



Evaluation of the Role of -137G/C Single Nucleotide Polymorphism (rs187238) and Gene Expression Levels of the IL-18 in Patients with Coronary Artery Disease

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ABSTRACT

Objectives: Interleukin-18 (IL-18) is a proinflammatory and proatherogenic cytokine, and its genetic variations may contribute to the development of coronary artery disease (CAD). We sought to investigate the role of -137G/C polymorphism and gene expression levels of IL-18 in patients with CAD. **Methods:** The study population included 100 patients with angiographically proven CAD and 100 matched controls. Total RNA and DNA were extracted from leukocytes using appropriate kits. The genotype of -137G/C polymorphism and gene expression level of IL-18 was determined using allele-specific polymerase chain reaction (PCR) and real-time (RT)-PCR assay, respectively. **Results:** The genotypic and allelic distribution of IL-18 -137G/C polymorphism was not significantly different between the two groups ($p > 0.050$). Moreover, the -137G/C polymorphism did not increase the risk of CAD in dominant and recessive genetic models ($p > 0.050$). However, subgroup analysis of CAD patients revealed that the IL-18 -137G/C polymorphism was significantly associated with increased risk of CAD in hypertensive patients (odds ratio (OR) = 7.51; 95% confidence interval (CI): 1.24–25.17; $p = 0.019$) and smokers (OR = 4.90; 95% CI: 1.21–19.70; $p = 0.031$) but not in the diabetic subpopulation ($p = 0.261$). The genotype distribution of IL-18 -137G/C genetic polymorphism was significantly different among patients with one, two, and three stenotic vessels ($p < 0.050$). The gene expression level of IL-18 was significantly higher in the CAD group than the control group ($p < 0.001$). Moreover, the carriers of CC genotype had significantly lower gene expression levels of IL-18 than carriers of GG genotype ($p < 0.050$). **Conclusions:** The -137G/C polymorphism of IL-18 may be associated with the CAD risk in hypertensive and smoker subgroup of CAD patients. The -137G/C polymorphism seems to play an important role in determining the severity of CAD. Increased IL-18 gene expression level is a significant risk factor for the development of CAD. The CC genotype of -137G/C polymorphism is associated with lower IL-18 gene expression levels.

Coronary artery disease (CAD) is a multifactorial disease characterized by the formation of atherosclerotic plaques in the coronary vessels.¹ According to a recently published study, cardiovascular disease is the most common cause of death in low- and middle-income countries.² Numerous risk factors including hypertension, dyslipidemia, male sex, diabetes mellitus, smoking, obesity, and family history have been proposed for the development of

CAD.^{3,4} Furthermore, immune system deregulation and altered activity of T-helper cell subtypes were shown to be involved in the occurrence of CAD.⁵ Inflammation and immune system activation are the two key players involved in initiation, progression, final destabilization, and rupture of atherosclerotic plaques.⁶ Several lines of evidence support the involvement of innate and adaptive immune system in the development, progression, and severity of CAD.⁷ Interleukin-18 (IL-18) is a pleiotropic

proinflammatory cytokine, which is mainly produced by macrophages and plays a central role in the inflammatory cascade.⁷ IL-18 contributes to the development of CAD by inducing the production of interferon (IFN)- γ , IL-6, and IL-1 by respective cells.⁸ IL-18 also significantly upregulates the expression of CD36 and matrix metalloproteinase (MMP)-9 by activating the nuclear factor (NF- κ B) pathway, and these mediators are involved in the progression and destabilization of atherosclerotic plaques.^{8,9}

Elevated levels of IL-18 have been linked with the formation and progression of unstable atherosclerotic plaques, which is associated with the development of CAD.¹⁰⁻¹² The plasma level of IL-18 is influenced by several factors including genetic variations in its promoter region.^{13,14} Genetic polymorphisms may affect gene expression and circulating levels of IL-18 and thereby modify the risk of CAD.^{13,15}

