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Human *AGT*-p.Met268Thr and coronary heart disease risk: a case-control study and meta-analysis

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A – Study Design, B – Data Collection, C – Statistical Analysis, D – Data Interpretation, E – Manuscript Preparation, F – Literature Search, G – Funds Collection

Summary **Background.** Polymorphisms in genes, which is involved in the renin–angiotensin system, play an important role in the pathogenesis of coronary heart disease (CHD). Polymorphism of c.803T>C in the human *angiotensinogen* gene results in methionine (M) to threonine (T) substitution at codon 268 (p.Met268Thr), which traditionally has been known as M235T. This polymorphism may contribute to cardiovascular diseases.
Objectives. The aim of this study was to investigate the association between p.Met268Thr polymorphism in the *angiotensinogen* gene and coronary heart disease (CHD) through a case-control study, which is followed by a meta-analysis.
Material and methods. In the case-control study, c.803T>C genotyping of 217 subjects (102 CHD cases vs 115 controls) was investigated by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. In the meta-analysis, 31 studies were included, reflecting 12,028 people with CHD and 16,362 healthy controls.
Results. The data from the case-control study revealed that MT (OR, 1.875; 95%CI, 1.060–3.316; *p* = 0.031) and TT (OR, 3.389; 95%CI, 1.251–9.179; *p* = 0.016) genotypes are significantly associated with CHD. The meta-analysis revealed a significant association in the recessive model (OR, 1.156; 95%CI, 1.011–1.321; *p* = 0.034).
Conclusions. Although the pooled OR of the meta-analysis showed that there is an increased risk of CHD conferred by p.Met268Thr of the *AGT* gene, this association was weak, which could be attributed to a bias in publications.
Key words: coronary disease, angiotensinogen, genetic polymorphism, meta-analysis.

Background

Coronary artery disease (CHD) is a major health burden in both developed and developing countries, which leads to morbidity and mortality throughout the world [1]. Epidemiological studies demonstrated that a diet high in fat, lack of physical activity, smoking, cholesterol and hypertension are the major risk factors of development of CHD [2], although it has been speculated that CHD is a multifactorial disease with a great contribution from genetic factors [3]. Clinical observations in the 1950s demonstrated that the CHD risk is heritable. A scientific project in more than twenty thousand Swedish twins established the discovery of increased susceptibility of CHD among close relatives, and this project estimated a heritability of ~50% for CHD [4]. In addition, an updated genome-wide association approach similarly estimated the heritability of CHD at 40–50%. Such studies are the basis for the emergence of genetic approaches to comprehending the underlying genetic basis of CHD, to discover innovative structural biology and to interpret these discoveries into clinical training [5]. Despite the tendency to cluster in families, CHD is an intricate and common disease. Genotyping chips designed to identify common genetic variation have provided the basis for common polymorphism association studies and genome-wide association studies. Since common genetic variants happen relatively often, it is useful to

evaluate each variant separately by comparing its incidence in case studies and healthy controls [6]. One efficient description of 'common' is a variant with an allele frequency of more than 0.5% [7]. The first genetic association studies for CHD were reported in 2007, and 3 independent groups described common variations at chromosome 9 (9p21) that correlated with an increased risk of CHD. Further studies have repeated this outcome and extended the association to other vascular diseases, such as stroke, peripheral arterial disease, and carotid atherosclerosis [5]. Since 2007, increasingly greater sample sizes have been employed to investigate the genetic basis of CHD, making about 60 separate genetic locus for CHD. These accumulative experience results permit numerous conclusions. First, a large number of these variations have a minor allele frequency of more than 5% in the population, they are correlated with mild increases in CHD risk (e.g. < 20% alteration in CHD risk per allele), and the generally explain 30 to 40% of CHD heritability [5]. By contrast, fifteen low-frequency polymorphisms explained only 2% of CHD heritability [8].

