INTERNATIONAL JOURNAL OF NATURAL AND APPLIED SCIENCES (IJNAS), VOL. 4, NOS.1& 2 (2009); P. 164 – 172, 2 TABLES, 2 FIGS, 1 PLATE.

Angiotensin II type 1 receptor A1166C gene polymorphism and essential hypertension in the Efiks of Calabar.

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ABSTRACT

Hypertension is a multifactoral disorder because of the interaction of risk genes and environmental factors. The angiotensin II is a well known vasoconstrictor that exerts most of its influence through the angiotensin II type 1 receptor. The A1166C polymorphism is a single base substitution of adenine for cytosine at position 1166 in the 3¹ untranslated region of the gene. There are conflicting reports on the association of the A1166C polymorphism with cardiovascular diseases such as prevalent hypertension, left ventricular hypertrophy, and pregnancy induced hypertension. These variations were attributed to ethnic differences in different populations. We investigated the association of the A1166C polymorphism with hypertension in 100 individuals from the Efik tribe who were matched for gender and sample size. PCR-RFLP analysis was carried out to determine the allele frequency of the gene. The genotype frequencies were 48, 2 and 47, 3 for the AA, AC genotypes respectively in the patient and control groups. No individual with the CC genotype was observed in the study population. The frequency of the C allele was 0.03 and 0.02 in the control and the patient population. The genotype and allele frequency did not conform to the Hardy-Weinberg theory. Using regression analysis, age and diastolic were positive predictors for SBP, r=0.50; systolic was the only predictor for DBP in the patient group. Diastolic was the only predictor for SBP, r= 0.656 while age and systolic were positive predictors for DBP r= 0.718 in the control group. Gender, BMI, A1166C polymorphism and other independent variables were not predictors for SBP and DBP in the population. P= 0.05, odds ratio 0.65, 95% CI (0.13 to 3.44). The A1166C polymorphism is not an independent risk factor for essential hypertension in the study population.

INTRODUCTION

Hypertension is a multifactorial disorder because of the interaction of many risk genes such as molecular variants of the angiotensinogen gene, angiotensin converting enzyme gene, angiotensin II receptor I gene and the corin gene (Cooper *et al*, 2000; Hilgers *et a.*,, 1999; Dries *et al.*, 2005; Sethi *et al.*, 2003), and environmental factors such as obesity, body mass index (BMI), dietary salt intake, alcohol consumption, stress and high-density lipid (HDL) – cholesterol levels. Genes determine approximately 20 to 60% of the variability in blood pressure in different populations (Sethi *et al*, 2003, Cooper *et al*, 2000).

The angiotensin II is a well known vasoconstrictor that exerts most of its influence through the angiotensin II type 1 receptor (AT_1). Angiotensin II type 1 receptor (AT_1) is a membrane bound G protein coupled- receptor that mediates the effects of angiotensin II (De Gaspora *et al*., 2000). The highly polymorphic human AT_1R gene is 55kb long having five exons and four introns, $A^{1166}C$ polymorphism is a single base substitution of adenine for cytosine at position 1166 in the 3^1 untranslated region of the gene. The A allele is the larger fragment that lacks the restriction enzyme while the smaller fragment from the C allele has the restriction enzyme site.

The physiological significance of the polymorphism is uncertain because data on the function of the AT1R polymorphism is limited. Thus the mechanism responsible for the association of hypertension status with A1166C polymorphism has remained largely unknown and the amino acid sequence of the receptor is not altered. It is however thought to affect mRNA stability and transcription and is in linkage disequilibrium with some other polymorphism. It is also associated with some diseases (Bonnardeaux *et al.*, 1994; van Geel *et al.*, 2000; Stankovic *et al*., 2003; Lapierre *et al.*, 2006).

The A1166C polymorphism is associated with prevalent hypertension, increased aortic stiffness (Danser and Schunket, 2000; Benetos *et al*., 1996; Wang *et al*., 1997). This polymorphism has also been associated with other diseases such as left ventricular hypertrophy (Takami *et al.*,1998) pregnancy induced hypertension; early coronary disease and excessive vasoconstriction (Alvarez *et al*., 1998; van Geel *et al.*, 2000). Stankovic *et al*., 2003 reported a significant association between this polymorphism and hypertension in males but not females. The frequency of the C1166 allele was high among hypertensives (Rubattu *et al*., 2004; Dzida *et al*., 2001). Some other studies have also reported a negative association between the A1166C polymorphism and hypertension (Tiret *et al*, 1998; Takami *et al*., 1998; Kikuya *et al*., 2003).

