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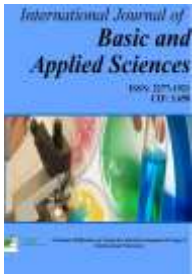

Full Length Research Paper

Genetic Association of Angiotensinogen (M235T) Gene Polymorphism with Essential Hypertension in Vindhyan Population of Madhya Pradesh, India.

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ARTICLE DETAILS

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ABSTRACT

Essential hypertension is common in the general population by its increasing incidences, thus recently researchers suggested that AGT (M235T) polymorphism may be the functional genotype in hypertensive patients. Therefore the study aimed to evaluate Angiotensinogen (AGT) gene polymorphisms (M235T) as well as its relation to serum level of renin and aldosterone among Sudanese hypertensive patients. The study concluded that there is no association between the polymorphism (M235T) of the AGT gene and essential hypertension, whereas association observed with serum renin and aldosterone levels. The descriptive data and comparison of biochemical parameters of essential hypertension patients versus healthy controls are presented in Table no. 4.2. As expected the essential hypertension patients had markedly higher levels of *HDL-C* (P<0.0001), *LDL-C* (P<0.0001), *TG* (P<0.0001), *Systolic BP* (P<0.0001) and *Diastolic BP* (P<0.0001) compared to that of control subject. Significant level of change has been seen in overall distribution of Angiotensinogen (M235T) genotypes in Healthy control group as compared to disease group although Healthy control group showed little increase in 'MM' genotype as compared to Patients of Essential hypertension (69.44% *vs* 56.25%). Similarly, mutant type 'TT' genotype was present in low frequency in Essential hypertension patients group 3.75% and also in control group 4.44% (χ^2 = 0.0243*, P= 7.436).

1. Introduction

Renin-angiotensin system (RAS) polymorphisms have been studied as candidate risk factors for hypertension with inconsistent results, possibly due to heterogeneity among various genetic and environmental factors (2). A case-control association study was conducted to investigate a possible involvement of polymorphisms of three RAS genes: *AGT* M235T (rs699) in essential hypertensive patients. Angiotensinogen (AGT) is a major precursor of the renin-angiotensin-system and its plasma levels have been shown to correlate with blood pressure. Variation in AGT gene and other genes of the RAS regulate blood pressure smooth muscle cell growth and cardiac remodeling (3). AGT variants coding region missense polymorphisms M235T the Methionine substituted to Threonine amino acid at the 235th position in the exon 2 of the AGT gene. It is suggested that AGT (M235T) polymorphism may be the functional genotype, as it affects the basal transcription rate of AGT, which could explain the association of the M235T genotypes with the plasma AGT concentration. Genetic polymorphisms in components of the RAS including AGT (M235T) are suggested to be associated with the pathogenesis of essential hypertension (1). The variant allele 235T is the most frequent one among African populations and it is not significantly associated with

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hypertension. It is reported that M235T mutants leads to small in- crease in concentration of polymorphic AGT rather than changes in function. Frequency distribution and disease association of M235T has been shown to vary between different ethnic groups and also within large ethnic groups (1-4).

The possible effects of two missense single nucleotide polymorphisms (SNPs) of the AGT gene on DN were investigated in this study. The rs699 in exon 2 is a T-to-C exchange at codon 268 resulting in a functional replacement of methionine (M) to threonine (T) (M268 T). The rs699 was previously located at amino acid 235, and, therefore, it is also named M235T. In Japanese obese women, the polymorphism in AGT (rs699) has been reported to be associated with visceral obesity and hyperinsulinemia (2,5). Another SNP on the AGT gene, rs4762, is a C-to-T substitution in exon 2 of the AGT gene at codon 207. As a result of this, the exchange of a functional threonine (T) to methionine (M), also known as T207M o T174M, occurs. The rs4762 may act as a predictor marker for the post-transplant diabetes mellitus development in addition to essential and pregnancy hypertension (6,8). Moreover, the risk for pregnancy-induced hypertension was also reported to be associated with rs4762 and rs699 haplotypes. Over the past few years, the role of the renin-angiotensin- aldosterone system (RAAS) in the development of essential hypertension has generated much interest across the world. The investigations were expanded to implicate that the RAAS in a variety of physiologic processes may play a significant role in the initiation and progression of atherosclerosis. The AGT M235T single nucleotide polymorphism (SNP) is a methionine (Met) to threonine (Thr) amino acid substitution at codon 235, designated the M and T alleles, respectively. However, published results have been inconsistent (6). To help clarify the inconsistent findings, with the publication of several more recent studies we conducted this meta-analysis of the M235T polymorphism in the AGT gene and risk of essential hypertension (5-9).

2. Materials and methods:

2.1 Study population:

The study population consisted of 340 unrelated subjects comprising of 160 Essential Hypertension patients and 180 ethnically matched controls of central Indian population were included in this study. In this region Hindu, Muslim and some Sikh peoples are mainly living but most people's belong to Hindu religion in this region.

2.2 Inclusion and Exclusion criteria for Cases:

Cases included consecutive patients who attended the Department of Medicine, Shyam Shah Medical College and Sanjay Gandhi Memorial Hospital, Rewa, Ayurveda Medical College, Rewa, Ranbaxy pathology Regional collection centre Rewa, District hospital Satna, Shahdol, Sidhi. Hypertension was diagnosed in accordance with World Health Organization (WHO Expert committee 2003) criteria.

