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Investigation of the Association between rs4977574 A > G Polymorphism in ANRIL Gene and Coronary Artery Disease in Iranian Population

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ABSTRACT

Background: Coronary Artery Disease (CAD) is an important disease where the arteries and vessels supplying oxygen and nutrients to the heart are narrowed or blocked. Early diagnosis and recognition of CAD leads to its complete treatment. Drug therapy, angiography, coronary angioplasty, and in advanced cases, coronary artery bypass surgery restore the normal flow of blood to the heart muscle.

Objectives: The present study aimed to identify the association between rs4977574 polymorphism in ANRIL gene and CAD in Iranian patients.

Materials and Methods: Blood samples were collected from 100 subjects with positive angiography (case group) and 93 ones with negative angiography (control group). Using Taq Man Real Time PCR, the extracted DNAs from the patients and controls were genotyped for rs4977574 polymorphism in ANRIL gene (applied biosystem, USA). Then, the genotypes and clinical parameters were compared by the SPSS statistical software, version 18 (Chicago, USA). The results were compared by one-way ANOVA, simple T-test, and Chi-square test and were presented as mean ± Standard Deviation (SD). P values < 0.05 were considered as statistically significant.

Results: The results showed a significant relationship between CAD and Diastolic Blood Pressure (DBP), Body Mass Index (BMI), uric acid, Low Density Lipoprotein (LDL), cholesterol, and triglyceride. However, no significant association was observed between rs4977574 polymorphism and biochemical characteristics in the two groups. Allele frequency was AA = 22%, AG = 44%, and GG = 34% in the case group and AA = 17%, AG = 44%, and GG = 32% in the control group.

Conclusions: The present study examined the association between rs4977574 polymorphism in ANRIL gene and CAD in a population of Iranian patients. The study findings revealed no direct relationship between rs4977574 polymorphism and the disease in Iranian population.

►*Implication for health policy/practice/research/medical education:*
This research aims to determine genetic markers for diagnosis of coronary artery disease or myocardial infarction and to assess the relationship between mutation in 9P21.3 locus and coronary artery disease. The results of this research can be used by cardiologists, specialists in medical genetics, medical universities, medical centers and laboratories, and welfare organizations.

1. Background

Over the past two centuries, there has been a steady increase in the frequency of Coronary Artery Disease (CAD). Currently, CAD is the main contributor to global death and disability. CAD accounts for 50% of total mortality in developed countries and more than 25% of deaths in developing countries (1). CAD is also the leading cause of death in Iran. Scientific reports have suggested excessive smoking, physical inactivity, lack of exercise, hypertension, and dyslipidemia as the most important

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factors contributing to heart disease (2). Recent studies have indicated that CAD usually occurs between the ages of 40 and 55 years; however, the molecular mechanisms responsible for the disease have yet to be identified. It appears that changes in the expression pattern of specific genes and proteins increases a person's susceptibility to the disease. Moreover, in addition to inherited and genetic risk factors, environmental factors influence the disease prevalence (3). CAD represents a prototype of multi-factorial disease, with contributions from life style, genetic, and environmental factors, all being used to demonstrate clinical symptoms. In the past decades, the pathophysiology of CAD has been defined as endothelial dysfunction, infiltration of inflammatory cells into the blood vessel wall, and coronary artery plaque formation. This subsequently leads to vascular fissure or rupture to develop thrombosis, which underlies Myocardial Infarction (MI) and coronary syndromes (4).

