A male-specific association between AGTR1 hypermethylation and coronary heart disease

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ABSTRACT

The AGTR1 gene encodes angiotensin II receptor type 1, which is involved in cardiovascular diseases such as coronary heart disease (CHD). In the current study, we analyzed AGTR1 promoter methylation level in a Han Chinese population by SYBR green-based quantitative methylation-specific PCR (qMSP). We collected blood samples from 761 CHD patients and 398 non-CHD controls at the Ningbo First Hospital. A data mining analysis was also performed to explore the association between AGTR1 methylation and AGTR1 gene expression, using datasets from the cBioPortal for Cancer Genomics and the Gene Expression Omnibus (GEO) database. Our results showed a significantly higher percentage of methylated reference (PMR) of AGTR1 in male CHD patients compared with male non-CHD controls (median PMR: 2.12% vs. 0.59%, p = 0.037). The data mining analysis showed that AGTR1 expression was significantly increased in human hepatoma HepG2 cells treated with the demethylation agent 5-aza-2'-deoxycytidine (fold = 3.12, p = 0.009). Further data mining analysis using the cholangiocarcinoma (TCGA, PanCancer Atlas) data indicated an inverse association between AGTR1 methylation and AGTR1 expression (r = -0.595, p = 1.29E-04). Overall, our results suggest that AGTR1 methylation is involved in the regulation of AGTR1 gene expression and that AGTR1 hypermethylation is associated with CHD in males. These findings may provide new clues about the pathogenesis of CHD.

KEYWORDS: Coronary heart disease; CHD; AGTR1; DNA methylation; males; angiotensin II receptor type 1; HepG2 cells; 5-aza-2'-deoxycytidine
INTRODUCTION

Coronary heart disease (CHD) is a type of cardiovascular disease, which poses a severe threat to public health (1, 2). As a complex disease, CHD is affected by the combinatory effect from both environmental and genetic factors. Previous studies implied that epigenetic modifications might be involved in the pathophysiological processes associated with CHD (3, 4).

As the most common epigenetic modification, DNA methylation is the process of converting cytosine to 5-methylcytosine, which usually occurs at the CpG site, playing an important role in regulating gene expression (5). Recent studies have shown that the methylation level of FOXP3, BAX and PON1 was significantly associated with CHD (6-8). Interestingly, CDKN2B methylation was specific for the risk of CHD in women, suggesting that CDKN2B promoter methylation might be related to sex dimorphism in the pathogenesis of CHD (9).

Angiotensin II is a potent vasopressor hormone that plays a vital role in blood pressure control and relates to the pathogenesis of CHD (10). Angiotensin II mainly acts through 2 types of receptors, including angiotensin II type 1 receptor (AGTR1) and angiotensin II type 2 receptor (AGTR2) (11). AGTR1 is a member of the G-protein-coupled receptor superfamily and encoded by a single copy gene on chromosome 3q (12). AGTR1 also mediates the significant cardiovascular effects of angiotensin II (12). Previous genetic studies found that AGTR1 was a candidate gene for CHD in Poles and Finnish (13, 14). Single-nucleotide polymorphisms (SNPs) of the AGTR1 gene were also found to increase the risk of CHD in Chinese populations (15, 16). However, our previous investigation of a CpG-SNP at the AGTR1 promoter was unable to find an association with CHD in Han Chinese population (17).

Aberrant AGTR1 methylation was found to be associated with multiple diseases. AGTR1 hypermethylation could increase the risk of oral cancer (18), and non-small cell lung cancer
A previous study found a significantly lower AGTR1 methylation in hypertension patients than in healthy controls (20). However, the relationship between AGTR1 methylation and CHD was still unknown. In light of previous findings, we aimed to explore the association of AGTR1 methylation with CHD.

