Lei Zhao, et al.: AHCY methylation increases the risk of ischemic stroke

DNA methylation of AHCY may increase the risk of ischemic stroke

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Genetic factors play an important role in the pathogenesis of ischemic stroke. Of these, epigenetic modifications provide a new direction for the study of ischemic stroke pathogenesis. This study aimed to determine the correlation between DNA methylation of the gene encoding S-adenosylhomocysteine hydrolase (AHCY) and the risk of ischemic stroke in 64 ischemic stroke patients and 138 patients with traumatic brain injury (control group). The methylation level of AHCY was analyzed using quantitative methylation-specific polymerase chain reaction. Statistically significant differences in AHCY methylation levels were observed between the case group [medians (interquartile range): 0.13% (0.09%, 0.27%)] and the control group [0.06% (0.00%, 0.17%), \( p < 0.0001 \)], and these associations remained significant in both male \( p = 0.003 \) and female \( p = 0.0005 \) subjects. A subgroup analysis by age revealed a considerably higher percentage of methylated AHCY in the case group than the control group in all age groups (age < 60 years, \( p = 0.007 \); age ≥ 60 years, \( p < 0.0001 \)). A receiver operating characteristic (ROC) curve analysis revealed a trend toward a role for AHCY methylation as an indicator of risk in all ischemic patients [area under the curve (AUC) = 0.70, \( p = 0.0001 \)], male patients (AUC = 0.67, \( p = 0.004 \)), and female patients (AUC = 0.75, \( p = 0.0002 \)). Our study confirmed a significant association between the AHCY DNA methylation level and the risk of ischemic stroke, suggesting that this gene methylation pattern may be a potential diagnostic marker of ischemic stroke.

**KEYWORDS:** Ischemic stroke; DNA methylation; S-adenosylhomocysteine hydrolase; sex; age
INTRODUCTION

Stroke is among the three leading causes of disease-related mortality in humans and a common cause of chronic disability in adults [1], and accordingly poses a serious risk to human health and places a heavy burden on society [2]. Stroke can be divided etiologically as ischemic or hemorrhagic stroke [3]. Regarding the former type, common risk factors such as diabetes, hypertension, and hyperlipidemia can explain only a small fraction of the actual risk [4]. Increasingly, studies have shown that genetic factors play key roles in the pathogenesis of ischemic stroke [5,6]. For example, genome-wide association studies have identified several genetic variations that contribute to the risk factors associated with ischemic stroke [7,8]. Other recent studies have indicated that epigenetic changes play important roles in stroke development [9,10], and even that the gap between environment factors and ischemic stroke may be bridged by an epigenetic mechanism [11].

DNA methylation is an epigenetic mechanism by which the spatial structure of a DNA molecule is transformed to affect the interactions between DNA and proteins. Potentially, DNA hypermethylation could induce the silencing of genes and the loss of gene function [12]. Aberrant DNA methylation has been observed in various vascular developmental diseases, including brain arteriovenous malformations [13], intracranial aneurysms [14], and cerebral infarctions [15]. Furthermore, the DNA methylation level was reported as a key regulator of vascular smooth muscle cell dedifferentiation and vascular remodeling [16].

S-adenosylhomocysteine hydrolase (AHCY) is the only enzyme known to catalyze the hydrolysis of S-adenosyl-L-homocysteine (SAH) to yield homocysteine and adenosine. Hence, this enzyme can relieve the inhibitory activities of SAH on S-adenosylmethionine (SAM)-dependent transmethylation reactions [17,18]. However, dysfunctional AHCY activity can result in serious pathological consequences, such as childhood death [19], Alzheimer's disease [20], Parkinson's disease [21], age-related diseases [22], neuroblastoma [23], and
large-artery atherosclerotic stroke [24]. Despite these negative outcomes, few studies have investigated the relationship between AHCY and the risk of ischemic stroke. As the homocysteine level is considered an independent predictor of severe neurological impairment in ischemic stroke patients, we hypothesized that DNA methylation of AHCY may play a key role in the pathological development of ischemic stroke. In this case-control study, we explored the role of DNA methylation of AHCY as a risk factor for ischemic stroke in a Chinese population.

