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Interferon Gamma Polymorphism and Expression Relationship with Severity of Coronary Artery Disease in Golestan, Iran

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ABSTRACT

Background: The Coronary artery disease (CAD) occurs as a result of atherosclerotic plaque formation. The interferon gamma (*IFNγ*) as a multifunctional cytokine is involved in inflammatory processes in atherosclerosis.
Objectives: We investigated the relationship between *IFNγ* (+874T/A) SNP with CAD. Moreover, we compared *IFNγ* mRNA expression in CAD patients and healthy controls.
Patients and Methods: This case-control study with randomized sampling included 300 patients with CAD and 301 normal controls. The SSP-PCR method was used for genotyping *IFNγ* (+874T/A) gene polymorphism. Quantitative Real-Time PCR was performed to measure *IFNγ* mRNA expression. All data was analyzed by GraphPad software. The chi-square and ordinal logistic regression tests were used to analyze differences in genotype frequencies.
Results: In this study, there was a significant association between male genders with CAD ($P < 0.001$). There was a significant association between genotype T/T and Allele T of *IFNγ* (+874T/A) polymorphism with CAD ($P = 0.021$ and $P = 0.022$, respectively). The inheritance model analysis showed that two copy of allele T is required for increased risk in CAD ($P = 0.031$). There was a significant association between the genotype T/T of *IFNγ* (+874T/A) polymorphism with CAD patients with double and triple vessel disease ($P = 0.030$ and $P = 0.013$, respectively). The *IFNγ* mRNA level in CAD group was significantly higher than control group ($P = 0.024$).
Conclusions: Conclusions: *IFNγ* gene functional polymorphism can be associated with incidence and severity of CAD. *IFNγ* mRNA level was also increased in CAD patients in comparison with controls. Therefore, *IFNγ* may play a role in predisposition to CAD.

1. Background

Coronary artery disease (CAD) is still considered as the first reason of death all over the world (1). As a polytransgenic and multifactorial disease, atherosclerosis is a chronic inflammatory condition, contributing to the development of cardiovascular diseases (CVD) (2). Based on the epidemiological studies, there are several important environmental and genetic risk factors which correlate with atherosclerosis (3, 4). Inflammatory factors such as cytokines have been involved in all stages of CVD. They are associated with endothelial dysfunction, leukocyte migration, extracellular matrix degradation, and platelet activation (5-8). Thus, genetic alteration leads to a change in the function or expression of cytokines and may be debatable in CVD. As previously reported, the levels of pro-inflammatory cytokines such as interferon gamma (*IFNγ*) and tumor necrosis factor-α (TNF-α) are higher than the anti-inflammatory mediators such as IL-4 (9).

Among the cytokines, *IFNγ* appears to serve as a key factor in pathogenesis of atherosclerosis (10, 11). *IFNγ* gene is located on the 12q24 chromosome, containing four exons and three introns (12). *IFNγ*-producing cells include the Th1 subpopulation of helper T cells, cytotoxic T cells and NK cells (10). This cytokine contributes to many processes such

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as stimulation of antigen presentation via induced expression of Class I and II major histocompatibility complex molecules on macrophage surfaces. It is also involved in processing of T-lymphocytes antigen, controlling Th1 and Th2 balance, activating macrophages, activating T-lymphocytes and NK cells, stimulating cytokine production in target cells, and recruiting the cells to the injury site via increased expression of chemokines and adhesion molecules (13).

Considering the effect of *IFNγ* in regulation of proliferation, differentiation and apoptosis, it seems that it affects plaque formation in the arteries (14). The functional Single Nucleotide Polymorphism (SNP) at position +874 of the *IFNγ* gene (rs2430561) maps to a putative nuclear factor-κB (NF - κB) binding site. The efficiency of NF-κB binding is enhanced by the presence of allele T in this functional variant, and this leads to an increased *IFNγ* expression in vitro (15-17). *IFNγ* (+874T/A) variant in the first intron of the *IFNγ* gene was associated with disease severity or drug resistance in various diseases. According to previous studies, there was an association between this polymorphism with the risk of many diseases, including autoimmune diseases and chronic inflammations due to hepatitis C virus, severe acute respiratory syndrome, and heart disease (18, 19).

