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ORIGINAL RESEARCH ARTICLE

Dual Effect of IL-6 -174 G/C Polymorphism and Promoter Methylation in the Risk of Coronary Artery Disease Among South Indians

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Abstract Inflammation plays an important role in the pathogenesis of atherosclerosis and coronary syndromes; moreover, various lines of evidence suggest that genetic factors do contribute to the risk of coronary artery disease (CAD). The proinflammatory cytokine IL-6 is a central mediator of inflammation associated with CAD. The present study is aimed to investigate the association of single nucleotide polymorphism in the promoter region of the IL-6 gene (-174 G > C) and methylation with the susceptibility of CAD. Genotyping of IL-6 -174 G/C polymorphism was performed by PCR–RFLP. Methylation-specific PCR method was used to study the IL-6 gene promoter methylation. Analysis of 470 subjects (265 CAD patients and 205 controls) showed association of the -174 G/C variant with the CAD risk in dominant model (OR 1.58, 95% CI, 1.024–2.23, $P = 0.04$). Further, the analysis of the distribution of genotypes and alleles of -174 G > C polymorphism according to clinical features of CAD, revealed significant association of genotype and allele (OR 1.86, 95% CI 1.18–2.84 $P = 0.01$, and OR 1.71, 95% CI 1.09–2.23 $P = 0.02$ respectively) with diabetes, and we found no association with hypertension (OR 0.95, 95% CI 0.57–1.59, $P = 0.8$). We also analyzed the methylation status of IL-6 promoter region between cases and controls showed significant hypo methylation in CAD subjects (OR 2.36, 95% CI 1.51–4.259, $P = 0.006$). Additionally, GC, CC genotypes and C allele carriers show hypomethylation in CAD cases compared to controls (54.58 vs. 76.85%, 29.83 vs. 40% respectively). In conclusion, the promoter polymorphism -174 G/C is associated with CAD risk and further carriers of 'C' allele at -174 locus showed significant hypo methylation which could contribute to increased risk of CAD. The present study highlights the association of allele and genotypes with differential DNA methylation of CpG islands in the IL-6 promoter region which may affect IL-6 gene regulation.

Keywords Coronary artery disease · IL-6 · Polymorphism · Methylation

Introduction

Coronary artery disease (CAD) is the leading cause of mortality and morbidity worldwide [1]. CAD is a major cause of death and disability in developed countries accounting for over one-third of total deaths and now became more epidemic in India [2]. The global burden of disease study estimate of age-standardized CAD death rate of 272 per 100,000 population in India is higher than the global average of 235 per 100,000 population [3]. CAD is a complex, multifactorial disorder that results from major risk factors such as dyslipidemias, diabetes, hypertension, obesity, smoking, stress, unhealthy diet and physical inactivity [4, 5]. In general, individuals with these risk factors are considered at high risk for development of CAD [6].

Atherosclerosis is considered as state of chronic low-grade inflammation occurring within the arterial wall [7], in which onset or progression of atherosclerotic manifestations is contributed by the pro-inflammatory cytokines

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including IL-6, IL-1, IL-8, IL-10 and TNF-α [8]. Cytokines play an important role in the inflammatory response and are involved in acute phase and chronic phase of the disease. Both the phases are characterized by increased blood flow, vascular permeability along with the accumulation of fluid, leukocytes, and inflammatory mediators such as cytokines finally promotes the pathogenesis of atherosclerosis [9].

The Interleukin-6 (IL-6) is a 26-kDa pleotropic inflammatory cytokine produced by many cell types, including fibroblasts, monocytes, adipocytes and endothelial cells and altered levels are associated with endothelial damage leading to initiation of atherosclerotic events [10]. The IL-6 contributes to CAD progression by affecting metabolic, endothelial and coagulant events, and is viewed as a local and circulating marker of coronary plaque inflammation [11]. The IL-6 was implicated in the pathogenesis of ischemic cardiovascular events, including unstable angina [11] and ACS [12]. IL-6 induces the expression of tissue factor, monocyte chemotactic protein-1, matrix-degrading enzyme, low-density lipoprotein receptors in macrophages which stimulates the aggregation of platelets, proliferation of vascular smooth muscle cells and production of C-reactive protein and fibrinogen that results in the plaque stability [13].

