Research Article

The insertion/deletion polymorphism of angiotensin-converting enzyme gene predisposes Cameroon female type 2 diabetes mellitus patients to essential hypertension

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ABSTRACT

The association between type 2 diabetes mellitus (T2DM) and essential hypertension (EH) is not well understood. Both conditions result from an interaction of multiple genetic (ethnic) and environmental (geographical) factors. One possible genetic determinant is the angiotensin-converting enzyme (ACE) insertion/deletion (I/D) gene polymorphism. Observations on the association between this polymorphism and EH in T2DM patients have been inconsistent in different populations. Given the high prevalence of EH in diabetic patients in the South West Region (SWR) of Cameroon, the aim of this work was to study the relationship between I/D polymorphism of the ACE gene and hypertension in T2DM patients. ACE I/D polymorphism was determined by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and D/D typing was further reconfirmed using insertion-allele-specific amplification. Analysis of ACE genotype and allele frequencies revealed statistically insignificant differences between the normotensive and hypertensive T2DM subjects. On the contrary, the frequency of the I allele was significantly lower in the normotensive than the hypertensive diabetic females. These findings suggest that the I allele of the ACE gene predisposes female T2DM patients to EH.

Key words: Diabetes, Hypertension, Angiotensin converting enzyme, Insertion deletion, Genetic polymorphism

RÉSUMÉ

L'association entre le diabète de type 2 (T2DM) et l'hypertension essentielle (EH) n'est pas bien comprise. Les deux conditions resultent d'une association de multiples facteurs génétiques (ethnique) et environementaux (géographique). Un facteur possible est le polymorphisme insertion/délétion (I/D) du gène convertisant l'enzyme angiotensine I en engiotensine II (ACE). Les observations sur l'association entre ce polymorphisme et l' EH chez les patients de T2DM ont été inconsistantes dans les defférentes populations. Etant donné que la prévalence de l'EH est très élevé (60%) chez les patients T2DM dans la Region du Sud Ouest du Cameroun, le but de ce travail est d'étudier le rapport entre le polymorphisme I/D du gène ACE avec l' EH chez les patients T2DM dans cette population. Ce polymorphisme a été déterminé par la méthode de 'Polymerase chain reaction-Restriction fragment length polymorphism' (PCR-RFLP). Le génotype DD a été confirmé en utilisant les amorces specifiques à l'allèle de délétion. L'analyse des fréquences des génotypes et alléles montre des differences statistiquement insignifiantes entre les T2DM sujets normotendus et hypertndus. Par contre, la fréquance de l'alléle I était statistiquement differente entre les femelles normotendues et hypertendues. Ces resultats suggèrent que l'allèle I du gène ACE prédispose les femelles T2DM à l'EH.

Mots clés: Diabète, Hypertension, Angiotensine, polymorphisme insertion/délétion.

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INTRODUCTION

Among the non-communicable diseases, diabetes mellitus (DM) and essential hypertension (EH) remain global challenges with increasing prevalence despite efforts made thus far in their prevention and control. On an increasing scale, the total number of deaths attributable to DM worldwide in 2012 was estimated at 1.5 million in the age group 20-79 years (WHO 2014). More than 80% of diabetes deaths occur in low- and middle-income countries (WHO 2014). Similarly, 9.4 million deaths each year (16.5% of all deaths) are attributed to high blood pressure (Lim et al. 2012). Of these deaths in both DM and EH, 80% come from the low-middle income countries (Mathers et al. 2006; WHO, 2011a) among which group Cameroon is classified (World Bank, 2012). EH has been labelled a co-morbidity of T2DM, accounting for increased mortality in DM patients. Although independent in its own natural history, each of these pathophysiological disease entities serves to exacerbate the other (Sowers et al. 2001). About 50-85% of people with DM die of cardiovascular diseases, primarily stroke and heart disease (Morrish et al. 2001; Arrauz-Pacheco et al. 2002).

DM and EH are both caused by the interplay of multiple genetic and environmental factors, with many of these factors common to both such as family history (genetic factor), increased Body Mass Index (BMI), sex, aging and lack of exercise (Staessen et al. 2003; Lyssenko et al. 2008). Although EH and T2DM co-exist and are caused by common factors, the pathophysiology of their association is poorly understood.

