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Increased plasma Angiotensinogen levels and its association with the M235T gene polymorphism and hypertension in Calabar and Uyo cities, Nigeria

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Abstract
Hypertension results from an interaction of many risk genes and environmental factors. The M235T allele of the angiotensinogen gene is thought to increase plasma levels of the angiotensinogen which is associated with hypertension. The angiotensinogen is an important substrate for renin in the RAAS that is finally converted into angiotensin II that plays a key role in the control of blood pressure. This study was designed to measure plasma angiotensinogen levels in an adult population in Calabar and Uyo (South-South), Nigeria in relation to the M235T allele and hypertension. Out of a large population of 1224 participants who had been genotyped for the M235T polymorphism, plasma was collected from a sample of 300 consisting of 150 patients and 150 controls. Protein A sandwich enzyme linked immunosorbent assay was carried out with the plasma samples to measure the angiotensinogen levels. Age, BMI, M235T allele, blood pressure and O.D values were compared between controls and patients using the independent t test. The absorbance values of plasma angiotensinogen were significantly higher in the patients (0.71) with M235T allele than in the controls (0.53). Further research still needs to be carried out to determine the actual concentration of the protein in the participants.

Keywords: angiotensinogen, M235T allele, hypertension, plasma.

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1. INTRODUCTION

The M235T polymorphism of the angiotensinogen gene has been associated with a 10% - 30% increase in plasma AGT. Chronic increases in plasma AGT concentration is believed to slightly increase blood pressure thus facilitating hypertension (Corvol and Jeunemaitre, 1997).

The renin-angiotensin (R-A) system is a powerful pressure system which influences salt and water homeostasis. Angiotensinogen (AGT) is a key component of this system, it is cleaved by renin to yield angiotensinogen 1 (AGT 1), which is cleaved by angiotensinogen converting enzyme (ACE) to yield angiotensinogen II (AGT II), responsible for carrying out a range of functions that include i) prompting the constriction of blood vessels causing a rise in blood pressure, ii) ensuring the release of aldosterone by the adrenal cortex which acts on the tubules causing absorption of more water and salt from urine. Blood volume increases so does blood pressure. Potassium ions are excreted from the tubules in exchange for sodium iii) mediates the release of antidiuretic hormone from the pituitary that enhances the reabsorption of water, it also increases an individual's appetite for salt and stimulates the sensation of thirst (Caulfield *et al.*, 1994). The measurement of angiotensinogen concentration has proved to be a convenient method for monitoring the activity of R-A system in human populations since it circulates at relatively constant level. In Nigerians, Rotimi *et al* (1997) reported a significant association between the presence of the M235T allele and high mean AGT concentration which was also significantly related to hypertension status, though Cooper *et al* (1999) reported a high level of plasma angiotensinogen but low hypertension status in Igbo Ora, Nigeria. There are no documented data on the levels of angiotensinogen in populations in the southern parts of Nigeria.

This research was carried out to determine angiotensinogen levels in hypertensive patients and controls in a population in South- south Nigeria.

METHODOLOGY

A total of 300 individuals,150 patients and 150 controls were selected from a adult population of 1224 who had been genotyped for the M235T angiotensinogen gene polymorphism by polymerase chain reaction(PCR) and restriction fragment polymorphism, these methods were described in detail (Kooffreh *et al*, 2012). Their plasma samples were screened using protein A sandwich ELISA for their angiotensinogen levels. The age, weight, height (was used to calculate Body mass index-BMI), Systolic and Diastolic blood pressure readings were also recorded.

Principle: The protein A sandwich ELISA procedure uses protein A to increase the sensitivity and specificity of the test by controlling the orientation of antibodies. The plate surface was coated with protein A followed by the trapping antibodies (polyclonal antibodies to angiotensinogen). Application of protein A increased the proportion of appropriately aligned antibody molecules. The plasma samples were then added, followed by the secondary antibody that was identical to the primary antibody (also polyclonal antibodies to angiotensinogen). The detecting agent was protein A conjugated to a marker enzyme (alkaline phosphatase). The protein A would only bind to the secondary antibody if the antibody was in the correct orientation. The substrate was added. Subjective analysis of ELISA results were achieved by quantifying the amount of light absorbed by the substrate. For p-nitrophenyl phosphate substrate, the substrate was exposed to light with a wavelength of 405nm and the absorbance was determined by measuring the amount of light that passed through the substrate which was quantified as the absorbance value (O.D.)

Method: Protein A was diluted 1 in 1000µl of coating buffer, 100µl of diluted protein A solution was added to each well of the ELISA plate. The plate was covered and incubated for 2 hours at 37ºC. The plate was washed vigorously with wash fluid PBS-Tween using a wash bottle. Each well was filled with PBS- Tween and left for three mins; the fluid was removed from the wells by snapping. The wash step was repeated three times and the plate was tapped dry on absorbent paper. Polyclonal angiotensinogen antibodies were diluted 1 in 1000µl of PBS-Tween, 100µl of diluted polyclonal anti AGT was added to each well. The plate was covered and incubated for 2 hours at 37ºC. The plate was removed and the wash procedure was repeated. Protein A alkaline phosphatase conjugate was diluted 1 in 15000µl of conjugate buffer, 100µl of diluted conjugate was added to the wells of the ELISA plate. The plate was covered and incubated for 2 hours at 37ºC. The wash procedure was repeated. p-Nitrophenyl phosphate substrate was diluted 0.01g in 10000µl of substrate buffer; 100µl of diluted substrate was added to each well. The plate was covered and incubated in the dark for 1 hour at room temperature. The bottom of the plate was wiped with absorbent paper and inserted into the microplate reader. The absorbance values were measured at 405nm and recorded after 1hour, 3 hours and overnight. The Statistical Package for Social Sciences - SPSS for windows® Version 16.0 was used to statistically analyze the data obtained. Genotypic frequencies in

control and hypertensive groups were compared by chi-square analysis. Continuous variables were compared between hypertensives and controls by independent t test. Means and standard deviation was used to describe results.

