Zagazig University Medical Journal
www.zumj.journals.ekb.eg
https://doi.org/10.21608/zumj.2022.161460.2636 Volume 30, Issue 3, May 2024
Manuscript ID ZUMJ-2209-2636 (R1)
DOI 10.21608/ZUMJ.2022.161460.2636

ORIGINAL ARTICLE

Evaluation of Cyclin-Dependent Kinase Inhibitor 2A and Hepatocyte Nuclear Factor-1A Gene Polymorphisms in Coronary Artery Diseases

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Submit Date 2022-09-09
Revise Date 2022-09-16
Accept Date 2022-09-18

Abstract

Background: Blood lipid-regulating and cell proliferation-regulating genes have both been connected to the development of coronary artery disease (CAD),we aimed to find the correlation between CDKN2A rs3088440, HNF1A rs55783344CT gene polymorphisms and CAD.

Methods : One hundred and six participants were subjected to this case-control study; case group I: patients with CHD and control group II: Normal healthy people. Subjects underwent a comprehensive history and physical examination, standard laboratory tests, and coronary angiograms. CDKN2A rs3088440 and HNF1A rs55783344CT gene polymorphisms was done by (PCR-RFLP).**Results:** CT genotype and CT+TT of HNF1AC were statistically Signiant higher in CAD cases than controls. T allele of HNF1AC was much higher in CAD cases than controls, with a significant difference between 2 groups. Proportion of those with the CDKN2A GA+AA genotype in the CAD patients was significantly higher than in the control group. Also, the frequency of the CDKN2A A allele was significantly different between CAD cases and controls.**Conclusions:** Coronary heart disease (CHD) has a complex etiopathogenesis and a multifactorial origin. SNPs are potential risk factors of CHD. CDKN2A rs3088440 (G>A) and HNF1A rs55783344CT gene polymorphisms be a factor in pathogenesis of CHD.

Keywords: Coronary Artery Diseases; Cyclin-Dependent Kinase Inhibitor 2A; Hepatocyte Nuclear Factor-1A.

INTRODUCTION

Angina pectoris, acute myocardial infarction, as well as sudden cardiac death are all clinical manifestations of coronary artery disease (CAD), commonly known as ischemic heart disease. Worldwide, coronary heart disease is the leading cause of death and disability. An estimated 8.9 million lives are lost, and 164 million people are disabled per year due to CAD in low and middle-income nations.[1]Coronary artery disease occurs due to atherosclerosis which characterized by formation of atheromatous plaque at specific sites of coronary artery walls leading to narrowing of vessel lumen and blocking of blood flow causing hypoxia, cardiac ischemia, as well as necrosis [2]Numerous studies have demonstrated the importance of genetic variables in the onset of CAD [3] . One of the SNPs which related to occurrence of atherosclerosis is cyclin-dependent kinase inhibitor 2A(CDKN2A) rs3088440 (G>A) gene polymorphism. CDKN2A gene codes for p14ARF and p16ink4a, which are inhibitors of cellular proliferation [4]CDKN2A has a

role in controlling atherosclerosis by affection of apoptosis and cell proliferation, so SNPs in the human CDKN2A rs3088440 (G>A) might increase the risk for atherosclerosis and cardiovascular disorders [5] .Hepatocyte nuclear factor-1 alpha (HNF1A) rs55783344CT gene polymorphism is another SNP that can raise the risk for CAD. Several hepatic genes are controlled by the protein HNF-1A, which is encoded by the HNF1A gene. Organs that express this gene include the pancreas, liver, gut, and kidneys[6].Hepatic biochemistry, including glucose metabolism, lipid metabolism, and serum protein synthesis, are all regulated by HNF-1A-bound genes. Therefore, SNPs in the HNF1A rs55783344CT will affect the levels of serum lipids as low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC) [7] .Therefore, pathogenesis of CAD includes SNPs of both CDKN2A gene (which affects cell proliferation) and HNF1A gene (which affects levels of serum lipids) so in our study we attempted to find the association between CDKN2A rs3088440, HNF1A rs55783344CT gene polymorphisms and CAD.

