

## Association of Angiotensinogen (M235T) Gene Polymorphism with Blood Pressure Lowering Response to Angiotensin Converting Enzyme Inhibitor (Enalapril)

Kamna Srivastava<sup>1</sup>, Sudhir Chandra<sup>1</sup>, Jagriti Bhatia<sup>2</sup>, Rajiv Narang<sup>3</sup>, Daman Saluja<sup>1</sup>

<sup>1</sup>Dr. B.R. Ambedkar Centre for Biomedical Research, University of Delhi, Delhi, India;

<sup>2</sup>Departments of Pharmacology and <sup>3</sup>Cardiology, All India Institute of Medical Sciences, New Delhi, India.

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### ABSTRACT

**Purpose:** It has been suggested that genetic backgrounds, which have an association with essential hypertension, may also determine the responsiveness to ACE inhibitor. We determined the association of angiotensinogen (M235T) gene polymorphism with essential hypertension and the relationship between polymorphism in the angiotensinogen (M235T) gene and blood pressure response to ACE inhibitor (Enalapril) in patients with essential hypertension from northern Indian subjects. **Methods:** 250 patients with essential hypertension and 250 normal healthy controls from Delhi and surrounding areas were recruited for the investigation. Blood pressure was recorded before and after 6 weeks of treatment with ACE inhibitors, Enalapril. Genotyping were carried out by polymerase chain reaction and Restriction fragment length polymorphism technique. **Results:** Statistically significant association of T allele was observed with essential hypertension [ $\chi^2 = 14.67$ ,  $p = 0.00013$ , Odds ratio = 1.76 (1.3-2.32) at 95% CI], the relative risk at 95% CI being 1.28 (1.2-1.54). The decrease in systolic blood pressure and diastolic blood pressure after six weeks of treatment of the patients carrying TT genotype (SBP =  $26 \pm 17.4$  mmHg, DBP =  $14.83 \pm 7.6$  mmHg) were greater than the groups carrying MT (SBP =  $3.0 \pm 7.8$  mmHg, DBP =  $6.2 \pm 3.0$  mmHg) and MM genotypes (SBP =  $1.2 \pm 0.8$  mmHg, DBP =  $0.10 \pm 12.1$  mmHg). **Conclusions:** The angiotensinogen (M235T) gene polymorphism is significantly associated with essential hypertension. Patients carrying TT genotype had higher blood pressure lowering response when treated with ACE inhibitor, Enalapril than those carrying MM and MT genotypes suggesting that the T allele may be a possible genetic marker for essential hypertension.

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### INTRODUCTION

Essential hypertension is directly responsible for 57% of all stroke deaths and 24% of coronary heart disease death in India (1). Essential hypertension is a multifactorial disease associated with the interaction of genetic and environmental factors and genes contribute 20-40 % of the pathogenesis of essential hypertension (2).

The renin-angiotensin system (RAS) contributes to the regulation of vascular tone, electrolyte and volume homeostasis (3), and their role in blood pressure regulation has been well established (4). Genes encoding components of RAS, including angiotensinogen, have been extensively investigated as genetic determinants of essential hypertension through genetic linkage studies, and by allelic association studies (5-6).

Three RAS gene polymorphisms- the angiotensinogen (AGT) M235T (7-13), the ACE insertion/deletion (I/D) (10, 13-19), and the AT1R A1166C (10, 20-21) polymorphisms—have been extensively studied. The gene that encodes angiotensinogen is found on chromosome 1q42 to 43 where a tyrosine for cytosine substitution in the second exon results in the substitution of threonine for methionine at amino acid position 235 (M235T) in the translated protein. This missense mutation of the angiotensinogen (M235T) gene has been associated with elevated

**Corresponding Author:** Dr. Kamna Srivastava, Molecular Cardiology Lab, Dr. B.R. Ambedkar Centre for Biomedical Research, University of Delhi, Delhi, India; E-mail: kamna\_srivastava@hotmail.com

levels of angiotensinogen, with 235 TT homozygotes having between 10 % and 20 % more plasma angiotensinogen than 235 MM individuals (6-7). Association (case-control) studies of the M235T polymorphism in essential hypertension have yielded conflicting results. Some found linkage or association in Nigerian (22), French (21), Han Chinese (23) and Malaysian populations (24), while others have not (25-26).

