Role of single nucleotide polymorphism rs2383206 on coronary artery disease risk among Saudi Population: a case-control study

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Abstract. – OBJECTIVE: We aim to investigate the relationship between genetic variation and biological function on a genomic scale, focusing on identifying genes responsible for complex diseases using single nucleotide polymorphisms. Specifically, the study explores the association between the rs2383206 gene located on chromosome 9p21.3 and the development of coronary artery disease (CAD) in a specific Saudi population.

PATIENTS AND METHODS: This case-control study was conducted between September 2013 and May 2015 at King Abdullah Medical City (KAMC) and Al-Noor Specialist Hospital targeting the Saudi Population residing in the western region of Saudi Arabia. The study enrolled 315 cases with documented CAD and 205 controls with normal coronary arteries on coronary angiography. Genomic DNA was extracted from peripheral blood samples of both groups, and genotyping of rs2383206 was performed using the tetra-primer amplification-refractory mutation system-polymerase chain reaction (ARMS-PCR) method.

RESULTS: In this study, the prevalence of the GG genotype in rs2383206 was found to be higher in patients with CAD than in controls, with an odds ratio of 1.997 [95% confidence interval (CI): 1.176-3.394, p = 0.007]. Additionally, individuals with the GG genotype who had sedentary lifestyles, hyperlipidemia, and smoked were found to be at a higher risk for developing CAD (p = 0.003, 0.009, and 0.003, respectively). The G allele also increased the risk of CAD with an odds ratio of 1.413 (95% CI: 1.099-1.817; p = 0.004).

CONCLUSIONS: In conclusion, this study demonstrated a significant association between the rs2383206 variant located on chromosome 9p21 and the development of CAD. The findings of this study provide valuable insights into the genetic susceptibility to CAD and highlight the potential of this variant as a target for future functional studies.

Key Words: Amplification-refractory mutation system-polymerase chain reaction, Coronary artery disease, Chromosome 9p21.3, Genotyping, rs2383206.

Introduction

Cardiovascular disease (CVD) is a significant contributor to worldwide mortality rates. While epidemiological and clinical research1-4 has demonstrated that coronary heart disease can be prevented, evidence suggests that the disease has a genetic component. In asymptomatic individuals, coronary artery calcification has been
found to be a strong predictor of mortality. Despite progress in treatment, coronary artery disease (CAD) continues to be associated with significant morbidity and mortality on a global scale with approximately 30% of deaths worldwide attributed to CAD.

CVD is an increasingly pressing public health issue in the Gulf Council countries, including Saudi Arabia, where it is estimated that CVD accounts for over 45% of all deaths. In the North Africa and Middle East regions, mortality rates for heart disease are 115 and 294 per 100,000, respectively. The exploration of genetic variation and biological function at the genomic level has provided new insights into biology, evolution, and pathophysiology. Single nucleotide polymorphisms (SNPs) have emerged as a powerful tool for identifying genes associated with complex diseases. As the most abundant genetic variation, SNPs are a valuable resource for mapping complex genetic traits. They are commonly used to identify disease-associated genes and to understand inter-individual variation in drug response, offering potential medical benefits.

Numerous genome-wide association studies and meta-analyses have identified multiple SNPs associated with CAD. Among these, one of the most consistently observed genetic associations for CAD has been located on chromosome 9p21. This locus contains very few coding genes with the nearest genes being CDKN2A and CDKN2B. These genes are cell cycle regulators that inhibit cell growth by transforming growth factor 1 and are associated with the development of atherosclerosis. There is growing evidence that long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) play a role in the pathogenesis of CAD. Studies have shown that ANRIL, an antisense non-coding RNA in the INK4 locus, is improved in 9p21 carriers and affects atherosclerosis. ANRIL is believed to facilitate the connection between the 9p21.3 risk locus and CAD susceptibility.

