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Research Article

Association Between Angiotensinogen Gene M235T Polymorphism and Plasma Angiotensinogen Level in Essential Hypertension

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Abstract

Studying the genetic of human renin angiotensin system, *AGT* M235T polymorphism has been associated with the mechanism of the pathophysiology of essential hypertension. The present study efforts on the association between the *AGT* M235T polymorphism and plasma angiotensinogen level of different genotypes in essential hypertension in local area by the molecular technique that dissects the understanding pathophysiology of essential hypertension in Myanmar population.

The study involved 144 subjects, 72 hypertensives and 72 normotensives. After getting informed consents, the *AGT* M235T genotypes were determined by PCR-RFLP followed by enzyme digestion and Enzyme-Linked Immunosorbant Assay (ELISA) was used to determine plasma angiotensinogen level.

In the TT and MT genotypes that were mutant homozygous and heterozygous genotypes, the plasma angiotensinogen level was significantly increased in hypertensives than those of normotensives ($p = 0.005$ and < 0.001 respectively). In the present study, subjects carrying the homozygous TT genotype possessed three folds increased in plasma angiotensinogen level than subjects carrying homozygous MM genotype in essential hypertension. There was significant difference between the genotypes of *AGT* M235T polymorphism and plasma angiotensinogen level in the study group $\chi^2 = 38.16$ and $\rho < 0.001$.

This study supported the significant association of the *AGT* M235T variant and plasma angiotensinogen level with essential hypertension in Myanmar. That may lead to indicate that *AGT* variants might play a critical role in the pathogenesis of essential hypertension in local area.

Keywords: *Plasma Angiotensinogen; M235T Polymorphism; Hypertension; Myanmar*

Introduction

The genetic makeup of hypertension ranges from 30% to 50%. Essential hypertension and its associated complications impact gigantic burden of morbidity and mortality to society and the health care system. In Myanmar, the prevalence of hypertension was 30.1% (95% CI: 28.4 - 31.8) in males and 29.8% (28.5 - 31.1) in females [1].

About 90 - 95% of hypertension is idiopathic and apparently primary or essential hypertension. Essential hypertension is a multifactorial disorder arising from the influences of genetic and environmental stimuli. To know the mechanism that related to pathophysiology of disease process in local area is the most important thing in essential hypertension. Identifying genes variants that contribute in hypertension may provide better understanding of the mechanism of essential hypertension and related risks [2]. Moreover, it may lead to new therapeutic approach of individualized medicine that concerned with treatment of hypertension in more effective and directed way to treat individual with specific genotypes [3].

The activation of Renin-Angiotensin-System (RAS) is one of the main pathogenesis of hypertension and there have been many studies about genes coding for the Renin-Angiotensin-System. The most inspected gene linked to the disease for essential hypertension was the angiotensinogen (*AGT*) gene and which produces the large precursor molecule, angiotensinogen that is the first and main component of the RAS system. The human *AGT*cDNA possess 1455 nucleotides long and is translated for a 485 amino acid protein [4]. Angiotensinogen is first converted by renin to produce the decapeptide AngI. Angiotensin I is then converted to the octapeptide angiotensin, AngII which is the most active vasopressor by the removal of a C-terminal dipeptide by angiotensin-converting enzyme (ACE) [5]. Nowadays, drugs used as RAS inhibitors have been proven effectively in controlling blood pressure in treatment of hypertension. This is why RAS system genes are the most interesting genes that control blood pressure regulation [6].

A single nucleotide polymorphism (SNP) that converts methionine to threonine at amino acid 235 was significantly increased in hypertensive subjects than in normotensive control [7]. This association has been confirmed by many case control studies worldwide [8-10].

Angiotensinogen (*AGT*) contributes to arterial pressure regulation through extravascular fluid volume control. A rise in plasma *AGT* level which forms a comparable increase in the formation of AngII advocates a direct involvement of plasma angiotensinogen in pathogenesis of essential hypertension. This is because the plasma concentration of *AG*T level is close to the Michaelis constant of the enzymatic reaction between renin and AGT [11].

