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GENDER SPECIFIC ASSOCIATION OF ANGIOTENSINOGEN GENE POLYMORPHISMS WITH ESSENTIAL HYPERTENSION

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ABSTRACT

Hypertension is a rapidly emerging pathophysiological condition caused by both genetic and environmental factors responsible for widespread morbidity and mortality and angiotensinogen is a key polypeptide in blood pressure physiology. In the present study M235T, T174M and G-6A polymorphisms of angiotensinogen gene have been studied in context of hypertension. Adult patients/hypertensives (n=246) and controls/normotensives (n=274) were enrolled. Their anthropometric, lifestyle data and blood samples were collected. Lipid profiling and genotyping were carried out. The parameters those were found to be higher in hypertensives compared to normotensives in the total group are alcohol consumption ($p<0.001$) and triglyceride levels ($p=0.031$). In males, M allele of M235T polymorphism ($p<0.001$), G allele of G-6A polymorphism ($p=0.001$) and alcohol consumption ($p<0.001$) and in females, the T allele of M235T polymorphism ($p=0.005$) and M allele of T174M polymorphism ($p=0.003$) were significantly high in the patient groups than the respective control groups. All the three polymorphisms were strongly linked to each other, the M235T and T174M being the most tightly linked (Total: D'=0.9987, $p<0.0001$, r^2= 0.4498; Males: D'=0.8828, $p<0.0001$, r^2=0.4200; females: D'=0.3890, $p=0.0019$, r^2=0.0592) followed by M235T and G-6G (Total: D'=0.9975, $p<0.0001$, r^2=0.5427; Males: D'=0.8553, $p<0.0001$, r^2=0.5747; females: D'=0.4614, $p=0.0012$, r^2=0.4837) and the least between T174M and G-6A (Total: D'=0.9985, $p<0.0001$, r^2=0.0110; Males: D'=0.9220, $p<0.0001$, r^2=0.0120; females: D'=0.3558, $p=0.0087$, r^2=0.0132). The TMG haplotype in the total population ($p=0.0036$) and females ($p=0.0039$) and the MTG haplotype in males ($p=0.0042$) were identified as risk haplotypes. The angiotensinogen gene polymorphisms were found to exert gender-specific effects on blood pressure regulation and hypertension pathophysiology.

Key words: Angiotensinogen; gene polymorphism; haplotype; hypertension; linkage.

INTRODUCTION

Hypertension is a major risk factor for cardiovascular diseases and essential hypertension accounts for 90-95% of cases. It is a complex pathophysiological condition involving both genetic as well as environmental factors. According to the World Health Statistics – 2012, one in three adults worldwide, has raised blood pressure – a condition that causes around half of all deaths from stroke and heart diseases. In India, it is directly responsible for 57% of all stroke deaths and 24% of coronary heart disease deaths (WHO, 2012).

The renin-angiotensin system (RAAS) has a central role in controlling blood pressure (BP) and sodium homeostasis and angiotensinogen is an important component of the system. Angiotensinogen is an α-2-globulin of hepatic origin, a plasma protein (also called as renin substrate) produced constitutively and released into the circulation mainly by the liver. Human angiotensinogen is 453 amino acids long, but other species have angiotensinogen of varying sizes. The first 12 amino acids are the most important for activity (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-...).It is acted on by an enzyme, renin to release a 10 amino acid protein called angiotensin I. It persists in the blood for 30 minutes to 1 hour and continues to form angiotensin II by the action of Angiotensin Converting enzyme. Angiotensin II stimulates vasoconstriction, vascular and cardiac hypertrophy and sodium and water retention leading to increase in blood pressure. The level of angiotensinogen has been observed to be high in hypertensive subjects compared to normotensive individuals. In humans, the angiotensinogen (*AGT*) gene is present in 1q42–43 locus and comprises of five exons and four introns spanning 13 kb of genomic sequence (Crisan & Carr, 2000).

Total three single-nucleotide polymorphisms (SNPs) have been observed to be associated with changes in serum anigiotensinogen levels. These are (i) M235T or T4072C (rs699) which replaces Met with Thr at amino

acid residue 235 (A**T**G→A**C**G), (ii) T174M or C3889T (rs4762) which replaces Thr with Met at amino acid residue 174 (A**C**G→A**T**G) and (iii) G-6A (rs5051) which is a point mutation in the promoter region (Karthikeyan et al., 2013, Li et al., 2012). Though several reports have described the association between these polymorphisms and essential hypertension in different populations, the results of which have been inconsistent (Karthikeyan et al., 2013, Li et al., 2012, Mohana et al., 2012, Niu et al., 1999, Macro et al., 2005 and Nejatizadeh et al., 2008), very few reports are available in India. Hence this study was carried out with a motive to investigate the possible association of these three polymorphisms with the clinical expression of essential hypertension in the population Odisha who belong to a distinct genetic group (Tartaglia et al., 1995).

MATERIALS AND METHODS

STUDY AREA, SUBJECT SELECTION AND DATA COLLECTION

The study was conducted in Odisha, an eastern Indian state situated along the Bay of Bengal with a total population of 42.5 million (Census, 2011). The subjects were recruited from the patients attending the medicine OPDs of Capital Hospital, Bhubaneswar and VSS Medical College and Hospital, Burla, about 300 km from Bhubaneswar towards north west, from January 2011 to August 2012. These two hospitals have been included because of their locality in two geographic zones of the state and people from all over the state come here for treatment, therefore subjects from these two hospitals can be considered as representative of the state population. A total 246 hypertensive (patients) and 274 normotensive (controls) individuals were enrolled in the study based on inclusion/exclusion criteria. Informed consent has been obtained from all individuals before enrollment. The study was approved by the institutional ethical committee of Regional Medical Research Centre, Bhubaneswar.

