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Preliminary study of the relationship between promoter methylation of the *ANGPTL2* gene and coronary heart disease

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Background: Coronary heart disease (CHD) is primarily caused by atherosclerosis of coronary arteries. It is largely an inflammatory disease of the vascular wall. The inflammation is related to DNA methylation. Angiopoietin-like protein 2 (*ANGPTL2*) has various functions in several chronic inflammatory diseases. Macrophage-derived ANGPTL2 was reported to accelerate CHD development. It is reported that DNA hypomethylation in the promoter region of *ANGPTL2* gene was associated with acute coronary syndrome (ACS), a type of CHD. Our objective was to explore the correlation between promoter methylation of the *ANGPTL2* gene and CHD, and to investigate the association between methylation status and clinical characteristics of CHD patients.

Methods: Firstly, we collected 122 CHD patients and 58 non-CHD participants from Han Chinese population and purified the peripheral blood DNA. The purified DNA was subjected to bisulfite modification. After bisulfite conversion, the target DNA locus was amplified using polymerase chain reaction (PCR), and the PCR products were measured by pyrosequencing. Finally, the methylation level was calculated according to the sequencing result, and the data were analyzed using xx software.

Results: CHD patients had a relatively lower methylation levels (*P*50: 7.67% [*P*25: 6.22%, *P*75: 10.43%]) in the *ANGPTL2* promoter region than did controls (*P*50: 8.25% [*P*25: 5.46%, *P*75: 17.98%], $P = 0.001$), indicating an association between *ANGPTL2* promoter methylation and CHD (OR: 0.890; 95% CI, 0.832-0.953; adjusted $P = 0.001$). A breakdown analysis by gender showed that *ANGPTL2* promoter methylation was associated with CHD in females (adjusted $P = 0.002$) but not in males (adjusted $P = 0.404$). We found no correlation between gene methylation and other clinical characteristics.

Conclusions: The present work provides evidence to support an association between *ANGPTL2* promoter DNA methylation status and the risk profile of CHD in females.

Abbreviations: A/G, albumin/globulin; ACS, acute coronary syndrome; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANGPTL2, angiopoietin-like protein; ApoAI, apolipoprotein AI; ApoB, apolipoprotein B; ApoE, apolipoprotein E; AST, aspartate aminotransferase; CAD, chronic coronary disease; CHD, coronary heart disease; GGT, glutamyl transpeptidase; GLB, globulin; HDL-C, high-density lipoprotein cholesterol; Hs-CRP, high-sensitivity C-reactive protein; LDL-C, low-density lipoprotein cholesterol; Lp, lipoprotein; LVEF, left ventricular ejection fraction; NSTEMI, non-ST-segment elevation myocardial infarction; PCI, percutaneous coronary angiography; STEMI, ST-segment elevation acute infarction; TC, total cholesterol; UA, unstable angina pectoris.

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Our data indicated that in females, promoter DNA hypomethylation of the *ANGPTL2* gene is associated with an increased risk of CHD.

KEYWORDS
ANGPTL2, bisulfite pyrosequencing, coronary heart disease, DNA methylation

1 | INTRODUCTION

Simultaneous with the development of society and the improvement of living standards, coronary heart disease (CHD) has become one of the leading causes of death in the world. Clinical physicians have divided CHD into acute coronary syndrome (ACS) and chronic coronary disease (CAD). The former includes unstable angina pectoris (UA), non-ST-segment elevation myocardial infarction (NSTEMI), and ST-segment elevation acute infarction (STEMI). The latter includes stable angina pectoris, asymptomatic myocardial ischemia, and ischemic heart failure. Coronary heart disease is primarily caused by atherosclerosis of coronary arteries. There are many well-established risk factors promoting atherosclerosis, including dyslipidemia, smoking, unhealthy diet, overweight and obesity, hypertension, diabetes mellitus, aging, genetic factors, and family history.[1-3] Furthermore, it has been recognized that atherosclerosis is primarily an inflammatory disease of the vascular wall.[4] Inflammatory responses are not only involved in the formation of atherosclerotic lesions, but are also associated with complications such as thrombosis and plaque rupture.

