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CDKN2A and *CDKN2B* methylation in coronary heart disease cases and controls

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Abstract. The aim of the present study was to investigate the association between cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and cyclin-dependent kinase inhibitor 2B (*CDKN2B*) methylation, and coronary heart disease (CHD), and to explore the interaction between methylation status and CHD clinical characteristics in Han Chinese patients. A total of 189 CHD (96 males, 93 females) and 190 well-matched non-CHD controls (96 males, 94 females) were recruited for the study. Methylation-specific polymerase chain reaction technology was used to examine gene promoter methylation status. Comparisons of methylation frequencies between CHD and non-CHD patients were carried out using the Chi-square test. Methylation levels of *CDKN2A* and *CDKN2B* genes were not found to be associated with the risk of CHD. However, the mean age of *CDKN2A*-hypermethylated participants was significantly lower than *CDKN2A*-unmethylated participants (58.73±5.88 vs. 62.62±5.36 years, adjusted P<0.001). Conversely, the mean age of *CDKN2B*-hypermethylated participants was significantly higher compared with *CDKN2B*-unmethylated participants (62.26±5.48 vs. 58.33±7.47 years, adjusted P=0.048). In addition, *CDKN2B* methylation frequencies were significantly increased in female participants compared with males (99.47 vs. 11.98%, P=0.032). In conclusion, the results indicated that *CDKN2A* and *CDKN2B* promoter methylation frequencies were significantly associated with age, and there was a gender dimorphism in *CDKN2B* methylation.

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Introduction

Coronary heart disease (CHD) is a complex chronic disease that is caused by an imbalance between blood supply and demand in myocardium. Various environmental and genetic factors are known to contribute to onset and development of CHD (1). As of 2010, CHD was the leading cause of mortality globally, resulting in over 7 million cases of mortality (2). Therefore, association studies for CHD biomarkers have been performed worldwide (3-5) for future forefront diagnostics for the early assessment of cardiac risks.

The genetic locus at chromosome 9p21 has been demonstrated to be strongly associated with the risk of CHD (6,7). Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and cyclin-dependent kinase inhibitor 2B (*CDKN2B*) genes both encode putative regulators of cyclin-dependent kinases on chromosome 9p21. Genome-wide association studies have identified that some *CDKN2A* or *CDKN2B* genetic variants are susceptible to CHD (8-10). As recently reported, many human diseases, including cardiovascular disease, could be influenced by aberrant DNA methylation modification (11). Aberrant methylation of cytosine-phosphate-guanine (CpG) islands in gene promoters is associated with transcription silencing and activity (3). However, the exact role of *CDKN2A* and *CDKN2B* methylation in cardiovascular system has not yet been fully elucidated.

CDKN2A gene is involved in the regulation of cell proliferation, cell aging and apoptosis (12). However, a bidirectional role of *CDKN2A* gene expression has been reported in previous studies. Knösel *et al* (13) reported that increased CDKN2A may be linked to oncogene-induced senescence, whereas the loss of CDKN2A contributes to malignant progression. Furthermore, Bayoglu *et al* (14) reported that increased *CDKN2A* gene expression in artery plaques may increase the risk of atherosclerosis and contribute to the development of carotid artery stenosis. Although methylation-induced CDKN2A downregulation is observed in multiple human cancer types (15-17), few studies have evaluated the epigenetic role of *CDKN2A* in CHD.

CDKN2B gene lies adjacent to *CDKN2A*, and the protein encoded by this gene is associated with controlling cell cycle

G1 progression (18). *CDKN2B* has been previously detected as a candidate gene in CHD (19-21). Kojima *et al* (22) demonstrated that loss of CDKN2B promoted advanced development of atherosclerotic plaques, which suggests a crucial role for CDKN2B in the initiation and development of CHD. An inverse correlation between *CDKN2B* hypermethylation and low expression has previously been found in CHD (23). However, the potential for attenuating *CDKN2B* expression in CHD patients differs in different CpG regions (23).

The current study aimed to evaluate whether DNA methylation of *CDKN2A* and *CDKN2B* genes is associated with the risk of CHD. The results of this study may help to provide a molecular marker for early detection and individual therapy among CHD patients.