2.3 Inclusion and Exclusion criteria for Controls:

Control group composed of non-diabetic healthy individuals that were collected during "Diabetes Awareness Camps" organized in urban regions in and around SSMC Rewa and many volunteers were also included to collect control sample. The control subjects were recruited from the regions that from homogenous cluster in Vindhyan region India in accordance with a recent report of genetic landscape of the people of India. (Indian Genome Variation Consortium 2008)

2.4 Anthropometric and Biochemical Measurements:

2.4.1 Anthropometry:

Height and Weight were measured in light clothes and without shoes in standing position as per standard guidelines. Body Mass Index (BMI) was calculated as weight in kilograms divided by height in meters squared. Waist circumference was measured in standing position midway between iliac crest and lower costal margin and hip circumference was measured at its maximum waist to hip ratio (WHR) was calculated using waist and hip circumferences. Systolic and diastolic blood pressures were measured twice in the right arm in sitting position after resting for at least 5 minute using a standard sphygmomanometer and the average of the two reading was used.

2.4.2 Biochemical Analysis:

Biochemical parameters related to type 2 diabetes were estimated for both cases and controls subjects. Measurement of Serum levels of Total cholesterol (TC), Triglycerides (TG), HbA1c, High density lipoprotein-cholesterol (HDL-C), Low density lipoprotein-cholesterol (LDL-C) and Urea were measured based on spectrophotometric method using automated clinical chemistry analyzer Cobas Integra 400 plus (Roche Diagnostics, Mannheim, Germany).

2.4.3 Blood collection and plasma/serum separation:
Venous blood samples were obtained from the subjects after 12 hours of overnight fasting in vacutainers with and without appropriate anti-coagulants. Immediately, plasma and serum from the respective vacutainers were separated by centrifuging the tubes at 1000 rpm for 10 min. at 4˚C

2.5 Molecular Laboratory Analysis:
2.5.1 Method for DNA isolation:
Genomic DNA was extracted from whole blood by the modification of salting out procedure described by Miller and coworkers (Miller *et al*. 1988). Frozen blood sample was thawed at room temperature. 0.5 ml. of whole blood sample was suspended in 1.0 ml. of lysis buffer (0.32 M Sucrose, 1 mM Mgcl2, 12 mM Tris and 1% Triton-X-100) in a 1.5 ml. microcentrifuge tubes. This mixture was mixed gently by inverting the tube upside down for 1 min. The mixture was than allowed to stand for 10 min. at room temperature to ensure proper lysis of cells. The mixture was centrifuged at 11,000 rpm for 5 min. at 4˚C to pellet the nuclei. The supernatant was discarded carefully in a jar containing disinfectant, as pellet formed is loosely adhered to the bottom of centrifuge tube. The pellet was resuspended in 0.2 ml. of lysis buffer and recentrifuge at 11,000 rpm for 5 min. The pellet was than dissolved in 0.2 ml. of deionized autoclaved water and mixed thoroughly on vortexer. The mixture was centrifuged at 14,000 rpm for 1 min. at 4˚C. Supernatant was discarded to gain an intact pellet. To the above pellet, 80 µl. of proteinase K buffer (0.375 M Nacl, 0.12 M EDTA, pH 8.0) and 10 µl. of 10% SDS (10% w/v SDS, pH 7.2) was added. Mixture was well frothed with the help of micro tip to allow proper lysis of pelleted nuclei. After digestion was complete, 100 µl. of saturated cold 5M Nacl was added and shaken vigorously for 15 sec. To the above mixture 0.2 ml. of deionized, autoclaved water and 0.4 ml. of phenol-chloroform (4:1 v/v) was added to remove most of the non nucleic acid organic molecules. Microcentrifuge tube was inverted upside down until the solution turned milky. Phases were separated by centrifuging the above mixture at 12,000 rpm for 10 min. at 4˚C. Aqueous (top) layer was saved and transferred in another microcentrifuge tube. Transferring of any interface layer was avoided. To the aqueous layer, 1 ml. chilled absolute ethanol was added and the tube was inverted several times until the DNA precipitated. DNA precipitates like thread. This was centrifuged at 14,000 rpm for 4 min. at 4˚C to pellet the DNA thread. Supernatant was discarded. The pellet was washed twice with 1 ml. of 70% alcohol. The mixture was again centrifuged at 14,000 rpm for 1 min. 4˚C. Supernatant was discarded and pellet was air dried for 10-20 min. The pelleted DNA was rehydrated in 100-200 µl. of TE buffer pH 7.4 (10 mM Tris-HCL pH 7.4, 1mM EDTA, pH 8.0). DNA was allowed to dissolve overnight at 37˚C before quantization.

2.5.2 Determination of quality and quantity of isolated DNA:
The isolated DNA is to be used for PCR based study. Therefore its suitability for PCR along with its size heterogeneity is among the most important criterion for purity. As a matter of general practice all DNA preparations were tested for quality and quantity measures, as described in the following paragraphs.