Atherosclerosis is the most common cause of CAD. The endothelium encompasses crucial functions in vascular regulation and is a layer of cells that lines the inner surface of blood vessels to form an interface between blood in the lumen and the vessel wall. Oxidized LDL-C particles, elevated plasma homocysteine levels, free radicals, and infectious microorganisms cause endothelial dysfunction followed by atherosclerosis (5). The endothelium initiates self-resorting process by attracting T lymphocytes, monocytes, and platelets to the location of injury. When the self-restorative mechanism fails, the endothelium becomes permeable, triggering lymphocyte and monocytes migration into the intima and attracts oxidized LDL-C particles. These particles are responsible for formation of visible atherosclerotic plaque lesions that are made up of fat. Furthermore, monocytes that later differentiate to macrophages and accumulation of macrophages recruit these oxidized LDL-C particles to the forming plaques. Media to intima migration of smooth muscle cells from the artery wall also participate in this process. Subsequently, monocytes and macrophages involved in the reaction die by apoptosis. This directs the formation of a fat-rich center with fibrous cap, generating atherosclerosis plaques (6). In the presence of increasing intraluminal pressure, the plaques become vulnerable to rupture or cracking. Moreover, damage and breaking of the vasa vasorum increases plaque rupture. After plaque rupture, its contents are released into the lumen of vessel and the result is formation of a thrombus (blood clot). Activation of this process could also be reflected on detachment of the endothelium layer of atherosclerotic plaque. Now, it is clear that formation of clot on ruptured atherosclerotic plaque or the plaque of which the endothelium layer has been detached are responsible for acute MI, unstable angina, and many cases of sudden cardiac deaths. In summary, acute coronary syndrome refers to this stated group of symptoms (7, 8).

Based on the Genome-Wide Association Studies (GWAS), one of the important loci associated with CAD is 9p21 locus, which has been introduced by deCODE database. ANRIL gene has also been named CDKN2B-AS in the National Center for Biotechnology Information (NCBI) database (9, 10). This gene consists of 19 exons with 126.3 kb length and one of its coding RNAs has 3834 bp (11). ANIRL is an antisense long non-coding RNA (lncRNAs) mapped on 9p21 locus (12). The gene contributes to epigenetic inhibition of transcription by regulating polycomb proteins (13). According to the published literature, this gene is involved in developing cardiovascular diseases, diabetes, Alzheimer, and several types of cancer (14-16). Maintaining genome integrity is crucial for proper functioning and survival of all living organisms. Human cells use fast and efficient responses to eliminate DNA damages. DNA damage response engages a complex network of cellular processes that mediate detection and repair of the damage. lncRNAs are of a new class of regulatory RNAs that may play an important role in this process. Studies have recently recognized a great number of lncRNAs in mammalian transcriptomes. However, the underlying mechanisms in regulation and function of lncRNAs in response to DNA damage are not clear. A study in 2013 reported that ANRIL was a specific lncRNA (17). It serves as a genetic risk factor for CAD and periodontitis (PD). Additionally, it is independently linked to other types of metabolism and immune disorders and many types of cancer (18). Congrains et al. (2013) showed that ANRIL connected to CBX7 and SUZ12 proteins to methylate histon 3 in nucleus and silence CDKN2A / B locus. A decrease in CDKN2A and CDKN2B product levels induced different effects, particularly in White Blood Cells (WBC) and Vascular Smooth Muscle Cells (VSCM), including changes in aging, cell death, and proliferation. Increased proliferation of smooth muscle cells and participating macrophages promotes the development of atherosclerotic plaques in which, smooth muscle cells form an atheroma and WBCs are responsible for formation of atherosclerotic plaque core. Macrophages and VSCM are released from atherosclerotic lesions. Furthermore, chemokines mediate an influx of monocytes to the site of inflammation. Macrophage migration increases the expression of cell adhesion molecules and causes rapid relocation of the inflammatory cells into the intima that is a cause of atherosclerosis and MI (18). ANIRL mediates increased cell proliferation and adhesion and decreased apoptosis through unknown molecular mechanisms, all being essential contributors to angiogenesis process (19). Association studies aim at evaluation of genetic association or genotype frequencies in a population.

2. Objectives

The present study also aims to identify the association between rs4977574 polymorphism in ANIRL gene and CAD incidence in a population of Iranian patients.

