Elevated methylation of cyclin dependent kinase inhibitor 2B contributes to the risk of coronary heart disease in women

XIAOMIN CHEN1*, DANJIE JIANG2*, LIMIN XU2*, LIYUAN HAN2, HAOCHANG HU1, YI HUANG2, DEYI LU3, HUIHUI JI2, BIN LI2, YONG YANG2, CONG ZHOU2, XUTING XI2, NAN WU1, XIAOFENG XI1, YAN XU3, YUSHENG SHEN2, JIYI LI4 and SHIWEI DUAN2

1Key Laboratory of Ningbo First Hospital and Cardiovascular Center of Ningbo First Hospital, Ningbo University, Ningbo, Zhejiang 315010; 2Medical Genetics Center, School of Medicine, Ningbo University, Ningbo, Zhejiang 315211, P.R. China; 3Richard and Loan Hill Department of Bioengineering, University of Illinois at Chicago, Chicago, IL 60607, USA

Received August 7, 2017; Accepted January 18, 2018

DOI: 10.3892/etm.2018.6920

Abstract. Cyclin dependent kinase inhibitor 2B (CDKN2B) encodes a cyclin-dependent kinase inhibitor that may enhance the formation of atherosclerotic plaques. The aim of the present study was to investigate the contribution of CDKN2B promoter methylation on the risk of coronary heart disease (CHD). The present results indicated a significant association between increased CDKN2B methylation and the risk of CHD (adjusted P=0.043). A breakdown analysis according to sex demonstrated that CDKN2B methylation was significantly associated with the risk of CHD in women (adjusted P=0.010), but not in men. A further breakdown analysis by age indicated a significant association of CHD in the women >60 years (P=0.024). Luciferase reporter gene assay results indicated that the CDKN2B promoter fragment significantly enhanced luciferase activity (P<0.001). In addition, CDKN2B transcription was significantly enhanced following treatment with 5-aza-2'-deoxycytidine methylation inhibitor in human aortic endothelial cells (HAEC) and human primary coronary artery smooth muscle cells (HPCASMC; P<0.05 and P<0.01), but not in 293 cells. Notably, estrogen treatment reduced CDKN2B methylation of several CpGs and significantly increased CDKN2B gene expression levels in HAEC, HPCASMC and 293 cells (P<0.05 and P<0.01). Additionally, treatment of HAEC and HPCASMC with simvastatin and γ-carboxy-L-glutamic acid reduced CDKN2B promoter methylation and increased CDKN2B transcription concomitantly. The present study suggests that CDKN2B promoter methylation may be associated with sex dimorphism in the pathogenesis of CHD.

Introduction

Coronary heart disease (CHD) is the leading cause of mortality in African-American men and women (1). Genetic factors have been demonstrated to serve a significant role in the development of CHD (2). In addition, the involvement of epigenetic modifications has been suggested in the development and the progression of CHD (3-6). DNA methylation is a stable epigenetic modification that results in the addition of a methyl group to the 5' carbon of cytosine and primarily occurs at CpG dinucleotide sequences in the mammalian genome (7). Notably, gene promoter hypermethylation typically silences gene transcription, and aberrant gene methylation has been indicated to be involved in the pathogenesis of various diseases, including CHD (4,8,9) and type 2 diabetes (10,11).

Cyclin dependent kinase inhibitor 2B (CDKN2B) is located on chromosome 9p21, which has been associated with CHD in a number of genome-wide association studies (GWASs) (12,13). CDKN2B encodes a cyclin-dependent kinase inhibitor that regulates cell cycle G1 progression (14,15). Cancer cells with hypermethylated CDKN2B are typically associated with aberrantly accelerated proliferation (16). Atherosclerotic plaques are the major contributing factor in CHD pathogenesis and are caused by overproliferation of vascular smooth muscle cells and macrophages (4,17). Notably, CDKN2B loss in mice promoted atherosclerosis by increasing the size and complexity of the lipid-laden necrotic core through impaired efferocytosis (17). Furthermore, CDKN2B has been suggested...
as a candidate gene of CHD (18,19). CDKN2B hypermethylation has been indicated to be significantly associated with the elevated expression of its antisense noncoding RNA, antisense noncoding RNA in the INK4 locus (ANRIL), and an increased risk of CHD (4). Several studies have revealed the potential roles of CDKN2B in CHD (14,17,20).