2.5.3 Quantitation by UV spectrophotometry:
The isolated genomic DNAs were then tested for purity by measuring their absorbance values at 230 nm, 260 nm, 280 nm and 300 nm using a UV visible spectrophotometer (Systronic, India). A DNA preparation was considered to be good if it had A 260 nm / A 280 nm ratio as approximately 1.8 and A 300 nm was 0.1 or lesser. The absorbance at 260 nm was used to calculate the amount of DNA, using the relationship that double stranded DNA at 50µg/ml concentration has an absorbance= 1.0 at 260 nm.

2.5.4 Agarose Gel Electrophoresis:
Gel electrophoresis of the genomic DNAs was carried out for qualitative estimation of samples prepared. A good DNA preparation appears as single band. A horizontal agarose slab gel electrophoresis apparatus (Bangalore Genei, Bangaore, India) was used. In brief, 4-5 µl of each genomic DNA was loaded on 0.8 agarose (0.8 % w/v, Sigma) containing ethidium bromide solution (0.5 µg/ml) and electrophoresis was done at 80 V in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). Lambda DNA *Eco*RI / *Hind* III double digest (Bangalore Genei, Bangalore, India) was used as molecular weight marker after completion of electrophoresis, the DNA bands were visualized and photographed using an UV transilluminator (312 nm) and gel documentation system (Vilber Lourmate, Cedex 1, France) respectively.

*2.5.5 Polymorphism screening***:**
In general, the genomic DNA extracted from peripheral blood of healthy individuals and diseased individuals was subjected to PCR followed by restriction digestion and electrophoresis to genotype both the groups for relevant gene of interest. All the PCRs were carried out in a PTC 200 thermal cycler (MJ Research Inc. USA). PCR is a rapid, inexpensive and simple mean of producing relatively large copy number of DNA molecules from the small amounts of source DNA material, even when the source DNA is of relatively poor quality. Due to the extreme sensitivity, precautions were taken against contamination of the reaction mixture with the trace amounts of DNA, which could serve as an unwanted template. Appropriate negative control

was included in each PCR run carried out for each gene, to monitor this contamination of PCR mix to avoid any false positive results. The negative control used for PCR contained whole PCR reaction mix except target DNA which was replaced by HPLC purified water free of RNase, DNase, and any contamination from any other source resembling the gene sequence. Subsequently restriction enzyme digestion was performed by incubating the double stranded DNA with appropriate amount of restriction enzyme, in its respective buffer as recommended by the supplier and at optimal temperature for that specific enzyme. A typical digestion includes one unit of enzyme per microgram of starting DNA. One enzyme unit is usually defined as the amount of enzyme needed to completely digest one microgram of double stranded DNA in one hour at the appropriate temperature. Their biochemical activity of the restriction enzyme is the hydrolysis of phosphodiester backbone at specific sites in a DNA sequence. Precaution was taken to avoid star activity of restriction enzymes. When DNA is digested with certain restriction enzymes under non-standard conditions, cleavage can occur at sites different from the normal recognition sequence. Such aberrant cutting is called "star activity" which can be due to high pH (>8.0) or low ionic strength, extremely high concentration of enzyme (>100 U/µg of DNA) and presence of organic solvents in the reaction (e.g. ethanol, DMSO). The PCR and restriction digestion conditions were optimized for specific locus of relevant segment of the gene to be studied. The PCR products as well as the digested products were separated on either agarose gel or polyacrylamide gel depending on their size. Gels were stained with ethidium bromide solution (0.5µg/ml) and subsequently visualized and photographed under UV transilluminator.

2.6 Detection of Angiotensinogen (M235T) Single Nucleotide Polymorphism:

Polymorphism in The angiotensinogen (AGT) gene M235T polymorphism is located in the SNP rs699 region of the gene. The AGT M235T single nucleotide polymorphism (SNP) is a methionine (Met) to threonine (Thr) amino acid substitution at codon 235, designated the M and T alleles, respectively.

2.6.1 Primer sequence: The oligonucleotides sequences (primers) used were those described by MM Shamaa *(Shamaa MM et. al. 2013).*

Forward primer: 5'- CCG TTT GTG CAG GGC CTGGCT CTC T -3'

Reverse Primer: 5' - CAG GGT GCTGTC CAC ACT GGA CCC C-3'

2.6.2 PCR Mix:

The PCR was carried out in a final volume of 25 µl, containing 50-100 ng of genomic DNA(4-5 µl), 2.5 µl of 10X *Taq* polymerase buffer (10 mM Tris HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.005% Tween-20, 0.005% NP-40; final concentration 1X; Genetix Biotech Asia Pvt. Ltd.,India), 1 µl of 10 mM dNTPs (Banglore Genei, Bangalore, India), 1 µl of 25 pmol/µl of forward and reverse primers specific for and 1 µl of unit of 1U/ µl Red Taq DNA polymerase (Bangalore genei).

2.6.3 PCR Thermal Program:

After an initial denaturation of 5 min at 95˚C, the samples were subjected to 35 cycles at 95˚C for 1 min, at 58˚C for 45 s, and 72˚C for 45 s, with a final extension of 10 min at 72˚C in a thermal cycler. A 100bp ladder with amplified product has been run under 2.5 % agarose gel electrophoresis.