3. Materials and Methods

Blood samples were collected from 100 patients older than 40 with positive angiography. This was based on the indicators of World Health Organization (WHO, 2007 Geneva) which are confirmed by coronary angiography and left ventriculography with at least one coronary artery with stenosis > 50%. In addition, 93 subjects with negative angiography were selected as the control group for further analysis. The control group had no previous history of cardiac infarction, familial CAD, and risk factors such

as smoking and diabetes. They were characterized by above 40 years of age with negative angiography result (≤ 50% coronary artery blockage). All the procedures were performed in accordance with the ethical standards of the responsible committee on human experimentation as well as with 1975 Declaration of Helsinki revised in 2008. The study was also approved by the local Ethics Committee. Besides, were obtained from all the participants. The patients with history of past angiography, pregnant and lactating women, patients suffering from heart, systemic, and kidney diseases, drug users, and those with genetic diseases influencing heart disease were excluded from the study. All the participants completed a standardized questionnaire, including information about name, occupation, age, ethnicity, family history of MI and stroke, and past medical history.

3.1. Laboratory Investigation

In this study, 5 mL peripheral blood was taken from the subjects' femoral vessels and were stored in EDTA containing tubes for genetic tests. The samples were then kept at -20°C and the sera were used for paraclinical tests, such as measuring Triglyceride (TG), Low Density Lipoprotein (LDL), High Density Lipoprotein (HDL), cholesterol, uric acid, and Fasting Blood Sugar (FBS). The participants' Diastolic Blood Pressure (DBP), Systolic Blood Pressure (SBP), Body Mass Index (BMI), age, gender, and height were evaluated, as well.

3.2. DNA Extraction and Determination of Genotypes

High pure PCR template preparation kit (Roche, USA) was used to extract DNA according to the manufacturer's instructions. Quality and quantity of the extracted DNA was evaluated by spectrophotometry (Nanodrop, Thermo 2000, USA) and 1% agarose gel electrophoresis. Based on the previous studies and NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/), rs4977547 polymorphism in ANRIL gene was selected for further analysis in CAD. Predesigned primers, probes, and master mix were ordered to ABI (applied biosystem, USA). The types of SNPs were determined by TaqMan SNP genotyping assays. PCR amplifications were performed by 96-well plate Roche Light Cycler 96 Real-Time PCR (Germany). PCR for the polymorphism of interest was conducted for each sample in a total volume of 25 µL, including 12.5 µL of real time premix, 10.25 µL of ddH2O, 1.25 µL of forward and reverse primers (10 pmol/µL), and 1 µL of genomic DNA. Amplification reaction consisted of an initial 10 min denaturation at 95ºC followed by 40 cycles of denaturation at 92ºC for 15 sec, and annealing and extension at 60ºC for 1 min. The genotypes were then analyzed by SDS software, version 1.3 (Applied Biosystems). It should be mentioned that genotyping was performed using TaqMan® SNP Genotyping Assay (Figure 1).

3.3. Statistical Analysis

Quantitative variables were presented as mean ± standard deviation, while qualitative ones were expressed as percentage. All the statistical analyses were performed using the SPSS statistical software, version 18 (Chicago, USA). Frequency of biochemical, environmental, and personal risk factors was statistically described in the case and control populations. Student's T-test was used to assess the significance of differences between the case and control groups regarding gene expression. Moreover, Chi-square test was used to compare the qualitative variables, and ANOVA was utilized to examine the possible changes in ANIRL gene expression levels in the presence of each