However, a study on the association of common variants in key genes involved in CHD could be helpful for screening projects. In humans, the rennin–angiotensin system (RAS) plays an important role in the maintenance of salt-water balance and controlling blood pressure [9, 10]. Several genes are participating in RAS pathways as *angiotensinogen* (*AGT*; OMIM: 106150)
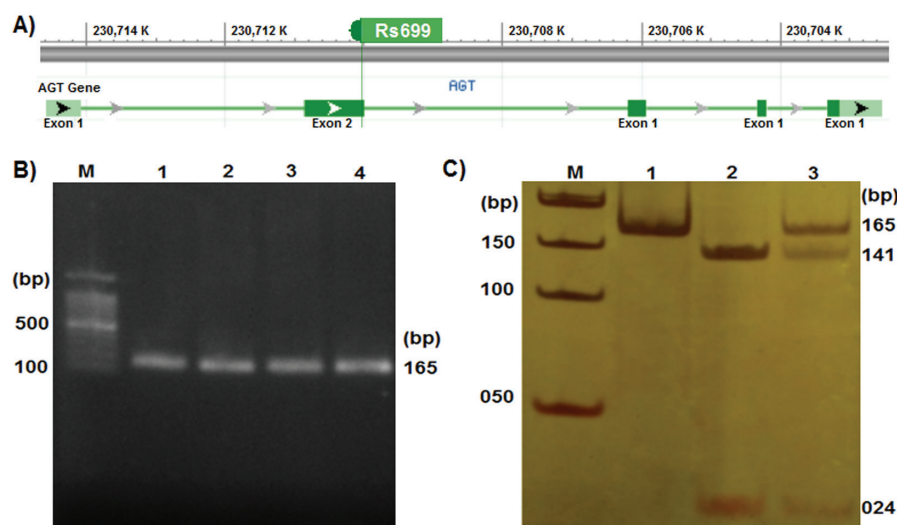
Figure 1. Human *AGT* gene map and p.Met268Thr genotyping

A) *AGT* gene map that the p.Met268Thr is located on exon 2; **B)** PCR product of exon 2 with 165-bp, which contains p.Met268Thr [lane M = 100-bp DNA ladder]; **C)** Digestion map for 165-bp PCR fragment by *Psy*I restriction enzyme [lane M = 50-bp DNA marker; lane1 = MM genotype; lane2 = TT genotype; lane3 = MT genotype].

[11], angiotensin I-converting enzyme (*ACE*; OMIM: 106180) [12] and angiotensin II type 1 receptor (*AGTR*1; OMIM: 106165) [13], which are noteworthy targets. These genes are considered to influence the inherited predisposition toward CHD [14]. There is a common single nucleotide polymorphism (SNP) at location 803 (c.803T>C; SNP ID: rs699) on exon 2 of the *AGT* gene (Figure 1A). This polymorphism resulted in substitution of methionine residue to threonine at codon 268 (p.Met268Thr), which is traditionally known as M235T [15]. In recent years, the correlation between the molecular variant of the *AGT* gene (p.Met268Thr) with CHD has started to receive attention. Numerous studies have investigated the association between this substitution and CHD in different populations [16–18]; however, the results are inconsistent.

Objectives

The aim of this study was to investigate the association between p.Met268Thr polymorphism and CHD through both a case-control study and a meta-analysis study.

Material and methods

Study design

In a case-control study, patients with diagnosed CHD and control subjects without any history of cardiovascular disease were included into the study.

Participants

Blood samples of 102 admitted CHD patients were collected from patients who were admitted to the Coronary Care Unit of the Shahid Beheshti Hospital (Kashan, Iran) between 2013 and 2015. 115 healthy individuals without a history of cardiovascular diseases were recruited from the Local Blood Donor Center in Kashan, Iran, as a control group. A complete description for subject selection was presented previously [19]. CHD was confirmed angiographically by stenosis severity 50% or more in at least one major coronary artery. The exclusion criteria were as follows: clinical symptoms of coagulopathy, collagenosis, cardiomyopathy and acute poisoning (such as amphetamine and CO). Exclusion criteria for the healthy blood donors were as follows: symptoms of myocardial infarction (MI), CHD, diabetes mellitus and other genetic and familial diseases. A signed informed consent was taken from all participants. Approval of the Local Ethics Committee was obtained from Kashan University of Medical Sciences. The demographics of study participants are summarized in Table 1.