The inclusion criteria for patients were systolic blood pressure (SBP) ≥140 mmHg and/or mean diastolic blood pressure (DBP) ≥ 90 mmHg (Chobanian et al.,2003) or current antihypertensive medication. The persons with secondary hypertension (hypertension due to secondary causes such as renovascular disease, renal failure, pheochromocytoma, aldosteronism, or other causes), diabetes or under any sort of lipid-lowering drugs were excluded from the study. The controls had no history of hypertension, diabetes or any other cardio-vascular disease and were not under any lipid-lowering drugs.

Data on age, sex, height, weight, family history, education, intake of additional salt during any sort of food intake, diet (vegetarian/non-vegetarian), tobacco and alcohol habits were recorded. Body Mass Index was calculated using the formula (weight in Kg)/ (height in meters)^2 and individuals with BMI ≥23kg/m^2 and ≥25kg/m^2 were classified as overweight and obese respectively (WHO, 2000). Ex-tobacco consumers and alcoholics were excluded from the study. Blood pressure was measured by the physician in a sitting position on the right arm.

BLOOD SAMPLE COLLECTION

At least 2 ml of venous blood was collected aseptically from each subject after overnight fasting in EDTA vials and was transported to the laboratory under cold conditions. From 1 ml of blood, plasma was separated within 3 hours by centrifuging the blood at 3000 rpm for 3 minutes and stored at -20°C for biochemical analysis.

BIOCHEMICAL ANALYSIS AND KIDNEY FUNCTION TESTS

The Adult Treatment Panel-III, 2002 criteria (NCEP, 2002) was used for stratifying the biochemical data. The lipid profile (total cholesterol, high density lipoprotein: HDL, low density lipoprotein: LDL, triglycerides) and the indicators of kidney function status (urea and creatinine) were analyzed by automatic analyzer (Cobas Integra 400, Roche Diagnostics, Germany) using the commercially available reagent kits supplied by the company.

GENOMIC DNA ISOLATION AND GENETIC ANALYSIS

The genomic DNA was extracted from the whole blood using the standard phenol–chloroform method (Sambrook et al., 2001). Briefly the cells were lysed using lysis buffer (2X concentration, 200mM NaCl, 50mM EDTA), the mixture was centrifuged and the pellet was incubated overnight with 1X lysis buffer (100mM NaCl, 25mM EDTA), 50 µl 10% SDS and 3 µl proteinase K. The next day phenol was added and then it was centrifuged at 10,000 rpm for 10 minutes at 4°C. The same step was repeated for the supernatant. Then chloroform-isoamyl alcohol was added in 24:1 ratio and again centrifuged at 10,000 rpm for 10 minutes at 4°C. The DNA was precipitated using chilled absolute ethanol. The extracted DNA was resuspended in 100 µl of DNase-free water and kept at -20°C until use.

The M235T and T174M polymorphisms were analysed together in a single PCR reaction using a forward primer: 5'GAT GCG CAC AAG GTC CTG-3' and a reverse primer 5'-CAG GGT GCT GTC CAC ACT GGC TCG C-3' (Caulfield et al., 1994). The PCR amplification for M235T and T174M polymorphisms was carried out in a 40µl reaction mixture containing 250nM each of forward and reverse primer, 100pM of each dNTP, 1X Taq buffer A [10X buffer A (Banglore Genei): 100mM Tris (pH 9.0), 500mM KCl, 15mM MgCl2, 0.1% Gelatin] 3U Taq DNA polymerase (Bangalore Genei) and 6 µl of template DNA. The reaction conditions included an initial denaturation for 10 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min and extension at 72°C for 1 min 30 sec, followed by a final extension for 10 min at 72°C. The resultant amplicon size was 303 base pairs. The mixture containing the amplicon was divided into two parts, one part was incubated with 2.5units of SfaNI and the other part with 2.5 units of NcoI overnight. On digestion of the amplicon with SfaNI, a 266bp band indicates the presence of M235 allele whereas the 303bp undigested product indicates the presence of 235T allele.

The T174M polymorphism was detected by digestion with NcoI. In presence of the 174M allele the product is cut into bands of 211bp and 92bp sizes and the T174 allele is identified by the uncut band.

The region containing the G-6A polymorphism was amplified using the flanking primer pair 5'-CCC TCA GCT ATA AAT AGA GCA TC-3' and 5'-GCA GGA AGA CCT GAC CAT CT-3' as forward and reverse primers (Prasad et al., 2006). The PCR reaction mixture volume was 20 µl, the composition of which was similar to the former with the only exceptions that 1.5 U Taq DNA polymerase and 3µl of template DNA were added. The cycling conditions were as follows: initial denaturation: 96°C for 10 min followed by 35 cycles of denaturation: 96°C for 1 min, annealing: 61°C for 1 min and 72°C for 1 min and final extension at 72°C for 10 min. The amplicon was incubated overnight with 2.5U of BstNI. Bands of 245 and 63 are obtained for the G allele whereas bands of 209, 36 and 63 are obtained for the A allele (Table 1).

The products were electrphoretically separated on 3% agarose gels and visualized under UV lamp after staining with ethidium bromide (0.5 µg/ml).

STATISTICAL ANALYSIS

Unpaired t-test or chi-square test or Fisher's exact test was used to compare the characteristics of two groups. Genotypes and alleles were compared using chi-square test or Fisher's exact test as applicable. Graph Pad version 5 was used for the above analysis. Linkage and haplotype analysis were done using SNPStats online software (http://bioinfo.iconcologia.net/SNPstats). The extent of linkage disequilibrium was determined by studying the D' value ($D' = D / D_{max}$, where D is the measure of linkage disequilibrium, i.e., the deviation of the observed frequency of a haplotype from the expected is a quantity and D_{max} is the theoretical maximum for the observed allele frequencies). Logistic regression analysis was carried out to identify the independent risk factors using SPSS version 17. For the analysis, dominant models were considered for M235T and T174M polymorphisms, but from the observations of univariate analysis, recessive model was considered for G-6A polymorphism.

