Association of the rs10757274 SNP with coronary artery disease in a small group of a Pakistani population

Syed Kashif Nawaz, Aasma Noreen, Asima Rani, Memoona Yousaf, Muhammad Arshad

Department of Biological Sciences, University of Sargodha; Sargodha-Pakistan

ABSTRACT

**Objective:** The present study aimed to investigate the association between the rs10757274 SNP (present on locus 9p21 in the gene for CDKN2B-AS1) and coronary artery disease (CAD) in a local population of Pakistan.

**Methods:** It was a case-control study. An allele-specific PCR-based strategy was used for the identification of genotypes. A total of 350 samples were used for the investigation, out of which 220 samples were CAD patients and 130 samples were normal healthy individuals. Effects of parameters, like family history of CAD, smoking, presence of diabetes, and hypertension, in changing the chances of CAD were studied. Odds ratio was estimated with 95% confidence interval.

**Results:** A strong association was observed between CAD and factors, like smoking (OR: 1.666; 95% CI: 1.042-2.664), presence of hypertension (OR: 26.55; 95% CI: 15.95-44.20), diabetes (OR: 3.009; 95% CI: 1.841-4.920), and family history of CAD (OR: 4.9; 95% CI: 2.965-8.099). Results for the association between the genotype on the basis of rs10757274 showed a strong association between the GG genotype and the occurrence of CAD (OR: 9.603; 95% CI: 5.746-16.05).

**Conclusion:** The present results suggest the importance of the 9p21 locus in modulating the chances of CAD. (Anatol J Cardiol 2015; 15: 709-15)

**Keywords:** CAD, genotype, rs10757274, Pakistan, hypertension, familial CAD, smoking, diabetes, SNP

Introduction

Coronary artery disease and its sequelae are affecting millions of people all over the world. The increased deaths due to CAD in the close relatives of CAD patients provide strong evidence showing the role of genetic factors in the onset of CAD (1). The human genome exhibits far more variability than expected (2, 3), and DNA sequence variations are present in more than 44% of annotated genes (3). According to an estimate, more than 40% of inter-individual variability is based on genetics (4). Many studies have investigated the association of specific parts of the genome with the onset of CAD, considering its pathophysiological contributing factors (arterial wall, myocardium, neurohormones, coagulation factors, etc) (5, 6), but the difficulty is always being faced while defining the specific genetic contribution to CAD due to inter-individual variations and polygenic inheritance (7). Additionally, the existence of a relationship between the related genes and the impact of the environment on gene expression also produce a complicated situation for understanding the genetic etiology of CAD.

The Wellcome Trust Case Control Consortium (WTCCC) reported one of the very important GWAS array analyses of CAD patients. Affymetrix 5.0 arrays, containing 5 million SNPs across the genome, were used, and the 9p21 locus was found to be associated with CAD (8). How this risk locus increases the susceptibility is still not clearly defined. The risk locus spans 58 kb and is without annotated genes, such as cyclin-dependent kinase inhibitors (CDKN2A and CDKN2B) and 5'-methylthioadenosine phosphorylase (MTAP). The expression of CDKN2B is increased due to TGF-β, which has its role in atherosclerosis (9). Through the mapping of expression sequence tags, it has been shown that the association region of CAD is overlapped by a non-coding RNA gene, ANRIL (10). The expression of ANRIL is high in cells that are involved in atherosclerosis, like vascular tissues, smooth muscle cells, and monocytes (11). ANRIL is thought to be involved in the expression regulation of neighboring protein-coding genes, like MTAP and CDKN2A and CDKN2B. ANRIL is also involved in the progression of atherosclerosis by playing a role in vascular remodeling, thrombogenesis, and...
plaque stability (12). The studies that have been conducted to know how the 9p21.3 locus affects the expression of various transcripts of ANRIL have mostly relied on data taken from circulating blood cells, and the results were contrasting (13, 14). So, the association of 9p21.3 with gene expression in atherosclerotic tissues needs to be defined.