2. Objectives

With regard to some recent studies which have described a relationship between *IFNγ* with heart diseases (6, 10, 11, 14, 15), in the present study, we investigated the relationship between *IFNγ* (+874T/A) between SNP and CAD. Moreover, we compared *IFNγ* mRNA expression in CAD patients and healthy controls.

3. Patients and Methods

2.1. Sample Preparation

This case-control study with randomized sampling was performed to evaluate the genetic association of *IFNγ* (+874T/A) polymorphism in patients with CAD. The study population consisted of 300 CAD patients and 301 normal individuals, who were selected by angiography test and approved by a cardiologist from March 2013 to March 2014 in Amiralmomenin hospital, Golestan province, Iran. Inclusion criterion for the patient group was having a stenosis less than 50% in at least one major coronary artery. Inclusion criteria for the control group were having normal electrocardiograms at rest, without symptoms of myocardial ischemia during exercise. In agreement with the Helsinki Declaration, all the participants were aware of the study details, and signed the relevant written informed consent. This study was approved by the approved by ethics committee ethics committee by Ethics Committee f Golestan University University of Medical Sciences ethics committee (code number: IR.GOUMS.REC.1395.15). Personal information including age, sex and ethnic group was also recorded for all participants.

2.2. Genotyping

The rs2430561SNP, located in 874 base pairs (bp) downstream the transcription start site, was Genotyped, using a sequence specific primers polymerase chain reaction (SSP-PCR) primers set.

The primers were designed by Gene Runner software (version 5.2; Hastings, USA) and using *IFNγ* gene sequence information (Table 1). The genomic DNA was extracted with a standard protocol as described previously (20). Extracted DNA was purified, quality-controlled and quantified by spectrophotometer (Biochrom, Cambridge, UK). All DNA samples had a 260 nm optical density (OD) of 0.5 to 1 micrograms per microliter, and a 260/280 ratio of 1.5 to 2. PCR reactions were performed (Eppendorf, Hamburg, Germany) with 100 ng DNA and a mastermix containing DNA polymerase (Roche, Woerden, Netherlands). A primer set was also used to amplify the human growth hormone (hGH) gene as internal control to confirm negative PCR (Table 1). PCR conditions consisted of 10 cycles (95°C for 15s, 54°C for 50s, 72°C for 40 s) followed by 20 cycles (95°C for 20s, 59°C for 50s, 72°C for 40s). PCR products were then electrophoresed on a 1.5% agarose gel (Merck, Darmstadt, Germany) stained in SYBR safe stain (Thermo Fisher Scientific, Massachusetts, USA), and observed under UV light. The sizes of PCR products were estimated according to 100-bp DNA ladder (Fermentas, Sankt Leon-Rot, Germany).

2.3. Quantitative Real-Time PCR

Total RNA was extracted from the peripheral blood by trizol regent (Invitrogen, Karlsruhe, Germany). The remaining genomic DNA was digested with RNase-free DNase (Fermentas, Sankt Leon-Rot, Germany); the quality and quantity of RNA extracted was assessed by spectrophotometer. All RNA samples had a 260 nm OD of 0.4 to 0.9 nanograms per microliter, and a 260/280 ratio of 1.8 to 2.2. The sample was then converted to cDNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Sankt Leon-Rot, Germany) according to the manufacturer's instructions. Quantitative Real-time PCR was performed

Table 1. Primers Used for Detection of IFNγ +874 T > A Polymorphism and mRNA Expression Analysis

