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Original Scientific Paper

ASSOCIATION OF RS1333049 POLYMORPHISM ON 9p21 LOCUS WITH CORONARY ARTERY DISEASE IN ALBANIAN PATIENTS

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Abstract

An association between rs1333049 polymorphism on Chr9p21 and coronary artery disease (CAD) has been reported in multiple studies. Rising trends in coronary heart disease in Albania requires in-depth analysis of socio-economic as well as genetic factors. The purpose of this study was to investigate the association between rs1333049 variant and the presence of CAD in Albanian patients. This was a case-control study involving 177 patients who underwent coronary angiography at the cardiology department at Hygeia Hospital in Tirana, Albania. Patients were divided into two groups; CAD and no-CAD, based on severity and the number of atherosclerotic lesions. Genotypes were determined by allele specific polymerase chain reaction. Allelic and genotype frequencies were determined and tested against Hardy-Weinberg equilibrium. Odds ratio (ORs) and 95% CI were analyzed by logistic regression for $p < 0.05$. The risk associated with rs1333049 polymorphism and CAD was evaluated and adjusted for risk factors. We replicated rs1333049 C-allele association with CAD (OR=1.71 [95%CI: 1.10-2.66], p=0.0165). In addition, under recessive model of inheritance, and after adjustment for covariates, we observed an association of the homozygous genotype (CC) of rs1333049 with coronary artery disease (OR adjusted=2.22 [95%CI: 1.09 – 4.54], p=0.02278). Similarly, under log-additive model of inheritance, homozygous carriers of rs1333049 C-allele were associated with increased risk of CAD (OR adjusted= 1.81 [95% CI; 1.12 – 2.94], p= 0.01360). Our results provide substantial evidence for the association of rs1333049 polymorphism with CAD in Albanian patients. Furthermore, CAD risk conferred by C-allele of rs1333049 polymorphism in our study was higher than the reported association of the risk allele-C with CAD in Caucasians of European origin. These results have significant importance in the light of increased incidence of coronary artery disease in Albania.

Keywords: rs1333049, Chr9p2, Albanian, CAD

1. Introduction

Coronary artery disease (CAD) is the leading cause of adverse cardiovascular events worldwide. This disease has multiple clinical manifestations ranging from asymptomatic atherosclerosis to stable/unstable angina pectoris, myocardial infarction and sudden cardiac death. Coronary artery atherosclerosis is associated with narrowing of the arterial lumen and formation of atherosclerotic plaques (Sanchis-Gomar et al., 2016; Sayols-Baixeras et al., 2014).A thorough understanding of the entire set of mechanisms involved in the pathogenesis of coronary artery disease is required to treat and prevent its devastating impact on health

Familial clustering of individuals with CAD indicates that genetic factors, among other, play a crucial role in the onset and progression of the disease (Bachmann et al., 2012). The heritability of coronary artery disease is reported to be approximately 50-60%. However, due to heterogeneous nature of clinical coronary artery disease and non-genetic physiological factors that rely on environmental factors it has proven difficult to pinpoint the exact role and involvement of genes in the pathogenesis of CAD (Dai et al., 2016).

To this date, genes suspected to be involved in the development of monogenic CAD such as CYP27A1 ST6GALNAC5 and MEF2A have been shown to be related to lipid metabolism as well as to molecular changes to endothelial walls(InanlooRahatloo et al., 2014; Wang et al., 2003; Zurkinden et al., 2014).

On the other hand, association studies have repeatedly shown a clear relationship between different genetic variants and coronary artery disease. Among important candidate single nucleotide polymorphisms (SNPs), the rs1333049 shows a significant association with CAD as well as its progression (Bown et al., 2008; Çakmak et al., 2015; Chen et al., 2009; Hinohara et al., 2008; Koch et al., 2011; Pinós et al., 2014; Samani et al., 2007; Schunkert et al., 2008; Yamada et al., 2008). The (rs1333049:C>G) polymorphism is located in the 9p21 locus which is not directly related to any known genes. The nearest protein coding genes (~100 Kb) to rs1333049 are CDKN2A and CDKN2B and a non-coding antisense RNA CDKN2BAS. CDKN2A and CDKN2B, which are known as cycling depended kinase inhibitors, are involved in cell cycle regulation which may be involved in cellular endothelial changes associated with progression of atherosclerosis (Cunnington et al., 2010; Helgeland et al., 2015; Yayla et al., 2016; Zhong et al., 2017). The non-coding region CDKN2BAS remains yet unclear in function however it has been hypothesized that it may play a role in the senescence of CDKN2A gene (Congrains et al., 2012).