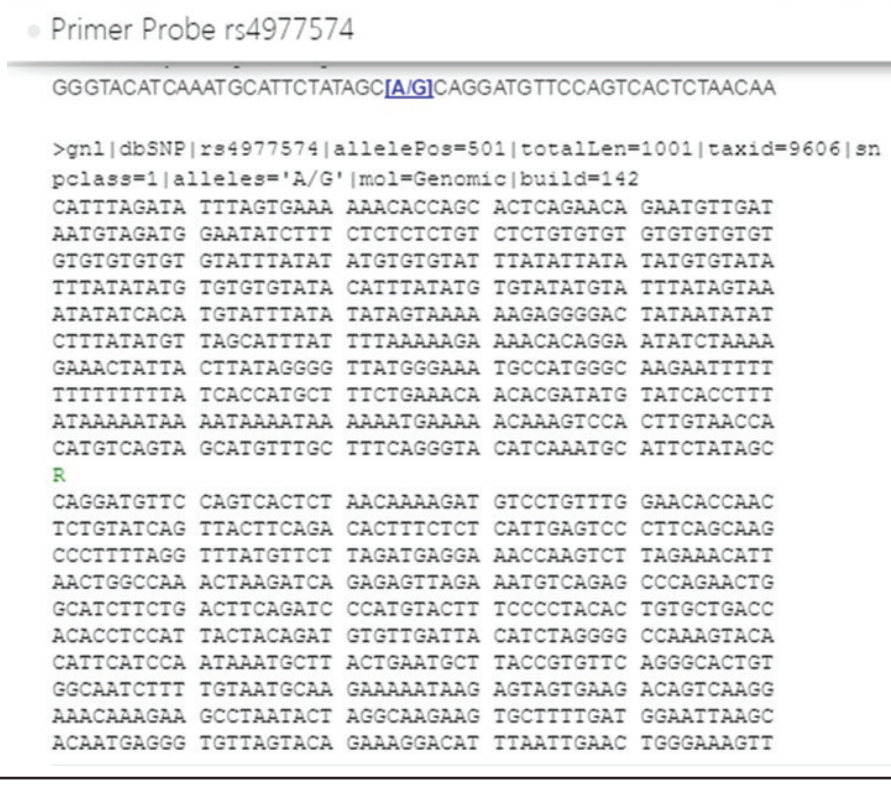

Figure 1. Genotyping Was Performed Using TaqMan® SNP Genotyping Assay and Showed the Forward and Reverse Sequence of the Primer Probe

genotype. P values < 0.05 were considered as statistically significant.

4. Results

In this study, the case group included 66 males (66%) and 34 females (34%) and the control group included 43 males (46%) and 50 females (53%). The two groups were compared regarding clinical and biochemical parameters and the results have been summarized in Table 1. Accordingly, a significant difference was found between the two groups concerning distribution of male participants, height, weight, DBP, BMI, LDL, cholesterol, and TG (P < 0.05). rs4977574 polymorphism in ANRIL gene was also studied in angio-positive (case) and angio-negative individuals (controls). According to the results, the control group included 17 individuals with allele AA, 44 ones with allele AG, and 32 ones with allele GG. In the case group, on the other hand, 22, 44, and 34 subjects had AA, AG, and GG alleles, respectively (Table 2). The study results revealed no significant difference between the control and patient groups in this regard (P > 0.05). Also, no significant difference was observed between the patients and controls with respect to rs4977574 polymorphism genotypes/alleles and clinical features (Table 3).

Table 1. Clinical and Biochemical Characteristics of the Patients in the Case Group (Angio-Positive) and the Control Group (Angio-Negative)

Baseline Characteristics	Case (Male/Female) N = 100	Control (Male/Female) N = 93	P value
Age (years)	63.55 ± 9.89	61.47 ± 10.97	0.165
Male (%)	66%	43%	0.016
Height (cm)	165.39 ± 10.76	161.39 ± 8.86	0.005
Weight (kg)	76.81 ± 13.71	69.77 ± 12.96	0.001
BMI (kg/m²)	28.02 ± 5.28	23.32 ± 4.68	0.017
SBP (mm Hg)	123.24 ± 19.01	122.80 ± 19.02	0.87
DBP (mm Hg)	81.35 ± 14.15	73.60 ± 12.91	0.001
TC (mg/dL)	174.54 ± 44.49	192.85 ± 27.75	0.001
TG (mg/dL)	142.92 ± 74.35	181 ± 54.20	0.001
FBS (mg/dL)	114.46 ± 87.84	122.62 ± 40.07	0.40
HDL (mg/dL)	41.82 ± 10.18	41.80 ± 5.92	0.98
LDL (mg/dL)	98.04 ± 36.24	115.10 ± 23.61	0.001
Uric acid (mg/dL)	6.10 ± 1.40	5.43 ± 0.73	0.001

