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ORIGINAL RESEARCH ARTICLE

Association of Serum Level and DNA Methylation Status of Brain-Derived Neurotrophic Factor with the Severity of Coronary Artery Disease

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Abstract New investigations suggest a pivotal role of brain-derived neurotrophic factor (BDNF) in cardiovascular homeostasis. However, no data could indicate the association between BDNF methylation status and the risk of coronary artery disease (CAD). The aim of the present study was to assess the association of BDNF methylation status and its serum level with the severity of CAD. According to the angiography report, a total of 84 non-diabetic CAD patients with at least 50% stenosis in one of the major coronary arteries were selected as the CAD group. For comparison, 62 angiographically proven non-CAD participants were selected as control. Additionally, subjects were categorized according to the Gensini Scoring system. Blood sample was used for genomic DNA isolation. Methylation status of the BDNF gene in exonic region was determined using the MS-PCR method and serum BDNF levels were measured with ELISA. BDNF gene methylation was significantly higher in the CAD group than in the non-CAD group. After adjustment for confounding factors, BDNF gene hypermethylation increases the risk of CAD in the total population (OR = 2.769; 95% CI, 1.033–7.423; $P = 0.043$). BDNF gene hypermethylation was higher in patients with severe CAD than patients with mild CAD. Additionally, the serum BDNF level was not different from non-diabetic CAD and control groups. Our findings indicate that BDNF hypermethylation was associated with an increased risk of CAD, which may help identify subjects being at the risk of developing CAD. In addition, BDNF hypermethylation shows a significant correlation with the severity of CAD.

Keywords Brain-derived neurotrophic factor · Coronary artery disease · DNA methylation

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Introduction

Coronary artery disease (CAD) is one of the leading causes of worldwide death [1]. For decades, dyslipidemia, diabetes, smoking, hypertension, obesity, and a sedentary lifestyle have been recognized as primary clinical risk factors for CAD. However, the cellular and molecular basis of CAD is complex and different pathways and molecules have been found to contribute to the pathogenesis of CAD [1, 2].

Brain-derived neurotrophic factor (BDNF) is a dimeric polypeptide which best characterized by its effect on differentiation, survival, plasticity, and myelination of neurons [3]. In addition to its pivotal role in the central nervous system, BDNF has important functions in cardiovascular homeostasis [4].

BDNF and its receptor, TrkB, are expressed in different non-neuronal tissues and cell types including heart, atherosclerotic vessels, macrophages, vascular smooth

muscle cells, and endothelial cells [5]. New emerging piece of evidence suggests a critical role of BDNF in regulating cardiac contraction force, and coronary blood vessel development [6].

Studies using heterozygous BDNF (±) mice have indicated that conditions linked to metabolic and cardiovascular dysfunction such as diabetes, obesity and heart disease can be altered through manipulation of BDNF in the brain and in the peripheral circulation [7]. BDNF and its receptor, have pivotal role in cardiovascular development. In BDNF knock-out mice, reduction in endothelial cell contacts in coronary arteries and intraventricular wall hemorrhage of the heart have been reported that cause early postnatal death [8]. It has been demonstrated that in response to hypoxic stimuli, BDNF promote neovascularization through acting on endothelial cells [9]. Therefore, it has been postulated that decrease in BDNF may involve in a poor prognosis of patients with ischemic heart disease and that BDNF-based therapy could be effective for patients with myocardial infarction [10]. Recent studies revealed that direct intramyocardial injection of BDNF greatly improve microvessel density and cardiac function in ischemic myocardium [11]. These findings suggest a possible role of BDNF in the pathogenesis of CAD.

It has been shown that low serum or plasma level of BDNF is associated with a higher prevalence of CAD risk factors and mortality [12]. However, Eriji et al. demonstrated that a high level of BDNF in coronary vasculature may detrimentally affect plaque stability [13].

Recently, accumulating evidence suggests an important role of epigenetics in the development of CAD [14]. Epigenetic mechanisms can change gene functions without altering the sequences of DNA. DNA methylation is the most widely studied epigenetic feature that occurs on cytosine residues within CpG islands by DNA methyltransferase enzyme [15]. DNA methylation is usually associated with suppression of gene expression through hindering the binding of transcription factors to their cis-DNA elements [16].

Aberrant DNA methylation is implicated in the development and progression of CAD and its underlying atherosclerosis [17]. Both candidate-gene approaches and global DNA methylation have been investigated in different atherosclerotic conditions [18].