The IL-18 gene located at 11q22.2-q23.3 has six exons, and its expression is regulated by two promoters upstream of exon 1 (Promoter 1) and exon 2 (Promoter 2).¹⁶ Several individual single nucleotide polymorphisms (SNPs) of the IL-18 gene were shown to have functional properties. SNPs rs1946518 (-607C/A) and rs187238 (-137G/C) located in the promoter region of IL-18 gene are associated with the altered transcriptional activity of the gene *in vitro*.¹⁷ Moreover, other SNPs, including +183A/G, +113T/G, and +127C/T have also been reported to affect gene expression activity and circulating levels of IL-18.^{15,18} The two promoter SNPs of rs1946518 and rs187238 have been widely studied in different diseases including CAD and cancer.¹⁹⁻²¹ IL-18 -137G/C polymorphism changes the histone 4 transcription factor-1 (H4TF-1) nuclear factor-binding site and alters the transcriptional activity of the IL-18 gene.¹⁶ According to some studies, the minor C allele of the -137G/C SNP has been associated with lower circulating levels of IL-18 and may confer some protection against the development of CAD.²² Other studies have not supported such an association.¹⁵

Several lines of evidence suggest that the synergistic effects of both genetic and environmental risk factors may significantly enhance the development and severity of CAD.¹ Therefore, identification of genetic risk factors in addition to environmental risk factors may have pivotal roles in the prevention, treatment, and prognosis of the disease, especially in populations with a high probability of CAD

development including hypertensive patients and smokers. Hence, the present study was aimed to investigate the role of -137G/C (rs187238) promoter polymorphism and gene expression levels of IL-18 in an Iranian subpopulation of CAD patients in Zanjan province of Iran.

METHODS

We included 100 patients with angiographically confirmed CAD and 100 ethnically matched healthy subjects as controls. The mean age of CAD patients and controls was 59.4 ± 13.5 and 56.7 ± 9.5 years, respectively. Both CAD patients and controls were selected from the Zanjan population with an Azeri ethnic background. The presence of CAD was diagnosed by angiography conducted by an expert cardiovascular specialist. All CAD patients who had at least 50% stenosis in one major coronary artery were included in the study. The severity of CAD was determined based on the number of stenotic vessels showing more than 50% stenosis. Accordingly, the patients were classified as single-, double-, and triple-vessel stenosis patients. CAD patients who were on lipid-lowering drugs or with lumen stenosis less than 50% were excluded from the study. CAD patients who had a personal or family history of autoimmune, metabolic and inflammatory diseases, as well as congenital heart failure and malignancy were excluded from the study. Control subjects were selected after careful examination by a cardiovascular specialist. Control subjects with any evidence of overt disease, including cardiac, renal, hepatic, autoimmune, metabolic and inflammatory diseases, and a family history of heart disease were excluded from the study. Appropriate information was collected from all participants regarding hypertension (as defined by systolic blood pressure > 140 mmHg and/or diastolic blood pressure > 90 mmHg), diabetes (as defined by the presence of fasting blood glucose > 126 mg/dL in study population), smoking habits, family history of disease, hyperlipidemia, and the presence of any other disease. This study was approved by the ethics committee of Zanjan University of Medical Sciences (Ethical code: ZUMS.REC.1395.241), Zanjan, Iran.

After 12 hours of fasting, 5 mL blood was collected in EDTA-containing tubes and immediately centrifuged. Plasma was separated and stored at -20 °C until biochemical analysis was

Table 1: Characteristics of the CAD and control group.

Variables	CAD group	Control group	p-value
Age, mean (range), years	59.4 (37–85)	56.7 (35–78)	0.475
Sex (M/F)	56/44	51/49	0.478
TG, mean \pm SD, mg/dL	180.4 \pm 95.6	169.4 \pm 75.7	0.395
TC, mg/dL	192.8 \pm 65.4	167.3 \pm 49.5	0.002
HDL-C, mean \pm SD, mg/dL	38.6 \pm 10.5	43.3 \pm 14.8	0.010
LDL-C, mean \pm SD, mg/dL	99.3 \pm 51.6	85.5 \pm 43.2	0.041
Hypertension, n %	21 (21.0)	9 (9.0)	0.017
Diabetes, n %	23 (23.0)	11 (11.0)	0.024
Smoking, n %	36 (36.0)	12 (12.0)	0.002
IL-18 -137G/C Genotype GG/GC/CC	57/39/4	48/46/6	0.417

CAD: coronary artery disease; TG: triglyceride; SD: standard deviation; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; IL-18: interleukin-18.

done. The cellular fraction was used for DNA and RNA extractions.

