Association between type 2 diabetes mellitus, biochemical factors and UCSNP-43 polymorphisms of CALP IN-10 gene in patients with atherosclerosis of coronary artery disease in Southern Iran population

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Introduction
Atherosclerosis is a major cause of death among adults in developed and developing countries. Atherosclerosis will lead to blocked coronary artery. Premature coronary heart disease (PCAD) is influenced by some genetic, environmental and metabolic disease factors. Metabolic syndrome is caused by some factors, the most important of which is diabetes mellitus (DM).¹ DM is the background of coronary artery disease (CAD).² Diabetes is an autoimmune disease and it emerges from defects in insulin secretion and action. Decreasing insulin hormone causes diabetic people to get energy from food.³ It is a group of metabolic diseases in which there are high blood sugar levels over a long period of time.⁴ In 2014, it was estimated that 387 million people have diabetes worldwide.⁵ 8.3% of the adult population have DM and men and women are equal in terms of the rate of the disease.⁶ More than 4 million Iranian adults have diabetes and it has increased by 35% over the 2005-2011.⁷ In Iranian adults, a striking prevalence of DM was 11.4% recorded and 14.6% of Iranian adults were diagnosed with impaired fasting glucose. It is significantly lower than the rates observed in the United States (14.6%); the prevalence of diabetes in Iran is significantly higher than its neighboring countries, such as Pakistan (6.7%) and Turkey (7.2),⁸ but it falls below the incidence of diabetes in Arab countries.⁹ Horikawa et al estimated the population-attributable risk of diabetes at 14%, whereas this risk among Caucasians was estimated only 4%.¹⁰ Although the physical activity and changes in lifestyle and diet can reduce the complications of type 2 diabetes, recognizing the genetic risk factors is very important for its prevention and treatment. Some genes are the basis for DM. One of the genes is calcium-activate neutral protease-10 (CALP IN-10). In addition, CALP IN-10 has an important role in proliferation and migration of vascular smooth muscle cell; platelet aggregation, degranulation, and spreading are the other factors.¹¹,¹² This gene is...
a member of the calpain family. CALPIN-10 gene located at 2q37 and spans 31 kb and contains 15 exons, constitut-
ed by 672 amino acids; it is in the third intron region.15
CALPIN-10 gene was the first diabetes gene identified by
cloning and encodes the calpain-10 protein.13 Calpain-10
protein is involved in the secreting pancreatic islands and
action of insulin, and is associated with adipocytes.
This gene encodes a nonlysosomal cysteine protease and
‘contributes to’ atherosclerosis and is associated with sev-
eral metabolic syndromes, for example diabetes, BMI, lip-
id profile (plasma cholesterol and triglyceride concentra-
tions); it finally leads to atherosclerosis of CAD.16,17
One of the variation of CALPIN-10 is SNP-43 that this
variant is the result of converting amino acids (arginine
into guanine). SNP-43 is an A-to-G variant in intron 3 of
the CALPIN-10 gene and is identified as a possible type 2
diabetes.18 Baier et al19 showed that the A allele had little
promoter activity than the G allele and G/G genotype. It
seems that it makes an association between the G/A and
A/A genotype and reduced CALPIN-10 mRNA expres-
sion. The G allele (G/G genotype) is more associated with
higher blood glucose than A allele (G/A and A/A).17
Although many studies on the correlation between
CALPIN-10, SNP-43 G>A gene polymorphism and DM have
been performed so far, existence of this relationship is not
clear to researchers. A significant correlation was observed in African-Americans,20 Caucasians, His-
panics,21 Japanese Americans,22 and Caucasians from the
United States,23 France,24 Finland,21,23-27 and the United
kingdom.28
Variation in the CALPIN-10 gene has been linked to 2.8-
fold increased risk for type 2 diabetes in Mexican-Amer-
icans and some European populations, but in some com-
"munities conflicting results have been produced, for ex-
ample Pima Indian and South Indian. In the present proj-
et, we plan to examine this relationship between SNP-43,
diabetes and the risk of early form of PCAD in Iran. To
this aim, we involved the patients and controls of CAD
and aimed to determine the relationship of CALPIN-10
SNP-43 G/A gene polymorphism and T2DM in the Irani-
an population. Also, we collected the information about
the patients, including age, sex, race, personal and family
history of diabetes, fasting blood sugar, and hyperlipid-
emia profile (total cholesterol, high-density lipoprotein
[LDL] cholesterol, and triglycerides). We used a modified
version of the questionnaire developed by Baecke et al.29