Description		Primer Name	Sequence (5' → 3')	Product Size (bp)	GenBank Number
SSP-PCR	+874 T > A	Forward A-allele	TTCTTACAACACAAAATCAAATCA	264	NG_015840.1
		Forward T-allele	TTCTTACAACACAAAATCAAATCT		
		Reverse generic	TCAACAAAGCTGATACTCCA		
	hGH	Forward	GCCTTCCCAACCATTCCCTTA	509	NG_011676.1
		Reverse	GAAGGACGGGCATTGGCTGTG		
Real-Time PCR	IFNγ	Forward	TCGGTAACTGACTTGAATGTCCA	93	NM_000619.2
		Reverse	TCGCTTCCCTGTTTTAGCTGC		
	PGK1	Forward	GCAGATTGTGTGGAATGGTC	101	NM_000291.3
		Reverse	CCCTAGAAGTGGCTTTCACC		

using Maxima SYBR Green/Rox QPCR Master Mix (2X) (Thermo Fisher Scientific, Heiligen, Germany) and by *IFNγ* expression primers (Table 1) in ABI7300 detection system (Applied Biosystems, Foster City, United States). The phosphoglycerate kinase 1 (PGK1) gene mRNA was amplified as an endogenous reference. Real-Time PCR was performed in accordance with the conditions previously described (21). The mRNA level was calculated, using the 2-ΔCt method (22).

2.4. Statistical Analysis

All data were analyzed using GraphPad software (version 6; San Diego, CA, United States). Data are presented as means ± SD for parametric variables and as percentages for non-parametric variables. The Chi-square and ordinal logistic regression tests were used to analyze the differences in genotype frequency between the patient groups with single, double, or triple vessel disease (VD).

Deviation from the Hardy - Weinberg equilibrium was checked for each polymorphism in patients and controls separately by Chi-square test. Allelic and genotypic frequencies were calculated and compared between groups by Fisher's exact test. A P value < 0.05 was statistically considered significant.

4. Results

In this study, we analyzed the association of gender status and ethnic subgroups with CAD disease. As Table 2 shows, there was a significant association between male gender and CAD [OR (95% CI): 2.64 (1.09 - 3.67), P < 0.001]. No significant association was observed between CAD and ethnic subgroups in comparison with Persian (Table 2).

In the present study, the Hardy-Weinberg equilibrium analysis showed no deviation in either CAD group ($\chi 2$ = 0801, df = 1, P = 0.879), or the control group ($\chi 2$ = 0.777, df = 1, P = 0.377). Genotypic and allelic frequencies of the rs2430561 SNP at *IFNγ* gene are presented in Table 3; it is indicated that there was a significant association between genotype T/T and CAD [OR (95%CI): 1.81 (1.12 - 2.93), P = 0.021]. The results also showed that allele T was significantly associated with the CAD [OR (95%CI): 1.31 (1.04 - 1.65), P = 0.022]. To investigate the inheritance model of rs2430561 SNP at *IFNγ* gene, three models, i.e. recessive, dominant and co- dominant, were analyzed (Table 3). The result of analysis in the recessive model showed that two copies of allele T was required for increased risk [OR (95%CI): 0.62 (0.40 - 0.95), P = 0.031]. No significant association was seen in inheritance of the dominant and co-dominant models.

In this study, *IFNγ* genotypic distributions were compared between patient groups with one, two, and three involved vessels that are shown in Table 4. Results indicated that there was a significant association between the genotype T/T of *IFNγ* (+874T/A) polymorphism with CAD patients with 2VD (P = 0.030) and 3VD (P = 0.013) (Table 4).

We also compared the *IFNγ* gene expression between the CAD and control groups, as shown in figure 1. A remarkable difference was noticed between the expression level of *IFNγ* gene in the control group and CAD patients in a way that it was higher in the latter.

Table 2. The Association Analysis of Gender Status and Ethnic Subgroups with CAD Disease

Features		Patients	Control	OR (95% CI)	P value
Gender	Female	122 (40.7%)	194 (64.4%)	1	< 0.001
	Male	178 (59.3%)	107 (35.6%)	2.64 (1.09 - 3.67)	
Ethnic subgroup	Persian	220 (73.3%)	199 (66.1%)	1	
	Turkmen	41 (13.7%)	57 (18.9%)	0.65 (0.42 - 1.02)	0.072
	Sistani	39 (13%)	45 (15%)	0.78 (0.49 - 1.25)	0.339

