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ORIGINAL ARTICLE

Association analysis of rs1049255 and rs4673 transitions in *p22phox* gene with coronary artery disease: A case-control study and a computational analysis

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Abstract
Background The *p22phox* gene encodes the main subunit of NADH/NADPH-oxidase. This enzyme is expressed in smooth muscle cells of arteries, and it produces the reactive oxygen species. On the other hand, oxidative stress plays a main role in the pathogenesis of coronary artery disease (CAD).
Aim The aim of this study is to evaluate the association between rs4673 and rs1049255 polymorphisms of *p22phox* gene with CAD in an Iranian population which was followed with a computational analysis approach.
Methods In a cross-sectional study, we collected blood samples of 302 Iranian Caucasian including 143 patients and 159 healthy controls. Genotype of the polymorphisms was detected through PCR-RFLP method. A computational analysis was also performed using SNAP, Polyphen-2, Chou-Fasman, RNAsnp, and miRNA SNP databases.
Results Data of case control study demonstrated that CT genotype ($R = 1.84$, 95% CI = 1.13–3.00, $p = 0.014$) and T allele (OR = 1.53, 95% CI = 1.09–2.15, $p = 0.013$) of rs4673 polymorphism, have a significant association with enhanced risk of CAD. But rs1049255 analysis demonstrated the absence of such an association with CAD. Indeed, in silico data analysis demonstrated that rs4673 transition could impact on function of p22phox protein (SNAP score 56, expected accuracy 75%; Polyphen-2 score 0.99, sensitivity 0.09, specificity 0.99). Data derived from miRNA SNP database demonstrated that rs1049255 polymorphism increases the affinity of attachment between has-miR-3689a-3b with 3′-UTR of *p22phox* gene.
Conclusion Our data demonstrated that rs4673 transition may be involved in susceptibility to CAD and could be applied as a potential biomarker for this disease.

Keywords Computational analysis · Coronary artery disease · Genetic association · *p22phox* gene

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Introduction

Coronary artery disease (CAD; OMIM: 608320), the most common manifestation of cardiovascular diseases, is a multifactorial disease with complex etiology in which various environmental and genetic factors impact on its incidence [1]. Thus, evaluation of causes of CAD should encompass through both risk factors as diabetes, hypertension, hyperlipidemia, and genetic susceptibility [2, 3]. Oxidative stress plays a fundamental role in development of CAD as a primary pathogenic step [4, 5]. Over production of reactive oxygen species (ROS) due to imbalance in oxidative anti-oxidant systems, results in subsequent inflammatory events in immune and endothelial cells [6]. NADH/NADPH oxidases are the most important enzyme involved in innate immune system which produces active oxygen species. They most often express in vascular smooth muscle cells (VSMCs), endothelial cell, and phagocytosis [7]. The phagocytic NADH/NADPH oxidase is a flavoprotein which includes two-transmembrane subunits

(gp91phox and p22phox) and three cytosolic proteins (p40phox, p47phox, and p67phox) [8]. Other NOX isoforms are expressed in thyroid and colon cells and pair with p22phox subunit [9]. Thus, p22phox is considered as an essential component for NADH/NADPH oxidase function [10]. Nicotinamide adenine dinucleotide phosphate [NAD(P)H] system might be involved in pathophysiology of CAD. This system is the predominant source of ROS production in vascular cells [11]. In addition, *p22phox* expression is more in human atherosclerotic vs. non-atherosclerotic coronary arteries [12].

The *p22phox* (*CYBA*) gene is located on long arm of chromosome 16 (16q24) and encodes p22phox subunit [13], and it has several polymorphisms [14]. Among them, rs4673 (c.214 T>C; also, it is traditionally known as C242T) and rs1049255 (c.*24G>A) polymorphisms have been widely investigated in cardiovascular complications, but results are still controversial [15]. The rs4673 polymorphism resulted in histidine to tyrosine substitution on codon 72 (Y72H) of protein, while rs1049255 polymorphism is located on 3′-UTR of *p22phox* gene (Fig. 1). This study aimed to evaluate the association between aforementioned single nucleotide polymorphisms (SNPs) and CAD in an Iranian population parallel with an in silico analysis.

