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Direct Bisulfite Sequencing and Methylation Specific PCR to Detect Methylation of p15INK4b and F7 genes in Coronary Artery Disease Patients

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Abstract

Genome-Wide Association Studies (GWAS) have identified genetic variants contributing to the risk of cardiovascular disease (CVD) at the chromosome 9p21 locus. The chromosome 9p21 is an important susceptibility locus for several multifactorial diseases like ischemic stroke, aortic aneurysm, type 2 diabetes mellitus and coronary artery disease (CAD). F7 gene because of its role in activating the extrinsic pathway by the exposure of tissue factor after plaque disruption is related to atherothrombosis. The aim of the present study was to evaluate methylation status of two CAD related genes, p15INK4b and F7, in Iranian patients with coronary artery disease (CAD). Thirty samples from 15 male and 15 female with diagnosed 3 vessels disease CAD and 60 Samples from 60 non-CAD controls who underwent coronary angiography was analyzed by MSP and direct sequencing. DNA methylation levels at p15INK4b gene increased significantly in CAD patients in comparison with control group (p-value<0.001). To quantitative analysis of methylation, direct bisulfite sequencing method revealed 6 methylated CpGs islands. No significant difference in F7 promoter methylation was observed between CAD patients and control group. Based on the findings of this study it is possible to assume, p15INK4b methylation is associated with pathophysiology of CAD.

Keywords: Coronary artery disease (CAD); Chromosome 9p21; p15INK4b gene; F7 gene; Epigenetics.

Introduction

The chromosome 9p21 is an important susceptibility locus for several multifactorial disease like ischemic stroke, aortic aneurysm, type 2 diabetes mellitus and coronary artery disease [1]. Genome-Wide Association Studies (GWAS) have demonstrated that single nucleotide polymorphisms (SNPs) on chromosome

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9p21 (Chr9p21) affect susceptibility to coronary artery disease (CAD) in Caucasian population and these associations have been reproduced in other populations [2-5]. $P^{15INK4b}$ belongs to INK4 family of protein kinase inhibitors [6-9]. Previous studies have shown that both deletion of Chr9p21 locus and repression of INK4/ARF or ANRIL expression have their impacts on atherosclerosis [10-12]. Atherosclerotic plaque also has characteristics of excessive vascular smooth muscle cells (VSMCs) and macrophages proliferation [13]. Another CAD marker is Plasma factor VII concentrations (FVIIa) that its elevation is a major risk factor for CAD and has an important function in the coagulation pathway [14-16]. F7 promoter methylation through epigenetic modifications at the promoter site may regulate transcription of F7 gene [16]. Epigenetics (mainly DNA methylation) is defined as stable and heritable changes that are not due to disrupting the coding sequences of disease genes [17, 13]. The alteration in the epigenome forms an interface between the genotype and the environment and a group of enzymes called methyltransferases add methyl groups covalently to the cytosine ring within CpG islands [18]. It has been shown that global DNA hypermethylation per se is associated with CAD [19]. Methylation-specific PCR (MSP) is sensitive and specific method which can assess the methylation of almost any group of CpG sites in CpG island [20]. Inspired by these evidences we decided to assess the association of $p15^{INK4b}$ and F7 promoter methylation with CAD susceptibility in a group of Iranian patients compare to normal controls.

Materials and Methods

Ethic statement

This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Science and Tehran Heart Center affiliated to Tehran University of Medical Sciences, Tehran, Iran. All patients gave written informed consent.

Study population

An aggregate of 30 patients who were analyzed as CAD by angiography and 60 sex- and age-coordinated members without CAD in angiography were enlisted in Department of Cardiology, Tehran Heart Center.

Coronary angiography

Quantitative appraisal of CAD was performed utilizing coronary angiography. Briefly, significant CAD was characterized as the vicinity of luminal width narrowing 50% in the left anterior descending artery, left circumflex vein, right coronary supply route and their primary branches (13). Left primary trunk stenosis was considered as two-vessel sickness. Seriousness of coronary atherosclerosis was further classified as 1-, 2- or ≥3-vessel ailment as per number of coronary vessels with critical stenosis, and afterward we selected just patients with 3 vessel malady (3VD) with CAD.

Sample collection

All peripheral venous blood (10 mL) samples were taken in the morning with patients fasting from midnight forward, and then drawn into adequate tubes from each subject. A white differential cell count on whole blood using automated counter was performed. Peripheral Blood Mononuclear Cells (PBMC) was extracted and stored in -70°C.