Abbreviations: BMI, Body mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; TG, Triglyceride; FBS, Fasting blood sugar; HDL, High density lipoprotein cholesterol; LDL, Low density lipoprotein cholesterol; TC, Total cholesterol

Table 2. Genotype and Allelic Frequencies of rs4977574 Polymorphism in ANRIL Gene in the Case and Control Groups

Model	Genotypes/Allele	Cases N = 100	Controls N = 93	OR (95% CI)	P value
Co-dominant	A/A	22	17	1 (reference)	
	A/G	44	44	0.77 (0.36 - 1.65)	0.50
	G/G	34	32	0.82 (0.37 - 1.81)	0.62
Dominant	A/A	22	17	1 (reference)	
	A/G-G/G	78	76	0.74 (0.39 - 1.60)	0.52
Recessive	A/A-A/G	66	61	1 (reference)	
	G/G	34	32	0.98 (0.54 - 1.78)	0.05
Over-dominant	A/A-G/G	56	49	1 (reference)	
	A/G	44	44	0.87 (0.5 - 1.54)	0.64

Table 3. Comparison of Clinical Features Based on rs4977574 Genotypes/Alleles in the Case and Control Groups

Clinical Features	Case (Male/Female)				Controls (Male/Female)			
Genotypes/Allele	AA	AG	GG	P value	AA	AG	GG	P value
Age (year)	62.61 ± 9.70	63.46 ± 11.12	64.28 ± 8.39	0.83	60.05 ± 11.25	61.18 ± 11.86	62.50 ± 11.28	0.75
Male (%)	11%	30%	25%	0.18	5%	18.2%	17%	0.34
Height (cm)	161.72 ± 15.63	166.54 ± 8.80	166.26 ± 8.90	0.19	160.82 ± 9.15	161.47 ± 9.11	160.38 ± 8.82	0.87
Weight (kg)	78.90 ± 12.77	78.31 ± 15.53	73.50 ± 11.34	0.22	66.94 ± 14.55	71.60 ± 12.10	67.19 ± 13.04	0.24
BMI (kg/m²)	29.28 ± 5.15	28.25 ± 5.53	26.90 ± 4.96	0.24	25.41 ± 4.71	27.04 ± 4.79	25.62 ± 4.58	0.31
TC (mg/dL)	168.00 ± 40.50	179.50 ± 46.71	172.12 ± 44.84	0.60	190.77 ± 22.28	194.50 ± 27.40	192.38 ± 33.75	0.89
TG (mg/dL)	153.71 ± 67.85	142.02 ± 84.97	135.00 ± 61.38	0.70	191.34 ± 62.66	186.36 ± 61.08	170.89 ± 44.34	0.37
FBS (mg/dL)	143.63 ± 17.2	108.45 ± 31.64	100.02 ± 15.33	0.18	122.56 ± 42.69	127.72 ± 45.09	119.23 ± 33.74	0.66
HDL (mg/dL)	40.76 ± 9.12	42.10 ± 12.70	42.28 ± 5.88	0.86	39.29 ± 5.05	41.36 ± 6.21	43.45 ± 6.02	0.064
LDL (mg/dL)	93.33 ± 29.31	101.00 ± 38.81	97.28 ± 38.11	0.73	114.41 ± 20.02	115.46 ± 25.31	115.80 ± 25.71	0.98
SBP (mmHg)	122.22 ± 17.50	122.40 ± 20.58	124.97 ± 18.25	0.81	121.76 ± 21.57	122.50 ± 17.9	125.31 ± 20.47	0.77
DBP (mmHg)	81.31 ± 9.98	81.84 ± 17.45	80.73 ± 11.83	0.94	71.76 ± 11.85	74.09 ± 14.19	75.00 ± 12.18	0.73

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; FBS, fasting blood sugar; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; TC, total cholesterol

5. Discussion

CAD is a complex genetic disease developed as a result of interaction among a number of genetic and environmental factors. A useful method to investigate potential genes in such multifactorial diseases is comparing allelic distributions and genotyping of two patient and healthy groups. The present study assessed the contribution of genetic variability of ANIRL gene to development of CAD. In the recent years, ANRIL gene has been introduced as an lncRNA that, by unknown molecular mechanisms, promotes gene expression, cell proliferation, and adhesion and suppresses apoptosis. ANIRL gene expression leads to INK4 production, implicated in severity of atherosclerosis in CAD. Despite Iranian population's high potential risk of cardiovascular diseases, the associations between CAD and variants at 9p21.3 have not been well investigated.

