. BDNF has shown early genomic response following ischemic injury in rat brain [32], and therefore the influence of BDNF genotype on BDNF secretion might be initiated at the acute phase of stroke. Furthermore, given that the BDNF met allele is associated with reduced activity-dependent secretion of BDNF [4], functional deficiency at the chronic phase of stroke might also be associated with less BDNF secretion. However, it is not known how BDNF methylation status influences BDNF secretion over time after stroke, and it is important to bear in mind that there were no significant genotype-methylation interactions (Table 5) and no direct associations between BDNF

Table 6. BDNF promoter methylation percentages by val66met polymorphism.

| Methylation site | Mean (SD) methylation percentage by val66met polymorphism | p-value* |
|------------------|----------------------------------------------------------|----------|
|                  | val/val (N = 66) | val/met (N = 157) | met/met (N = 63) |       |
| CpG 1            | 14.0 (3.6)      | 13.4 (3.5)      | 14.2 (3.2)     | 0.219 |
| CpG 2            | 97.6 (7.9)      | 96.7 (9.9)      | 98.7 (2.0)     | 0.115 |
| CpG 3            | 72.9 (6.7)      | 71.8 (6.9)      | 73.4 (4.1)     | 0.183 |
| CpG 4            | 30.6 (7.9)      | 29.6 (7.8)      | 31.3 (4.8)     | 0.221 |
| CpG 5            | 55.9 (11.3)     | 54.2 (11.3)     | 56.9 (13.1)    | 0.289 |
| CpG 6            | 17.8 (14.9)     | 21.6 (17.9)     | 23.0 (17.6)    | 0.188 |
| CpG 7            | 28.0 (18.4)     | 28.6 (19.9)     | 34.7 (24.6)    | 0.110 |
| CpG average      | 45.3 (6.0)      | 45.4 (6.1)      | 46.9 (5.8)     | 0.116 |

*p-value derived from ANOVA.

doi:10.1371/journal.pone.0051280.t006

Table 7. Scores on stroke assessment scales at three evaluation points.

| National Institutes of Health Stroke Scale | At admission (N = 286) | At 2 weeks (N = 286) | At 1 year (N = 222) |
|-------------------------------------------|------------------------|----------------------|---------------------|
| At admission (N = 286)                    | 3.4 (3.2; 0–18)        | 2.1 (2.2; 0–10)      | 1.1 (1.7; 0–7)      |
| Barthel Index                              | 80.1 (23.0; 0–100)     | 85.8 (18.4; 0–100)   | 93.1 (14.6; 25–100) |
| Mini-Mental State Examination              | 24.8 (4.0; 16–30)      | 25.3 (4.6; 3–30)*    |                     |

*Data were available in 201 patients.
Data are mean (SD; range).

doi:10.1371/journal.pone.0051280.t007
Figure 2. Comparing scores on stroke assessment scales between groups of BDNF val66met polymorphism over time.
Repeated measures ANOVA demonstrating the following: For NIHSS, no group effect of genotype (p-value = 0.976) or group by time interaction (p-value = 0.259); For BI, no group effect of genotype (p-value = 0.985) but a significant group by time interaction (p-value = 0.003); For MMSE, no group effect of genotype (p-value = 0.826) but a significant group by time interaction (p-value = 0.035) after adjustment for age and Hamilton Depression Rating Scale score.

doi:10.1371/journal.pone.0051280.g002

Table 8. Partial correlations between BDNF promoter methylation percentages and scores on stroke assessment scales.

| Methylation site | Partial correlation coefficient between methylation percentages and outcome measures |
|------------------|----------------------------------------------------------------------------------|
| CpG1             | Adjusted partial correlation coefficient on admission to 2 weeks (N = 286)        |
| CpG2             | Adjusted partial correlation coefficient on admission to 1 year (N = 222)         |
| CpG3             | Change in score from admission to 2 weeks (N = 286)                               |
| CpG4             | Change in score from admission to 1 year (N = 222)                                |
| CpG5             | Score at admission (N = 286)                                                      |
| CpG6             | Score at 2 weeks (N = 286)                                                        |
| CpG7             | Score at 1 year (N = 222)                                                         |
| CpG average      | Change in score from 2 weeks to 1 year (N = 201)                                  |

All data are adjusted for age and Hamilton Depression Rating Scale score.

doi:10.1371/journal.pone.0051280.t008
genotype and methylation percentages (Table 6) in our sample. This is discordant with a previous study which reported the BDNF methylation status was dependent on genotype, and consequently had a differential effect on major psychosis (Mill et al. 2008) [11]. However, further research into the role of BDNF in stroke patients is indicated.