RESULTS

For the genotyping of the angiotensinogen polymorphism, The normal individual M235M gives an undigested 165bp, a mutant individual M235T gives two fragments of 141bp and 24bp. Recessive individual T235T gives a 141bp fragment. However agarose gel allows the visualization of a 165bp fragment for M235M, a 141bp fragment for T235T, a 165bp and 141bp for the M235T individuals respectively. Figs 1 and 2 (Procopciuc *et al*, 2002)

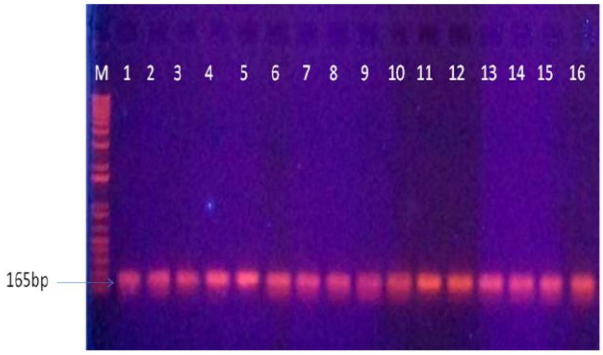

Figure: 1 Gel electrophoresis showing 165 bp PCR product after amplification of the angiotensinogen gene

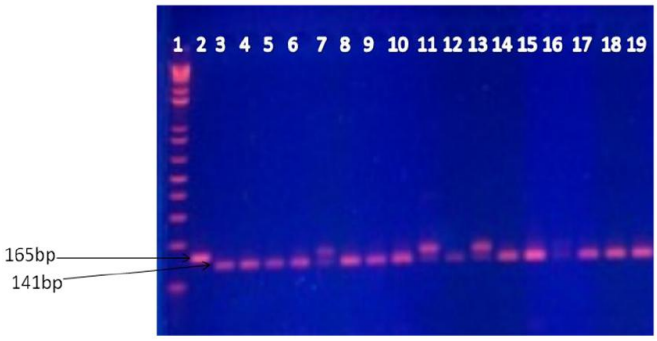

Figure: 2 Agarose gel electrophoresis showing the amplification of the 165 bp fragment after enzymatic digestion with the Tth 111l restriction endonuclease enzyme

The frequency of the genotypes M235M, M235T, T235T of the M235T allele for the Efiks were 0.4, 7.7, 92 % in patients and 0, 6, 94 % in controls; for the Ibibios were 0.5, 1.2, 87 % in patients and 0, 7, 93 % in controls. The genotype frequencies in the patient and control population did not conform to the Hardy-Weinberg equilibrium. There were no significant differences between the genotype frequencies of patients and controls. In the control group, the mean O.D value for plasma angiotensinogen was 0.53 in individuals with the M235T variant; 0.49 in individuals with the T235T variant. There was only one individual with the M235M variant (O.D value 0.28). In the patients, the mean O.D value was 0.71 in individuals with the M235T variant; 0.66 in individuals with the T235T variant. There was also only one individual with the M235M variant (O.D value 0.41) among the patient group Table 1.

		Controls	Patients
O.D values for ELISA	Ihr	0.16 ± 0.06	0.17 ± 0.05
	3Hrs	0.26 ± 0.12	0.38 ± 0.20
	Overnight	0.50 ± 0.21	0.66 ± 0.33
Genotype	TT	0.49 ± 0.20	0.66 ± 0.33
	MT	0.53 ± 0.27	0.71 ± 0.33
	MM	0.28	0.41
Age		34.56 ± 10.27	53.45 ± 14.14
BMI		23.05 ± 6.51	26.31 ± 5.78
Systolic Blood pressure		115.60 ± 9.63	163 ± 23.87
Diastolic blood pressure		71.39 ± 8.50	94.87 ± 13.59

Table 1 Plasma angiotensinogen ELISA values and demographic characteristics among patients and controls

DISCUSSION

A sample population of individuals who had been genotyped for the M235T allele of the angiotensinogen gene were investigated using a protein A sandwich ELISA to measure plasma angiotensinogen levels in relation to the M235T allele. The T allele was associated with increased plasma AGT (Bloem *et al*, 1997, Rotimi *et al*, 1997). Elevation in plasma levels of angiotensinogen has been associated with hypertension. The protein A sandwich ELISA was sensitive enough to identify the presence of the angiotensinogen in the plasma of patients and controls, the mean O.D values for plasma angiotensinogen was significantly higher in the patients than the controls with the mutant T allele implying an association with hypertension which is in line with literature (Jeunemaitre *et al*, 1992 and Corvol *et al*, 1999). Corvol and Jeunemaitre (1997) reported that the M235T allele was associated with a 10-30% increase in plasma angiotensinogen which is able to increase blood pressure, thus facilitating hypertension. The elevation of O.D. values of angiotensinogen may suggest a role of the M235T genotype in hypertension however this needs further confirmation in larger sample size of this population.

CONCLUSION

The absorbance values of plasma angiotensinogen were significantly higher in the patients with M235T allele than in the controls. further research still needs

to be carried out to determine the actual concentration of the protein in the participants.

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