2.6.4 Restriction digestion By PsyI:

PCR amplification of Angiotensinogen (M235T) Gene with specific primers gave 165-bp undigested product which was digested with *PsyI* enzyme (New England Biolabs, Boverly, MA) for 16 h at 37˚C. The wild-type genotype (MM) was not digested, whereas the mutated homozygous genotype (TT) was cut as a doublet of 141 bp and 24 bp. The heterozygous genotype (TC) was represented as 2 fragments of 165 bp and 141 bp whereas 24 bp of DNA fragments are run out from the gel because of its too short fragments the digestion products were then separated by electrophoresis on a 2.5% agarose gel. The results were documented by digital camera and further saved by gel documentation system.

2.7 Statistical Analysis of Genotype:

Statistical analysis was done by comparing the distribution of genotype frequencies, allele frequencies and carriage rates of all the four polymorphism in diseased and control group. Disease group included Diabetic patients whereas control group included all healthy controls (HC) enrolled in the study. The proportions of different genotypes for a gene in a population are known as genotype frequencies.

The proportion of genotype in a sample will be the ratio of the number of individuals having that genotype to the total number of individuals in the sample. The proportions of different alleles for a gene present in a population are known as allele frequencies. The proportion of an allele in a sample will be the ratio of number of occurrences of the investigated allele in the population to the total number of alleles. The carriage rate was calculated as the number of individuals carrying at least one

copy of the test allele divided by the total number of individuals. Data was analyzed using Microsoft Excel 2002, Microsoft Corporation. Only the biochemical parameters, for difference between obese diabetic patients and the obese non-diabetic controls were assessed using the student's t test. Similarly the biochemical parameters between the normal weight patients and normal weight non-diabetic controls were also assessed. The P-values calculated using t test along with the mean (inter-quartile range) were presented. Statistical analyses were performed using statistical package, Prism 3.0, Prism 5.1 version.

3. Results

3.1 Results:

The descriptive data and comparison of anthropometric and biochemical parameters of essential hypertension patients versus controls are presented in Table no. 4.1. The age, sex, BMI, WHR were the parameters. As expected the essential hypertension patients had markedly higher levels of weight of men (P=0.3916) then women (P=0.0723) and BMI of Women (P=0.2247) and Men (P=0.3499) but both was not significantly different between patient and healthy population. Thus WHR in Women (P=0.1741) and Men (P=0.0973) were not found significantly different between case and control group.

Table 1. Comparison of anthropometric parameters of essential hypertension patients and healthy controls

Characteristics	Cases (160)	Controls(180)	P-value
n(Men/Women)	160(104/56)	180(118/62)	
Age(years)	52.5±12.5	52.6±12.4	0.9411,ns
Height(m)	162.50±11.3	161.2±12.4	0.3152,ns
Weight (Kg)			
Women	62.5 ±4.7	61.6 ± 4.5	0.0723,ns
Men	68.4±5.6	67.8±7.1	0.3916,ns
BMI (kg/m^2)			
Women	25.6±3.1	26.1 ± 4.3	0.2247,ns
Men	24.6±4.7	25.1± 5.1	0.3499,ns
Waist circumference (cm)			
Women	92.5±6.2	93.6±6.7	0.1186,ns
Men	90.0±7.0	89.0±6.0	0.1571,ns
Hip (cm)			
Women	95.9±2.4	96.1±2.2	0.4233,ns
Men	90.8±4.3	91.2±1.5	0.2426,ns
WHR			
Women	0.97±0.05	0.98±0.08	0.1741,ns
Men	0.98±0.08	0.99±0.01	0.0973,ns

(*denotes level of significant change between case and control)

3.2 Biochemical and clinical findings:

Biochemical test performed in the blood sample for following clinical parameters and the findings were tabulated. Statistical analysis was done by using student's t-test and p value suggests the level of significant were changed here. The descriptive data and comparison of biochemical parameters of essential hypertension patients versus healthy controls are presented in Table no. 2. As expected the essential hypertension patients had markedly higher levels of *HDL-C* (P<0.0001), *LDL-C* (P<0.0001), *TG* (P<0.0001), *Systolic BP* (P<0.0001) and *Diastolic BP* (P<0.0001) compared to that of control subject. Whenever, rests of parameters were not significantly different between patient and healthy population.

Table 2. Comparison of Biochemical & clinical findings of essential hypertension patients and healthy controls.

Characteristics	Cases (160)	Controls(180)	P-value
Post-Prandial Glucose (mg/Dl)	118.7±12.4	119.4±11.6	0.5912,ns
HbA1C(%)	5.9±0.7	5.8±0.8	0.2235,ns
HDL-C(mmol/L)	122.8±12.2	109.3±11.6	P<0.0001***
LDL-C (mg/dL)	49.1±2.6	41.8±3.7	P<0.0001***
TG(mg/dL)	132.9±13.2	126.2±12.2	P<0.0001***
Systolic BP (mmHg)	145.4±8.1	124.8±5.7	P<0.0001***
Diastolic BP (mmHg)	97.1±5.8	86.5±6.2	P<0.0001***
Blood Urea(mg/dL)	16.68±1.7	16.80±1.8	0.5293,ns
Urinary Citrate (mmol/24 h)	2.58±0.96	2.62±0.57	0.6365,ns
Serum calcium (mg/dl)	9.42±0.32	9.46±0.38	0.2978,ns

