

ORIGINAL ARTICLE

Angiotensinogen Gene (M235T) Polymorphism and Coronary Artery Disease in the Egyptian Population. A genetic Association Study

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Background	The etiology of coronary artery disease (CAD) involves both environmental and genetic factors; the former group was intensively studied. Angiotensinogen (AGT) is important in cardiovascular remodeling and control of blood pressure.
Methods	we investigated role of M235T variant of AGT gene in development of CAD. M235T refers to substitution of cytosine into thymine at codon 235 that encode for theronine instead of methionine at exon 2 of AGT gene.
Results	Seventy male patients were diagnosed as myocardial infarction (35) and unstable angina (35) were analyzed against healthy control male subjects (60) to evaluate gene polymorphism M235T and plasma angiotensinogen level. The distribution of genotype or paired allele among patients and control for 235TT variant was 23% and 3.3% and Odds Ratio (OR) was 8.83, for MT variant was 50% and 33.3% OR=2 and for MM was 27% and 63.3%, OR=0.21 respectively, there was significant difference between them as regard TT, MM genotype with P <0.01 and P <0.001 respectively. There was a significant increase in frequency of T allele distribution among cases than control P <0.001. There was a significant increase of plasma angiotensinogen level in patient than control P <0.01. Positive correlation was observed between TT genotype, age and diastolic blood pressure in CAD group $r=0.34$, P <0.001 and $r=0.25$, P <0.05 respectively. A significant increase of plasma angiotensinogen and cholesterol levels in CAD group than control was also observed.
Conclusions	T polymorphism of AGT gene was significantly associated with CAD and was correlated positively with blood pressure.
Keywords	Coronary artery diseases, Angiotensinogen gene, Gene Variant. (Heart Mirror J 2009; 3(2): 86-91)

INTRODUCTION

Atherosclerotic coronary artery disease (CAD) is the leading cause of death worldwide (1). Coronary artery disease is a complex disorder with both environmental and genetic determinants. The mode of inheritance of CAD is polygenic with a complex interaction between multiple genes and multiple environmental risk factors. The traditional risk factors of diabetes, hypertension, dyslipidemia and family history account for 80% of cases of CAD (2). Disease prevention is an important strategy for reducing the overall burden of CHD and identification of biomarkers for disease risk is the key, both for risk prediction and for potential intervention to reduce the chance of future events. Angiotensinogen (AGT) is the precursor of angiotensin I. Angiotensin-converting enzyme

converts angiotensin I to angiotensin II which is the crucial biologically active product of the renin angiotensin system which plays a significant role in the regulation of blood pressure. It has direct toxic effects on myocardial cells, activates the sympathetic nervous system, stimulates fibroblast proliferation and vasoconstricts coronary vessels (3). Many recent studies showed that polymorphisms involving the genes encoding components of RAS have been associated with elevated blood pressure and increased risk of coronary artery disease (4-7). Angiotensinogen plasma levels have been shown to correlate with the blood pressure. The human AGT gene polymorphism has been identified. The T allele of the polymorphism, which encodes theronine instead of methionine at position 235

Abbreviations and Acronyms

AGT : Angiotensinogen
CAD : Coronary artery disease
MI : Myocardial infarction
SBP : Systolic blood pressure
DBP : Diastolic blood pressure

(M235T), has been shown to be associated with increased circulating AGT concentrations and increased risk of essential hypertension. Apart from hypertension, which is a well established risk factor for CHD, several studies have reported a significant association with risk of CHD or myocardial infarction among individuals with the TT genotype (3). However, the distribution of AGT genotypes differs among races, suggesting that the clinical implication of genetic variant that may also differ (7, 8). So, we estimated the presence of this M235–T variant in Egyptian patients suffered from CAD either angina pectoris or myocardial infarction (MI). We also measured the plasma AGT level to study its relation to the gene variant plus the development of hypertension which is frequently present in those patients.