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These variations were attributed to ethnic differences in the various populations (Agachan *et al*, 2003, Kikuya *et al*, 2003).

There is no published data on this allele among the Efiks and since various published reports suggest the existence of ethnic differences, the aim of this study was therefore, to investigate the distribution and association of the A1166C polymorphism among a sample population of the Efiks living within Calabar town.

MATERIALS AND METHODS

The research was a population based case control study of 100 individuals, 50 patients and 50 controls with 25 males and females in each group. Patients were selected from the hypertension clinics in the hospital while the controls were from the general population. Venous blood (3ml) was collected from each participant after they had given informed consent, into bottles containing anticoagulant EDTA. DNA was extracted from blood for A1166C gene genotyping. Subjects included in the study gave informed consent and ethical approval for the study was obtained from the University Teaching Hospital, Calabar, and the General Hospital, Calabar.

Questionnaires were developed in English to assist in acquiring information on family history for hypertension and socio-demographic data, age, sex, dietary habits, physical activity, smoking habits; alcohol consumption. Weight was measured in kilograms and height in metres; Readings were taken using a sphygmomanometer in millimetres of mercury by certified medical personnel for the patients in the clinics and a certified nurse for the controls in the general population. Systolic and diastolic BP values were recorded. Before taking the measurement, the respondent was advised to sit quietly for 5 mins, with the legs uncrossed and the right hand free from clothing. The right hand was placed on the table with the palm facing upwards. The appropriate cuff size was selected and the cuff wrapped and fastened securely. The cuff was kept at the same level as the heart during measurement. The upper reading, the systolic blood pressure (SBP) and lower reading the diastolic blood pressure (DBP) recorded, the first and second readings were taken twice and the average of the two used for the analysis.

DNA was extracted according to Dellaporta, 1983 with little modification. Genomic DNA (2µl) was amplified in a 25µl PCR reaction mix containing Promega flexi green buffer 5µl, dNTPs 0.5µl, upstream and downstream oligonucleotide primers 0.5µl each, magnesium chloride 1.5µl, 12.88µl of nuclease-free water and Taq DNA polymerase 0.06µl.

AT1R gene: Cycling conditions include an initial denaturation of 94^{o}C for 2 mins, followed by 40 cycles of a further denaturation at 94^{o}C for 1 min, annealing at 60^{o}C for 1 min, extension 72^{o}C for 2mins, and a final extension of 72^{o}C for 10 mins.

A1166C Polymorphism of the Angiotensin 11 Type 1 Receptor primer sequence

5' - AAT GCT TGT AGC CAA AGT CAC CT- 3'

5' - GGC TTT GCT TTG TCT TGT TG -3'

A cocktail of 0.25µl of the Dde1 enzyme, 1µl of the 10 x buffer D; 0.1µl of acetyl BSA and 8.5µl of sterile water was added to 10µl of the PCR product. The enzyme digestion was performed in a final volume of 19.85µl at 37^{o}C for 4 hours. For the Rsa1 enzyme, the same concentrations was use in preparing the cocktail but Rsa1 enzyme and 10x buffer E were substituted. The digested products were separated on 2% agarose gel stained with 10µl of ethidium bromide for 30 mins at 125V.

For the Dde1 RFLP, the enzyme cuts the PCR product into two pieces, 600bp and 250bp in the A variant. An additional Dde1 recognition site is created in the C variant at nucleotide 1166 located within the 250bp fragment. The homozygous CC individual produces three bands (600bp, 140bp and 110bp long). The homozygous AA individual produces two (600bp and 250bp long). The heterozygous individual produces four bands (600bp, 250bp, 140bp and 250bp long (Plate 1), Lapierre *et al*, 2006).

The Statistical Package for Social Sciences (SPSS) for Windows®, Version 16.0, was used to statistically analyze the data obtained. Descriptive statistics was used to analyze all variables studied which include marital status, educational attainment, sex, age, BMI and the genotype frequencies of the A1166C polymorphisms in the study population. Genotype frequencies in control and hypertensive groups were compared by chi-square analysis. Continuous variables were compared between hypertensives and controls by independent t-test.

Multiple regression analysis was also carried out using SBP or DBP as dependent variable, while sex, age, BMI and other variables were used as independent variables. $P < 0.05$ was considered statistically significant.

