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Angiotensin Gene Polymorphisms (T174M and M235T) are Significantly Associated with the Hypertensive Patients of Tamil Nadu, South India

M. Karthikeyan[1, 2], V. Shridevi[2], Rajiv Rose[2], B. Anandan, Kh. Dhanachandra Singh[1], S. Shanmugasundaram[3], D. Mohan[4], A. Ramesh[2] and G. Jayaraman[2]

[1]Department of Bioinformatics, Alagappa University, Karaikudi 630 004, Tamil Nadu, India
[2]Department of Genetics, Dr. ALMPGIBMS, University of Madras, Taramani Campus, Chennai 600 113, Tamil Nadu, India
[3]K.S Hospital, Kilpauk, Chennai, Tamil Nadu, India
[4]Govt. Hospital, Head quarters, Dindigul 624001, Tamil Nadu, India

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ABSTRACT Hypertension is complex multi factorial disease; the development and progression of hypertension varies from population to population. The T174M and M235T polymorphisms of the *AGT* gene have been reported to be associated with essential hypertension in many populations. The researchers analyzed the possible association of T174M and M235T polymorphisms of *AGT* gene in essential hypertension patients (n=254) of Tamil Nadu, India along with equal number of age and sex matched control subjects. Results revealed that 235T mutation is an independent risk factor for essential hypertension in this study group (RR = 2.52; χ^2 = 39.32; CI = 1.89-3.371). It is a risk factor for both younger (RR = 2.72; χ^2 = 16.39; CI = 1.689-4.380) and older age (RR = 2.48; χ^2 = 16.53; CI = 1.60-3.839) groups. 174M is a more potent risk allele for younger onset (RR = 1.54; χ^2 = 3.97; CI = 1.01-2.34) of hypertensive patients than in the older (RR = 1.09; χ^2 = 0.14; CI = 1.42-1.67) essential hypertensive patients. Further both T174M and M235T polymorphisms were observed to be high risk factors in females than in males. Our finding suggested that both polymorphisms (M235T and T174M) are independent risk factors for essential hypertension while homozygous TT235 has greater effect than the MM174 mutation.

INTRODUCTION

Essential hypertension is a leading cause for human cardiovascular mortality and morbidity with a prevalence rate of 25-30% among adults of industrialized societies (Lifton et al. 2003). Blood pressures are known to be regulated by the renin-angiotensin system by controlling peripheral vascular resistance fluid and electrolyte homeostasis (Pilbrow et al. 2007). The effecter molecule of the renin angiotensin system (RAS) is angiotensin II. Angiotensin II can cause toxic effect on myocardial cells through the activation of sympathetic nervous system fibroblast proliferation and vasoconstriction of the coronary vessels (Pilbrow et al. 2007). Angiotensin II can cause vasoconstriction of the coronary vessels fibroblast proliferation and activation of sympathetic nervous system.

Address for correspondence:
Dr. M. Karthikeyan,
Assistant Professor,
Department of Bioinformatics, Science Block,
Alagappa University, Karaikudi 630 004,
Tamil Nadu, India
Telephone (Off): +91-4565-223100 Ext. 366
Fax (Off): +91-4565-225202
E-mail: mkbioinformatics@gmail.com

Angiotensinogen (AGT) is a major precursor of the renin-angiotensin-system and its plasma levels have been shown to correlate with blood pressure (Goldenberg et al. 2006). Variation in *AGT* gene and other genes of the RAS regulate blood pressure smooth muscle cell growth and cardiac remodeling (Sarzani et al. 2001). Among the *AGT* variants two coding region missense polymorphisms M235T (Methionine to Threonine amino acid substitution at the 235th position) and T174M (Threonine to Methionine amino acid substitution at the 174th position) in the exon 2 of the *AGT* gene have been significantly associated with hypertension in different ethnic populations (Jeunemaitre et al. 1992; Jeunemaitre et al. 1997; Corvol et al. 1999; Nakajima et al. 2004; Gopi et al. 2011; Charita et al. 2012; Mohana et al. 2012). Several studies found that G-6A polymorphism was in complete linkage disequilibrium with M235T and the haplotype with both polymorphisms was associated with hypertension (Chaves et al. 2002; Karthikeyan et al. 2009; Corvol et al. 1999; Nakajima et al. 2004).