MATERIALS AND METHODS

Samples and clinical data

Approval of the study procedure was provided by the Ethics Committee of the Ningbo First Hospital. The investigation included 64 ischemic stroke patients and 138 controls (patients with traumatic brain injury without any vascular lesions) that visited the Stroke Center, Ningbo First Hospital, between September 2013 and December 2015. In all patients, fulfillment of the diagnostic criteria based on international standardized definitions was confirmed using magnetic resonance imaging and cranial computed tomography scans. Any subject presenting with a serious liver or kidney disease or cardiovascular disease was excluded.

Peripheral venous blood was collected from all patients in the morning after fasting and stored in tubes containing EDTA (anticoagulant). General information, including sex, age, and the levels of triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), apolipoprotein A (ApoA), apolipoprotein B (ApoB) and apolipoprotein E (ApoE), was collected for all patients. Bioindicators were measured using an automatic biochemical analyzer (AU2700; Olympus Corp., Tokyo, Japan).

SYBR Green-based quantitative methylation-specific PCR (qMSP)
Genomic DNA was extracted from peripheral blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The DNA bisulfite conversion was assessed using the EZ DNA Methylation - Gold™ Kit (Zymo Research, Orange, CA, USA). The qMSP assay was performed using a LightCycler480 device (Roche Diagnostics, Mannheim, Germany). Human ACTB was used as the internal reference gene to standardize the amount of target DNA. The percentage of methylated reference (PMR) of the genes in each sample was calculated using the 2–ΔΔCt quantification approach [25]. The sequences of specific primers were as follows: AHCY forward, 5ʹ-GGTCGTAATCGGTTGAT-3ʹ and reverse, 5ʹ-CAATTCCCTATTCCCAACTAAA-3ʹ; ACTB forward, 5ʹ-TGGTGATGGAGGAGGTTTAGTAAGT-3ʹ and reverse, 5ʹ-AACCAATAAAAACCTACTCCTCCCTTAA-3ʹ. The entire protocol for qMSP was previously described [26].

Statistical analyses
Statistical data were analyzed and plotted using SPSS Version 20.0 (IBM Corp, Armonk, NY, USA) and GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, USA). Measured data were assessed using the one-sample Kolmogorov–Smirnov test for normality, and normally distributed data were assessed using the independent samples t-test. The data are presented as means ± standard deviations. Nonparametric tests were used to assess data that did not conform to a normal distribution, and these data are expressed as medians (interquartile range). A receiver operating characteristic (ROC) curve analysis was performed to evaluate the sensitivity of AHCY DNA methylation as an indicator of an ischemic stroke diagnosis. All statistical tests were bilateral. Statistical significance was defined as a p value < 0.05.

RESULTS
The clinical characteristics of patients in the ischemic stroke and control groups are shown in Table 1. The HDL level was considerably lower in stroke patients (1.03 ± 0.27) than in controls (1.13 ± 0.26, p = 0.022). No significant differences were observed with respect to the other demographic and clinical characteristics (age, sex, hypertension, diabetes, alcohol consumption, smoking, TG, TC, LDL, ApoA, ApoB, and ApoE; p > 0.05) between the two groups.

Target sequences (Chr20: 34303211–34303334) located in the CGI region of AHCY were selected to determine the DNA methylation level (Fig. 1). As shown in Fig. 2, the PMR level of AHCY was significantly higher in ischemic stroke patients [0.13% (0.09%, 0.27%)] than in controls [0.06% (0.00%, 0.17%), p < 0.0001]. A subgroup analysis stratified by sex similarly demonstrated significantly higher PMR levels of AHCY in both male [0.12% (0.09%, 0.24%)] and female ischemic stroke patients [0.13% (0.10%, 0.33%)] relative to controls [0.06% (0.00%, 0.16%), p = 0.003 and 0.05% (0.00%, 0.17%), p = 0.0005, respectively]. A subgroup analysis stratified by age similarly revealed a significantly higher PMR level of AHCY in the ischemic stroke patients relative to the controls, regardless of age group [<60 years, 0.13% (0.10%, 0.29) vs 0.07% (0.00%, 0.17%), p = 0.007; age ≥60 years, 0.13% (0.07%, 0.27%) vs 0.04% (0.00%, 0.17%), p < 0.0001; Fig, 3].