Through several GWASs, it has been demonstrated that the risk allele of SNPs in the 9p21 locus increases the susceptibility to CAD. This locus association is independent of all other risk phenotypes, such as smoking, obesity, age, gender, hypertension, and cholesterol level. It is also notable that 9p21.3 is expressed in various phenotypes, and this is the reason for its association with other diseases, like arterial stiffness (15), myocardial infarction (16), ischemic stroke (17, 18), glioma (19), and aortic aneurysm (20).

The reported SNPs with a CAD association are being investigated in different populations all over the world and also in association with other diseases to learn about not only the genetic etiology of CAD but also the pathophysiological pathway of contributing risk factors. The present research work investigated the association of an SNP, rs10757274, in CAD patients of a local population of Pakistan. This is a kind of independent association study and an initial step to study the reported genetic variants of CAD. The findings of this study will be helpful in understanding the genetic predisposition of CAD in a local population of Pakistan.

Methods

Study design
It was a case control study.

Study subjects
All procedures were in compliance with the Declaration of Helsinki. The patients in this study were enrolled from Civil Hospitals, Sargodha, and people, mostly from rural areas, approach these hospitals, even when the conditions get worse. The research work started after approval of the study protocol by the Advance Studies and Research Board, University of Sargodha. Prior permission was also taken from the Ethical Committee of the University of Sargodha. The subjects included in this study ranged from 12-65 years. District civil hospitals of the Sargodha division were visited for the collection of data and samples. All participants belonged to different areas of the Sargodha division, Pakistan. Written approval for using DNA and other obtained data for research purposes was collected from each participant. Individuals with a history of hepatitis and AIDS were excluded from both categories. Antigen antibody reaction based devices were used for the detection of HCV, HBSAg and HIV. Two study groups were differentiated for the analysis of data on the basis of the presence of disease. One group contained CAD patients, and the other group consisted of healthy volunteers. The CAD patients who were included in the studies were angiographically confirmed by hospitals. The individuals who were apparently healthy (without CAD symptoms) were placed in the control group. The group of CAD patients was named CAD patients. The data for smoking, hypertension, and diabetes were taken for all participants. CAD family history was also recorded. The participants having first-degree relatives with CAD were considered to have a positive family history for CAD. If the blood sugar level of a sample was 120 mg/dL (at fasting) (21), then the donor was known as a diabetic patient. Individuals with consistent who had a diastolic pressure value of more than 90 and systolic pressure of more than 140 were designated hypertensive persons (21). Blood pressure readings were taken for three different timings. A person who smoked at present and had a smoking history of more than 5 years was considered a smoker.

Sample collection
The sample collection was performed during September 2012 to February 2013. 3 mL of blood from peripheral circulation was drawn by puncturing the cubital vein. Tubes containing EDTA (BD, USA) were used for the collection of whole blood. An ice box with a temperature of 0°C to -2°C was used for the transportation of blood samples. In the laboratory, these samples were stored at -20°C for further analyses.

Genetic analysis of rs10757274
The major techniques used in the genetic analysis included genomic DNA isolation, agarose gel electrophoresis, and allele-specific PCR. The standard protocol of the Vivantis DNA extraction kit (Cat# GF-BD-100) was used for the isolation of genomic DNA. Amplification of the required genomic sequence was carried out in 200-µL PCR tubes using an allele-specific PCR strategy. The volume of the reaction mixture was 50 µL. PCR Master Mix (Invitrogen #12532-016) was used for the amplification. The primers used in the study were synthesized by Invitrogen, USA. The primer sequences are shown in Table 1.

