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Polymorphism of the endothelial nitric oxide synthase gene is associated with diabetic retinopathy in a cohort of West Africans

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Purpose: In addition to chronic hyperglycemia, there is increasing evidence that genetic factors may be important in the development of diabetes retinopathy (DR). Specifically, polymorphisms of the endothelial nitric oxide synthase gene (eNOS) have been reported to be associated with multiple health conditions including DR, hypertension, nephropathy, and cardiovascular diseases in several ethnic groups. However, there is a paucity of similar data in African Americans and other African populations. To address this issue, we investigated the potential association between polymorphisms of the eNOS gene and diabetes-related phenotypes in 384 persons with type 2 diabetes and 191 controls from two West African countries (Ghana and Nigeria).

Methods: We genotyped the deletion/insertion (4a/b) and the G894T polymorphisms of eNOS gene in a total of 575 persons.

Results: The b/b genotype of the polymorphism was associated with a 2.4 fold increased risk of DR (95% CI 1.39-4.09). In contrast, we did not observe any association between the genotypes or alleles of G894T polymorphism with DR, hypertension, or nephropathy.

Conclusions: We observed a significant association between the 4a/b polymorphism of the eNOS and DR in our West African cohort.

Diabetic retinopathy (DR) is the leading cause of blindness in adults between the ages of 20 and 65 in industrialized countries [1,2]. About 21-29% of patients with type 2 diabetes (T2D) in the United States and Europe have retinopathy at the time of diagnosis [3-5]. In Africa, the reported prevalence of retinopathy varies from 9% to 55% in persons with diabetes [6-9]. T2D duration and blood glucose level are considered the major risk factors for DR [10,11]. Interestingly, despite poor glycemic control of African diabetic patients, we observed a prevalence of DR of 17% in an earlier study of West Africans [12]. A review of the literature indicated that 17% is in the lower range of reported estimates for some African populations. Given this earlier observation and also recognizing that chronic hyperglycemia, although a major risk factor for DR, is usually not sufficient to produce severe DR in majority of patients with diabetes [13], we investigated the potential role of polymorphisms in the endothelial nitric oxide synthase gene (eNOS), a known candidate gene, in the pathophysiology of DR in our West African cohort.

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Nitric oxide (NO), synthesized continuously in the endothelium from L-arginine by eNOS, plays an important role in maintaining basal vascular tone through its effect on the soluble guanylate cyclase (GS) signaling pathway [14,15]. It also inhibits platelet as well as leukocyte adhesion to vascular endothelium and inhibits proliferation of smooth muscle cells via a GS-independent mechanism [16,17]. It has been demonstrated, in vitro and in vivo, that overproduction of NO may induce oxidative stress in retinal, endothelial, and glomerular cells [18,19]. Also, it has been experimentally shown that NO contributes to the angiogenic properties of vascular endothelial growth factor (VEGF), a molecule implicated in the development of proliferative retinopathy [20]. Intravenous infusion of VEGF can acutely impair endothelial cell barrier functional integrity through a mechanism involving activation of eNOS [21], but more important, VEGF-induced angiogenesis and vascular permeability was abolished in eNOS knockout mice [22,23].

eNOS is a constitutively expressed enzyme of 135 kDa in vascular endothelial cells (b1-2). The eNOS gene, located on chromosome 7q35-36, is composed of 26 exons, and spans 21 kb. A commonly reported polymorphism of the eNOS gene is the 27 bp deletion (allele a) and insertion (allele b) polymorphism in intron 4- the 4a/b polymorphism. The 4a allele has been reported to be associated with a number of pheno-

types including hypertension among persons with T2D [24], diabetic nephropathy [25,26], end-stage renal disease [27,28], ischemic heart disease [29], and coronary artery disease [30]. The deletion allele (a allele) was also reported to be associated with increased risk of macular edema among Japanese subjects with T2D [31]. However, Taverna et al (2002) reported that the a/a genotype was associated with non-severe diabetic retinopathy in patients with type 1 diabetes; interestingly, they observed a more than two-fold increased risk when they compared the frequency of eNOS4b/b in patients with severe DR to controls [13]. This intriguing observation was subsequently replicated by a German study that reported the b/b genotype of eNOS was associated with a 2.4 fold increased risk of DR in type 1 diabetic subjects [32].