Methods and materials

Subjects

In this case-control study, total of 302 Iranian Caucasians including 143 patients with CAD and 159 age-matched healthy subjects were participants. Case group were selected from CAD patients admitted to cardiology department of Shahid Beheshti hospital (Kashan, Iran) with the age more than or equal to 55 years old, between 2014 and 2015. CAD was confirmed angiographically by stenosis severity 50% or more in at least on coronary artery. Patients with history of diabetes mellitus, malignancy, clinical evidence of coagulopathy, collagenosis, acute intoxication (amphetamine and carbon oxide), and cardiovascular, renal and hepatic disorders were excluded from this study. Control group included healthy subjects referred to hospital for routine check-up tests with exclusion criteria including signs and symptoms of acute myocardial infarction, diabetes mellitus, CAD, and other familial and genetic disorders. Some demographic data and biochemical features of participants are included in Table 1. After getting signed informed consent, 2 ml of blood was drawn from all participants and collected in CBC tubes and stored at −20°C.

SNP genotyping

Genomic DNA was isolated from blood samples by a DNA extraction kit (Bioneer Co., Korea). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used for genotyping of rs4673 and rs1049255 polymorphisms. For this purpose, the fragments containing aforementioned SNPs were amplified by specific primers and condition which are given in Table 2. PCR was performed by Eppendorf thermal cycler (Eppendorf Co., Germany) in 25 μl total volume containing 0.25 μM each of forward and reverse primers, 1 mM $MgCl_2$, 100 μM dNTP mixture, 1 unit of *Taq* polymerase, and 50 ng of DNA template. All of PCR reagents were purchased from Fermentas Company (Fermentas, Leon-Rot, Germany). About 10 μl (≈0.1 μg) of the PCR products containing c.214 T>C and c.*24G>A polymorphisms were respectively treated with 5 units of *Rsa*I (Fermentas) and *Dra*III (Fermentas) restriction enzymes according to the protocol (Table 3). Genotypes of rs4673 and rs1049255 polymorphisms were detected by 1% agarose gel electrophoresis.

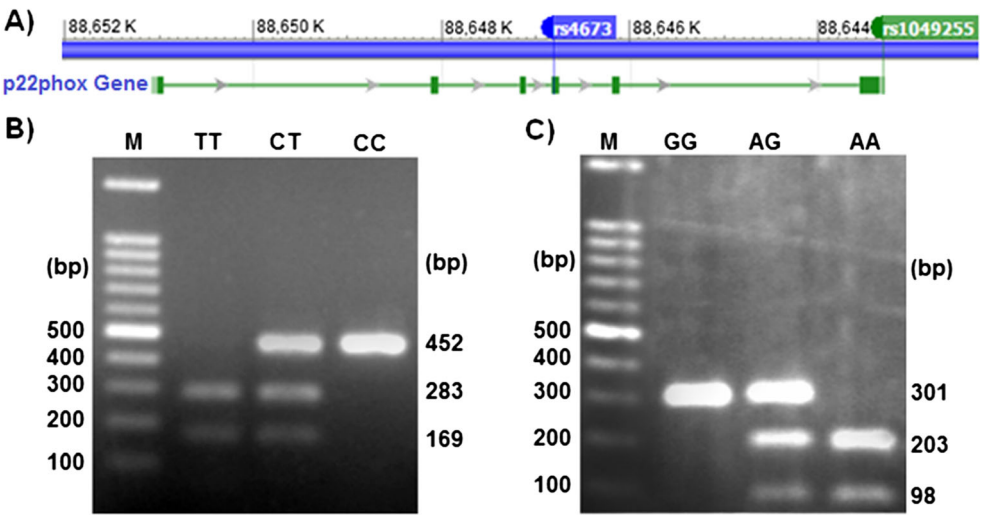


Fig. 1 The *p22phox* gene map and PCR-RFLP results. Human *p22phox* gene map was obtained from NCBI which rs4673 and rs1049255 are located on exons 4 and 6, respectively (**a**). Restriction enzyme map of the 452-bp fragment following *Rsa*I treatment (**b**). Restriction enzyme map of the 301-bp fragment following *Dra*III treatment (**c**)