Recently, several lines of evidence indicated that inflammation is related to DNA methylation.[5] DNA methylation often occurs in the CpG dinucleotides and tends to be associated with gene transcription silencing. Loss of methylation up-regulates gene expression.[6] Nevertheless, the epigenetic changes are not permanent and are influenced by smoking,[7] hypertension,[8] obesity, and environment.[9-11]

Angiopoietin-like protein 2, originating from various cells, has several functions in various chronic inflammatory diseases. Macrophage-derived ANGPTL2 was reported to accelerate atherosclerosis and CHD development,[12] while endothelial cell-derived ANGPTL2 accelerates vascular inflammation and leads to the development of endothelial dysfunction and atherosclerosis.[12] Circulating ANGPTL2 levels might reflect the severity of atherosclerosis, not lipid metabolic disorders. ANGPTL2 is a proinflammatory mediator and accelerates the development of systemic insulin resistance, endothelial dysfunction, and atherosclerosis in mice.[13-15] Abundant expression of ANGPTL2 was detected in adipose tissue and other organs, including skeletal muscle, heart, endothelial cells, lung, and kidney.[14,16] Early reports state that post-ACS patients in the cardiovascular prevention center of the Montreal Heart Institute had elevated circulating levels of ANGPTL2.[17] ANGPTL2 expression is regulated by DNA methylation. Nguyen et al have shown that DNA hypomethylation in the promoter region of *ANGPTL2* was associated with post-ACS differently from that of age-matched healthy controls.[17] Nevertheless, to the best of our knowledge, no experiments have been conducted to demonstrate the correlation between the promoter methylation of the *ANGPTL2* gene and the risk of CHD in a Han Chinese population. The aim of the present study was to assess whether aberrant promoter methylation of the *ANGPTL2* gene was associated with the risk of CHD in Han Chinese on the coast of China and to explore the interaction between methylation levels and clinical characteristics.

2 | MATERIALS AND METHODS

2.1 | Participants and clinical data

Between 1st of November 2015 and 1st of September 2017, 180 patients were under the care of our Cardiology department in the Ningbo Lihuili Hospital. All of them were Han Chinese originated from Ningbo city in Eastern China. Among these patients, 122 met the inclusion criteria for CHD group and 58 met the inclusion criteria for non-CHD group. Patients were divided into case group and control group by standardized coronary angiography according to the Seldinger's method.[18] Every patient was diagnosed by at least two independent cardiologists. The inclusion criteria of CHD cases selected patients with coronary lesions greater than 50% or with history of PCI or CABG. Non-CHD patients had a <50% obstruction in any major coronary for PCI and did not have any atherosclerotic vascular disease. The exclusion criteria were as follows: (a) Cancer. (b) Diabetes. (c) Cardiogenic shock. (d) Heart failure patients. (e) Severe ventricular arrhythmias. (f) Aortic dissection. (g) Rheumatic heart disease. (h) Familial hyperlipidemia. (i) Pregnant and lactating woman. (j) Autoimmune diseases. (k) Active infections. (l) Chronic inflammatory diseases. All of the experiments were approved by the Ethical Committee of Ningbo Lihuili Hospital, and written informed consent was obtained from all the subjects. Blood samples were collected into EDTA two sodium anticoagulant tubes and then stored at −80°C.