MATERIALS AND METHODS

Patients

Methylation assay of AGTR1 involved with 761 CHD patients (501 men and 260 women, median age: 62 years) and 398 non-CHD controls (220 men and 178 women, median age: 60 years). We collected blood samples from Ningbo First Hospital. The samples included CHD patients who were diagnosed with coronary artery stenosis greater than 50% in one or more major coronary artery. In addition, all controls were obtained from the inpatients with coronary artery stenosis less than 50% in major coronary arteries and without any atherosclerotic vascular disease. Standardized coronary angiography by Seldinger’s method was applied on all subjects (21, 22). The results of the angiography were independently judged by at least two cardiologists. None of the involved inpatients had histories of congenital heart disease, cardiomyopathy and severe liver or kidney disease. The institutional review board of Ningbo First Hospital and Ningbo University approved the current study (NBU-CHD-20180305). All the participants provided the written informed consent forms.

SYBR green-based quantitative methylation-specific PCR (qMSP)

As previously described, we extracted human genomic DNA from peripheral blood samples (23). The details of bisulfite conversion and qMSP procedures were available in our previous studies (24, 25). We used the percentage of methylated reference (PMR) to represent gene methylation and its calculation formula (24, 25). The primer sequences of qMSP were 5’-GGAGGAGGAGGGAATGTAA-3’ for the forward primer, and 5’-CCTATCACTCGCTACTACCT-3’ for the reverse primer.
Data mining analysis

A bioinformatics analysis was performed using the cholangiocarcinoma (TCGA, PanCancer Atlas) data to explore the association between AGTR1 methylation and AGTR1 gene expression. The data was downloaded through the cBioPortal for Cancer Genomics (http://www.cbioportal.org) (26). AGTR1 methylation and AGTR1 gene expression were determined with Infinium Human Methylation 450 Bead Array and RNAseq, respectively. We performed the correlation test of AGTR1 methylation with AGTR1 expression using the data in human hepatoma cells from the Gene Expression Omnibus (GEO) database (GSE5230).

Statistical analysis

PASW statistics 18.0 software (SPSS, Inc., Somers, NY, USA) was used to analyze all the data. We compared continuous variables between the two groups by either a T-test or a non-parametric test. Univariate linear regression analysis was used to find the appropriate factors, then a regression analysis of multivariate was used to explore the association between these factors and CHD. A Spearman’s correlation test assessed the association tests between AGTR1 methylation and each of the 9 metabolic phenotypes that included low-density lipoprotein (LDL), total cholesterol (TC), triglyceride (TG), apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), apolipoprotein E (ApoE), lipoprotein A [Lp(a)], and high sensitivity C reactive protein (hs-CRP). A two-tailed $p < 0.05$ was considered to be significant.

RESULTS

In the current study, we recruited 761 CHD subjects (mean age: 58.52 ± 9.52) and 398 controls (mean age: 61.41 ± 9.29) in the present study (Table 1). As shown in Figure 1, the chosen region for the AGTR1 methylation assay locates in a transcription factor-binding site. The methylation level of an AGTR1 CpG was measured to represent the amplified fragment
(142 bp) in the CpG island of AGTR1 (Figure 1).

As shown in Table 1, we collected the demographic and biochemical characteristics, including LDL, TC, TG, ApoA1, ApoB, ApoE, Lp(a), and hs-CRP. Our univariate regression analyses showed that age, gender (male), smoking, diabetes, TG, and hs-CRP were risk factors of CHD (all \( p < 0.05 \), Table 1). A further multivariate regression analysis showed that four factors (age, gender (male), diabetes, and TG) and Lp(a) were the risky and protective factors of CHD, respectively (all \( p < 0.05 \), Table 2). However, AGTR1 methylation was not associated with CHD in the subgroup tests by diabetic status (\( p > 0.05 \), data not shown). We found no significant relationships between AGTR1 methylation and clinical indexes, including age, TG, and Lp(a) (\( p > 0.05 \), data not shown).