Table 1 Demographic and biochemical features of the subjects

Variables	Case (n = 143)	Control (n = 159)	p value
Age (years)	62.32 ± 3.05	61.64 ± 3.96	0.099
Gender (m/f)	95/48	112/47	0.454
Smoking (y/n)	64/79	64/95	0.429
BMI (kg/m^2)	24.608 ± 2.46	24.33 ± 2.34	0.320
HDL (mg/dl)	42.61 ± 4.55	42.11 ± 4.44	0.333
LDL (mg/dl)	116.21 ± 14.70	112.80 ± 19.23	0.082
TG (mg/dl)	134.35 ± 23.94	129.07 ± 25.65	0.066
TC (mg/dl)	136.80 ± 24.28	140.97 ± 23.19	0.129

The data are expressed as mean ± standard deviation

M male, *F* female, *Y* yes, *N* no, *BMI* body mass index, *HDL* high density lipoprotein, *LDL* low-density lipoprotein, *TG* triglycerides, *TC* total cholesterol

Computational analysis

Complete coding sequence of *p22phox* gene was obtained from NCBI database (Accession number: NC_000016). The impact of rs4673 polymorphism on function and structure of protein was evaluated using SNAP [16] and PolyPhen-2 [17] software. Indeed, the impact of this transition on protein hydrophobicity was evaluated through Kyte-Doolittle scale [18]. The possible impacts of Y72H substitution on secondary structure of protein was evaluated using Chou-Fasman method [19]. The impact of rs4673 and rs1049255 transitions on mRNA structure was evaluated using RNAsnp web server [20]. The miRNA SNP ver2.0 database was used for evaluation of miRNA interaction with 3′-UTR of *p22phox* transcript [21]. In order to predict the impact of rs1049255 on affinity between 3′-UTR of *p22phox* gene with miRNAs, the above mentioned server was applied.

Statistical analysis was performed using SPSS ver.19 software (SPSS Inc., IBM Corp Armonk, NY, USA). The numerical variables were compared by independent *t*-test whereas qualitative variables were compared by chi-squared test. Hardy–Weinberg equilibrium (HWE) was calculated for both case and control groups through chi-squared test. The association of various genotypes and alleles with CAD risk was assessed by odds ratio (OR) and 95% confidence interval (CI) which were calculated by binary logistic regression. A p value less than 0.05 was considered statistically significant.

Results

PCR-RFLP

Human genome showed a principal band with low mobility on agarose gel after electrophoresis. While human genome was applied as template in PCR, *p22phox* fragments including rs4673 and rs1049255 were produced with 452- and 301-bp length, respectively. After electrophoresis of digested products of rs4673, CC, CT, and TT genotypes showed one (452-bp), three (452-, 283-, and 169-bp), and two (169- and 83-bp) bands on agarose gel, respectively. But regarding rs1049255 polymorphisms, GG, AG, and AA genotypes showed one (301-bp), three (301-, 203-, and 98-bp) and two (203- and98-bp) bands on agarose gel, respectively.

