Original Article
The SNP rs915014 in MTHFR regulated by miR-661 associates with atherosclerosis

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Abstract: Genetic polymorphisms of methylene tetrahydrofolate reductase (MTHFR) were associated with atherosclerosis. This study analyzed MTHFR polymorphisms at the 3'-untranslated region for association with risk and outcome of atherosclerosis in a Chinese Han population. 500 patients and 600 healthy volunteers were enrolled for MTHFR rs915014 genotyping identified based on bioinformatics approach. The binding of miR-661 to MTHFR rs915014 was determined by luciferase assay, MTHFR expression was assessed using qRT-PCR, and plasma homocysteine levels were assayed by ELISA. Cigarette smoking, alcohol consumption, diabetes, hypertension and low levels of serum high-density lipoprotein-C were associated with an increased risk of developing ischemic stroke. MTHFR rs915014 AG and GG genotypes were significantly associated with increased risk of rs915014 compared with the GG genotype. The qRT-PCR confirmed that MTHFR rs915014 AG or GG genotypes could facilitate miR-661 binding leading to low MTHFR levels in cells. In addition, patients carrying the MTHFR rs915014 AG or GG genotypes were associated with accumulation of serum tHcy and a poor atherosclerosis outcome. In conclusion, this study demonstrates that the MTHFR rs915014 SNP is associated with increased risk in developing atherosclerosis, miR-661 binding, low MTHFR levels in cells.

Keywords: Atherosclerosis, MTHFR, polymorphism, homocysteine, miR-661

Introduction
Atherosclerosis is a devastating and life-threatening disease that is characterized by an accumulation of fibrous elements and lipids in large arteries and is the leading cause of mortality and morbidity worldwide [1-3]. Unstable atherosclerotic plaques are characterized by a relative preponderance of inflammatory cells and heightened proteolytic activity. It is believed that endothelial cells apoptosis results in the denudation or dysfunction of the intact endothelial monolayer, which causes lipid accumulation, monocyte adhesion, and inflammatory reactions leading to atherosclerotic lesion [4, 5].

Methylenetetrahydrofolate reductase (MTHFR) is the key enzyme involved in plasma homocysteine (tHcy) metabolism by catalyzing the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a methyl donor during tHcy remethylation [6]. The tHcy is a crucial intermediate in methionine metabolism and causes excessive production of reactive oxygen species (ROS) [7]. This plays an important role in the regulation of cell signaling and homeostasis. MTHFR is localized at chromosome 1 p36.3 of human genome, and to date there are over 40 point mutations or SNPs in the MTHFR gene identified [8-10]. The polymorphism 1801133 involves substitution of C to T at position 677 (C677T), causing the conversion of alanine to valine. This missense mutation will result in approximately 70% and 35% reductions of normal MTHFR enzymatic activity in TT and CT genotype carriers [11].

To date, non-coding RNAs (ncRNAs) including long non-coding RNAs (lncRNAs) and microRNAs (miRNAs), have gained increasing attention in tumor malignant processes [12, 13]. MicroRNAs (miRNA) are small, non-coding RNA molecules. MicroRNAs (miRNAs) are a family of small noncoding RNAs with 20-25 nucleotides, which function through binding to their comple-
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Bioinformatic analysis of miRNAs binding to MTHFR SNP and linkage disequilibrium analysis among MTHFR polymorphisms

Bioinformatics software (http://www.bioguo.org/miRNASNP/) was used to detect the candidate SNPs which could affect MTHFR gene regulation via miRNAs.

Cell line, culture, and miR-661 transfection

Embryonic Kidney 293T cells were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in RPMI-1640 (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in a humidified incubator with 5% CO₂ at 37°C. The miR-661 mimics and control were obtained from GenePharma (Shanghai, China) and transfected into 293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

Construction of luciferase-based reporter plasmids and luciferase reporter assay

MTHFR 3'-UTR fragments containing either A or G alleles were amplified using PCR from genomic DNA and cloned into pGL3-promoterless luciferase-based plasmids (Promega, Madison, WI, USA) multiple cloning sites. MTHFR 3'-UTR fragments potentially binding to miR-661, predicted by bioinformatic analysis or a mutated sequence with the predicted target sites, were cloned using PCR from genomic DNA and inserted into the pGL3 promoter vector (Genscript, Nanjing, China). The cloned plasmids were amplified and verified by DNA sequencing. Next, 293T cells were plated onto 24-well plates and transfected with 100 ng of pGL3-MTHFR wild, pGL3-MTHFR mutant, and miR-661 mimics, respectively. A Renilla luciferase vector pRL-SV40 (5 ng) was also co-transfected as a normalization and transfection efficiency control. Each experiment was performed in triplicate and luciferase activity was assessed 48 h after transfection using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