To the best of our knowledge, there have been no reports studying the methylation status of BDNF gene in CAD in human beings or animals. Therefore, the aim of the present investigation was to study the gene methylation status and serum level of BDNF in non-diabetic CAD patients and its association with CAD risk and CAD severity. Additionally, the association between the aforementioned methylation and some clinical risk factors of CAD were explored.

Subjects and Methods

Study Subjects

This study was conducted in the Cardiology Department of Shahid Mohammadi Hospital of Hormozgan University of Medical Sciences. Study subjects were selected among those individuals who suffered from possible cardiac symptoms like dyspnea and chest pain. They were the first time candidates for coronary angiography.

Exclusion criteria are listed as following: being younger than 18 years old, presence of any malignancy, chronic liver or kidney diseases, chronic inflammatory diseases, having major surgery in the last two months, any history of cardiovascular disease, thyroid disorders, mental diseases, using immune suppression drugs, and antidepressant or tranquilizers. All CAD patients and control subjects were checked for the presence of diabetes and those with diabetes were also excluded. Diabetes mellitus was defined if subjects were previously diagnosed as diabetic, used antidiabetic medications, and had fasting plasma glucose $\geq$ 126 mg/dl on at least two occasions or positive results of an oral standard glucose tolerance test.

Coronary Angiography and Severity of Coronary Atherosclerosis

Among participants, those subjects who had at least one major coronary artery with more than 50% stenosis confirmed by angiography were recruited as CAD group (n = 84). The non-CAD control group (n = 62) consisted of those subjects who had a completely normal angiogram and they had no coronary stenosis at all.

The Gensini scoring system was used to determine the severity of coronary artery disease [19]. This method defines stenosis of the coronary artery lumen as 1 for 1–25% stenosis, 2 for 26–50%, 4 for 51–75%, 8 for 76–90%, 16 for 91–99%, and 32 for total occlusion. Then, this score is multiplied by a factor representing the importance of the lesion's location in the coronary artery system. For the location scores, 5 points were given for left main lesion; 2.5 for proximal left anterior descending (LAD) or left circumflex (LCX) artery; 1.5 for the mid-segment LAD and LCX; 1 for the distal segment of LAD and LCX, first diagonal branch, first obtuse marginal branch, right coronary artery, posterior descending artery, and intermediate artery; and 0.5 for the second diagonal and second obtuse marginal branches. The participants were divided into three groups according to Gensini score: those without any stenosis in the coronary artery (non-CAD; Gensini score, 0; n = 62), those with mild (n = 37;

Gensini score, 1–25; mild CAD) and severe (n = 47; Gensini score, > 25; severe CAD) stenosis.

The body mass index (BMI), waist circumference, waist to hip ratio (WHR), systolic, and diastolic blood pressure of all the participants were measured before angiography. Data regarding medication, gender, age, and smoking habit were also recorded. All subjects were from Hormozgan Province with similar ethnicity. The local ethics committee of our university approved our study procedures and all the subjects granted their informed consents prior to their participation in this study.

Blood Collection and Biochemical Measurements

Before the injection of the contrast agent, fasting peripheral venous blood samples were collected between 8:00–9.00 a.m. to minimize the effects of a possible circadian rhythm of BDNF concentration as reported previously [20]. All samples were allowed to clot for 1 h at room temperature before being centrifuged at 2,000 g for 10 min at 4 °C [21] and the serum was transferred to the Eppendorf tube and stored at – 80 °C until analysis time. Also, blood samples with anticoagulant (K_2EDTA 1 mg/ml) were stored at − 80 °C for genomic DNA extraction and DNA methylation analysis.

Serum levels of triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and fasting blood glucose (FBS) were measured by standard laboratory techniques on Hitachi 7600 Automatic Biochemical Analyzer (Hitachi Company, Japan). Low-density lipoprotein cholesterol (LDL-C) was estimated by the Friedewald formula for those subjects with TG lower than 400 mg/dl. LDL-C was directly measured using LDL-C kit for those subjects with TG higher than 400 mg/dl. Platelet counts were analyzed in EDTA whole blood samples using standard automated hematological methods. Serum BDNF levels were measured using commercially available human BDNF ELISA kits (ZellBio GmbH, Germany) following the instructions suggested by the manufacturer. The sensitivity and assay range of the kit were 0.01 ng/ml and 0.05–10 ng/ml, respectively.