Sex-specific associations have been indicated in various aspects of CHD. For example, women have been demonstrated to have a proportionally lower prevalence of disease and tend to develop it later in life compared with men, and the difference of incidence, development and surgical treatment of CHD between males and females was indicated in previous studies (21-23). Prior to menopause, women have relatively more protection against CHD compared with men of the same age range (24-26). A previous GWAS indicated sex differences in DNA methylation on 470 autosomal sites, including sites in CDKN2B (27). These epigenetic differences are associated with differential mRNA and microRNA expression levels and organ functions (27). In addition, conventional cardiovascular pharmacological agents have been indicated to induce their therapeutic effects on CHD through various mechanisms, for example, by affecting serum levels of vascular calcification inhibitors, which reduce cardiac workload and increase coronary blood flow (28,29).

In the present study, a case-control study was performed to investigate whether CDKN2B promoter methylation contributes to the risk of CHD in a sex-dependent pattern, and whether estrogen and conventional cardiovascular pharmacological agents are able to recover CDKN2B expression by reversing CDKN2B promoter methylation.

Materials and methods

Samples. CHD and non-CHD control samples were obtained from patients at Ningbo First Hospital (Ningbo, China) between May 2008 and April 2010. A total of 36 CHD cases (18 males and 18 females, mean age, 62.5±5.5) and 36 age- and sex-matched controls were included in the present study. All individuals were Han Chinese from Ningbo city in Eastern China diagnosed according to the World Health Organization criteria (30). The inclusion criteria utilized were as follows: Angiographic evidence of >50% stenosis in one or more major coronary arteries. Patients were excluded from the current study if they had congenital heart disease, autoimmune disease, cardiomyopathy or severe liver or kidney disease. All peripheral blood samples from patients (5 ml) were collected in 3.2% citrate sodium-treated tubes and stored at -80℃. The study protocol was approved by the Ethics Committee in Ningbo First Hospital and all methods were performed in accordance with the relevant guidelines and regulations. Written, informed consent forms were obtained from all subjects.

Bisulfite pyrosequencing. Human blood genomic DNA was extracted and quantified as described previously (31). The DNA methylation assay comprised of sodium bisulfite DNA conversion (Epitech Bisulfite kits; Qiagen AB, Sollentuna, Sweden), polymerase chain reaction (PCR) amplification (Pyromark PCR kit; Qiagen) and pyrosequencing (Pyromark Gold Q24 Reagents; Qiagen AB), which were performed in accordance with the manufacturer's protocol. The Pyromark PCR Master Mix was used in PCR amplification. PCR primers were designed using PyroMark Assay Design software v2.0.1.15 (both Qiagen). The sequences of the primers utilized were as follows: CDKN2B forward, 5'-TAGGGGGAGGATTTAAG GGG-3' and reverse, 5'-biotin-ACA CTCTTCCTTCTTTTC C-3'; CDKN2B sequencing primer, 5'-GGGGTAGTGGAG ATT-3'. The thermocycling conditions were as follows: 1 cycle at 94℃ for 15 sec, 45 cycles at 94℃ for 20 sec, 58℃ for 30 sec, 72℃ for 60 sec and an extension stage at 72℃ for 3 min.

Cell lines. Recent studies have suggested that CDKN2B is associated with the occurrence and development of several types of cancer (32,33). Notably, 293 cells are typically applied to study the transforming and oncogenic properties of cancer-associated genes as a model (34). Thus, 293 cell lines were selected for the present study. Three cell lines, including 293 cells (https://www.atcc.org/Products/All/CRL-11268.aspx), human aortic endothelial cells (HAEC; https://www.atcc.org/Products/All/PCS-100-011.aspx) and human primary coronary artery smooth muscle cells (HPCASMC; https://www.atcc.org/Products/All/PCS-100-021.aspx) were used in the present study. Cell lines were purchased from American Type Culture Collection (Manassas, VA, USA) and were cultured using dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in an incubator at 37℃ with 5% CO₂ for 24 h.