The IL-6 gene, located at chromosome 7p21–24 is composed of 4 introns and 5 exons. Polymorphic variants in the IL-6 gene promoter may affect the expression and secretion of IL-6 and subsequently altered circulating levels might result in relevant biological responses, that leads to the pathogenesis of various diseases such as CAD, myocardial infarction (MI) and ischemic stroke [14]. Polymorphisms in the promoter region (G-597A, G-572C, G-174C) of IL-6 gene have been well studied [15–17], among which, the single nucleotide change from G to C at position -174 (rs1800795) is a crucial polymorphism that affects IL-6 production, therefore predispose an individual to cardiovascular events [18, 19]. Several studies have reported that IL-6 -174 polymorphism has been associated with CAD [18, 19], type II diabetes mellitus [17]. In a meta-analysis, reported the significant association between the incidence of stroke and IL-*6* -174G/C polymorphism [20, 21].

The DNA methylation is an epigenetic modification that plays a crucial role in controlling gene expression in the genome [22] and may significantly contribute to the risks of many complex diseases, including cardiovascular, cancer, and metabolic diseases [22, 23]. Aberrant promoter methylation of several genes has been associated with the development and progression of coronary heart disease (CHD) [24]. As with many inflammatory mediators, IL-6 is also known to be regulated through epigenetic mechanisms. Several studies have been reported the correlation between the methylation of different CpG sites in the promoter region of IL-6 and its mRNA expression [25, 26]. Abnormal methylation patterns in the CpG islands of disease-associated genes might be involved in CAD pathogenesis [27]. Hence the present study is aimed to investigate the association of SNPs in the promoter region of the IL-6 gene (174 G > C) and effect of methylation with the susceptibility to CAD.

Methods

Study Subjects and Sampling

We have enrolled 470 subjects, which includes 265 angiographically documented CAD patients and 205 age, gender and ethnicity-matched healthy controls from Nizam's Institute of Medical Sciences (NIMS), Hyderabad, India. Patients who had malignancies, myocardial spasms, myocardial bridges, as well as those suffering from autoimmune diseases, congenital heart diseases, or end-stage kidney or liver diseases were excluded from the study. Healthy control subjects were hospitals staff and voluntary blood donors who were screened for dyslipidemia, liver and kidney function and other investigations to demonstrate CAD risk and found to be normal. Baseline characteristics of all of the patients and controls were obtained using a self-designed questionnaire and medical records. Data on gender, age, body mass index, alcohol consumption, tobacco consumption, and hypertension and diabetes were collected from the self-designed questionnaire. Total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) levels were obtained from medical records. Institutional Ethical committee of Nizam's Institute of Medical Sciences, Hyderabad, India has approved the study. Informed consent was obtained from all the study subjects.

Genotyping of -174 G/C Polymorphism

Five milliliters (5 ml) of blood was collected from each subject in an EDTA vacutainer. The genomic DNA was extracted from whole blood by using the phenol–chloroform extraction protocol. Genotyping for IL-6 -174G/C polymorphism was performed by PCR–RFLP. Primers used for PCR amplification was shown in Table 1. PCR was carried out on an Eppendorf thermol cycler under the following conditions: 94 °C for 5 min, followed by 40 cycles of 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C for extension, followed by a final extension of 10 min at 72 °C. The PCR products were then digested with 1 U of NlaIII restriction enzyme (reaction volume 10 µl) at 37 °C, for 4 h. The enzyme cut the 198 bp PCR product into four fragments 168, 119, 49 and 30 bp in length. Fragments size of 119 and 49 bp indicates the presence of a mutant homozygous CC genotype, two 168 bp and 30 bp fragments displayed the