DNA Extraction, Bisulfite Modification and Methylation-Specific PCR

Genomic DNA was extracted from whole blood according to the salting out standard method [22]. Purity and concentration of the extracted DNA were measured using the Nanodrop spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific, USA). 2 µg of extracted DNA was modified chemically with bisulfite [23], then methylation-specific polymerase chain reaction (MS-PCR) was performed. The basis of bisulfite modification is the conversion of unmethylated cytosine to uracil. Modified DNA was purified using a commercial column (Roche Company, Germany) and methylation status of exonic region of the BDNF gene was assessed by MS-PCR method using specific primers set for methylated and unmethylated DNA sequences [24].

Completely methylated and completely unmethylated DNA (Zymo Research Company, USA) were used as a standard positive and negative control, respectively.

PCR reaction mix for the methylated primer in 50 µl volume contains 5 µl PCR buffer 10X, 2 µl $MgCl_2$ 50 mM, 1 µl dNTP 10 mM, 0.3 µl Taq polymerase 5U/µl, 2 µl of forward and reverse primer 10 µM, and 3 µl of modified DNA. Thermocycler condition was: 1) 95 °C for 3 min; 2) 36 cycles of 95 °C for 15 s, 58 °C for 30 s, 72 °C for 30 s and 3) 72 °C for 7 min. PCR reaction mix for the unmethylated primer in 50 µl volume contains 5 µl PCR buffer 10X, 4.5 µl $MgCl_2$ 50 mM, 1 µl dNTP 10 mM, 0.3 µl Taq polymerase 5 U/µl, 2.5 µl of forward and reverse primer 10 µM, and 3 µl of modified DNA. Thermocycler condition was: 1) 95 °C for 3 min; 2) 36 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s and 3) 72 °C for 7 min.

PCR products were run on 2% agarose gel, stained with GelRed and visualized under UV illumination (Fig. 1).

Statistical Analyses

The normality of distribution was assessed for all continuous variables using the Kolmogorov–Smirnov test. Continuous variables were expressed as the mean ± SE, skewed data were presented as medians and interquartile range. All the parametric data were compared using an independent t-test and one-way ANOVA. When the data were not normally distributed, the Mann–Whitney U test and Kruskal–Wallis were used. Categorical variables were presented as number and percentage, Chi-square test was used to evaluate categorical variables. Because of the skewed distribution, serum BDNF was transformed as 1/sqrt in the analyses according to the gladder command in Stata software. To identify the association between BDNF methylation and clinical variables, each variable was separately entered in a univariate logistic regression model; then, the variables which had a $p < 0.2$ were selected and entered into a multiple regression model by using forward stepwise method and Odds Ratios (OR), 95% Confidence Intervals (CI) and p-value were estimated. All statistical analyses were performed using SPSS software version 16.0 and Stata version 12. P values less than 0.05 were considered statistically significant.

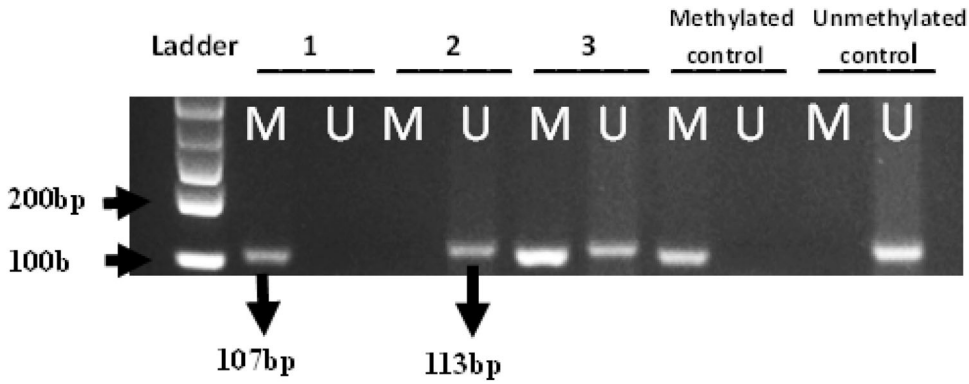

Fig. 1 The methylation analysis results of the BDNF gene by methylation-specific polymerase chain reaction (MS-PCR). Lanes 1–3 show methylation status of BDNF gene for three subjects. Commercially methylated and unmethylated DNA control were used as a standard positive and negative control, respectively. M: methylated band, U: unmethylated band

Results

Primer Characteristics

In Table 1 the characteristics of methylated and unmethylated primers for the BDNF gene are shown [24]. The amplicon were located in: Human Genome 19 assembly – chr11: 27,720,802–27,720,909 for methylated DNA sequence and chr11: 27,720,799–27,720,912 for unmethylated DNA sequence (GRCh37/hg19).