2.2 | Biochemical analyses

Genomic DNA was extracted from peripheral blood samples using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). DNA concentrations were measured by the ultramicro nucleic acid ultraviolet tester (NANODROP 1000, Wilmington, DE, USA). Plasma levels of TG, TC, HDL, and LDL were measured using an

enzymatic end point assay.[19] The ApoA, ApoB, and ApoE levels were measured by the transmission turbidimetric method.[20] The plasma Lp(a) concentrations were determined by a sandwich enzyme-linked immunosorbent assay method [Macra-Lp(a); SDI, Newark, DE, USA]. The concentrations of ALT, AST, ALP, and GGT in plasma were measured by the IFCC reference measurement systems.[21-23] The ALB level was worked through the bromocresol green method.[24] All the mentioned procedures were followed the standard procedures recommended by the manufacturers. The purified DNA was subjected to bisulfite modification using the EpiTect Bisulfite Kit (Qiagen, Hamburg, Germany) following the manufacturer's protocol. Genomic DNA was chemically modified by sodium bisulfite to convert all unmethylated cytosines to uracils while the methylated cytosines remained unchanged. After bisulfite conversion, polymerase chain reaction (PCR) was used to amplify the target DNA locus. The primers were designed by PyroMark Assay Design software 2.0 (Qiagen, Hamburg, Germany). The sequences of the PCR primers are listed in Table 1. The PCR products were measured by pyrosequencing which was conducted by Q96 machine and reagents (PyroMark Q96 ID; Qiagen, Hamburg, Germany).

TABLE 1 List of all primers used for PCR

Gene	Primer sequence	Modification
Forward primer	GGGAGGGGTTAGGTTAGT	5'-Biotin
ANGPTL2 Reversed primer	TTCTCTCCACCCACCAATAC	
Sequencing primer	GGGTTAGGTTAGTTGG	

TABLE 2 Characteristics of all subjects according to subgroup analysis by CHD status and gender

Characteristics	Subgroup analysis by CHD status			Subgroup analysis by gender		
	CHD (n = 122) (Mean ± SE)	Non-CHD (n = 58) (Mean ± SE)	*P* value*	Male (n = 111) (Mean ± SE)	Female (n = 69) (Mean ± SE)	*P* value*
Age (years)	63.48 ± 10.08	59.83 ± 8.94	**0.020**	61.73 ± 10.28	63.22 ± 9.13	0.613
Gender (M/F)	(83/39)	(28/30)	0.464	NA	NA	NA
Smoking, n (%)	58 (47.5)	17 (29.3)	**0.004**	74 (66.7)	1 (1.4)	**<0.001**
Hypertension, n (%)	74 (60.7)	23 (39.7)	**0.027**	62 (55.9)	35 (50.7)	0.642
TC (mmol/L)	4.39 ± 1.27	4.40 ± 1.00	0.479	4.31 ± 1.22	4.53 ± 1.12	0.487
TG (mmol/L)	1.38 (0.98, 1.81)	1.38 (0.90, 1.95)	0.852[a]	1.39 (0.90, 1.91)	1.37 (0.98, 1.74)	0.677[a]
HDL-C (mmol/L)	1.13 ± 0.25	1.21 ± 0.25	0.189	1.10 ± 0.24	1.25 ± 0.26	0.156
LDL-C (mmol/L)	2.60 ± 0.99	2.48 ± 0.74	0.200	2.57 ± 0.99	2.55 ± 0.80	0.462
ApoAI (g/L)	1.14 ± 0.22	1.21 ± 0.23	0.376	1.10 ± 0.22	1.25 ± 0.20	**0.001**
ApoB (g/L)	0.90 ± 0.28	0.88 ± 0.26	0.446	0.89 ± 0.29	0.89 ± 0.24	0.845
ApoE (g/L)	3.26 ± 1.05	3.60 ± 0.91	0.254	3.24 ± 1.06	3.62 ± 0.90	0.274
Lp(a) (g/L)	0.14 (0.05, 0.28)	0.09 (0.05, 0.17)	**0.032**[a]	0.13 (0.05, 0.22)	0.12 (0.05, 0.23)	0.989[a]
Hs-CRP (mg/L)	3.60 (1.80, 7.53)	2.95 (1.08, 6.83)	0.084[a]	3.80 (1.80, 7.30)	2.40 (1.00, 7.10)	**0.017**[a]
ALB (g/L)	40.98 ± 3.46	42.00 ± 3.43	0.122	41.15 ± 3.72	41.56 ± 3.05	0.267
GLB (g/L)	26.55 ± 3.93	26.39 ± 4.40	0.435	25.62 ± 3.73	27.92 ± 4.22	**0.009**
A/G	1.60 (1.40, 1.70)	1.60 (1.40, 1.73)	0.206[a]	1.60 (1.40, 1.80)	1.50 (1.30, 1.70)	**0.004**[a]
ALT (IU/L)	20.00 (14.00, 29.25)	20.00 (14.00, 33.25)	0.635[a]	21.00 (14.00, 35.00)	19.00 (14.00, 25.00)	0.215[a]
AST (IU/L)	22.00 (18.00, 31.00)	21.00 (17.00, 25.25)	0.271[a]	22.00 (17.00, 32.00)	21.00 (18.00, 26.50)	0.449[a]
ALP (IU/L)	77.83 ± 27.61	79.53 ± 27.46	0.731	76.56 ± 29.17	81.30 ± 24.48	0.525
GGT (IU/L)	26.00 (17.00, 43.25)	22.00 (17.00, 36.00)	0.466[a]	28.00 (19.00, 50.00)	21.00 (15.00, 31.00)	**0.001**[a]