Studies from different ethnic populations show variably associations between hypertension and the M235T, T174M variants. Highest fre-

quency of 235T polymorphism has been observed in Africans (84-94%) followed by Asians (70-73%) and Caucasians (20-45%) (Jeunemaitre et al. 1997; Sato et al. 2000; Nakajima et al. 2002). Though the variant allele 235T is the most frequent one among African populations and it is not significantly associated with hypertension (Cooper et al. 2000; Rotimi et al. 1997). It is reported that M235T mutants leads to small increase in concentration of polymorphic AGT rather than changes in function (Zhou et al. 2012; Kim et al. 1995). In a study from the Western part of India (Mumbai) neither of these polymorphisms was significantly associated with hypertension (Nair et al. 2003).

Frequency distribution and disease association of M235T and T174M has been shown to vary between different ethnic groups and also within large ethnic groups. To identify the specific frequency distribution and to elucidating the role of these variants in causation of essential hypertension in South India, we screened the *AGT* gene for M235T and T174M variants among hypertensive patients and controls from Tamil Nadu, South India.

METHODS

Selection criteria defined in the 7th JNC report (2003) and a WHO-ISH guideline for management of hypertension (Chalmers et al. 1997) was used for inclusion of hypertensive individuals. All the cases included in the present analysis met at least one of the following two criteria for the diagnosis of essential hypertension: prior diagnosis of essential hypertension by a physician and use of prescribed antihypertensive medication and/or having a systolic blood pressure (SBP) ≥140 mmHg and diastolic blood pressure (DBP) ≥90 mmHg. Any major illnesses in the past six months at the time of sample collection were excluded from the study. The institutional Human Ethical Committee of Dr. ALMPGIBMS, University of Madras, Taramani Campus, Chennai approved the research work prior to collection of start of the study. Informed consent from each individual was obtained before clinical screening and collection of blood samples. Five millilitre of venous blood was collected from hypertensive patients (n = 254) and age sex and location matched controls control subjects (n = 254) (127 males and 127 females each). Blood samples were collected from two different areas of Tamil Nadu, South India: 1. Rural-Government Hospital Head Quarters Dindigul, Tamil Nadu (patients n = 155; controls n=161) and 2. Urban- K.S. Hospital, Kilpauk, Chennai, Tamil Nadu (patients n = 99; controls n=93).

DNA was extracted using the protocol of Miller et al. (1988). DNA was quantified spectrophotometrically and quality checked using agarose gel electrophoresis. Allele specific primer polymerase chain reaction was used to estimate a T174M and M235T polymorphisms of the angiotensinogen gene. Allele specific primers; 5´AGGCCCAGCTGCTGCTGTCCAC 3´ (forward) for the 174T (1) allele, 5´AGGCCC AGCTGCTGCTGTCCAT 3´(forward) for the 174M(2) allele. 5´GTGCTG TCCACACT GGCTCCCA 3´(reverse) for the 235M(3) allele and 5´GTGCTGTCCACACTGGCTCCCG 3´ (reverse) for the 235T(4) allele were custom synthesized from (Sigma, Mumbai, India). These primer sequences were adapted from Iso et al. (2000). The four primer combi-nations can discriminate all possible haplotypes 1-3 (174T-235M), 1-4 (174T-235T), 2-3 (174M-235M) and 2-4 (174M-235T). The expected amplicon size is 226 bp. Each reaction was performed in duplicates for confirmation of the genotypes. One amplicon from each haplo-type was re-amplified, column purified and sequenced commercially (Genotypic India (p) Ltd. Bangalore, India) using both forward and reverse primers by via dye termination chemistry and read using the 96 capillary 3730x1 DNA analyzer (Applied Bio-systems, Carlsbad, CA).