Materials and methods

Patient samples. A total of 189 CHD cases and 190 non-CHD controls were selected from Ningbo First Hospital (Ningbo, China) between June 2013 and December 2015. All the participants had undergone coronary angiography and were reviewed by at least two independent cardiologists. Those that had ≥50% diameter stenosis in any of the main coronary arteries, or a history of prior angioplasty, or coronary artery bypass surgery were placed in the CHD group. Those who had <50% diameter stenosis in the major coronary artery, or no history of atherosclerotic vascular disease were placed in the non-CHD group (24). Demographic data (age and gender) were collected by researchers. The mean age of CHD patients was 62.25±5.55 years, including 96 males and 93 females. The mean age of non-CHD controls was 62.07±5.58 years, including 96 males and 94 females. Biochemical indices [triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) in blood serum] were enzymatically measured using a CX7 biochemical analyzer (Beckman Coulter, Inc., Brea, CA, USA). Ethical approval was provided by the Ethics Committee at Ningbo First Hospital. All patients provided written informed consent.

DNA extraction and bisulphite conversion. DNA extraction and quantification was performed as described previously (25). DNA samples were converted using an EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions.

Methylation-specific polymerase chain reaction (MSP). The methylation status of *CDKN2A* and *CDKN2B* was determined by MSP, as described previously (26). Polymerase chain reaction (PCR) products were considered as methylated or unmethylated when clearly visible peaks were produced by a Qsep100 DNA Analyzer (BiOptic, Inc., Taipei, Taiwan). Further sequencing results indicated a successful bisulphite conversion and amplification (Fig. 1). The primer sequences of methylated and unmethylated primers were as follows: *CDKN2A* methylated, forward 5'-GTAGGGTTTAGAGTC GTTTCGA-3' and reverse 5'-AACTACAAACTAAAACCC ACGC-3'; *CDKN2A* unmethylated, forward 5'-CGTAGG GTTTAGAGTTGTTTTGA-3' and reverse 5'-AACTACAAA CTAAAACCCACACA-3'; *CDKN2B* methylated, forward 5'-GCGTTCGTATTTTGCGGTT-3' and reverse 5'-CGTACA ATAACCGAACGACCGA-3'; and *CDKN2B* unmethylated, forward 5'-TGTGATGTGTTTGTATTTTGTGGTT-3' and reverse 5'-CCATACATAACCAAACAACCAA-3'. The total amplification involved a reaction volume of 20 μl, containing 0.5 μl forward and reverse primers, 1.6 μl bisulphate-converted DNA, 10 μl ZymoTaq™ PreMix (Zymo Research) and 7.4 μl DNase/RNase-free water. The annealing temperatures were 55˚C for *CDKN2A* methylation and unmethylation PCR, 55˚C for *CDKN2B* methylation PCR and 57˚C for *CDKN2B* unmethylation PCR.

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analyses were performed using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). The mean subgroup differences for clinical characteristics were compared using Student's t-test. P-values were adjusted by age, gender, TG, TC, HDL-C and LDL-C using logistic regression. The Chi-square test was used to determine the association between promoter methylation and CHD. Two-sided P<0.05 was considered to indicate a statistically significant result.

Results

Patient characteristics. Baseline characteristics of CHD cases and non-CHD controls are shown in Fig. 2. There was no significant difference between the age of CHD cases (62.25±5.55 years) and non-CHD controls (62.07±5.58 years). There were also no significant differences between levels of TG, TC, HDL-C or LDL-C between CHD cases and non-CHD controls. Subsequently, subgroup analysis was performed by gender. The TG level was significantly higher in males compared with females in CHD cases (2.42±0.92 vs. 1.68±1.05 mmol/l; P<0.001) and non-CHD controls (2.52±0.80 vs. 1.39±0.69 mmol/l; P<0.001). The TC level was significantly lower in CHD males compared with CHD females (4.25±1.08 vs. 4.70±1.14 mmol/l; P<0.009). The HDL-C level was significantly lower in males compared with females both in CHD (1.06±0.27 vs. 1.17±0.29 mmol/l; P=0.009) and non-CHD (1.06±0.27 vs. 1.21±0.29 mmol/l; P=0.001). The LDL-C was significantly lower in males compared with females both in CHD (1.96±2.77 vs. 2.78±1.00 mmol/l; P=0.016) and non-CHD (1.66±0.96 vs. 2.77±0.88 mmol/l; P<0.001).