A/G, Albumin/Globulin; ALB, Albumin; ALP, Alkaline phosphatase; ALT, alanine aminotransferase; ApoAI, Apolipoprotein AI; ApoB, Apolipoprotein B; ApoE, Apolipoprotein E; AST, aspartate aminotransferase; CHD, coronary heart disease; GGT, Glutamyl transpeptidase; GLB, Globulin; HDL-C, high-density lipoprotein cholesterol; Hs-CRP, High-sensitivity C-reactive protein; LDL-C, low-density lipoprotein cholesterol; Lp, Lipoprotein; Non-CHD, non coronary heart disease; TC, total cholesterol; TG, triglyceride.

P-value: adjusted for age, gender, smoking (smoker vs never smoker), lipid level, history of hypertension, and history of diabetes by logistic regression.

Values are given as mean ± SE; NA denotes not applicable.

[a]Nonparametric rank test was applied, and values are given as P50 (P25, P75).

*Statistically significant *P* values are highlighted in bold type.

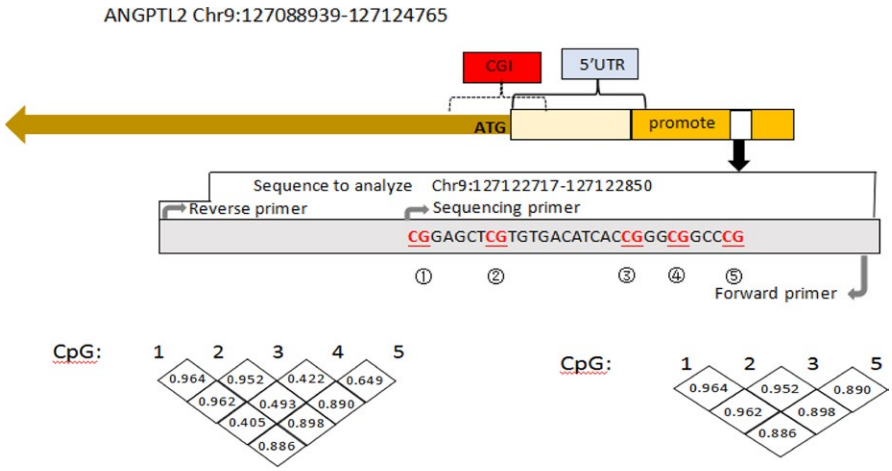

FIGURE 1 Information of CpG sites for the *ANGPTL2* gene

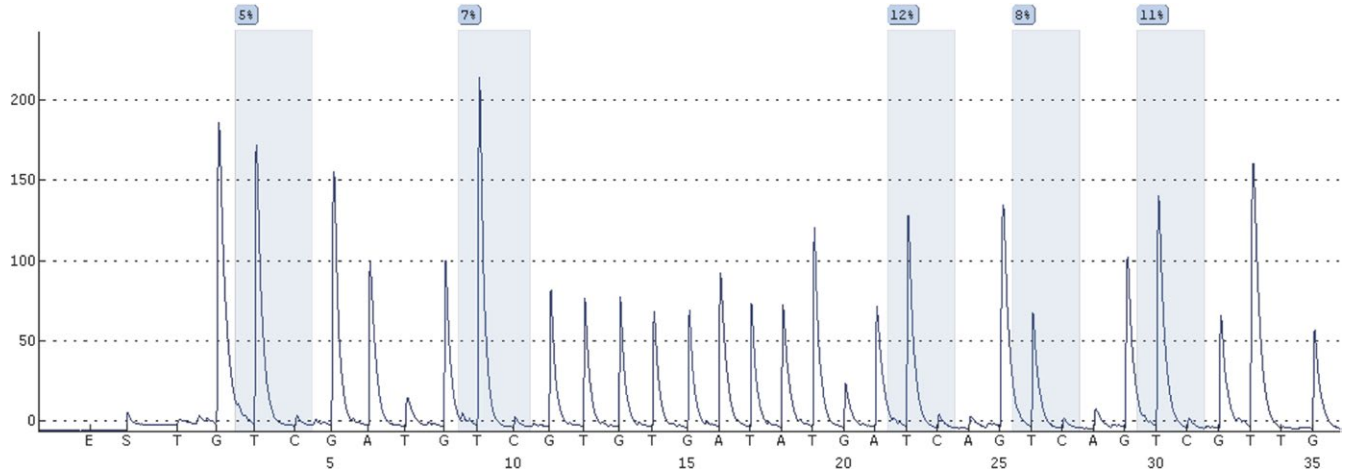

FIGURE 2 The methylation levels of five CpG sites on *ANGPTL2* gene promoter of one sample

2.3 | Statistical analysis

All the statistical analyses were performed using the SPSS package version 18.0 (SPSS Inc, Chicago, IL, USA) and Graph Pad Prism 5 Software (Graph Pad Software Inc., San Diego, CA, USA). If data follow normal distribution, it is presented as means ± SD. If data do not conform to the normal distribution, the rank and test are used to indicate the median and quartile (*P*50, [*P*25, *P*75]). SPSS package is recruited to evaluate the difference in the DNA methylation frequency of *ANGPTL2* gene promoter and various biochemical factors between case group and control group. All the *P* values are adjusted for the traditional risk factors, such as gender, age, smoking, lipid level, and hypertension by SPSS package with binary logistic regression. A two-tailed *P* value <0.05 was considered to be significant.

3 | RESULTS

Clinical and biochemical characteristics of the study population are displayed in Table 2. There are 122 CHD patients with a mean age of 63.48 ± 10.08 years. The non-CHD participants had a mean age of 59.83 ± 8.94 years. The CHD patients were older than the non-CHD participants. Furthermore, there were more males in the CHD group than in the control group. The number of smokers in the CHD group was greater than those in the non-CHD group. The CHD group had significantly more hypertension than did the control group ($P < 0.05$). When we analyzed all the participants according to gender, the mean age for males was 61.73 ± 10.28 years old and mean age for females was 63.22 ± 9.13 years. There was no statistically significant difference in age between the male and female groups. The percentage of smokers was higher in the male group than in the female group (66.7% vs 1.4%, $P < 0.001$).