The present study efforts on the association between the *AGT* M235T polymorphism and plasma angiotensinogen level in essential hypertension in local area by the molecular technique that dissects the understanding pathophysiology of essential hypertension in Myanmar population.

Materials and Methods

We have analyzed total 144 subjects. Hypertensive subjects were recruited from the outpatient department of Medical Units, Mandalay General Hospital. Healthy volunteers were obtained from Mandalay area and they were age, sex matched with essential hypertensive subjects. After well explanation about the research, all case and control subjects gave informed consents. The ethical approval for the study was obtained by the ethical board of University of Medicine, Mandalay. All hypertensive subjects are primary, essential hypertension; prior diagnosed by physicians and excluded from the secondary hypertension. The criteria for hypertensives were systolic blood pressure (SBP) > 140 mmHg and/or diastolic blood pressure (DBP) > 90 mmHg, for at least two consecutive blood pressure measurements or the subjects taken antihypertensive therapy. The normotensives were SBP ≤ 120 mmHg and DBP ≤ 80 mmHg and no family history of hypertension and without diabetes mellitus.

All cases and control subjects were completed the standard questionnaire with history taking. The blood pressure determination was taken using sphygmomanometer in milliliters of mercury level and systolic and diastolic blood pressures were recorded. Blood pressure was measured twice with the subject seated with a 5 min interval between measurements. And then determination of BMI was done and recorded. Peripheral blood samples were collected from all subjects and routine investigations were done for determination of plasma angiotensinogen level and genotyping.

Genotyping

DNA extraction

DNA samples were isolated from peripheral lymphocytes by the salting out DNA extraction method [12]. The DNA were re-suspended in 75 µl of water and stored in -20 freezer. And then checking DNA on 1% agarose gel and checking DNA by Nano Drop (Thermo Fisher Scientific).

Preparation for Primer [13]

Forward Primer

5´-CAG-GGT-GCT-GTC-CAC-ACT-GGA-CCC-C-3´

The forward primer was made mismatched in the forward primer at the indicated position with red.

Reverse Primer

5´-CCG-TTT-GTG-CAG-GGC-CTG-GCT-CTC-T-3´

The reason for making mismatched is to create the part of the restriction site for

*T^th^ 111*I which is

5´……GACNNNGTC……3´

3´……CTGNNNCAG……5´

AC

Primer 5´……..ACTGG CCCA/GTCAG…….3´

Template 3´……..TGACC GGGT/CAGTC…….5´

GA

"M" allele

ACTGGACCC**A**TCA

TGACCTGGG**T**AGT

"T" allele

ACTGGACCCCGTCA

TGACCTGGGGCAGT

Polymerase Chain Reaction

Master mix was prepared by containing distilled water 37.3 µl, 5 µl 10X PCR buffer with $(NH4)2SO_4$, 2.5 µl $MgCL_2$, 0.2 µl of Taq polymerase (Thermo Fisher Scientific), 1 µl of each deoxynucleotide triphosphate, 1 µl of each primer and 1 µl of genomic DNA in a final volume of 50 µl. Individual pipette tips were used for all additions to prevent cross- contamination of the samples. The reaction tubes were placed in the heat block in thermocycler and were proceed with the following PCR temperature profile for M235T angiotensinogen gene. Heat denaturation was done at 94°C for 4 minutes. Then, 35 cycles of 94°C 1 minute, 69°C 1 minute, 72°C 1.5 minute were followed by final extension at 72°C for 8 minutes and were hold at 4°C. The PCR reaction products were analyzed by 3% agarose gel electrophoresis and were visualized under ultraviolet illumination and the presence of a 165 base pairs band in PCR for M235T angiotensinogen gene.