Allelic and genotypic frequency distribution

Statistical analysis demonstrated that frequency distribution of rs4673 (χ^2 = 0.642, p = 0.422) and rs1049255 (χ^2 = 2.100, p = 0.147) genotypes in control group, were in the Hardy–Weinberg equilibrium. Allelic and genotypic frequency distribution of mentioned polymorphism in a case and control groups are shown in Tables 4 and 5. Regarding rs4673 polymorphism, frequency distribution of CC, CT, and TT genotypes in case group were 35.66, 50.35, and 13.99%, respectively. While these values for control groups were 50.94, 38.99, and 10.07%, respectively. Genotype analysis revealed a significant association between CT genotype and CAD risk (OR = 1.84, 95% CI = 1.13–3.00, p = 0.014). Indeed, T allele carriers (CT + TT) showed increased risk for development of CAD (OR = 1.87, 95% CI = 1.18–2.97, p = 0.008). Allelic analysis demonstrated a significant association between T allele and CAD risk (OR = 1.53, 95% CI = 1.09–2.15, p = 0.013). Genotype evaluation of rs1049255 polymorphism demonstrated that frequency of AA, AG, and GG genotypes in case group, were 42.66, 39.86, and 17.48%, respectively. While these ratios in control group, were 49.06, 38.36, and 12.58%, respectively. Statistical analysis revealed absence of any association between AG (OR = 1.19, 95% CI = 0.73–1.96, p = 0.479) and GG (OR = 1.60, 95% CI = 0.81–3.14, p = 0.174) genotypes with CAD risk. Indeed, G allelic of rs1049255 polymorphism was not associated with CAD risk (OR = 1.28, 95% CI = 0.92–1.80, p = 0.145).

Table 2 Primer sequences and polymerase chain reaction conditions

SNP (rs no.)	Primer sequence (5′→3′)	PCR conditions	Product size
c.214 T>C (rs4673)	5′-TGGAGCGCTGGTGAGTCTCC 5′-GTGGCTCCTGTCCAGGCAGC	30 cycles of 94 °C (30 s), 59 °C (45 s), and 72 °C (45 s)	452-bp
c.*24G>A (rs1049255)	5′-CCGGGAGCGGCCGCAGATCG 5′-AGGCCTCGGGAACCATCGCT	30 cycles of 94 °C (30 s), 63 °C (45 s), and 72 °C (45 s)	301-bp

Table 3 Digestion conditions of the restriction enzymes

SNP (rs no.)	Restriction enzyme	Restriction site	Digestion products (bp)		
			Wild type	Mutant type	Heterozygote
c.214 T>C (rs4673)	*Rsa*I	GT^AC	452	283, 169	452, 283, 169
c.*24G>A (rs1049255)	*Dra*III	CACNNN^GTG	203, 98	301	301, 203, 98

In silico analysis

Bioinformatics analysis demonstrated a significant impact of rs4673 polymorphism on structure and function of *p22phox* protein. Data derived from SNAP server demonstrated that histidine to tyrosine substitution at codon72 of p22phox protein might be considered as a functional mutation for protein (with score: 56; expected accuracy: 75%; Fig. 2). Data from Polyphen-2 software in both HumDiv (score, 1.00; sensitivity 0.00; specificity 1.00) and HumVar (score, 0.99; sensitivity 0.09; specificity 0.99) algorithm demonstrated significant impact of rs4673 polymorphism on protein function (Fig. 2). Hydrophobicity analysis demonstrated reduced rate of hydrophobicity at codon 72 from −0.311 to −0.522 consequent to Y72H substitution (Fig. 3). Chou-Fasman analysis revealed altered secondary structure of protein by rs4673 polymorphism (Fig. 3). This analysis demonstrated reduced percentage of sheet structures in mutated state from 57.9 to 55.4%.

Effects of rs4673 and rs1049255 polymorphisms on mRNA structure were evaluated using RNAsnp server. Absence of any impact on mRNA structure by rs4673 (distance 0.0122; *p* value 0.823) and rs1049255 (distance 0.0324; *p* value 0.648) polymorphisms was observed (Fig. 4). In this study, we have evaluated the effects of rs1049255 polymorphism which is located on 3′-UTR of gene, on interaction between miRNA and 3′-UTR of *p22phox* gene. Data derived from miRNA SNP database v2.0 demonstrated that rs1049255 polymorphism results in increased strength of affinity between has-miR-3689a-3b and 3′-UTR of *p22phox* gene. The energy derived from attachment of wide type and mutant mRNA with this miRNA was −24.5 and −25.0 kcal/mol, respectively (Fig. 4).