METHODS

At the Zagazig University Department of Medical Biochemistry and Department of Cardiology we conducted this case-control study. One hundred and six participants were enrolled. IRB approval and informed permission were obtained from the university and the patients before the study was conducted. Written informed consent was obtained from all participants, as study was approved by the research ethical committee of faculty of medicine Zagazig university. The study was done according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving human. There were two sets of subjects: case group I: patients with CHD and control group II: Normal healthy people.

Sample size statistics: assuming that percentage of CDKN2A rs3088440 (G>A) and HNF1A rs55783344CT Polymorphisms cases versus control group were(72.2 % vs 43.8 %) sample was calculated to be 106 participants (53 in each group), using open EPI program with test power 80%, and CI 95%.

Inclusion criteria*: Cases:** 53 patients admitted to cardiology department and undergone coronary angiography and diagnosed to have coronary artery disease with angiographic evidence of narrowing of one or more major coronary arteries greater than 50% of lumen stenosis. **Control:** 53 age and sex matched people who attend outpatient clinics for routine care.Exclusion criteria*:** Patients with the following conditions: mental health problems, physical disabilities, infectious diseases, chronic inflammatory disease other than atherosclerosis, advanced hepatic and renal disorders, and cancer.All cases of the study were subjected to the following: full history taking, complete general examination , Electrocardiography, Coronary angiography, blood tests to determine Fasting blood glucose level , high density lipoprotein-cholesterol (HDL-c), total cholesterol (TC), and triacylglycerol (TAG) levels.Blood sampling**:**Subjects fasted for 12 hours before having 5 ml of venous blood collected from a sterile vein puncture in a completely aseptic environment and divided into three samples, two ml were collected in sterile ethylene diamine tetra acetic acid, dipotassium salt, dihydrate (K2EDTA-2H2O) treated tubes and genomic DNA extraction samples were kept at -20 degrees Celsius. One ml collected in sodium fluorid /potassium oxalate for estimation of fasting blood glucose level. Two ml were collected in plain tubes then left for 20 minutes until clot retraction then separation of serum and stored at -20 °C for estimation of fasting lipid profile.

Special Investigations:

Detection of HNF1A rs55783344 (C>T) :Isolation of DNA An industrially accessible DNA extraction kit (G-spin TM Total DNA Extraction Kit) was used to separate genetic material from whole blood samples (iNtron bio-tehnology, Seongnam-Si, Gyeonggi-do, Korea). Genotyping of HNF1A rs55783344CT **:**PCR-RFLP for detection HNF1A rs55783344CT gene polymorphism was done using the primers as follow: Forward primer: (5′- ACC

GGC GCA AAG AAG AAG-3′) and Reverse primer: (5′- GAG TGA TAA GGA GTG GCA TGA A-3′). PCR was performed at 94°C for 1 min followed by 35 cycles of at 94°C for 30s , 61°C for 30s, 72°C for 1 min and finally 72°C for 5 min. Gel electrophoresis was done using (Tris acetate EDTA (TAE) 50X). Target bands were visualized via UV illuminator. The size of any resultant band was compared to a 100 bp M.W marker. Wild type CC genotype showed one band (235 bp) , whereas homozygous mutant TT genotype showed two bands (190,45 bp) and heterozygous CT genotype showed three bands (235, 190, and 45 bp).Detection of CDKN2A rs3088440 (G>A): single nucleotide gene polymorphism by melting curve analysis using real-time PCR. Detection of CDKN2A rs3088440 single nucleotide gene polymorphism was carried out by Melting curve analysis, using Real-Time PCR detection system ((Rotor-Gene Q 2 Plex (Qiagen, Hilden, Germany) using primers as follow : Forward primer: (5′- TTAGATCATCAGTCACCGAA-3′) and Reverse primer: (5′- GGACATTTACGGTAGTGGGG-3′).