The involvement of the angiotensin-converting enzyme (ACE) gene polymorphism as a possible genetic determinant of the association of EH and T2DM remains controversial. This polymorphism results to three different genotypes II, ID and DD, where I and D stand for insertion and deletion alleles respectively (Rigat et al. 1990; Baroudi et al. 2009). Some studies on the association of ACE I/D gene polymorphism with T2DM have shown that the DD genotype is associated with increased risk of developing T2DM (Feng et al. 2002; Badr et al. 2012). Other studies have proposed that the DD genotype increases the incidence of EH (Higaki et al. 2000; Di Pasquale et al. 2005), while others have not found a significant association (Schmidt et al. 1993; Mondry et al. 2005). These results suggest that ethnic and geographical variations may be responsible for the discrepancy in findings by different researchers.

Considering the high prevalence (60%) of EH in T2DM patients in the SWR of Cameroon (Unpublished data from the diabatic & hypertension clinic, Buea) the aim of this study was to investigate the relationship that exists between the ACE gene polymorphism and risk of developing EH in T2DM patients in this region of Cameroon. Establishment of the relationship between I/D polymorphism of the ACE gene and hypertension in diabetics in this population could reveal new insights on the co-existence of EH and T2DM in this Cameroonian population.

MATERIALS AND METHODS:

Study population

One hundred and eleven adults (51 normotensive T2DM subjects and 60 hypertesnsive T2DM patients) visiting the Buea Diabetic and Hypertension Clinic were randomly recruited for this study from March to April 2015. The diagnosis of diabetes was based upon the WHO criteria. Normotensive T2DM subjects were patients who had a fasting blood glucose value of at least 110 mg/dl (WHO, 2006) and/or are on anti-diabetic drugs. These patients had a systolic blood pressure (SBP) of less than 140mmHg and a diastolic blood pressure (DBP) of less than 90 mmHg (WHO, 2013). Hypertensive T2DM subjects were patients who had an SBP of at least 140mmHg, and a DBP of at least 90 mmHg or are on hypertensive medication. These hypertensive T2DM patients

were subjects who had a fasting blood glucose value of at least 110 mg/dl and/or are on anti-diabetic drugs. They must have developed EH after the onset of diabetes. Hypertensive T2DM patients who were diagnosed to have had diabetis after the onset of EH were excluded from the study. The hypertensive T2DM patients were thse whose parents and/or siblings had a family history of EH and were not obese (body mass index [BMI] <25 kg/m^2). All subjects were aged above 25 years and were out patients visiting the Buea diabetic and hypertension clinic of the Buea Regional Hospital. Ethical clearance and administrative authorisation for this investigation was respectively obtained from the Faculty of Health Sciences Institutional Review Board, University of Buea and the Regional Delegation of Health for SWR of Cameroon. Only patients who gave their informed consent participated in the study.

Collection of anthropometric, biochemical and biological data

A certified nurse used structured questionnaire for data collection of anthropometric measurements (height, weight & waist circumference WHO 2011), family history, sex, age, and duration of T2DM or EH. BMI was calculated according to Quetelet equation (BMI = weight in kilograms/height in metres squared) (Lacksmi et al, 2015). Fasting blood sugar (FBS) was determined using OneTouch UltraMini strip ™ and analyzer (Carlifornia, USA).

Two morning systolic (SBP) and diastolic (DBP) blood pressure measurements were taken using a sphygmomanometer (Omron health care, Illinois, USA) as described by Sharman et al, (2015). A third measurement was taken only when the difference between the two measurements was greater than 5 mm Hg, and the readings were averaged for analysis. A 5-min relaxation period between measurements was maintained for all subjects.

ACE gene I/D polymorphism

Two millilitres of whole blood were collected by venepuncture from patients in EDTA-microtainer tubes and preserved at 4°C for less than 12 hours until DNA extraction. Genomic DNA was extracted manually from blood leucocytes by the Phenol/Chloroform method as described by Saker et al. (2005). Genomic DNA obtained was resuspended in 0.5 ml of 0.1 M TE buffer and stored at at -20 °C untill used for polymerase chain reaction (PCR).