Table 1. Demographics characteristics of CHD and control groups

Parameters		Controls (*n* = 115)	CHD (*n* = 102)
Gender	male	76	65
	female	39	37
Age, mean ± SD	years	61.09 ± 5.09	50.42 ± 7.39
BMI, mean ± SD	kg/m^2	29.99 ± 2.67	32.64 ± 2.60
Smoking	yes	38	57
	no	77	45
Triglyceride, mean ± SD	mg/dL	120.13 ± 40.46	129.33 ± 35.64
High-density lipoprotein, mean ± SD	mg/dL	38.87 ± 5.18	43.07 ± 6.46
Low-density lipoprotein, mean ± SD	mg/dL	106.93 ± 27.65	111.27 ± 18.12

Setting

We examined just one gene polymorphism (*AGT*-p.Met268Thr) by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). Total genomic DNA was isolated from blood samples using a DNGplus buffer (Cinnagen Co., Tehran, Iran). *AGT*-p.Met268Thr gene polymorphism was genotyped by the PCR-RFLP method and primers sequences, which were described by Nalbantoglu et al. [20]. (All of the PCR reagents were purchased from Fermentas Co., Sankt Leon-Rot, Germany). *AGT* fragments with p.Met268Thr polymorphism were amplified by the following PCR program: initial denaturation at 94°C for 5 min. and 35 repetitive cycles 94°C for 30 sec., 59°C for 45 sec., and 72°C for 1 min. followed by final extension at 72°C for 10 min. in a Mastercycler gradient (Eppendorf Co., Hamburg, Germany). The 165-bp amplified fragment (Figure 1B) was treated by a *Psy*I restriction enzyme (Fermentas) at 37°C for 16 h. The digested mixture was electrophoresed in 8% polyacrylamide gel and visualized by silver nitrate (AgNO$_3$; Cinnagen) staining [21]. The 268TT homozygous genotype produced two fragments of 24-bp and 141-bp. The 268MT heterozygote genotype produced three fragments of 24-bp, 141-bp, and 165-bp, whereas the 268MM homozygous wild-type produced one fragment of 165-bp (Figure 1C). The validity of PCR-RFLP was confirmed by DNA direct sequencing, performed by the Bioneer Company (Daejeon, South Korea).

Meta-analysis

The literature included in this analysis was selected using PubMed, Google Scholar, Elsevier, Springer and Link databases

with the keywords 'angiotensinogen', '*AGT* gene', 'M235T', 'polymorphism' and 'coronary heart disease'. All extracted articles were studied carefully. Any experimental studies were included if they applied the following criteria: The case-control design had been used to provide p.Met268Thr genotype frequencies; Genotype frequency in the control group should be in the Hardy–Weinberg equilibrium; Applicable data was accessible to calculate the odd ratio (OR) and its 95% CI. According to the previous characteristics, the data, including name of first author, year of publication, ethnicity and frequency of genotypes, was extracted from the papers by two students.

Statistical methods

Statistical analysis was done using SPSS ver. 19 software (SSPS Inc., IBM Corp Armonk, NY, USA). The Hardy–Weinberg equilibrium (HWE) was evaluated using the Chi-square test. Differences in the p.Met268Thr genotypes and allele frequencies between the controls and cases were compared by this test. The association of the genotypes and alleles with CHD risk was expressed in terms of the odds ratios (OR) and 95% confidence interval (CI). A *p*-value less than 0.05 ($p < 0.05$) was considered statistically significant.

In the meta-analysis, we first estimated the OR and 95% CI for each of the studies. The following four genetic models were then used: MT+TT vs MM as a dominant; TT vs MT+MM as a recessive; TT vs MM and TM vs MM as co-dominants. The values of each study were combined by Mantel–Haenszel fixed effects [22] or random effects models [23]. The I^2 statistic was calculated to measure the consistency between trials [24]. The fixed effect model was used when the heterogeneity was non-significant ($p < 0.1$); otherwise, the random effect model was used. A funnel plot was applied to calculate publication bias. The Comprehensive Meta-Analysis (Biostat, Inc., Englewood, NJ, USA; https://www.meta-analysis.com/) and Open Meta Analyst (Tufts University, Medford, MA, USA; http://www.cebm.brown.edu/openmeta/) software were used for all calculations in the meta-analysis.