McPherson et al. identified two SNPs (rs1075724 and rs2383206) on chromosome 9p21 in 2007, while Helgadottir et al. established two additional SNPs (rs2383207 and rs10757277) in 2008. These SNPs were confirmed in other genome-wide linkage analyses for CAD, making the 9p21.3 locus the most well-documented locus associated with CAD susceptibility.

This study aims to examine the influence of SNP rs2383206 at the 9p21.3 loci on susceptibility to CAD in the Saudi Arabian population and correlate this genetic variant with various disease risk factors. To our knowledge, this research represents the first investigation of this SNP in Saudi Arabia.

Patients and Methods

Study Design and Participants

This prospective study was conducted between September 2013 and May 2015 at two medical facilities in the western region of Saudi Arabia: King Abdullah Medical City (KAMC) and Al-Noor Specialist Hospital. The study population was divided into two groups: cases and controls. The cases included patients with documented CAD, defined as more than 50% stenosis confirmed on coronary angiography in one of the main coronary arteries. The controls were age-matched individuals without evidence of CAD, stroke, or peripheral vascular diseases. Coronary angiography was performed for chest pain or before valvular open-heart surgery and confirmed no significant coronary stenosis in the controls. Patients with a malignancy or autoimmune disease history were excluded from the study.

Sample Size

The sample size for this study was determined using the open-source Epi version 6 (available at: https://www.cdc.gov/epiinfo/support/downloads/previous/ct6.html), a standard analysis tool for managing, summarizing, and analyzing data. The calculation was based on a power of 80%, a 95% confidence level, and using 39% of the total number of patients with ischemic heart disease reported in the Ministry of Health Statistical Yearbook, which was 167,499 people. The mutant genotype frequency in the Eastern region of Saudi Arabia was found to be around 50%, and an additional 10% sample was added to compensate for potential dropouts and missed cases.

Data Collection Tool

a) Patients’ demographics and baseline characteristics, such as age, sex, smoking, diet, height, and weight, were collected on the first day of enrolment.

b) Biochemical assays: venous blood samples of 10 ml were collected from each recruited participant, and 5 ml of the blood was drawn into ethylenediaminetetraacetic acid (EDTA) tubes for DNA extraction. The remaining 5 ml was used to analyze glucose, lipids, and cardiac markers. The serum was separated and aliquoted into su-
Role of single nucleotide polymorphism rs2383206 on coronary artery disease risk in Saudi Arabia

Biochemical quantification of the markers was conducted on fully automated chemical analyzers (Life Technologies, Foster City, CA, USA), from Siemens at the clinical chemistry laboratories of King Abdullah Medical City and Al-Noor Specialist Hospital in Mecca.

c) DNA extraction, PCR amplification, and genotyping: genomic DNA was extracted using the MagMax-96 Multi-Sample Kit (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -20°C. Genotyping for rs2383206 was done in 45 s by tetra-primer amplification-refractory mutation system-polymerase chain reaction (T-ARMS-PCR) as described previously. Four primers were used as follows\textsuperscript{36,37}:

- Forward outer primer: 5'-CTAACTTTAAGCCACCAAGAGAGGAG-3';
- Reverse outer primer: 5'-AGCAAAAATTTTTTGTGTGAATTCA-3';
- A alprimer: 5'-GTTCAGGATTCAGGCCATCTTGCA-3';
- G alprimer: 5'-TGTCTTTTCCTTAGAAATGTTATTGTATTG-3'.