DNA extraction

Genomic DNA was separated from whole blood cells, neutrophilis and lymphocytes, using salting out method and subsequently stored at -70°C until analysis. Nanodrop was recruited to determine the concentration of DNA for all samples.

Bisulfite treatment

Genomic DNA was then artificially changed by sodium bisulfite to change over every unmethylated cytosine to uracils while leaving methylcytosines unaltered (EZ Zymo Methylation Kit, Zymo Research, CA, USA). Treated DNA samples were stored at -22°C until MSP analysis.

Methylation-Specific PCR (MSP)

A modified DNA was used for two separate MSP reactions, both for the $p15^{INK4b}$ and F7 genes: one reaction particular for methylated DNA and other particular for unmethylated DNA. The primer sequences described in Table 2 [20].

For all reactions the amplification conditions were: Bisulfite-modified DNA (0.5 µL); master mix Ampliqone™ (12.5 µL), H_2O (11 µL), and 0.5 microliter of each primer (10 pico M). The PCR conditions were 95 °C for 5 min followed by 35 cycles (30 s at 95 °C, 30 s at 60 °C for Annealing, 30 s at 72 °C), and a final extension step of 4 min at 72 °C. Each PCR product was analyzed using 2% agarose gel. , stained with Simply Safe dye™ and directly visualized under UV illumination.

Bisulfite Direct Sequencing

To validate the MSP results, sequencing was performed. Bisulfite-treated DNA was amplified by a nested-PCR protocol for both genes ($p15^{INK4b}$ and F7),

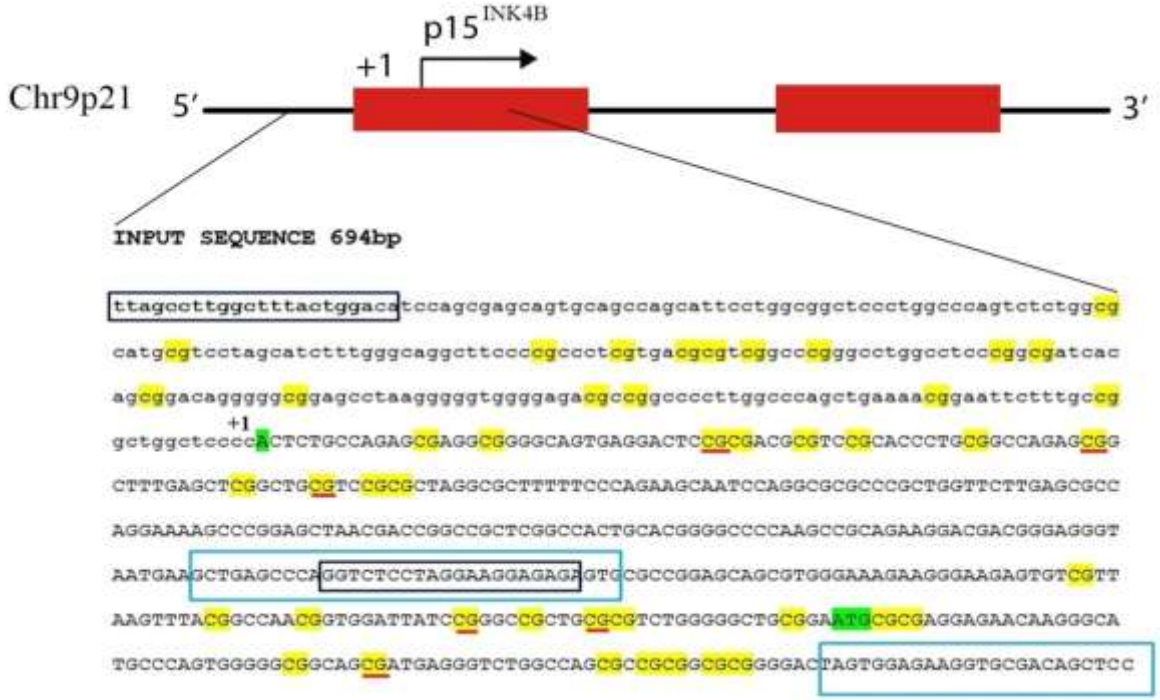

Figure 1. CDKN2B gene input sequence 694bp analyzed by DBS and MSP, 498 bp and 226 bp CpG islands that analyzed by direct sequencing is highlighted in yellow. The region amplified by MSP is also in the region. Intron sequences, in short letters, exon sequences, in capital letters, and analysed CpG dinucleotides in yellow and significant methylated CpGs with Red line under them numbering from top left. RefSeq accession NM_004936.3

Following the first amplification, an aliquot of the PCR products was used as a template DNA for the nested PCR and PCR products were diluted 20-fold and 1µl were used for second reaction with using the primer pairs described in Table 1 [21].