Material and methods
Study population
This is a case-control study on 452 subjects with an age
range of 22-81 and mean age of 52.58 ± 12.88 years. They
had undergone coronary angiography. Positive angiog-
raphy has been explained as coronary diameter decrease
greater than 50% as described by a cardiologist while con-
trol subjects had fewer than 30% stenosis in all major ves-
sels. A total of 224 patients were diagnosed with positive
angiographic stenosis (cases) and 228 with negative an-
 giographic stenosis (controls). We matched them for age,
sex and ethnicity. We excluded the patients with a history
of using drugs for blood pressure, blood sugar, those were
fat, and those who smoked more than one pack a day (for
professionals). The questionnaire was filled out by two
trained interviewers about cardiovascular disease such as
family history, cigarette smoking, age, and hypertension.

Biomedical measurement

Ten milliliters of the whole blood samples was taken from
the brachial vein of each subject with suspected CAD in
the early morning fasting (12 hours). One of two test tubes
contained EDTA (about 5 mL) and the other without
EDTA (about 5 mL) and in 1 hour were transferred to the
medical lab. To measure the glucose, triglyceride (TG),
high-density lipoprotein (HDL), low-density lipoprotein
(LDL), and cholesterol concentration, we used samples of
blood poured into tubes without anticoagulant. Standard
enzymatic assay (Pars Azmoonen, Diagnostic Kit) was used
to measure the biochemical parameters. High standards
for blood pressure, sugar and fat were calculated as fol-
ows: hypertension (>130/85 mm Hg), HDL low levels
(<40 mg/dl in men and <50 mg/dl in women), high tri-
glyceride levels (>150 mg/dl), LDL high levels (>160 mg/
dl) and hyperglycemia (>100 mg/dl), and diabetes accord-
ing to the criteria of the American diabetes Association
(ADA) (blood glucose level >126 mg/dl).

Genomic DNA was extracted from 5 ml blood samples
with EDTA by salting out method. The analysis of the
SNP-43 polymorphism in the CALPIN-10 gene was per-
formed by polymorphism chain reaction (PCR) coupled
restriction fragment length polymorphism (RFLP) meth-
od.30 A PCR was performed with 20 ng DNA using the
following primers:
Sense 5’ GCTGGCTGGTACATCAGT3’
Antisense 5’ TCAAGTTCATCCTTCCTGCAG3’
The resulting 144-bp product was digested with 1 ml Nsi
(Cinnagene, Iran) at 37°C for overnight (15-18 hours).
We identified the digested products with electrophoresis
on 3.5% agarose gel followed by ethidium-bromide stain-
ing and visualized by UV light to identify SNPs-43 and
visualized by ultraviolet light to determine the SNPs. The
percentage of genotype(s) for all the three loci performed
under co-dominant, dominant and recessive models was
134 bp for allele 1 (G) and 152 bp for allele 2 (A).

Statistical analysis

Statistical analysis was carried out using SPSS version
11.5 for Windows (SPSS Inc, Chicago, IL, USA). P < 0.05
was considered as statistically significant. Comparisons
of frequencies between qualitative variables were carried
out by chi-square test. Also, to assess the Hardy-Weinberg
equilibrium (HWE), a logistic regression model was fitted
to examine the independent impact of different clinical
and biochemical factors on CAD. To determining distri-
bution alleles in the groups (case and control), we used
chi-square test. The frequencies of different genotypes
were obtained; then allele frequencies were calculated in
cases and controls and then tabulated. The normality of

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distributing the investigated variables was assessed using the Kolmogorov–Smirnov criterion. Since TG, Total cholesterol (TC) and HDL were not normally distributed, the results of these variables were assessed using non-parametric tests (Mann-Whitney and Kruskal-Wallis). For normal parameters, such as fasting blood sugar (FBS) and LDL, parametric method was used (t test and ANOVA). Respective odds ratios (OR) were calculated for an unadjusted analysis and for an adjusted model which had been controlled for parameters that may contribute to the risk of CAD, such as CALPIN-10 genotype, smoking status, diabetes, hypertension, lipid profile, and familial history.

Results
Clinical and baseline characteristics and of cases and controls are summarized in Table 1. CALPIN-10 mutation frequency is presented in Table 1 for the case and control groups. The frequency of genotypes GG, GA and AA patients and healthy individuals was 66.9%, 24.75% and 8.25% (for patients) and 67.9%, 20.7%, 11.4% (for normal people). There was no significant difference between two groups regarding genotype and allele frequencies. The genotype frequencies of G/G, A/G, and A/A were 67.3%, 23.1%, and 9.5%, respectively in the whole population and not according to Hardy-Weinberg equilibrium ($\chi^2$) (Table 2).