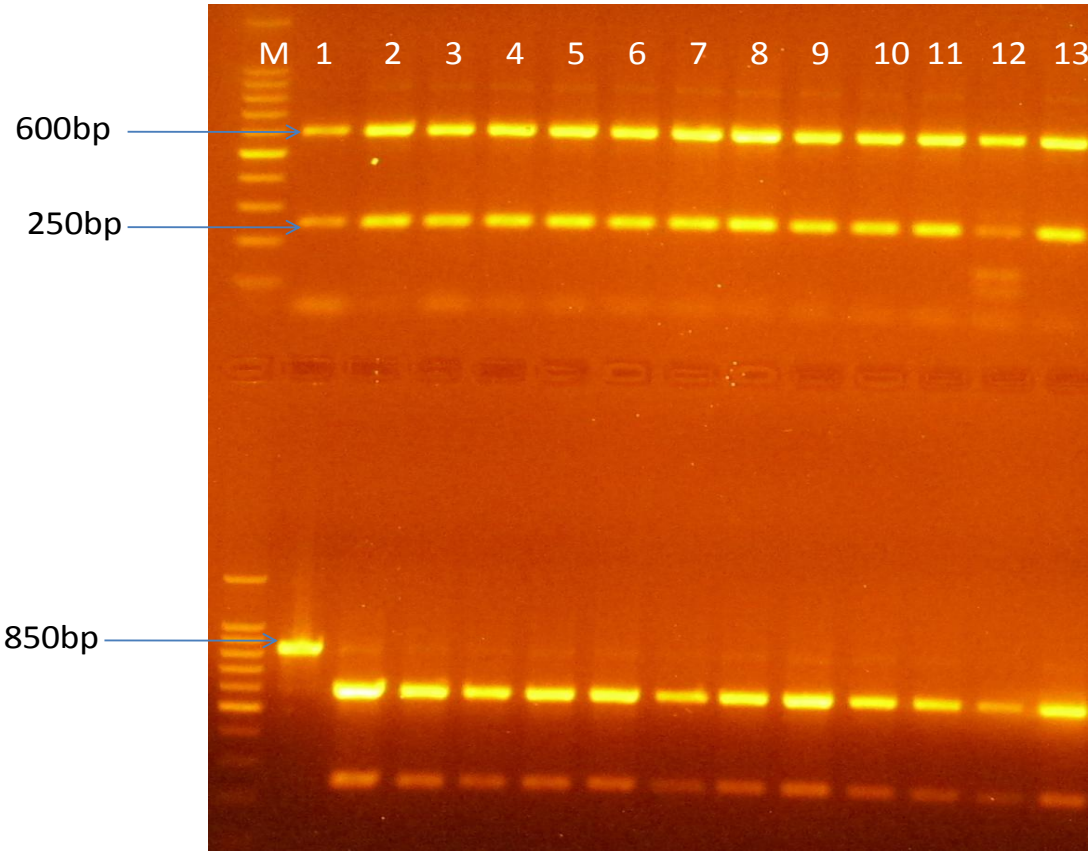

Plate 1. Agarose gel electrophoresis showing digestion of the 850bp PCR product by the Dde1 restriction endonuclease

Legend:
Lane 0 contains the 100bp DNA ladder for both rows
Lane 1 of row 1 contains homozygous AA individual (600,250bp)
Lane 12 of row 1 contains heterozygous AC individual
(600,250,140 and110bp)
Lane 1 of row 2 contains an undigested amplified PCR product

RESULTS

This study was aim at genotyping a sample population of 100 individuals to determine the frequencies of the A1166C of the AT1R gene and associate these alleles with hypertension status. The study group was from the Efik tribe and was matched for gender and sample size. Polymerase chain reaction and enzymatic digestion was performed on the 50 control and 50 patient samples collected from Calabar to determine the frequency of the A1166C variant and its relationship with hypertension status. Table 1 illustrates the genotype and allele frequencies observed among controls and patients. For the angiotensin 11 type 1 receptor gene polymorphism, the genotype frequencies were 48, 2 and 47, 3 for the AA and AC genotypes in the patient and control groups, the CC

genotype was also not observed in this study population. The C allele frequency was 0.03 and 0.02 in the control and patient population. The frequency of the allele in the study population did not conform to the Hardy Weinberg equilibrium theory.

Table 2 gives the mean characteristics of the study population. The patients' ages were 23 to 80years with a mean of 56.1 years while the ages of the controls were 18 to 73years with a mean of 34.1 years.

In the patient group, more individuals were overweight with 19(38%) individuals overweight and 14(28%) individuals were obese. More 30(60%) individuals among the controls were normal weight, 10(20%) individuals were overweight and 10(20%) individuals were obese.