Table 1 Primer sequence information used for IL-6 -174 G/C polymorphism and methylation

Primer name	Sequence	PCR product
IL-6 rs1800795	5′TGACTTCAGCTTTACTCTTTG3′ 5′CTGATTGGAAACCTTATTAAG3′	198 bp [42]
IL-6 M-MSP	5′GAAATTTTTGGGTGTCGACGC3′ 5′AAAACTACGAACGCAAACACG3′	67 bp [62]
IL-6 U-MSP	5′GAAATTTTTGGGTGTTGATGT3′ 5′AAAACTACAAACACAAACACA3′	67 bp

presence of homozygous GG genotype and three fragments of 168, 119 and 49 bp indicated the presence of heterozygous GC genotype. The resulting products were separated by 3.0% agarose gel electrophoresis. The products were visualized with Ultraviolet light by using Gel documentation system.

Analysis of IL-6 DNA Methylation

Sodium bisulfite conversion procedure was done using the EpiMark bisulfite conversion kit (NEW ENGLAND Bio-LABS) according to the manufacture's protocol. Subsequently, 10–60 ng of treated DNA was used for methylation specific PCR (MSP) reaction. The primers for MSP reaction was shown in Table 1. Each PCR reaction was performed with a total volume of 10 µl, which contained 5 µl of Hot-Star Taq Master Mix (Ampliqon), 4 µl of bisulfite- treated DNA template, and 0.3 µl of each primer pair. The reaction mixture was incubated at 95 °C for 5 min, followed by 40 cycles of 95 °C for 45 s, 54 °C for 45 s, 72 °C for 40 s and a final extension at 72 °C for 10 min. The PCR products were checked on 2.5% agarose gels with ethidium bromide staining. MSP products were analyzed for methylation index.

Statistical Analysis

Allele and genotype frequencies between CAD cases and controls were compared in a 2 × 2 contingency table using Fisher's exact test. All *P* values were two-sided and differences were considered statistically significant for $P < 0.05$. Odds ratio (OR) at 95% confidence intervals (CI) was determined to describe the strength of association. Logistic regression analysis was used to assess the independent effect of each risk factor on CAD.

Table 2 Demographic details of CAD subjects

Variables	CAD patients (n = 265)
Age (mean ± SD), years	56.00 ± 12.00
Gender	
Male	193 (72.83)
Female	72 (27.16)
BMI	
Male (kg/m^2)	25.01 ± 4.64
Female (kg/m^2)	25.95 ± 4.66
Diabetes mellitus	
Yes (%)	144 (55.3)
No (%)	121 (45.66)
Hypertension	
Yes (%)	168 (63.39)
No (%)	97 (36.6)

Results

Demographic Characteristics of the Study Population

A total of 470 subjects are recruited for the current study, among which 265 (193 male and 72 female) are CAD patients and 205 (105 male and 100 female) are control group. Mean age of the CAD group is 56.0 ± 12.0 years with 25.26 ± 4.66 BMI kg/m^2. Out of 265 CAD patients, 144 (55.3%) had diabetes and 168 (63.39%) had hypertension. The demographic characteristics of the study population are given in Table 2.