Variation in patient's response to therapy is a well-known phenomenon in clinical practice. This discrepancy in patients's response most of the times lead to therapy discontinuation and/or underdosing because of poor efficacy or unwanted side effects. Genetics might be playing some role in the variability in individual response to therapy (2-7). Association between a genetic polymorphism and the response to treatment may provide insight in the pathogenetic mechanisms. The inter-individual's variation in response to the rennin-angiotensin system (RAS) blockade may partly be genetically determined. We hypothesize here that the genetic backgrounds, which have an association with essential hypertension, may also determine the responsiveness to ACE inhibitor. We chose angiotensinogen (M235T) gene polymorphism as we are aware that their role in the pathogenesis of Essential hypertension is fully elucidated. Little information is available on its possible association with the responsiveness to ACE inhibitor therapy.

Hence, present study was undertaken to determine the possible association of the genetic variants of angiotensinogen (M235T) gene polymorphism with Essential hypertension in Asian Indian subjects. Knowledge of Intergenotypic variations in angiotensinogen (M235T) gene might help to predict blood pressure response to ACE inhibitor therapy in individual patients. Hence, we have made our second objective to determine the association between polymorphism in the angiotensinogen (M235T) gene and blood pressure response to ACE inhibitor (Enalapril) in patients with Essential hypertension from northern Indian subjects.

## **MATERIALS AND METHODS**

**The three study groups are:**

### **(1) Patients with essential hypertension to**

### **study angiotensinogen M235T gene polymorphism**

The criteria of selection of patients were as follows: Patients (N=250) suffering from Essential hypertension was recruited from department of cardiology, AIIMS, New Delhi. Age (25-60 years), Onset (25-60 years), Systolic BP > 140 mmHg and a diastolic BP > 90 mmHg on 2 consecutive visits for those untreated, absence of secondary forms of hypertension. Subjects with a history of diabetes mellitus, renal failure and major infectious disease were excluded. They had no metabolic or endocrine disorder or any acute illness. They were not on any antihypertensive treatment and were drug naive patients.

At this stage of screening the blood were withdrawn from the patients in order to study the Association of angiotensinogen (M235T) gene polymorphism with essential hypertension.

### **(2) Patients with essential hypertension with ACE inhibitor treatment**

Above mentioned patients (group 1) were then receiving ACE inhibitor Enalapril for six weeks thereafter and their Blood pressure response on angiotensinogen (M235T) gene polymorphism, were recorded. In our work, we limited patient's recruitment, to only those who were never treated before in order to avoid the influence of any previous treatment.

### **(3) Controls (Normal healthy volunteers)**

Controls (n=250) which were age and sex matched normal healthy volunteers from the similar population group recruited from the staff of AIIMS and residents of Delhi and surrounding areas. The screening of controls was carried out by the same clinician as in patients. Blood pressures were measured in all three groups of study subjects using sphygmomanometer at least three times at an interval of five minutes in accordance with the procedures recommended by JNC VII (The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High blood pressure) criteria. The subjects were seated in a chair with back support and their feet on the ground and the arm placed comfortably on a table at heart level. There was 5 min of rest in a quiet room preceding the blood pressure measurements. The appropriate-sized cuff, based on the patient's arm

circumference was placed on the upper arm, 5 cm above the ulnar head. The blood Pressure measurements were always performed by the same doctor between 8.00 and 10.00 am about 24 hour after the last Enalapril dosage.

Various baseline characteristics and clinical parameters including age, gender, Body mass index, smoking and blood urea, serum creatinine and lipid profile were measured. During the study period, patient medication and dietary intake for sodium was not changed. Prior informed consent from both the study groups was obtained. An approval of study protocol by the ethical committee of AIIMS, New Delhi was obtained prior to the start of the study.

### Sample collection and processing

The peripheral venous blood was drawn from the study subjects after 12 hours of fasting and packed cells were used for DNA isolation using the QIAamp® DNA Blood Mini Kit by QIAGEN®.