PCR amplification of the ACE gene was done using a pair of primers flanking the ACE gene as described by Nakhjavani et al. (2007). The reaction was performed in 100 µL PCR tubes with 5 µL of 100 µM of each primer (sense: 5'-CTG GAG ACC ACT CCC ATC CTT TCT-3' and antisense primer: 5'-GAT GTG GCC ATC ACA TTC GTC AGAT-3') in a final volume of 24 µL, containing 1.5 µL genomic DNA (0.2 µg) suspension and 12.5 µL of PCR Master Mix (Sigma). PCR master mix contained other components of the PCR reaction (Tag polymerase, $MgCl_2$, deoxy-Nucleotide Phosphates). PCR was done with an initial denaturation at 94°C for 1 min Then the DNA was amplified for 30 cycles with denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 min followed by a final extension at 72°C for 5 min PCR products were analysed on 3 % agarose gel. Mistyping of the ID genotype for DD genotype due to preferential amplification of the D (190 bp) allele over the I allele (490 bp) was verified using a second set of insertion allele-specific primers (sense primer: 5'-TCG GAC CAC AGC GCC CGC CAC TAC-3' and antisense primer: 5'-TCG CCA GCC CTC CCA TGC CCA TAA-3'). This second PCR was done for samples that showed the DD genotype. PCR conditions were identical except for an annealing temperature of 67 °C. The reaction yielded a 335 bp amplicon only in the presence of the I allele and no product when the samples were homozygous for DD.

Statistical analysis

Data were analysed with SPSS (Version 17.0) statistical program. All tests were two-sided and $p<0.05$ was considered statistically significant at 95% confidence interval. Significance of differences between group means was tested by the Student-t test and differences in proportions were assessed by the Chi-square test to determine variables that could be considered risk factors of EH in T2DM patients.

Results

Clinical investigation:

In total, 111 patients with T2DM were examined, 51 of them being normotensive diabetics, and 60 hypertensive diabetics. Clinical parameters of these groups were compared (Table 1). Age, diabetes duration, SBP, DBP, SBP and were significantly higher in hypertensive T2DM patients than in normotensive T2DM patients while FBS was significantly higher in normotensive T2DM than hypertensive T2DM. On the other hand, sex and BMI were not significantly different in the two study groups.

Table 1: Characteristics and statistical comparison of clinical groups

Parameter	Normotensive Diabetics	Hypertensive Diabetics	p value
Age (years)	51.2 ± 12.9	58.7 ± 9.84	0.04*
Diabetes Duration (years)	4.3 ± 3.8	6.7 ± 6.3	0.03*
Systolic BP (mm Hg)	123.5 ± 8.9	158.7 ± 16.3	0.02*
Diastolic BP (mm Hg)	78.1 ± 7.6	87.9 ± 15.6	0.03*
FBS (mg/dL)	245.7 ± 96.0	206.3 ± 82.9	0.03*
Sex ratio (male : female)	17 : 34	17 : 43	0.19
BMI(Kg/m²)	28.24 ± 7.82	28.32 ± 5.67	0.21

*signifies statistical significance according to the student t-test

To find out if the prevalence of hypertension was related to age and sex, diabetics of different ages were grouped into normotensive and hypertensive male or females and their blood pressures compared (Table 2).

Table: 2: Distribution of hypertensive and normotensive individuals by age category and sex

Age group (Years)	Normortensive Diabetics		Hypertensive Diabetics		Total
	Males n (%)	Females n (%)	Males n (%)	Females n (%)	
25-40	2 (11.8)	7 (20.6)	1 (5.8)	1 (2.3)	11
41-60	12 (70.6)	20 (58.8)	9 (53.0)	22 (51.2)	63
> 60	3 (17.6)	7 (20.6)	7 (41.2)	20 (46.5)	37
Total	17	34	17	43	111

Analysis of results by the student t-test revealed that the proportion of male to females among the normotensive diabetics (17/34) was not significantly different ($p > 0.05$) from that of hypertensive diabetics (17/43). Also, the proportion of normotensive diabetics was higher in the 25-40 age group as compared to the hypertensive diabetics (9/3). The proportion was 32/31 for the 41-60 age group and 10/30 in the > 60 age group.

Genotype and allele frequencies of ACE I/D gene polymorphisms

The 51 normotensive T2DM and 60 hypertensive T2DM patients were genotyped and deletion polymorphism was characterized by a 190-bp fragment, whereas the presence of the insertion was observed as a 490-bp fragment (Figure1).