Abbreviations: OR, odds ratio; CI, confidence interval

Table 3. The Genotype and Allele Distributions of IFNγ +874 T > A Polymorphism in the CAD Group and Control Groups

Characteristic		Patients	Control	Odds Ratio	P value
Genotypes	A/A	88 (30.3%)	108 (35.9%)	1	-
	A/T	150 (50.0%)	151 (50.2%)	1.21 (0.85 - 1.74)	0.312
	T/T	62 (20.7%)	42 (13.9%)	1.81 (1.12 - 2.93)	0.021
Alleles	A	326 (54.3%)	367 (60.5%)	1	-
	T	274 (45.7%)	235 (39.5%)	1.31 (1.04 - 1.65)	0.022
Model of inheritance	Recessive (T/T vs. A/T + A/A)			0.62 (0.40 - 0.95)	0.031
	Dominant (A/T + T/T vs. A/A)			1.34 (0.95 - 1.90)	0.098
	Co-dominant (A/T vs. A/A + TT)			1.00 (0.73 - 1.38)	1.000

Abbreviations: OR, odds ratio; CI, confidence interval

Table 4. The Genotype Distributions of IFNγ +874 T > A Polymorphism in the CAD Subgroups (Based on the Number of Vessels Involved) and Control Group. The Chi-Square Test Was Used for Analysis

Genotype	Control	One Vessel	P value	Two Vessel	P value	Three Vessel	P value
A/A	107 (35.5%)	40 (35.7%)	-	19 (21.6%)	-	30 (29.5%)	-
A/T	151(50.2%)	56 (48.7%)	1	39 (52.7%)	0.238	55 (49.1%)	0.532
T/T	43 (14.3%)	16(15.7%)	1	18 (25.7%)	0.030	27 (21.4%)	0.013

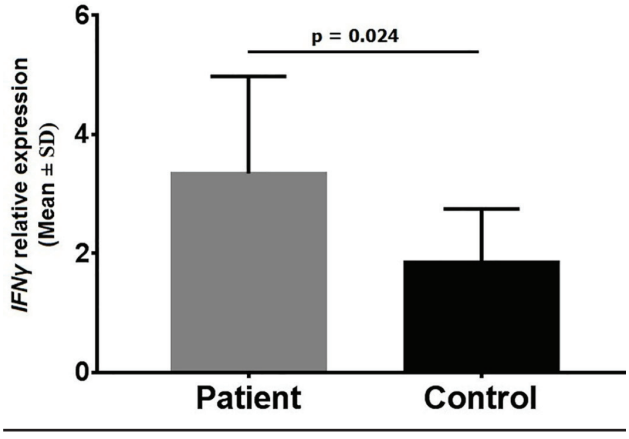

Figure 1. Relative Expression of IFN-Gamma Gene in Patient and Control Groups

5. Discussion

Several genetic studies have found the association between several cytokine genes with heart disease and its severity (5, 9). Among them, *IFNγ* significantly contributes to atherosclerosis development due to its central role in inflammation. *IFNγ* plays a role in atherosclerosis via the effect on the superoxide radicals, endothelial damage, and deposition and activation of cellular elements in the artery walls (9-11, 14). Among *IFNγ* gene polymorphisms, the SNP (+874T/A) is located on the transcription initiating site in intron 1, and it has been shown that allele T of this SNP is associated with the NF-κB binding site (15-17). NF-κB is considered as the key transcription factor in inflammatory responses involved in regulation of the immune and inflammatory genes, apoptosis and cell proliferation. It also controls the transcription of a large number of genes involved in atherosclerosis (cytokines, chemokines, adhesive molecules, acute phase proteins, apoptosis proteins, and cell proliferation regulators). NF-κB strongly affects various stages of atherosclerosis, LDL oxidation, chemotaxis and adherence. Moreover, NF-κB induces the expression of TH1 cytokines such as *IFNγ* and Interleukin-10 (23, 24). In this study, the distribution of allelic frequencies indicated that there was a significant association between allele T with CAD. In agreement with our study, previous studies have shown that a substitution A for T in +874T/A position has been associated with heart disease. Balci et al.'s study (25) showed that the genotypes of T/T and T/A of *IFNγ* (+874T/A) were significantly associated with dilated cardiomyopathy. Kim et al. (6) investigated the distribution of the SNP rs2430561 (+874T/A) and microsatellite CAn repeat rs3138557 in the *IFNγ* gene and its association with the amount of thrombosis in CAD patients. Their results, as the combination of genotypes, indicated that the CA12/TT and CA13/TT genotypes were associated with the disease. Therefore, this genotype can be considered as a predisposing factor in CAD. The analysis of *IFNγ* mRNA level showed a 1.4-fold increase in the disease group. Although few studies have investigated the *IFNγ* expression level in CAD, such as Enayati et al.'s study (26), indicating that no observed difference was seen in *IFNγ* expression level between CAD patients and controls. Our study was limitedto a small sample size; however, greater sample sizes would help to confirmed our findings. Our results needs to be put together. The differences observed in our study could be explained by differences in genetic background of the Iranian population. Further studies on other genetic polymorphisms may reveal other candidate genes involved in pathogenesis of CAD, clarifying the complex interactions between the genes and environmental factors.