The first region analyzed (498bp) after bisulfite treatment of the DNA for $p15^{INK4b}$ gene is found roughly -234 to +264bp from the transcription start site, However, the CpG dinucleotides analyzed by direct sequencing contained only the region located -242 to +42bp (283bp) from the transcription start site that shows 22 CpG dinucleotides. The second region is located +227 to +453 which contain 21CpGs [Figure 1]. To minimize the heterogeneity among samples, 10

Table 1. PCR primers used for MSP and nested PCR and sequencing

Primer set (5`→3`)	Forward	Reverse	Size bp	Anneal temp	Source
p15-W	CGCACCCTGCGGCCAGA	AGTGGCCGAGCGGCCGG	137	65	
Pl5-M	GCGTTCGTATTTTGCGGTT	CGTACAATAACCGAACGACCGA	148	60	(20)
p15-U	TGTGATGTGTTTGTATTTTGTGGTT	CCATACAATAACCAAACAACCAA	154	60	
CDKN2B-S1	GGTTGAAGGAATAGAAATTT	ACACTCTTCCCTTCTTTCCC	651	55	
CDKN2B-S2	TTAGTTTTGGTTTTATTGGA	TCTCTCCTTCCTAAAAAACC	498	55	(21)
CDKN2B-S3	GTTGAGTTTAGGTTTTTTAGGAAGG AGAGAGTG	AAAACTATCRCACCTTCTCCACTA ATCCCC	226	68	-
F7-M	ACGTGGATTGTCGTTAGTCGG	AAATAAAATTTCACCGTATTAAC CAAACT	130	60	
F7-U	TGTGGATTGTTGTTAGTTGGGTATA GT	CAAAAATAAAATTTCACCATATT AACCAAAC	132	60	(16)
F7-S1	TGTTGGGTAATTTAGGAGGTTTGG AT	CAAACAACACCCCCATTCTC	525	60	
F7-S2	TTTGGGGTGGTTAGTTATTATAGTT TGTGGTTTGGG	CTCCTAACCTCAAATAATCCACA TACCTCAACCTCCC	150	68	-

Table 2. Clinical characteristic of patients with and without coronary artery disease

	CAD (n=30)	Control (n=60)	p-value
Age	46.5 ± 5.6	46.5 ± 6.9	0.204
Male/female	15/15 (50%/50%)	30/30 (50%/50%)	1
BMI (kg/m^2)	28.7 ± 4.5	30.4 ± 5.2	0.205
Smoking	9 (30%)	15 (25%)	0.337
Opium	6 (20%)	2 (3.3%)	<0.001
Hypertension	16 (53.3%)	19 (31.7%)	0.044
Diabetes mellitus	11 (36.7%)	10 (16.7%)	<0.001
FBS (mg/dL)	120.6 ± 50.5	106.2 ± 26.4	0.034
TG (mg/dL)	187.7 ± 64	184 ± 128.9	0.128
TC (mg/dL)	200.6 ± 57.8	189.9 ± 46.9	0.187
LDL-C (mg/dL)	136.3 ± 50	116.8 ± 40.8	0.574
HDL-C (mg/dL)	36.3 ± 7.7	43.1 ± 11.4	0.065
Family history	15 (50%)	15 (25%)	0.002
MI history	15 (50%)	2 (3.3%)	<0.001

Data for age, BMI, FBS, TG, TC, LDL-C and HDL-C, are presented as means and 95% CIs.
Abbreviations: CAD= coronary artery disease; BMI= Body Mass Index; FBS = Fasting blood sugar; TG= Triglyceride; TC= Total cholesterol; LDL-C = low-density lipoprotein cholesterol; HDL-C= high-density lipoprotein cholesterol; MI= Myocardial Infarction.

males (5 CAD patients and 5 control individuals) and 10 females (5 CAD patients and 5 control individuals) who were non-hypertensive, non-diabetic were chosen for the direct bisulfite sequencing for both regions 498bp and 226bp from $p15^{INK4b}$ gene and 150bp from F7-S2 (nested PCR) segment to avoid the confusing effects of established risk factors on DNA methylation.