### Angiotensinogen (M235T) gene polymorphism

Genomic DNA was amplified by polymerase chain reaction (PCR) to amplify part of the exon 2 gene with primers designed to insert a half restriction endonuclease site into the product. Primers were designed using Gene Runner programme and the designed sequences were: sense-primer 5' GAT GCG CAC AAG GTC CTG TC 3' and antisense primer 5' CAG GGT GCT GTC CAC ACT GGA CCC C 3'. Amplified 303 bp products of the angiotensinogen gene were digested using the *Pst*I (Fermentas life sciences) and the resultant fragments resolved on 2.7 % agarose gel to give 2 fragments, the longer fragment, 279 bp and the shorter 24 bp. Individuals homozygous for allele, ie MM, a 303-bp band appeared on the gel, homozygous TT (only a single 279-bp band appeared on the gel) and heterozygous MT (303-bp band and a 279-bp band appeared on the gel).

### STATISTICAL ANALYSIS

All computations were carried out with STATA program, version 8. Results are expressed as mean  $\pm$  sd (standard deviation). Sample size was adequate for the present study determined by using standard statistical method at the ratio of 1:1 for case control groups, at the significance level of 0.05 at power 80% on the basis of prevalence of minor alleles referred from previous studies (6, 27). Chi-square goodness of fit was

used to verify the agreement of observed genotype frequencies with those expected (Hardy–Weinberg equilibrium). Difference between genotype groups were tested with analysis of variance (ANOVA) using Bonferroni's method for multiple comparisons between genotype classes. The multilinear stepwise regression was made to assess the factors responsible for the reduction of blood pressure.  $P < 0.05$  was considered statistically significant.

Calculation of mean arterial pressure is done by the formula given below:

$$\text{Equation: MAP} = [(2 \times \text{diastolic}) + \text{systolic}] / 3$$

Where, diastole counts twice as much as systole because 2/3 of the cardiac cycle is spent in diastole. The usual range of MAP is 70-110 and an MAP of about 60 is necessary to perfuse coronary arteries, brain and kidneys.

## RESULTS

### Baseline characteristics of the study subjects

A total of 250 hypertensive patients and 250 controls were enrolled in our study. Average age of the controls and patients was comparable. Number of male volunteers was comparable to female in both the study subjects. The mean values of body mass index (BMI), heart rate, different biochemical parameters and Systolic and diastolic blood pressures of study subjects were comparable in both the study subjects (Table 1).

### Distribution of genotypes and alleles of angiotensinogen (M235T) gene polymorphism in the study subjects

The distribution of genotypes (MM, MT and TT) in the controls and patients did not differ significantly from that expected under Hardy-Weinberg equilibrium. In the total of 250 patients, the MM genotype was observed in 112 patients, the MT genotype was observed in 120 patients and the TT genotype in 18 patients. The observed genotypes in controls were 154 MM, 90 MT, 6TT genotype. The allele frequencies in patients were found to be 0.69 and 0.31 for M and T allele respectively whereas 0.80 and 0.20 for M and T in controls. We found significant association of angiotensinogen genotypes (MT+TT) with essential hypertension as the observed difference in genotypes between the controls and patients was statistically significant. [ $\chi^2 = 13.9$ ,  $p = 0.00016$ , Odds ratio = 1.97 (1.35-2.85) at 95% CI], the relative risk at 95% CI was

1.3 (1.15-1.6) . Statistically significant association of T allele was observed with essential hypertension [ $\chi^2 = 14.67$ ,  $p = 0.00013$ , Odds ratio = 1.76 (1.3-2.32) at 95% CI], the relative risk at 95% CI being 1.28 (1.2-1.54) (Table 2). Odds ratio and relative risk adjusted for age and sex in both genotypes and alleles.

**Comparison of Reductions of Blood pressure among different genotypes of angiotensinogen (M235T) gene in the patients receiving ACE inhibitor Enalapril**