SUBJECT AND METHODS

This study included 70 male patients with CAD who were recruited from January 2009 till July 2009. Inclusion criteria were unstable angina or myocardial infarction. Patients were divided into two groups. *Group (1)*: 35 patients with from unstable angina pectoris diagnosed as (9) angina occurring at rest and prolonged, new-onset angina of at least CCS class III severity or previously diagnosed angina that has become distinctly more frequent, longer in duration, or lower in threshold (i.e., increased by 1 or more CCS class to at least CCS class III severity). *Group (2)*: 35 patients with myocardial infarction (MI) diagnosed as (10) rise and/or fall of cardiac biomarkers with at least one value above the 99th percentile of the upper reference limit together with evidence of myocardial ischemia with at least one of the following: symptoms of ischemia, new ST-T wave changes or new left bundle branch block, development of pathological Q waves in the ECG and/or imaging evidence of new loss of viable myocardium or new regional wall motion abnormalities. The control group consisted of 60 healthy males with no symptoms or manifestations of CAD.

METHODS

An informed consent was obtained from all study recruits. Five ml of blood were taken under aseptic condition and were collected on EDTA. Two ml of them were used for DNA analysis. Three ml was used for determination

of cholesterol, triglycerides, glucose and angiotensinogen concentration in plasma.

1. DNA extraction:

DNA was extracted using the PURGENE kit from Gentra Systems (Minneapolis, MN, USA), and stored at -80°C in aliquots until required.

2. PCR for angiotensinogen genotyping:

PCR was performed according to Ishigami, et al. (11). The PCR mixture was formed of DNA solution (200 ng), Two specific primers with sequences as follows: upstream 5'CGTTTGTGCAGGGCCTGGCTCTC3' & downstream, 5'AGGGTGCTGTCCACACTGGACCC3'

The final volume was 20 µl. The PCR cycling condition was as follows, an initial denaturation step at 90°C for 3 min, 10 cycles at 94°C for 1 min, 68°C for 1 min, and 72°C for 1 min, and 30 cycles at 90°C for 30 seconds and 68°C for 30 seconds and 72 for 30 seconds and final extension at 72°C for 10 min.

3. Allelic polymorphism using restriction endonuclease:

Four µl of PCR product was digested with 4 µl of enzyme (Tth III), the mixture was incubated at 65°C for 2 hours. The digested products were electrophoresed on 2% agarose gel stained with ethidium bromide. M 235 T polymorphism of Angiotensinogen gene was diagnosed by UV. Homozygous for M was detected at 163 bp, heterozygous MT was detected at 163 and 140 bp and homozygous for T was detected at 140 bp.

4. Quantitation of plasma angiotensin I level:

Angiotensin concentration in plasma was measured as described by Umemura, et al. (12). Angiotensin I was measured by RIA [Renin RIA BEAD, Ang I Kit, Dainabat-Ltd).

Echocardiography was performed using a commercial machine Philips Sonos 2000 with a 2.5 MHz probe. Dimensions were taken from the standard parasternal short axis and long axis views. Ejection fraction was calculated for all patients using the bi-plane Simpson's rule from the apical four chambers and apical two chamber views. Doppler analyses of mitral and aortic valves were obtained from the apical windows. Left ventricular systolic dysfunction was diagnosed as an ejection fraction less than 50%. Regional wall motion abnormalities were diagnosed and quantified according to the 16-segment model of the American Society of echocardiography. Hypokinesis was defined as less than 30% increase of wall thickness during systole. Akinesis was defined as absence of wall thickening in systole. Dyskinesis was defined as systolic thinning (13).

Statistical analysis was performed using Microsoft Win PEP statistical program. For comparison of parametric data we used mean±SE and student-t-test for numerical data. Non-parametric data were analyzed by Chi-Square test, Odds ratio and 95% confidence interval (CI) which is the range of scores within which the percentage will be found.