Genomic DNA was isolated from blood leukocytes using a commercially available kit (GG2001, Viogene, Poland) according to instructions of the kit. Total RNA was extracted from blood leukocytes using TRIZOL reagent (Invitrogen, USA) according to kit instructions.

Genotyping of -137G/C polymorphism was performed using allele-specific polymerase chain reaction (PCR), as previously described with slight modifications.²⁰ Two separate sets of PCR were performed for each sample. Each reaction contained one of the two allele-specific forward primers (F wild: 5'-CCC CAA CTT TTA CGG AAG AAA AAC-3' or F mutant: 5'-CCC CAA CTT TTA CGG AAG AAA AAG-3') and a common reverse primer (R: 5'-AGG AGG GCA AAA TGC ACT GG-3') that amplified a 261 bp fragment. A control forward primer (F control: 5'-CCA ATA GGA CTG ATT ATT CCG CA-3) was used in each reaction as an internal control to amplify a 446 bp fragment covering the polymorphic site. The PCR products were analyzed on 2% agarose gel.

Initially, cDNA was constructed from 500–1000 ng of total RNA in a total volume of 20 μ L according to instructions of a commercially available kit (dART RT, EURx Ltd. 80-297 Gdansk Poland). Quantitative real-time (RT)-PCR was performed using high ROS SYBR Green PCR Master Mix Kit (Ampliqon, Denmark) on an ABI 7300 instrument (Applied Biosystems, Foster City, USA). RT-PCR conditions were as follows: initial denaturation at

95 °C for 15 minutes, and 35–40 subsequent cycles of denaturation at 95 °C for 30 seconds, 64 °C for 30 seconds, and 72 °C for 30 seconds. All samples were run in duplicate, and β 2-microglobulin (β 2-M) was amplified as an internal control to normalize the expression of target gene. The sequence of RT-PCR primers for the IL-18 gene was: forward: 5'-GTG GCT GGT ACA TGA GCA CT-3'; reverse: 5'-GTC TTT CCA CAG GGA CGA GG-3' and for β 2M were forward: 5'-TCT TTC TGG CCT GGA GGC TAT C-3'; reverse: 5'-CGG ATG GAT GAA ACC CAG ACA C-3'. Fold changes in mRNA expression levels among different samples were determined by the $2^{-\Delta\Delta CT}$ method.

Total cholesterol (TC), triglycerides (TG), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), and fasting glucose levels in plasma were measured using commercially available enzyme assay kits (Pars Azmoon Co, Tehran, Iran) using Mindray auto-analyzer (BS-200).

The statistical analysis was performed using SPSS Statistics (SPSS Inc. Released 2007. SPSS for Windows, Version 16.0. Chicago, SPSS Inc). The chi-square test (χ^2) was used to test for consistency of the genotype frequencies with the Hardy-Weinberg Equilibrium (HWE). Continuous variables were compared using unpaired Student's *t*-test or Mann-Whitney test, and categorical variables were compared using chi-square test or Fisher's exact test when appropriate. Binary logistic regression analysis was used to assess the independent association between the genotype and CAD

Table 2: Genotype and allele frequency of IL-18 -137G/C polymorphism in CAD and control group.

IL-18 -137G/C polymorphism	CAD group n = 100, %	Control group n = 100, %	OR	95% CI	p-value
Genotype					
GG	57 (57.0)	48 (48.0)	1.00	Ref	-
GC	39 (39.0)	46 (46.0)	0.71	(0.40–1.26)	0.307
CC	4 (4.0)	6 (6.0)	0.56	(0.14–2.10)	0.512
Dominant model					
GG	57 (57.0)	48 (48.0)	1.00	Ref	-
GC+CC	43 (43.0)	52 (52.0)	1.43	(0.82–2.50)	0.257
Recessive model					
GC+GG	96 (96.0)	94 (94.0)	1.00	Ref	-
CC	4 (4.0)	6 (6.0)	0.65	(0.17–2.38)	0.747
Allele					
G	153 (76.5)	142 (71.0)	1.00	Ref	-
C	47 (23.5)	58 (29.0)	0.75	(0.48–1.17)	0.255

IL-18: interleukin-18; CAD: coronary artery disease; OR: odds ratio; CI: confidence interval; GG: wild-type; GC: heterozygote; CC: homozygote.

status and to detect the independent effect of each risk factor on CAD development. The statistical significance was set at $p < 0.050$.