To analyze the data for descriptive statistics and computation of means and standard deviations, SPSS 10.0 version (SPSS Inc. Chicago Illinois, USA) was used. χ^2 statistics were computed for significance of differences in the distribution of genotypes between cases and controls. The frequencies of the marker alleles were estimated by allele counting method and tested for Hardy-Weinberg (HW) equi-librium. Relative risks (RR) and 95% confidence intervals (CI) were computed for different combinations of genotypes to estimate their relative risk for developing EHT.Haplotype frequencies were estimated using SNPHAP software (http://www.gene.cimr.cam.ac.uk/clayton).

RESULTS

Genotype and Gene Frequency of AGT T174M Polymorphism

For analyses of the genotype and gene frequency distribution of the T174M polymorphism

the T allele is represented as allele 1 and M allele is represented as allele 2 (Table1). Among both controls and patients the distribution of genotype frequencies was independent of the residential status and gender ($p > 0.05$). Frequency of allele 1 is higher in control subjects (0.555) versus the patients (0.488) ($p < 0.05$). All RR estimates are significantly greater than one ($p \leq 0.05$) (Table 2). The genotype 22 has the greatest risk compared to the genotype 11 (RR=1.81; 95% CI =0.91 -2.98) and 12 (RR = 1.59; CI =0.98-2.47) (Table 2). Similarly, RRs were estimated for T174M polymorphism based on the sex (Appendix Table 1) residential status (Appendix Table 2) and age (Appendix Table 3). Results indicate that females urban population and younger age group (≤50 years) are more prone to hypertension when compared to male rural population and older age group (≥51 years).

Table 1: Genotype and gene frequencies of *AGT* gene (T174M polymorphism)

Group	*N*	*T174M polymorphism*				
		Genotype frequency (%)			*Gene frequency (%)*	
		11	*12*	*22*	*1*	*2*
Patients	254	24.4	47.6	28.0	48.8	51.2
Controls	254	29.9	51.2	18.9	55.5	44.5

11= TT; 12 = TM; 22= MM genotypes and 1 = T; 2 = M allele of T174M polymorphism

Table 2: Relative Risk (RR) for developing hypertension of the T174M genotypes and alleles

Comparison between	*T174M polymorphism*		
	RR	χ^2	*95 % of CI*
22 Vs 11	1.81	5.52*	0.91-2.98
22 Vs 12	1.59	4.30*	0.98-2.47
22 Vs 11+ 12	1.67	5.75*	1.10-2.53
2 Vs 1	1.34	5.39*	0.96-1.72

11= TT; 12 = TM; 22= MM genotypes and 1 = T; 2 = M allele of T174M polymorphism
χ^2 = Chi-Square with 1 degree of freedom; * p< 0.05. ** p< 0.01; ***p < 0.001.

Genotype and Gene Frequency of AGT M235T Polymorphism

For analyses of the genotype and gene frequency distribution of the M235T polymorphism the M allele is represented as allele 3 and T allele is represented as allele 4 (Tables 3 and 4). The frequency distribution of genotypes is independent of residential status and gender in essential hypertension patients ($p>0.05$) but not in control subjects ($p < 0.05$). The frequency of the allele 3 among the control subjects is twice the value (0.36) observed in patients (0.18). Individuals with the genotype 44 (or TT) are at greater risk of developing hypertension compared to the individuals with the other two genotypes i.e. 33(RR=2.29; 95% CI=1.37-3.85) and 34 (RR=5.53; 95% CI=3.46-8.92) (Table 4). Compared to 34+33 genotypes the RR of the individuals with genotype 44 is 3.61 (95% CI = 2.46-5.29). The heterozygous individuals are less predisposed to develop hypertension compared to individuals with either of the homozygous genotypes. The allele 4 (or T) relative to the allele 3 (or M) has a RR of 2.52 (95% CI = 1.89-3.37). The RRs were estimated based on the sex (Appendix Table 4) residential status (Appendix Table 5) and age (Appendix Table 6) except for few combinations (mentioned in the Appendix Tables 4, 5, 6) of this polymorphism (M235T) all are risk factor for hypertension.