RESULTS

There were no significant differences in mean age between the control and CAD groups (Table 1) However, there were a significant differences in DBP (P <0.001), serum cholesterol (P <0.001) and angiotensin 1 levels (p <0.01). (Table 2) showed the parameters shared as risk factors. There were significant differences in the number of patients who their ages exceed 45 years between control and CAD groups (p <0.05), but Hypertension (DBP >80), smoking, hypercholesterolemia and fasting blood glucose >120) showed non significant differences. (Table 3) showed the frequencies and differences of paired alleles, TT, MT and MM were significantly higher in CAD 23%, 50% and 27% respectively compared to 3.3%, 33.4% and 63.3% in control group respectively. The frequency of T was 0.54 and 0.66 in control and CAD group respectively, while M allele frequency was 0.83 and 0.84 in control and CAD patient respectively. (Figure. 1) The distribution of T allele among cases was significantly higher than control. By comparing the genotype distribution between diseased group we found non-significant differences between TT, MT, and MM, p >0.05 as shown in (Table 4). Analysis showed that homozygous TT was the high risk group and

heterozygous MT was at risk while homozygous MM controls was not at risk as shown in (Table 5). On the other hand a positive correlation was found between diastolic blood pressure and genotype TT and age with p <0.00 and 0.05 respectively (Table 6).

Table 1: Statistical analysis of age (Years), diastolic blood pressure (mmHg) and biochemical parameters in control (N=60) and CAD (N=70) groups:

	Control (n=60) Mean±SE	CAD (n=70) Mean±SE	P
Age (years)	48.3±5.2	52.4±2.1	>0.05
DBP (mmHg)	78.5±5.27	90.2±6.2	<0.001*
Fasting Blood glucose (mg/dl)	104.3±3.3	112.6±2.5	>0.05*
Cholesterol (mg/dl)	163.2±3.7	195.1	<0.001*
Angiotensin 1(ug/ml)	47.1±2.9	55.3±1.2	<0.01*

Table 2: Comparison between control and CAD as regard to parameters shared as risk factors [Age >45 years), Hypertension (DBP >80), smoking, hypercholesterolemia and fasting blood glucose (>120)]:

	Control (N=60)	CAD (N=70)	X2	P
Age (>45 years)	24/60	46/70	5.83	0.015*
Hypertensive (DBP>80)	18/60	23/70	0.101	0.749
Smokers	28/60	40/70	1.097	0.294
Hypercholesteremia	15/60	25/70	0.635	0.425
Fasting Blood Glucose (>120)	10/60	14/70	0.145	0.703

* Significant P <0.05

Table 3: Frequency of genotype in control and CAD patients:

	Control (n=60)	CAD (n=70)	X ²	P	Odds Ratio O.R.	(95% CI)
TT	2 (3.3%)	16 (22.9%)	5.16	<0.0165*	8.83	1.1-189.3
MT	20 (33.4%)	35 (50%)	2.25	0.134	2	0.73-5.52
MM	38 (63.3%)	19 (27.1%)	11.31	<0.001*	0.21	0.07-0.59

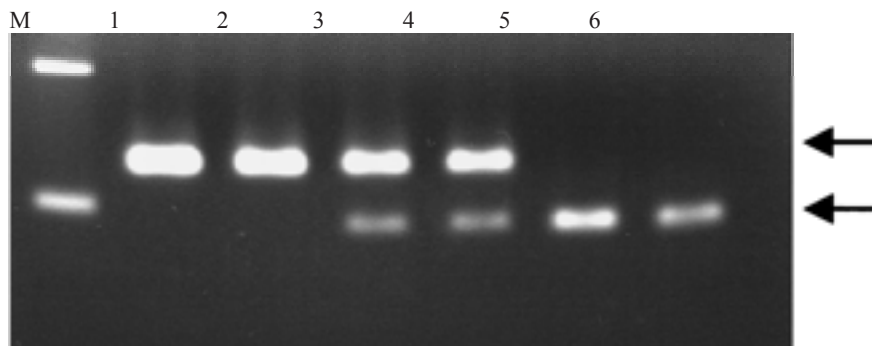


Figure 1: Representative agarose gel electrophoresis, findings of M235-T polymorphism of AGT gene. Marker (100 bp), Lanes 1, 6, 7 Homozygous for M, Lanes 2, 4, 5 Heterozygous MT, Lane 3 Homozygous for T.