There are five CpGs sites in the promoter region (hg, chr9: 127122717-127122850) of the *ANGPTL2* gene (Figure 1). According to the DNA sequence of this region, the primers were designed for bisulfite pyrosequencing (Figure 2). The methylation levels of the five CpG sites on the *ANGPTL2* promoter were measured using the Pyromark Q96 (Qiagen, Hamburg, Germany), and then, we evaluated the methylation levels of the *ANGPTL2* gene promoter. Except for the fourth CpG site, the other four CpGs significantly correlated with

TABLE 3 Comparison of the average DNA methylation levels of *ANGPTL2* gene's CpG1, CpG2, CpG3, CpG5 and CPG4 within subgroups and gender separately

Sites	Gene	Male (n = 111)	Female (n = 69)	*P*	CHD (n = 122)	Non-CHD (n = 58)	*P*
Average of CpG1, 2, 3, and 5[b]	ANGPTL2	7.34 (6.08, 10.36)	8.82 (6.06, 16.69)	0.299[a]	7.67 (6.22, 10.43)	8.25 (5.46, 17.98)	**0.001**[*,a]
CpG4[c]	ANGPTL2	7.99 ± 2.49	8.46 ± 2.50	0.569	8.26 ± 2.64	7.98 ± 2.18	0.382

P-value: adjusted for age, gender, smoking (smoker vs never smoker), lipid level, history of hypertension, and history of diabetes by logistic regression.

[a]Nonparametric rank test was applied.

[b]Values for CpG1, CpG2, CpG3, and CpG5 are given as P50 (P25, P75).

[c]Values for CpG4 are given as mean ± SD.

[*]Statistically significant *P* values are highlighted in bold type.

DNA methylation levels (Figure 2, $r > 0.8$, $P < 0.0001$). Therefore, these four average methylation levels can be used to indicate the degree of methylation of these four sites. There was no statistical difference between gender for the promoter DNA methylation levels of *ANGPTL2* gene (Table 3, average methylation of CpG1, 2, 3, and 5 $P = 0.299$; CpG4 $P = 0.569$). Except for CpG4, the average DNA methylation of the four CpGs showed significantly higher promoter DNA methylation of *ANGPTL2* gene in non-CHD cases (8.25% [5.46%, 17.98%]) than that in the CHD cases (Table 3, 7.67% [6.22%, 10.43%], adjusted $P = 0.001$). The CpG4 DNA methylation levels showed no differences between the CHD and non-CHD groups (adjusted $P = 0.382$). According to gender (83 male and 39 female) in the coronary heart patient group, the average DNA methylation level in the CpG1, CpG2, CpG3, and CpG5 of the *ANGPTL2* promoter region showed no difference ($P = 0.983$). In addition, there was no difference in the CpG4 methylation status of the *ANGPTL2* promoter region between male and female groups ($P = 0.401$).

On analysis of the male cases and male controls, the adjusted *P* value was not statistically significant. Nevertheless, when we conducted a case and control analysis for female, we observed that the adjusted *P* value reached significance ($P = 0.002$, Figure 3). It is interesting that we observed a significant association only in the female subgroup (Figure 3, females: adjusted $P = 0.002$; males: adjusted $P = 0.404$). However, Figure 3 shows that control groups' data were distributed at both ends of the mean. Then, we divided the control data into two groups: one group was the data above the mean value and the other was the mean value below. We tested the clinical characteristics as we collected (age, gender, smoking, hypertension, blood lipid, hs-CRP, ALB, GLB, ALT, AST, ALP, and GGT), and there were no statistical differences between the two groups. Therefore, we speculate that some factors most likely exist to influence the control data distribution. Comparing of *ANGPTL2* gene's CpG4 DNA methylation levels for case and control analysis, there was no significant difference between the groups ($P = 0.382$). The results are confirmed by binary logistic regression adjusting for age, gender, smoking (smoker vs non-smoker), lipid level, history of hypertension, and history of diabetes. Nevertheless, further analysis showed that CHD cases and non-CHD controls had no differences between genders by comparing *ANGPTL2* gene's CpG4 DNA methylation levels (Figure 4, females: adjusted $P = 0.614$; males: adjusted $P = 0.322$). Last but not least, we explored the association between the DNA methylation in the promoter region of *ANGPTL2* gene and clinical characteristics as we collected (age, gender, smoking, hypertension, blood lipid, hs-CRP, ALB, GLB, ALT, AST, ALP, and GGT). Unfortunately, we found no correlation between gene methylation and clinical characteristics.