Table 3 shows distribution of biochemical risk factors studied between CALPIN-10 genotypes in the total subjects. As shown in Table 3, none of the risk factors and CALPIN-10 genotypes in all subjects showed a significant relationship. A significant relationship was seen between the high levels of blood glucose (diabetes) in patients and genotype CALPIN-10 (Table 4).

Between clinical and biochemical risk factors with different genotypes there was no significant relationship in the control group (Table 5).

There was a significant correlation in the level of clinical characteristics (FBS, TG, cholesterol, and HDL) between the control and patient groups (Table 6).

Discussion
 Coronary heart disease is a major cause of mortality and morbidity in Middle East populations. A few convincing functional researches supported that some genes affect atherosclerosis and diabetes. On the other hand, CAD and T2DM have the same genetic backgrounds. They mostly happen with each other, and some records suggest this relation. Now, several genes recognized which influence diabetes and atherosclerosis.

Research on genetic factors of CAD is being conducted in Iranian populations. In most studies, it is shown that hereditary susceptibility explains about 50% of predisposition to CAD. One of the reasons of CAD is atherosclerotic plaque and this stenosis is caused by high blood sugar levels.

So far, many experimental studies have suggested a pathogenic role for CALPIN-10 in various complications of CAD and diabetes. Calpain-10 protein (intracellular Ca$^{2+}$ dependent cysteine protease) is the risk factor of increasing blood sugar but this relevance has not been confirmed by many other researchers. In other words, the role of this polymorphism in development of diabetes in different populations is still controversial.

A gene sequence of a G/G combination at this sequence is the abnormal gene sequence polymorphism, and the normal sequence is the A/G combination. This research provided the opportunity to look at the effect of this genetic factor (SNP-43 polymorphisms) in T2DM risk on coronary artery patients potential interactions of extrinsic factors that are known to influence T2DM risk with the SNP-43 polymorphisms in coronary artery patients. We studied about SNP-43 polymorphism in controls and...
patients with coronary heart disease and correlation between SNP-43, G>A gene polymorphism and diabetes. In this study, we showed G allele of CALPIN-10, SNP43 gene polymorphism did not increase the susceptibility to T2DM in the control groups of Iranian population (our data confirmed that the GG allele genotype slightly raised the fasting glucose level in the control group), but in the CAD patients, SNP-43 was significantly associated with T2DM.

There seems that not being a G/G polymorphism leads to high blood sugar levels in normal Iranian population but the correlation between gene polymorphism and CAD disease in patients is caused by high mean blood sugar (diabetes) as a risk factor for heart disease.

Our results support the idea that most of the genetic markers for type 2 diabetes (such as SNP-43) and metabolic syndrome are not independent pathogenic agents, but the markers associated with unhealthy environmental factors (high body mass index [BMI], unhealthy diet, bad lifestyle) can predispose one to diabetes. In other words, it does not seem that any relationship the UCSNP-43 polymorphism alone with diabetes involves impairment of insulin secretion in Iranian population. Similarly, this SNP-43 polymorphism was not associated with T2DM in recent studies in the United Kingdom,30 Pima Indians, German Caucasians,31 and Caucasian population32 in the south eastern areas. Also, our results are consistent with the observations. The similar result might be due to the type of studies or correlation of polymorphisms between race and geographical location. The Iranian population is Caucasian and in this respect, genetic polymorphism of Iranian population is similarly close to other countries with the same race. Probably, we can offer this gene as a good marker for anthropological studies.

In another study on Northern Iranian population, the results showed that among the population of Azerbaijan (North Iran) G allele increased the risk of type 2 diabetes compared with allele A in diabetic patients. Also, this result is in the same line with those of Northern European, South Indians, and African-Americans (Table 7).12,33,34

In the past, some studies used the case-control method and others used the cross-sectional method. One of the strengths in this case-control study is the strong solidar-

<table>
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<th>Table 4. Distribution of qualitative information based on clinical and biochemical case group</th>
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<tr>
<td>Risk factors</td>
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<td>FBS (&gt;100 mg/dl)</td>
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<td>TG (&gt;200 mg/dl)</td>
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<td>Cholesterol (&gt;200 mg/dl)</td>
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<tr>
<td>HDL (&lt;35 mg/dl)</td>
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<td>LDL (&gt;130 mg/dl)</td>
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<td>Hypertension</td>
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<tr>
<td>Smoking</td>
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<tr>
<td>Family history</td>
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Abbreviations: FBS, fasting blood sugar; HDL, high density lipoprotein; LDL, low density lipoprotein; mg/dl, milligram per deciliter; TG, triglyceride.