Urinary potassium (mmol/24 h)	64.21±4.7	64.39±4.3	0.7125,ns
Urinary Phosphate (mmol/24 h)	27.45±4.2	26.81±3.3	0.1172,ns

(denotes the level of significant change between case and control)*

3.3 Detection of Genetic Polymorphism in Angiotensinogen (M235T) Gene:

PCR amplification with specific primers gave 165 bp product which was digested with *PsyI* enzyme (New England Biolabs, Boverly, MA) for 16 h at 37˚C. The wild-type genotype (MM) was not digested, whereas the mutated homozygous genotype (TT) was cut as a doublet of 141 bp and 24 bp. The heterozygous genotype (TC) was represented as 2 fragments of 165 bp and 141 bp whereas 24 bp of DNA fragments are run out from the gel because of its too short fragments.

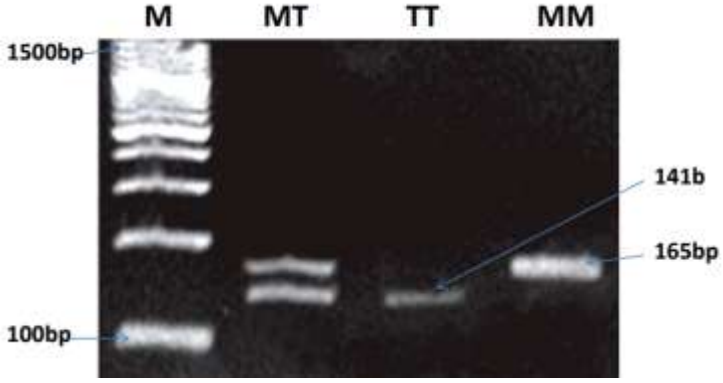


Fig-1: Representative gel picture of Angiotensinogen (M235T) polymorphism. Lane M represents 1500 bp molecular marker, Lane MM Wild type genotype, and Lane MT heterozygous genotype and Lane TT variant genotype**.**

The distribution of the polymorphisms of Angiotensinogen (M235T) was consistent with Hardy- Weinberg equilibrium (HWE) in healthy controls. The observed genotype frequencies, allele frequencies and carriage rates for Angiotensinogen (M235T) polymorphism are depicted in table 4.5 and table 4.6 and Graph 1, 2, 3. Significant level of change has been seen in overall distribution of Angiotensinogen (M235T) genotypes in Healthy control group as compared to disease group although Healthy control group showed little increase in 'MM' genotype as compared to Patients of Essential hypertension (69.44% *vs* 56.25%). Similarly, mutant type 'TT' genotype was present in low frequency in Essential hypertension patients group 3.75% and also in control group 4.44% (χ^2 = 0.0243*, P= 7.436). 'TT' genotype is higher in control group and may be protective in our population and statistically significantly different between both groups.

An odds ratio of MM genotype is 0.5657 which indicates little protective effect whereas an odds ratio of MT genotype is 1.887 of Essential hypertension patients group respectively indicate little or no effect and association of this mutant genotype with the Essential hypertension susceptibility. Overall allele 'M' was found little lower frequency in disease group as compared to HC group whereas allele 'T' was present in little high frequency in the disease group but the difference is nominal and significant (χ^2 = 0.0437*, P=4.069). Carriage rate of allele 'M' was slightly high in essential hypertension group as compared to healthy control (75.77% Vs 68.75%) whereas carriage rate of allele 'T' was approximately similar in both control and disease group and no significant level of change has been seen. The pattern of genotype distribution, allele frequency and carriage rate in disease and control group suggests Angiotensinogen (M235T) polymorphism is significantly associated with Essential hypertension in our population.

Table 3. Frequency distribution and association of Genotype, allele frequency and carriage rate of Angiotensinogen (M235T) gene polymorphism in population of Vindhyan region using Chi Square Test

Angiotensinogen (M235T) GENE	**CASE N= 160**		**CONTROL N=180**		**CHI SQUARE VALUE χ^2 (P Value)**
	N	**%**	**N**	**%**	
Genotype					
MM	90	56.25	125	69.44	
MT	64	40.00	47	26.11	7.436 (0.0243*)
TT	6	3.75	8	4.44	

Allele					
M	244	76.25	297	82.50	4.069 (0.0437*)
T	76	23.75	63	17.50	
Carriage Rate					
M	154	68.75	172	75.77	2.774 (0.0958ns)
T	70	31.25	55	24.22	