Treatment with 5-aza-2'-deoxycytidine (DAC), estrogen and cardiovascular pharmacological agents. Cells were cultured at a density of 1x10⁵ cells/well in 6-well plates at 37℃ for 24 h and the media (DMEM with FBS and penicillin/streptomycin) was replaced following 4-8 h. To determine the potential regulatory roles of DNA methylation in CDKN2B gene transcription, HAEC, HPCASMC and 293 cells were treated with DAC (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 0.5, 1.0 and 2.0 µM at 37℃ for 3 days. Following this incubation step, total RNA was isolated from the cells and subjected to reverse transcription. To examine the effects of estrogen treatment, the cells were treated with estrogen (17-β-estradiol; Sigma-Aldrich; Merck KGaA) at different concentrations (10, 100 and 1,000 nM), and the total RNA and genomic DNA were isolated. In aforementioned comparisons, these three cell lines with the EtOH treatment were considered as the control. In addition, 10 µM simvastatin, 10 µM trimetazidine dihydrochloride and 50 µM γ-carboxy-L-glutamic acid (all Sigma-Aldrich; Merck KGaA) at different concentrations (10, 100 and 1,000 nM), and the total RNA and genomic DNA were isolated. In aforementioned comparisons, these three cell lines with the EtOH treatment were considered as the control. In addition, 10 µM simvastatin, 10 µM trimetazidine dihydrochloride and 50 µM γ-carboxy-L-glutamic acid were used to treat HAEC and HPCASMC for 1, 6, 12 and 24 h. Notably, γ-carboxy-L-glutamic acid is an effective ingredient of isosorbide mononitrate, which is a common cardiovascular drug (35,36). Following treatments, total RNA and genomic DNA were extracted from cells. 293 cells were not subjected to treatment as HAEC and HPCASMC were more specific to cardiovascular disease and thus would produce more meaningful results.

Total RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). TRIzol reagent (Invitrogen; Thermo
Fisher Scientific, Inc.) was used for total RNA isolation. Subsequently, 1 µg RNA was treated with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific, Inc.) for 2 h at 37°C for cDNA synthesis according to the manufacturer’s protocol. qPCR was performed using SYBR green master PCR mix (Applied Biosystems, Thermo Fisher Scientific, Inc.). CDKN2B transcription was normalized to GAPDH transcription levels and the PCR products were quantified using the 2^ΔΔCq method (37). The thermocycling conditions were as follows: denaturation at 94°C for 5 min, followed by 40 cycles of amplification (30 sec of denaturation at 94°C, 30 sec of annealing at 58°C and 30 sec of extension at 72°C). The following primers were used: CDKN2B forward, 5'-GGG GCA GTG ACCGG-3' and reverse, 5'-ACCTTCTCCACTAGTCCC-3'; and GAPDH forward, 5'-TGGTATGGAAGGACTCA-3' and reverse, 5'-CCAGTAGGCGAGGGATGAT-3'.

Bisulfite sequencing. Genomic DNA was isolated from cells with or without estrogen (10, 100 and 1,000 nM) and cardiovascular drug treatments (10 µM simvastatin, 10 µM trimetazidine dihydrochloride and 50 µM γ-carboxy-L-glutamic acid) using a genomic DNA isolation kit (Qiagen, Inc., Valencia, CA, USA). Bisulfite conversion reagents (EpiTect Bisulfite kit; Qiagen, Inc.) were used to convert genomic DNA. Products were amplified with the following primers for CDKN2B promoter: CDKN2B_F, 5'-TTGTTTAGTTGAAAAAYGGA TT-3'; and CDKN2B_R, 5'-AACRCCTAAACRCAACRCAAC C-3'. PCR was performed as follows: 95°C for 5 min; followed by 35 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec, with a final extension of 2 min at 72°C. PCR products were cloned using a commercial CloneJET PCR Cloning kit (Thermo Fisher Scientific, Inc.) according to manufacturer’s instructions. A total of 15-20 independent bacterial clones were isolated and sequenced for each PCR fragment to calculate the cytosine methylation levels.

Luciferase reporter gene assays. The CDKN2B promoter fragment containing seven CpG sites was generated using PCR as aforementioned, and the primer sequences of CDKN2B were as follows: Forward, 5'-GGGGCGATGT AGGACT-3' and reverse, 5'-GCCTGAGTGCTCTC-3'. The subsequent PCR product was cloned into pCR2.1 (included in the kit) using a T-A Cloning Kit (Invitrogen; Thermo Fisher Scientific, Inc.) and sequenced. Plasmids containing CDKN2B promoter region were amplified and digested with XhoI and KpnI (New England Biolabs, Ipswich, MA, USA). The target DNA fragment containing the reporter gene was cloned into pGL3-Luciferase reporter plasmid (Promega Corporation, Madison, WI, USA). Subsequently, constructed pGL3-CDKN2B-Luciferase plasmid was transfected into 293 cells using Lipofectamine 2000 Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer’s protocol. Luciferase and β-galactosidase activities were measured using a Luciferase Assay System (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to manufacturer’s protocol. β-Galactosidase activity was used to normalize transfection efficiency.