Table 3: Genotype and gene frequencies of *AGT* gene (M235T polymorphism)

Group	*N*	*M235T polymorphism*				
		Genotype frequency (%)			*Gene frequency (%)*	
		33	*34*	*44*	*3*	*4*
Patients	254	12.2	11.8	76.0	18.1	81.9
Controls	254	16.5	38.6	44.9	35.8	64.2

33= MM; 34 = MT; 44= TT genotypes and 3 = M; 4 = T allele of M235T polymorphism

Table 4: Relative Risk (RR) for developing hypertension of the M235T genotypes and alleles

Comparison between	*M235T polymorphism*		
	RR	χ^2	*95% of CI*
44 Vs 33	2.29	9.77**	1.37-3.85
44 Vs 34	5.53	50.87***	3.46-8.92
44 Vs 34+33	3.61	43.28***	2.46-5.29
4 Vs 3	2.52	39.32***	1.89-3.37

33= MM; 34 = MT; 44= TT genotypes and 3 = M; 4 = T allele of M235T polymorphism
χ^2 = Chi-Square with 1 degree of freedom; * p< 0.05. ** p< 0.01; ***p < 0.001.

Genotype frequencies of neither polymorphism T174M / M235T were in Hardy-Weinberg equilibrium. The frequency distribution of genotypes significantly differed between the patient

and control groups (χ^2 with $5_{df} = 69.5$; $p < 0.001$). This may be due to the size of population.

Allele 2 of T174M and Allele 4 of M235T are significantly associated with hypertension. The two allelic variants at each of these sites (1. 2 and 3. 4) form four haplotypes *viz.* 13. 14. 23 and 24 which result in ten possible genotypes (Appendix Table 7a and 7b). Most frequent genotypes among patients were 14/24 and 24/24 while in the control group genotypes 13/13 and 13/24 were most frequent. Haplotype 13 is statistically significant in controls (p value of ≤ 0.001; χ^2 with 3df are 17.78) and haplotypes 14 and 24 are statistically significant in the patients (p value of ≤ 0.001 χ^2 with 3_{df} 24.37 and 17.85 respectively) for the allele 23 is not statistically significant (p value of = 0.05; χ^2 with 3df = 4.94).

DISCUSSION

Hypertension is a multifactor genetic disease which is influenced by number of gene polymorphisms. Hypertension associated gene polymorphisms were varied in different ethnic groups. These variations may be due to genetic makeup and selective pressure of the populations (Cooper et al. 2000). Among the different gene polymorphisms of *AGT* M235T and T174M were significantly associated in some studies (Goldenberg et al. 2006; Yanai et al. 1996; Sethi et al. 2001). However, studies in African and Asian populations could not equally support these findings (Jeunemaitre et al. 1992; Rotimi et al. 1997; Nair et al. 2003). The most important finding of the present study is that there is a strong association between the homozygotes 174MM and 235TT of the *AGT* gene. However, these two polymorphisms independently associated with hypertension which correlates with earlier reports (Pilbrow et al. 2007). Further, the frequency of double heterozygote (TMMT or 1234) is significantly greater in control subjects compared to the hypertensive patients (seven times higher). The frequency of 1234 genotype among hypertensive subjects is 3.5 percent. This implies that double heterozygote individuals are less predisposed to hypertension. Another notable observation is the significant association of 174MM genotype with hypertension as reported in other populations (Agachan et al. 2003; Yamagishi et al. 20054). On the contrary however, in some studies association has been reported with the 174TT genotype (Iso et al. 2000; Rotimi et al. 1997; Martinez et al. 2002). Association of hypertension with TT genotype at 235 amino acid position of AGT in the present study is that concurs with majority of the studies that reported a positive association with the M235T locus.