We also investigated the potential association between another polymorphism (G894T) of the eNOS gene with DR in our cohort. This polymorphism is a G to T transversion at nucleotide position 894, resulting in a GAG to GAT substitution in exon 7 with the replacement of glutamine by aspartate (Glu298Asp). A review of the literature showed that this polymorphism has been observed to be associated with multiple diseases outcomes, including essential hypertension, coronary artery disease, ischemic heart disease, myocardial infarction (MI), and end stage renal disease [28,33-35]. However, these observations have not been consistently reported by all groups [36-38]. We therefore investigated the potential role of two important polymorphisms (a 27-bp repeat at intron4 and Glu298Asp) in the etiology of diabetes related complication including DR in West African subjects with T2D and controls.

METHODS

Subjects: Individuals included in the present study were enrolled and examined as part of an international collaboration of Unite States and West African scientists to study the epidemiology and genetics of T2D in West Africa. A detailed description of the parent study, the Africa America Diabetes Mellitus (AADM) Study, has been published [9]. Briefly, the AADM study enrolled and examined 420 sibling pairs (840 individuals) and 191 unaffected spouse controls from multiple West African ancestral populations of African Americans. The three centers in Nigeria (Enugu, Ibadan and Lagos) enrolled two major ethnic groups-Ibos (28%) and Yorubas (28%); the two centers in Ghana also enrolled two ethnic groups, Akan-Ashante (25%) and Gaa (11%), which accounts for 92% of all the subjects in this cohort. We randomly selected one case from each family along with all the controls in the above four ethnic groups for genotyping. As a result, a total of 383 unrelated subjects with diabetes and 191 unaffected controls were selected for this association study.

Diagnosis of T2D was based on the criteria established by the American Diabetes Association Expert Committee as follows: a fasting plasma glucose concentration >126 mg/dl (7.0 mmol/l) or a 2 h post load value in the OGTT >200 mg/dl (11.1 mmol/l) on more than one occasion. Alternatively, diagnosis of T2D was accepted if an individual was on pharmacological treatment for T2D and review of medical records indicated adequate justification for that therapy. The detection of autoantibodies to glutamic acid decarboxylase antibody as well as a fasting C-peptide <0.03 nmol/l were used to exclude probable cases of type 1 diabetes. Healthy controls were required to have fasting plasma glucose <110 mg/dl. Hypertension was defined as >140 mmHg systolic blood pressure, diastolic >90 mmHg blood pressure, or taking anti-hypertensive medication. Serum leptin level was measured using DSL-10-23100 Active Human Leptin Enzyme linked Immunosorbent (ELISA) kit (Diagnostic Systems Laboratories, Inc., Webster, TX). All procedures were approved by the Institutional Review Boards of the five West African universities and of Howard University and all subjects gave informed consent.

Eye examination and diagnosis of diabetic retinopathy: Eye examination was part of a comprehensive physical examination of each participant in the study. Each participant had the following ocular examinations: visual acuity, ocular alignment and motility, pupil reactivity and function, visual fields, intraocular pressure, slit lamp examination of the cornea, iris, lens, and vitreous, dilated fundus examination (see Rotimi et al 2003 for more detailed description of the physi-