The observed changes in systolic and diastolic blood pressure were analyzed to determine their association with genotypes at the angiotensinogen gene locus. There was statistically significant reduction in systolic blood pressure in patients with MM, MT and TT genotypes,  $1.2 \pm 0.8$  mmHg,  $3.0 \pm 7.8$  mmHg,  $26 \pm 17.4$  mmHg, respectively ( $p < 0.0001$ ) when treated with ACE inhibitor, Enalapril for a duration of 6 weeks. Similarly, there was significant reduction in diastolic blood

pressure in patients with MM, MT and TT genotypes, ie,  $0.10 \pm 12.1$  mmHg,  $6.2 \pm 3.0$  mmHg and  $14.83 \pm 7.6$  mmHg, respectively ( $p < 0.0001$ ) when treated with ACE inhibitor, Enalapril. The decrease in systolic blood pressure and diastolic blood pressure after six weeks of treatment of the patients carrying TT genotype (SBP =  $26 \pm 17.4$  mmHg, DBP =  $14.83 \pm 7.6$  mmHg) were greater than the groups carrying MT (SBP =  $3.0 \pm 7.8$  mmHg, DBP =  $6.2 \pm 3.0$  mmHg) and MM genotypes ( $1.2 \pm 0.8$  mmHg, DBP =  $0.10 \pm 12.1$  mmHg).

The mean arterial pressure (MAP) before Enalapril treatment in patients with three genotypes ie MM, MT and TT were comparable ( $p = 0.06$ ). In our study, the mean arterial pressure in patients with TT genotypes ( $116.9 \pm 15.4$  mmHg) was higher than the normal range of 70 - 110 mmHg and there was significantly greater reduction in MAP after treatment with Enalapril as compared to patients with MT ( $p = 0.03$ ) and MM genotypes ( $p = 0.001$ ) (Table 3).

**Table 1.** Baseline characteristics of the study subjects.

Parameters	Patients	Controls	p value
Number (N)	250	250	
Sex (M/F)	164/86	170/80	0.85
Age (years)	$52.2 \pm 5.8$	$49.7 \pm 11.1$	0.21
BMI, (Kg/m <sup>2</sup> )	$18.58 \pm 2.9$	$18.6 \pm 3.4$	0.95
Heart Rate, (Beats/min)	$74.5 \pm 10.6$	$71.8 \pm 5.8$	0.86
Blood glucose (mg/dl)	$92.54 \pm 18.3$	$91.9 \pm 14.3$	0.78
Blood Urea (mg/dl)	$20.7 \pm 4.3$	$18.8 \pm 3.7$	0.63
Serum Creatinine	$0.98 \pm 0.2$	$0.96 \pm 0.25$	0.72
LDL cholesterol (mg/dl)	$86.3 \pm 21.5$	$92.3 \pm 27.3$	0.11
HDL cholesterol (mg/dl)	$41.4 \pm 6.2$	$38.3 \pm 6.2$	0.08
Triglyceride (mg/dl)	$167.1 \pm 30.3$	$169.9 \pm 20$	0.78
Total cholesterol (mg/dl)	$149 \pm 45$	$158 \pm 44$	0.08
Systolic blood pressure(SBP) mm Hg,	$152.0 \pm 13.0$	$120 \pm 3.3^*$	0.0001
Diastolic blood pressure(DBP) mm Hg,	$94.6 \pm 8.7$	$80.6 \pm 2.8^*$	0.0001

\*Significant difference between groups.

**Table 2.** Genotype and Allele Frequencies of M235T variant of the angiotensinogen gene in the study subjects.

Subject	Genotypes*			Allele Frequency**	M	T
	MM(%)	MT(%)	TT(%)			
Controls (n=250)	154 (61.60)	90 (36.0)	6 (2.4)	$\chi^2 = 2.94$ , $p > 0.05$ (DF = 1)	0.8	0.2
Patients (n =250)	112 (44.8)	120 (48.0)	18 (7.2)	$\chi^2 = 3.49$ , $p > 0.05$ (DF=1)	0.69	0.31

\*Unadjusted Odds Ratio at 95% CI 1.98 [1.3 – 2.8],  $\chi^2 = 14.17$ ,  $p = 0.00017$ ,

\*Adjusted Odds Ratio at 95% CI 1.97 [1.35 – 2.85],  $\chi^2 = 13.9$ ,  $p = 0.00016$ ,

\*Adjusted Relative Risk at 95% CI 1.3 [1.15 – 1.6],

\*Unadjusted Odds Ratio at 95% CI 1.8 [1.32 – 2.36],  $\chi^2 = 14.67$ ,  $p = 0.00013$ ,

\*\*Adjusted Odds Ratio at 95% CI 1.76 [1.3 – 2.32],  $\chi^2 = 14.6$ ,  $p = 0.00013$ ,

\*\*Adjusted Relative Risk at 95% CI 1.28 [1.2 – 1.54],

Patients groups were compared with controls with chi-square ( $\chi^2$ ) test at one degree of freedom with Odds ratio and relative risk adjusted for age and sex in both genotypes and alleles.  $P < 0.05$  is considered to be significant

**Table 3.** Intergenotypic (M235T variant of the angiotensinogen gene) variations in systolic and diastolic blood pressure response when treated with ACE inhibitor (Enalapril) in the patients with essential hypertension.