Table 4 Genotype and allele frequencies of c.214 T>C in cases and controls

Genotype/allele	No. and percentage		OR (95% CI)	*p* value
	Control (*n* = 159)	Case (*n* = 143)		
CC	81 (50.94%)	51 (35.66%)	–	–
CT	62 (38.99%)	72 (50.35%)	1.84 (1.13–3.00)	*0.014*
TT	16 (10.07%)	20 (13.99%)	1.99 (0.94–4.18)	0.071
CT + TT	78 (49.06%)	92 (64.34%)	1.87 (1.18–2.97)	*0.008*
C	224 (70.44%)	174 (60.83%)	–	–
T	94 (29.56%)	112 (39.17%)	1.53 (1.09–2.15)	*0.013*

Significant differences between the case and control groups are italicized

OR odds ratio, *CI* confidence interval

Discussion

Nowadays, CAD is considered as a great clinical problem, worldwide [22]. Despite of advances made in this filed, prevention remained still the best challenging with this disease. Thus, identification of CAD risk factors is paramount value. Several genetic factors involved in enhanced susceptibility to CAD [23, 24]. Polymorphism of genes involved in reactive oxygen species system such as *p22phox* gene could be considered as important risk factors for CAD [25]. In this study, the association between rs4673 and rs1049255 polymorphism with CAD in an Iranian population was evaluated. In our study, CT genotype and T allele of rs4673 polymorphism showed significant association with CAD risk. But analysis of rs1049255 polymorphism demonstrated that GG and AG

Table 5 Genotype and allele frequencies of c.*24G>A in cases and controls

Genotype/allele	No. and percentage		OR (95% CI)	*p* value
	Control (*n* = 159)	Case (*n* = 143)		
AA	78 (49.06%)	61 (42.66%)	–	–
AG	61 (38.36%)	57 (39.86%)	1.19 (0.73–1.96)	0.479
GG	20 (12.58%)	25 (17.48%)	1.60 (0.81–3.14)	0.174
AG + GG	81 (50.94%)	82 (57.34%)	1.29 (0.82–2.04)	0.266
A	217 (68.24%)	179 (62.59%)	–	–
G	101 (31.76%)	107 (37.41%)	1.28 (0.92–1.80)	0.145