Table 4: Distribution of genotype of M235T in CAD group:

	Angina Pectoris (n=35)	MI (n=35)	X ²	P	OR	O.R. (95% CI)
MM	10 (28.6%)	9 (26%)	0.039	0.84	1.12	0.3-4.13
MT	15 (42.8%)	20 (57%)	1.714	0.190	0.94	0.15-1.6
TT	10 (28.6%)	6 (17%)	1.79	0.18	2.27	0.57-9.3

Table 5: Effects of T allele on other allele variants of CAD group:

Effect	Odds ratio (95% CI)	P
Recessive (TT vs MT and MM)	11.28 (1.4-24.32)	<0.006*
Dominant (TT and MT vs MM)	4.675 (1.69-13.6)	<0.001*
Additive (TT vs MT)	4.67 (0.51-106.9)	0.13
(TT vs MM)	4.67 (0.51-106.9)	<0.002*

Table 6: Rank between DBP and studied parameters in patient group:

Univariate correlation model	r	r ²	F	P
Genotype (TT)	0.43	0.19	13.45	<0.001*
Age	0.25	0.06	3.87	<0.05*
Fasting Blood glucose (mg/dl)	0.14	0.019	0.10	>0.05
Cholesterol	0.07	0.01	0.32	>0.05
Smoking	0.12	0.01	0.84	>0.05
Angiotensin 1	0.06	0.01	0.36	>0.05

DISCUSSION

Human angiotensinogen a hormone precursor with a molecular mass of 61.400, is predominantly synthesized in liver under estrogens, glucocorticoids, thyroid hormones and angiotensin. It is cleaved by the enzyme renin to produce angiotensin I, which is further converted into a vasoconstricting peptide angiotensin II (14). The concentration of AGT is a rate limiting in production of angiotensin I and therefore plays an important role in the production of Angiotensin II . The angiotensinogen gene is expressed in multiple tissues including liver, adipose tissue, heart, blood vessels, brain and kidney. Among the cell types expressing AGT are epithelial cells of the renal proximal tubule, hepatocytes of the liver, adipocytes in fat and astrocytes and selected neurons of the brain. The human AGT gene, a member of the serpin gene super family, stretches over only 12kb on chromosome 1(1q42-q43) and contains 5 exons interrupted by four intervening sequences and spans 12 Kb. In its 5 flanking region multiple regulating elements as well as the major control elements are present (5, 15). The aetiology of coronary heart disease (CHD) involves both environmental and genetic factors. Much attention has been given to the contribution of smoking, hypertension and hyperlipidaemia. The renin

angiotensin system is not only essential in controlling cardiovascular haemodynamics and homeostasis, but also plays an important role in the development of CHDiv. Earlier studies showed that angiotensin II has a role in the maintenance of vascular structure. Recently variants of AGT gene were found to be associated with increased risk of hypertension and CAD (3, 16). Many studies were done on male subject only (17, 18) since male are more exposed to CAD. Others showed that the genetic variant of AGT (M235-T) contributes to the presence of coronary heart disease independently of blood pressure profile. So, we investigated the association of molecular variant of AGT (M235-T) in coronary heart disease patients to evaluate its role in development of the disease and to estimate its relationship to other risk factors

The results of this study showed that the frequency of TT genotype is significantly elevated in CAD group than control (P <0.01) while the MM genotype is significantly elevated in control group than CAD (P <0.001), the heterozygous form is none significantly different between both groups (P >0.05). The distribution of T allele among cases was significantly higher in cases than control .The level of plasma angiotensinogen was significantly elevated in CAD group than control (P <0.01). A significant positive correlation was detected between the DBP and either TT genotype (P <0.001) or age (P <0.05) in CAD group only.