Statistical analysis. SPSS package software (version 16.0; SPSS, Inc., Chicago, IL, USA) was used to determine the association between CDKN2B promoter methylation and CHD and various biochemical factors. Comparisons of CDKN2B methylation were performed using GraphPad Prism 5 Software (GraphPad Software, Inc., La Jolla, CA, USA). Correlations of CDKN2B methylation with age and biochemical indicators were performed using R statistical software (R V.3.3.2; https://www.r-project.org/). All P-values were adjusted for age, smoking, diabetes and hypertension. Relative expression data were presented as the mean ± standard deviation. Statistical analysis for RT-qPCR data was performed using one-way analysis of variance followed by a Dunnett’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Significant association of CDKN2B promoter methylation with CHD in women. The bisulfite pyrosequencing assay was used to measure a total of seven CpGs on the CDKN2B promoter among 36 cases and 36 matched controls. Significant correlations were observed among DNA methylation levels of the seven CpGs (r=0.7, Fig. 1A). Subsequently CDKN2B methylation was represented as the mean methylation level of the seven CpGs. As indicated in Table I and Fig. 1B, significantly increased CDKN2B methylation levels (7.66±3.02%) were observed in patients with CHD compared with non-CHD subjects (6.43±2.23%, adjusted P=0.043). In addition, a significant association of CHD with sex was indicated regarding CDKN2B methylation (adjusted P=0.048; Fig. 1B). Further analysis demonstrated a significant female-specific association of CDKN2B methylation with CHD [women with CHD (7.21±2.40%) compared with women without CHD (4.83±1.31%), adjusted P=0.010; Fig. 1B]. However, there was no significant correlation between CDKN2B methylation and age in the whole cohort (total, r=0.041, adjusted P=0.811; Fig. 1C), further analysis regarding age indicated a significant association with CHD in women >60 years old (women, r=0.672, adjusted P=0.024; Fig. 1C).

CDKN2B promoter fragment enhances luciferase gene activity. The dual-luciferase reporter assay is a standard method that utilizes Firefly and Renilla luciferase to explore the promoter activity of target fragment (38). The CDKN2B promoter fragment containing seven CpG sites was amplified and cloned into pGL3 luciferase plasmid in the present study. The construct was transfected into 293 cells. Notably, HPCASMC and HAEC were difficult transfect and were therefore excluded from this experiment. Results suggested that the CDKN2B promoter fragment significantly enhanced the luciferase activity by >200 fold compared with pGL3-Basic in 293 cells (P<0.001; Fig. 2A). The present results indicated that CDKN2B promoter enhanced the luciferase activity in 293 cells.

Methylation inhibitor DAC enhances CDKN2B gene transcription. Endogenous CDKN2B transcription levels were determined in HPCASMC, HAEC and 293 cells (Fig. 2B). Furthermore, cell lines were incubated with different concentrations of DAC, a DNA methyltransferase
inhibitor. Results revealed that DAC (0.5 µM/l) significantly upregulated CDKN2B transcription levels compared with EtOH treatment in HPCASMC (P<0.05; Fig. 2C). In addition, DAC (1 µM/l and 2 µM/l) significantly upregulated CDKN2B transcription levels compared with EtOH in HAEC (1 µM/l, P<0.01 and 2 µM/l, P<0.01; Fig. 2C). However, no significant differences in CDKN2B transcription levels were detected in 293 cells, which may have been due to the high endogenous CDKN2B expression. In light of these findings, it was speculated that DNA methylation may serve an important role in the regulation of CDKN2B transcription in HAEC and HPCASMC.

Table I. Comparison of cyclin dependent kinase inhibitor 2B methylation levels within subgroups.

| DNA methylation | CHD (n=36) mean ± SD | Non-CHD (n=36) mean ± SD | P-value | Sex subgroup interaction P-value |
|-----------------|----------------------|------------------------|---------|---------------------------------|
| Variable        |                      |                        |         |                                 |
| DNA methylation site |                 |                        |         |                                 |
| CpG 1           | 8.89±3.97            | 7.44±2.99              | 0.066   | 0.058                          |
| CpG 2           | 5.94±2.03            | 5.14±1.78              | 0.042   | 0.239                          |
| CpG 3           | 6.11±2.81            | 4.61±1.68              | 0.012   | 0.088                          |
| CpG 4           | 4.83±2.25            | 4.00±1.64              | 0.057   | 0.168                          |
| CpG 5           | 9.19±4.03            | 7.97±3.07              | 0.149   | 0.278                          |
| CpG 6           | 9.03±3.19            | 8.11±2.97              | 0.142   | 0.005                          |
| CpG 7           | 9.61±4.09            | 7.72±3.53              | 0.031   | 0.023                          |
| Mean ± SD DNA methylation (%) | 7.66±3.02 | 6.43±2.23              | 0.043   | 0.048                          |

P-values were adjusted by age, history of smoking, diabetes and hypertension. CHD, coronary heart disease; SD, standard deviation.