Association analysis between CHD and methylation of CDKN2A and CDKN2B. In the present study, MSP was used to estimate the methylation status of *CDKN2A* and *CDKN2B* gene promoters in 189 CHD patients and 190 non-CHD controls. No associations were found between *CDKN2A*/*CDKN2B* gene promoter methylation and CHD in the total samples or in gender subgroups (Table I).

Association analysis between age and methylation of CDKN2A and CDKN2B. In all participants, the mean age of *CDKN2A*-methylated participants was significantly lower compared with *CDKN2A*-unmethylated participants (58.73±5.88 vs. 62.62±5.36 years; P<0.001; adjusted P<0.001; Fig. 3). Conversely, the mean age of *CDKN2B*-methylated

Table I. Methylation frequencies of *CDKN2A* and *CDKN2B* promoters in CHD cases and non-CHD controls.

Gene	CHD	Non-CHD	P-value	Odds ratio (95% confidence interval)
Total samples				
CDKN2A (M/U)	38/151	29/161	0.217	1.397 (0.821-2.378)
CDKN2B (M/U)	184/5	186/4	0.751	0.791 (0.209-2.994)
Male				
CDKN2A (M/U)	12/84	11/85	0.842	1.104 (0.462-2.640)
CDKN2B (M/U)	92/4	92/4	1.000	1.000 (0.243-4.119)
Female				
CDKN2A (M/U)	26/67	18/76	0.156	1.638 (0.826-3.250)
CDKN2B (M/U)	92/1	94/0	0.497	0.495 (0.428-0.572)

CDKN2A, cyclin-dependent kinase inhibitor 2A; *CDKN2B*, cyclin-dependent kinase inhibitor 2B; M, methylation-specific primer; U, unmethylation-specific primer; CHD, coronary heart disease.