<table>
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<th>Table 5. Distribution of qualitative information based on clinical and biochemical control group</th>
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<tr>
<td>Risk factors</td>
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<td>FBS (&gt;100 mg/dl)</td>
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<td>TG (&gt;200 mg/dl)</td>
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<td>Cholesterol (&gt;200 mg/dl)</td>
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<tr>
<td>HDL (&lt;35 mg/dl)</td>
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<td>LDL (&gt;130 mg/dl)</td>
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<td>Hypertension</td>
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<td>Smoking</td>
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<td>Family history</td>
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Abbreviations: FBS, fasting blood sugar; HDL, high density lipoprotein; LDL, low density lipoprotein; mg/dl, milligram per deciliter; TG, triglyceride.

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<th>Table 6. Demographic and clinical characteristics in the control and patient groups</th>
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<td>Risk factors</td>
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<td>FBS (&gt; 100 mg/dl)</td>
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<td>TG (&gt;200 mg/dl)</td>
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<tr>
<td>Cholesterol (&gt;200 mg/dl)</td>
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<tr>
<td>HDL (&lt;35 mg/dl)</td>
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<td>LDL (&gt;130 mg/dl)</td>
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Abbreviations: FBS, fasting blood sugar; HDL, high density lipoprotein; LDL, low density lipoprotein; mg/dl, milligram per deciliter; TG, triglyceride.
Association FBS and Lipid profile and UCSNP43- polymorphism with CAD

The allele frequencies of SNP-43 polymorphisms in CALPIN-10 gene in the earth's population

<table>
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<tr>
<th>Continent and region</th>
<th>Populations</th>
<th>SNP-43 Alleles</th>
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<tbody>
<tr>
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<td></td>
<td>G</td>
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<tr>
<td>Africa</td>
<td>Baik (N=138)</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>Mbuti (N=70)</td>
<td>0.97</td>
</tr>
<tr>
<td>Europe</td>
<td>Europeans (N=180)</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>Danes (N=98)</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>Druzel (N=126)</td>
<td>0.90</td>
</tr>
<tr>
<td>Asia</td>
<td>Chinese (N=110)</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Japan (N=72)</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>Yakut (N=75)</td>
<td>0.85</td>
</tr>
<tr>
<td>Pacific Islands</td>
<td>Nasioi (N=44)</td>
<td>0.90</td>
</tr>
<tr>
<td>North and South</td>
<td>Maya (N=94)</td>
<td>0.69</td>
</tr>
<tr>
<td>America</td>
<td>Surui (N=92)</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>Eastern Azerbaijan province (N=202)</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>Fars province (N=452)</td>
<td>0.79</td>
</tr>
</tbody>
</table>

ity and in the present research we used the case-control method.

There are possible explanations to reconcile our findings with other studies inconsistent results as below:
First, the main reason for this difference is probably the ethnic variability and different nature of the genetic predisposing populations. Second, this research focused on a population with normal coronary artery rather than random normal population. Third, this study was conducted on a population that had no HWE. Other findings of this project is investigating the relationship between SNP-43 and clinical characteristic factors. In the control and CAD patients groups, our data confirmed that no significant relationship existed among UCSNP-43 and hyperlipidemia, smoking, hypertension and family history, but in the patients group the level of this biochemical factor was higher than normal population. So these extrinsic risk factors do not play a major role in G/G homozygosity with CAD. Another research has shown the relationship between this gene and the high level of cholesterol, Triglyceride free fatty acid. Therefore, to understand the relationship and interaction between genes and the metabolic syndrome (e.g., diabetes), we should not overlook the role of nutrition and other factors.

High blood sugar was a strong risk factor for CAD in the south of Iran. One of the strengths in this study was the use of a case-control design. Hyperlipidemia profile especially HDL is a strong factor in coronary disease in Iranian population. Briefly, we showed that this variation of CALPIN-10 was separately accompanied with clinical atherosclerosis of T2DM. In the present study, We managed to genotype 452 families with several generations of conflict with coronary heart disease in Southern Iranian descent. We showed CALPIN-10, SNP-43 contributes to lead to coronary artery atherosclerosis in Iranian population diagnosed with atherosclerosis and insulin resistance but according to the survey results, G allele of SNP-43 CALPIN-10 polymorphism is not a risk factor for type 2 diabetes in the normal population of southern Iran. This increased risk in our families, which were ascertained to a strong history of T2DM.

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Ethical issues
The study was approved by the Ethics Committee of Shiraz University of Medical Sciences.

Competing interests
Authors declare no conflict of interest in this study.

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