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Short Communication

Association of *ANRIL* polymorphism (rs1333049:C>G) with myocardial infarction and its pharmacogenomic role in hypercholesterolemia

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ABSTRACT

Single nucleotide polymorphisms (SNPs) of non-coding RNA in the INK4 locus (*ANRIL*) have been found to be associated with myocardial infarction (MI). However, the effect of rs1333049:C>G in INK4 locus in familial hypercholesterolemia patients and on lipid profile of the patients has not been studied in Pakistan. We therefore investigated the association of SNP rs1333049:C>G with MI as well as familial hypercholesterolemia patients and also determined the effect of genotype on lipid levels in a northern Pakistani population. A case–control association study was performed in which 611 individuals (294 patients, 290 healthy controls and 27 patients from hypercholesterolemia families) were genotyped for rs1333049:C>G, using an Allele specific polymerase chain reaction. We found a significant association of rs1333049:C>G with MI ($\chi^2 = 22.3$, $p<0.001$). The frequency of risk genotype CC was significantly different from the healthy controls ($p<0.001$, $\chi^2 = 22.3$). The risk allele C was at a higher frequency in the MI patients as compared to the controls (odds ratio [OR] = 1.55 (95% confidence interval [CI] = 1.22–1.96), $p<0.001$). The logistic regression analysis for the genotype distribution resulted in strong association of risk allele C with MI under recessive model (OR = 3.17 (95% CI = 1.85–5.44) $p<0.001$). When the data were further analyzed along the lines of gender, a significant association with both males and females was observed.

The pleiotropic role of rs1333049 was revealed further when CC genotype hypercholesterolemic individuals on statins were found to have a significantly lower TC, LDL-C and Tg levels as compared to the CG and GG individuals ($p<0.05$). The current study demonstrates a strong association of the *ANRIL* SNP (rs1333049) with MI as well as familial hypercholesterolemia patients in a northern Pakistani population and could be used as a useful genetic marker for the screening of MI in the general Pakistani population.

1. Introduction

Myocardial infarction (MI) is one of the major causes of morbidity and mortality worldwide. The conventional risk factors (hypertension, smoking, hypercholesterolemia and diabetes) contribute 50–60% toward disease susceptibility, while genetic variations account for predisposition in 40–50% of the sporadic cases (Baudhuin, 2009). Recently a number of genome wide association studies (GWAS) have identified a locus on chromosome 9p21 spanning a 58 kb region to be associated with coronary artery disease (CAD) and MI (Cunnington et al., 2010; Jarinova et al., 2009). Although this locus has no prominent atherosclerosis associated genes, but in this region an antisense non-coding RNA in the INK4 locus (*ANRIL*) resides within the vicinity of the cell cycle regulating genes, and this locus is also in strong linkage disequilibrium (LD) with the cell proliferation genes including cyclin dependant kinase inhibitors 2A and 2B (CDKN2A and CDKN2B) (Cunnington et al., 2010). The *ANRIL* locus has been shown to modulate the expression of neighboring genes by presumably acting through different mechanisms like RNA interference, gene silencing, chromatin remodeling or DNA methylation (Jarinova et al., 2009). Till date the function of *ANRIL* is not clear and there is no direct involvement of the above mentioned proteins in

Abbreviations: ANRIL, antisense non-coding RNA in the INK4 locus; MI, myocardial infarction; GWAS, genome wide association studies; CAD, coronary artery disease; LD, linkage disequilibrium; CDKN2A, cyclin dependant kinase inhibitor 2A; CDKN2B, cyclin dependant kinase inhibitor 2B; T2D, type 2 diabetes; CPK, creatine phosphokinase; CK-MB, creatine kinase -MB; MLR, multiple linear regression; UM, unaffected males; UF, unaffected females; AM, affected males; AF, affected females; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TC, total cholesterol; Tg, triglycerides; ncRNAs, non-coding RNAs; TDGF1, teratocarcinoma-derived growth factor 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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atherosclerosis but they play a vital role in cell proliferation, aging, apoptosis and also function as tumor suppressors (Ding et al., 2009; Farzaneh-Far et al., 2009).

Samani and Schunkert (2008) had indicated a major role of 9p21 chromosomal region in the manifestation of CAD in Europeans, as they found it to contain a risk haplotype, which also included a single nucleotide polymorphism (SNP) rs1333049:C>G. In addition this region was found to be in LD with type 2 diabetes (T2D) in a population specific manner (Cheng et al., 2011; Zeggini et al., 2007).

Based upon the results of recent studies, which had shown a strong association of rs1333049:C>G with CAD and MI (Helgadottir et al., 2007; Holdt et al., 2010), we investigated its role in the onset of MI in a northern Pakistani population and also statistically determined its effect on the lipid profile of the patients.

2. Methods

2.1. Ethics declaration

This study conforms to the tenants of the Helsinki declaration and was approved by the Ethics Committee and Institutional Review Board of Shifa College of Medicine/Shifa International Hospital, Islamabad. All the patients and healthy controls were informed about the significance of the study in their local language and informed written consent was taken from them.