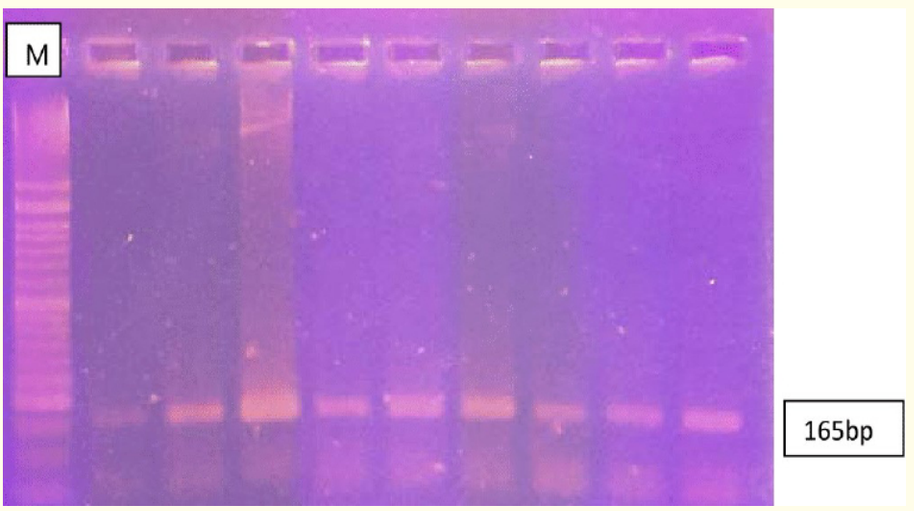

Figure 1: *PCR products of AGT M235T gene, 165 bp using 50 bp ladder with lane M.*

Amplification fragments cut by *T^th^ 111*I (BioLabs, New England)

A cocktail of 0.25 µl of the *T^th^ 111*I enzyme, 1.5 µl of 10Xbuffers and 3 µl of sterile water was added to 10 µl of PCR products. The enzyme digestion was performed in a final volume of 14.5 µl at 65°C for 3 hours. The digested products were separated on 4% agarose gel stained with ethidium bromide for 30 minutes at 125V.

The PCR products were digested with restriction enzyme *T^th^ 111*I. And then, the digested PCR products were separated and identified by agarose gel electrophoresis (4%) and three different genotypes were noted as shown in figure 2.

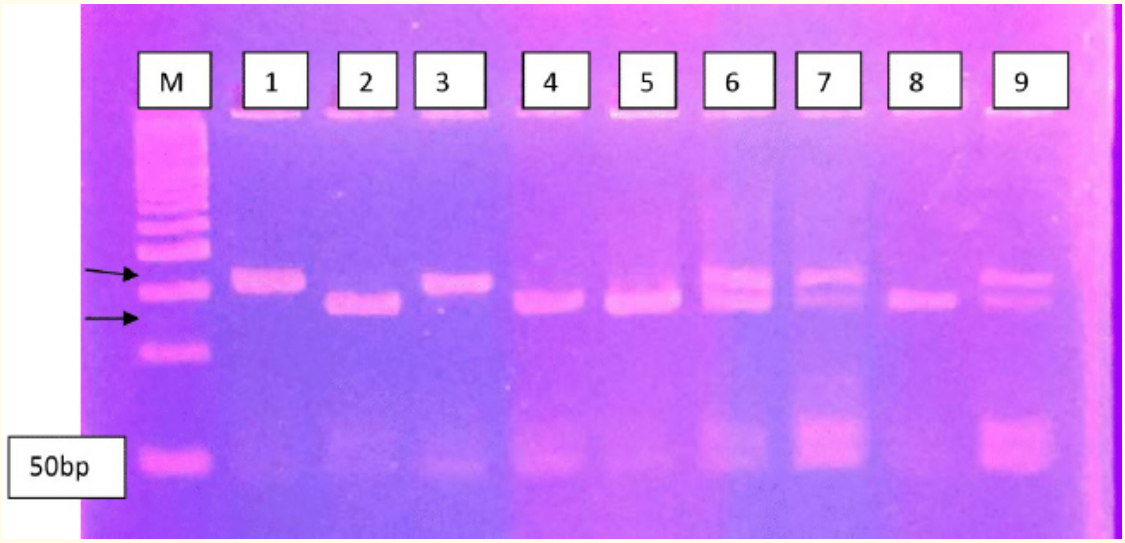

Figure 2: *Agarose gel with electrophrosis showing fragment of the 165 bp & 141 bp after enzymatic digestion with the T^th^ 111I restriction endonuclease enzyme.*

Lane 2, 4, 5 and 8 showed homozygous (TT) genotypes which were seen as a single band and position at 141 bp. Lane 6,7 and 9, heterozygous (MT) genotypes were seen as two bands at 165 bp and 141 bp. Lane 1 and 3 showed wild type (MM) genotypes which were seen at 165 bp.