Baseline Characteristics

A total of 146 subjects took part in the present research project and all of them were non-diabetic. Out of 146 subjects 84 patients (59 male and 25 female) who had more than 50% stenosis in at least one major coronary artery were classified as the CAD group. 62 subjects (30 male and 32 female) whose coronary angiographies were completely normal were classified as the non-CAD control group. Demographic, clinical, and biochemical characteristics of the study population are shown in Table 2. Our data reveal that the percentage of those men who were affiliated with CAD was higher than with those men who did not have CAD ($p < 0.05$). The average age and smoking percentage in CAD patients were more than the non-CAD group ($p < 0.05$). In Fig. 1, the methylated and unmethylated pattern of BDNF gene on agarose gel was observed. As shown, the size of methylated and unmethylated bands were 107 and 113 bp, respectively. In Table 2 it has been reported that BDNF gene was hypermethylated in 88.1% of CAD patients and 72.6% of non-CAD subjects ($p = 0.017$). The values of serum BDNF concentration were not statistically different between subjects with or without CAD (1.77 [1.55–2.05] vs 1.71 [1.43–2.04] ng/ml). The mean values for age, BMI, waist circumference, waist to hip ratio (WHR), systolic blood pressure, total cholesterol (TC), and HDL-C were significantly higher in women than men. On the other hand, a higher percentage of men were smoker than women ($p < 0.05$). BDNF methylation was detected in 80.9% male and 82.5% female ($p = 0.813$). Additionally, no statistically significant difference was found between serum BDNF levels in men and women (1.78 [1.54–2.05] vs 1.72 [1.43–2.01]). When serum levels of BDNF in men and women in CAD and non-CAD groups were compared, no statistically significant differences were observed (data are not shown).

Table 3 shows the demographic and clinical characteristics of the study groups according to the Gensini score classification. When the severity of CAD increased, consequently the male to female ratio, percent of smokers, and mean of age are increased significantly ($p < 0.05$). BDNF gene methylation in patients with severe CAD is significantly higher than the patients with mild CAD. Interestingly, individuals with mild CAD also have a significantly higher percentage of BDNF gene methylation than the non-CAD control group. Moreover, serum BDNF level was not

Table 1 The characteristic of primers that used for methylation specific PCR

Gene	Forward primer	Reverse primer	Product size (bp)	Annealing temperature
BDNF M	5′-TCG GTA ATG GGT AAT TTT TTC-3′	5′-CCT TAC GAT TTA TCA CGT ACG-3′	107	58 °C
BDNF U	5′-TTA TTG GTA ATG GGT AAT TTT TTT-3′	5′-CTT CCT TAC AAT TTA TCA CAT ACA-3′	113	60 °C

BDNF M, BDNF methylated primer; *BDNF U*, BDNF unmethylated primer

Table 2 Demographic, clinical and biochemical characteristics of all subjects according to subgroup analysis by CAD status and gender