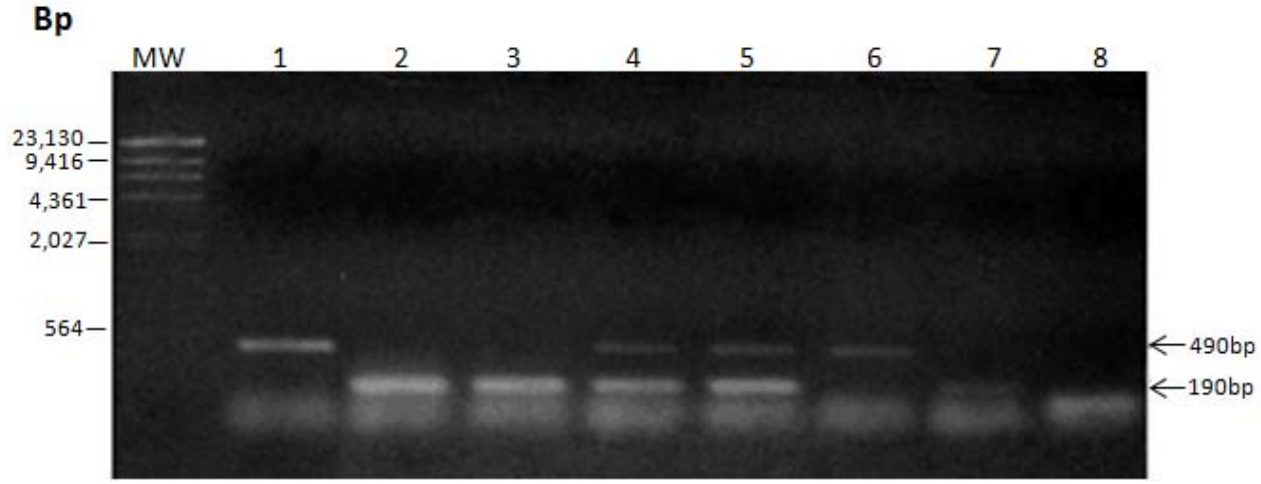

Figure 1. The ACE I/D genotype. Lane 1: Molecular weight marker. Lanes 1&6: Genotype II (single band of 490 bp); Lanes 2, 3 & 7: Genotype DD (single band of 190 bp); Lanes 4 & 5: Genotype ID (Two bands of 490 and 190 bp). Lane 8: Negative control.

The frequency of the polymorphic forms DD, ID and II of the ACE gene in the normotensive diabetic population (47.1%, 19.6%, 33.3%) was not significantly different ($\chi^2 = 3.8$; $p > 0.05$) from that of the hypertensive diabetics (30.0%, 31.7%, 38.3%). Allele frequencies for D and I alleles in normotensive diabetics (56.9% and 43.1%) were also comparable to that of hypertensive diabetics (45.9% and 54.1%) ($\chi^2 = 3.6$; $p > 0.05$).

Table 2: Distribution of ACE genotypes and allele frequencies in normotensive and hypertensive patients

	Normotensive diabetics		Hypertensive diabetics	
Genotypes	N	%	n	%
DD	24	47.1	18	30.0
ID	10	19.6	19	31.7
II	17	33.3	23	38.3
Allele				
I	44	56.9	65	45.9
D	58	43.1	55	54.1

Data were compared between normotensive diabetics and hypertensive diabetics by chi-squared test. N=number of individuals. ($\chi^2 = 3.8$, $p > 0.05$ for genotype frequencies and $\chi^2 = 3.6$, $p > 0.05$ for allele frequencies).

Considering that neither genotype nor allele frequency differences were observed between the normotensive diabetics and the hypertensive diabetics, patients were separated with respected to sex to find out sex-related differences (Table 3).

Table 3: **Genotype and allele distribution with respect to sex**

			Genotype			Allele	
			DD	**ID**	**II**	**D**	**I**
Sex	**Male**	**Normotensive diabetics**	**N**	6	3	8	15	19
			%	35.3	17.7	47	44.1	55.9
		Hypertensive diabetics	**N**	5	4	8	14	20
			%	29.4	23.5	47.1	41.2	58.8
	Female	**Normotensive diabetics**	**N**	18	7	9	43	16
			%	52.9	20.6	26.5	72.9	27.1
		Hypertensive diabetics	**N**	11	16	16	38	48
			%	25.6	37.2	37.2	44.2	55.8

Analysis of results of Table 3 by Chi squared test shows that the I allele is significantly higher in the female hypertensive diabetics than the female normotensive diabetic patients ($p = 0.04$)); No significant differences existed between male and female hypertensives ($p = 0.24$); the D allele is significantly higher in the female normotensive diabetic than the male normotensive diabetic patients ($p = 0.03$).