Association of -174 G > C Polymorphism with CAD

The frequency distribution of the genotype and alleles of IL-6 in the CAD and healthy control groups are shown in Table 3. The distribution of genotypes of IL-6 gene polymorphism at -174 G > C among healthy controls was in accordance with of Hardy–Weinberg equilibrium. The statistical analysis revealed that differences in the distribution of the IL-6 gene -174 G > C polymorphism between the CAD patients and healthy control groups (OR

Table 3 The genotype and allele frequencies of promoter polymorphisms in IL-6 gene in CAD patients and healthy control subjects

IL-6 SNP	Genotype and allele	Cases (n = 265)	Controls (n = 205)	OR	95% CI	*P* value
rs1800795 (G/C)	GG	163 (61.50%)	145 (70.73%)	1.58	1.024–2.23	0.04*
	GC	99 (37.35%)	57 (27.80%)			
	CC	03 (1.13%)	03 (1.46%)			
	G	425 (80.18%)	347 (84.63%)	1.36	0.96–1.91	0.08
	C	105 (19.81%)	63 (15.36%)			

*P value < 0.05 is considered as significant

Table 4 The genotype and allele frequencies of promoter polymorphisms in the IL-6 gene in CAD patients with and without risk factors

Clinical variable	Genotype			OR (95% CI)	*P*	Allele		OR (95% CI)	*P*
	GG	GC	CC			G	C		
CAD T2DM(+) (n = 144)	79 (54.86%)	62 (43.04%)	03 (2.08%)	1.86 (1.01–2.84)	0.01*	220 (76.36%)	68 (23.64%)	1.71 (1.09–2.66)	0.02*
CAD T2DM(−) (n = 121)	84 (69.49%)	37 (30.5%)	0 (0%)			205 (84.715)	37 (15.285)		
CAD HTN(+) (n = 168)	104 (61.9%)	61 (36.3%)	03 (1.78%)	0.95 (0.5–1.5)	0.8	269 (80.05)	67 (19.94)	1.02 (0.65–1.59)	1.00
CAD HTN(−) (n = 97)	59 (60.83%)	38 (39.17%)	0 (0%)			156 (80.41%)	38 (19.58%)		

*P value < 0.05 is considered as significant

1.58, 95% CI 1.024–2.23, = 0.04). And also higher frequency of allele "C" allele at -174 G > C was found in patients with CAD compared to the controls (19.81 vs. 15.36), but failed to reach statistical significance ($P = 0.08$, OR 1.36, 95% CI 0.96–1.91).

Further, we analyzed the distribution of genotypes and alleles of -174 G > C polymorphism in association with clinical features of CAD. The genotype frequency distribution between CAD with diabetes and without diabetes revealed the significant distribution with allele and genotypes (OR 1.86, 95% CI 1.18–2.84, $P = 0.01$ and, OR 1.71, 95% CI 1.09–2.23, $P = 0.02$ respectively). However, -174 G > C polymorphism did not show association with hypertension (OR 0.95, 95% CI 0.57–1.59 $P = 0.8$) and with other clinical features (Table 4). Further, logistic regression analysis also revealed association of CAD with type 2 diabetes (OD: 1.99, CI at 95%:1.10–3.58, $P = 0.02$) and no association with other variables such as age ($P = 0.99$), sex ($P = 0.42$), BMI ($P = 0.50$) and hypertension ($P = 0.20$) (Table 5).

DNA Methylation of IL-6 Promoter

The percentage of methylation of IL-6 promoter was quantified using the MSP method in 97 CAD cases and 81 controls. Methylation status between cases and controls revealed significant hypomethylation in CAD subjects (OR 2.36, 95% CI 1.51–4.259, $P = 0.006$). Regardless of case–control status, carrier of homozygous "GG" had 45.16%, "GC" had 50% and individuals homozygous for the variant genotype "CC" had 4.8% methylation. We also found "C allele" carriers showed hypomethylation in both cases and control group (Cases G: 70.16% vs. C 29.83%, Controls G: 60% vs. C: 40%) (Fig. 1).