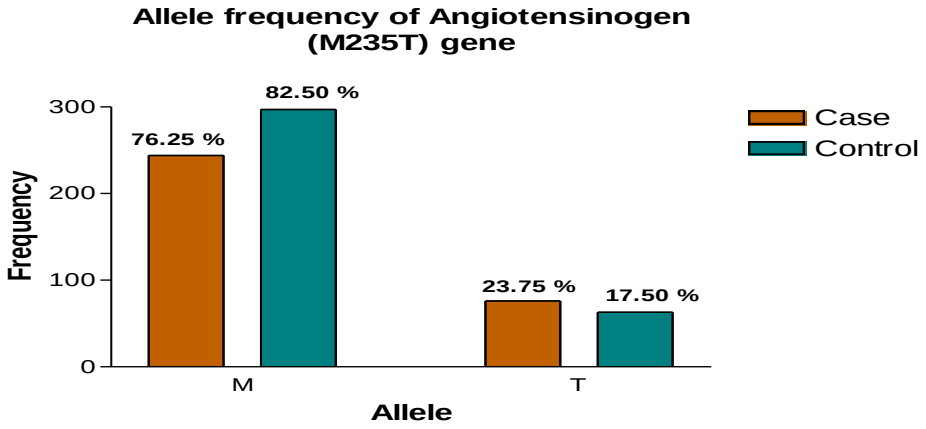
(- denotes the level of significant association between case and control.)*
(N – Number of individuals in study group.)
(% - Genotype allele frequency and carriage rate expressed in percentage.)

Table 4. Fisher Exact Test values of Angiotensinogen (M235T) gene polymorphism

Angiotensinogen (M235T) GENE	**CASE N= 160**		**CONTROL N=180**		**P Value**	**Odds Ratio (95% confidence interval)**
	N	**%**	**N**	**%**		
Genotype						
MM	90	56.25	125	69.44	0.0133*	0.5657 (0.3624 to 0.8830)
MT	64	40.00	47	26.11	0.0077**	1.887 (1.192 to 2.986)
TT	6	3.75	8	4.44	0.7914ns	0.8377 (0.2842 to 2.469)
Allele						
M	244	76.25	297	82.50	0.0459*	0.6810 (0.4683 to 0.9904)
T	76	23.75	63	17.50		1.468 (1.010 to 2.135)
Carriage Rate						
M	154	68.75	172	75.77	0.1144ns	0.7035 (0.4646 to 1.065)
T	70	31.25	55	24.22		1.421 (0.9388 to 2.152)

(- denotes the level of significant association between case and control.)*
(N – Number of individuals in study group.)
(%-Genotype allele frequency and carriage rate expressed in percentage.)

Graph No.-1: Genotype Frequency of Angiotensinogen (M235T) gene.

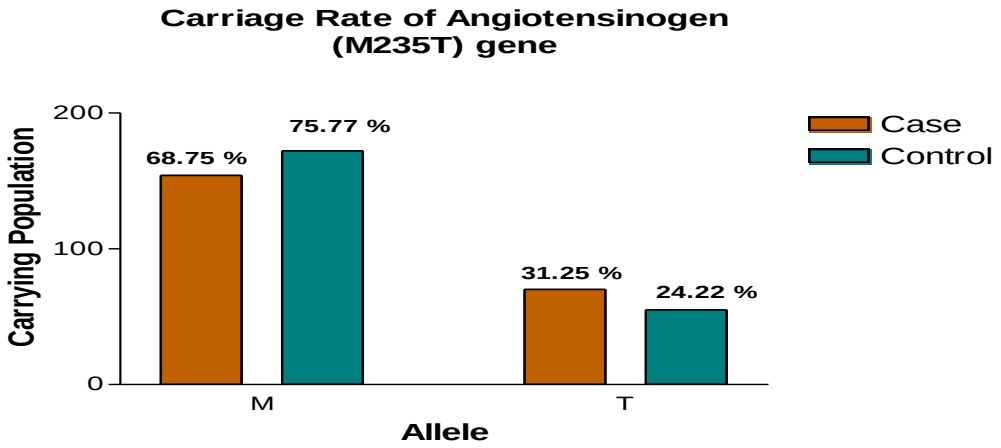


4. Discussion:

The genetic basis of common essential HTN on the other hand is only just becoming accessible through high-throughput approaches. Unbiased genome-wide analyses of BP genomics have identified 43 genetic variants associated with systolic, diastolic BP, and HTN (11). It is highly likely based on current findings that there are hundreds of such loci with small effects

on BP, opening a perspective on the genetic architecture of BP that was unknown before. A powerful interaction between the autonomic and the immune systems plays a prominent role in the initiation and maintenance of hypertension and significantly contributes to cardiovascular pathology, end-organ damage and mortality (7,14). The sympathetic nervous system, a major determinant of hypertension, innervates the bone marrow, spleen and peripheral lymphatic system and is proinflammatory, whereas the parasympathetic nerve activity dampens the inflammatory response through α7-nicotinic acetylcholine receptors. The neuro-immune synapse is bidirectional as cytokines may enhance the sympathetic activity through their central nervous system action that in turn increases the mobilization, migration and infiltration of immune cells in the end organs (13). Kidneys may be infiltrated by immune cells and mesangial cells that may originate in the bone marrow and release inflammatory cytokines that cause renal damage. Hypertension is also accompanied by infiltration of the adventitia and perivascular adipose tissue by inflammatory immune cells including macrophages. Increased cytokine production induces myogenic and structural changes in the resistance vessels, causing elevated blood pressure (10-14).

Graph No.-2: Allele Frequency of Angiotensinogen (M235T) gene.