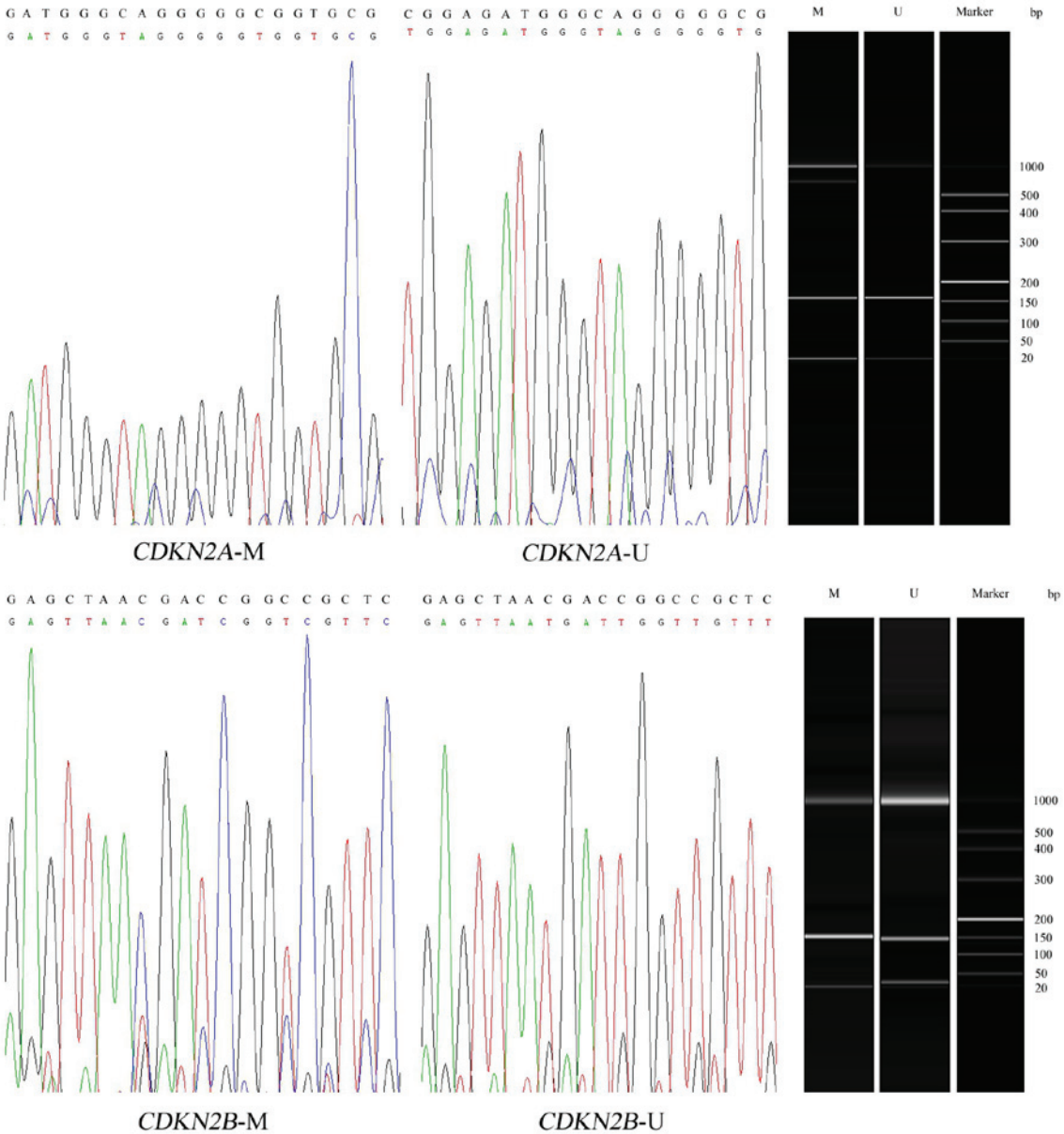

Figure 1. Typical methylation analysis result of sequencing validation and methylation-specific polymerase chain reaction for the *CDKN2A* and *CDKN2B* gene promoter regions. The top row of the sequence represents the original sequences of genes and the bottom row shows the converted sequences. *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *CDKN2B*, cyclin-dependent kinase inhibitor 2B; M, methylation-specific primer; U, unmethylation-specific primer.

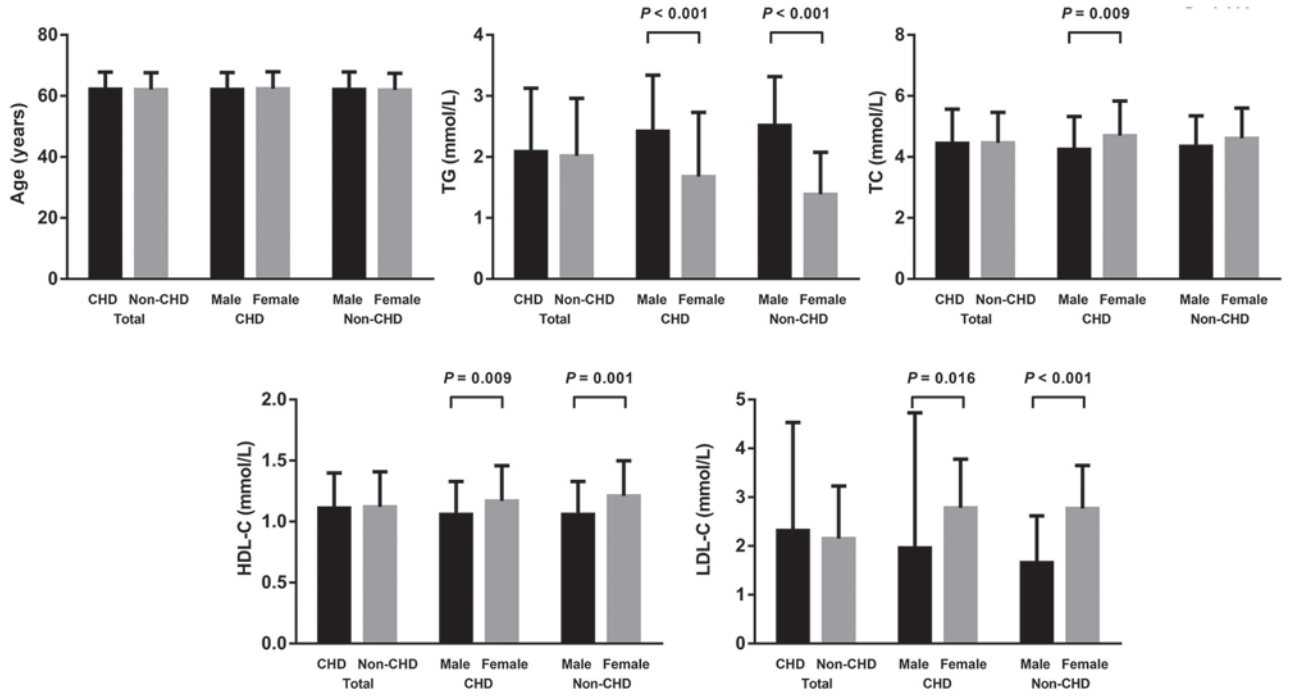

Figure 2. Clinical characteristics of all subjects according to subgroup analysis by CHD status and gender. CHD, coronary heart disease; TG, triglyceride; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