Schmidt et al. (1995) reported a significant association of 235T allele with individuals with an early onset hypertension (≤ 50 years) and a positive family history. Whereas in present study we found that 235T variant was significantly associated with all the age group. Iso et al. (2000) earlier reported that the haplotype 174M–235T was significantly higher among hypertensive subjects of rural Japan which is similar to the observation in the present study. However, Iso et al. (2000) found a significant association of hypertension with the 174M allele and not the 235T allele. Compared to 174T allele the estimated risk of hypertension to 174M allele was 80 percent greater. In an earlier study from Japan. Morise et al. (1995) also made a similar observation with respect to these two variants of the AGT protein. Yamagishi et al. (2004), also from Japan provided further support to the hypothesis of Iso et al. (2000). Overall there was no difference in the estimated risk between TT and (MM+MT) genotypes of the T174M polymorphism. In younger subjects (30-64 years) a significant elevation of diastolic blood pressure but not of systolic blood pressure was observed only if the subjects were not over weight and/or had higher urinary sodium excretion. It is also reported that 235T variant had a strong association with high plasma *AGT* level (Schmidt et al. 1995). And hence these polymorphisms could be an important genetic marker in predicting essential hypertension.

CONCLUSION

In the present study, both T174M and M235T polymorphisms of the *AGT* gene have been found to predispose subjects of South Indian origin to hypertension. Judged by the magnitude of the relative risks (RRs), the risk are much greater for the latter variant than the former variant. Further, the risks are much greater among females than in males. Another important finding in this study is the heterozygous advantage of the genotype 1234 (TMMT). Individuals with this genotype are least susceptible to hypertension.

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APPENDIX

Appendix Table 1: Sex specific relative risk estimation for the AGT gene (T174M polymorphism)

Group	*Males*			*Females*		
Comparison between	*RR*	χ^2	*95 % CI*	*RR*	χ^2	*95% CI*
22 Vs 11	1.12	0.10	1.81-2.28	2.84	8.88**	1.50-5.65
22 Vs 12	1.45	1.30	1.31-2.73	1.64	2.58	0.90-3.00
22 Vs 11+ 12	1.33	0.87	1.68-2.43	1.99	5.77*	0.88-3.52
2 Vs 1	1.32	0.98	1.31-2.29	1.76	3.97*	1.01-3.06

11= TT; 12 = TM; 22= MM genotypes and 1 = T; 2 = M allele of T174M polymorphism
χ^2 = Chi-square with 1 degree of freedom; * p< 0.05, ** p< 0.01

Appendix Table 2: Residential habitation based relative risk (RR) estimation for the *AGT* gene (T174M polymorphism)

Group	*Rural*			*Urban*		
Comparison between	*RR*	χ^2	*95% CI*	*RR*	χ^2	*95% CI*
22 Vs 11	1.24	0.45	1.51-2.28	3.34	8.36**	1.47-7.55
22 Vs 21	1.50	2.46	1.11-2.65	1.69	1.93	1.24-3.56
22 Vs 11+ 21	1.43	1.88	0.86-2.38	2.19	4.79*	0.08-1.48
2 Vs 1	1.13	0.37	1.30-1.65	1.91	5.11*	1.09-3.32

11= TT; 12 = TM; 22= MM genotypes and 1 = T; 2 = M allele of T174M polymorphism
χ^2 = Chi-square with 1 degree of freedom; * p< 0.05, ** p< 0.01

Appendix Table 3: Age specific relative risk (RR) estimation of AGT gene (T174M polymorphism)

Group	*Younger age patients (≤ 50 years)*			*Older age group patients(≥ 51 years)*		
Comparison between	*RR*	χ^2	*95% CI*	*RR*	χ^2	*95% CI*
22 Vs 11	2.25	3.91*	1.01-5.00	1.67	2.66	0.90-3.10
22 Vs 21	2.05	3.40	0.96-4.38	1.44	1.77	0.84-2.44
22 Vs 11+ 21	2.13	4.45*	1.06-4.31	1.50	2.49	1.03-2.49
2 Vs 1	1.54	3.97*	1.01-2.34	1.09	0.14	1.42-1.67