2.2. Selection criteria and sample collection

A total of 294 MI patients were enlisted from local hospitals, who were clinically classified on the basis of standard World Health Organization criteria, which included; typical chest pain for more than 20 min, elevated cardiac specific markers including Troponin T and ST changes on electrocardiogram (deWerf et al., 2008). The patients included in the current study were index MI cases with no previous history of an episode, in addition the patients had cardiac marker serum ranges of Creatine PhosphoKinase (CPK) > 196 and Creatine Kinase (CK-MB) > 25. The patients were on Atorvastatin and had stabilized lipid levels at the time of blood sampling. Patients with normal ECG regardless of chest pain were excluded from the study. In addition to patients, 290 healthy control individuals and a panel of 27 males suffering from familial hypercholesterolemia were also selected based on criteria described by Marks et al. (2003). The anthropometric information about the MI cases and healthy control individuals are summarized in Table 1.

DNA isolation and lipid profile determination of the 611 individuals were performed as described previously (Ajmal et al., 2011).

2.3. Genotyping

Allele specific primers for the ancestral (C) and derived (G) alleles of rs1333049:C>G, were designed to selectively amplify the relevant target sequences (Little, 2001). The sequence of the forward primer for the C allele was; 5′-TCC TCA TAC TAA CCA TAT GAT CAA CAG TTC-3′ and for G allele the sequence was: 5′-TCC TCA TAC TAA CCA TAT GAT CAA CAG TTG-3′. The internal control primer sequence was: 5′-GAA GAT CAT ACC CGA AGT AGA GCT GC-3′. For all the forward primers a common reverse primer was used, with the sequence: 5′-ATA CCA CAG TGA ACA TAA TTG TGC ATA CAT-3′.

Amplification of ancestral and derived alleles was performed separately in a total reaction volume of 25 μl each, containing 0.2 μM deoxynucleotide triphosphates, 1× Taq buffer, 3 mM $MgCl_2$, 0.2 μM allele specific forward primer, 0.3 μM reverse primer, 0.1 μM internal control primer and 1.0 U Taq polymerase. Thermocycling consisted of an initial denaturation step of 95 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 59 °C for 30 s, 72 °C for 30 s and a final extension cycle of 72 °C for 5 min.

The amplified products consisting of a 280 bp fragment for the allele specific primers and 500 bp for the internal control were electrophoretically separated on 2% agarose gels, DNA bands were visualized by UV trans-illumination, the image documented using the BioCap MW software (ver. 11.01, Vilber Lourmat, France) and the genotype data were then calculated.

2.4. Statistical analysis

The data were statistically analyzed using Chi-Square and multiple linear regression (MLR) analysis to determine the statistical significance of the difference in frequencies between patients and healthy population, a $p<0.05$ was taken as statistically significant.

3. Results

3.1. The anthropometric data of the cases and controls

The mean age of MI cases was 53.1 years, there were more smokers among the MI cases as compared to the healthy controls, which was significantly different ($p<0.0001$, $\chi^2=18.3$; odds ratio [OR] = 2.67 [95% confidence interval (CI) = 1.69–4.62]). Body mass index and obesity were not found to be risk factors for MI in the current study as no significant difference was observed between the MI cases and healthy controls. However, diabetes mellitus was found to be significantly associated with the disease ($p<0.0001$, $\chi^2=34.9$, OR = 12.3 [95% CI = 4.4–34.4]). Hypertension, another risk factor for MI, was also significantly associated with the cases $p<0.0001$, $\chi^2=42.2$, OR = 6.84 (95% CI = 3.61–12.96). Breathlessness after mild exertion was also observed to be significantly higher in MI cases as compared to the healthy controls ($p<0.0001$, $\chi^2=16.98$, OR = 3.3 [95% CI = 1.83–5.95]). A positive coronary artery disease family history was not associated with MI cases as compared to the healthy controls ($p>0.05$, $\chi^2=0.13$, OR = 0.92 [95% CI = 0.61–1.40]; Table 1).

3.2. Genetic analysis

In the MI patients (male + female) the genotype distribution of rs1333049:C>G was significantly different ($\chi^2=22.3$, $p<0.001$; Table 2) from the controls (male + female). The difference between the two groups is the higher proportion of CC risk genotype in patients as compared to healthy controls. Logistic regression analysis also revealed association of the genotypes with MI under the recessive model (OR = 3.17 [95%; CI = 1.85–5.44], $p<0.001$).

The frequency of risk allele C was also significantly different between MI patients as compared to the healthy individuals ($\chi^2=13.5$, $p<0.001$, OR = 1.55 [95% CI = 1.22–1.96], $p<0.001$; Table 2).

3.3. Gender based analysis

The rs1333049:C>G was further analyzed for any gender specific association; the CC genotype was found to be associated with MI in

Table 1
The anthropometric information about the MI cases and healthy control individuals.

	Controls	MI cases	OR (95% CI)	p (χ^2)
Age	40 ± 12	53.1 ± 11.3		
Male (%)	59.8	70.1		>0.05
BMI	24.1 ± 3.2	24.6 ± 4.2		>0.05
Smoker (%)	22.2	51.2	2.67 (1.69–4.62)	<0.0001 (18.3)
Obesity (%)[a]	7.4	9.4	1.69 (0.71–3.99)	>0.05 (1.5)
Diabetes (%)	3.4	30.6	12.3 (4.4–34.4)	<0.0001 (34.9)
Hypertension (%)	10.5	45.3	6.84 (3.61–12.96)	<0.0001 (42.2)
Breathlessness (%)	18.8	43.4	3.3 (1.83–5.95)	<0.0001 (16.9)
CAD history (%)	44.4	42.54	0.92 (0.61–1.40)	>0.05 (0.13)

MI = myocardial infarction, BMI = body mass index, CAD = coronary artery disease.
[a] Persons having >30 BMI were considered to be obese.