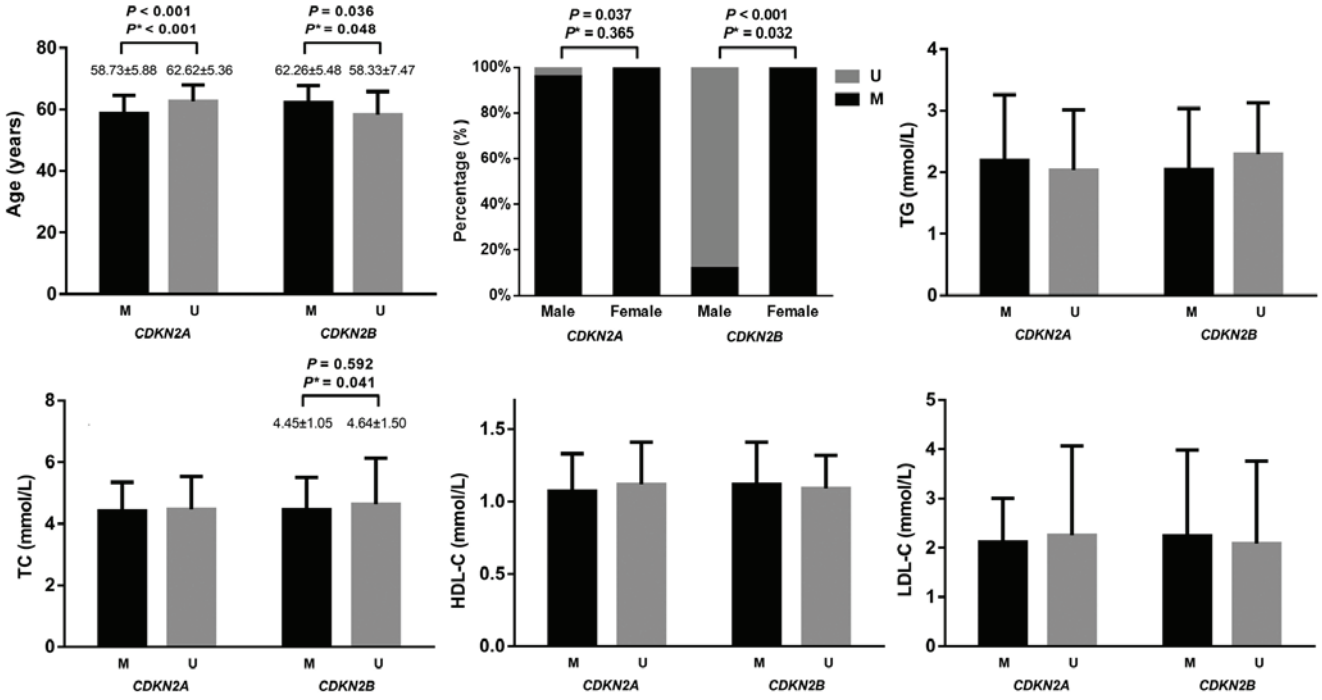

Figure 3. Association between *CDKN2A* and *CDKN2B* methylation and clinical characteristics in all subjects. P* is adjusted by age, gender, TG, TC, HDL-C and LDL-C using logistic regression. *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *CDKN2B*, cyclin-dependent kinase inhibitor 2B; M, methylation; U, unmethylation; TG, triglyceride; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

participants was significantly higher compared with *CDKN2B*-unmethylated participants (62.26±5.48 vs. 58.33±7.47 years; P=0.036, adjusted P=0.048).