Statistical analysis

Data were collected and analyzed using SPSS software (IBM, Version 20.0). For characterization of quantitative data (IQR), mean, range, median, and standard deviation were used. And for categorical variables, the Chi-square test was utilized, one way ANOVA test followed by Turkey's test for multiple comparisons for three group. All the tests were two-sided. p-value < 0.05 was considered statistically significant, p-value ≥ 0.05 was considered statistically insignificant

RESULTS

The mean age in controls and CAD cases was 60.1 ± 2.8 and 59.3 ± 4.7 respectively. Most of subjects were male in controls (66%) and CAD cases (62%). 28% of controls were smokers while 26% of CAD cases were smokers. In terms of demographics, neither group differed significantly from the other.Cases of coronary artery disease (CAD) had higher levels of low-density lipoprotein (LDL), total cholesterol, fasting blood glucose (FBG) as well as triglycerides (TG) than controls. Significantly reduced levels of HDL-C were seen in CAD cases compared to controls, (table 1).The CT genotype and CT+TT of HNF1AC were significantly superior in cases of CAD compared to control, with a significant variation between the groups. Further, the T allele of HNF1AC was much greater in CAD cases than controls, further highlighting the distinction between the two groups, as shown in (table 2).The GA+AA genotype of CDKN2A was significantly greater in cases of CAD compared to control, with a significant variation between the groups. The A allele of CDKN2A was also much more common in CAD cases than controls, adding more evidence of a group difference (table 3).Compared to CAD cases with the CT or CC genotype of HNFC1, those with the TT genotype of HNFC1 had higher levels of low-density lipoprotein (LDL), total cholesterol, fasting blood glucose (FBG) as well as triglycerides (TG) (table 4).Neither demographic data nor laboratory examinations indicated a correlation between CDKN2A genotype and coronary artery disease (table 5).

Table (1): Fasting blood glucose and lipid profile among the studied groups

	Controls (N=53)	CAD cases (N=53)	t	P value
FBG (mg/dl)	104.3 ± 19.1	116.5 ± 29.1	-2.5	0.01*
Total cholesterol (mg/dl)	175.9 ± 12.1	207.5 ± 47.2	-4.5	<0.001*
TG (mg/dl)	136.1 ± 21.6	170.4 ± 50.1	-4.5	<0.001*
LDL-C (mg/dl)	102.1 ± 11.5	128 ± 42.6	-4.2	<0.001*
HDL-C (mg/dl)	46.7 ± 5.1	43.2 ± 7.9	2.7	<0.001*

Data are represented as mean ± SD. Data are analyzed using independent student t test, FBS:fasting blood suger;TG:triglycerides;LDL-C:low denisty lipoprotien –cholesterol;HDL-C:high denisty lipoprotien –cholesterol

Table (2): Genotype and allele frequency distribution of HNF1AC in CAD cases and control subjects.

HNF1AC	Controls (N=53)	CAD cases (N=53)	OR (95%CI)	P- value
CC	41 (77%)	29 (55%)	**Ref (1)**	
CT	10 (19%)	18 (34%)	**2.5 (1.02-6.3)**	**0.04***
TT	2 (4%)	6 (11%)	**3.9 (0.7-20.9)**	**0.1**
CT+ TT	12 (23%)	24 (45%)	**2.8 (1.2-6.5)**	**0.01***
***C* allele**	**92 (87%)**	**76 (72%)**	**Ref (1)**	
***T* allele**	**14 (13%)**	**30 (28%)**	**2.5 (1.2-5.2)**	**0.007***

Data are represented as number (%). Data are analyzed using odd ratio

Table (3): Genotype and allele frequency distribution of CDKN2A in CAD cases and control subjects.