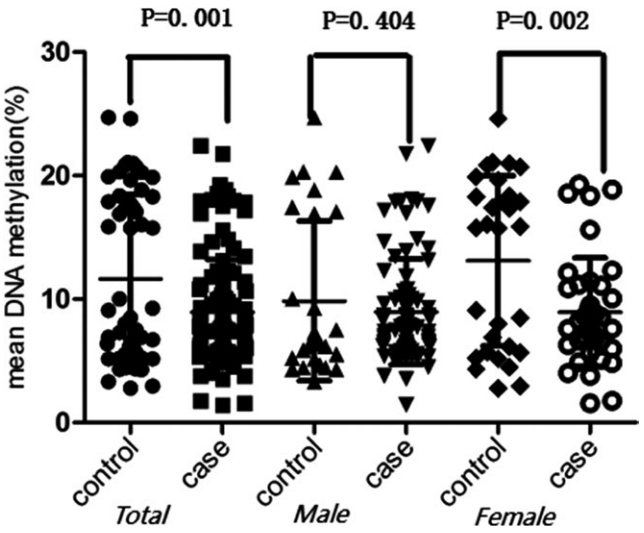

FIGURE 3 Comparison of the average DNA methylation levels of *ANGPTL2* gene's CpG1-5 between cases and controls

4 | DISCUSSION

Previous studies have shown that global DNA methylation and locus-specific methylation were related with atherosclerosis.[25] High levels of methylation in the promoter region of lipid-related genes, including *ABCA1* and *ABCG1*, lead to various concentrations of high-density lipoprotein cholesterol (HDL-C), while HDL-C concentration is associated with CHD.[26,27] ANGPTL2, a member of the ANGPTL family, promotes chronic inflammation and irreversible pathological tissue remodeling.[14,28] Studies have shown that ANGPTL2 protein induces a vascular inflammatory response through the integrin

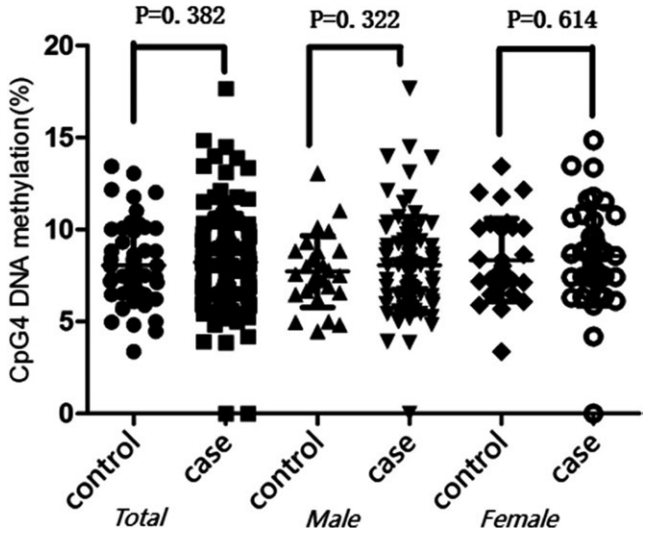

FIGURE 4 Comparison of *ANGPTL2* gene's CpG4 DNA methylation levels between cases and controls

α5β1/Rac1/NF-κB pathway, leading to atherosclerosis.[29] Studies found that ANGPTL2 was active during various stages of atherosclerosis and promoted leukocyte adhesion to inflamed pre-atherosclerotic and dysfunctional endothelia, in turn contributing to lesion formation.[30] Therefore, our effort to study CHD therapeutic strategies was focused on vascular inflammation that is recognized as a critical pathology driving atherosclerosis progression.[15]