Association analysis between gender and methylation of CDKN2A and CDKN2B. A significantly larger proportion of female participants were found to be *CDKN2B*-methylated compared with male participants (99.47 vs. 11.98%; P<0.001, adjusted P=0.032; Fig. 3). Furthermore, 99.47% of female participants were *CDKN2A*-methylated and 95.83% of male participants were *CDKN2A*-methylated (P=0.037; adjusted P=0.365).

Association analysis between blood cholesterol level and methylation of CDKN2A and CDKN2B. No significant associations were observed between the plasma levels of TG, HDL-C and LDL-C and *CDKN2A*/*CDKN2B* methylation status (Fig. 3). In addition, no significant association was observed between TC level and *CDKN2A* methylation status. TC level was significantly lower in methylated *CDKN2B* compared with unmethylated *CDKN2B* (4.45±1.05 vs. 4.64±1.50 mmol/l; adjusted P=0.041; Fig. 3).

Discussion

The purpose of this study was to investigate the association between *CDKN2A* and *CDKN2B* promoter methylation and CHD risk. Through a series of statistical analyses, no notable relationship was found between the methylation status of *CDKN2A* or *CDKN2B* and CHD. However, it was noteworthy that the methylation of *CDKN2A* and *CDKN2B* promoters was associated with age in all participants. *CDKN2A* methylation was associated with younger age, whereas *CDKN2B* methylation was associated with older age. Moreover, female participants were found to be more frequently *CDKN2B*-methylated compared with male participants.

DNA methylation is one of the major epigenetic modifications (3). Accumulating studies have indicated that DNA methylation changes are associated with an increased risk of CHD (27-29). *CDKN2A* and *CDKN2B* genes have been previously reported as hypermethylated tumor suppressor genes in leukemia (30), parathyroid tumor (31) and breast cancer (32), suggesting a potential epigenetic regulation on cell proliferation and apoptosis. Using pyrosequencing and MethyLight methods, Zhuang *et al* (23) demonstrated that $p15^{INK4b}$ and $p16^{INK4a}$ methylation was an important event in CHD. However, the current data indicated that the methylation of *CDKN2A* and *CDKN2B* genes was not significantly associated with the risk of CHD, which might be explained by different target fragments and testing methods.

In the present study, it was demonstrated that age was associated with gene promoter methylation changes. Alterations of epigenetic marks such as DNA methylation have been linked to cancer in older patients (33). Age-dependent gene methylation may also contribute to the phenotypic changes associated with skin aging (34). A previous study demonstrated that age-related DNA methylation affected the essential hypertension status (25). For the *CDKN2A* gene, older patients were more likely to be unmethylated in the present study, even when assessed independent of blood cholesterol and gender. An elevated level of CDKN2A in artery plaques may increase the risk of atherosclerosis (14); it is hypothesized that this may result from the regulatory effect of demethylation on gene active expression, or from dysregulation of DNA integrity and function. In the current study, *CDKN2B* gene methylation was associated with older age, which is in accordance with the hypothesis that the pathogenic role of this cancer suppressor gene in vascular disease may be associated with its DNA methylation.

Gender is a variable that must be taken into consideration in studies of chronic diseases, including CHD (35). The prevalence and incidence of cardiovascular events are different between males and females (36). A previous study reported that women with a low TG/HDL ratio have substantially lower CHD rates compared with men with a low TG/HDL ratio (37). *CDKN2B* polymorphism was found to be independently associated with increased TG/HDL ratio change (38). In the present study, it was indicated that methylation of the promoter of *CDKN2B* was significantly more likely in females compared with males. No gender dimorphism was observed for methylation of the *CDKN2A* gene.

There were some limitations to the present study. Firstly, the study involved 189 CHD patients and 190 non-CHD controls. However, power analysis indicated insufficient powers (5.0-29.4%) for overall test and gender subgroup analyses. A lack of power existed in the current study due to small sample size, thus further replication studies with larger sample sizes are required. Secondly, only Chinese Han people were recruited, therefore validations of the findings are required in other ethnic populations. Furthermore, DNA methylation status was measured using a qualitative method, and a quantitative method should be explored in the future.

In conclusion, the present study indicates that there is an association between age and *CDKN2A* and *CDKN2B* gene promoter methylation status, as well as an association between gender and *CDKN2B* methylation. However, no association was observed between the methylation of these genes and the risk of CHD. Further investigations are needed to verify these results and explore the role of DNA methylation in CHD in more detail.

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