The PCR amplification was performed in a Veriti thermal cycler (Life Technologies, Foster City, CA, USA). DNA was amplified by employing the GoTaq Green Master Mix (Promega) (Life Technologies, Foster City, CA, USA), and pursuing this order: initial denaturation at 95°C for 5 min, then 35 cycles at 95°C for 30 s, 52°C for 30 s, and 72°C for 45 s. Finally, an extension step at 72°C for 7 minutes. These primers produced PCR fragments of 428 bp, 270 bp for the A allele, and 215 bp for the G allele. The products were then resolved on a 2% agarose gel and visualized using ethidium bromide and ultraviolet light. We visualized 428 bp and 270 bp bands for the AA homozygotes, 428 bp and 215 bp for the GG homozygotes, and 428 bp, 270 bp, and 215 bp for the AG heterozygotes\textsuperscript{42}.

d) Capillary sequencing\textsuperscript{43}: The variant rs2383206 found by the T-ARMS-PCR method was checked with capillary sequencing. PCR was performed with the HotStarTaq Plus Kit (Qiagen, Hilden, Germany). A magnetic bead separation was employed to cleanse PCR amplicons (Agencourt AMPure kit; Beckman Coulter, Brea, CA, USA). The cleansed PCR products were set to use the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The capillary sequencing was done on an ABI 3500 genetic analyzer (Applied Biosystems, Foster City, CA, USA), and the final analysis was performed with the Sequence Analysis Software v. 5.4 (Applied Biosystems, Foster City, CA, USA) (Figure 1). Results are shown in Figure 2.

Statistical Analysis

Genotype frequencies were examined for Hardy-Weinberg equilibrium. The Kolmogorov-Smirnov normality test examines whether quantitative variables are normally distributed. The mean, standard deviation (SD) of quantitative variables with a normal distribution was used to compare them using the Student’s t-test. While
non-normally distributed variables were represented as the median, interquartile range (IQR) and tested using the Mann-Whitney U test, categorical variables were presented as frequencies and percentages and compared using the 2-test. Odds ratios (ORs) were derived using a two-way contingency table. Multivariable logistic regression analysis was used to check the association. An ANOVA test was used to test the difference among the three genotypes regarding risk factors and clinical parameters. SPSS v. 21 (IBM Corp., Armonk, NY, USA) was used in the analysis. Associations with a two-tailed \( p < 0.05 \) were considered statistically significant, and the lower the \( p \)-value, the higher the significance level.

Results

The study comprised 520 Saudi participants, including 315 individuals with angiographically documented CAD and 205 controls. The outcome parameters in patients and the diagnosis of CAD were determined in adherence to established clinical guidelines.

Clinical Data and Biochemical Characteristics of Study Subjects

The mean age of CAD patients was 59.7 years (SD = 10.87), and 65% were males. Among the controls, 58% were males. Table I displays the demographic and clinical baseline characteristics.
of the participants. Significant differences were observed between cases and controls regarding BMI, body weight, waist circumference, systolic and diastolic blood pressure, and biochemical parameters.

Comparison of Risk Factors and Comorbidities
The study assessed risk factors and found that 59% of the CAD cases had diabetes, 70% had hypertension, and 66% had dyslipidemia. Statistical analysis showed significant differences between cases and controls for lack of exercise, obesity, dyslipidemia, smoking, diabetes, and hypertension (Table II). Prior cardiovascular events among cases were also evaluated and presented in Table II.

Genotype and Allele Frequencies in the Study Population
Results showed no evidence of divergence from the Hardy-Weinberg equilibrium in either of the two groups ($p > 0.05$). Genotypic as well as allelic frequencies of the studied SNP are presented together with the ORs, which show an increased risk of CAD in individuals having the $GG$ genotype (OR: 1.997, CI: 1.176-3.394) and heterozygous genotype (OR: 1.852, CI: 1.227-2.796) with a $p$-value of 0.007 and 0.002, respectively (Table III). Subjects carrying the $G$ allele are more likely to develop CAD (OR: 1.413, CI: 1.099-1.817, $p = 0.004$).

Logistic Regression Analysis
Our study found that when age and gender were taken into account, as well as the effects of other risk factors, the SNP rs2383206, smoking, and lack of exercise were all independent predictors of CAD (Table IV).