RESULTS

CHARACTERISTICS OF SUBJECTS

A total of 246 hypertensives (159 males and 87 females) and 274 normotensives (158 males and 116 females) individuals were included in the study. The mean age of patients was 49.47 ± 10.38 years and that of controls was 48.82 ± 11.04 years. The mean age of male patients and controls were 49.20 ± 9.76 years and 47.17 ± 9.25 years and that of female patients and controls were 49.87 ± 11.47 years and 51.30 ± 13.15 years respectively. All the subjects were age and sex matched. Systolic blood pressure (SBP), diastolic blood pressure (DBP), frequency of family history and overweight were higher in patients in total as well as male and female groups. Body Mass Index (BMI), triglycerides and alcohol consumption rate were higher in patients of the total group whereas high density lipoprotein (HDL) levels were high in the control group. In males, the triglyceride levels and alcohol consumption rate were high and in females BMI, creatinine levels and frequency of hyperlipidemia were high in the patient groups compared to the controls (Table 2).

GENOTYPING RESULTS

The genotype distributions of M235T polymorphism were in Hardy-Weinberg equilibrium in both patients and controls in the total population as well as in males and females. No difference was observed in the genotype distributions or allele frequencies in any group. In univariate analysis, in females the T allele was observed to increase the chances of risk in additive (TT vs MM) and dominant models (MT/TT vs MM) whereas in males the M allele increased the risk in recessive model (TT vs MT/MM) but after bonferroni correction was applied (p-value= 0.05/3= 0.017), the associations were not significant.

In case of the T174M polymorphism, the genotype distributions did not deviate from Hardy-Weinberg equilibrium and no significant difference was observed in their pattern. The homozygous mutant frequency (MM) was very low; therefore it was merged with the heterozygous genotype (TM) for analysis. In the total group and in males, no association could be detected. In females, although the M allele was higher and increased the risk for hypertension in the dominant model (MM/TM vs TT), the association was not significant after bonferroni correction.

The genotype distributions of the G-6A polymorphism deviated from Hardy-Weinberg equilibrium in all groups of patients and controls except in male controls. Only in males, the distribution pattern of the genotypes was significantly different (p-value=0.0094) and associations were observed in additive (GG vs AA) (p=0.0025, Odds ratio: 3.114, 95% CI: 1.46-6.63) and recessive (GG vs AG/AA) models (p=0.0027, Odds ratio: 2.917, 95% CI: 1.42-5.99) and the G allele was significantly higher in the male subjects (p=0.0029, Odds ratio: 1.743, 95% CI: 1.21-2.51). The results have been depicted in Tables 3 and 4. The gel photographs have been shown in Figure 1.

LOGISTIC REGRESSION ANALYSIS RESULTS

From logistic regression analysis, it was found that in the total population alcohol consumption ($p<0.001$) and high triglyceride levels (p=0.031), in males, MM and MT genotypes of M235T polymorphism ($p<0.001$) GG genotype of G-6A polymorphism (p=0.001) and alcohol consumption ($p<0.001$) and in females, the MT and TT genotypes of M235T polymorphism (p=0.005) and the TM and TT genotypes of T174M polymorphism (p=0.003) were associated with hypertension. No polymorphism could be identified as risk factor in the total group. Low HDL/LDL levels were high in the total group (p=0.022) and in males (p=0.001) which may be linked to higher levels of LDL in the respective control groups (Table 5).

Table 1- Genotyping protocols

Polymorphism	Primers	Cycling conditions	Product size	Restriction enzyme	Resultant product size after digestion
M235T	Forward primer: 5'GAT GCG CAC AAG GTC CTG-3' Reverse primer: 5'-CAG GGT GCT GTC CAC ACT GGC TCG C-3'	Initial Denaturation: 94°C for 10 min 30 cycles: Denaturation: 94°C for 1 min Annealing: 61°C for 1 min Extension: 72°C for 1 min 30 sec Final Extension: 10 min at 72°C	303 bp	SfaNI	266bp, 37bp bands: M235 allele 303bp band: 235T allele.
T174M				NcoI	211 bp, 92bp bands: 174M allele 303 bp: T174 allele
G-6A	Forward primer: 5'-CCC TCA GCT ATA AAT AGA GCA TC-3' Reverse primer: 5'-GCA GGA AGA CCT GAC CAT CT-3'	Initial Denaturation: 96°C for 10 min 35 cycles: Denaturation: 96°C for 1 min Annealing: 61°C for 1 min Extension: 72°C for 1 min Final Extension:72°C for 10 min.	308	BstNI	245bp, 63bp bands: G allele 209bp, 36bp 63bp: A allele.