Second region primers for $p15^{INK4b}$ gene and F7 gene were described in Table 1 designed through Zymo Research website (marked by -).

PCR was performed in a solution containing bisulfite-modified DNA (1 µL); master mix Ampliqone™ (12.5 µL), H_2O (10 µL), and 1 microliter of each primer (10 pico M). The PCR conditions were: 94 °C for 5 min followed by 35 cycles (94 °C for 1 min, 68 °C for 1 min, 72°C for 1 min, and final 72 °C for 10 min.

PCR products were separated on 1% agarose gels (peQlab), Excised carefully through trans-illuminator, and then purified with GeneAll® Combo GP kit according to manufacture structure, then the purified products were sequenced directly by Macrogen Korea.

Results

All the baseline demographic and biochemical characteristics of patients have been summarized in Table 2.

Methylation specific PCR analysis

For $p15^{INK4b}$ gene, the methylated band was observed in both CAD and control samples, which was not significantly different between two groups. Although methylated bands were sharper in CAD samples [Figure 2]. For F7 gene, both methylated and unmethylated band was seen in both CAD and control samples, which was not significantly different between two groups [Figure 3].

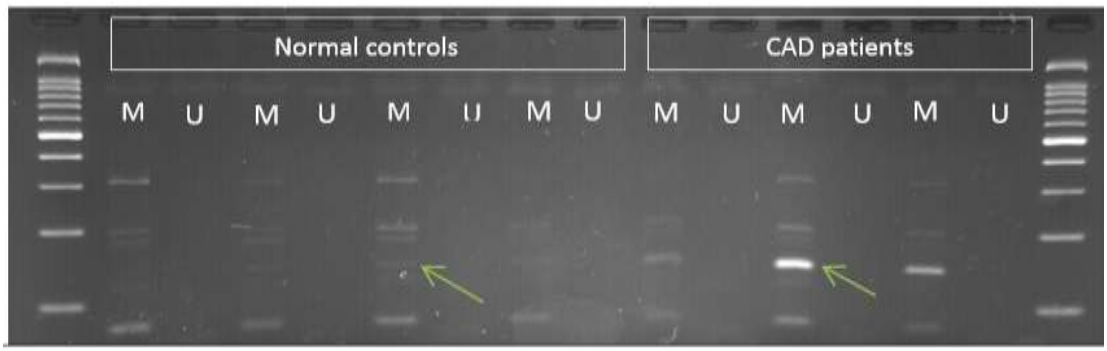

Figure 2 . MSP of CDKN2B. Primer sets used for amplification are designated as methylated (M) or unmethylated (U). The CAD samples are represented in Roman numbers. Samples amplified with methylated and unmethylated primer sets.

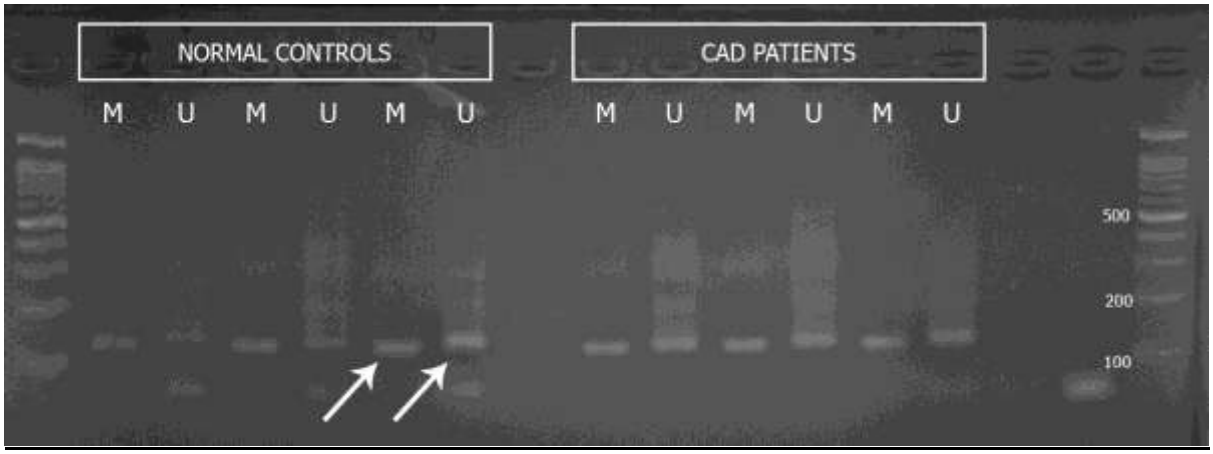

Figure 3 . MSP of F7 gene. Primer sets used for amplification are designated as methylated (M) or unmethylated (U). The CAD samples are represented in Roman numbers. Samples amplified with methylated and unmethylated primer sets.