Amplification of DNA was performed in a thermal cycler (BIOTEC TECHNOLOGY CO., LTD., TC-XP-G, China). The program used for the PCR included an initial denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 30 seconds, 30 cycles of annealing at 56.4°C for 30 seconds, and 30 cycles of extension at 68°C for 30 seconds. The final extension was accomplished at 68°C for 12 minutes. A gel apparatus (Thermo Scientific, model no. ECX001L2) was used for the agarose gel electrophoresis. PCR products were detected using a 2% agarose gel, and bands were visualized under a UV transilluminator. The presence or absence of PCR products provided the informa-

| Table 1. Primers used for amplification |
|----------------------------------------|----------------------------------|
| Primers                  | Sequences                      |
| Forward 1 (F1)           | 5CAAATCTAAGCTGAGTGTA3          |
| Forward 2 (F2)           | 5CAAATCTAAGCTGAGTGTA3          |
| Reverse primer (R)       | 5CTTAGTGCAATGTAGGTA3           |
Table 2. Baseline characteristics

| Characteristics | CAD patients (n=220) | Control (n=130) | Total (n=350) | P |
|-----------------|----------------------|----------------|---------------|---|
| Age, yearsa     | 50.94±9.107          | 49.98±10.483   | 50.58±9.637   | P=0.368 |
| Gender, Male (% age)b | 98 (44.54%) | 65 (50%) | 163 (46.57%) | P=0.322 |
| Smokers, Yes (% age)c | 92 (41.81%) | 38 (29.2%) | 130 (37.1%) | P=0.018* |
| Diabetic, Yes (% age)d | 107 (48.61%) | 32 (24.61%) | 139 (39.7%) | P<0.001** |
| Hypertensive, Yes (% age)e | 187 (85%) | 42 (32.31%) | 229 (65.41%) | P<0.001** |
| Family history, Yes (% age)f | 131 (59.54%) | 29 (22.31%) | 160 (45.7%) | P<0.001** |

a Student’s t-test was used for the comparison of groups. b Chi-square test for the comparison of groups. *p<0.05. **p<0.01

Table 3. Genotype and allele frequency

| ALLELE | CAD (n=220) | Control (n=130) | Total (n=350) |
|--------|------------|----------------|---------------|
| AA     | 16 (7.27%) | 20 (15.38%) | 36 (10.28%) |
| GG     | 175 (79.54%) | 36 (27.69%) | 211 (60.28%) |
| AG     | 29 (13.18%) | 74 (56.92%) | 103 (29.42%) |
| A      | 0.14       | 0.44          | 0.25          |
| G      | 0.86       | 0.56          | 0.75          |
| HWE (P) | 44.17 (P<0.001) | 3.16 (P<0.05) | 16.21 (P<0.001) |

CAD - coronary artery disease; HWE - Hardy Weinberg equilibrium; (p), statistical P value; <, value less than

Table 4. Association of genotype with CAD

| Genotypes | AA (n=36) | GG (n=211) | AG (n=103) |
|-----------|----------|-----------|------------|
| Control   | 20       | 36        | 74         |
| OR        | 0.431    | 10.15     | 0.114      |
| (95% CI)  | (0.214-0.866) | (6.128-16.82) | (0.068-0.193) |

CAD - coronary artery disease; OR - odds ratio; χ² - Chi-square; 95% CI - 95% confidence interval

Results

The present study was designed for the investigation of the association between genetic variants for rs10757274 and CAD. Baseline characteristics of the samples are shown in Table 2.

There was no significant difference in age between the two categories (p>0.05). The groups were similar on the basis of age and percentage of gender (p>0.05). A difference between groups was noticed for diabetes (p<0.01), hypertension (p<0.01), and family history (p<0.01). Smoking habit was also considered significantly different in these groups (p<0.05). Experiments for the optimization of PCR indicate that the appropriate annealing temperature was 56.4°C. On the basis of allele-specific PCR, the genotype for each sample was detected. Table 3 shows the genotype frequency in the CAD and control groups, frequencies of G and A alleles in both groups, and the results of Hardy-Weinberg equilibrium (HWE). The GG genotype was higher in CAD patients, and its occurrence was also higher in the whole population as compared to other genotypes. The AG genotype showed a greater percentage in the control group. The AA genotype was less in both groups. The results indicate that G allele frequency was higher in both groups and also in the population. The A allele was comparatively smaller in CAD patients and showed a small frequency in the population. Results for the HWE estimation show that the allele frequencies in CAD patients were deviant from HWE.