Variables	Non-CAD (62)	CAD (84)	*P*	Male (89)	Female (57)	*p*
Gender (M/F)	30/32	59/25	0.007	–	–	–
Male, n (%)	30 (48.4)	59 (70.2)	–	–	–	–
Female, n (%)	32 (51.6)	25 (29.8)	–	–	–	–
Age (year)	51.61 ± 1.19	54.89 ± 1.01	0.037	51.29 ± 0.94	56.75 ± 1.24	< 0.001
BMI (kg/m^2)	26.06 ± 0.59	25.23 ± 0.50	0.279	24.56 ± 0.46	27.17 ± 0.61	0.001
Waist circumference (cm)	99.73 ± 1.46	96.48 ± 1.26	0.093	95.22 ± 1.17	101.97 ± 1.49	< 0.001
WHR	0.98 ± 0.007	0.96 ± 0.006	0.086	0.95 ± 0.006	0.98 ± 0.007	0.003
Systolic blood pressure (mmHg)	131.05 ± 3.02	135.23 ± 2.34	0.269	127.88 ± 1.90	142.44 ± 3.44	< 0.001
Diastolic blood pressure (mmHg)	79.53 ± 1.46	81.93 ± 1.15	0.195	79.99 ± 1.11	82.42 ± 1.55	0.195
Blood pressure history, n (%)	32 (51.6)	49 (58.3)	0.419	46 (51.7)	35 (61.4)	0.249
Dyslipidemia history, n (%)	40 (64.5)	65 (77.4)	0.087	63 (70.8)	42 (73.7)	0.704
Smoking, n (%)	27 (43.5)	55 (65.5)	0.008	58 (65.2)	24 (42.1)	0.006
Aspirin medication, n (%)	10 (16.1)	15 (17.9)	0.784	73 (82)	48 (84.2)	0.732
Statin medication, n (%)	39 (62.9)	58 (69)	0.437	59 (66.3)	38 (66.7)	0.963
BDNF methylation, n (%)	45 (72.6)	74 (88.1)	0.017	72 (80.9)	47 (82.5)	0.813
Serum BDNF (ng/ml)	2.02 ± 0.14	2.20 ± 0.16	0.180	2.24 ± 0.14	1.92 ± 0.16	0.084
	1.71 [1.43–2.04]	1.77 [1.55–2.05]		1.78 [1.54–2.05]	1.72 [1.43–2.01]	
FBS (mg/dl)	90.46 ± 1.46	91.58 ± 1.36	0.576	91.38 ± 1.38	90.61 ± 1.39	0.703
TG (mg/dl)	120[92–181]	139[100–181.5]	0.276	142[97–183.75]	126.5[92–161.25]	0.373
	150.82 ± 13.84	151.81 ± 8.40		151.50 ± 8.53	151.26 ± 14.20	
Total cholesterol (mg/dl)	167.33 ± 6.08	162.98 ± 4.61	0.563	157.53 ± 4.06	176.94 ± 6.88	0.010
HDL-C (mg/dl)	44.53 ± 1.59	41.71 ± 1.46	0.198	40.29 ± 1.30	47.23 ± 1.75	0.002
LDL-C (mg/dl)	93.77 ± 5.18	89.28 ± 4.38	0.509	87.58 ± 3.79	97.28 ± 6.27	0.161
Platelet (× 10^3/µl)	228.06 ± 9.49	229.06 ± 7.82	0.936	225.52 ± 7.56	234.17 ± 10.03	0.492

Data are presented as mean ± SE or number (%) unless indicated otherwise. *BDNF*, brain-derived neurotrophic factor; *BMI*, body mass index; *CAD*, coronary artery disease; *FBS*, fasting blood sugar; *HDL-C*, high-density lipoprotein-cholestrol; *LDL-C*, low-density lipoprotein-cholestrol; *TG*, triglyceride; *WHR*, waist to hip ratio

significantly different in subjects with varying degrees of coronary stenosis (Table 3).

Association Between BDNF Methylation Status, Serum BDNF Level and CAD Risk Factors

The association between BDNF methylation pattern and study variables is shown in Table 4. 62.18% of the participants with the methylated status of BDNF gene had CAD, while only 37.04% of the subjects with the unmethylated form of BDNF had the disease. Interestingly, after adjustment for potential confounders such as age, gender, smoking, and lipid profile these differences between the groups were still significant. The mean of age and serum TG level in subjects with the BDNF gene hypermethylation were markedly higher than those participants without hypermethylation and the difference was statistically significant before and after adjustment of aforementioned confounding factors. The differences between mean of serum BDNF levels in participants with and without hypermethylation were not statistically significant.

The percentage of men with BDNF gene hypermethylation in the CAD group was significantly higher than the non-CAD group but no significant differences in this regard were seen among women (Table 5).

The association between the BDNF hypermethylation and severity of CAD is shown in Table 6. BDNF hypermethylation in patients with severe CAD was significantly higher than the normal group ($p < 0.05$ before and after the adjustment of mentioned confounding factors). Furthermore, in the mild CAD group hypermethylation was higher than the control group but the differences weren't statistically significant.

Table 3 Demographic, clinical and biochemical characteristics of all subjects according to Gensini score