Hypertension, the leading risk factor for cardiovascular disease, originates from combined genetic, environmental, and social determinants. Environmental factors include overweight/obesity, unhealthy diet, excessive dietary sodium, inadequate dietary potassium, insufficient physical activity, and consumption of alcohol (15). Prevention and control of hypertension can be achieved through targeted and/or population-based strategies. For control of hypertension, the targeted strategy involves interventions to increase awareness, treatment, and control in individuals. The Chronic Care Model, a collaborative partnership among the patient, provider, and health system, incorporates a multilevel approach for control of hypertension. Optimizing the prevention, recognition, and care of hypertension requires a paradigm shift to team-based care and the use of strategies known to control BP (17). Essential hypertension illustrates the formidable task presented by the identification of genetic determinants of common disease. Making an initial genetic inference may prove difficult enough; the subsequent demonstration of functional significance at various levels of biological integration may be even more challenging. We review three instances in which an initial genetic inference has led to the development of testable hypotheses pursued at increasingly higher levels of biological organization (18). Men had significantly higher BMI than women. Average BMI was significantly higher in participants with elevated cholesterol levels compared to participants with normal cholesterol levels. Majority of participants consumed less than five meals per day with no major differences between students according to residence and sex. Men had significantly higher protein intake and consumed at least four meals daily compared to woman who had three or less meals daily with no differences in intake according to residence. Students with normal lipid profile consumed more carbohydrates than students with increased cholesterol (15-19).

Our study suggested that the essential hypertension patients had markedly higher levels of weight of men (P=0.3916) then women (P=0.0723) and BMI of Women (P=0.2247) and Men (P=0.3499) but both was not significantly different between patient and healthy population. Thus WHR in Women (P=0.1741) and Men (P=0.0973) were not found significantly different between case and control group. Biochemical test performed in the blood sample for following clinical parameters and the findings were tabulated. Statistical analysis was done by using student's t-test and p value suggests the level of significant were changed here. The descriptive data and comparison of biochemical parameters of essential hypertension patients versus healthy controls are presented in Table no. 2. As expected the essential hypertension patients had markedly higher levels of

HDL-C (P<0.0001), *LDL-C* (P<0.0001), *TG* (P<0.0001), *Systolic BP* (P<0.0001) and *Diastolic BP* (P<0.0001) compared to that of control subject. Whenever, rests of parameters were not significantly different between patient and healthy population.

The M235T polymorphism is the most important angiotensinogen (AGT) genetic variant. The relationship between AGT gene polymorphisms and CAD has been investigated in only a few studies, however, with conflicting results. In most of these studies, not all participants underwent coronary angiography to determine the existence of coronary artery stenosis. In this study, we tested this relationship again in Taiwanese subjects who underwent coronary angiography. This study enrolled 576 patients who underwent coronary angiography, including 362 patients with CAD (the CAD group) and 214 without CAD (21). The angiotensinogen gene has been linked to the development of essential hypertension, and a M235T variant of this gene, associated with increased plasma levels of angiotensinogen, is more common in hypertensives than in normotensive controls in various populations. The present study was conducted to examine whether the M235T variant of the angiotensinogen gene may be a risk factor for the development of hypertension in patients undergoing renal transplantation (23). Presence of hypertension and graft survival was analysed by blinded review of all case records over a follow-up period up to 30 months. Angiotensinogen genotype was determined by a mutagenically separated allele-specific polymerase chain- reaction technique. While post-transplant hypertension was present in 78% of all patients, no relationship was found between either donor or recipient genotype and the presence or severity of post-transplant hypertension. Furthermore, there was no relationship between Angiotensinogen genotype and graft survival during the course of the study. These findings do not support the hypothesis that the M235T variant of the Angiotensinogen gene is a risk factor for the development of post transplant hypertension (20-24).

Essential hypertension is a common, polygenic, complex disorder resulting from interaction of several genes with each other and with environmental factors such as obesity, dietary salt intake, and alcohol consumption. Since the underlying genetic pathways remain elusive, currently most studies focus on the genes coding for proteins that regulate blood pressure as their physiological role makes them prime suspects (25). The present study examines how polymorphisms of the insertion/deletion (I/D) ACE and M235T AGT genes account for presence and severity of hypertension, and embeds the data in a meta-analysis of relevant studies. The I/D polymorphisms of the ACE and M235T polymorphisms of the AGT genes were determined by RFLP (restriction fragment length polymorphism) and restriction analysis in 638 hypertensive patients and 720 normotensive local blood donors (26).Severity of hypertension was estimated by the number of antihypertensive drugs used. No difference was observed in the allele frequencies and genotype distributions of ACE gene polymorphisms between the two groups, whereas AGT TT homozygotes were more frequent in controls (4.6% vs. 2.7%, P = .08). This became significant (p = 0.035) in women only (24-27).