Based on hospitalization rates, there is an increasing trend of coronary artery disease in Albania (Nichols et al., 2014), which apart from socioeconomic factors, could be attributed to genetic predispositions. Hence, in the present study, we aimed to investigate the association between rs1333049 variant and the presence of coronary artery disease in Albanian patients.

2. Materials and methods

2.1 Study design and eligibility criteria

This was a case-control study involving 177 randomly selected patients who underwent coronary angiography (CAG) at the cardiology department of Hygeia Hospital during 2015-2016 in Tirana, Albania. The study protocol was in accordance with guidelines proposed by Helsinki Declaration. Each patient gave written informed consent. All patients were admitted following positive stress test or clear clinical indication of coronary artery disease. Patients with malignancies, hematological diseases, severe chronic obstructive lung disease and severe infections were excluded from the study. Patient characteristics, including clinical information regarding presence of hypertension (HTN), diabetes mellitus (DM) and other important medical history data were carefully recorded. Blood samples for biochemical analysis were carried out, in accordance to hospital guidelines, prior to coronary angiography procedures. All biochemical analysis was performed in an ISO15189 accredited laboratory. Diagnostic coronary angiography by the Judkins technique was used by two independent invasive cardiologists to evaluate for the presence of CAD. Diagnosis of coronary artery disease was made according to American College of Cardiology Clinical Data Standards (Cannon et al., 2001). Severity and the number of atherosclerotic lesions were assessed for each patient undergoing CAG. Thereafter patients were divided into two groups (according to stenosis), group 1: (No-CAD) patients with insignificant luminal stenosis <50% and group 2:(CAD) patients with significant luminal stenosis ≥50% of at least one major coronary artery (Harris et al., 1980).

2.2 Molecular genotyping

All genotyping was performed at GJENOMA, Molecular Genetics Laboratory, Tirana, Albania. Genomic DNA was extracted from whole K3EDTA blood samples using PureLink™ Genomic DNA Mini Kit (Invitrogen©2012 Life Technologies Corporation). Patient genotypes were determined by allele specific polymerase chain reaction (ASPCR) with specific primers for the ancestral (C) and derived (G) alleles of rs1333049: C > G. Primer selection and PCR protocol was selected according to Ahmed et al (2013). A modification to the PCR reaction adopted from the literature was used to amplify the selected region separately for both alleles. A total reaction volume of 25µl containing 0.4 mM deoxynucleotide triphosphates, 1× Taq

buffer, 1.5 mM MgCl2, 0.2 µM allele specific forward primer, 0.2 µM reverse primer, and 1 U AmpliTaq Gold® DNA Polymerase was employed throughout the experiment. Amplification was carried on an Eppendorf® Mastercycler Personal using a three step PCR reaction consisting of an initial denaturation step of 10 minutes followed by 35 cycles of 94°C for 30 seconds, 59 °C for 30 seconds, 72°C for 30 seconds and a final extension cycle of 72 °C for 10 minutes. A 280 bp fragment expected to be amplified by allele specific primers was separated on a 2% agarose gel. The visualization of DNA fragments was done via a UV trans-illuminator from which were derived the respective genotypes.

2.3 Statistical analysis

Statistical analysis was performed on MedCalc Statistical Software version 14.8.1 (MedCalc Software, Ostend, Belgium; http://www.medcalc.org; 2014), R package version 1.1.456 (R-package.org) and 'SNPassoc Version 1.9-2 (CRAN.R-project.org). Genotype frequencies were tested against Hardy-Weinberg equilibrium to evaluate genotype distribution. The association with CAD was evaluated under 5 genetic models (Co-dominant, Dominant, Recessive, Over-dominant and Log-additive) using C allele as reference and adjusting for risk factors such as age, diabetes mellitus and hypertension. Continuous variables were presented as mean ± standard deviation and were analyzed using non-parametric Mann-Whitney test for independent samples. Categorical data were presented as percentages (%) and compared using Chi-square test or Chi-square for trend (Cochran-Armitage test for trend) when needed. Odds ratio (ORs) and 95% CI were analyzed by logistic regression using Hosmer-Lemeshow test as a statistical test for goodness of fit. Statistical significance was considered for $p < 0.05$