Results

Main results

The distribution of *AGT* genotypes for p.Met268Thr polymorphism was in the Hardy–Weinberg equilibrium in the control groups ($\chi^2 = 0.272$; $p = 0.602$). The *AGT*-p.Met268Thr allele and genotype frequencies for both control and CHD groups are shown in Table 2. The TT genotype was significantly higher in patients with CHD than the healthy control group (OR, 3.389; 95% CI, 1.251–9.179; $p = 0.016$). The carriers of the MT genotype were at a high risk for CHD (OR, 1.875; 95% CI, 1.060–3.316; $p = 0.031$). Carriers of T allele (MT+TT) were significantly higher in patients than in the healthy control group (OR, 2.071; 95% CI, 1.199–3.578, $p = 0.005$). Similarly, the frequency of T allele was significantly higher in cases than in the control group (OR, 1.787; 95% CI, 1.191–2.682; $p = 0.005$).

Table 2. Genotype and allele frequencies of p.Met268Thr in cases and controls

Genotype/ /Allele	No. and Percentage		OR (95% CI)	*p*
	Control (*n* = 115)	Case (*n* = 102)		
MM	61 (53.04%)	36 (35.29%)	–	–
MT	47 (40.87%)	52 (50.98%)	1.875 (1.060–3.316)	0.031
TT	7 (06.09%)	14 (13.73%)	3.389 (1.251–9.179)	0.016
MT+TT	54 (46.96%)	66 (64.71%)	2.071 (1.199–3.578)	0.005
M	169 (73.48%)	124 (60.78%)	–	–
T	61 (26.52%)	80 (39.22%)	1.787 (1.191–2.682)	0.005

OR – odds ratio; CI – confidence interval. Significant differences between the case and control groups are in bold.

Other analyses

Based on the inclusion criteria, a total of 30 studies were retrieved [16–18, 25–50]. The data from our study was also added to the meta-analysis (Figure 2). Finally, 31 studies were included in the meta-analysis, reflecting 16,362 healthy controls and 12,028 subjects with CHD. The control group in these 31 studies revealed no deviation from the Hardy–Weinberg equilibrium. The total results of the meta-analysis of these 31 studies, which involved 8 studies of Asian, 20 of Caucasian and 3 other ethnicities populations, are shown in Table 3 and 4. The meta-analysis revealed that the p.Met268Thr substitution is associated with CHD in the recessive model with OR, 1.156; 95% CI, 1.011–1.321; $p = 0.034$ (Figure 3).
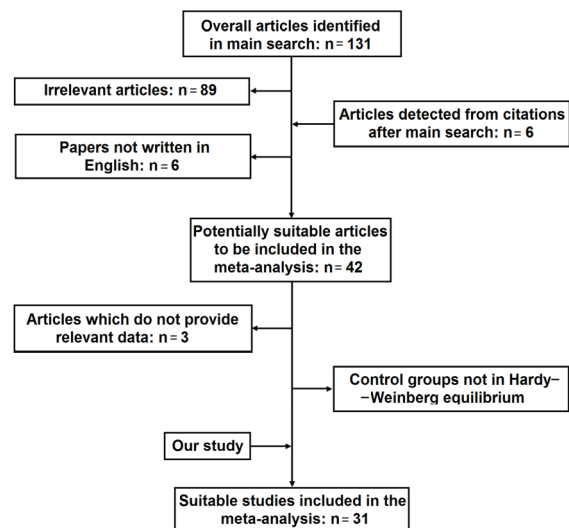

Figure 2. Flow chart representing the results of the search strategy and the details for exclusion.

Table 3. Results of meta-analysis for *AGT* gene p.Met268Thr polymorphism and CHD risk in Asian, Caucasian and total populations

Group	TT vs MM				MT vs MM				MT + TT vs MM				TT vs MM + MT			
	OR (95% CI)	*p*	*ph*	*I²*	OR (95% CI)	*p*	*ph*	*I²*	OR (95% CI)	*p*	*ph*	*I²*	OR (95% CI)	*p*	*ph*	*I²*
Total	1.14 (0.96–1.35)	0.13	< 0.001	60%	1.00 (0.89–1.13)	1.00	< 0.001	50%	1.11 (0.96–1.29)	0.17	< 0.001	73%	1.16 (1.01–1.32)	0.03	< 0.001	65%
Asian	0.72 (0.50–1.04)	0.08	0.66	0%	0.70 (0.48–1.01)	0.06	0.77	0%	0.73 (0.51–1.03)	0.08	0.68	0%	0.96 (0.83–1.10)	0.54	0.21	28%
Caucasian	1.10 (0.93–1.30)	0.27	0.00	55%	0.99 (0.92–1.07)	0.80	0.13	28%	1.01 (0.94–1.08)	0.80	0.05	39%	1.20 (0.99–1.45)	0.06	< 0.001	73%

OR – odds ratio; CI – confidence interval; *ph*, *p*-values for heterogeneity from the Q test.