Genotype Distribution among Different Risk Factors
Modifiable risk factors for CAD, such as lack of exercise, smoking, and dyslipidemia, significantly differed among the three genotypes in the case group ($p = 0.006, 0.015,$ and 0.012, respectiv-
Gender (a non-modifiable risk factor) was not statistically different across genotypes. Other modifiable factors, such as hypertension and diabetes, were not significantly different among the three genotypes (Table V).

**Lipid Profile Stratification by Genotype**

In the case group, the genotype **GG** was significantly linked to the lowest HDL-c values and the highest levels of total cholesterol, triglycerides, and low-density lipoprotein cholesterol (LDL-c) \((p > 0.001)\). Also, people with the heterozygous **GA** genotype had much lower high-density lipoprotein cholesterol (HDL-c) and higher LDL-c levels than people with the wild-type **AA** genotype (Table VI).

**Combined Risk Factor Analysis**

We found a significant association between the genotype **GG** and dyslipidemia of LDL-c 160 mg/dl (OR: 2.87, CI: 1.43-5.76, \(p = 0.003\)). Similarly, TG 240 mg/dl and genotype **GG** are associated with odds of 5.16 folds (CI: 2.37-11.28, \(p = 0.003\)). We analyzed the CAD cases for the combined risk due to different risk factors and genotypes using the dominant genetic model (**GG** vs. **GA** + **AA**). Sedentary behavior and the **GG** genotype are linked by a 2.2-fold odds ratio (CI: 1.2-3.96; \(p = 0.009\)). Smokers with the **GG** genotype have a 2.36-fold higher risk of CAD (CI: 1.3-4.29, \(p = 0.003\)) (Table VII).

**Discussion**

Substantial evidence \(5,18,24,35,44\) supports the notion that genetic predisposition is a significant independent risk factor for CAD. Numerous genome-wide association studies \(5,18,24,35,44\) have identified various polymorphisms in the intergenic re-

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**Table IV. Logistic regression analysis of associated risk factors.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>(\rho)</th>
<th>Adj OR</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2383206</td>
<td>322</td>
<td>0.036</td>
<td>2.580</td>
<td>1.287 3.771</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>57</td>
<td>0.141</td>
<td>945</td>
<td>903   989</td>
</tr>
<tr>
<td>Smoking</td>
<td>952</td>
<td>0.000</td>
<td>2.592</td>
<td>1.600 4.199</td>
</tr>
<tr>
<td>Lack of exercise</td>
<td>-327</td>
<td>0.023</td>
<td>1.487</td>
<td>1.010 2.377</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.582</td>
<td>928</td>
<td>206</td>
<td>122   348</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>158</td>
<td>421</td>
<td>1.203</td>
<td>767   1.887</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>254</td>
<td>317</td>
<td>776</td>
<td>472   1.276</td>
</tr>
<tr>
<td>DM</td>
<td>1.210</td>
<td>406</td>
<td>298</td>
<td>174   511</td>
</tr>
<tr>
<td>Blood Glucose</td>
<td>109</td>
<td>194</td>
<td>897</td>
<td>864   931</td>
</tr>
<tr>
<td>TG</td>
<td>10</td>
<td>291</td>
<td>990</td>
<td>986   995</td>
</tr>
<tr>
<td>TC</td>
<td>9</td>
<td>516</td>
<td>991</td>
<td>987   995</td>
</tr>
<tr>
<td>LDL-c</td>
<td>14</td>
<td>742</td>
<td>986</td>
<td>979   993</td>
</tr>
<tr>
<td>HDL-c</td>
<td>-10</td>
<td>93</td>
<td>1.010</td>
<td>984   1.037</td>
</tr>
</tbody>
</table>

*p* in bold means there was a statistically significant difference.