CDKN2A	Controls (N=53)	CAD cases (N=53)	OR (95%CI)	P- value
GG	40 (75%)	30 (57%)	**Ref (1)**	
GA	10 (19%)	16 (30%)	**2.1 (0.8-5.3)**	**0.1**
AA	3 (6%)	7 (13%)	**3.1 (0.7-13.03)**	**0.1**
GA+AA	13 (25%)	23 (43%)	**2.3 (1.03-5.4)**	**0.04***
***G* allele**	**90 (85%)**	**76 (72%)**	**Ref (1)**	
***A* allele**	**16 (15%)**	**30 (28%)**	**2.22 (1.12-4.3)**	**0.02***

Data are represented as number (%). Data are analyzed using odd ratio

Table (4): Demographic data and laboratory investigations of different HNFC1 genotypes among CAD cases.

	CC (N=29)	CT (N=18)	TT (N=6)	F/ X^2	P Value
Age (years)	58.6 ± 5.2	60.1 ± 4.06	59.8 ± 4.3	**F= 0.58**	**0.55**
Gender **Male** **Female**	 18 (62%) 11 (38%)	 10 (56%) 8 (44%)	 5 (83%) 1 (17%)	**X^2= 1.4**	**0.47**
Smoking	7 (24%)	4 (22%)	3 (50%)	**X^2= 1.9**	**0.48**
FBG (mg/dl)	111.08 ± 24.9	115.1 ± 29.4	147.1 ± 33.1 ab	**F= 4.3**	**0.01***
Total cholesterol (mg/dl)	193.1 ± 35.5	206.2 ± 46.8	281.3 ± 52.3 ab	**F= 11.2**	**<0.001***
TG (mg/dl)	156.8 ± 32.4	165.4 ± 43.5	250.8 ± 69.6 ab	**F= 12.9**	**<0.001***
LDL-C (mg/dl)	114.6 ± 31.3	128.2 ± 42.4	191.7 ± 37.1 ab	**F= 11.3**	**<0.001***
HDL-C (mg/dl)	43.8 ± 8.6	42.9 ± 7.2	41.1 ± 6.9	**F= 0.28**	**0.7**

Data are represented as mean ± SD or number (%). Data are analyzed using Chi square (X^2) or One way ANOVA followed by Tukey s test.A significant from CC. b significant from CT.

Table (5): Demographic data and laboratory investigations of different of CDKN2A genotypes among CAD cases.

	GG (N=30)	GA (N=16)	AA (N=7)	F/X^2	P Value
Age (years)	59.6 ± 4.9	59.1 ± 5.2	58.4 ± 3.4	**F= 0.18**	**0.83**
Gender **Male** **Female**	 18 (60%) 12 (40%)	 10 (63%) 6 (37%)	 5 (71%) 2 (29%)	**X^2= 0.31**	**0.85**
Smoking	5 (17%)	6 (38%)	3 (43%)	**X^2= 3.4**	**0.17**
FBG (mg/dl)	115.3 ± 28.2	112.7 ± 28.3	130.1 ± 34.8	**F= 0.92**	**0.4**
Total cholesterol (mg/dl)	201.7 ± 44.3	203.5 ± 47	241.2 ± 65.8	**F= 1.9**	**0.14**
TG (mg/dl)	163.6 ± 42.4	166.1 ± 41.8	209.2 ± 81.4	**F= 2.5**	**0.08**
LDL-C (mg/dl)	122.1 ± 39.1	125.2 ± 39.3	159.5 ± 56	**F= 2.3**	**0.1**
HDL-C (mg/dl)	43.1 ± 8.4	44.8 ± 7.5	40.1 ± 6.1	**F= 0.91**	**0.4**

Data are represented as mean ± SD or number (%). Data are analyzed using Chi square (X^2) or One way ANOVA followed by Tukey s test. FBS:fasting blood suger;TG:triglycerides;LDL-C:low denisty lipoprotien –cholesterol;HDL-C:high denisty lipoprotien –cholesterol