11= TT; 12 = TM; 22= MM genotypes and 1 = T; 2 = M allele of T174M polymorphism
χ^2 = Chi-square with 1 degree of freedom; * p< 0.05

Appendix Table 4: Sex specific relative risk (RR) estimation for the AGT gene (M235T variant)

Group	*Male*			*Female*		
Comparison between	*RR*	χ^2	*95% CI*	*RR*	χ^2	*95% CI*
44 Vs 33	1.32	0.57	0.64-2.73	4.00	13.50***	0.65-2.12
44 Vs 34	2.81	10.76**	1.52-5.21	7.40	30.49***	3.64-15.07
44 Vs 33 +34	2.09	8.00**	0.80-3.50	11.13	44.16***	5.47-22.65
4 Vs 3	1.51	1.65	1.24-2.83	3.85	15.84***	1.98-7.47

33= MM; 34 = MT; 44= TT genotypes and 3 = M; 4 = T allele of M235T polymorphism
χ^2 = Chi-square with 1 degree of freedom; ** p< 0.01; ***p< 0.001.

Appendix Table 5: Residential habitation based relative risk (RR) estimation for the *AGT* gene (M235T polymorphism)

Group	*Rural*			*Urban*		
Comparison between	*RR*	χ^2	*95% CI*	*RR*	χ^2	*95% CI*
44 Vs 33	1.26	0.41	1.61-2.56	4.17	12.56***	1.90-9.19
44 Vs 34	10.04	49.83***	5.29-19.04	1.96	3.31	1.05-4.04
44 Vs 33+ 34	4.69	38.62***	2.88-7.64	2.81	11.66**	1.55-5.09
4 Vs 3	2.21	5.80*	1.16-4.22	2.80	10.28**	1.49-5.25

33= MM; 34 = MT; 44= TT genotypes and 3 = M; 4 = T allele of M235T polymorphism
χ^2 = Chi-square with 1 degree of freedom; * p < 0.05; ** p< 0.01; ***p< 0.001.

Appendix Table 6: Age specific relative risk (RR) estimation of *AGT* gene (M235T polymorphism)

Group	*Younger age patients (≤ 50 years)*			*Older age group patients(≥ 51 years)*		
Comparison between	*RR*	χ^2	*95% CI*	*RR*	χ^2	*95% CI*
44 Vs 33	3.15	7.84**	1.41-7.01	1.80	2.66	0.92-3.51
44 Vs 34	3.54	10.41**	0.61-7.62	6.95	43.26***	3.90-12.4
44 Vs 33+ 34	3.39	14.54***	1.81-6.34	4.37	37.12***	2.72-7.027
4 Vs 3	2.72	16.93***	1.69-4.38	2.48	16.53***	1.60-3.84

33= MM; 34 = MT; 44= TT genotypes and 3 = M; 4 = T allele of M235T polymorphism
χ^2 = Chi-square with 1 degree of freedom; ** p< 0.01; ***p< 0.001.

Appendix Table 7: Genotype and haplotype frequencies at the T174M and the M235T polymorphisms of *AGT* gene

a. Genotype frequencies

Genotype	*EHP N*	*%*	*ControlN*	*%*
13/13	23	9.1	33	13.0
13/14	11	4.3	31	12.2
14/14	28	11.0	14	5.51
13/23	6	2.4	8	3.15
13/24	9	3.5	60	23.6
14/23	0	0.0	2	0.78
14/24	105	41.31	60	23.6
23/23	2	0.79	3	1.18
23/24	11	4.3	5	1.97
24/24	59	23.2	38	14.96
Total	254	100.0	254	100.0

Appendix Table 7: Contd.....

b. Haplotype frequencies

Haplotypes	*Haplotype frequencies*	
	EHP	*Controls*
13	0.1385	0.3146
14	0.3438	0.2383
23	0.0426	0.0403
24	0.4751	0.4068

N = Sample size; EHP=essential hypertensive patients