**Table V. Risk factors distribution across genotypes.**

<table>
<thead>
<tr>
<th></th>
<th>GG</th>
<th>GA</th>
<th>AA</th>
<th>(\chi²) or ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>60.23 (11.99)</td>
<td>59.42 (10.71)</td>
<td>59.93 (10.29)</td>
<td>0.860</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>29 (4)</td>
<td>29.18 (4.33)</td>
<td>29.72 (3.65)</td>
<td>0.557</td>
</tr>
<tr>
<td>Gender (male n, %)</td>
<td>40 (60.6)</td>
<td>120 (66.67)</td>
<td>46 (66.67%)</td>
<td>0.655</td>
</tr>
<tr>
<td>Systolic BP mmHg</td>
<td>147.52 (10.34)</td>
<td>145.35 (8.03)</td>
<td>143.13 (9.02)</td>
<td>0.371</td>
</tr>
<tr>
<td>Diastolic BP mmHg</td>
<td>93.03 (8.45)</td>
<td>91.23 (7.34)</td>
<td>89.23 (5.67)</td>
<td>0.279</td>
</tr>
<tr>
<td>Pulse/min</td>
<td>80.8 (2.3)</td>
<td>79.3 (3.6)</td>
<td>784 (2.8)</td>
<td>0.263</td>
</tr>
<tr>
<td>Blood Glucose mg/dl</td>
<td>142.25 (2.8)</td>
<td>141.3 (2.1)</td>
<td>140.47 (1.9)</td>
<td>0.324</td>
</tr>
<tr>
<td>Sedentary life, n (%)</td>
<td>23 (34.85%)</td>
<td>41 (22.78%)</td>
<td>8 (11.59%)</td>
<td>0.006</td>
</tr>
<tr>
<td>Overweight, n (%)</td>
<td>42 (63.6)</td>
<td>142 (78.9)</td>
<td>47 (68.1)</td>
<td>0.562</td>
</tr>
<tr>
<td>Dyslipidemia, n (%)</td>
<td>51 (77.3)</td>
<td>129 (71.7)</td>
<td>29 (42)</td>
<td>0.012</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>48 (72.73)</td>
<td>94 (55.95)</td>
<td>38 (46.9)</td>
<td>0.015</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>48 (72.72)</td>
<td>122 (67.78)</td>
<td>51 (73.91)</td>
<td>0.560</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>42 (63.64)</td>
<td>100 (55.56)</td>
<td>45 (65.22)</td>
<td>0.278</td>
</tr>
</tbody>
</table>

*\(\chi²\) and ANOVA tests. “*p*” in bold indicates a statistically significant difference.
Role of single nucleotide polymorphism rs2383206 on coronary artery disease risk in Saudi Arabia

The region of chromosome 9p21.3, such as rs10757274, rs2383206, rs10757278, and rs1333049, which have been shown to confer varying degrees of risk for CAD across different ethnic groups. Our study, which included a cohort of 315 patients, revealed that the SNP rs2383206 is significantly associated with CAD in the Western Region of Saudi Arabia.

The minor G allele frequency of rs2383206 was observed in approximately 49.5% of the study population, consistent with the findings of other studies in various populations. For instance, the G allele frequency ranges from a minimum of 41% in African Americans to a maximum of 73% in CAD cases in the Egyptian population. Studies conducted on South Koreans and Han Chinese reported a G allele frequency of 50.6% and 50.5%, respectively, while the Iranian and Italian populations have a similar G allele frequency of 69% and 62%, respectively.

Our study found that the GG genotype of the SNP rs2383206 was significantly more prevalent in CAD patients than in controls. Interestingly, these comorbidities were not significantly distributed across different genotypes.

This study found a strong association between the risk genotype GG of SNP rs2383206 and smoking and physical inactivity. However, a detailed patient history and clinical examination revealed several comorbidities that indicated an atherogenic milieu in the study cases with combined risk factors. Interestingly, these comorbidities were not significantly distributed across different genotypes.