RESULTS

The demographic, clinical, and biochemical parameters of the CAD group and control group are presented in Table 1. The two groups were similar in terms of mean age ($p = 0.475$), sex distribution ($p = 0.478$), and TG levels ($p = 0.395$). However, significant differences were observed regarding the plasma levels of TC ($p = 0.002$), LDL-C ($p = 0.041$), and HDL-C ($p = 0.010$) between the two groups. Moreover, the prevalence of diabetes ($p = 0.024$), hypertension ($p = 0.017$), and smoking ($p = 0.002$) were significantly higher in the CAD group compared with the control group [Table 1].

The genotype distribution in both CAD group ($p = 0.397$) and control group ($p = 0.241$) was in accordance with the HWE. Furthermore, statistical analysis using chi-square test indicated no significant differences in the genotype distribution of IL-18 -137G/C polymorphism between the two groups ($p = 0.417$, $\chi^2 = 1.748$) [Table 1]. In the univariate analysis, using the GG (wild-type) genotype as a reference, neither GC (heterozygote) genotype ($p = 0.307$) nor CC (homozygote) genotype ($p = 0.512$) was significantly associated with the risk of CAD. Additionally, analyzing the IL-18-137G/C polymorphism under dominant

($p = 0.257$), recessive ($p = 0.747$), and allelic ($p = 0.255$) models did not reveal any significant association between this polymorphism and the risk of CAD [Table 2].

Multiple logistic regression analysis was done to investigate the independent association of each risk factor with CAD. Results indicated five independent risk factors for CAD, including TC ($p = 0.004$), HDL-C ($p = 0.024$), LDL-C ($p = 0.048$), smoking ($p = 0.005$), and hypertension ($p = 0.022$). However, age, sex, TG, diabetes, GC genotype, and CC genotypes of IL-18 -137G/C

Table 3: Results of multiple binary logistic regression analysis.

Covariates	OR	95% CI	p-value
Age	1.03	0.98–1.05	0.475
Sex (M/F)	0.95	0.67–1.52	0.901
Smoking	3.83	1.47–10.68	0.005
TG	1.00	1.00–1.01	0.076
TC	1.00	1.00–1.01	0.004
HDL-C	0.95	0.91–1.02	0.024
LDL-C	1.00	1.00–1.01	0.048
Diabetes	2.12	0.98–5.82	0.078
Hypertension	3.42	1.63–9.12	0.022
CC vs. GG	0.44	0.23–2.88	0.076
GC vs. GG	0.37	0.16–2.67	0.083

OR: odds ratio; TG: triglyceride; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; CC: homozygote; GG: wild-type; GC: heterozygote; CI: confidence interval.

Table 4: Interaction between IL-18 -137G/C polymorphism and demographic characteristics of subjects in the risk of coronary artery disease.

Variables	IL-18 -137G/C polymorphism						
	GC+CC		GG		OR*	95% CI	p-value
	Case	Control	Case	Control			
Hypertension							
Yes	40 (50.63)	47 (51.6)	39 (49.3)	44 (48.3)	1.04	0.56-1.90	0.991
No	3 (14.28)	5 (55.5)	18 (85.7)	4 (44.4)	7.51	1.24-25.17	0.019
Smoking							
Yes	35 (54.69)	45 (51.1)	29 (45.3)	43 (48.8)	0.86	0.45-1.65	0.742
No	8 (22.22)	7 (58.3)	28 (77.7)	5 (41.6)	4.90	1.21-19.70	0.031
Diabetes							
Yes	36 (46.75)	46 (51.6)	41 (53.2)	43 (48.3)	1.21	0.66-2.24	0.537
No	7 (30.43)	6 (54.5)	16 (69.5)	5 (45.4)	2.75	0.62-12.08	0.261

Data given as n (%); *Adjusted for age and sex; GC: heterozygote; CC: homozygote; GG: wild-type; OR: odds ratio; CI: confidence interval; IL-18: Interleukin-18.