DISCUSSION

Both in underdeveloped and advanced societies, coronary artery disease (CAD) and its life-threatening clinical consequences continue to be the leading cause of death. Atheromatous plaque builds up in certain areas of the coronary artery walls, narrowing the lumen and blocking blood flow.[8].The development of CAD is affected by various of hereditary and environmental variables. While obesity, dyslipidemia, hypertension, and diabetes mellitus are all recognized risk factors for developing CAD, new data from GWAS is indicating a substantial link between specific genetic variations on chromosome 9p21 and CAD predisposition.[9].Furthermore, Armendariz demonstrated that The importance of HNF1A in coronary artery disease, atherosclerosis, and lipid homeostasis has been well established. Armendariz had demonstrated that hypercholesterolemia occurs from HNF1A loss [10] .Our study was aimed to achieve early detection of susceptibility to CAD via evaluation of cyclin-dependent kinase inhibitor 2A (CDKN2A) and hepatocyte nuclear factor-1A (HNF1A) gene polymorphisms in CAD.Regarding the demographic data in our study, the mean age in controls and CAD cases was 60.1 ± 2.8 and 59.3 ± 4.7 respectively. Most of subjects were male in controls (66%) and CAD cases (62%). 28% of controls were smokers while 26% of CAD cases were smokers. In terms of demographics, neither group differed significantly from the other (p value >.0.05).In line with Zhou et al. [11] Five hundred sixty individuals with coronary artery disease (CAD) were selected from the inpatient population at the First Affiliated Hospital of Guangxi Medical University and included in the study. Everyone who was enrolled had a coronary angiogram because they were worried, they might have CAD. Both controls and CAD patients had similar distributions of age and sex. Cigarette smokers were the vast majority of coronary artery disease patients. With the MASHAD cohort trial, Sadegh et al. [12] enrolled 291 participants with CVD and 218 healthy controls for a case-control study. These healthy individuals served as controls, and they were chosen at random from a pool of people of the same sex and age as the CAD patients.Cases with CAD had greater levels of low-density lipoprotein (LDL), total cholesterol, fasting blood glucose (FBG) as well as triglycerides (TG) than controls did. Significantly reduced levels of HDL-C were seen in CAD cases compared to controls. This came in agreement with Ghaznavi et al. [13] who reported that FBG, total cholesterol, TG and LDL were statistically greater in CAD patients

than in control subjects. HDL-C was significantly lower in CAD cases than controls. While, in disagreement with our study, Zhong et al. [11] observed no statistically significant variations in total cholesterol, HDL cholesterol, low-density lipoprotein cholesterol, or triglyceride levels between the two groups.In our study, we analyzed allele frequency distribution and alleles of HNF1AC in CAD cases and control subjects and found that The CT genotype and CT+TT of HNF1AC, which were both significantly higher in CAD cases, were statistically different from those in the control group (34% and 45% respectively) than controls (19% and 23% respectively) (p value 0.04 and 0.01 respectively). Also, Higher rates of the HNF1AC T allele were found in CAD cases (28 percent) compared to controls (13 percent).(p value 0.007). In agreement with our study, Hsu et al. [14] noted that the Polymorphisms in the HNF1A gene, namely rs55783344CT, have been linked to an increased risk of coronary artery disease. HNF1A rs55783344CT, rs1169288, rs2464196, and rs2650000 were each shown to be independently linked with CAD risk, and this association was further strengthened by an interlocus interaction.Zhou et al. [11] found that this was the case because the connection was moderated by other traditional risk factors including blood pressure and body mass index, and by lipid characteristics like high-density lipoprotein cholesterol, triglyceride levels, and other CAD biomarkers. Furthermore, any gene-gene interactions, ethnic differences in the phenotypes, and genetic/environmental linkages must be considered. The complex interplay between HNF1A haplotypes and other environmental factors that either raise or lower CAD risk need to be elucidated. Among all polymorphisms studied, rs55783344CT has been found to have the strongest connection with CAD in previous research [15] .