Note: bp: base pairs

Table 2- Characteristics of patients and controls

Variables	Hypertensives (n=246)	Normotensives (n=274)	Male Hypertensives (n= 159)	Male Normotensives (n=158)	Female Hypertensives (n=87)	Female Normotensives (n=116)
Age	49.47 ± 10.38	48.82 ± 11.04	49.20 ± 9.76	47.17 ± 9.25	49.87 ± 11.47	51.30 ± 13.15
SBP (mmHg)	148.4 ± 18.40	116.2 ± 5.40***	149.8 ± 17.11	115.9 ± 5.47 ***	146.5 ± 20.03	116.5 ± 5.33 ***
DBP (mmHg)	93.18 ± 9.94	78.14 ± 4.24***	93.34 ± 10.28	78.19 ± 4.57 ***	92.96 ± 9.50	78.07 ± 3.85 ***
Family history of HTN (%)	42.22	12.5***	46.67	11.11***	33.33	14.29**
BMI (kg/m^2)	24.23 ± 3.99	23.16 ± 1.94**	24.15 ± 4.34	23.49 ± 2.09	24.40 ± 3.18	22.72 ± 1.63 ***
Overweight/obese (%) (BMI ≥23 kg/m^2)	63.01	40.98***	63.27	40.00***	62.50	42.31**
TC (mmol/L)	4.54 ± 0.82	4.45 ± 0.90	4.71 ± 0.80	4.66 ± 1.01	4.34 ± 0.82	4.25 ± 0.72
HDL (mmol/L)	0.99 ± 0.23	1.09 ± 0.34*	1.01 ± 0.18	1.09 ± 0.33	0.96 ± 0.28	1.08 ± 0.35
LDL (mmol/L)	2.67 ± 0.61	2.64 ± 0.68	2.70 ± 0.54	2.77 ± 0.77	2.62 ± 0.71	2.49 ± 0.54
TG (mmol/L)	1.94 ± 0.92	1.60 ± 0.87*	2.17 ± 1.07	1.73 ± 0.88 *	1.65 ± 0.58	1.46 ± 0.85
HDL/LDL	0.3961 ± 0.16	0.4477 ± 0.21	0.3880 ± 0.10	0.4375 ± 0.22	0.4070 ± 0.23	0.4587 ± 0.20
Urea (mmol/L)	7.20 ± 3.18	6.95 ± 2.10	7.28 ± 3.46	7.19 ± 2.39	6.73 ± 2.84	6.70 ± 1.73
Creatinine (µmol/L)	78.72 ± 24.75	77.17 ± 20.33	88.03 ± 28.75	84.86 ± 27.40	66.30 ± 19.45	71.54 ± 11.49*
Hyperlipidemia (%)	76.19	64.52	66.67	68.75	88.89	60.00*
Smoking (%)	20.73	18.33	32.08	34.92	0	0$^{\#}$
Chewable tobacco	25.61	21.67	35.85	34.38	6.90	7.14

consumption (%)						
Alcohol consumption (%)	19.51	10.00**	30.19	18.75*	0	0 [#]
Intake of additional salt (%)	28.57	22.22	25.00	19.05	35.29	26.67
Education ≥10th (%)	81.40	76.56	86.21	78.38	71.43	74.07
Diet (Non-Vegetarian) (%)	89.09	84.78	94.29	90.32	80.00	75.00

Note: Continuous variables are expressed in Mean ± Standard Deviation and frequency data are expressed in percentage.
BMI: Body Mass Index, SBP: Systolic Blood Pressure, DBP: Diastolic Blood Pressure, TC: Total Cholesterol, HDL: High Density Lipoprotein, LDL: Low Density Lipoprotein, TG: Triglycerides, HDL/LDL: High Density Lipoprotein/Low Density Lipoprotein ratio, mmol/L: millimoles/Liter.
***: p<0.0001 **: p<0.01 *: p<0.05
[#]: Females were not smokers or alcoholics

Table 3- Genotype and allele frequencies of M235T, T174M and G-6A polymorphisms

	All subjects		**Males**		**Females**	
	Hypertensives	**Normotensives**	**Hypertensives**	**Normotensives**	**Hypertensives**	**Normotensives**
M235T						
Genotypes						
MM	17 (8.25%)	23 (9.83%)	15 (10.87%)	11 (7.91%)	2 (2.94%)	12 (12.63%)
MT	90 (43.69%)	84 (35.90%)	60 (43.48%)	48 (34.53%)	30 (44.12%)	36 (37.89%)
TT	99 (48.06%)	127 (54.27)	63 (45.65%)	80 (57.55%)	36 (52.94%)	47 (49.47%)
p-value	0.2459		0.1376		0.0905	
Alleles						
M	124 (30.10%)	130 (27.28%)	90 (32.61%)	70 (25.18%)	34 (25.00%)	60 (31.58%)
T	288 (69.90%)	338 (72.22%)	186 (67.39%)	208 (74.82%)	102 (75.00%)	130 (68.42%)
OR (95%CI), p-value	0.893 (0.67-1.20), 0.4487		0.696 (0.48-1.01), 0.0537		1.385 (0.84-2.27), 0.1960	
T174M						
Genotypes						
TT	156 (76.10%)	180 (80.36%)	108 (81.81%)	92 (81.42%)	48 (65.75%)	88 (79.28%)
TM	45 (21.95%)	43 (19.20%)	21 (15.90%)	20(17.70%)	24 (32.88%)	23 (20.72%)
MM	4 (1.95%)	1 (0.44%)	3 (2.27%)	1 (0.88%)	1 (1.37%)	0(0.0%)
p-value	0.2562		0.6584		Not possible	
Alleles						
T	357 (87.07%)	403 (89.96%)	237 (89.77%)	204 (90.27%)	120 (82.19%)	199 (89.64%)
M	53 (12.93%)	45 (10.04%)	27 (10.23%)	22 (9.73%)	26 (17.81%)	23 (10.36%)
OR (95%CI), p-value	1.33 (0.87-2.03), 0.1849		1.056 (0.58-1.91), 0.8562		1.875 (1.02-3.43), 0.0396	

G-6A						
Genotypes						
AA	87 (43.28%)	110 (50.69%)	57 (43.18%)	71 (54.20%)	30 (43.48%)	39 (45.35%)
AG	69 (34.33%)	68 (31.34%)	45 (34.09%)	48 (36.64%)	24 (34.78%)	20 (23.26%)
GG	45 (22.39%)	39 (17.97%)	30 (22.73%)	12 (9.16%)	15 (21.74%)	27 (31.40%)
p-value	0.2847		0.0094		0.2081	
Alleles						
A	243 (60.45%)	288 (66.36%)	159 (60.23%)	190 (72.52%)	84 (64.87%)	98 (56.98%)
G	159 (39.55%)	146 (33.64%)	105 (39.77%)	72 (27.48%)	54 (39.13%)	74 (43.02%)
OR (95%CI), p-value	1.291 (0.97-1.71), 0.0760		1.743 (1.21-2.51), 0.0029		0.8514 (0.54-1.34), 0.4890	

Note: OR: Odds ratio, 95% CI: 95% Confidence Interval
Results were derived using chi-square test.