Figure 1. Sex dimorphism in the association of CDKN2B promoter methylation with CHD and age. (A) Significant association among the seven CDKN2B promoter CpGs. All r-values are presented in the squares (all P<0.05). (B) Significant differences of CDKN2B methylation between CHD and non-CHD subjects. (C) Correlation between CDKN2B promoter methylation and age. P, P-value adjusted according to age, history of smoking, diabetes and hypertension. CHD, coronary heart disease; CDKN2B, cyclin dependent kinase inhibitor 2B.
**Estrogen increases CDKN2B transcription and alters its promoter methylation.** Cells were incubated with different concentrations of estrogen. Results suggested that estrogen significantly upregulated CDKN2B transcription levels in HAEC and 293 cells compared with EtOH treatment (HAEC, 100 nM/ł: P<0.01 and 1,000 nM/ł: P<0.01; 293, 10 nM/ł: P<0.05, 100 nM/ł: P=0.015 and 1,000 nM/ł: P=0.030; Fig. 2D). Specific concentrations of estrogen also significantly increased CDKN2B transcription levels in HPCASMC (HPCASMC, 10 nM/ł: P=0.015 and 1,000 nM/ł: P=0.030; Fig. 2D).

Methylation levels of seven CDKN2B CpGs were varied in HAEC, HPCASMC and 293 cells. There were four fully methylated CpGs (CpG-1, 5, 6 and 7) and three unmethylated CpGs (CpG-2, 3 and 4) in 293 cells, and four fully methylated CpGs (CpG-1, 2, 3 and 6) and three unmethylated CpGs (CpG-4, 5 and 7) in HPCASMC. The methylation levels of the seven CpGs were <0.3 in HAEC (Fig. 3). In some cases, estrogen treatment reduced the methylation levels of previously hypermethylated CpGs and increased the methylation levels of previously hypomethylated CpGs in HPCASMC and 293 cells (Fig. 3). Notably, estrogen treatment was able to increase the methylation levels of the majority of CpG sites in HAEC.

Although estrogen treatment did not result in a similar methylation pattern among the three cell lines, the results suggested that estrogen was able to increase CDKN2B gene transcription among the three cell lines. The findings indicated that estrogen may increase CDKN2B transcription by altering CDKN2B methylation.

Cardiovascular pharmacological agents increase CDKN2B transcription and reduce CDKN2B promoter methylation concomitantly. Three cardiovascular pharmacological agents, including simvastatin, γ-carboxy-L-glutamic acid and trimetazidine dihydrochloride, were selected to explore their regulatory effects on CDKN2B. Results revealed that 24-h treatment with these agents significantly altered CDKN2B transcription levels in HPCASMC and HAEC, with the exception of HAEC treated with simvastatin and γ-carboxy-L-glutamic acid (P<0.05 and P<0.01; Fig. 4A). Notably, CDKN2B transcription levels in HPCASMC increased 63.3-fold following 24 h treatment with simvastatin, 26.5-fold following 24 h treatment with γ-carboxy-L-glutamic acid and 41.9-fold following 24 h treatment with trimetazidine dihydrochloride, respectively (Fig. 4A). CDKN2B transcription levels in HAEC increased 2.0-fold following 12 h treatment with simvastatin and 40.0-fold following 6 h treatment with γ-carboxy-L-glutamic acid treatment, respectively (Fig. 4A). Conversely, CDKN2B transcription levels significantly decreased 2.2-fold following 24 h treatment with trimetazidine dihydrochloride treatment in HAEC (P<0.01; Fig. 4A). Methylation assays demonstrated that the cardiovascular pharmacological agents reduced CDKN2B methylation levels in HAEC and HPCASMC (Fig. 4B). Treatment with simvastatin, γ-carboxy-L-glutamic acid and trimetazidine dihydrochloride reduced CDKN2B promoter methylation and increased CDKN2B transcription concomitantly in HPCASMC. Additionally, treatment with simvastatin and γ-carboxy-L-glutamic acid reduced CDKN2B promoter methylation and increased CDKN2B transcription concomitantly in HAEC.