Discussion

In recent years, the disease susceptibility has been increased when genomic factors combined with the environmental factors [18, 19]. IL-6 is a pleiotropic cytokine with a broad range of humoral and cellular immune effects relating to inflammation, host defense, and tissue injury [28].The SNPs associated with the disease might alter the gene transcription and expression by modulating transcription factor (TF) binding site [29, 30]. Functional variation in the promoter region of the IL-6 gene can influence gene transcription [31]. This would affect plasma or tissue levels of IL-6 which in turn would influence plasma levels of cardiovascular disease risk factors and thus leads to cardiovascular disease [31]. The G (-174) C SNP in the promoter of IL-6 gene is considered a key expression regulator of the gene [32]. Several studies have assessed the relationship between the IL-6 gene

Table 5 Logistic regression analysis of association between the IL-6-174 G/C genotypes and risk of coronary artery disease

Variable	B	SE	Wald	Odds ratio	95% CI	*P*
Age	− 0.00001	0.011	0.000001	1.0	0.97–1.02	0.99
Sex	− 0.247	0.308	0.642	0.78	0.42–1.42	0.42
BMI	0.017	0.026	0.435	1.0	0.96–1.07	0.50
HTN	− 0.403	0.315	1.636	0.66	0.36–1.23	0.20
DM	0.688	0.299	5.271	1.99	1.10–3.58	0.021*

B = coefficient, *SE* std. error, *HTN* hypertension, *DM* diabetes mellitus

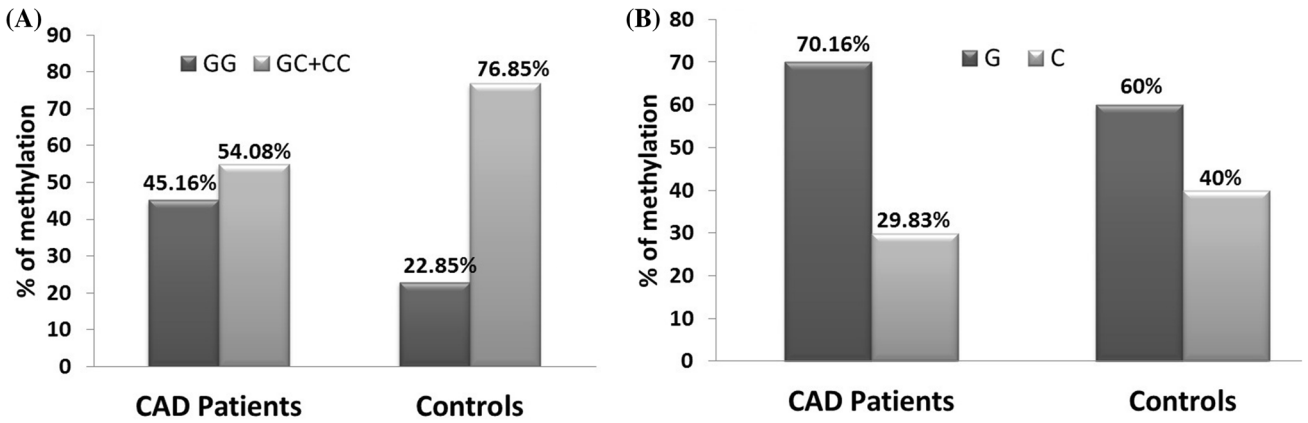

Fig. 1 Extent of IL-6 promoter methylation levels according to rs1800795 -174 G/C. IL-6 promoter methylation was determined using methylation-specific PCR. DNA methylation results are sorted according to rs1800795 -174 G/C genotype and alleles. **a** The rs1800795 GC + CC genotypes show hypomethylation in CAD cases compared to controls (54.58 vs. 76.85%). **b** Similarly, methylation results are sorted based on the rs1800795 alleles. "C allele" carriers also displayed hypomethylation in CAD subjects compared to the controls (29.83 vs. 40%). The data shows significant hypomethylation in IL-6 promoter in carriers of "GC" and "CC" genotypes in CAD cases when compared to controls (n = 97 CAD cases and 81 controls)