ANIRL gene includes several polymorphisms responsible for CAD development, but rs4977574 polymorphism could increase the risk of disease occurrence. Because of ANIRL function in angiogenesis processes and previous studies confirming its role in CAD, this study examined the ANIRL rs4977574 polymorphism in Iranian patients. However, the polymorphism was not significantly associated with CAD among the Iranian subjects. Similar results were also obtained in the reports with larger number of cases in 2014, such as the studies performed by Bai et al. and Lee et al. on Chinese patients (19, 20). Nonetheless, controversial results have been reported in this regard up to now. For instance, a GWA study by Lee et al. (2013) genotyped more than 2000 Korean CAD cases and identified three critical loci associated with the disease, including rs4977574. They used SNP Array chips for a relatively large sample size that may explain the discrepancy found with our results (21). Another association study of the 9p21.3 locus with risk of MI was performed by Saleheen et al. (2010) on Pakistani patients. They assessed several SNPs among which, rs4977574 was significantly associated with MI. The presence of ethnic-mediated diversities in disease susceptibility, differences in case backgrounds, and inclusion of early-onset MI patients may clarify the reason for dissimilar results from our study (22). The studies conducted by Sakalar et al. on Turkish population, River et al. on Canadian population, and Wang et al. on Chinese patients also showed a significant association between this polymorphism and severity of CAD occurrence, being inconsistent with the results of the present study (23-26).

The findings of the current study indicated no significant association between rs4977574 polymorphism and clinical (age, gender, height, and weight) and biochemical (FBS, BMI, HDL, LDL, TG, cholesterol, and uric acid) parameters in the case and control groups. In the same line, a GWA study in an American population demonstrated no significant association between this polymorphism and blood pressure and lipid level (27). However, the study by Hamrefors et al. (2014) showed a significant association between rs4977574 polymorphism and BMI, age, sex, and SBP, which is inconsistent with the results of the present study. Nevertheless, they could not demonstrate a significant association between rs4977574 and smoking (28). Furthermore, significant relationships were detected between atherosclerosis and CAD, and biochemical variables including LDL, cholesterol, BMI, TG, and uric acid. Three independent studies have also shown that atherosclerosis and CAD were significantly associated with TG and cholesterol (29-31). The results of these investigations were in agreement with our results, suggesting a significant association between CAD and uric acid level (32, 33). Similarly, Achari et al. (2003) and Holmes et al. (1981) reported a significant association between CAD and high levels of LDL and cholesterol, low HDL levels, diabetes, and blood pressure (34, 35).

The difference between the results of the current study and other researches might be attributed to the fact that we did not investigate other polymorphisms in our study. However, given that the sample size of this research was smaller than that of other similar studies, this difference could be partially justified. The discrepancies between the results of studies could also be related to racial differences and using diverse assessment techniques. Moreover, the results could be affected by the study design, parameters involved in atherosclerosis and CAD, and study subjects' age distribution. Additionally, interaction with other genes involved in etiology of atherosclerosis and CAD and environmental factors may partially explain the inconsistency among the results. It should, too, be noted that this study was performed for the first time on Iranian patients to find a possible association between rs4977574 polymorphism in ANIRL gene and CAD. Thus, to determine the molecular mechanisms by which ANIRL gene affects the development and severity of CAD, further comprehensive studies should be performed on larger sample sizes.

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Authors' Contribution

Study concept and design, Critical revision of the manuscript for important intellectual content: Sayyed Mohammad Hossein Ghaderian. Analysis and interpretation of data, Drafting of the manuscript, Statistical analysis: Sareh Sepahvand Hossein Beigi. Study supervision: Abbas Doosti

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