There were no significant differences between the genotype frequencies of hypertensive and the control groups by $\chi 2$ analysis for the polymorphisms under consideration in this study. When continuous variables were compared between hypertensive and control groups using the independent t test, significant differences existed between the age, BMI, systolic and diastolic blood pressure of controls and patients.

By multiple regression analysis, age and diastolic was the positive predictors for SBP r=0.502 and systolic was the only positive predictor for DBP r=0.502 in patients. Diastolic was the only predictor for SBP r= 0.656 while age and systolic were predictors for DBP r= 0.718 in the controls. Gender, body mass index, A1166C polymorphism and other independent variables were not predictors for SBP and DBP in the hypertensive group (p=0.05)

In the control group, 28(56%) persons were married and 22 (44%) persons were singles. For the patients, 45(90%) persons were married and 4 (8%) were singles. There was 1 (2%) widow among patients. Fig 1 shows the marital status in the study population. Among the patient group, 18(36%) individuals attended only primary school, 16(32%) individuals attended secondary, 12(24%) individuals attended tertiary institution and 4(8%) individuals had no form of formal education. Among the control group, individuals who attended primary schools were 12(24%); secondary schools were 16(32%); tertiary institutions were 20(40%) and individuals that had no formal education were 2(4%), Fig 2.

Table 1. Genotype and allele frequencies of the A1166C polymorphism in the study population

Groups	N	Genotype Frequencies			Allele Frequencies			AA		AC		CC	
		AA	AC	CC	A	C		M	F	M	F	M	F
Patients observed frequency	50	48	2	0	92	2	Patients	25	23	0	2	0	0
		0.96	0.04	0	0.98	0.02							
Hardy Weinberg prediction expected		0.92	0.08	0.0004									
Controls observed frequency	50	47	3	0	97	3	Controls	24	23	1	2	0	0
		0.94	0.06	0	0.97	0.03							
Hardy Weinberg prediction expected		0.94	0.06	0.0009									
							Total	49	46	1	4	0	0

N= Total number of individuals

AA= Dominant individuals

AC= Heterozygous individuals

CC= Recessive individuals

A= Dominant allele

C= Recessive allele

M= Male

F= Female

Table 2. Characteristics of the control and patient groups

Variables	Controls Mean±SD	Patients Mean±SD
Age (years)	34.18 ± 12.80	56.16 ± 13.82
BMI (kg/m2)	25.47 ± 5.63	27.82 ± 5.80
SBP (mm Hg)	118.0 ± 8.99	158.0 ± 20.06
DBP (mm Hg)	73.56 ± 8.91	90.60 ± 10.18