The Quantitative Determination of Plasma Angiotensinogen Concentration (Cloud-Clone, USA)

Principle

Enzyme Linked Immunosorbent Assay (ELISA) is a very sensitive immunochemical technique which is used to access the presence of specific protein (antigen or antibody) in a given sample and it's quantification. An enzyme conjugated with an antibody reacts with a colorless substrate to generate a colored reaction product.

The plate was pre-coated with an antibody specific for human Angiotensinogen. The capture antibody could bind to the human Angiotensinogen in the standard and samples. After washing the plate of any unbound substances, an antibody-HRP conjugate against human Angiotensinogen was added to the wells. After the last wash to remove any unbound enzyme, a substrate solution was added to the wells and color develops in direct proportion to the amount of human Angiotensinogen bound in the standard solutions or samples. A standard curve could be established and sample values could be read off the standard curve.

Procedure

1. All reagents, samples and standards were prepared;
2. 100 µl of standard or sample were added to each well and incubated 2 hours at 37°C;
3. 100 µl prepared Detection Reagent A was aspirated and added and incubated 1 hour at 37°C;
4. There were aspirated and washed 3 times with the washing solution about 300 µl per well;
5. 100 µl prepared Detection Reagent A was aspirated and added and incubated 1 hour at 37°C;
6. There were aspirated and washed 5 times.
7. 90 µl of Substrate Solution was added and incubated 15 - 25 hour at 37°C;
8. 50 µl of Substrate Solution was added and read at 450 nm immediately.

Data Management

Statistical Analysis was carried out using SPSS version 15.0 software and the following tests were used for data analysis: All the data were expressed as Mean ± SD. Chi squared (χ^2) test, t-test was used for comparison between means.

Results

In a total of 72 hypertensive subjects, (34.72%) had homozygous (TT) genotype, (62.50%) had heterozygous (MT) genotype and (2.78%) had wild type (MM) genotype for *AGT* M235T. In a total of 72 normotensive subjects, (9.72%) had homozygous (TT) genotype, (66.67%) had heterozygous (MT) genotype and (23.61%) had wild type (MM) genotype for *AGT* M235T. Moreover, the heterozygous (MT) was common in both groups which was (62.59%) in hypertensives and (66.67%) in normotensives.

Characteristics of Subjects (n)	Hypertensives (72) n (%)	Normotensives (72) n (%)
Female	44 (61.11)	44 (61.11)
Mean age (years)	49.77 ± 6.14	48.14 ± 6.57
BMI	30.12 ± 8.82	24.00 ± 3.44
Male	28 (38.89)	28 (38.89)
Mean age (years)	49.60 ± 6.5	49.88 ± 6.77
BMI	26.02 ± 5.66	23.39 ± 3.13

***Table 1**: General characteristics and clinical data of hypertensives and normotensives.*

	Hypertensives (72)	Normotensives (72)
No. of cases	72	72
MM genotype, n (%)	2 (2.78)	17 (23.61)
MT genotype, n (%)	45 (62.50)	48 (66.67)
TT genotype, n (%)	25 (34.72)	7 (9.72)

***Table 2**: The distribution of genotype frequencies of AGT M235T in hypertensives and normotensives.*

Table 3 showed the comparison of plasma angiotensinogen level in hypertensives and normotensives with different *AGT* M235T genotypes. In the TT and MT genotypes that were mutant homozygous and heterozygous genotypes, the plasma angiotensinogen level was significantly increased in hypertensives than those of normotensives (p = 0.005 and < 0.001 respectively). In MM genotype, wild type, there was no significantly difference of plasma angiotensinogen level between hypertensives and normotensives (p = 0.3).