AGTR1 methylation was shown to be significantly higher in male CHD patients than in male controls (mean PMR: 2.12 versus 0.59, \( p = 0.037 \), Table 3). Furthermore, GEO data analysis showed a significantly decreased AGTR1 expression in HepG2 cells treated with a demethylation agent (5’-aza-2’-deoxycytidine, 5’-AZA, fold = 3.12, \( p = 0.009 \), Figure 2). Further bioinformatics analysis using the cholangiocarcinoma (TCGA, PanCancer Atlas) data indicated a significant inverse association between AGTR1 methylation and AGTR1 gene expression (\( r = -0.595 \), \( p = 1.29E-04 \), Figure 3). All the above evidence suggested a pivotal role of AGTR1 methylation in the regulation of AGTR1 gene expression.

In our previous study, we found no association of CHD with a CpG-SNP (rs275653) at the AGTR1 promoter (17). Here, we performed an interaction test between rs275653 and AGTR1 methylation. However, we were unable to observe a significant interaction between them (data not shown).

**DISCUSSION**

In the present study, we explored the association between AGTR1 methylation and CHD. Our
research found four risk factors (age, male, diabetes, and TG) and one protective factor [Lp(a)] of CHD. Although there was no association of AGTR1 methylation with CHD in the total samples, we found elevated AGTR1 methylation in male CHD patients compared with male non-CHD controls. Moreover, data mining analyses showed that a demethylation agent could induce higher AGTR1 expression and that AGTR1 expression was inversely associated with AGTR1 methylation. All the above evidence suggested that AGTR1 methylation might contribute to the risk of CHD in males through its regulation of AGTR1 gene expression.

Gender differences existed in the incidence and development of CHD as well as CHD related genes (27). The incidence of CHD was higher in all age groups in men than in women (28). Men had a higher prevalence of ST-elevation myocardial infarction but were less likely to have heart failure than women (29). There was a shift in the gender distribution of CHD, and this might result from different lifestyles between males and females, such as smoking, alcohol consumption, high sodium diet, and physical activity (30-33).

Previous study showed that males with the angiotensin-I converting enzyme (deletion/deletion) genotype shared a higher risk for premature myocardial infarction (34), suggesting a possible sex difference in the effect of the ACE gene variability on the CHD risk. In addition, sex hormones had been provided to regulate specific DNA methylation alterations (35). An early study only observed lower AGTR1 methylation in male hypertension when compared to matched controls (20). Our study demonstrated that gender was a risk factor in CHD, and AGTR1 hypermethylation only existed in CHD males. Therefore, gender differences of AGTR1 methylation levels might reflect the gender differences in lifestyles and sex hormones. Moreover, AGTR1 was a mandatory mediation in the significant cardiovascular effects (36), and our data mining found that AGTR1 hypermethylation could suppress gene expression. Our results indicated that gender difference implicated in CHD, and that AGTR1 hypermethylation might increase the risk of
CHD via its regulation on AGTR1 gene expression.

Some studies have shown that elevated Lp(a) level is an independent risk factor for CHD (37, 38). High levels of Lp(a) are independently associated with CHD-induced recurrent heart failure in patients with chronic heart failure (39). Interestingly, our results suggest that Lp(a) may be a protective factor for CHD. Our findings are contrary to previous conclusions. Therefore, the role of Lp(a) in CHD needs to be explored in the study of larger samples in the future.

Although our findings provided an association between AGTR1 methylation with CHD in males, the cause-effect relationship between AGTR1 methylation and CHD remained unclear. Therefore, we might need a more convincing method, such as a longitudinal cohort study in the future. Besides, we measured AGTR1 methylation in peripheral blood samples. DNA methylation profile might vary among different tissues. Further studies were required to confirm our findings in other tissues.

In summary, our results suggested that AGTR1 methylation was related to CHD in males. These findings were likely to provide new clues to elaborate the pathogenesis of CHD.