Table 4- Association of different genotypes of M235T, T174M and G-6A polymorphisms with hypertension tested under different genetic models

	All subjects		Males		Females	
	p-value	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**
M235T						
MT vs MM	0.2928	1.450 (0.72-2.90)	0.8438	0.917 (0.39-2.18)	0.0373	5.000 (1.04-24.12)
TT vs MT	0.1158	0.7276 (0.49-1.08)	0.0712	0.630 (0.38-1.04)	0.7995	0.919 (0.48-1.76)
TT vs MM	0.8780	1.055 (0.53-2.08)	0.1995	0.578 (0.25-1.35)	0.0434	4.596 (0.97-21.85)
MT/TT vs MM	0.5659	0.212 (0.63-2.34)	0.3990	0.705 (0.31-1.60)	0.0443	4.771 (1.03-22.07)
TT vs MT/MM	0.1931	0.780 (0.54-1.14)	0.0475	0.620 (0.39-0.996)	0.6624	1.149 (0.62-2.14)
T174M						
TM vs TT	0.4311	1.208 (0.75-1.93)	0.7450	0.894 (0.46-1.75)	0.0564	1.913 (0.98-3.74)
MM/TM vs TT	0.2848	1.285 (0.81-2.04)	0.9354	0.9735 (0.51-1.86)	0.0409	1.993 (1.02-3.88)
G-6A						
AG vs AA	0.2638	1.283 (0.83-1.99)	0.5703	1.168 (0.68-1.995)	0.2508	1.56 (0.73-3.34)
GG vs AG	0.6434	1.137 (0.66-1.96)	0.0126	2.667 (1.22-5.84)	0.0795	0.463 (0.19-1.10)
GG vs AA	0.1480	1.459 (0.87-2.43)	0.0025	3.114 (1.46-6.63)	0.4191	0.722 (0.33-1.59)
GG/AG vs AA	0.12295	1.347 (0.92-1.98)	0.0739	1.557 (0.96-2.53)	0.8159	1.079 (0.57-2.04)
GG vs AG/AA	0.2603	1.317 (0.81-2.13)	0.0027	2.917 (1.42-5.99)	0.1789	0.607 (0.29-1.26)

Note: OR: Odds ratio, 95% CI: 95% Confidence Interval
Results were derived using chi square test when frequencies were ≥5 and Fisher's exact test when frequencies were <5.

Table 5-Results of Logistic Regression Analysis

Factors	All subjects				Males				Females			
			95% C.I. for Exp(B)				95% C.I. for Exp(B)				95% C.I. for Exp(B)	
	p-value	Exp(B)	Lower	Upper	p-value	Exp(B)	Lower	Upper	p-value	Exp(B)	Lower	Upper
M235T	.699	.980	.885	1.085	.000	.743	.633	.872	.005	1.171	1.049	1.308
T174M	.845	1.009	.922	1.104	.056	.804	.643	1.006	.003	1.239	1.077	1.426
G-6A	.978	.999	.934	1.069	.001	1.264	1.103	1.449	.771	1.015	.920	1.119
BMI	.712	.983	.899	1.075	.133	1.110	.969	1.273	.135	.908	.800	1.031
Smoking	.052	.527	.276	1.006	.055	.512	.258	1.014				
Smokeless Tobacco	.646	1.116	.700	1.779	.642	1.135	.666	1.933	.007	2.027	1.211	3.391
Alcohol	.000	4.170	2.110	8.242	.000	4.443	2.094	9.425				
TC	.713	1.206	.445	3.267	.149	3.597	.633	20.424	.079	.185	.028	1.218
HDL	.900	1.048	.503	2.186	.054	3.511	.981	12.560	.232	.462	.130	1.640
LDL	.708	.866	.408	1.839	.252	.365	.065	2.047	.253	1.841	.647	5.239
Triglycerides	.031	2.579	1.088	6.114	.109	5.479	.686	43.777	.041	4.290	1.061	17.355
HDL/LDL ratio	.022	.304	.110	.844	.001	.034	.005	.233	.804	1.204	.277	5.231
Constant	.749	.847			.610	.738			.113	3.369		

Note: Abbreviations used are same as in table 2.
Exp(B): exponentiation of the Beta coefficient, is an odds ratio.
Females were not smokers or alcoholics

Table 6: Linkage analysis

	All subjects		Males		Females	
	M235T	**T174M**	**M235T**	**T174M**	**M235T**	**T174M**
T174M	D'=0.9987, p<0.0001 r^2= 0.4498	-	D'=0.9975 p<0.0001 r^2=0.4200	-	D'=0.9985 p<0.0001 r^2=0.0592	-
G-6A	D'=0.8828 p<0.0001 r^2=0.5427	D'=0.3890 p=0.0019 r^2=0.0110	D'=0.8553 p<0.0001 r^2=0.5747	D'=0.4614 p=0.0012 r^2=0.0120	D'=0.9220 p<0.0001 r^2=0.4837	D'=0.3558 p=0.0087 r^2=0.0132

Note: $D' = D / D_{max}$
Where D is the measure of linkage disequilibrium, i.e., the deviation of the observed frequency of a haplotype from the expected is a quantity and D_{max} is the theoretical maximum for the observed allele frequencies