Table 4 describes the results for the association between the rs10757274 polymorphism and CAD (p<0.01). It was noticed that a strong association was present between the polymorphism and coronary artery disease. The same table also illustrates the association between genotypes (AA, AG, GG) and CAD. It was found that the AA genotype acts as a weak protective factor. Its presence decreased the chances of CAD 0.431 times (OR: 0.431, 95% CI: 0.214-0.866). Similarly, the GG genotype increased the risk of CAD 10.154 times (OR: 10.154, 95% CI: 6.128-16.823). Surprisingly, the AG genotype also showed protective effects. Its presence decreased the chances of CAD 0.114 times. Table 5 shows the results for the association between different factors (smoking, diabetes, hypertension, family history of CAD) and CAD. It was observed that smoking showed a marginal association with CAD (p<0.05). Smokers had a 1.74-fold increased chance of CAD as compared to non-smokers.

The association was found to be significant (p<0.01) between diabetes with CAD. Diabetes increased the chances of CAD by 2.899. The results of the association between hypertension and CAD describe that there exists a strong association between hypertension and CAD (p<0.01). Hypertensive individuals had an 11.87 times greater risk of developing CAD as compared to normotensive persons. The results for the association of family history with CAD showed a strong association of family history with CAD (p<0.01). CAD family history increased the onset of CAD by 5.126 times as compared to that of persons with no family history of CAD.

Statistical analysis

Hardy-Weinberg equilibrium was analyzed using the chi-square test. Gene frequencies, allele frequencies, and differences in genotype and allele frequencies between different groups were also examined. Chi-square test and other non-parametric tests were applied by SPSS® software, version 18 for Windows (SPSS Inc., Chicago Illinois, USA) and MINITAB Student Version, release 12 for Windows (Minitab Inc.) Odds ratios were calculated using an online calculator (22).
Table 5. Effect of different factors on CAD occurrence

| Factors                        | CAD (n=220) | Control (n=130) | OR (95% CI) | Chi-square (P-value) |
|-------------------------------|-------------|-----------------|-------------|----------------------|
| Smoking                       | Yes         | 92              | 38          | 1.74 (1.094-2.765)   | 5.546 (P=0.018) |
|                               | No          | 128             | 92          |                      |                     |
| Diabetes                      | Yes         | 107             | 32          | 2.899 (1.796-4.680)  | 19.69 (P<0.001)    |
|                               | No          | 113             | 98          |                      |                     |
| Hypertension                  | Yes         | 187             | 42          | 11.87 (7.047-20.00)  | 100.3 (P<0.001)    |
|                               | No          | 33              | 88          |                      |                     |
| Family history of CAD         | Yes         | 131             | 29          | 5.126 (3.131-8.392)  | 45.65 (P<0.001)    |
|                               | No          | 89              | 101         |                      |                     |

95% CI - 95% confidence interval. CAD - coronary artery disease
Multinomial logistic regression was performed using SPSS

Table 6. Effect of polymorphism on CAD occurrence considering various covariates

| Effects of polymorphism | Odds ratio | 95% Confidence interval |  
|-------------------------|------------|-------------------------|
|                         | Lower bound| Upper bound              |
| AA                      | 1.331      | 1.186-1.494             |
| GG                      | 1.222      | 1.126-1.327             |
| AG                      | 1.612      | 1.430-1.817             |

Smoking, hypertension, diabetes, and family history were adjusted as covariates.

Discussion

Our study aimed to investigate the association of the rs10757274 SNP with CAD. The strategy that was adopted for the identification of genotypes was based on allele-specific PCR. rs10757274 is one of the members of a group of SNPs in the 9p21.3 locus, which has been found to be in a strong association with CAD in various studies. The chromosomal region having this SNP includes genes for cyclin-dependent kinase inhibitors, such as CDKN2A, including its alternative reading frame (ARF) transcript, and CDKN2B. Recently, a non-coding RNA gene, CDKN2BAS, or ANRIL (antisense noncoding RNA in the INK4 locus), was also discovered. The polymorphism in this SNP affects the differential expression in ANRIL and neighboring genes (CDKN2A and CDKN2B). Transcripts of ANRIL have also been observed to be associated with phenotypes of many diseases (22).