Although the GG genotype of SNP rs2383206 is an independent risk factor for CAD, its impact is even more remarkable when combined with other risk factors. Gioli-Pereira et al conducted a Cox proportional hazards model adjusted for age, previous myocardial infarction, diabetes, smoking, and type of coronary anatomy (double- or triple-vessel). They found that rs2383206 was still significantly associated with a 1.75-fold increased risk of overall mortality in CAD patients.

Hu et al also found an effective combination of rs2383206 and other risk factors in cases with ischemic stroke.

In our study, we observed that individuals with the GG genotype of SNP rs2383206 had lower levels of HDL-c and higher levels of total cholesterol, triglycerides, and LDL-c compared to other genotypes. Chen et al also investigated plasma lipids’ response to fluvastatin treatment based on rs2383206 genotypes and found no significant difference in changes in plasma lipid levels among the genotypes, except for plasma triglyceride levels. Additionally, treatment with fluvastatin and baseline HDL-c levels were independent predictors of coronary atherosclerosis progression.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Sedentary life</th>
<th>Smoking</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GG/GA+AA</td>
<td>GG/GA+AA</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>273.38 ± 55.82</td>
<td>252.58 ± 55.43*</td>
</tr>
<tr>
<td>LDLc (mg/dL)</td>
<td>126.31 ± 36.84**</td>
<td>130.17 ± 41.64**</td>
</tr>
<tr>
<td>HDLc (mg/dL)</td>
<td>43.04 ± 6.75</td>
<td>27.63 ± 4.69**</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>201.71 ± 52.59</td>
<td>253.61 ± 75.96**</td>
</tr>
</tbody>
</table>

*ANOVA test p-value < 0.05. **p-value < 0.001. “p” in bold indicates a statistically significant difference.

Table VII. Genotypes distribution and association with different risk factors.

<table>
<thead>
<tr>
<th>OR (CI)</th>
<th>TG ≥ 240 mg/dl</th>
<th>Sedentary life</th>
</tr>
</thead>
<tbody>
<tr>
<td>15/25</td>
<td>0.003</td>
<td>23/49</td>
</tr>
<tr>
<td>2.87 (1.4-5.76)</td>
<td>5.16 (2.37-11.28)</td>
<td>2.2 (1.2-3.96)</td>
</tr>
<tr>
<td>0.003</td>
<td>0.000</td>
<td>2.36 (1.3-4.29)</td>
</tr>
</tbody>
</table>

*p in bold indicates a statistically significant difference.
The clinical and biochemical assessment of our sample sets allowed the identification of the independent nature of the risk association of SNP rs2383206, despite the presence of traditional coronary risk factors. Furthermore, combining two or more risk factors, including the SNP, may increase the risk for CAD and potentially accelerate the atherogenic process of the coronary arteries. This relationship between polymorphisms and premature onset of CAD could be attributed to the additive effects of these risk factors.

Limitations
While the sample size was relatively large and diverse, it was limited to one Saudi Arabian province. Therefore, it may not be representative of the entire Saudi Population or other populations around the world. Additionally, only one SNP was studied, and other SNPs on chromosome 9p21 may affect CAD risk differently. A comparison study of multiple SNPs would provide more robust evidence of the association between genetic factors and CAD risk.

Conclusions
This study suggests that rs2383206 on chromosome 9p21 is significantly associated with CAD in Saudi Arabian population. The study also highlights the importance of understanding gene-environment interactions in the development of CAD and the need for more effective preventive strategies. The findings can aid in the pre-symptomatic detection of CAD in families at higher risk and the introduction of personalized medicinal modalities as prevention and therapeutic measures in high-risk individuals based on interactive genetic and environmental predisposition via pharmacogenetic research on the 9p21.3 loci. Further research in this area can lead to the development of more effective strategies for preventing and managing CAD.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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Role of single nucleotide polymorphism rs2383206 on coronary artery disease risk in Saudi Arabia

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Role of single nucleotide polymorphism rs2383206 on coronary artery disease risk in Saudi Arabia