**Discussion**

In the present study, it was identified that there was a female-specific association of elevated CDKN2B promoter
Figure 3. Methylation levels of CDKN2B in HPCASMC, HAEC and 293 cells treated with estrogen. The cells treated with EtOH were used as the control. CDKN2B, cyclin dependent kinase inhibitor 2B; HAEC, human aortic endothelial cells; HPCASMC, human primary coronary artery smooth muscle cells.

Figure 4. Methylation and relative transcription levels of CDKN2B in HPCASMC and HAEC treated with simvastatin, trimetazidine dihydrochloride and γ-carboxy-L-glutamic acid. (A) Relative CDKN2B transcription levels in the cell lines treated with the three cardiovascular pharmacological agents at 0, 1, 6, 12 and 24 h. *P<0.05, **P<0.01. (B) Methylation levels of CDKN2B CpGs in the cell lines treated with the three cardiovascular pharmacological agents. Cell lines with the EtOH treatment were considered as the control. CDKN2B, cyclin dependent kinase inhibitor 2B; HAEC, human aortic endothelial cells; HPCASMC, human primary coronary artery smooth muscle cells.
methylation with CHD. Subsequent functional experiments indicated that CDKN2B promoter methylation is important for gene expression, which was further demonstrated to be susceptible to estrogen and conventional cardiovascular pharmacological agents.

CDKN2B has been suggested to regulate efferocytosis and atherosclerosis (39). The deletion of CDKN2B promotes the advanced development of atherosclerotic plaques (40). Furthermore, CDKN2B expression is reduced in atherosclerotic plaques, indicating that CDKN2B serves an essential role in the formation of atherosclerotic plaques (39). In the present study, the CDKN2B promoter fragment was able to significantly enhance luciferase reporter gene activity. Furthermore, methylation inhibitor DAC increased CDKN2B transcription levels. These results suggest that CDKN2B promoter methylation may be important in the regulation of CDKN2B gene function.

Sex dimorphism of CHD has been observed in the prevalence and the onset age of CHD (21,41). Notably, the onset age of CHD in women is typically 10 years later than that in men (21). Furthermore, non-obstructive CHD and angina are more frequently identified in women than in men (42,43). Previous results suggested an interaction between sex and age impacted DNA methylation (44,45). Furthermore, age has been indicated to increase the risk of CHD in women compared with men (46). CHD risk in men plateaus at the age of 45-50, whereas in women, CHD risk continues to increase sharply until the age of 60-65 (47). In the present study, a female-specific association of elevated CDKN2B promoter methylation was indicated with the risk of CHD. Further analysis by age suggested that the women >60 years of age had significantly higher CDKN2B promoter methylation levels.

Studies have indicated that estrogen may inhibit atherosclerotic plaque progression and vasodilation through its anti-oxidative and anti-inflammatory properties (21,43,48). Furthermore, it has been identified that estrogen may influence neoplastic diseases via its effects on the levels of gene expression and DNA methylation (49,50). In the present study, CDKN2B transcription levels were upregulated following estrogen treatment in HPCASMC, HAEC and 293 cells, in addition with promoter cytosine modifications in the promoter region of CDKN2B. The present findings suggest that estrogen may exert its regulatory role through promoter methylation modification.

Cardiovascular pharmacological agents include angiotensin-converting enzyme inhibitors, nitrates, statins and β-adrenergic blockers (51-54). Abnormal gene methylation may influence the curative effect of various kinds of drugs, including anti-tumor drugs and chemotherapeutic drugs (55-57). In the present study, treatment with three types of cardiovascular pharmacological agents increased CDKN2B transcription levels and reduced CDKN2B methylation levels concomitantly in HPCASMC. Similar results were identified in HAEC, except trimetazidine was demonstrated to decrease CDKN2B transcription in HAEC and further study should be performed to verify this result. This suggests that these agents may deliver their effects through CDKN2B gene silencing.

In conclusion, the present study demonstrated the role of DNA methylation in the regulation of CDKN2B transcription and that CDKN2B transcription may be affected by estrogen and cardiovascular pharmacological agents. Furthermore, the present results provided an improved understanding of the mechanisms by which CDKN2B may contribute to the risk of CHD in women. Due to the moderate sample size, future studies with extended samples are required to assess the significant association of CDKN2B promoter methylation with CHD in females.

Acknowledgements

Not applicable.

Funding

The present study was supported by grants from the National Natural Science Foundation of China (grant no. 81371649), the Natural Science Foundation of Zhejiang Province (grant no. LR13H020003) and from K. C. Wong Magna Fund in Ningbo University (Ningbo, Zhejiang, China).