Our study has several strengths, as well as being the first to report on associations between BDNF methylation status and stroke outcomes. Stroke outcomes were assessed at a similar time point (two weeks and one year after stroke) in all participants, which reduced the risk of error arising from heterogeneous examination times. Participants were recruited consecutively from all eligible patients with a recent stroke at the study hospital, which reduced the likelihood of selection bias and increased the potential generalizability. In addition, a range of covariates were considered in the analyses, and the follow-up rate was reasonable and not apparently differential with respect to risk factors of interest.

Our study also has some limitations. Blood samples were obtained in only 68% of the total stroke sample in the parent study, although there were no significant differences in demographic and clinical characteristics between those with and without samples. Second, methylation status was investigated with only one CpG island of the BDNF gene. Further studies of other CpG islands for this gene, and for genome-wide DNA methylation are therefore needed. Third, the BDNF promoter methylation profile could be tissue specific. Although our results on methylation status in genomic DNA isolated from leukocytes with longterm stroke outcomes have some prognostic value, it is not clear whether this would be the relevant tissue/cell type to infer BDNF expression of most importance for stroke. Fourth, the sample size was limited for detecting associations particularly with BDNF genotype and gene-methylation interactions. Another important consideration is that participants with severe cognitive impairment or aphasia were excluded due to the particular study design, and therefore the present findings can only be assumed to apply to people with mild to moderate stroke severity without these deficits. Lack of associations with the scores on NIHSS might be related to this issue.

In conclusion, our findings support a role for BDNF in poststroke recovery and have several potential implications. Considering the significant morbidty poststroke, more careful evaluation and management are indicated for those with increased genetic vulnerability, particularly for East Asian populations who had higher met allele frequency. The DNA promoter methylation profile of the BDNF might be a prognostic biomarker for long-term stroke outcomes. However as with any first report, our findings need further replication by other study groups. Methylation tests might have clinical utility because they are non-invasive, DNA based analyses are convenient to conduct due to the amplifiable and stable nature of DNA, and are advantageous over blood BDNF levels which poorly accurately reflect brain BDNF status after stroke [27]. There are also potential treatment implications. Development of a new drug, which could increase BDNF release and regulate BDNF promoter methylation, may be helpful for enhancing stroke recovery [33]. In essence, epigenetics is at an embryonic stage, although it is a potentially promising area of enquiry in stroke research. We believe that our study represents an important first step to elucidate the role of epigenetic mechanisms in stroke recovery, and as such is a reference point for future research.

Supporting Information

Figure S1 Primary experimental data for BDNF val66met genotyping. Left panel: BDNF val66met amplification products using 3% agarose gel. Lane M-50 bp DNA ladder. Lane1-4-PCR-BDNF product of 171 bp. Right panel: BDNF val66met genotyping using 3% agarose gel. Lane M-50 bp DNA ladder. BDNF val/val genotype was presented with 99 and 72 bp (lane 2, 5, and 10); val/met genotype with 171, 99, and 72 bp (lane 1, 3, 4, 6, 8, and 9); and met/met genotype with 171 bp bands (lane 7 and 11).

(TIF)

Figure S2 Primary experimental data for BDNF DNA promoter methylation analysis. Seven CpG sites methylation percentages of the BDNF promoter region in a patient sample.

(TIF)

Author Contributions

Conceived and designed the experiments: JMK. Performed the experiments: JMK MSP KHC. Analyzed the data: HJK SYK SWK ISS JSY. Contributed reagents/materials/analysis tools: HRK MGS. Wrote the paper: JMK. Interpreted the data: RS. Revised the article critically: RS.

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