In a prospective meta-analysis, Schunkert et al. [16] in Germany and the UK genotyped the rs55783344CT polymorphism in 7 case-control studies involving 4645 patients with MI or CAD and 5177 healthy controls. The results showed that rs55783344CT was consistently linked to CAD across all studies. There was a significant different variation between the CAD cases and controls in our study in terms of the GA+AA genotype of CDKN2A, with 43% of CAD cases having this genotype compared to 25% of controls (p value 0.04). Further, the A allele of CDKN2A was much more common in CAD cases (28%) than controls (15%), indicating a significant difference between the two groups (p value 0.02).In disagreement with our study, Sakalar et al. [17] found that in the Turkish population, the frequency of the G allele of the CDKN2A polymorphism was considerably greater in CAD patients than in controls. While Sadegh et al. [12] found that those who carry the CDKN2A G allele are more likely to develop coronary artery disease, as well as people who have the GG genotype being more likely to acquire hypertension and obesity.In addition, Li et al. [18] proved that Subgroup analysis revealed a stronger association between the CDKN2A rs3088440 polymorphism and CHD in the Asian subgroup than in the Caucasian subgroup, despite the fact that heterogeneity was lower in the Asian subgroup. The many ethnic groups may account for the diversity.Pourgholi et al. [19] indicated that the A allele of CDKN2A's rs3088440 polymorphism increased prevalence of coronary artery disease in Iran. According to their assessment of the data, people who have the A allele of rs3088440 are at far greater risk of developing coronary artery disease.Mehramiz et al. [20] demonstrated that both the total TG/HDL ratio as well as TG level were considerably greater in obese cases with the TT or CT genotypes versus participants who have wild-type genotype. Patients carrying the TT genotype were more likely to have hypercholesterolemia and insulin resistance, both of which are risk factors for the development of coronary atherosclerotic plaques. [20]. To the contrary, Samani et al. [15] found in 2007 research of the British population that the frequency of the G allele was much lower in CHD patients than in controls. [15]In the current study, FBG, total cholesterol, TG and LDL-C were higher in CAD cases with TT genotype of HNFC1 than CT and CC genotypes of HNFC1 while None of the demographic variables, including age, gender,

smoking history, or high-density lipoprotein concentration, were significantly different. In agreement with our study, Colclough et al.[21] found that the High plasma concentrations of high-density lipoprotein cholesterol (HDL-C) have been linked to HNF1A SNPs that increase the risk of high levels of low density lipoprotein cholesterol (LDL-C), total cholesterol (TC), and triglycerides (TG).Cholesterol oxidation important hepatic enzymes CYP27A (ceramide synthase), as well as CYP7A1 (cholesterol 7a-hydroxylase), are being regulated by HNF1A. In response to inflammation and cholestasis, these genes and bile acid transporters appear to be tightly regulated by the transcription factor HNF1A.[22]

In our study, no significant difference was found between different CDKN2A genotypes among CAD cases regarding to demographic data and laboratory investigations. The fact that this SNP contributes to the emergence of CAD separately from other factors might help to explain this outcome. Differences in phenotype among individuals exposed to the same risk factors may be at least in part explained by the relationship between this genetic variation and coronary artery disease and associated risk factors. In agreement with our study, Pourgholi et al. [19] found that no significant correlations were found between age, CAD risk factors, and biochemical markers and the distribution of the rs3088440 (G>A) genotypes (p0.05). Despite our findings, another investigation identified a significant correlation between gender and genotypes in the CAD population.

Limitation of the study

Unfortunately, we were unable to determine whether or whether any other SNPs in the CDKN2A gene locus were associated with CAD risk in the research population. CDKN2A expression in the study population was not assessed. Furthermore, the role of CDKN2A polymorphism on CVD processes was not explored in this study.