Table 5: IL-18 -137G/C genotypes according to patients with 1, 2, and 3 stenotic coronary arteries.

-137G/C genotypes	1 Stenotic vessel n = 37	2 Stenotic vessels n = 41	3 Stenotic vessels n = 22	p-value (2 vs. 1 stenotic vessel)*	p-value (3 vs. 1 stenotic vessel)*
GG	6 (16.2)	33 (80.4)	18 (81.8)	Ref	Ref
GC	29 (78.3)	7 (17.0)	3 (13.6)	< 0.001	< 0.001
CC	2 (5.4)	1 (2.4)	1 (4.5)	0.087	0.201
GC+CC	31 (83.7)	8 (19.5)	4 (18.1)	< 0.001	< 0.001

Data given as n (%); *Values were calculated by Fishers exact test; GG: wild-type; GC: heterozygote; CC: homozygote; IL-18: interleukin-18.

Table 6: Gene expression levels of IL-18 across different genotypes of IL-18 -137G/C polymorphism.

Genotype	GG	GC	CC	p-value GC vs. GG	p-value CC vs. GG
Means of 2 ^{-ΔΔCT} in CAD group	1.00 ± 0.06	0.72 ± 0.05	0.56 ± 0.04	0.031	0.001
Means of 2 ^{-ΔΔCT} in control group	1.00 ± 0.08	0.79 ± 0.04	0.61 ± 0.05	0.045	0.008

GG: wild-type; GC: heterozygote; CC: homozygote; CAD: coronary artery disease; IL-18: interleukin-18.

polymorphism were not significant risk factors for CAD [Table 3].

We also conducted gene-environmental analysis to investigate the association between IL-18 -137G/C polymorphism and demographic characteristics of subjects for the risk of CAD. The results indicated significant interaction of GC+CC genotype with hypertension and smoking habits, but not with diabetes in conferring the increased risk of CAD.

The adjusted odds ratio (OR) (95% confidence interval (CI) were 7.51 (1.24–25.17) in hypertensive

and 4.90 (1.21–19.70) in smoker CAD patients, respectively [Table 4].

To assess the association of IL-18 -137G/C polymorphism with the severity of CAD, the genotype distribution of IL-18 -137G/C genetic polymorphism among patients with one, two, or three stenotic vessels was investigated. Results indicated significant differences between them, which revealed the important effect of this common polymorphism in determining the severity of CAD [Table 5].

We observed significantly increased gene expression levels of IL-18 in the CAD group compared

with control group (1.70 ± 0.26 vs. 1.00 ± 0.07) ($p < 0.001$). The gene expression levels of IL-18 showed significant inter-genotypic differences in the CAD patients and control subjects so that the carriers of GC and CC genotypes had significantly lower gene expression levels than carriers of GG genotype ($p < 0.050$) [Table 6].

DISCUSSION

IL-18 is a proinflammatory and proatherogenic cytokine that plays an important role in the development of CAD through the induction of Th1 immune responses.⁸ Carriers of CC and GC genotypes of IL-18 -137G/C polymorphism have significantly lower gene expression levels of IL-18 than carriers of the GG genotype. Therefore, harboring of CC and GC genotypes may confer some protection against CAD development, and the decreased frequency of CC and GC genotypes of IL-18 -137G/C polymorphism could contribute to the development of CAD.

Our study indicated no significant differences in the genotype distribution of IL-18 -137G/C polymorphism between the CAD group and control group. However, in subgroup analysis, a significant association was seen between IL-18 -137G/C polymorphism and CAD risk in hypertensive and smoker subpopulations, which could be explained by a decreased frequency of CC+GC genotype in these subpopulations. The association between IL-18 -137G/C polymorphism with the risk of CAD has been studied extensively; however, conflicting results have been reported. A study by Jabir et al,¹¹ conducted in a Saudi Arabian population observed no significant differences in the genotype and allele distribution of IL-18 -137G/C polymorphism between CAD and control groups. Also, Kariž et al,¹⁶ found no significant association between IL-18 -137G/C polymorphism and risk of myocardial infarction in Caucasian patients with type 2 diabetes. Moreover, Shayan et al,²⁰ investigated the role of IL-18 -137G/C polymorphism in patients with and without CAD and found no significant association between them. On the contrary, the study by Liu et al,²² found a significant difference in the genotype distribution of IL-18 -137G/C polymorphism between CAD patients and control group. The study by Ansari et al,²³ suggested a significantly higher prevalence of IL-18 rs187238

gene promoter polymorphism in the premature-CAD cases compared to control.