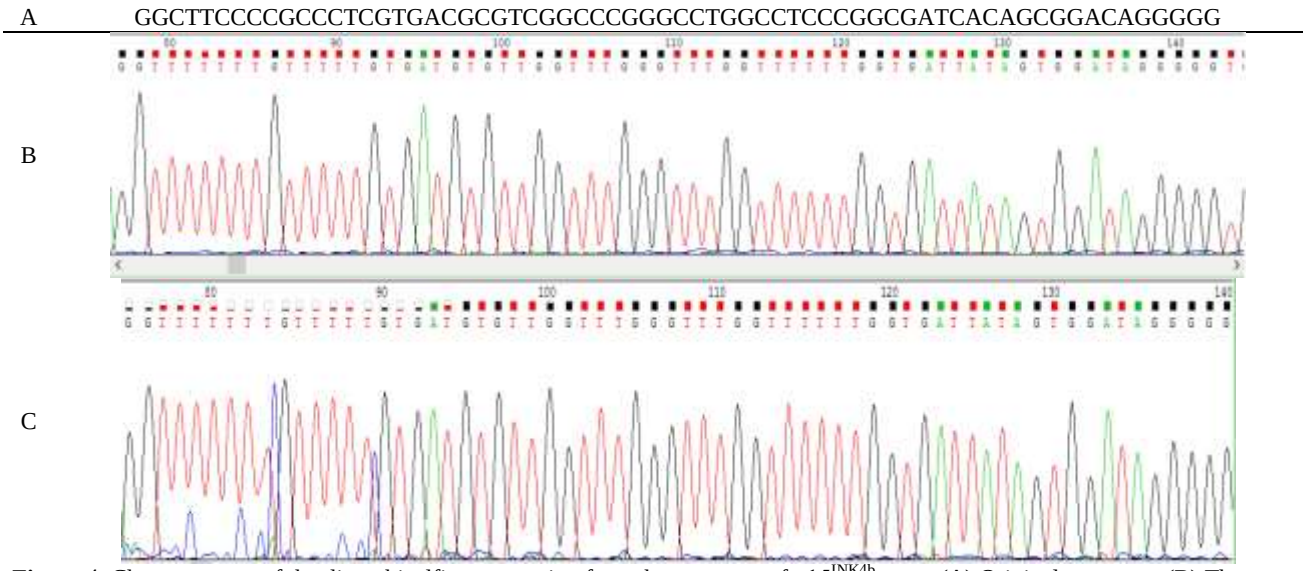

Figure 4. Chromatogram of the direct bisulfite sequencing from the promoter of $p15^{INK4b}$ gene: (A) Original sequence. (B) The same region of promoter in a normal control; (C) a part of the promoter, which is hemimethylated in two sites in a CAD patient.

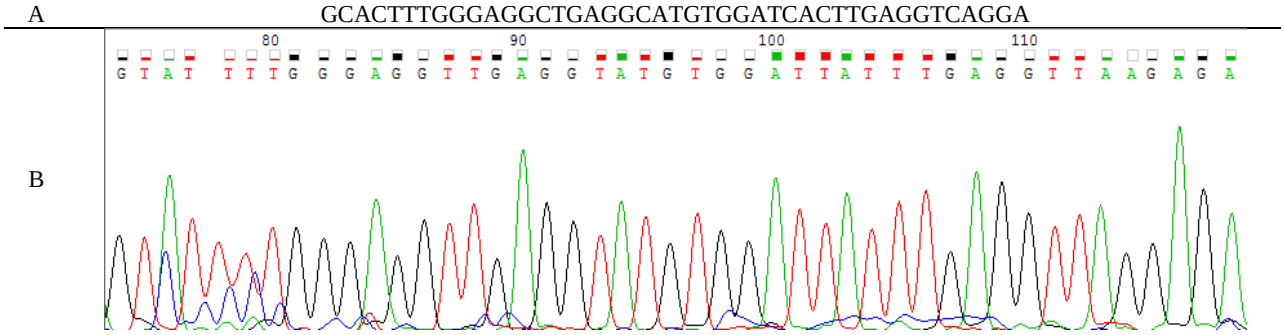

Figure 5. Chromatogram of the direct bisulfite sequencing from the promoter of F7 gene: (A) Original sequence. (B) Part of promoter in a normal control and CAD patient.