Availability of data and materials

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

Authors' contributions

XC and SD conceived and designed the current study. YY, HJ, CZ, BL, XaX, XiX, NW, YX, JL and YS performed the experiments. DJ, LX, LH, YH, DL and YX performed the analyses. CZ, BL, XuX, XiX, NW, YX, JL and YS performed the experiments. XC, DJ, LX and HH prepared all figures and tables. XC wrote the manuscript. All the authors reviewed the manuscript and agreed to its publication.

Ethics approval and consent to participate

The protocol of the current study was approved by the Ethics Committee of Ningbo First Hospital (Ningbo, China) and all methods were performed in accordance with the relevant guidelines and regulations.

Patient consent for publication

Written informed consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

References

1. Lettre G, Palmer CD, Young T, Ejebe KG, Allayee H, Benjamin EJ, Bennett F, Bowden DW, Chakravarti A, Dreisbach A, et al: Genome-wide association study of coronary heart disease and its risk factors in 8,090 African Americans: The NHLBI CARe Project. PLoS Genet 7: e1001300, 2011.
2. Kitzmazarzyk PT, Perusse L, Rice T, Gagnon J, Skinner JS, Wilmore JH, Leon AS, Rao DC and Bouchard C: Familial resemblance for coronary heart disease risk: The HERITAGE family study. Ethn Dis 10: 138-147, 2000.
3. Locsaldo J and Handy DE: Epigenetic modifications: Basic mechanisms and role in cardiovascular disease (2013 Grover Conference series), Pulm Circ 4: 169-174, 2014.

4. Zheugoue XT, Li W, Li X, Li W and Xu Y: Methylation of p15INK4B and expression of ANRIL on chromosome 9p21 are associated with coronary artery disease. PLoS One 7: e47193, 2012.

5. Baccarelli A, Rienstra M and Benjamin EJ: Cardiovascular epigenetics: Basic concepts and results from animal and human studies. Circ Cardiovasc Genet 5: 567-573, 2012.

6. Turunen MP, Aavik E and Yli-Herttuala S: Epigenetics and atherosclerosis. Biochim Biophys Acta 1790: 886-891, 2009.

7. Feinberg AP: Phenotypic plasticity and the epigenetics of human disease. Nature 447: 433-440, 2007.

8. Ma S, Lotto V, Girelli D, Pinotti M, Guarinì U, Udali S, Pattini P, Pizzolo F, Martinelli N, et al: Promoter methylation in coagulation F7 gene influences plasma FVII concentrations and relates to coronary artery disease. J Med Genet 49: 192-199, 2012.

9. Guay SP, Brisson D, Munger J, Lamarche B, Gaudet D and Bouchard L: ABCA1 gene promoter DNA methylation is associated with HDL particle profile and coronary artery disease in familial hypercholesterolemia. Epigenetics 7: 464-472, 2012.

10. Dayeh TA, Olsson AH, Volkov P, Almgren P, Ronn T and Ling C: Identification of CpG-SNPs associated with type 2 diabetes and differential DNA methylation in human pancreatic islets. Diabetes Obes Metab 13: 1046-1056, 2011.

11. Yang M, Sun JZ, Sun YL, You W, Dai J and Li GS: Association between leptin gene promoter methylation and type 2 diabetes mellitus. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 29: 474-477, 2012 (In Chinese).

12. Sanovic N, Zhu J, Zhang H, Hengstenberg C, Mangino M, Mayer B, Dixon RJ, Meijting T, Braund P, Wichmann HE, et al: Genomewide association analysis of coronary artery disease. N Engl J Med 357: 443-453, 2007.

13. Wellcome Trust Case Control Consortium: Genomewide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447: 661-678, 2007.

14. Pilbrow AP, Folkers LM, Pearson JF, McNoe L, Wang NM, Sweet W, Tang WH, Black MA, Troughton RW, et al: The chromosome 9p21.3 coronary heart disease risk allele is associated with altered gene expression in normal heart and vascular tissues. PLoS One 7: e39574, 2012.

15. Soto JL, Cabrera CM, Serrano S and Soto JL, Cabrera CM, Serrano S and: Mutation data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.

16. Xu YZ, Kanagaratham C, Jancik S and Radzioch D: Promoter deletion analysis using a dual-luciferase reporter system. Methods Mol Biol 977: 97-99, 2013.

17. Kojima Y, Downing K, Kundu R, Miller C, Dewey F, Lancero H, Kakizuka A, Pericin M, Hedlin U, Schadt E, et al: Cyclin-dependent kinase inhibitor 2B regulates efferocytosis and atherosclerosis. J Clin Invest 124: 1083-1097, 2014.