These results coincide with that reported by Sarzani, et al. (19) and Jurkovicova, et al. (16). While a contradictory results were obtained by Sekuri, et al. (4), and Marciante, et al. (6). Rodriguez, et al. (20) confirm the association between CAD (MI and unstable angina) and AGT M235-T polymorphism by finding a significant increase in T235 allele in CAD than control but presence of hypertension is not related to the disease. They also proved that the genetic variation of AGT gene (M235T), but not ACE (insertion-deletion) genotypes contribute to the presence of CAD. Sarzani, et al. (19) showed that the AGT promoter G-6 variants did not affect the cardiovascular parameters in young adults but an effect of this polymorphism on cardiovascular phenotype in older adults can not be excluded. Sethi, et al. (21) showed that M 235-T single nucleotide polymorphism (SNP) was associated with elevated angiotensinogen levels in both genders and 30% of elevated blood pressure was found in women homozygous for 235 T, however, the risk of CAD and ischemic cerebro-vascular disease (ICVD) were not associated

with variation in genotype. The same investigator (21) proved that 235 TT was associated with 5-10% increased level of angiotensinogen and 10-20% increased in systolic and diastolic blood pressure in white patients while in Asians the risk of hypertension increases to 20-30 % but there was no risk of ischemic heart disease or ICVD. Buraczynska, et al. (22) showed that molecular variation of angiotensinogen gene was associated with increased risk of CHD more frequently with hyper lipidemia and it increases the risk of MI only in smokers. Also Goldenberg, et al. (3), put the hypothesis that homogenous M235T mutation of angiotensinogen gene may be a risk factor for cardiac events after discharge from the first MI attack. But Gross, et al. (23) found no association between AGT gene polymorphism and recurrent stenosis after stent insertion.

Winkelmann, et al. (17), showed that plasma Angiotensinogen was the most predictor of DBP followed by body mass index, they proved also that AGT M235T gene mutation was significantly positively associated with DBP and the predictive power of this mutation remains unchanged when the variables of age, smoking, and body mass index were entered in the multivariate analysis. On the other hand some investigator showed that the significant elevation of angiotensinogen in plasma was insignificantly associated with M235T mutation but with other type of mutation (C235T mutation in exon 2 of angiotensinogen gene (8).

Recently, the TT genotype of M235T polymorphism has been associated with high blood pressure in Brazilian, Russian, Romanian, Spanish, Turkish, Mongols, Taiwanese and Pakistani populations (24). Our study showed a significant elevation of angiotensinogen level, serum cholesterol and DBP in CAD than control, however non significant positive correlation was detected between them and DBP, however significant positive correlation was detected between TT mutation, age and DBP.

Liu, et al. (25) studied six polymorphism of AGT gene at position -217GA,-152GA,-20AC and-6GA in the promoter region and T174 Mand M235T in exon 2 in essential hypertension and showed that -152A,-20C, -6A and 235 Talleles were significantly increased in essential hypertensive patients than control, earlier in Chen, et al. found that genotype of AGT gene at exon-2 might be associated with increased risk of essential hypertension (26).

Recently many studies was concerned with investigating the contribution of different single locus polymorphism, genotypic interactions and haplotypes towards hypertension and coronary heart disease (7, 27, 28). Eventually this study confirmed the role of 235TT molecular variant in occurrence of CAD. More studies must be done on the Egyptian population to estimate the effect of other SNP of AGT gene in those patients and link M235T variant with them if present.

STUDY LIMITATIONS

The causes of discrepancies among genetic association studies may include statistical artifacts due to small sample size, population stratification, different population histories and genetic backgrounds in different ethnic populations, epistatic gene-gene interaction when the effect of the studied gene is masked by the effect of other susceptible loci, or that a specific multi-locus haplotype, rather than any of the single loci which define the haplotype, is more significant in determining the association.

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