In conclusion, our findings suggest that allele T had a significant relationship with the incidence and severity of CAD. Our data also showed that *IFNγ* mRNA level was increased in CAD patients in comparison with the controls. Therefore, *IFNγ* may play a role in predisposition to CAD.

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Authors' Contribution

Majid Shahbazi initiated the research program. Farnoosh Shateri genotyped the patients and controls. Reza Salehi Manzari, Marzieh Attar and Majid Shahbazi accumulated and banked all of the DNA samples. Touraj Farazmandfar carried out the statistical analyses. Majid Shahbazi supervised the project. Farnoosh Shateri and Touraj Farazmandfar wrote the paper. Ali Sharifian was the clinician involved in sample collection, clinical assessment, and data recording.

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References

1. Mehta JL, Saldeen TG, Rand K. Interactive role of infection, inflammation and traditional risk factors in atherosclerosis and coronary artery disease. *Journal of the American College of Cardiology*. 1998;**31**(6):1217-25.
2. Sitia S, Atzeni F, Sarzi-Puttini P, Di Bello V, Tomasoni L, Delfino L, *et al*. Cardiovascular involvement in systemic autoimmune diseases. *Autoimmunity reviews*. 2009;**8**(4):281-6.
3. Lusis AJ. Genetics of atherosclerosis. *Trends in genetics : TIG*. 2012;**28**(6):267-75.
4. Wong ND. Epidemiological studies of CHD and the evolution of preventive cardiology. *Nature reviews Cardiology*. 2014;**11**(5):276-89.
5. Garg PR, Saraswathy KN, Kalla AK, Sinha E, Ghosh PK. Pro-inflammatory cytokine gene polymorphisms and threat for coronary heart disease in a North Indian Agrawal population. *Gene*. 2013;**514**(1):69-74.
6. Kim HJ, Kang SW, Chung JH, Kim SJ, Choe BK. Polymorphisms of the Interferon gamma gene and coronary artery disease in the Korean population. *Molecular biology reports*. 2012;**39**(5):5425-32.
7. Libby P, Ridker PM, Hansson GK, Leducq Transatlantic Network on A. Inflammation in atherosclerosis: from pathophysiology to practice. *Journal of the American College of Cardiology*. 2009;**54**(23):2129-38.
8. Shah PK. Molecular mechanisms of plaque instability. *Current*