BP (mmHg)	ACE genotypes			P-value
	MM	MT	TT	
SBPB	146.9±3.8	149.4±27.5	159.8±27	0.043
SBPA	145.7± 3.0	146.4±19.7	133.9±9.6	0.0002
SBPΔ	1.2± 0.8	3.0±7.8	26±17.4	0.0001
DBPB	94.34±18.8	91.8±11.9	95.38±9.2	0.05
DBPA	94.24±6.7	85.7±8.9	80.55±1.6	0.0001
DBPΔ	0.10±12.1	6.2±3.0	14.83±7.6	0.0001
MAPB	111.9±13.8	111.0±17.1	116.9±15.4	0.06
MAPA	111.4±6.5	105.9±12.5	98.3±13.5	0.0001
MAPΔ	0.5± 7.3	5.1± 4.6	18.6±1.9	0.0001

  

Comparison of Genotypes*	p- values					
	SBPB	SBPA	DBPB	DBPA	MAPB	MAPA
MM vs MT	1.0	0.016	0.22	0.0001	0.012	0.0001
MM vs TT	0.049	0.0001	1.00	0.0001	0.72	0.001
MT vs TT	0.071	0.017	0.10	0.036	1.0	0.03

SBP, DBP and MAP were compared with respect to genotypes with t-test of significance test at one degree of freedom adjusted for age and sex. P< 0.05 is considered to be significant; \*analysis of variance (ANOVA) using Bonferroni's method for multiple comparisons between genotype classes.

## DISCUSSION

Several studies have reported association between essential hypertension and angiotensinogen (M235T) gene polymorphism. These associations were reported in the French (21), Han Chinese (23), and Malaysian populations (24), whereas others have found no association (25-26). In German population, Ortlepp et al. (28) reported that the effect of angiotensinogen (M235T) gene polymorphism on blood pressure regulation is detectable very early in life far before the onset of arterial hypertension. In Japanese populations (29-30), some studies found a positive association of TT genotype with essential hypertension. A study from India, reported in small sample size was lacking the association of this gene polymorphism with essential hypertension (31). We conducted a case-control study in North Indian population to find any association between M235T gene polymorphism and essential hypertension. In our study, a codominant pattern of inheritance was seen in both the study subjects as the genotype frequencies in both groups were all in accordance with the Hardy-Weinberg equilibrium. Results from our study have shown the distribution of M235T genotypes among patients in the order of MT>MM>TT, which was similar to French (21) and East Anglians (32) and quite different from the Asian populations

reported ie, Han Chinese (23) and Japanese (25). The frequency of T allele among controls in our study, which was 0.20, is 2 times lower than the study in French population (6, 21) and approximately three times lower than Japanese (30) population. Multivariate logistic regression analysis (regressed for age and sex) revealed that individuals with the "T" allele were at 1.8 times higher odds [1.76(1.3-2.32) at 95% CI, X<sup>2</sup>=14.6, p=0.00013] to develop Essential hypertension. Our study showed a higher Odds Ratio (O.R.) of 1.76 (95% CI, 1.3–2.32) compared to a recent meta-analysis of 12 studies in the Whites which indicated that T235 is associated with a 20% increase risk of hypertension (O.R. = 1.22, 95% CI, 1.10–1.29) (9). The relative risks for hypertension are 1.3 for subjects carrying the TT phenotype and 1.28 for those having allele T of the angiotensinogen (M235T) gene polymorphism. This study provides the normal distribution of the genotypes and alleles in this polymorphism among Asian Indians from Northern region of India which can be used as the baseline data to elucidate the contribution of this polymorphism in the disease state. It suggests that genetic factors have an important role to play in the etiology of Essential hypertension.