polymorphisms and pathogenesis of CAD and showed significant association with the CAD susceptibility [33–38]. The SNPs in the promoter region and intron 3 of the IL-6 gene have shown to play a role in the blood pressure regulation and progression of atherosclerosis in the Japanese [28]. Galimudi et al. [39] reported that CC genotype of IL-6 -174G/C gene was associated with development of CAD. In present study, we also found that IL-6 -174G/C was associated with CAD in dominant model with 1.58-fold risk for developing CAD. However, few studies have reported that IL-6 rs1800795 is not associated with an increased risk of CAD in Tunisians, Chinese population and in Isfahan population [40–42]. But in a recent meta-analysis on 50 studies suggested that the IL-6 − 174 G > C polymorphism was positively associated with susceptibility to CAD [18], which is in line with the results of our study. Hou et al. conducted a meta-analysis with 42 studies including 15,145 cases and 21,496 controls, and they reported that C allele of IL-6 -174G/C was correlated with an increased risk of CAD in Caucasians [43]. Song et al. [44] found that the CC genotype of IL-6 -174G/C was related to the onset of cardiovascular events. The C allele of G (− 174) C polymorphism was determined as a risk factor for MI [45]. In patients with type 2 diabetes, rs1800795 SNP of the IL-6 gene is significantly related to increased risk of CVD [46]. In the sub group analysis, we also observed that the significant association of CC genotype and C allele in CAD patients with diabetes. The carriers of the C allele were at increased risk of developing CAD in patients with type 2 diabetes. Based on these evidences, we speculate that polymorphisms in the promoter region may result in variation in transcriptional regulation and altered gene expression which in turn could affect the disease phenotypes and influence an individual's risk of disease.

The hypomethylation is a feature of transcriptionally active genes [47]. The expression of IL-6 gene is regulated by both transcriptional and post-transcriptional mechanisms through epigenetics which leads to the pathogenesis of inflammatory diseases including CAD [48]. The methylation level of IL-6 promoter has been associated with air pollution exposure, [49] which has been known to increase cardiovascular morbidity and mortality. In addition, serum IL-6 level has been associated with increased risk of mortality in patients with CHD [50]. Zuo et al. [51] have observed that hypomethylation in IL-6 promoter was

strongly associated with risk estimation for AMI. Several studies have observed the correlations of DNA methylation in IL-6 promoter with diet and environmental factors, which would confound the associations between IL-6 promoter hypo methylation and risk for CHD [48, 52–54]. The implication of IL-6 in the pathogenesis of CHD and the inverse correlation of IL-6 promoter methylation with CHD risk factors have been demonstrated [55]. Previous studies have been reported that hypomethylation of IL-6 promoter was associated with the pathogenesis of systemic lupus erythematous, rheumatoid arthritis, chronic periodontitis and CHD [56–60]. Gene-specific DNA methylation profiles and LINE-1 hypo methylation are associated with myocardial infarction risk [61]. In the present study, the methylation in the promoter region of the IL-6 gene showed significant association with the CAD, particularly in patients with CC genotype and C allele have shown hypomethylation in cases which may lead to increased transcription activity and subsequently up regulation of IL-6 production in targeted tissues finally contribute to the susceptibility to CAD. These observations are consistent with the results of other studies [57–59]. Suggesting that hypomethylation status is associated with the over transcription of genes that is related to the pathogenesis of inflammatory diseases.

Conclusion

In conclusion, our results revealed that IL-6 rs1800795 – 174G (G/C) might contribute to the risk of CAD. IL-6 hypo methylation was observed in CAD cases which may help in identifying individuals at risk of developing CAD. Further − 174 G/C polymorphism have influence on CAD patients with diabetes. Our study demonstrates that genetic and epigenetic variability plays an important role in the disease pathogenesis.

Author Contributions L.K performed clinical investigations and contributed samples. V.K.K provided research material. B.I and S.K.K conducted experiments and analyzed the data. B.I, S.K.K and V.K.K wrote and reviewed the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors have no conflicts of interest to declare.

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