Moreover, in a recently published study by Dong et al,²¹ it was reported that the GG genotype of IL-18 -137G/C polymorphism was a significant risk factor for CAD in Asians but not Caucasians. The reasons for inconsistent results of genetic association studies remain unclear. However, numerous factors, including variability in phenotype definitions across independent samples, variable selection criteria for the study population, differences in study design, heterogeneity in sample size, and the presence of gene-gene and gene-environmental interactions may explain the discrepancy of genetic association studies.²⁴

We found a significant association between IL-18 -137G/C genetic polymorphism with the risk of CAD only in hypertensive and smoker subgroups of patients. This finding may be explained by the fact that the pathogenesis of CAD is a complex and multifactorial process, not only influenced by the genetic profile but also by environmental factors that could play an important role in this respect. Therefore, the interaction of genetic and environmental factors can significantly enhance the development of CAD, as seen in this study.

The identification of genetic variants associated with CAD development could improve our knowledge regarding the etiopathogenesis of this disease, and could consequently reduce the burden of disease at both individual and population levels. Furthermore, genetic association studies provide useful information that could potentially reduce the burden of disease in three main ways. That includes improvements in the identification of high-risk individuals, identification of new pharmacologic targets, and pharmacogenomics.²⁵

In addition, previous studies have demonstrated the interactions between IL-18 with several CAD risk factors (including smoking) in enhancing the CAD risk.¹⁰ Recently, in a study by Hernesniemi et al,¹³ it was shown that the interaction between hypertension and GG homozygote genotype of IL-18 -137G/C genetic polymorphism significantly increased the risk of sudden cardiac death and CAD development. Also, the study by Evans et al,²⁶ indicated that GC genotype carriers of the -137G/C polymorphism in the IL-18 gene had significantly elevated blood pressure levels in a sample of the South African population. IL-18 is

a Th1 cytokine that induces the production and release of several inflammatory cytokines including IFN γ , TNF α , IL-6, and IL-1 by macrophages and lymphocytes.^{27,28} These inflammatory cytokines may act on blood pressure by upregulation of gene expression levels of angiotensin and deregulation in the inflammatory response that collectively causes high blood pressure.²⁹ Our study is also in agreement with previous reports related to a significant increase in IL-18 gene expression levels in CAD patients.³⁰ Similar to previous studies, the present study reported significantly decreased gene expression levels of IL-18 in C allele carriers of IL-18 -137G/C polymorphism than non-carriers.³¹ Interestingly, in another study lower promoter activity was observed for IL-18 -137C allele and this allele was proposed as to be a 'low activity' allele.¹⁸ Other studies did not report such an association.¹⁵ Even though the IL-18 -137G/C genetic polymorphism could change the transcriptional regulation and gene expression pattern of IL-18, its genetic association with CAD risk is not definitive. It should be noted that both genetic and environmental factors determine IL-18 levels. It seems that the interaction between both factors are necessary for the induction of CAD since our results indicated the significant association of IL-18- 137G/C genetic polymorphism with CAD in hypertensive and smoker subpopulations.^{8,32}

Our study had some limitations. We did not determine the protein levels of IL-18 and other polymorphisms of IL-18 such as -607C/A and its possible interaction with IL-18- 137G/C genetic polymorphism.

CONCLUSION

We found a possible association between IL-18 -137G/C polymorphism and the risk of CAD in hypertensive and smoker subgroups of CAD patients. C allele carriers of IL-18 -137G/C polymorphism had significantly decreased gene expression levels of IL-18, which may have protected them against CAD development. The decreased frequency of CC+GC genotypes in hypertensive and smoker subgroups of CAD patients may have a role in CAD pathogenesis.

Disclosure

The authors declared no conflicts of interest. No funding was received for this study.

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