There is general agreement that the T allele of angiotensinogen (M235T) gene is associated with increased plasma angiotensinogen level (6-7).

Angiotensin-converting-enzyme (ACE) inhibitors are recommended for managing cardiovascular diseases, such as hypertension and heart failure (33). However, there is substantial variability in individual responses to these agents. For example, fewer than 50% of hypertensive patients achieve adequate blood pressure control with ACE inhibitor monotherapy (34). An increasing number of studies have indicated that patients from different ethnic groups of population have different responses to ACE inhibitors. Heterogeneity in the individual blood pressure response to ACE inhibitor therapy still represents an obstacle in the treatment of Essential hypertension. Comparison of genotypes has shown a significant difference in the frequencies of angiotensinogen (M235T) gene polymorphisms differ significantly by race (6, 22, 24, 30). Thus, it is possible that the ethnic diversity in genotypes contributes to the observed variability in ACE inhibitor responses among races, although no data pertaining to this hypothesis are available.

Hingorani et al. (26) have reported association of 235 T allele associated with greater blood pressure response in patients essential hypertension when treated with ACE inhibitor Enalapril for a duration of 4 weeks. They further added that blood pressure response was dependent on angiotensinogen genotype in the Caucasians with captopril, enalapril, lisinopril and perindopril. In a separate study, Hingorani et al. (32) have concluded that the angiotensinogen M235T gene polymorphism is not a marker for blood pressure level in these East Anglian subjects. Mondorf et al. (35) found no relationship between the polymorphism and blood pressure response to an ACE inhibitor (captopril) in white subjects. This inconsistency in results might be explained by the genetic and environmental heterogeneity among different ethnic groups, and differences in ACE inhibitors (36) used and study sample size. This inconsistency in results led us to determine the relationship between the Angiotensinogen M235T gene polymorphism and blood pressure response to an ACE inhibitor (Enalapril) in an appropriate sample size of 250 patients from North India. To the best of our knowledge, no such study exists in Asian Indian context.

The major finding of our work is the significantly greater blood pressure fall observed in carriers of genotype TT compared with individuals with MT and MM genotypes in systolic as well as diastolic blood pressures ( $p < 0.0001$ ). Our results are in accordance with the

study conducted by Hingorani et al. (26) in which they have reported association of 235 T allele associated with greater blood pressure response in 125 subjects with previously untreated essential hypertension when treated with ACE inhibitor Enalapril for a duration of 4 weeks. However, in our study subjects, the number of patients was comparatively higher than previously studies reported so far (32, 35) and we have also reported a significant association of angiotensinogen (M235T) gene with essential hypertension. We have also determined the mean arterial pressure in the study subjects. The mean arterial pressure represents the average arterial pressure throughout the cardiac cycle, and is the force that drives blood through the vasculature. In our study, the mean arterial pressure in patients with TT genotypes was higher than the normal range of 70 -110 mm Hg and there was significantly greater reduction in MAP after treatment with Enalapril as compared to patients with MT ( $p = 0.03$ ) and MM genotypes ( $p = 0.001$ ). Our study can provide a baseline data to predict response to ACE inhibitors in a patient population.

## CONCLUSION

We have found that the individuals with the T allele of the angiotensinogen (M235T) gene were strongly associated with essential hypertension in northern Indians. Our findings also provide the direct evidence of gene disease association of this region and higher predisposition of Asian Indians to the essential hypertension. Our findings also suggest that angiotensinogen (M235T) gene polymorphisms may help in predicting the intergenotypic variations in blood pressure response to ACE inhibitor, Enalapril in patients with essential hypertension in northern Indian subjects, also it can provide a baseline data to predict response to ACE inhibitors in a patient population. Patients carrying TT genotype had higher blood pressure lowering response when treated with ACE inhibitor, Enalapril than those carrying MM and MT genotypes, suggesting that T allele may be the biomarker of patients with essential hypertension. The effect of different angiotensinogen (M235T) genotypes on ACE inhibitor therapy may offer some clinical clues to the individual therapy for essential hypertension. This information will be of immense use in personalization of drug therapy to patients with hypertension based on the angiotensinogen (M235T) genotypes.

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