OR odds ratio, *CI* confidence interval

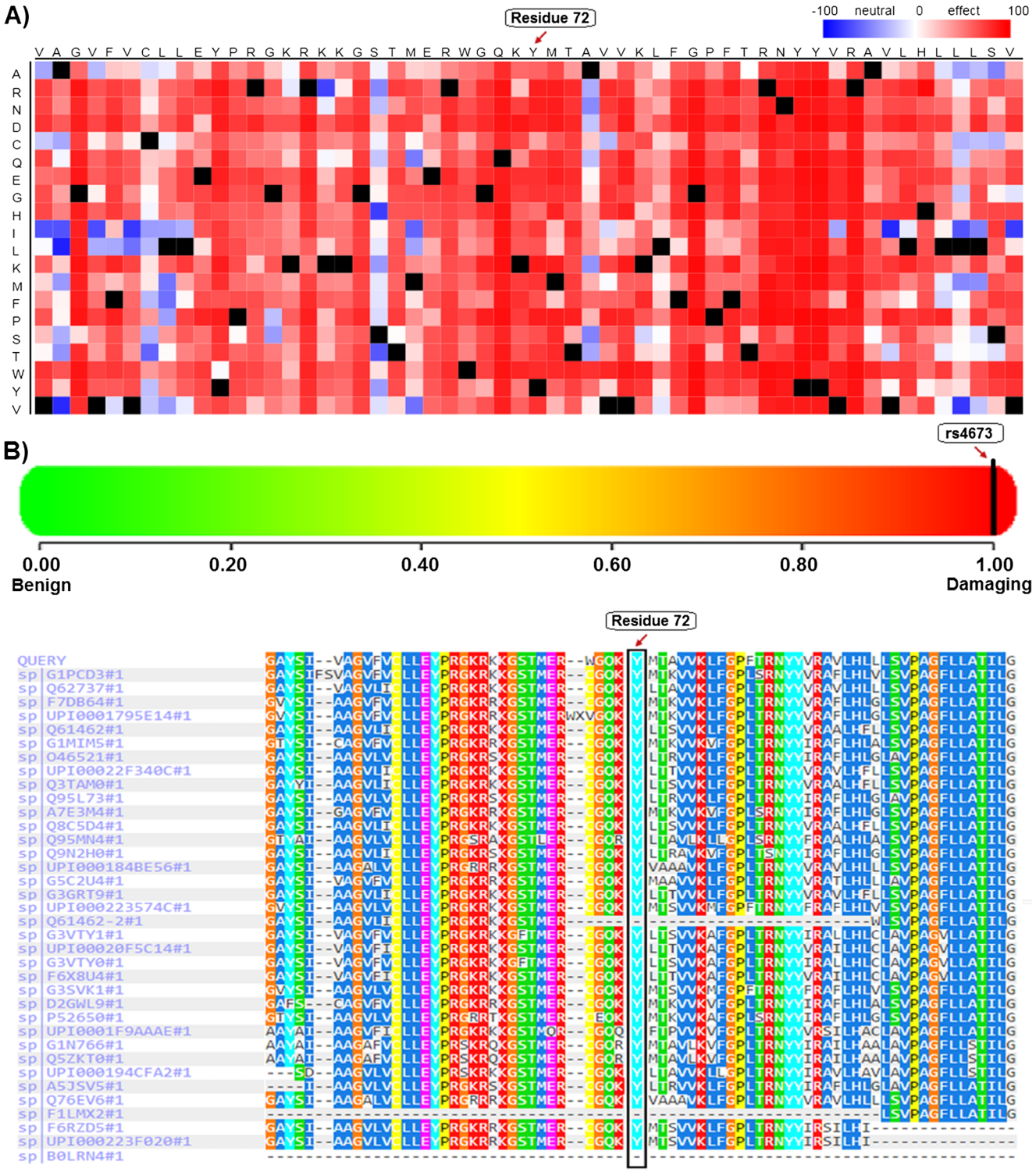


Fig. 2 SNAP and Polyphen2 prediction results. SNAP predicted Y72H substitution as a functional mutation (**a**). Polyphen-2 predicted Y72H mutation to be damaging according to homology search (**b**). The residue 72 is shown by *arrowhead*

genotypes and G allele, had no significant association with CAD risk. By now, several investigations have been performed regarding association between rs4673 and rs1049255 polymorphisms of *p22phox* gene and CAD risk. Recently, results of these studies have been evaluated in two meta-analysis [15, 25]. Wu et al. (2013) have evaluated the association between rs4673 polymorphism of *p22phox* gene with CAD risk in a meta-analysis approach. They revealed the association between rs4673 polymorphism and CAD risk under the recessive genetic model in Caucasian population [25]. But