Variables	Non-CAD (62)	Mild CAD (37)	Severe CAD (47)	*P*
Gender (M/F)	30/32	24/13	35/12	0.005
Male, n (%)	30(48.4)	24(64.9)	35(74.5)	–
Female, n (%)	32(51.6)	13(35.1)	12(25.5)	–
Age (year)	51.61 ± 1.19	53.14 ± 1.67	56.22 ± 1.22	0.037
BMI (kg/m^2)	26.06 ± 0.59	25.77 ± 0.78	24.80 ± 0.65	0.353
Waist circumference (cm)	99.73 ± 1.46	97.89 ± 2.13	95.36 ± 1.50	0.148
WHR	0.98 ± 0.007	0.95 ± 0.010	0.96 ± 0.008	0.162
Systolic blood pressure (mmHg)	131.05 ± 3.02	134.03 ± 3.31	136.15 ± 3.28	0.496
Diastolic blood pressure (mmHg)	79.53 ± 1.46	82.14 ± 1.90	81.77 ± 1.44	0.428
Blood pressure history, n (%)	32(51.6)	22(59.5)	27(57.4)	0.520
Smoking, n (%)	27(43.5)	24(64.9)	31(66)	0.016
Aspirin medication, n (%)	52(83.9)	28(75.7)	41(87.2)	0.715
Statin medication, n (%)	39(62.9)	23(62.2)	35(74.5)	0.225
BDNF methylation, n (%)	45(72.6)	30(81.1)	44(93.6)	0.005
Serum BDNF (ng/ml)	2.02 ± 0.14	2.16 ± 0.24	2.23 ± 0.212	0.416
	1.71 [1.43–2.04]	1.78[1.56–2.08]	1.77[1.55–2.05]	
FBS (mg/dl)	90.46 ± 1.46	93.09 ± 2.34	90.59 ± 1.64	0.570
TG (mg/dl)	120[92–181]	137.5[109–195]	140[91–180]	0.349
	150.82 ± 13.84	156.65 ± 13.45	148.32 ± 10.81	
Total cholesterol (mg/dl)	167.33 ± 6.09	162.32 ± 6.05	163.45 ± 6.70	0.841
HDL-C (mg/dl)	44.53 ± 1.59	40.59 ± 2.31	42.52 ± 1.88	0.350
LDL-C (mg/dl)	93.77 ± 5.18	88.04 ± 6.56	90.15 ± 5.91	0.782
Platelet (× 103/µl)	228.06 ± 9.49	224.12 ± 13.13	232.64 ± 9.69	0.334

BDNF, brain-derived neurotrophic factor, body mass index; *CAD*, coronary artery disease; *FBS*, fasting blood sugar; *HDL-C*, high density lipoprotein-cholesterol; *LDL-C*, low density lipoprotein-cholesterol; *TG*, triglyceride; *WHR*, waist to hip ratio

Discussion

In recent years, BDNF has been the main area of interest because of its role in promoting growth, survival, and regeneration of the neurons. However, the reports about the role of BDNF outside the nervous system are limited. New evidence indicates that BDNF exerts essential cardiovascular functions [25].

A growing body of reports has been focused on the role of DNA methylation alterations in CAD development, prognosis, and diagnosis [18]. Despite the important role of BDNF in cardiovascular homeostasis, to the best of our knowledge, no report was available regarding BDNF gene methylation status in CAD patients and its association with CAD risk. Previous investigations demonstrated that methylation alterations in some genes like ABCG1 (ATP-binding cassette subfamily G member 1), and GALNT2 (Polypeptide N-acetylgalactosaminyltransferase 2) [26] have a key role in CAD pathogenesis.

In the present study, the association between the BDNF gene methylation status with the CAD risk and CAD severity among non-diabetic CAD patients was examined. The results of the present study have revealed that the BDNF gene methylation was markedly higher in the CAD group than the non-CAD control group. The BDNF gene hypermethylation significantly increases the risk of CAD in the total population (OR = 2.796; 95% CI, 1.178–6.636; *P*, 0.017 and OR = 2.769; 95% CI, 1.033–7.423; *P**, 0.043), (*P**adjusted for age, sex, smoking habit, and lipid profile). Hypermethylation of the BDNF gene was associated significantly with CAD risk in the male (OR = 3.714; 95% CI, 1.243–11.102; *P*, 0.015 and OR = 4.090; 95% CI, 1.200–13.937; *P**,0.024) but not rather than in the female group (OR = 2.053; 95% CI, 0.473–8.920; *P*, 0.331 and OR = 1.301; 95% CI, 0.207–8.162; *P**,0.779) (Table 5). Interestingly, when participants were classified according to the Gensini score, BDNF hypermethylation in severe CAD group was more pronounced than patients with mild CAD and in the mild CAD group was higher than non-CAD group. In other words, with increasing the severity of the CAD, the methylation of the BDNF gene was markedly increased (Table 6). A growing body of evidence has suggested that stressful events can change the BDNF gene methylation in blood or brain [27]. Furthermore, antipsychotic drugs can influence DNA methylation in blood. Guidotti et al. have shown that clozapine and quetiapine