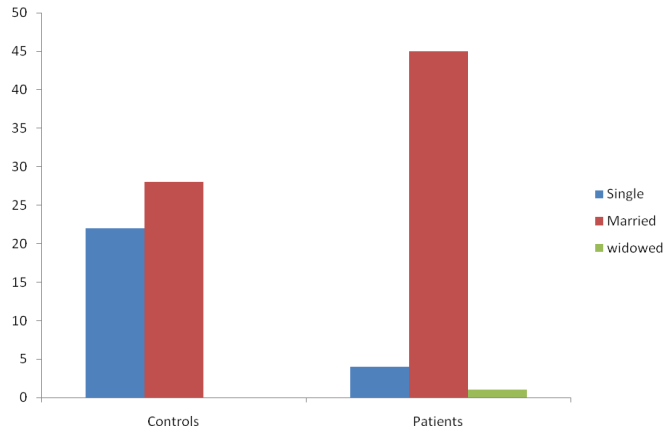

Fig. 1. Marital status among study population

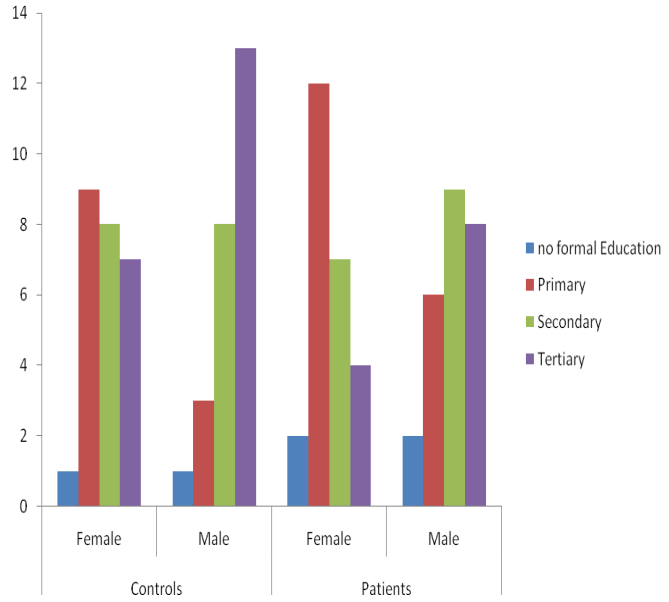

Fig. 2. Level of education among study population

DISCUSSION

The aim of this study was to investigate allele and genotype frequencies of the A1166C polymorphism among 50 individuals with hypertension and 50 healthy individuals of the Efik tribe in Calabar (Nigeria) and the association of the A1166C allele with hypertension. 95% of the population had the AA wild type allele, 5% of individuals had the A1166C heterozygous allele. This is similar to what is reported in some studies. Lee and Kim (2003) observed 96% and 6% for the A and C allele respectively of the angiotensin 11 type 1 receptor polymorphism in Korea. Zhenni *et al.*, 2001 observed only two genotypes AA and AC of the AT1IR polymorphism but reported a higher frequency of the A allele among patients than controls. Liu *et al.*, 2002 reported a high frequency of the A1166C allele among the Han, Yi and Tibetian Chinese populations and also concluded that the A allele may be a predisposing factor for essential hypertension in Tibetian males.

The CC genotype of the AT1R gene was not observed in this study population. La pierre *et al*, 2006 in a study carried out in San Luis (Argentina) observed a frequency of 74.2% for the AA, 24.2% for AC and 1.6 for CC genotypes respectively but also observed a higher frequency of the 1166C allele (0.19) among patients with hypertension than control group (0.06).These results suggest an association between the A1166C allele and hypertension in agreement with earlier studies carried out in Caucasian population (Bonnardeaux *et al.*, 1994; Alvarez *et al.*, 1998) A high prevalence of the CC genotype was observed in Chinese hypertensives than controls (Jiang *et al.*, 2001). The average C allele frequency reported in the Chinese population is 0.11. Liu et al (2002) made a comparison of the A1166 allele frequency in different ethnic groups, and reported, the frequency of CC genotype in Asians to be lower (1.4%) than in the Caucasians (1.7 to 13%). The frequency of the C allele in this study was very low(0.02 and 0.03 in patients and controls) compared to the frequency in Caucasian and Asian population (Morisawa *et al.*, 2001; Agachan *et al.*, 2003; Liu *et al.*, 2002). The A1166C polymorphism was not associated with hypertension and is not a risk factor for the disease in the study population using multiple regression, odds ratio is 0.65, 95%CI(0.13 to3.44). In a sample of Swedish twins, Iliadou *et al*.(2002) did not observe any association between the ACE I/D polymorphism or AT1R A1166C polymorphism and blood pressure. Schmidt *et al*. (1997) also did not detect any association between the A1166C allele and hypertension but a decreased prevalence of C allele was observed among hypertensives. Tiret *et al*.(1998) reported a higher prevalence of the C allele among female hypertensives than controls but no such observation among men. Stankovic *et al*. (2003) reported the C allele to be significantly associated with hypertension in males with an odds ratio of 2.56 among the Serbian population.

Generally large interethnic differences in the frequencies of genotype polymorphisms of the renin angiotensin system exist in different populations.

When subjects were grouped according to their educational levels, more patients 18(36%) were at the primary level of education but the number of controls 20(40%) was higher at the tertiary level of education. This result suggests that low levels of education are inversely associated with the development of hypertension but the sample population is small for this result to be generalized. Education plays an important role in guarding against disease influenced by one's lifestyle (Liberatos *et al.*, 1988; Vargas et al. 2000). Tedesco *et al. (*2001) reported low knowledge of hypertension and its risk factor among the uneducated, making symptomless patients unwilling to alter their lifestyle, take medication and visit health facilities when necessary to forestall some poorly perceived danger while the educated subjects were more likely to consider the health care need as a priority. This fact highlights the need for increased awareness campaigns to enlighten the less educated and make them aware of the disease and the health facilities available to them. Adedoyin *et al*. (2005) reported low socio-economic status to have an inversely significant effect on systolic, heart rate and pulse rate thus implicating socio economic status in the development of hypertension among sedentary Nigerian adults.

CONCLUSION

The A1166C polymorphism is not associated with hypertension in the study population. The results suggest that low educational levels are inversely associated with the development of hypertension.

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