Our study on polymorphism Angiotensinogen (M235T) revealed that the PCR amplification with specific primers gave 165 bp product which was digested with *PsyI* enzyme (New England Biolabs, Boverly, MA) for 16 h at 37°C. The wild-type genotype (MM) was not digested, whereas the mutated homozygous genotype (TT) was cut as a doublet of 141 bp and 24 bp. The heterozygous genotype (TC) was represented as 2 fragments of 165 bp and 141 bp whereas 24 bp of DNA fragments are run out from the gel because of its too short fragments. The distribution of the polymorphisms of Angiotensinogen (M235T) was consistent with Hardy- Weinberg equilibrium (HWE) in healthy controls. The observed genotype frequencies, allele frequencies and carriage rates for Angiotensinogen (M235T) polymorphism are depicted in table 4.5 and table 4.6 and Graph 1, 2, 3. Significant level of change has been seen in overall distribution of Angiotensinogen (M235T) genotypes in Healthy control group as compared to disease group although Healthy control group showed little increase in 'MM' genotype as compared to Patients of Essential hypertension (69.44% *vs* 56.25%). Similarly, mutant type 'TT' genotype was present in low frequency in Essential hypertension patients group 3.75% and also in control group 4.44% (χ^2 = 0.0243*, P= 7.436). 'TT' genotype is higher in control group and may be protective in our population and statistically significantly different between both groups. An odds ratio of MM genotype is 0.5657 which indicates little protective effect whereas an odds ratio of MT genotype is 1.887 of Essential hypertension patients group respectively indicate little or no effect and association of this mutant genotype with the Essential hypertension susceptibility. Overall allele 'M' was found little lower frequency in disease group as compared to HC group whereas allele 'T' was present in little high frequency in the disease group but the difference is nominal and significant (χ^2 = 0.0437*, P=4.069). Carriage rate of allele 'M' was slightly high in essential hypertension group as compared to healthy control (75.77% Vs 68.75%) whereas carriage rate of allele 'T' was approximately similar in both control and disease group and no significant level of change has been seen. The pattern of genotype distribution, allele frequency and carriage rate in disease and control group suggests Angiotensinogen (M235T) polymorphism is significantly associated with Essential hypertension in our population. The angiotensinogen gene M235T polymorphisms that has an effect on the activity of the renin-angiotensin-aldosterone system are related to the high hypertension risk. The aim of this study was to find out the association between angiotensinogen M235T gene polymorphism and the risk of developing hypertension. A total of 306 samples - 153 patients with hypertension and 153 age- and sex-matched healthy controls were selected using a simple

random sampling technique. Clinical and biochemical variables were measured to assess the associated risk factors. Blood samples from the patients and matched controls were used to isolate deoxyribonucleic acid. The AGT M235T genotypes were identified using polymerase chain reaction and analyzed by agarose gel electrophoresis (22). Logistic regression with a 95% confidence interval (CI) was employed to assess the risk correlations of AGT gene M235T polymorphisms with hypertension. This study also identified the clinical risk factors for hypertension, such as, total cholesterol, triglycerol, low density lipoprotein-cholesterol, and high density lipoprotein-cholesterol levels, which were significantly higher in patients compared to controls (P< 0.001). The AGT M235T genes of the TT genotype and the T allele are associated with an increased risk of hypertension among the Ethiopian patients. A population-based epidemiological study is needed corroborate the association between AGT and HTN. Gene polymorphisms linked to the renin–angiotensin (AGT) aldosterone system (RAAS) were broadly inspected in patients with diabetic nephropathy (DN) and hypertension (25).

The pathogenesis of essential hypertension (EH) is affected by genetic and environmental factors. Mutations in hypertension-related genes can affect blood pressure (BP) via alteration of salt and water reabsorption by the nephron. The genes of the renin-angiotensin system (RAS) have been extensively studied because of the well documented role of this system in the control of BP. It has been previously shown that angiotensinogen (AGT) gene polymorphism could be associated with increased risk of EH (27).The current study evaluated the frequency of AGT (M235T) polymorphism in relation to EH in a group of Egyptian population. Restriction fragment length polymorphism- Polymerase chain reaction (RFLP-PCR) was used for the analysis of M235T polymorphism of AGT genes in peripheral blood samples of all patients and controls (26). The results revealed that there was a positive risk of developing EH when having the T allele whether in homozygous or heterozygous state. It was concluded that there was an association between AGT (M235T) gene polymorphism and the risk of developing EH (26-28).

5. Conclusion:

The renin-angiotensin system (RAS) is a group of related hormones that act together to regulate BP by maintaining vascular tone and the balance of water and sodium. Angiotensinogen (AGT) is among the components that are involved in the activation/effector cascade of the RAS. Genetic polymorphisms in components of the RAS including AGT (M235T) are suggested to be associated with the pathogenesis of EH. Significant level of change has been seen in overall distribution of Angiotensinogen (M235T) genotypes in Healthy control group as compared to disease group although Healthy control group showed little increase in 'MM' genotype as compared to Patients of Essential hypertension (69.44% *vs* 56.25%). Similarly, mutant type 'TT' genotype was present in low frequency in Essential hypertension patients group 3.75% and also in control group 4.44% (χ^2 = 0.0243*, P= 7.436). 'TT' genotype is higher in control group and may be protective in our population and statistically significantly different between both groups. Finally, we summarized, some biochemical and clinical parameters expected the essential hypertension patients had markedly higher levels of HDL-C, LDL-C, TG, Systolic BP and Diastolic BP compared to that of control subject was significantly associated with Essential hypertension. Here, data from results reveals Angiotensinogen (M235T) gene was significantly associated with Essential hypertension.

6. References:

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