opinion in lipidology. 2007;**18**(5):492-9.
9. Tedgui A, Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiological reviews*. 2006;**86**(2):515-81.
10. McLaren JE, Ramji DP. Interferon gamma: a master regulator of atherosclerosis. *Cytokine & growth factor reviews*. 2009;**20**(2):125-35.
11. Voloshyna I, Littlefield MJ, Reiss AB. Atherosclerosis and interferon-gamma: new insights and therapeutic targets. *Trends in cardiovascular medicine*. 2014;**24**(1):45-51.
12. Pravica V, Perrey C, Stevens A, Lee JH, Hutchinson IV. A single nucleotide polymorphism in the first intron of the human IFN-gamma gene: absolute correlation with a polymorphic CA microsatellite marker of high IFN-gamma production. *Human immunology*. 2000;**61**(9):863-6.
13. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. *Journal of leukocyte biology*. 2004;**75**(2):163-89.
14. Harvey EJ, Ramji DP. Interferon-gamma and atherosclerosis: pro- or anti-atherogenic? *Cardiovascular research*. 2005;**67**(1):11-20.
15. Garcia-Bermudez M, Lopez-Mejias R, Gonzalez-Juanatey C, Corrales A, Robledo G, Castaneda S*, et al.* Analysis of the interferon gamma (rs2430561, +874T/A) functional gene variant in relation to the presence of cardiovascular events in rheumatoid arthritis. *PloS one*. 2012;**7**(10):e47166.
16. Manne M, Gunde S, Kondreddy RK, Thurlapati N, Tirunilai P. Association of IFN-g+874(T/A) polymorphism with female patients of age-related cataracts. *Oman journal of ophthalmology*. 2012;**5**(1):32-6.
17. Sun Y, Lu Y, Li T, Xie L, Deng Y, Li S*, et al.* Interferon Gamma +874T/A Polymorphism Increases the Risk of Hepatitis Virus-Related Diseases: Evidence from a Meta-Analysis. *PloS one*. 2015;**10**(5):e0121168.
18. Chong WP, Ip WK, Tso GH, Ng MW, Wong WH, Law HK*, et al.* The interferon gamma gene polymorphism +874 A/T is associated with severe acute respiratory syndrome. *BMC infectious diseases*. 2006;**6**:82.
19. Dai CY, Chuang WL, Hsieh MY, Lee LP, Hou NJ, Chen SC*, et al.* Polymorphism of interferon-gamma gene at position +874 and clinical characteristics of chronic hepatitis C. *Translational research : the journal of laboratory and clinical medicine*. 2006;**148**(3):128-33.
20. Zamani M, Mehri M, Kollaee A, Yenki P, Ghaffarpor M, Harirchian MH*, et al.* Pharmacogenetic Study on the Effect of Rivastigmine on PS2 and APOE Genes in Iranian Alzheimer Patients. *Dementia and geriatric cognitive disorders extra*. 2011;**1**(1):180-9.
21. Janbabai G, Oladi Z, Farazmandfar T, Taghvaei T, Naghshvar F. The prognostic impact of EGFR, ErbB2 and MET gene amplification in human gastric carcinomas as measured by quantitative Real-Time PCR. *Journal of cancer research and clinical oncology*. 2015;**141**(11):1945-52.
22. Shahbazi M, Abdolmohammadi R, Ebadi H, Farazmandfar T. Novel functional polymorphism in IGF-1 gene associated with multiple sclerosis: A new insight to MS. *Multiple sclerosis and related disorders*. 2017;**13**:33-7.
23. de Winther MP, Kanters E, Kraal G, Hofker MH. Nuclear factor kappaB signaling in atherogenesis. *Arteriosclerosis, thrombosis, and vascular biology*. 2005;**25**(5):904-14.
24. Hoesel B, Schmid JA. The complexity of NF-kappaB signaling in inflammation and cancer. *Molecular cancer*. 2013;**12**:86.
25. Balci SO, Col-Araz N, Baspinar O, Sever T, Balat A, Pehlivan S. Cytokine Gene Polymorphisms in Childhood Dilated Cardiomyopathy: Interferon- gamma, Tumor Necrosis Factor-alpha and Transforming Growth Factor - beta 1 Genes Are Associated with the Disease in Turkish Patients. *Iranian journal of pediatrics*. 2013;**23**(5):603-4.
26. Enayati S, Seifirad S, Amiri P, Abolhalaj M, Mohammad-Amoli M. Interleukin-1 beta, interferon-gamma, and tumor necrosis factor-alpha gene expression in peripheral blood mononuclear cells of patients with coronary artery disease. *ARYA atherosclerosis*. 2015;**11**(5):267-74.