Table 7: Haplotypes and their association with hypertension

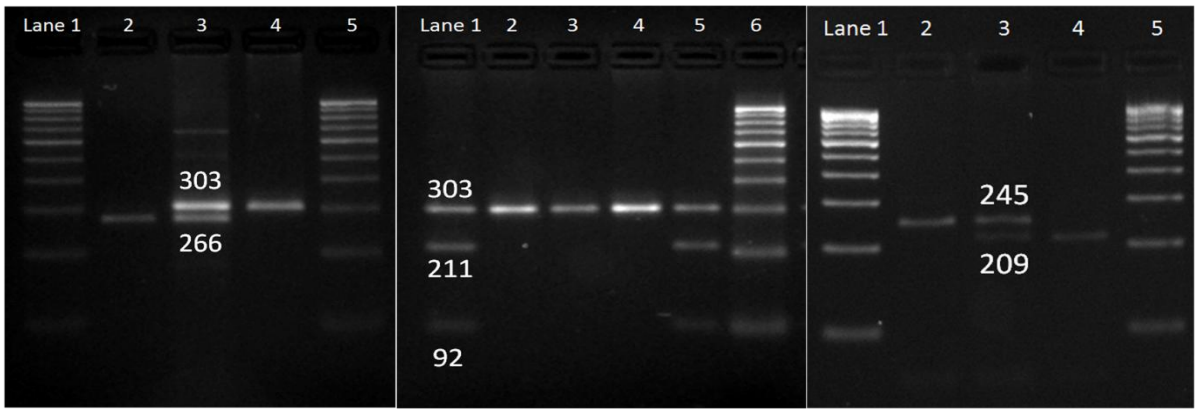
	SNP1 M235T	SNP2 T174M	SNP3 G-6A	OR (95% CI)	p-value	Adjusted OR (95%CI)	Adjusted p-value
All subjects							
1	T	T	A	1.00	---	1.00	---
2	M	T	G	1.26 (1.01 - 1.58)	0.045	1.10 (0.86 - 1.41)	0.46
3	T	M	A	1.40 (0.93 - 2.11)	0.11	0.74 (0.46 - 1.18)	0.21
4	T	T	G	0.81 (0.54 - 1.21)	0.3	0.74 (0.44 - 1.25)	0.26
5	T	M	G	2.48 (1.16 - 5.28)	0.019	3.12 (1.45 - 6.70)	0.0036
6	M	T	A	0.34 (0.13 - 0.92)	0.033	0.25 (0.09 - 0.70)	0.0091
rare	*	*	*	0.00 (-Inf - Inf)	1	0.00 (-Inf - Inf)	1
Males							
1	T	T	A	1.00	---	1.00	---
2	M	T	G	1.64 (1.23 - 2.20)	0.001	1.57 (1.16 - 2.14)	0.0042
3	T	M	A	1.51 (0.87 - 2.64)	0.14	1.31 (0.73 - 2.34)	0.37
4	T	T	G	1.28 (0.74 - 2.22)	0.38	1.12 (0.63 - 1.97)	0.7
5	T	M	G	2.16 (0.67 - 7.00)	0.2	3.43 (0.88 - 13.39)	0.077
6	M	T	A	0.73 (0.24 - 2.28)	0.59	0.51 (0.16 - 1.55)	0.23
rare	*	*	*	0.00 (-Inf - Inf)	1	0.00 (-Inf - Inf)	1
Females							
1	T	T	A	1.00	---	1.00	---
2	M	T	G	0.88 (0.60 - 1.30)	0.53	0.87 (0.63 - 1.19)	0.38
3	T	M	A	1.36 (0.71 - 2.60)	0.36	1.38 (0.80 - 2.38)	0.25
4	T	T	G	0.47 (0.22 - 1.02)	0.056	0.51 (0.28 - 0.96)	0.036
5	T	M	G	2.92 (1.13 - 7.53)	0.027	3.02 (1.43 - 6.38)	0.0039
6	M	T	A	0.00 (-Inf - Inf)	1	0.00 (-Inf - Inf)	1

Note: OR: Odds ratio, 95% CI: 95% Confidence Interval

LINKAGE DISEQUILIBRIUM AND HAPLOTYPE ANALYSIS RESULTS

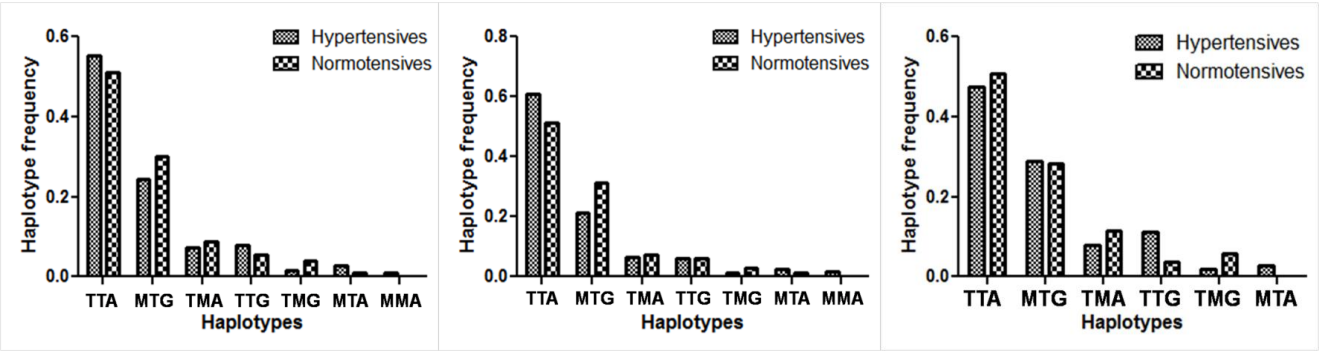
By linkage analysis it was observed that all the three polymorphisms are in linkage disequilibrium, The strongest linkage was between M235T and T174M (Total population: D'=0.9987, p<0.0001, r^2= 0.4498; Males: D'=0.8828, p<0.0001, r^2=0.4200; females: D'=0.3890, p=0.0019, r^2=0.0592), followed by that between M235T and G-6A (Total: D'=0.9975, p<0.0001, r^2=0.5427; Males: D'=0.8553, p<0.0001, r^2=0.5747; females: D'=0.4614, p=0.0012, r^2=0.4837) and the least between T174M and G-6A (Total: D'=0.9985, p<0.0001, r^2=0.0110; Males: D'=0.9220, p<0.0001, r^2=0.0120; females: D'=0.3558, p=0.0087, r^2=0.0132) (Table 6).