The mortality rate is high due to CAD among men and women all over the world. Women develop disease after 10 years of men. The reason is not clearly understood. Some CAD risk factors have been found to affect the onset of disease in men and women separately. The high intensity of risk factors (like hypertension, diabetes, and physical activity) at a younger age in males increases the chances of CAD (23). The CAD patients included in the present study were from both sexes. The percentage of women patients was high (55.5%). Our most rural areas are tied in various customs and traditions based on gender discrimination. Women in these areas are overburdened and have to face serious health issues. Poor living standards, interfamily marriages, and the absence of health care are making the women more susceptible to CAD and other heritable and non-heritable diseases. These factors may be the reason of the higher percentage of women CAD patients as compared to men.

Smoking is considered a major cardiovascular risk factor. Sometimes, it is taken as a habit, but in fact, nicotine changes the neurophysiology, and the smoker feels comfort with inhaled nicotine. A smoker with nicotine also inhales chemicals that influence vascular dysfunction, oxidation of lipids, and thrombosis. Smoking results in vascular damage in the case of both active and passive smoking. Cessation of smoking significantly reduces the chances of readmission to hospitals, progression of disease, and mortality in CAD patients (24). Smoking in association with CAD is considered a risk factor for the onset of disease, because oxidative stress is involved in the progression of CAD. Cigarette smoke causes oxidative stress. In young adult (age 32-35) patients with CAD with heavy smoking, oxidative stress indices are significantly associated (0.01) with CAD severity (25). Our results strengthen the information about the causative role of smoking for CAD (p=0.018). Wilson and his colleagues have also reported that treatment of cardiovascular risk factors, like smoking and hypertension, plays a critical role in the delayed onset and progression of CAD in diabetic patients (26).

CAD and diabetes are pathologically interrelated diseases. The severity of CAD is higher in diabetic patients (27). Genetic
variations in the 9p21.3 locus are associated not only with CAD but also with diabetes. The association of this region with CAD varies in diabetic and nondiabetic patients (28). It is well known that oxidative stress has a critical role in the early onset and progression of CAD. In diabetic patients, the anti-oxidant system becomes weak, and the number of free radicals starts increasing. Measures of increased oxidative stress and decreased anti-oxidants in diabetes may be beneficial for the clinical diagnosis (29). The present study results are inconsistent with the reported. Diabetes is significantly associated with CAD (p<0.01). A high odds ratio indicates the strength of the association with CAD in studied subjects. The endothelium protects the vascular lining by releasing some chemicals, like nitric oxide (NO). In case of risk factors, like diabetes, hypertension, and hyperlipidemia, this endothelium is damaged, causing vascular dysfunction. It has been found that insulin resistance before the onset of diabetes is associated with vascular dysfunction. This provides evidence that atherosclerosis may start with insulin resistance (30). According to Haffner et al. (32), insulin resistance is significantly associated with C-reactive protein that is produced due to inflammation. C-reactive protein is a predictor of diabetes. It may be concluded that atherosclerosis due to insulin resistance starts before the onset of CAD and that diabetes is a major risk factor in the development of CAD.

The study subjects included both male and female aged ≤65 years. The mean age was 50.9 and 49.9 for male and female CAD patients, respectively. The onset of disease at an early age increases the chances of genetic factor involvement. With increasing age, other risk factors also start contributing to the development of CAD. The relationship between genetic factors and CAD development has been described by many investigators. In the Korean population, genes of endothelial function showed an association with CAD, but an analysis for age gave significant results only in patients aged less than 51 years (32). The disease diagnosis at early age (≤54) depicts CAD family history as the sole and unique risk factor, which is masked by age more than 62 years. Smoking is also found to be associated with the diagnosis of CAD at an early age (33). In the present study, the mean age for patients and controls was the most suitable for exploring the association of risk factors with CAD. It has been reported in the Rotterdam study, based on an elderly population (aged ≥65), that the rs10757274 and rs10757278 SNPs are not associated with coronary heart disease and MI (34). These SNPs are reported in many GWASs to be associated with CAD and MI, but the absence of association may be due to the possibility that different genes may be involved in the onset of disease in young and old people.