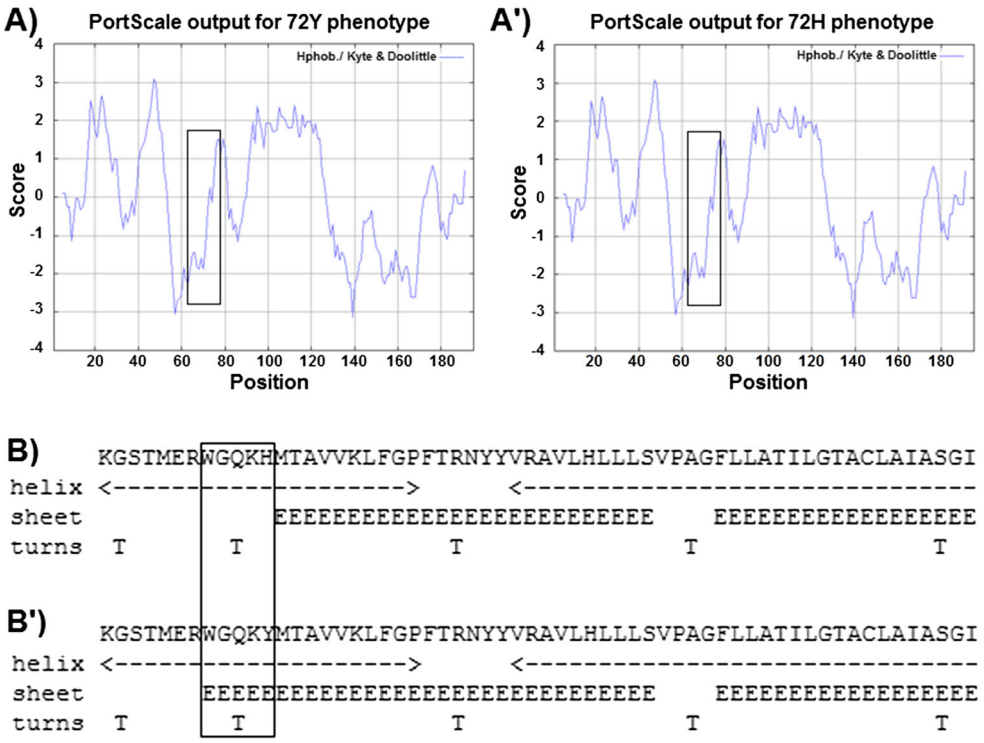
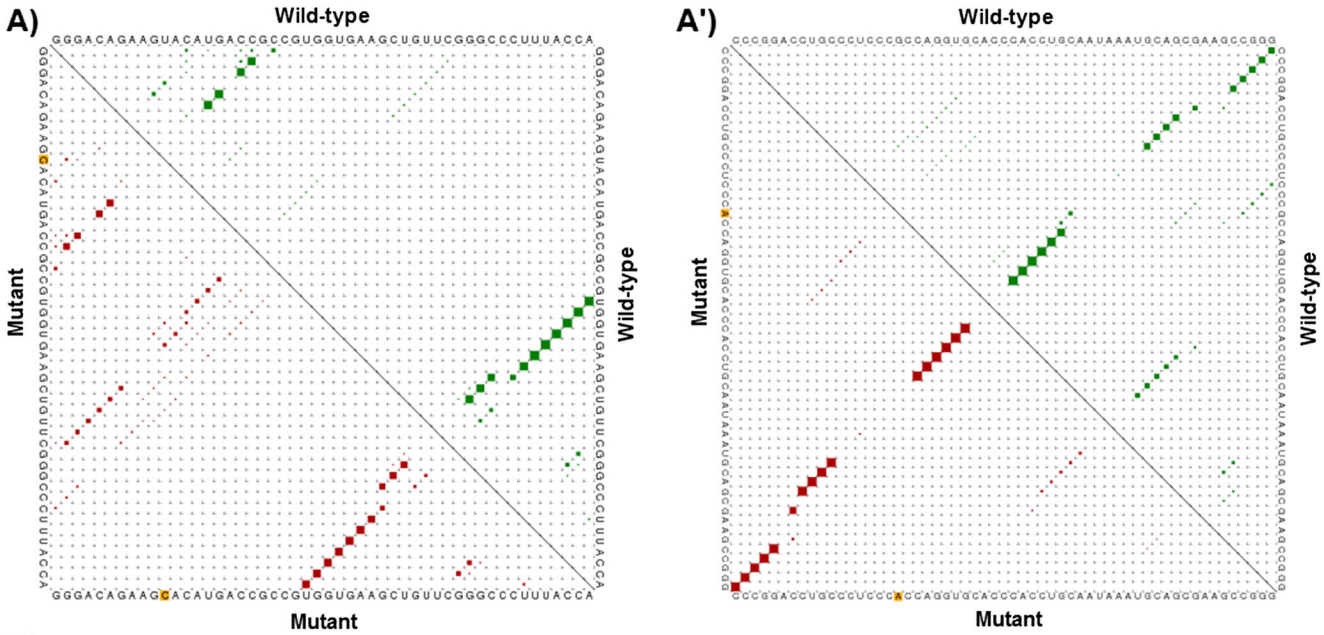


Fig. 3 Hydrophobicity and secondary structure predictions. Hydrophobicity plot for 72Y and 72H phenotypes, respectively (*A* and *A′*). ChoueFasman's secondary structure for 72Y and 72H phenotypes, respectively (*B* and B′). The residue 72 is shown by *boxes*