As shown in Fig 4, a ROC analysis demonstrated a trend toward AHCY methylation as an indicator of the ischemic stroke risk in all ischemic patients [area under the curve (AUC) =0.70, p = 0.0001], as well as in male (AUC = 0.67, p = 0.004) and female patients (AUC = 0.75, p = 0.0002).

**DISCUSSION**

The development of the field of epigenetics has elicited significant interest in the correlations between human diseases and epigenetic factors. Particularly, epigenetic modifications provide a new direction for studies of the pathogenesis of ischemic stroke. Although
epigenetic changes can result in heritable changes in gene expression, the underlying DNA sequence is not altered. Rather, these modifications enable a more selective expression or suppression of genes. DNA methylation is a highly studied epigenetic mechanisms that plays important roles in regulating gene expression. As noted previously, abnormal DNA methylation can induce related diseases such as stroke [27], cardiovascular disease [28], and malignancies [29]. However, the role of DNA methylation in the pathogenesis of ischemic stroke is yet to be fully elucidated.

In this study, we evaluated the potential association of AHCY methylation with ischemic stroke and determined a significantly higher frequency of this epigenetic modification among ischemic stroke patients relative to controls, irrespective of sex and age. AHCY, which is located on chromosome 20, contains 10 exons and encodes AHCY. This broadly expressed enzyme hydrolyzes SAH, which is produced by the demethylation of S-adenosylmethionine (SAM), a molecule that participates in the methylation of DNA and histones [30]. AHCY catalysis produces homocysteine from the metabolic precursor, SAH, and therefore the inhibition of AHCY can result in an accumulation of SAH in cells. Additionally, SAH is a potent feedback inhibitor of most SAM-dependent transmethylation reactions, including those related to the methylation of cellular components such as DNA, RNA, lipids, proteins, and neurotransmitters [17,31]. Therefore, AHCY methylation may affect AHCY expression and activity, leading to disordered SAH metabolism, with negative impacts on the regulation of biological processes. A previous study demonstrated that mutations in AHCY were associated with prognosis in neuroblastoma patients [32], suggesting that this gene could be considered as a potential prognostic factor in this context [23]. Giusti et al. identified a potentially significant difference in the allelic frequency of the AHCY polymorphism rs819146 between ischemic stroke patients and control subjects according to the recessive model [33]. Furthermore, the AHCY haplotype was reported as a susceptibility factor for
abdominal aortic aneurysm, and this association is independent of the homocysteine modulatory role of this enzyme [34]. Therefore, AHCY methylation may regulate the enzymatic activity of AHCY, resulting in increased homocysteine levels in patients with cerebrovascular disease.

Many biomarkers of brain diseases involve DNA methylation. Jakubowski et al. [35] reported that DNA methylation may be a novel biomarker with potential etiological relevance in Parkinson's disease. Thon et al. suggested that the O6-methylguanine-DNA methyltransferase promoter methylation status is crucial in glioblastoma treatment [36]. Moreover, TRIM59 and KLF14 hypermethylation in blood cells may be a valuable predictor of the molecular processes underlying familial Alzheimer's disease [37]. Recent studies suggested that age-related DNA methylation changes were independent predictors of ischemic stroke outcomes [38,39]. Li et al. [40] demonstrated a significant difference in the AHCY methylation levels between ischemic stroke patients and controls older than 60 years of age. We similarly demonstrated a significant difference in the AHCY methylation levels between ischemic cases and controls, although this result was consistent in all age groups. Our ROC analysis further confirmed that AHCY methylation is a significant predictor of the risk of ischemic stroke.

Our study had some limitations of note. First, our analysis of the association between AHCY DNA methylation and the risk of ischemic stroke focused on only one portion of the CGI. Therefore, our results may not be applicable to all AHCY sequences. Second, we did not analyze the transcription or translation of AHCY. Therefore, a mechanistic study of the association between AHCY methylation and ischemic stroke is needed. Third, the sample size of this study was small. Further evaluations with larger samples are needed to elucidate the relationship between AHCY methylation and ischemic stroke, particularly in various ethnic populations.
CONCLUSION

Our findings confirm a significant correlation between the AHCY DNA methylation level and the risk of ischemic stroke, and suggest that AHCY methylation may be a useful diagnostic biomarker of ischemic stroke.