Genotypes	Plasma angiotensinogen level (ng/ml) (Mean ± SD)		P-value
	Hypertensives (n = 72)	Normotensives (n = 72)	
TT	84.70 ± 24.42	55.14 ± 17.73	0.005
MT	55.77 ± 23.22	22.89 ± 9.23	< 0.001
MM	26.35 ± 6.65	18.02 ± 13.26	0.3

Table 3: *Comparison of plasma angiotensinogen level with different genotypes of AGT M235T in hypertensives and normotensives.*

According to the table 4, the plasma angiotensinogen level was divided into two groups, below medium (36.7 ng/ml) and above medium (36.7 ng/ml). In the homozygous wild MM genotype of AGT M235T polymorphism and there was 17 below medium score and there was 2 above the medium score level of the plasma angiotensinogen. In the heterozygous MT genotype of *AGT* M235T polymorphism and there was 53 below medium score and there was 40 above the medium score level of the plasma angiotensinogen. In the homozygous mutant TT genotype of *AGT* M235T polymorphism and there was 2 below medium score and there was 30 above the medium score level of the plasma angiotensinogen. There was significant difference between the genotypes of *AGT* M235T polymorphism and plasma angiotensinogen level in the study group $\chi^2 = 38.16$ and $p < 0.001$.

Genotypes	Plasma angiotensinogen level (ng/ml)		χ^2	P-value
	Below medium < 36.7 ng/ml	Above medium ≥ 36.7 ng/ml		
MM	17	2	38.16	< 0.001
MT	53	40		
TT	2	30		

Table 4: *Association between genotypes of AGT M235T and plasma angiotensinogen level in all groups.*

Discussion

Renin-Angiotensin System is the most important mechanism that regulates blood pressure in our body. The genes coding for RAS system have been extensively studied [14] because it is also major target for antihypertensive therapy. This study is correlated between the M235T polymorphism of the gene and plasma angiotensinogen level using age, sex matched individuals with two groups.

In the present study, we showed difference genotypes responded with different plasma angiotensinogen levels. In the TT and MT genotypes that were mutant homozygous and heterozygous genotypes, the plasma angiotensinogen level was significantly increased in hypertensives than those of normotensives (p = 0.005 and < 0.001 respectively). That indicated *AGT* M235T polymorphism was significantly associated with increased plasma angiotensinogen level hypertensives than normotensives.

The *AGT* M235T polymorphism is located on the nucleotide position 704 and exon 2 of the angiotensinogen gene. Actually it is far away from the cleavage site of renin enzyme. This gene variant itself directly acts upon the physical effect or acts as a marker for causative mutation is yet uncertain [15].

Over twenty five years, Jeunemaitre., *et al*. provided genetic evidence of *AGT* M235T was established initially in both Utah and French Caucasians in 1992. This association was confirmed in unselected French hypertensives in the clinical trial [16]. And then, Jeunemaitre and coworkers followed *AGT* gene locus and essential hypertension. They observed that the polymorphism occurring six residues upstream from the initiation site of transcription was in very strong linkage disequilibrium with M235T polymorphism of angiotensinogen gene. The frequencies of the A-6 and the T235 alleles are almost identical with > 97% concordance between two polymorphisms [17].

It was stated that G (-6) A variant controls the expression of *AGT* gene. They performed *in vitro* tests of promoter activity and DNA binding with nuclear proteins and reported that the nucleotide substitution affects the basal transcription rate of *AGT* gene in various cell lines. This could explain the association between *AGT* "T" allele and increased plasma angiotensinogen level [18].

To find out association between different genotypes of *AGT* M235T and plasma angiotensinogen concentration, the plasma angiotensinogen level was divided into two groups, below medium (36.7 ng/ml) and above medium (36.7 ng/ml). In the homozygous mutant TT genotype of *AGT* M235T polymorphism, the majority are above the medium score level of the plasma angiotensinogen, however, the homozygous wild type (MM) group, the majority are below the medium score level. There was significant difference between the genotypes of *AGT* M235T polymorphism and plasma angiotensinogen level in the study group $\chi^2 = 38.16$ and $p < 0.001$.

Conclusion

This study supported the significant association of the *AGT* M235T variant with essential hypertension in Myanmar. In the present study, subjects carrying the homozygous TT genotype possessed three folds increased in plasma angiotensinogen level than subjects carrying homozygous MM genotypes in essential hypertension. That may lead to indicate that *AGT* variants might play a critical role in the pathogenesis of essential hypertension in local area.

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