Table 1. Characteristics of the West African subjects with type 2 diabetes

Variable	Diabetes with retinopathy (68)	Diabetes without retinopathy (306)	p-value	Subjects with diabetes (384)	Subjects without diabetes (191)	p-value
Sex (M/F)	34/34	147/159		185/199	74/117	
Age	54.7±9.3	52.7±10.9	0.1574	53.1±10.7	49.4±11.6	0.0002
Body mass index	25.3±4.4	26.3±5.1	0.1375	26.1±5.0	26.4±5.5	0.5757
Waist-hip ratio	0.92±0.06	0.91±0.08	0.0963	0.91±0.07	0.87±0.07	<0.0001
Insulin	25.1±28.4	21.3±29.7	0.3649	22.4±29.8	16.7±15.2	0.0095
C-peptide	1.16±0.71	1.30±0.87	0.2032	1.28±0.84	1.26±0.78	0.7812
Glucose	215.1±86.8	191.6±86.8	0.0648	196.5±88.3	94.0±18.8	<0.0001
Leptin	12.1±13.3	16.3±19.9	0.0324	15.6±18.8	23.8±25.1	<0.0001
HDL	48.2±17.3	46.2±25.8	0.4811	46.7±25.1	61.5±39.6	0.0002
LDL	139.0±42.0	121.4±49.6	0.0134	118.3±48.9	105.8±50.0	0.0007
TG	87.0±44.0	89.0±54.0	0.7897	88.1±51.9	69.3±41.8	0.0001
Urine creatinine	98.0±70.0	130.7±94.5	0.0036	123.7±90.4	124.4±70.1	0.9332
Systolic BP	147±26	135±24	0.0002	137±25	131±22	0.0045
Diastolic BP	86±13	83±13	0.0963	84±13	83±13	0.3243

This table provides the means and standard deviations (SD; means±SD) of each of the phenotypes. P-value was calculated by comparing cases and controls, i.e. diabetes with retinopathy vs diabetes without retinopathy and diabetics vs non-diabetic controls.

cal eye examination in the AADM study [9]). Diagnosis of DR was made only when a participant had a minimum of one microaneurysm in any field as well as hemorrhages (dot, blot or flame shaped) and maculopathy (with or without clinically significant edema). In the interest of reproducibility, no attempts were made to classify retinopathy into the conventional stages of non-proliferative and proliferative maculopathy with or without edema.

Genotyping: Twenty mini-liter of blood was collected and processed on site. Buffycoat and plasma were shipped to the core laboratory of the National Human Genome Center at Howard University. Genomic DNA was extracted from buffy coats, using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN), according to the standard protocol. Genomic DNA was amplified using the GenomiPhi™ DNA Amplification kit (Amersham Biosciences, Piscataway, NJ). Briefly, about 10 ng of the original genomic DNA in 1 µl TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) was used as the template for the whole genome amplification (30 °C, 18 h). About 4-6 µg amplified DNA was diluted with TE buffer, pH 8.0, to a final concentration of 30 ng/ µl. One µl or 30 ng of this amplified DNA stock was used for subsequent genotyping. Genotyping PCR was carried out using high fidelity ThermalAce- DNA Polymerase (Invitrogen, Carlsbad, CA) and using touchdown condition, as follows: one cycle at 95 °C for 1.5 min, five cycles at 95 °C for 20 s, 65 °C for 20 s, and 74 °C for 20 s, five cycles at 95 °C for 20 s, 60 °C for 20 s, 74 °C for 20 s, five cycles at 95 °C 20 s, 55 °C 20 s, 74 °C 20 s, five cycles 95 °C for 20 s, 50 °C for 20 s, 74 °C 20 s, 74 °C for 7 min.

For the 27 bp I/D polymorphism, the following PCR primers were used: 5'-GTT ATC AGG CCC TAT GGT AGT GCC TTG-3', and 5'-GCC AGA GGG AGG AGG AAA CAT GTG TCA-3'. For Glu298Asp polymorphism, these PCR primers were used: 5'-GAG ATG AAG GCA GGA GAC AGT GGA T-3', and 5'-TCC ATC CCA CCC AGT CAA TCC CTT T-3' (biotin labeled).

Genotyping of the 27 bp I/D polymorphism was carried out by gel electrophoresis on 2% agarose gel with 1% ethidium bromide after PCR. All gels were read independently by two lab personnel to avoid misreading. An automated pyrosequencing instrument, PSQ96 (Pyrosequencing AB, Uppsala, Sweden) was used to genotype the Glu298Asp SNP. The following pyrosequencing primers were used 5'-TGC TGC TGC AGG CCC CAG AT-3'.

Statistical analysis: Haplotypes were inferred by the program SIMWALK2 [22]. All statistical analyses were performed using the SAS statistical package (SAS Institute, Inc., Cary, NC). Frequencies of allele, genotype and constructed haplotypes of the two polymorphisms were tested for statistical significance using the chi^2 test. To test the potential interaction between the two investigated polymorphisms, we constructed diplotypes (i.e., combined individual genotypes for the two polymorphisms).