B)

SNP in 3'UTR of CYBA gene	miRNA	SNP location and Target site on UTR	Energy change (kcal/mol)	miRNA/SNP-target duplexes	Effect by SNP on 3'UTR
rs1049255 (G/A)	hsa-miR-3689a-3p	23 4-24	Wild: -24.30 Mutant: -25.00	miRNA: 3'ugGUGCUAUAGUGUGGAGGGUc 5' UTR: 5'gcCCCGG-ACCUGCCCUCCCAc 3' rs1049255: G --> A	gain

Fig. 4 Results of RNAsnp and miRNA SNP databases. Confined area with determined differences in mutant and wild-type *p22phox*-mRNA for rs4673 (*A*) and rs1049255 (*A′*). The probabilities of wild-type and mutant sequences are presented in *upper* and *lower triangle* of the plots, respectively. The polymorphic position is highlighted in *yellow* (*A* and *A′*). The rs1049255G>A transition increases the affinity of has-miR-3689a-3b with 3′-UTR of *p22phox* (*B*)

Xu et al. (2014) showed protective effect of rs4673 polymorphism on CAD risk in Asian population. Decreased risk of CAD was also seen with rs1049255 polymorphism [15]. These controversial results from similar studies could be attributed to racial and environmental factors.

The relationship between *p22phox* gene and CAD risk could be explained through some molecular mechanisms. Some empirical findings are in favor of physiologic roles of some of the *p22phox* gene polymorphisms, in which some of them are able to modify expression and activity of NADPH oxidase in the cardiovascular disease [26]. In an animal model of essential hypertension, enhanced activity of aortic NADPH oxidase and increased expression of *p22phox* in spontaneously hypertensive vs. normotensive rats were seen which suggest that altered p22phox expression might be regulated through genetic variants of *p22phox* [27]. Our previous genetic association studies demonstrated that in silico analysis is an appropriate approach for evaluation of polymorphism effect on mRNA structure [28, 29], protein function [30, 31], and gene expression [32]. The rs4673 polymorphism is a nonsynonymous single nucleotide polymorphisms (nsSNPs) with histidine to tyrosine substitution on codon 72 of p22phox protein with the ability to alter structure and function of *p22phox* protein. Savas et al. 2006, have demonstrated that this polymorphism is possibly damaging for protein structure [33]. Najafi et al. (2012) have identified the absence of any effect of this polymorphism on secondary structure of protein [14]. Our bioinformatics analysis have demonstrated any impact of rs4673 polymorphism on mRNA structure, but secondary structure and function of protein were both altered in this setting. Indeed, we detected no effect by rs1049255 polymorphism on 3′-UTR of gene on mRNA structure of *p22phox* gene. But this polymorphism alters the binding affinity of has-miR-3689a-3b with 3′-UTR which might impact the expression of the gene. In an experimental study, Bedard et al. (2009) created reporter constructs containing luciferase coding sequence and variants (c.*24G and c.*24A) of the *p22phox* 3′-UTR. They observed that the construct with 3′-UTR containing c.*24G variant had an activity similar to the control construct. But, the construct with 3′-UTR containing c.*24A variant had a substantially decreased reporter gene activity [34]. With regard to rs4673 polymorphism, our in silico analysis revealed that this polymorphism has a significant effect on the *p22phox* protein function. By the same token, Zhu et al. (2006) reported that this SNP might influence protein function because it located in an important region for maturation of the protein [35].

One limitation of this study is the lack of angiographic results of control subjects. Some other limitations of our study include small sample size, and further studies with larger sample size and different races and considering gene-gene and gene-environmental interaction are needed.

Conclusions

Our data demonstrated that rs4673 polymorphism may be potential risk factor for CAD in Iranian populations. Although this association was not seen with rs1049255 polymorphism, this polymorphism could alter the affinity for attachment of miRNA with 3′-UTR of gene.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Informed consent Informed consent was obtained from all individual participants included in the study.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the medical ethic committee of research council of Kashan University of Medical Sciences and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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