CONCLUSIONS

We concluded that coronary heart disease (CHD) has a complex etiopathogenesis and a multifactorial origin. Single nucleotide polymorphisms (SNPs) are potential risk factors of CHD. CDKN2A rs3088440 (G>A) and HNF1A rs55783344CT gene polymorphisms play a role in pathogenesis of CHD. We recommend further studies to be done to analyze the role of single nucleotide polymorphisms (SNPs) in the etiopathogenesis of CAD and thus help physicians to formulate new ideas while outlining the recent guidelines for diagnosis and management of CAD considering the fact that an early diagnosis leads to better outcomes.

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To Cite :

ibrahimm, M., sherif, A., mohamed, D., samy, W. Evaluation of Cyclin-Dependent Kinase Inhibitor 2A and Hepatocyte Nuclear Factor-1A Gene Polymorphisms in Coronary Artery Diseases. Zagazig University Medical Journal, 2024; (859-868): -. doi: 10.21608/zumj.2022.161460.2636

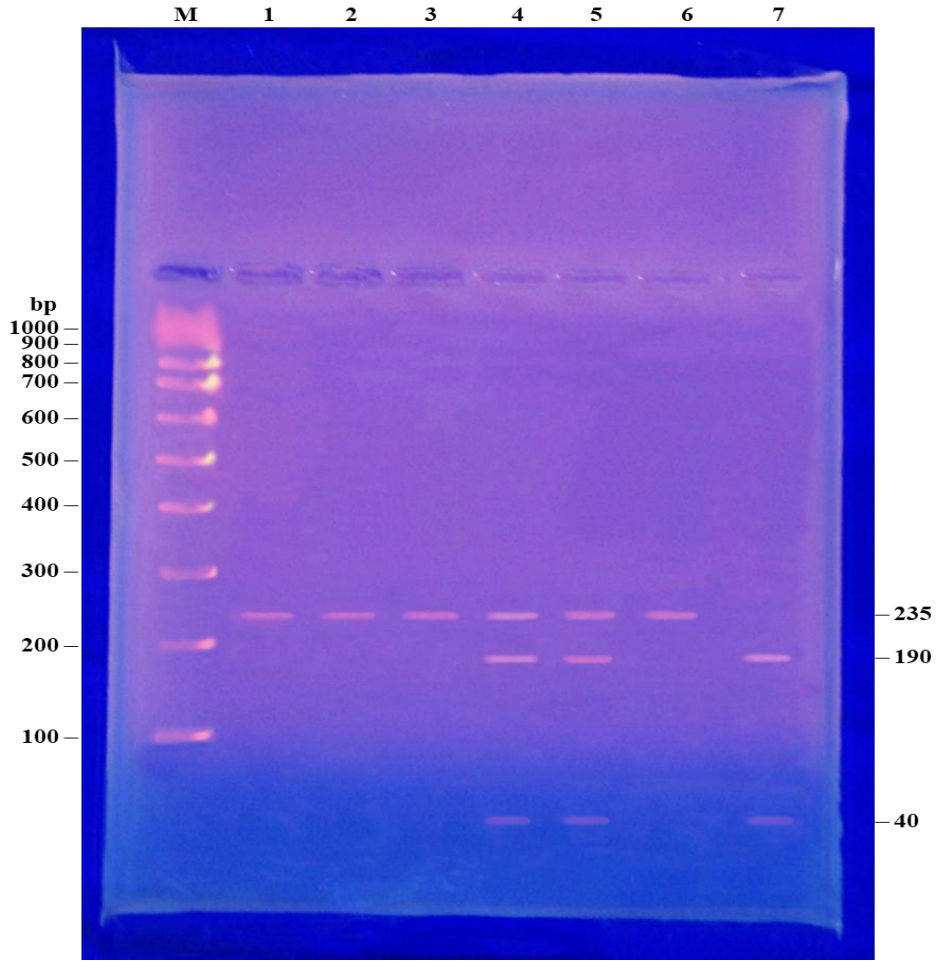

Figure S 1: The ethidium bromide-stained agarose gel electrophoresis image demonstrates the restriction enzyme digestion of the PCR result (Rsal) in which there was the analysis of the HNF1A rs55783344 CT gene polymorphism in CAD patients . DNA size marker (100bp) ladder. Lanes (1,2,3,6): CC genotype showing the presence of one band 235 bp. Lanes (7): TT genotype showing the presence of two bands 190+40 bp. Lanes (4,5): CT genotype showing the presence of three bands 235+190+40 bp.