Table 4 Association of the BDNF gene methylation status with clinical and biochemical parameters

Variables	Methylation status		Univariate logistic regression		Multiple logistic regression	
	Methylated (119)	Unmethylated (27)	OR (95%CI)	*p*	OR (95% CI)*	*P**
Age	54.46 ± 0.87	49.00 ± 1.51	1.071 (1.018–1.127)	0.008	1.087 (1.026–1.150)	0.004
Gender (M/F)	72/47	17/10	0.901 (0.380–2.136)	0.813	–	–
CAD (yes/no)	74/45	10/17	2.796 (1.178–6.636)	0.017	2.967 (1.057–8.329)	0.039
BMI	25.28 ± 0.40	26.90 ± 1.07	0.928 (0.849–1.015)	0.102	0.922 (0.829–1.025)	0.131
Waist circumference	96.85 ± 0.98	102.30 ± 2.72	0.958 (0.922–0.996)	0.030	0.956 (0.913–1.001)	0.055
Systolic blood pressure	134.36 ± 2.03	129.28 ± 4.64	1.011 (0.990–1.032)	0.299	–	–
Diastolic blood pressure	81.25 ± 0.99	79.36 ± 2.31	1.016 (0.976–1.058)	0.429	–	–
Blood pressure history (yes/no)	68/51	13/14	1.436 (0.621–3.318)	0.396		
Smoking (yes/no)	65/54	17/10	0.708 (0.299–1.674)	0.430	–	–
Aspirin medication (yes/no)	100/19	21/6	1.504 (0.536–4.218)	0.436	–	–
Statin medication (yes/no)	79/40	18/9	0.988 (0.407–2.395)	0.978	–	–
Serum BDNF	2.12 ± 0.20	2.65 ± 0.50	4.752 (0.351–64.314)	0.241	–	–
	1.72[1.44–1.93]	1.61[1.48–2.59]				
TG	140[96–186]	101[75–134]	1.011 (1.002–1.020)	0.022	1.012 (1.001–1.022)	0.028
	159.16 ± 8.60	113.35 ± 9.88				
Total cholesterol	167.75 ± 4.09	151.28 ± 8.17	1.010 (0.999–1.021)	0.088	1.008 (0.995–1.022)	0.215
HDL-C	42.66 ± 1.20	43.90 ± 2.50	0.992 (0.960–1.026)	0.658	–	–
LDL-C	92.94 ± 3.67	83.14 ± 7.97	1.007 (0.995–1.019)	0.258	–	–

BDNF, brain-derived neurotrophic factor; body mass index; *CAD*, coronary artery disease; *FBS,* fasting blood sugar; *HDL-C*, high density lipoprotein-cholesterol; *LDL-C*, low density lipoprotein-cholesterol; *TG*, triglyceride; *WHR*, waist to hip ratio. * adjusted for age, gender, smoking and lipid profile

Table 5 Association between the BDNF gene methylation status and CAD risk according to subgroup analysis by gender

Methylation status	CAD	Non-CAD	OR (95%CI)	*p*	OR (95%CI)*	*P**
Study participants						
M	74	45	2.796 (1.178–6.636)	0.017	2.769 (1.033–7.423)	0.043
U	10	17				
Sex (male)						
M	52	20	3.714 (1.243–11.102)	0.015	4.090 (1.200–13.937)	0.024
U	7	10				
Sex (female)						
M	22	25	2.053 (0.473–8.920)	0.331	1.301 (0.207–8.162)	0.779
U	3	7				

*Adjusted for age, gender, smoking and lipid profile. M: methylated, U: unmethylated

reduced DNA methylation, but haloperidol and risperidone had no effect on DNA methylation [28]. Because of the nature of our cross-sectional study, we couldn't follow the stressful events in our patients; however, we excluded all patients with any history of mental disorders or subjects who took antidepressant or tranquilizer medications.