3. Results

3.1 Data analysis of baseline characteristics

As shown in Table 1, the control group (No-CAD) consisted of 64 (36.16%) patients with a mean age of 62.84±9.20 whereas 113 (63.84%) patients were categorized in the CAD group with a mean age of 64.56±7.87. As indicated, there was no statistical significance regarding age between controls and cases (p=0.0701). However, the number of male patients categorized in CAD vs no-CAD group was significantly different (p<0.0001). Of all 177 patients included in the study, 78 (44.06%) were diagnosed with diabetes mellitus, and 102 (57.62%) were hypertensive. There was significant CAD risk associated with diabetes (p=0.0045, OR=2.58 [95%CI=1.3495 – 4.9619]). Hypertension, another risk factor for CAD, was significantly associated with cases (p<0.0133, OR=2.20 [95% CI= 1.1787 – 4.1159]).

Table 1: Baseline characteristics of the all patients included in the study (n=177)

	CONTROLS (no-CAD)	**CASES (CAD)**	**OR(95% CI)**	**p**
AGE	62.84±9.20	64.56±7.87		p = 0.0701
GENDER				
Males (%)	33 (51.5%)	82 (72.5%)		p<0.0001
Females(%)	31 (48.4%)	31 (27.4%)		p=0.898
DIABETES (%)	19 (29.6%)	59 (52.2%)	2.58 (1.3495 - 4.9619)	p=0.0045
HIPERTENSION (%)	29 (45.3 %)	73 (64.6%)	2.20 (1.1787 – 4.1159)	p=0.0133

3.2 Genetic data analysis

As shown in table 2, genotyping of rs1333049:C>G revealed frequencies of CC, CG and GG of 25%, 53.1% and 21.9% in controls and 41.6%, 46%, and 12.4 % in cases. There was no significant departure from Hardy-Weinberg equilibrium in control group (p=0.2584).

3.3 Association between coronary artery disease and rs1333049 polymorphism based upon genetic models.

Based on Akaike Information Criterion (AIC) in Table 2, the best models to describe the association between genotype and development of CAD are consistent with a log-additive and a recessive model of inheritance. Assuming log-additive model, CC carriers had a high risk of developing CAD (OR=1.75 [95%CI: 1.09 - 2.77] p=0.01543). Similarly, consistent with the recessive model, there was a significant association of CC carriers with coronary artery disease (OR=2.12 [95%CI: 1.08 – 4.16] p= 0.02465).

Table 2: Association of rs1333049 with coronary artery disease (CAD)

SNP:rs1333049								
	Control	**%**	**Cases**	**%**	**OR**	**Lower-upper**	**p**	**AIC**
Allele-G	62	48.43	80	35.39				
Allele-C	66	51.57	146	64.61	**1.71**	**1.10 - 2.66**	**0.0165**	
Codominant								
C/C	16	25.0	47	41.6	1.00		**0.0156***	231.6
C/G	34	53.1	52	46.0	0.52	0.26 – 1.06		
G/G	14	21.9	14	12.4	0.34	0.13 - 0.87		
Recessive								
C/C	16	25.0	47	41.6	**2.12**	**1.08 – 4.16**	**0.02465**	230.6
C/G-G/G	48	75.0	66	58.4	1.00			
Dominant								
C/C-C/G	50	78.1	99	87.6	1.96	0.87 – 4.54	0.10199	233.0
G/G	14	21.9	14	12.4	1.00			
Over dominant								
C/C-G/G	30	46.9	61	54.0	1.33	0.71 – 2.43	0.36329	234.8
C/G	34	53.1	52	46.0	1.00			
Log-additive								
0,1,2	64	36.2	113	63.8	**1.75**	**1.09 – 2.77**	**0.01543**	229.8

p-values were obtained from logistic regression analysis. **OR**, odds ratio; **Lower –Upper**, CI 95%; **AIC**, Akaike information criterion. All genetics models of inheritance were calculated for the reference allele "C". * Cochran-Armitage test for trend