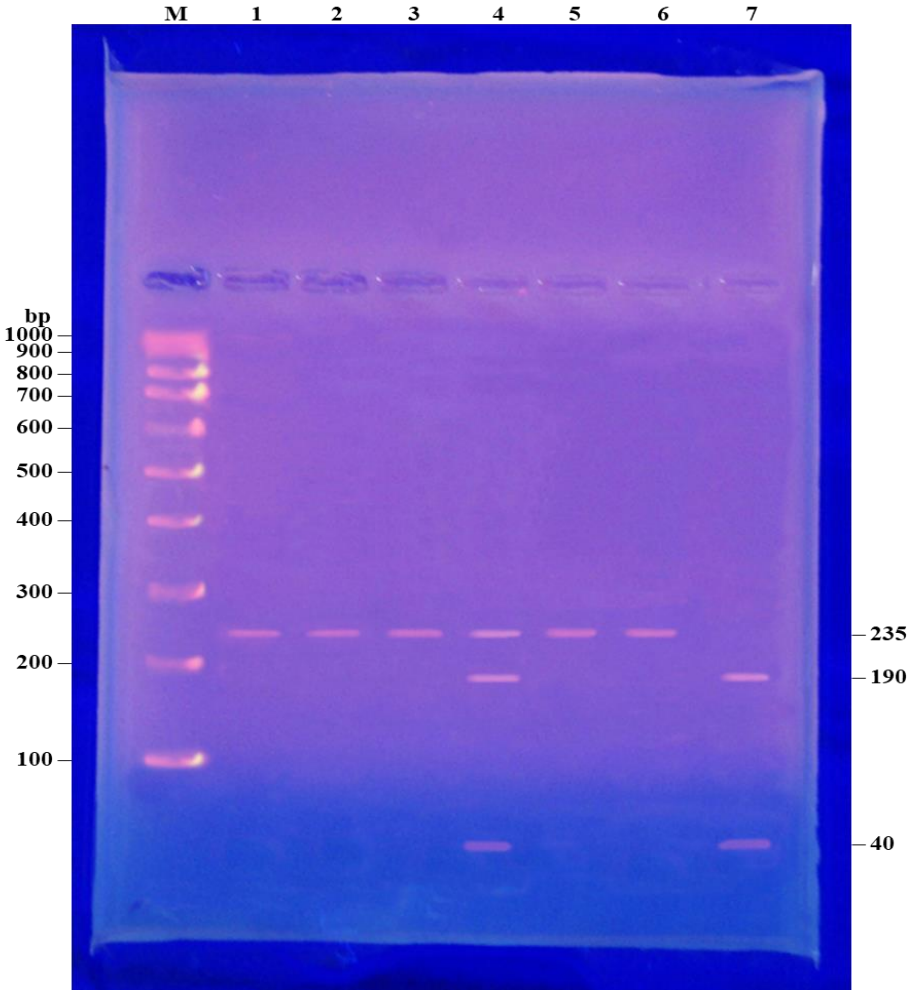

Figure S 2: The PCR result after restriction enzyme digestion, as shown by ethidium bromide staining of an agarose gel electrophoresis image (Rsal) in which there was the analysis of the HNF1A rs55783344 CT gene polymorphism in control cases. DNA size marker (100bp) ladder. Lanes (1,2,3,5,6): CC genotype showing the presence of one band 235 bp. Lanes (7): TT genotype showing the presence of two bands 190+40 bp. Lanes (4): CT genotype showing the presence of three bands 235+190+40 bp