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REFERENCES


TABLES AND FIGURES

**TABLE 1.** Clinical characteristics of all participants

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls</th>
<th>Stroke cases</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>63.44± 10.31</td>
<td>61.97 ± 11.50</td>
<td>0.81</td>
<td>0.417</td>
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<tr>
<td>Sex (M/F)</td>
<td>81/57</td>
<td>35/29</td>
<td>0.16</td>
<td>0.691</td>
</tr>
<tr>
<td>Hypertension (n)</td>
<td>52</td>
<td>26</td>
<td>0.01</td>
<td>0.902</td>
</tr>
<tr>
<td>Diabetes (n)</td>
<td>44</td>
<td>19</td>
<td>0.01</td>
<td>0.942</td>
</tr>
<tr>
<td>Drinking (n)</td>
<td>39</td>
<td>18</td>
<td>0.02</td>
<td>0.883</td>
</tr>
<tr>
<td>Smoking (n)</td>
<td>22</td>
<td>13</td>
<td>0.20</td>
<td>0.659</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.32 ± 1.09</td>
<td>1.56 ± 1.18</td>
<td>1.32</td>
<td>0.188</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>4.56 ± 1.25</td>
<td>4.29 ± 1.36</td>
<td>-1.34</td>
<td>0.191</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.13 ± 0.26</td>
<td>1.03 ± 0.27</td>
<td>-2.30</td>
<td>0.022</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.85 ± 0.90</td>
<td>2.55 ± 0.98</td>
<td>-1.97</td>
<td>0.051</td>
</tr>
<tr>
<td>ApoA (mg/dL)</td>
<td>1.11 ± 0.21</td>
<td>1.11 ± 0.24</td>
<td>0.13</td>
<td>0.895</td>
</tr>
<tr>
<td>ApoB (mg/dL)</td>
<td>1.09 ± 0.34</td>
<td>1.01 ± 0.39</td>
<td>-1.41</td>
<td>0.161</td>
</tr>
<tr>
<td>ApoE (mg/dL)</td>
<td>46.83 ± 20.42</td>
<td>43.05 ± 15.19</td>
<td>-1.24</td>
<td>0.218</td>
</tr>
</tbody>
</table>

Male (M), female (F), triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), apolipoprotein A (ApoA), apolipoprotein B (ApoB) and apolipoprotein E (ApoE).
FIGURE 1. Characteristics of target sequences in AHCY
FIGURE 2. Comparison of $AHCY$ methylation levels between cases and controls in a sex-stratified subgroup analysis.

(A): The PMR levels of $AHCY$ in male ischemic cases [0.12% (0.09%, 0.24%)] were higher than those in male controls [0.06% (0.00%, 0.16%), $p = 0.003$].

(B): The PMR levels of $AHCY$ in female ischemic cases [0.13% (0.10%, 0.33%)] were higher than those in female controls [0.05% (0.00%, 0.17%), $p = 0.0005$].

(C): The PMR level of $AHCY$ in all ischemic stroke cases [0.13% (0.09%, 0.27%)] was significantly higher than in the controls [0.06% (0.00%, 0.17%), $p < 0.0001$].
FIGURE 3. Comparison of AHCY methylation levels in a subgroup analysis stratified by age.

(A): In the <60 years age group, the PMR levels of AHCY were higher in the ischemic cases than in the controls [0.13% (0.10%, 0.29) vs 0.07% (0.00%, 0.17%), p = 0.007].

(B): In the ≥60 years age group, the PMR levels of AHCY were higher in the ischemic cases than that in the controls [0.13% (0.07%, 0.27%) vs 0.04% (0.00%, 0.17%), p < 0.0001].
FIGURE 4. Receiver operating characteristic (ROC) curve analysis of the association between \textit{AHCY} DNA methylation and ischemic stroke.

(A): In male patients, \textit{AHCY} methylation tended to indicate a diagnosis of ischemic stroke [area under curve (AUC) = 0.67, 95\% confidence interval (CI) = 0.59–0.79, \( p = 0.004 \)].

(B): In female patients, \textit{AHCY} methylation also tended to indicate a diagnosis of ischemic stroke (AUC = 0.75, 95\%CI = 0.65–0.85, \( p = 0.0002 \)).

(C): In all patients, \textit{AHCY} methylation tended to indicate a diagnosis of ischemic stroke (AUC =0.70, 95\%CI = 0.63–0.77, \( p = 0.0001 \)).