The heterogeneity test exhibited a true heterogeneity between studies for p.Met268Thr in the TT vs MM, MT vs MM, MT+TT vs MM, and TT vs MM+MT models with $P_{heterogeneity}$ < 0.001 (Table 3). On the other hand, the shape of the funnel's plot showed obvious evidence of asymmetry (Figure 4). Analysis of potential publication bias suggested the presence of a publication bias, based on Egger's test and the Begg–Mazumdar test, with *p*-values = 0.012 and 0.045, respectively. Subgroup analysis of the different ethnic groups showed that there is no significant association between p.Met268Thr and CHD in the Asian and Caucasian subgroups (Table 3).
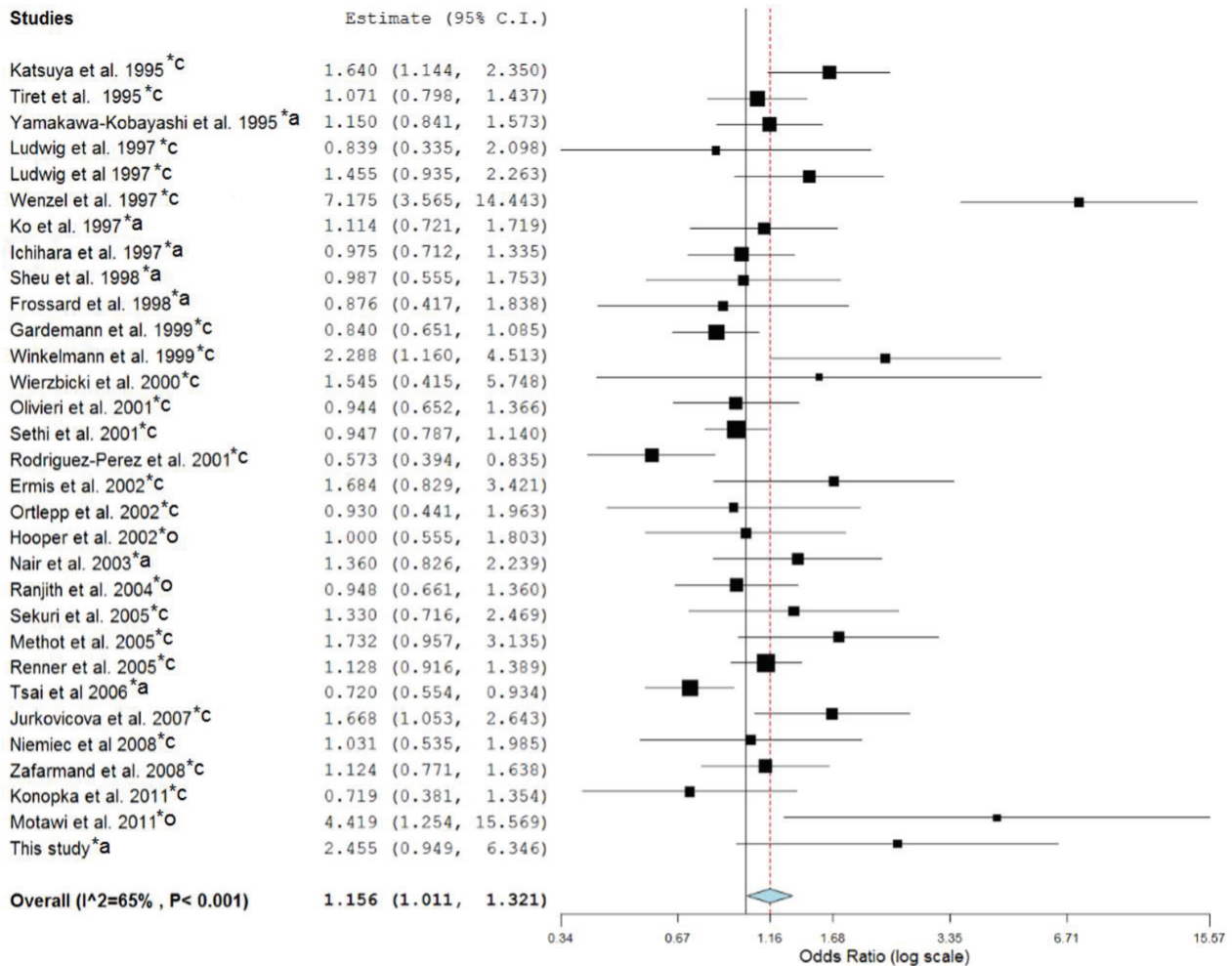
Studies	Estimate (95% C.I.)
Katsuya et al. 1995 [*c]	1.640 (1.144, 2.350)
Tiret et al. 1995 [*c]	1.071 (0.798, 1.437)
Yamakawa-Kobayashi et al. 1995 [*a]	1.150 (0.841, 1.573)
Ludwig et al. 1997 [*c]	0.839 (0.335, 2.098)
Ludwig et al 1997 [*c]	1.455 (0.935, 2.263)
Wenzel et al. 1997 [*c]	7.175 (3.565, 14.443)
Ko et al. 1997 [*a]	1.114 (0.721, 1.719)
Ichihara et al. 1997 [*a]	0.975 (0.712, 1.335)