Some studies investigated the association between BDNF methylation and depression or suicidal tendencies in acute coronary syndrome patients. Kim et al. in the K-DEPACS study evaluated the association between BDNF gene hypermethylation and depressive disorder and response to treatment in patients with acute coronary syndrome (ACS). Their results demonstrated that BDNF hypermethylation was associated with the susceptibility to early depressive disorder and better antidepressant treatment response in ACS population [29]. In another investigation led by Kim et al. on the same population, they

Table 6 The association between BDNF methylation status and severity of CAD

Groups	Methylation status		OR (95%CI)	*P*	OR (95%CI)*	*P**
Normal	M	45	1	–	1	–
	U	17				
Mild	M	30	1.565 (0.578–4.239)	0.342	1.859 (0.573–6.034)	0.290
	U	7				
Severe	M	44	5.541 (1.516–20.246)	0.010	5.175 (1.258–21.296)	0.023
	U	3				

*Adjusted for age, gender, smoking and lipid profile. M: methylated, U: unmethylated

reported that BDNF hypermethylation was associated with ACS prognosis in patients without depression [30].

In the present study, we reported that BDNF hypermethylation is positively related to the age of participants. In agreement with our findings, Ihara et al. suggested that methylation of 11 CpG sites of the BDNF gene was insignificant positive association with the age of healthy Japanese female subjects [31]. In contrast, Kim et al. were not able to show any association between BDNF methylation status and age [30].

In the present investigation, we couldn't find any association between circulating BDNF level and its gene methylation status. So far, no study has evaluated the relationship between BDNF methylation status and its serum level in CAD patients. In a previous report on healthy Japanese women (age 20–80 years), it has been shown that DNA hypermethylation of the BDNF gene was not significantly correlated with serum BDNF level. They concluded that hypermethylation of BDNF gene could not directly lead to the decrease in serum BDNF level [31].

We found no significant differences between serum BDNF levels in CAD patients and control subjects. Also, no differences were seen in serum BDNF between subjects with different severity of CAD (Table 3). Additionally, no association between serum BDNF level and CAD risk factors including lipid profile, hypertension, BMI, and smoking habits were observed (data are not shown). Data regarding the serum BDNF level in CAD patients are limited and inconclusive. Ejiri et al. examined BDNF level in the coronary circulation and peripheral vein of 38 unstable angina patients and 45 stable angina patients as well as 24 control group [13]. They found no significant difference in the level of BDNF in the peripheral vein sample among the 3 groups. However, in patients with unstable angina, a significant increase in the BDNF level in the coronary circulation has been observed compared with individuals with stable angina or control group. This suggested that BDNF may be involved in the instability of plaques [13]. In contrast, a study by Jiang et al. on patients with angina pectoris, lower plasma BDNF concentration was found in comparison to control healthy subjects [12]. Their findings indicated that BDNF has a protective role in the pathogenesis of CAD and a higher plasma concentration of BDNF was associated with a lower risk of future coronary events and mortality [12]. In Jiang et al. study [12] the control group was selected among healthy subjects just through routine physical examination and they couldn't rule out the presence of CAD in these individuals. But in our work, normal individuals without any angiographically confirmed coronary stenosis were considered as the control group. Additionally, all subjects with diabetes were excluded from the present investigation but in previous studies [12, 13], a significant percentage of CAD patients were diabetic. It has been reported that BDNF levels in serum and plasma were significantly decreased in type 2 diabetes compared to the control subjects [32]. In a case–control study, Lee et al. excluded diabetic patients and measured serum BDNF level in men with and without metabolic syndrome. Their results showed no differences between serum levels of BDNF in the two groups [33].

In Ejiri et al. research, the increased expression of BDNF in atherosclerotic plaques was reported [13], but to date, it remains unclear to what extent BDNF-expression in atherosclerotic plaques contributes to the overall BDNF circulatory levels. In another report platelet count was considered as the most important predictor of circulating BDNF level in children and adolescents [34]. In Jiang et al. study, low BDNF concentration in patients with angina pectoris was mainly attributed to the diminished release of the peptide from platelets [12]. Here, we observed approximately the same platelet counts in blood samples of subjects with mild or severe CAD as well as control non-CAD group (Tables 2 and 3). Our findings also could not show any association between the platelet count and the serum BDNF level.

Our study has certain limitations. First, we had a relatively small sample size (N = 146). Further new studies with larger groups are needed to confirm our findings. Second, our study participants took some medications such as aspirin or statins, which might have influenced our results. However, no statistically significant difference was observed in this regard between CAD and control groups. Finally, we were not able to consider all confounding factors that can affect the serum BDNF level.

Conclusion

In conclusion, our findings indicate that BDNF hypermethylation was associated with the increased risk of CAD, which may help identify subjects at risk of developing CAD. In addition, BDNF hypermethylation showed a significant correlation with the severity of CAD. No association was found between serum BDNF level and the risk or severity of CAD.

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Data availability All data are presented in the manuscript.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to content of this article.

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