ACKNOWLEDGMENTS

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.
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TABLES AND FIGURES

**TABLE 1.** Univariate logistic regression analysis of environmental factors that may influence CHD in Han Chinese population*

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>Case (n = 761)</th>
<th>Control (n = 398)</th>
<th>β</th>
<th>S.E.</th>
<th>p</th>
<th>Odds ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>58.52 ± 9.52</td>
<td>61.41 ± 9.29</td>
<td>0.033</td>
<td>0.007</td>
<td><strong>1.04E-06</strong></td>
<td>1.034</td>
<td>1.020−1.047</td>
</tr>
<tr>
<td>Gender (F/M)</td>
<td>503/260</td>
<td>220/178</td>
<td>0.448</td>
<td>0.126</td>
<td><strong>3.96E-04</strong></td>
<td>1.565</td>
<td>1.222−2.006</td>
</tr>
<tr>
<td>Smoking (Y/N)</td>
<td>295/467</td>
<td>125/273</td>
<td>0.328</td>
<td>0.131</td>
<td><strong>0.012</strong></td>
<td>1.389</td>
<td>1.075−1.794</td>
</tr>
<tr>
<td>Hypertension (Y/N)</td>
<td>427/336</td>
<td>204/194</td>
<td>0.189</td>
<td>0.124</td>
<td>0.127</td>
<td>1.209</td>
<td>0.825−1.421</td>
</tr>
<tr>
<td>Diabetes (Y/N)</td>
<td>155/608</td>
<td>41/356</td>
<td>0.737</td>
<td>0.184</td>
<td><strong>6.31E-05</strong></td>
<td>2.09</td>
<td>1.456−2.999</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.52 ± 0.92</td>
<td>2.56 ± 0.93</td>
<td>0.052</td>
<td>0.067</td>
<td>0.442</td>
<td>1.053</td>
<td>0.923−1.201</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>4.30 (3.67, 4.99)</td>
<td>4.25 (3.54, 5.06)</td>
<td>0.044</td>
<td>0.054</td>
<td>0.413</td>
<td>1.045</td>
<td>0.940−1.162</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.04 (0.90, 1.20)</td>
<td>1.10 (0.955, 1.28)</td>
<td>0.012</td>
<td>0.026</td>
<td>0.638</td>
<td>1.012</td>
<td>0.962−1.065</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.39 (1.01, 1.97)</td>
<td>1.31 (0.93, 1.81)</td>
<td>0.191</td>
<td>0.067</td>
<td><strong>0.005</strong></td>
<td>1.211</td>
<td>1.061−1.382</td>
</tr>
<tr>
<td>ApoA1 (mmol/L)</td>
<td>0.95 (0.82, 1.09)</td>
<td>0.99 (0.83, 1.12)</td>
<td>-0.087</td>
<td>0.159</td>
<td>0.585</td>
<td>0.917</td>
<td>0.671−1.253</td>
</tr>
<tr>
<td>ApoB (mmol/L)</td>
<td>0.74 (0.56, 0.93)</td>
<td>0.75 (0.52, 0.96)</td>
<td>-0.052</td>
<td>0.152</td>
<td>0.73</td>
<td>0.949</td>
<td>0.705−1.278</td>
</tr>
<tr>
<td></td>
<td>Lower Limit</td>
<td>Upper Limit</td>
<td>Difference</td>
<td>Mean</td>
<td>Standard Deviation</td>
<td>Minimum</td>
<td>Maximum</td>
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<tr>
<td>ApoE (mmol/L)</td>
<td>4.00 (3.10, 5.06)</td>
<td>4.03 (3.10, 5.01)</td>
<td>-0.006</td>
<td>0.009</td>
<td>0.553</td>
<td>0.994</td>
<td>0.976~1.013</td>
</tr>
<tr>
<td>Lp(a) (mmol/L)</td>
<td>0.28 (0.10, 0.62)</td>
<td>0.30 (0.10, 30.75)</td>
<td>-0.002</td>
<td>4.91E-04</td>
<td>1.73E-04</td>
<td>0.998</td>
<td>0.997~0.999</td>
</tr>
<tr>
<td>Hs-CRP (g/L)</td>
<td>2.30 (0.60, 5.20)</td>
<td>1.70 (0.10, 4.05)</td>
<td>0.012</td>
<td>0.006</td>
<td>0.043</td>
<td>1.012</td>
<td>1.000~1.024</td>
</tr>
</tbody>
</table>