Sheu et al. 1998 [*a]	0.987 (0.555, 1.753)
Frossard et al. 1998 [*a]	0.876 (0.417, 1.838)
Gardemann et al. 1999 [*c]	0.840 (0.651, 1.085)
Winkelmann et al. 1999 [*c]	2.288 (1.160, 4.513)
Wierzbicki et al. 2000 [*c]	1.545 (0.415, 5.748)
Olivieri et al. 2001 [*c]	0.944 (0.652, 1.366)
Sethi et al. 2001 [*c]	0.947 (0.787, 1.140)
Rodriguez-Perez et al. 2001 [*c]	0.573 (0.394, 0.835)
Ermis et al. 2002 [*c]	1.684 (0.829, 3.421)
Ortlepp et al. 2002 [*c]	0.930 (0.441, 1.963)
Hooper et al. 2002 [*o]	1.000 (0.555, 1.803)
Nair et al. 2003 [*a]	1.360 (0.826, 2.239)
Ranjith et al. 2004 [*o]	0.948 (0.661, 1.360)
Sekuri et al. 2005 [*c]	1.330 (0.716, 2.469)
Methot et al. 2005 [*c]	1.732 (0.957, 3.135)
Renner et al. 2005 [*c]	1.128 (0.916, 1.389)
Tsai et al 2006 [*a]	0.720 (0.554, 0.934)
Jurkovicova et al. 2007 [*c]	1.668 (1.053, 2.643)
Niemiec et al 2008 [*c]	1.031 (0.535, 1.985)
Zafarmand et al. 2008 [*c]	1.124 (0.771, 1.638)
Konopka et al. 2011 [*c]	0.719 (0.381, 1.354)
Motawi et al. 2011 [*o]	4.419 (1.254, 15.569)
This study [*a]	2.455 (0.949, 6.346)
Overall (I^2=65% , P< 0.001)	1.156 (1.011, 1.321)