Haplotype analysis revealed that after crude analysis none of the haplotypes were linked to hypertension except in males wherein the MTG haplotype was significantly high in hypertensives (Odds ratio: 1.64, 95% CI: 1.23-2.20, p=0.001) (p<0.007 considered significant: 0.05/number of haplotypes, i.e., 0.05/7). However, after adjustment for risk factors, the TMG haplotype was found to be associated with hypertension in the total (Odds ratio: 3.12, 95% CI: 1.45-6.70, p=0.0036) and female populations (Odds ratio: 3.02, 95% CI: 1.43-6.38, p=0.0039) and the MTG haplotype in the male population (Odds ratio: 1.57, 95% CI: 1.16-2.14, p=0.0042) (Table 7, Figure 2).



(a) (b) (c)

Figure 1- Gel photographs showing banding patterns of a) M235T polymorphism: Lanes 1 and 5: Ladder, Lane 2: MM, Lane 3: MT, Lane 4: TT; b) T174M polymorphism: Lanes 1: TM, Lane 2, 3, 4: TT, Lane 5: MM, Lane 6: Ladder; c) G-6A polymorphism: Lanes 1 and 5: Ladder, Lane 2: GG, Lane 3: AG, Lane 4: AA.



(a) (b) (c)

Figure 2- Frequencies of different haplotypes in the a) total group b) males c) females

DISCUSSION

Genetic studies are essential to understand pharmacogenetics of essential hypertension because knowledge on genetic or genomic basis of a disease would help to develop highly targeted therapies. We have carried out this study amongst the Odisha population since no report is available on these polymorphisms in this population. Besides as stated earlier, people of Odisha belong to a genetically distinct group (Tartaglia et al., 1995), therefore, such studies can add to the existing genetic data bank.

In the present study though M235T gene polymorphism has not been found to be associated with hypertension in whole population, but gender specific analysis revealed that both homozygous and heterozygous state of M allele (MM and MT genotypes) was associated with high risk of developing disease and T allele in double dose to be protective in case of males. On the contrary, both in homozygous and heterozygous states of the T allele conferred risk (MT and TT genotypes) in females and M allele in double dose was protective. Similar to our observation, T allele have been reported to be associated with hypertension in the pooled study group of Tamil Nadu, India (Karthikeyan et al., 2013) and in a han population of China (Cai et al., 2004) whereas association of M allele has been found with ischemic heart disease in

the total group and male patients in Netherlands (Plat et al., 2009) and in female hypertensives of Hyderabad, India (Mohana et al., 2012). Absence of such associations were, however, reported among the populations of London, UK (Caulfield et al., 1994), Hyderabad, India (Charita et al., 2012) and in either gender in Anqing, China (Niu et al., 1999). Ethnic differences have been reported in the frequencies of the variant allele. The frequencies of T allele were approximately 0.77 and 0.72 in patients and controls in the present study, which are quite similar to those observed among Chinese (0.80 and 0.72) (Chen et al., 2003) and Tamilian Indians (0.82 and 0.64) (Karthikeyan et al., 2013). Lower frequencies have been however, reported in white Caucasians (0.47 and 0.38) and south Indians of Andhra Pradesh (0.41 and 0.45) (Charita et al., 2012).

The TM and MM genotypes of T174M polymorphism have been identified as risk factors in females in the present study, i.e., the M allele have been found to confer risk in both homozygous and heterozygous states in females but not in males or in the total group. This allele was significantly high in hypertensives in a Chinese population comprising both males and females (Gu et al., 2012) and in south Indian females (Mohana et al., 2012). On the contrary, the TT genotype of T174M was observed to be positively associated with hypertension in the population of Spain (Macro et al., 2005). But no difference could be observed in either males or females in Anqing, China (Niu et al., 1999). In our population group, the frequencies of M allele were 0.13 and 0.10 for hypertensives and normotensives respectively similar to 0.11 and 0.12 in French Caucasians and 0.12 and 0.08 in Japanese (Jeunemaitre et al., 1997).

The GG genotype of the G-6A polymorphism was significantly high in male hypetensives in the present study, i.e., the GG homozygosity conferred risk of hypertension in the male gender, but this association was not observed in the total group or in females. The G allele was reported to be the risk factor in Li and Mongolian ethnicities in China but no significant association was found in other ethnic groups such as Han, Tibetan, Kazakh, Bai and Yi (2012). Similarily, in Tamil Nadu, India, too, no association of this polymorphism with hypertension was reported (Karthikeyan et al., 2009). Contrary to our findings, the A allele was the risk factor in north India (Nejatizadeh et al., 2008) and in a Danish female population but not in Danish males (Sethi et al., 2003). The frequencies of G allele in our population were 0.40 in hypertensives and 0.34 normotensives, but frequencies as low as 0.085 and 0.092 were reported in Tamil Nadu (Karthikeyan et al., 2009) and 0.18 and 0.16 in Taiwan, China (Wu et al., 2004) whereas, higher frequencies have been reported in Europeans like 0.49 and 0.52 in Spain (Rodriguez et al., 2000) and 0.63 and 0.64 in Italy (Bengra et al., 2002).