*p-value less than or equal to 0.05 is in bold; LDL: Low-density lipoprotein; TC: Total cholesterol; HDL: High-density lipoprotein; TG: Triglyceride; ApoA1: Apolipoprotein A; ApoB: Apolipoprotein B; ApoE: Apolipoprotein E; Lp(a): Lipoprotein A; Hs-CRP: High-sensitivity C-reactive protein.
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<td>0.067</td>
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<td>1.211</td>
<td>1.061~1.382</td>
</tr>
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<td>ApoA1 (mmol/L)</td>
<td>-0.087</td>
<td>0.159</td>
<td>0.585</td>
<td>0.917</td>
<td>0.671~1.253</td>
</tr>
<tr>
<td>ApoB (mmol/L)</td>
<td>-0.052</td>
<td>0.152</td>
<td>0.730</td>
<td>0.949</td>
<td>0.705~1.278</td>
</tr>
<tr>
<td>ApoE (mmol/L)</td>
<td>-0.006</td>
<td>0.009</td>
<td>0.553</td>
<td>0.994</td>
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<td>Lp(a) (mmol/L)</td>
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</tr>
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<td>Hs-CRP (g/L)</td>
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*p* value less than or equal to 0.05 is in bold; TG: Triglyceride; Lp(a): Lipoprotein A; Hs-CRP: High-sensitivity C-reactive protein.
TABLE 3. The comparisons of AGTR1 methylation between CHD and non-CHD in Han Chinese population*

<table>
<thead>
<tr>
<th></th>
<th>CHD (N, Median (25%, 75%))</th>
<th>Non-CHD (N, Median (25%, 75%))</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>761, 1.89 (0.00, 26.43)</td>
<td>398, 1.72 (0.00, 25.63)</td>
<td>0.263</td>
</tr>
<tr>
<td>Male</td>
<td>501, 2.12 (0.00, 26.29)</td>
<td>220, 0.59 (0.00, 23.55)</td>
<td><strong>0.037</strong></td>
</tr>
<tr>
<td>Female</td>
<td>260, 1.76 (0.00, 27.12)</td>
<td>178, 2.74 (0.00, 31.68)</td>
<td>0.579</td>
</tr>
</tbody>
</table>

*N stands for the number of participators; CHD stands for subject with coronary heart disease and Non-CHD stands for subject without coronary heart disease; p value less than or equal to 0.05 is in bold. The percentage of methylated reference (PMR) is represented by median and percentiles 25 and 75.
FIGURE 1. The tested DNA fragment in the methylation assay of AGTR1. A: The genomic position of the amplified fragment at AGTR1 gene CpG island; F and R stood for forward and reverse primer, respectively; B: DNA sequencing indicated a good bisulfite conversion; C: Gel electrophoresis showed a correct DNA length of the amplified fragment (142 bp).
FIGURE 2. Our data-mining analysis showed that demethylation agent significantly increased *AGTR1* gene expression.*

*: The 5'-AZA-treated cells showed a significantly higher *AGTR1* expression than controls (fold change = 3.12, *p* = 0.009).
FIGURE 3. Our data-mining analysis showed that the association between AGTR1 methylation and gene expression *

*: Spearman’s rank correlation was used to test the relationship between methylation and expression. The x-axis showed the levels of AGTR1 methylation. The y-axis showed a log-transformed AGTR1 gene expression values.