Discussion

Co-existence of T2DM and hypertension remains a puzzle as the pathophysiology of this association is not well understood. In this present study we examined whether ACE I/D, polymorphism could significantly predispose T2DM patients to hypertension since understanding of this relationship could provide new insights necessary for better management and control of this chronic disease.

Hypertensive T2DM patients were significantly older than normotensive T2DM patients ($p<0.05$). This is consistent with WHO facts of increased risk of EH with age due to the hardening of arteries with increased resistance to blood flow (WHO 2013, Arfa et al, 2010). Age is therefore a risk factor for EH in T2DM patients of the SWR of Cameroon contrary to the case in a group of Irarian T2DM patients where age was not a determinant of EH (Nakhjavani et al. 2007).

Hypertensive diabetics suffered from T2DM for a significantly longer period compared to normotensive diabetics ($p<0.05$). This finding is partly explained by the fact that prolonged high plasma levels of insulin in patients with T2DM characterised by insulin resistance results in dyslipidaemia –increasing the risk of EH in diabetics (Chapman et al. 2008). A corresponding finding by Nakjavani et al. is reported with diabetics in Iran (Nakhjavani et al. 2007).

FBS levels have been found to positively correlate with EH in Mexican Americans (Mitchell et al. 1999). In our case, the situation was different. Significant difference in fasting blood sugar (FBS) was observed with normotensive T2DM patients showing higher FBS levels as compared to hypertensive T2DM patients ($p < 0.05$). Higher FBS levels in the normotensive T2DM group could be explained partly by the fact that these subjects suffered from diabetes for a significantly shorter time (4.3 ± 3.8 years) compared to the hypertensive diabetics (6.7 ± 6.3 years). Normotensive T2DM subjects probably had lesser experience in managing their sugar levels resulting in the observation of higher FBS levels.

Considering the clinical parameters, sex was not associated with EH in T2DM patients in this study. This finding was similar with reports in an Iranian population study (Nakhjavani et al. 2007). In contrast, Sprafka et al. (1988) identified female T2DM in Minnesota, USA to be at greater risk of EH compared to T2DM males.

Also, results showed no statistically significant difference in BMI, thereby indicating that it is not a potential determinant of EH in this study population ($p>0.05$). Similar findings were observed in Iranian and Malaysian diabetics (Nakhjavani et al. 2007 Ramachandran et al. 2008). However, a contrast is observed in Minnesota where BMI is strongly associated with hypertension in diabetics (Sprafka et al. 1988).

These results suggest that determinants of hypertension in T2DM subjects are based on ethnicity and geographical location.

ACE Genotype and allele frequencies did not reveal any statistically significant difference between normotensive and hypertensive T2DM patients ($p>0.05$). These results suggest that this gene polymorphism may not be associated with EH in T2DM patients of the SWR. This finding is consistent with observations in Tunisian (Arfa et al. 2010) and Turkish (Degirmenci et al. 2005), German (Mondry et al. 2005) and Taiwanese (Chuang et al. 1997) diabetes patients. Observations in this study on association of ACE gene polymorphism and EH in T2DM patients in the SWR were contrary to findings of other researchers in different populations of Iran (Nakhjavani et al. 2007) and Malaysia (Ramachandran et al. 2008) where an association was observed. These results further support the fact that association of ACE I/D gene polymorphism with EH varies with ethnicity and geographical location.

Although generally, ACE I/D polymorphism was not associated with EH in the T2DM patient population, this polymorphism was observed to be associated with EH in females. Whereas genotype and allele frequencies between males of normotensive T2DM group and the hypertensive T2DM group remained the same, the I allele was significantly higher in the female hypertensive T2DM group than the female normotensive T2DM patients (Table 3). These results suggest that the allele I of the ACE gene pre-disposes female diabetics to EH.

CONCLUSION

Age, duration of T2DM, SBP, DBP and FBS are associated with EH in T2DM patients of the SWR. The I allele of the ACE I/D polymorphism predisposes females of this population to EH. These findings can be exploited in the formulation of public health strategies for the management of T2DM, provide risk assessment measures for the development of EH in the T2DM female population and its prevention through lifestyle adjustments.

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