ELISA of plasma homocysteine levels

Plasma levels of tHcy in patients was assessed using a tHcy ELISA kit (Green Stone, Bern, Switzerland), according to the manufacturer’s
SNP in MTHFR regulated by miR-661

Table 1. Frequency distributions of selected variables in patients and healthy controls

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cases (n=500)</th>
<th>Control (n=600)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>64 (49-79)</td>
<td>65 (51-78)</td>
<td>0.51</td>
</tr>
<tr>
<td>Sex (Male/Female)</td>
<td>329/171</td>
<td>399/201</td>
<td>0.81</td>
</tr>
<tr>
<td>Smoking (Yes/No)</td>
<td>367/133</td>
<td>344/256</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Drinking (Yes/No)</td>
<td>307/193</td>
<td>301/299</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes (Yes/No)</td>
<td>299/201</td>
<td>109/491</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension (Yes/No)</td>
<td>402/98</td>
<td>111/489</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.01 (4.04-5.39)</td>
<td>4.59 (4.05-5.34)</td>
<td>0.21</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.38 (1.22-1.49)</td>
<td>1.23 (1.11-1.52)</td>
<td>0.02</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>2.58 (2.54-3.12)</td>
<td>2.54 (2.22-3.11)</td>
<td>0.49</td>
</tr>
</tbody>
</table>

Table 2. SNPs located in the MTHFR gene 3'-UTR and the predicted miRNAs

<table>
<thead>
<tr>
<th>SNP</th>
<th>Chromosome</th>
<th>HGVS Names</th>
<th>miRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs55763075</td>
<td>1:11790377</td>
<td>XM_005263460.1:c.*303G&gt;A</td>
<td>miR-34b</td>
</tr>
<tr>
<td>rs915014</td>
<td>1:11789412</td>
<td>XM_005263458.1:c.*1268A&gt;G</td>
<td>miR-661</td>
</tr>
<tr>
<td>rs868014</td>
<td>1:11789390</td>
<td>XM_005263459.1:c.*1150T&gt;C</td>
<td>miR-1203</td>
</tr>
<tr>
<td>rs114290429</td>
<td>1:11788822</td>
<td>XM_005263460.1:c.*303G&gt;A</td>
<td>miR-521</td>
</tr>
<tr>
<td>rs4846048</td>
<td>1:11786195</td>
<td>XM_005263459.1:c.*4345C&gt;T</td>
<td>miR-522</td>
</tr>
</tbody>
</table>

Table 3. Genotype frequencies of the MTHFR rs915014 polymorphism among atherosclerosis patients and control group

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cases (n=500)</th>
<th>Control (n=600)</th>
<th>OR (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>rs915014</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>301</td>
<td>60.2</td>
<td>415</td>
<td>69.2</td>
</tr>
<tr>
<td>AG</td>
<td>127</td>
<td>25.4</td>
<td>122</td>
<td>20.3</td>
</tr>
<tr>
<td>GG</td>
<td>72</td>
<td>14.4</td>
<td>63</td>
<td>10.5</td>
</tr>
<tr>
<td>G carrier</td>
<td>199</td>
<td>39.8</td>
<td>185</td>
<td>30.8</td>
</tr>
</tbody>
</table>

*The ORs, 95% CIs and P value were calculated after adjusting for age, gender, parental smoking, drinking and other characteristics listed in Table 1.

Results

Characteristics of participants

Characteristics of patients with atherosclerosis and healthy controls are shown in Table 1. Specifically, the controls were matched with cases for age and gender. Cigarette smoking (P<0.001), alcohol consumption (P<0.001), diabetes and hypertension (P<0.001), a low level of serum high-density lipoprotein-C (HDL-C; P=0.02) were significantly associated with ischemic stroke. However, BMI and total serum cholesterol concentration were not associated with ischemic stroke (Table 1).

Identification of MTHFR polymorphisms in 3'-UTR

In this study, we mainly focused on the relationship of the SNPs in the MTHFR 3'-UTR to ischemic stroke risk and outcome. We first searched the GenBank of Single Nucleotide Polymorphism (SNP) database (https://www.ncbi.nlm.nih.gov/snp) to identify potential MTHFR genetic variants in the 3'-UTR using the following parameters: Organism (Homo sapiens); Function Class (3'-UTR); Global MAF (0.05-0.1); Validation Status (by-1000 Genomes). We identified five MTHFR polymorphisms (Table 2).