All the three polymorphisms were strongly linked to each other, the strongest linkage was between M235T and T174M, followed by M235T and G-6G and the least was between T174M and G-6A. Because haplotypes allow a more accurate and sensitive analysis compared to individual polymorphisms alone, therefore we carried out haplotype analysis form which it was found that the TMG haplotype conferred about 3 times greater risk in the total population and females whereas in males the MTG haplotype conferred about 1.6 times greater risk. Many studies have reported linkage between M235T and G-6A like those in Denmark (Sethi et al, 2003) and Hyderabad, India (Charita et.al, 2012), between T174M and M235T in European Americans and African Americans in USA (Zhu et al., 2003) and in Denmark (Sethi et al, 2003) and between G-6A and T174M in Denmark (Sethi et al, 2003). But no linkage was observed between G-6A and T174M in Hyderabad, India (Charita et al., 2012) and between T174 M amd M235T in an African population (Robinson et al., 2004). Besides, in north India, A/174T, 174T/235T, A/235T, and A/174T/235T in hypertensive patients and G/174T, 174T/235M, G/235M, and G/174T/235M in controls were identified as risk and protective haplotypes, respectively (Nejatizadeh et al., 2008). The variation in results of association of the polymorphisms or haplotypes with hypertension from different regions may be due to differences in environmental conditions, lifestyle attributes and overall genetic makeup of the study population.

The effect of M235T polymorphism on blood pressure is controversial. The 235 residue occurs in a region of the protein that displays little conservation in distant mammals such as humans and mice and appears distant from the site cleaved by renin, when one considers the structure of the homologous protein α_1-antitrypsin. Although the Met→Thr substitution at position 235 alters the immunological recognition of the protein, no difference in glycosylation, secretion, or enzymatic properties, between the two recombinant angiotensinogens have been found in expression studies (Jeunemaitre et al., 1997, Karthikeyan et al., 2009, Sethi et al., 2003, Wu et al., 2004, Rodriguez et al., 2000, Bengra et al., 2002, Zhu et al., 2003, Robinson et al., 2004 and Inoue et al., 1997). It has been hypothesized that its effect is because of strong LD between this variant and the promoter A-6G polymorphism (Inoue et al., 1997). Further 235T may not be able to act uniquely as a surrogate marker for one or more molecular variants of *AGT*, indicating additional complexity for the understanding of the molecular basis of interactions among *AGT* polymorphisms in vivo (Pereira et al., 2008). Therefore the functional role of the substitution at residue 235 in *AGT* cannot be either established or ruled out on the basis of the available experimental evidence (Inoue et al., 1997).

The effect of T174M polymorphism is not yet clear. The 174T allele is highly conserved even in species that diverged from humans ~450 million years ago. Also, this substitution is predicted to be probably damaging, suggesting that the codon 174 might be under an evolutionary selective pressure and, thus, that the 174M variant is likely to be functional. Although no functional evidence is available, it has been speculated based on modeling analysis that the T174M polymorphism may lead to abnormal function of the human angiotensinogen

protein (Pereira et al., 2008). The exact mechanism is uncertain but it is unlikely that a blood pressure increase is mediated by increased plasma angiotensinogen concentration, because previous studies have shown no association between T174M polymorphism and plasma angiotensinogen concentrations (Jeunemaitre et al., 1992). However, this does not negate a potential effect of T174M polymorphism on local production of angiotensinogen, which may affect blood pressure levels. It is also possible that other functional polymorphisms, in linkage disequilibrium with the T174M polymorphism, may contribute to the development of salt-sensitive hypertension (Yamagishi et al., 2004).

The promoter polymorphism *AGT* G (–6)A is located in region AGCE1 (hAG core promoter element 1 in position –25 to –1 bp) which binds the ubiquitously expressed nuclear factor AGCF1. The factor plays a major role in mediating the *AGT* enhancer function. Substitution mutation in this location affects the promoter activity (Yanai et.al,. 1996) and *AGT* gene promoter with −6A has increased promoter activity compared with −6G leading to increased transcription (Inoue et al., 1997).

The T235 and A-6 alleles mark the original form of the gene since the T allele occurred at higher frequency in the Caucasian, Japanese, African-Americans, American Hispanics, and Native American and M235 occurred in association with a much more restricted range of marker alleles than T235. Hence it is assumed that T235 marks the original form of the gene (Hata et al., 1994). Besides, in a study in USA, there was greater genetic diversity in *AGT* genes carrying T235 compared to their M235 counterparts and T235 and A(-6) were present in the *AGT* gene of all species of primates. Therefore it was concluded that M235 and G(-6) are neomorphs, i.e., mutations which arose recently in the course of human evolution; the persistence of their association may reflect either chance or correlated response to directional selection (Inoue et al., 1997).

Interestingly, however, we observed a gender specific association of the polymorphisms with hypertension. It is established that hypertension is a gender dependent trait and is influenced directly and indirectly by gonadal hormones. The gender specific effects of the polymorphism are due to the differential regulation of angiotensinogen gene expression by sex hormones. Estrogen increases angiotensinogen mRNA in the liver, whereas androgens can induce renal expression of angiotensinogen. Besides, modulation of plasma angiotensinogen levels may be due to differential binding of the estrogen receptor in competition with other transcription factors to an estrogen receptor response element overlying −20 which affects the level estrogen induced *AGT* promoter activity. It was also observed that a binding of transcription factors Upstream stimulatory Factors 1 and 2 effect *AGT* expression and consequently blood pressure differentially since expression in females is dependent on USF 1 and in males upon both USF1 and 2 (Park et al., 2012).

There are certain limitations of our study. First is the relatively small sample size. A greater sample size could have given more accurate results. Secondly, we could not quantify the angiotensinogen levels in the plasma which could have helped in estimating possible effects of mutations on the biochemical expression of the gene.

CONCLUSIONS

From the results, it can be concluded that in our the total group, alcohol consumption and high triglycerides, in males the M allele of M235T polymorphism, G allele of G-6A polymorphism and alcohol consumption and in females the T allele of M235T polymorphism and M allele of T174M polymorphism were risk factors for essential hypertension. The TMG haplotype conferred higher risk in the total group and females and the MTG haplotype in males. The angiotensinogen gene polymorphisms studied showed a gender specific effect which may be due to gonadal hormones or differential binding of transcription factors in males and females.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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