At the cardiovascular prevention center of the Montreal Heart Institute, by measuring 33 patients after an ACS and 20 age-matched controls, it was found that circulating levels of ANGPTL2 in the ACS group (3.35 ± 0.67 ng/mL) were higher than those in the control group (1.8 ± 0.42 ng/mL). Of the six CpG islands, two CpG sites (CpG5: cg13662634 and CpG6: 14281592) had methylation differences between the ACS group and the control group. The degree of CpG5 methylation in the ACS group (34.7% ± 1.4%; $P < 0.05$, n = 21) was significantly lower than that of the control group (45.8% ± 1.5%, n = 14). When analyzing the younger control group (66.6% ± 0.9%, n = 14), the CpG6 sites in the ACS group showed lower methylation levels (60.4% ± 1.5%, $P < 0.05$, n = 21); however, there was no significant difference in the age-matched control group (61.4% ± 2.0%, n = 12). Nevertheless, we found five CpGs sites (from CpG1 to CpG5) of the *ANGPTL2* gene promoter region Chr9:127122717-127122850. Our data suggest that four specific methylation sites, CpG1, CpG2, CpG3, and CpG5, are hypomethylated in CHD participants compared to non-CHD cases. The *P*25, *P*50, and *P*75 values of CHD group were 6.22%, 7.67%, and 10.43%, in contrast to 5.46%, 8.25%, and 17.98% of non-CHD group, respectively, indicating a significant difference between the groups ($P = 0.001$). Although we selected five CpG sites that were different from Nguyen et al's study, we drew a similar conclusion that CHD participants are hypomethylated in the *ANGPTL2* gene promoter region compared with levels in non-CHD participants. Therefore, differential CpG1, CpG2, CpG3, and CpG5 methylation patterns may predict patients at risk of cardiovascular events. In the case-control analysis, the methylation level of ANGPTL2 gene was significantly different in the female group ($P = 0.002$). Figure 3 shows that, in the female group, methylation levels of the control group were higher than those of the CHD group; however, we could not find a methylation level difference between the case and control subgroups of the male group; therefore, the total case-control differences primarily derive from females. To the best of our knowledge, for the first time, we found that low methylation levels of the *ANGPTL2* gene promoter region in the plasma of Han Chinese patients with coronary atherosclerotic heart disease were associated with the risk of CHD. Previous studies demonstrated that the *ANGPTL2* gene was highly methylated in ovarian cancer cell lines, where *ANGPTL2* was silenced.[31] By contrast, in human osteosarcoma cell lines, the *ANGPTL2* gene promoter region was hypomethylated and the expression of *ANGPTL2* gene was elevated.[32] Therefore, we drew a conclusion that CHD cases might have much higher circulating ANGPTL2 concentrations than controls. This is in line with the previous studies demonstrating that circulating ANGPTL2 concentration accelerated plaque vulnerability and inflammation.[33,34]

There exist some limitations in our study. The sample size we collected is small. Moreover, we collect the whole peripheral blood which is a mixed population of cells, such as leukocytes, lymphocytes, and other cells. Although the association between methylation level of *ANGPTL2* gene and CHD has been clarified, the mechanisms are still not clear.

In conclusion, we have revealed that in females, lower methylation of CpG1, CpG2, CpG3, and CpG5 in the promoter region (Chr9:127122717-127122850) of the *ANGPTL2* gene is associated with CHD. The results may improve the current clinical diagnosis and facilitate the treatment of CHD.

CONFLICT OF INTEREST

The authors declare that they have no competing interest.

AUTHORS CONTRIBUTIONS

ZJQ, LJF, and CL conceived and designed the experiments and wrote the manuscript. ZJQ performed the experiments and analyzed the data. LJF contributed to the quality control of data and algorithms. All authors read and approved the final manuscript.

DATA ACCESSIBILITY

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

PUBLICATION ETHICS

The study was approved by the Ethics Committee of LiHuili Hospital of Ningbo Medical Center. All screened individuals consented to the use of their anonymity clinical data of all screening program for the purpose of research.

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