The significance seen in recessive and log-additive models of inheritance was re-evaluated after adjustment for other known risk factors such as hypertension, diabetes and age. As shown in table 3, association of CC genotype with CAD under the recessive and log-additive model remained significant (ORrecessive model=2.22 [95%CI: 1.09- 4.54] p=0.02278), (ORlog-additive model=1.81 [95%CI: 1.12 – 2.94] p=0.01360)

Table 3. Association of rs1333049 with coronary artery disease (CAD) (adjusted for; HTN, DM, Age)

SNP:rs1333049								
	Control	**%**	**Cases**	**%**	**OR**	**Lower-upper**	**p**	**AIC**
Codominant								
C/C	16	25.0	47	41.6	1.00		**0.04519***	225.1
C/G	34	53.1	52	46.0	0.50	0.24 – 1.06		
G/G	14	21.9	14	12.4	0.32	0.12 – 0.84		
Recessive								
C/C	16	25.0	47	41.6	**2.22**	**1.09 – 4.54**	**0.02278**	224.1

C/G-G/G	48	75.0	66	58.4	1.00			
Dominant								
C/C-C/G	50	78.1	99	87.6	2.08	0.89 – 5.00	0.09050	226.4
G/G	14	21.9	14	12.4	1.00			
Overdominant								
C/C-G/G	30	46.9	61	54.0	1.35	0.70 – 2.56	0.36763	228.5
C/G	34	53.1	52	46.0	1.00			
Log-additive								
0,1,2	64	36.2	113	63.8	**1.81**	**1.12 – 2.94**	**0.01360**	223.2

pvalues were obtained from logistic regression analysis. **OR**, odds ratio; **Lower –Upper**, CI 95%; **AIC**, Akaike information criterion; HTN, Hypertension; DM, Diabetes mellitus. All genetics models of inheritance were calculated for the reference allele "C". * Cochran-Armitage test for trend

4. Discussion and conclusion

In resemblance to other studies conducted on several other populations, we have showed a significant association between rs1333049 polymorphism and development of coronary artery disease in the Albanian patients. As shown in our study, the frequency of risk allele C was higher in CAD patients (64.61%) as compared to no-CAD group (51.57 %). Whereas, G allele frequency was higher in no-CAD (48.43 %) vs CAD group (35.39%). Furthermore, our data indicate that the magnitude of CAD risk conferred by C allele of rs1333049 (OR=1.71 [95%CI: 1.10-2.66], p=0.0165) was higher than the risk reported by Schunkert et al., 2008 (OR=1.29 (95%CI, 1.22 - 1.37; p=0.0001) in a study of individuals of European origin. In addition, under recessive model of inheritance, we observed an independent association of the homozygous genotype CC of rs1333049 with development of CAD among Albanian patients. The association was evaluated before and after adjustment for other covariates such as, hypertension, diabetes and age (ORunadjusted=2.12 *vs* ORadjusted=2.22). Similarly, under log-additive model of inheritance, the association of homozygous risk genotype CC with development of CAD remained comparable (ORunadjusted=1.75 *vs* ORadjusted= 1.81). The slight increasing trend between adjusted *vs* unadjusted odds ratio (ORs) can be attributed to relatively small sample size and uneven distribution of patients according to gender. Nevertheless, the main advantage of the study remains in the rigorous selection criteria of case *vs* control group which was based on angiographic data rather than being limited to patient medical history. Nonetheless, a larger sample size is required to evaluate the frequency of risk allele-C in Albanian population. In addition, evaluation of other proxy polymorphisms to rs1333049 as well as adjustment for other covariates such as lipid profile would provide greater understanding between the association of this polymorphism and development of CAD in Albania.

Our findings provide substantial evidence for the involvement of rs1333049 polymorphism in coronary artery disease in Albanian population. CAD risk conferred by C-allele of rs1333049 polymorphism in our patients was higher than the reported association of the risk allele-C with CAD in Caucasians. Therefore, these results have significant importance in the light of marked prevalence of coronary heart disease in Albania

Nomenclature

CAD	Coronary artery disease
HTN	Hypertension
DM	Diabetes mellitus
CAG	Coronary angiography
PCR	Polymerase chain reaction
ASPCR	Allele specific polymerase chain reaction

OR	Odds ratio
AIC	Akaike information criterion
CI 95%	95%Confidence interval

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