18. Svensson PA, Wahlstrand B, Olsson M, Borg F, Falchi M, Bergman RN, McTernan PG, Hedner T, Carlsson LM and Jacobson P: CDKN2B expression and subadipose tissue expandability: Possible influence of the 9p21 atherosclerosis locus. Biochem Biophys Res Comm 446: 1126-1131, 2014.

19. Zhong J, Chen X, Ye H, Wu N, Chen X and Duan S: CDKN2A and CDKN2B methylation in coronary heart disease cases and controls. Exp Ther Med 15: 690-6908, 2018.

20. Vaccarino V: Ischemic heart disease in women: Many questions, few facts. Circ Cardiovasc Qual Outcomes 3: 111-115, 2010.

21. Pepeine CJ, Balaban RS, Bonow RO, Diamond GA, Johnson BD, Johnson PA, Mosca L, Nissen SE and Pohost GM: National Heart, Lung and Blood Institute; American College of Cardiology Foundation: Women’s Ischemic Syndrome Evaluation: Current status and future research directions: Report of the National Heart, Lung and Blood Institute workshop. October 2-4, 2002: section 1: Diagnosis of stable ischemia and ischemic heart disease. Circulation 109: e44-e46, 2004.

22. Takasugi M, Hayakawa K, Ariai D and Shiota K: Age- and sex-dependent DNA hypomethylation controlled by growth hormone in mouse liver. Mech Ageing Dev 134: 331-337, 2013.

23. Kolpek JC, Desai VG, Moland CL, Branham WS and Fusco JE: Age and sex dependent changes in liver gene expression during the life cycle of the rat. BMC Genomics 11: 675, 2010.
46. Jousilahti P, Vartiainen E, Tuomilehto J and Puska P: Sex, age, cardiovascular risk factors, and coronary heart disease: A prospective follow-up study of 14 786 middle-aged men and women in Finland. Circulation 99: 1165-1172, 1999.

47. Jousilahti P, Vartiainen E, Tuomilehto J and Puska P: Twenty-year dynamics of serum cholesterol levels in the middle-aged population of eastern Finland. Ann Intern Med 125: 713-722, 1996.

48. Mendelsohn ME and Karas RH: Molecular and cellular basis of cardiovascular gender differences. Science 308: 1583-1587, 2005.

49. Bredfeldt TG, Greathouse KL, Safe SH, Hung MC, Bedford MT and Walker CL: Xenooestrogen-induced regulation of EZH2 and histone methylation via estrogen receptor signaling to PI3K/AKT. Mol Endocrinol 24: 993-1006, 2010.

50. Kulig E, Landefeld TD and Lloyd RV: The effects of estrogen on prolactin gene methylation in normal and neoplastic rat pituitary tissues. Am J Pathol 140: 207-214, 1992.

51. Wiggins BS, Saseen JJ, Page RL II, Reed BN, Sneed K, Kostis JB, Lanfear D, Virani S and Morris PB; American Heart Association Clinical Pharmacology Committee of the Council on Clinical Cardiology; Council on Hypertension; Council on Quality of Care and Outcomes Research; and Council on Functional Genomics and Translational Biology: Recommendations for management of clinically significant drug-drug interactions with statins and select agents used in patients with cardiovascular disease: A scientific statement from the American Heart Association. Circulation 134: e468-e85, 2016.

52. Frishman WH: β-Adrenergic blockade in cardiovascular disease. J Cardiovasc Pharmacol Ther 18: 310-319, 2013.

53. Horinaka S: Use of nicardipine in cardiovascular disease and its optimization. Drugs 71: 1105-1119, 2011.

54. Ujhelyi MR, Ferguson RK and Vlasses PH: Angiotensin-converting enzyme inhibitors: Mechanistic controversies. Pharmacotherapy 9: 351-362, 1989.

55. Liu MZ, He FZ and Zhang W: Epigenetic research progress of anti-tumor drugs. Yao Xue Xue Bao 48: 1629-1636, 2013 (In Chinese).

56. Stone A, Valdés-Mora F, Gee JM, Farrow L, McClelland RA, Fieg H, Dutkowski C, McClay RA, Sutherland RL, Musgrove EA and Nicholson RI: Tamoxifen-induced epigenetic silencing of oestrogen-regulated genes in anti-hormone resistant breast cancer. PLoS One 7: e40466, 2012.

57. Baker EK, Johnstone RW, Zalberg JR and El-Osta A: Epigenetic changes to the MDR1 locus in response to chemotherapeutic drugs. Oncogene 24: 8061-8075, 2005.