RESULTS

A total of 384 T2D cases and 191 controls were selected for this study. Clinical characteristics of all study participants are summarized in Table 1. T2D patients were on average about four years older and had few women (52% versus 61%) compared to the group without diabetes. As expected, T2D participants were more likely to have higher waist-hip ratio, systolic blood pressure, and increased risk of dyslipidemia compared with the controls. Interestingly, leptin level was lower among the cases despite similar body mass indices. This, we believe, was due to the higher number of women in the control group. Our group has in the past found much higher levels of leptin in West African women compared to their male counterparts [39].

We observed a significant association between the bb genotype of the 4 a/b polymorphism and increased risk of DR with OR=2.4 (95% CI: 1.39-4.09) in our T2D cohort. Allelic specific association was also observed with the b allele expressing increased risk of DR with OR=1.7 (95% CI: 1.11-2.50; Table 2). Three of the four most frequently occurring diplotypes constructed from the 4 a/b polymorphism and the single nucleotide polymorphism G894T were also associated with DR. Among them, bG/bG and bG/bT diplotypes were

Table 2. Association study of eNOS gene polymorphisms and diabetic retinopathy

Genotypes and alleles	Diabetes with retinopathy	Diabetes without retinopathy	OR (95% CI)	p-value
4a/b				
Genotypes	68	301		
aa	10 (14.7)	48 (16.0)	0.9 (0.43-1.90)	0.7995
ab	17 (25.0)	136 (45.2)	0.4 (0.22-0.73)	0.0023
bb	41 (60.3)	117 (38.9)	2.4 (1.39-4.09)	0.0013
Alleles a	37 (27.2)	232 (38.5)		
b	99 (72.8)	370 (61.5)	1.7 (1.11-2.50)	0.0131
Glu298Asp				
Genotypes	68	305		
GG	57 (83.8)	263 (86.2)	1.2 (0.59-2.49)	0.6074
GT	11 (16.2)	38 (12.5)	1.4 (0.65-2.81)	0.4119
TT	0 (0)	4 (1.31)	1.0 (1.00-1.02)	0.3424
Alleles G	125 (91.9)	564 (92.5)	1.1 (0.54-2.14)	0.8280
T	11 (8.1)	46 (7.5)		

Odds ratio (OR) and 95% confidence interval (CI) were calculated from the 2x2 table of one genotype or allele versus the rest of all the other genotypes or alleles comparing with diabetes with retinopathy versus diabetes without retinopathy.

Table 3. Diplotype distribution in subjects with diabetes

Diplotypes and haplotypes	Diabetes with retinopathy	Diabetes without retinopathy	OR (95% CI)	p-value
Diplotype(n)	68	300		
aG/bG	17 (25.0)	133 (44.3)	0.4 (0.23-0.76)	0.0034
bG/bG	31 (45.6)	94 (31.3)	1.8 (1.07-3.14)	0.0250
aG/aG	9 (13.2)	46 (15.3)	0.8 (0.39-1.82)	0.6613
bG/bT	10 (14.7)	21 (7.0)	2.3 (1.03-5.12)	0.0389
aG/aT	1 (1.5)	2 (0.7)	2.2 (0.20-24.9)	0.5056
aT/bT	0	2 (0.67)	-	-
bT/bT	0	2 (0.67)	-	-
Haplotype				
bG	89 (65.4)	342 (57.0)	1.4 (0.97-2.11)	0.0712
AG	36 (26.5)	227 (37.8)	0.6 (0.39-0.90)	0.0125
bT	10 (7.4)	27 (4.5)	1.7 (0.80-3.57)	0.1692
aT	1 (0.7)	4 (0.67)	1.1 (0.12-9.95)	0.9300

Diplotypes (i.e., combined individual genotypes for the two polymorphisms 4a/b and G894T). Odds ratio (OR) and 95% confidence interval (CI) were calculated from the 2x2 table of one diplotype or haplotype versus the rest of all the other dipolotypes or haplotypes comparing with diabetes with retinopathy versus diabetes without retinopathy.

associated with increased risk of diabetic retinopathy with OR=1.8 (95% CI: 1.07-3.14) and OR=2.3 (95% CI: 1.03-5.12), respectively, whereas the aG/bG was associated with a significant decreased risk of DR with OR=0.4 (95%CI: 0.23-0.76, see Table 3).