Figure 3. Meta-analysis for 31 studies of p.Met268Thr in the total population

The 8 studies of Asian, 20 of Caucasian and 3 other ethnic populations were included, which are labeled with *a, *c and *o, respectively.
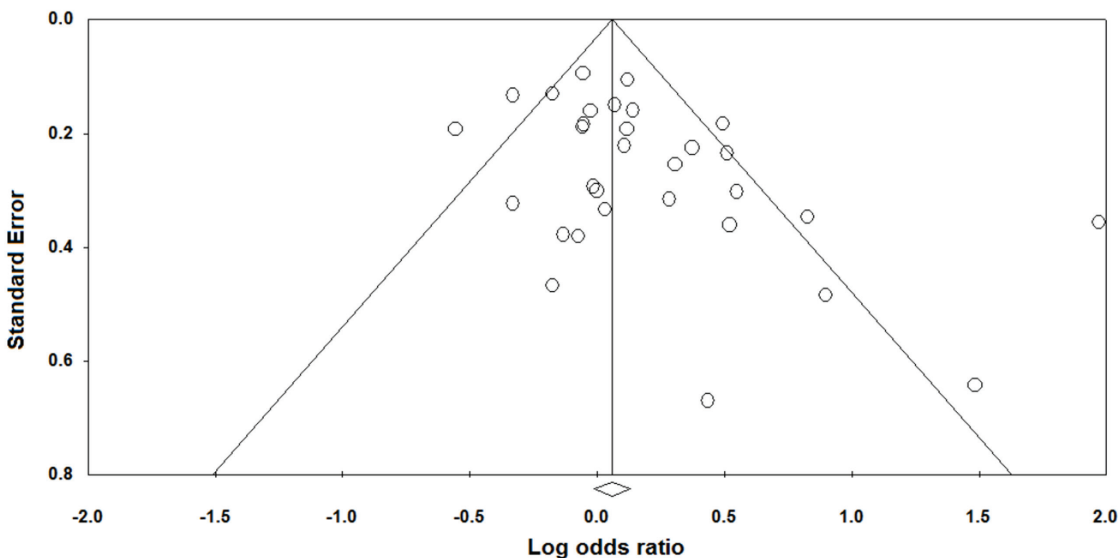

Figure 4. Begg's funnel plot for the recessive model in the overall analysis

Table 4. Summary OR and 95% CI adjusted for multiple testing using the BH-FDR method								
Group	TT vs MM		MT vs MM		MT+TT vs MM		TT vs MM+MT	
	OR (95% CI)	p_{FDR}	OR (95% CI)	p_{FDR}	OR (95% CI)	p_{FDR}	OR (95% CI)	p_{FDR}
Total	1.14 (0.96–1.35)	0.227	1.00 (0.89–1.13)	1.000	1.11 (0.96–1.29)	0.227	1.16 (1.01–1.32)	0.120
Asian	0.72 (0.50–1.04)	0.107	0.70 (0.48–1.01)	0.107	0.73 (0.51–1.03)	0.107	0.96 (0.83–1.10)	0.540
Caucasian	1.10 (0.93–1.30)	0.800	0.99 (0.92–1.07)	0.800	1.01 (0.94–1.08)	0.800	1.20 (0.99–1.45)	0.800

OR– odds ratio; CI – confidence interval; FDR: *p*-value from the Benjamini–Hochberg method control for the false discovery rate.

Discussion

Polymorphism in some key genes, such as *nitric oxide synthase* (OMIM: 600720) [36] and *p22phox* (OMIM: 608508) [51], might be genetic risk factors for cardiovascular disease. Genes involved in the renin–angiotensin system, such as angiotensinogen [11], angiotensin I-converting enzyme [12] and angiotensin II type 1 receptor [13], are good candidates to study CHD genetic risk factors. This system is well known to be involved in the control of blood pressure and plays an autocrine or paracrine role in cardiac remodeling and contributes to the pathophysiology of CHD [52, 53].

In the present study, we evaluated the association between *AGT*-p.Met268Thr polymorphism and coronary heart disease, followed by a meta-analysis. The genetic association study showed that there is a significant association between *AGT*-p.Met268Thr polymorphism and coronary heart diseases in the Iranian population. Our data introduced the 268T allele as a genetic risk factor for CHD in the study population (*p* = 0.005). To date, several studies have evaluated the association between *AGT*-p.Met268Thr polymorphism and CHD [27, 28, 36], but some studies did not detect any association [29, 39, 45]. To clarify this, we performed a meta-analysis via combining more eligible studies, enlarging the sample size and performing a subgroup analysis. Our meta-analysis of the Asian population revealed that the 268MT genotype was more frequent in cases vs controls, which was statistically non-significant (OR, 0.696; 95% CI, 0.479–1.011; *p* = 0.057). But a meta-analysis in the overall model revealed that *AGT*-p.Met268Thr polymorphism is associated with CHD in the recessive model (OR, 1.156; 95% CI, 1.011––1.321; *p* = 0.034). The inconsistent data from multiple studies suggests that the role of *AGT* polymorphism in CHD may depend on ethnic and geographic factors.