Direct bisulfite sequencing analysis

Since MSP is a qualitative method, we also used Direct Bisulfite Sequencing which is a straightforward way to detect methylated cytosine. For p15INK4b gene,

Table 3. Six CpGs islands were significantly methylated in case group versus

Methylated CpG	Case = n (%)	Control = n (%)	p-value
CpG 19	5 (50%)	0 (0%)	0.033
CpG 24	5 (50%)	0 (0%)	0.033
CpG 27	7 (70%)	1 (10%)	0.008
CpG 32	9 (90%)	2 (20%)	0.005
CpG 34	7 (70%)	1 (10%)	0.020
CpG 40	5 (50%)	0 (0%)	0.033

six CpGs islands were significantly different between case and control arms; include CpG 19, 24, 27, 32, 34, 40 which were shown in Table 3. These results are in agreement with previous study that found the CDKN2B gene to be methylated CAD patients [13]. For F7 gene, no meaningful methylation change was seen between two groups (data not shown) which were presented in Figure 5.

Pyrosequencing can examine frequency of methylation for an exact CpG island with high accuracy, but requires careful optimization and lack of pyrosequencing was a pitfal in this study.

Discussion

Epigenetic changes have a pivotal role in gene imprinting, embryonic development, X-chromosome gene silencing, and cell cycle regulation [22]. Epigenetic alterations in atherosclerotic tissues had been already reported in previous studies, Coronary atherosclerotic tissues showed higher methylation levels of estrogen receptor β gene, which has essential roles in vascular function and evidence of epigenetic dysregulation of estrogen receptor β in atherosclerosis and vascular aging was observed [22]. The 9p21 locus harbors variants associated with coronary artery disease. The 9p21 locus lies adjacent to the cyclin-dependent kinase inhibitors, CDKN2A and CDKN2B [3].

In this study, we sought to determine whether DNA methylations of selected CpG islands, in INK4B locus and promoter of F7 gene were involved in CAD. $P15^{INK4B}$ methylation through MSP method were investigated in many cancers, including leukemia [21], but this is the first study to used qualitative method to evaluate methylation status of $P15^{INK4B}$ in CAD patients. Via MSP method for $p15^{INK4b}$ gene, the methylated band was observed in both groups, although it was sharper in CAD samples. To validate the MSP results, Direct Bisulfite Sequencing was performed, which revealed significant different between case and control. These findings are in line with Zuhang et al [13] study that shown this association in twenty-two CAD patients. The exact mechanism of the effect of $p15^{INK4b}$ gene methylation on CAD is not clarified yet.

For the other gene (F7), methylated and unmethylated bands with equally sharpness was observed in both CAD and control samples, with MSP and direct bisulfite sequencing method; While Friso et al [16] discovered association of F7 promoter methylation with CAD in specific genotype (A1A1). One reason of this conflict is due to their comprehensive genotyping along with measurement of Plasma factor VII concentrations (FVIIa).

One limitation of our study was the small sample population, because 3VD occlusion is not frequent. Another limitation of this study was the lack of next generation sequencing (NGS) for precision assessment. Regardless of this, we did not evaluate all samples for DBS, in three targeted segments.

The current study had several strengths including evaluation of two CAD related genes in 3VD CAD patients and usage of both quantitative and qualitative methods. On the other hand, to our knowledge, this was the first study which assessed methylation status of $p15^{INK4b}$ and F7, in Iranian patients with coronary artery disease.

In conclusion, the present study shows that the $p15^{INK4b}$ promoter region in PBMC DNA has an elevated methylation status in coronary artery disease patients. Further studies are necessary to ascertain the relationship of methylation of $p15^{INK4b}$ and pathophysiology of atherosclerosis in CAD patients. Moreover, studies with larger samples will be required to elucidate the exact mechanism by which this locus modulates CAD risk.

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Conflict of interest

The authors report no conflict of interest.

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