The present study examined rs10757274. This SNP exhibited a strong association with CAD in a population of Sargodha, Pakistan. Variations of two SNPs (rs10757274 and rs2383207) in association with CAD were studied in different populations. A significant association was observed in all populations (p<0.05). The heterozygous genotype was high in the control group (35). These results are similar to the present study, having a high percentage of heterozygotes (56.9%). A highly significant association was found between rs10757274 and CAD (36). The reported associated SNP was selected for the current study to confirm the association in the Sargodha, Pakistan population, and to my knowledge, the present study was the first attempt to study such an association in a local population.

The frequency of the G allele was very high in our studied population. In CAD subjects, the G allele frequency was 86%. In the control group, the frequency of G was also high (56%) as compared to that of the A allele. With this high frequency, the contribution of risk factors in the onset of CAD within a population has become more critical. rs10757274 has been found to be strongly associated with CAD (p<0.01). In an Irish population study, a family-based approach was used with at least one member having early onset of disease. The three SNPs, rs10757274, rs2383206, and rs1333049, were found to be strongly associated with coronary heart disease (p<0.01) (37). The analysis for the association of family history with CAD in the current investigation has given significant results (p<0.01) with an odds ratio (OR) of 5.1263 and 95% confidence interval (CI) of 3.1313-8.3924. Several studies have already demonstrated that CAD family history is responsible for early onset of the disease. Sunman et al. (25) studied the role of family history in suspected CAD patients. The family history was associated with the extent and severity of coronary atherosclerotic plaque formation in premature CAD (38). The effects of rs10757274 were investigated by Shanker et al. (40). Their study indicated the importance of this SNP due to its involvement in the expression of the EU741058 and p16INK4a genes, modulating the chances of CAD. The same SNP also showed its association with peripheral artery disease in a Han Chinese population (40). These findings are in agreement with our results, obtained without the consideration of covariates.

### Study limitations

The strength of the study is the stringent criteria used for the sample selection for the study. However, the small sample size (350) may confine the potential of making a detailed confounding adjustment for all factors essential for the study of coronary artery disease. Apparently, the study seems to be regional due to the usage of a local population, but history tells that the population of Pakistan is composed of several ethnic groups. The caste system and increased trend of cousin marriages have saved the evidence of the genetic relationships of these groups with the populations of Arab, Iran, Central Asia, Turkey, Afghanistan, and India. On the basis of the ethnic origins of the population of Pakistan, the findings of the present study may be used in understanding the genetic etiology of coronary artery disease in other related populations. The study also did not investigate the detailed information of smoking-e.g., number of cigarettes consumed per day, smoking before or after taking meal, etc. The genotyping was performed by primer extension method. The validity of the method demands confirmatory tests by using restriction fragment length polymorphism, sequencing,
or Taqman assay. Another independent study may be designed for this purpose. The present study is also confined due to the investigation of a single SNP. A haplotype-based analysis may better unveil the genetic basis of CAD in the local population of Pakistan.

**Conclusion**

Without considering the impact of covariates, the studied SNP, rs10757274, in the 9p21.3 locus was observed to show a strong association with the onset of CAD. The G risk allele frequency was high in the population. It increased the chances of CAD in the local population. Risk factors, like smoking, diabetes, hypertension, and family history, also showed a strong association with CAD. Despite the presence of the G allele with high frequency in the Sargodha population, modifiable risk factors also play an important role in the onset of CAD in the local population of Pakistan. When the impact of the SNP was re-investigated after considering the covariates, the results were different. The results before and after consideration of the covariates indicate the challenges involved in the analysis of complex traits. The present finding suggests that thers10757274 SNP may play its role in the occurrence of CAD. However, the independent effects of this SNP are weak as compared to the other factors affecting the onset of CAD.

**Conflict of interest:** None declared.

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