SNPs may occur in coding or noncoding sequences. SNPs in noncoding sequences, as well as synonymous SNPs, can disrupt the gene expression or mRNA splicing [54, 55]; however, missense mutations are responsible for various attributed single gene disorders. Some studies reported that non-synonymous SNPs (nsSNP) are harmful for protein and mRNA structures [56, 57]. Most nsSNPs disturb the protein function through alteration of protein hydrophobicity [58, 59] or affecting the three--dimensional structure of protein [60, 61]. The exact pathogenic mechanisms of *AGT* deficiency by p.Met268Thr polymorphism in CHD are still unclear, but there are some speculated hypotheses in this regard. The p.Met268Thr as an nsSNP may alter the AGT function. Previous studies investigated the impact of *AGT* gene polymorphisms on the protein structure with a computational approach [10, 62]. Singh et al. reported that eight SNPs in the coding region of *AGT* may be deleterious for the structure of protein [10]. In addition, Raygan et al. reported that p.Met268Thr has a significant impact on the protein structure of AGT [62]. They reported that Met268Thr substitution may affect the hydrophobicity properties of AGT [62]. p.Met268Thr polymorphism of the human *AGT* gene has been associated with a variation of serum *AGT* concentration. Between 10% and 20% more plasma AGT concentration was seen in 268TT homozygotes compared to 268MM individuals [44, 63].

Xu et al. in 2007 performed a meta-analysis on the association of both p.Thr207Met and p.Met268Thr polymorphisms in the *AGT* gene with CHD. They found no association between p.Thr207Met and CHD, but they reported a weak association between p.Met268Thr and CHD [64]. Zafarmand et al. in 2008 also reported that there is an increased risk of CHD by *AGT* p.Met268Thr polymorphism. However, they state that this association is weak and might be due to publication biases and HWE violation [50]. However, Li et al. in 2012 performed a meta-analysis on the association of *AGT* gene polymorphisms (p.Met268Thr, p.Thr207Met) with coronary heart disease solely in the Chinese population [65]. The meta-analysis showed significant associations of *AGT* gene polymorphisms (p.Met268Thr, p.Thr207Met) with CHD in the Chinese population [65]. Our data was consistent with Xu et al. [64] and Zafarmand et al. [50].

Pharmacogenomics is the study of the role of genetics in the response to a therapeutic intervention. Single nucleotide polymorphisms play an essential role in an individual's susceptibility to different diseases and variable responses to drugs. There is a continuous approach to detect the common and functional SNPs related to various diseases [66]. *AGT*-p.Met268Thr polymorphism is frequently assessed in pharmacogenomics studies of the renin–angiotensin system (RAS). Evidence has exhibited that AGT variants influence the risk of hypertension. Different plasma concentrations of angiotensinogen were also observed in hypertensive patients with different *AGT* variants [67]. Thus, *AGT* gene variants could be a probable candidate for pharmacogenomic RAS blockage intervention [66].

This topic has been addressed exclusively in literatures, but this case control is the first which investigates the association of this particular polymorphism with CHD in the Iranian population. However, this study suffered from an inadequate control group and lack of evaluations of gene–gene and gene–environment interactions. There are more updated methods for SNP genotyping, such as TaqMan probes, Sequenom MassARRAY system, SNaPshot, etc., but our funds for the experimental study were limited. Therefore, we applied the PCR-RFLP method as an inexpensive technique for SNP genotyping. Some other potential limitations of this study are as follows: first, our results were calculated based on unadjusted data regardless of confounding factors, such as age, sex, etc. Furthermore, we did not have access to the diet of subjects (such as salt and fat consumption), which may modulate the effects of p.Met268Thr polymorphism in CHD. Second, limiting the meta-analysis to English language papers may potentially lead to language bias. This meta-analysis also lacks sufficient data from African populations.

Conclusions

The pooled data in the meta-analysis showed a weak association between *AGT*-p.Met268Thr and CHD, which may due to publication bias. Thus, we conclude that p.Met268Thr substitution might be a risk factor for CHD in susceptible individuals. In addition, we suggest further *in vitro* and *in vivo* analysis to find out the exact role of p.Met268Thr mutation in CHD.

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