We then assessed genotype frequencies of these five MTHFR SNPs in 500 atherosclerosis patients and 600 healthy controls. The level of plasma tHcy was normalized to the kit standard and the data were summarized as Mean ± SD.

Statistical analysis

The association between different genetic variants and ischemic stroke risk was evaluated by calculation of the odds ratios (ORs) and 95% confidence intervals (CIs) using univariate and multivariate logistic regression analysis. The difference in association of MTHFR mRNA levels with three MTHFR genotypes and of the relative luciferase activity between the wild and mutant genotype were evaluated by using an independent-sample t test. All statistical analyses were two-sided and P<0.05 was considered statistically significant using SPSS 13.0 (SPSS, Chicago, IL, USA) or SAS software (version 9.1.3; SAS Institute, Cary, NC, USA).
patients and 600 healthy controls. We found that the MTHFR SNP rs915014 had a statistically different distribution between atherosclerosis patients and healthy controls. The Chi-square test confirmed that the genotype of rs915014 was in Hardy-Weinberg equilibrium distribution pattern in the healthy control group (P=0.32). The logistic regression analysis revealed that MTHFR rs915014 AG and GG genotypes significantly associated with increased risk of developing of atherosclerosis compared with the AA genotype (OR: 1.44; 95% CI: 1.01-2.19 for AG genotype, while OR: 1.51; 95% CI: 1.09-1.98 for GG genotype; Table 3). A higher number of G-alleles were also associated with an increased risk of ischemic stroke (OR: 1.68; 95% CI: 1.29-2.65).

**Association of the MTHFR rs915014 SNP with a poor atherosclerosis outcome**

Next, we assessed the association of the MTHFR rs915014 SNP with atherosclerosis outcome. The Gensini score system was applied to evaluate the outcome of atherosclerosis. We found that patients carrying either the MTHFR.
rs915014 AG or GG genotype had an increased Gensini score. Moreover, we assessed levels of MTHFR mRNA in patients carrying the MTHFR rs915014 AA, AG and GG genotypes. Our data showed that patients carrying the MTHFR rs915014 AG or GG genotypes had a higher level of MTHFR compared with patients with the AA genotype. We then assayed levels of serum tHcy in patients and found that patients carrying the MTHFR rs915014 AG or GG genotype were associated with the accumulation of serum tHcy (Table 4).

Discussion

MTHFR is an important enzyme in the generation of ROS and subsequent cell damage. Indeed, in ischemic conditions, ROS generation is enhanced and can lead to damage to various types of cells, including brain endothelial cells, leading to formation of thrombosis and loss of brain function. Our current study provides direct evidence showing that this novel MTHFR rs915014 polymorphism increases the risk of atherosclerosis compared with healthy controls. Mechanistically, the MTHFR rs915014 polymorphism alters the binding of miR-661 to negatively regulate MTHFR expression, providing an epigenetic mechanism of gene regulation and expression. In this study, it was the first time to reveal the functional SNP located in the 3'UTR of MTHFR acting as a risk factor for the atherosclerosis. Besides, miR-661 was frequently documented in human malignant tumors especially the lung cancer, here we first demonstrate the involvement of miR-661 in atherosclerosis. This might providing demystify for the detailed mechanism study in future.

There is accumulating evidence demonstrating that SNPs localized at miRNA binding sites (miRSNPs) could affect the binding of miRNAs to the target genes and in turn result in reduction or increase in translation of the target mRNA and altered susceptibility to cancer. For example, previous studies showed that the rs2910164 polymorphism harboring the sequence for miR-146a could influence susceptibility to gastric cancer in a Chinese population, while rs4143815 and rs4819388 SNPs in the 3'-UTR of B7-H1 and B7-H2 genes, respectively, associated with development of gastric cancer.

In summary, our current study demonstrated that the function SNP rs915014 in the 3'UTR of MTHFR AG and GG genotypes were associated with increased risk of atherosclerosis compared with the GG genotype. The miR-661 is able to bind to MTHFR with G allele and down-regulate MTHFR expression resulting in an increased level of serum tHcy and a poor atherosclerosis outcome. The detection of rs915014 in atherosclerosis combining with tHcy might be a possible prognosis biomarker in the future.

Disclosure of conflict of interest

None.

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