Also performed was an association study between each genotype of both 4a/b and G984T polymorphisms and diabetes or other quantitative traits such as creatinine clearance, dyslipidemia, microalbuminurea, but no significant associations were observed (data not presented).

DISCUSSION

In the present study, we investigated two polymorphisms 4a/b and G894T of eNOS gene for their association with T2D and diabetes related phenotypes. We observed that the bb genotype of the 4a/b polymorphism was associated with 2.4 fold increased risk of DR in our diabetic cohort. These results are consistent with previous reports that the b/b genotype was associated with DR in Caucasian subjects with diabetes [13,32]. We also observed a positive significant association between constructed diplotypes (bG/bG: OR=1.8; and bG/bT: OR=2.3) of the 4a/b and G894T polymorphisms in subjects with DR compared to controls. Together with previous reports, our results suggest that the 4a/b polymporphism of the eNOS may play an important role in the development of DR in patients with T2D.

The consistencies of our results with those from other studies conducted in different ethnic groups are particularly encouraging. Together, these findings provide significant evidence that the 4a/b polymorphism may play an important role in the pathogenesis of DR in both type 1 and type 2 diabetes.

DR is characterized by loss of pericytes around capillaries in the retina followed by development of microaneurysm and fluid leakage from capillaries, ischemia and infarction, neovacularization and residual scarring [40]. Increased retinal oxidative stress is believed to play an important role in DR development. A number of reports have suggested that, in diabetics, increased level of NO in the retina causes oxidative stress and subsequent pathological changes resulting in DR. Clinical studies have demonstrated that, in patients with DR, NOS activity was increased [41,42] as were levels of NO or NO derivatives in plasma [43,44], vitreal fluid [45,46], and aqueous fluid [46,47]. However, although eNOS was localized in Muller cells and vascular endothelium of retina [48,49], both in vivo and in vitro studies revealed that the level of the constitutively expressed eNOS was reduced in the retinal vascular endothelial cells when diabetes was present [50]. It was suggested that both high glucose levels and osmotic stress in retinal endothelial cells of diabetics may increase NOS activity and generate an “uncoupled” eNOS [51], resulting in the overproduction of NO and subsequently its derivatives, such as peroxynitrite a normal NO derivative with high oxidative activity. Excess amount of peroxynitrite and other NO derivatives in diabetes may result in oxidative stress and generating cytotoxic effects by increasing DNA damage, stimulating lipid peroxidation and depleting glutathione levels [51,52]. Furthermore, NO is also known to increase vascular permeability and angiogenesis by increasing the activity of VEGF [53], a major feature of advanced DR [40] and interaction with prostaglandin cyclooxygenase pathways [54]. Thus, in diabetics, high glucose levels and osmotic stress may directly cause down-regulated expression of eNOS in subjects with genotype 4b/b, which subsequently activates an “uncoupled” overproduction of NO and its highly reactive oxidants. In subjects with genotype 4a/a, NO is produced mainly from the constitutively expressed eNOS with the uncoupled expression relatively suppressed.

On the other hand, Wang et al. (2000) [55] demonstrated that repeats of the 27-bp insert of the 4b can bind to a nuclear protein and act as a cis-acting factor of the eNOS promoter and regulate the transcription efficiency at a haplotype-specific fashion with the T-786C variant at the promoter region. Therefore, the b/b genotype probably acts both independently and in coordination with the functional SNP T786C at the promoter region of eNOS to regulate of the expression of eNOS gene in pathogenesis of DR as well as in physiological conditions. If correct, this process makes the regulation of eNOS expression more complicated. In the future, we plan to conduct association studies to investigate the potential combined effect of 4a/b and T786C polymorphisms and DR in this cohort of West Africans with T2D. In conclusion, we observed that the b/b genotype of the 4a/b polymorphism of eNOS gene is associated with a significant increased risk of DR.

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