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ORIGINAL ARTICLES

Association between Erythropoietin Gene Polymorphisms and Diabetic Retinopathy

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ABSTRACT

Objective: To determine whether sequence variation in the erythropoietin gene *(EPO*) is associated with the development of diabetic retinopathy (DR). **Methods:** Subjects and Methods: This was a multicenter study based on 100 subjects with long-standing diabetes mellitus (DM), 40 subjects with non proliferative DR, 60 with proliferative DR. Subjects with end-stage renal disease were excluded Study groups consisted of 50 control subjects with no DR.. DNA extracted from blood of each subject was genotyped for 3 *EPO* single-nucleotide polymorphisms (SNP). **Results:** All 3 SNPs in *EPO* were associated with overall DR status in the DM group in the multivariate analysis. The GCC haplotype was also associated with overall DR status in the combined DM (*P*=.008) by multivariate analysis. All SNPs and the GCC haplotype were also associated with proliferative DR. **Conclusion:** Sequence variation in *EPO* is associated with the risk of DR independent of duration of DM, degree of glycemic control, and nephropathy. could lead to the possibility of developing novel treatments or preventive therapies

Key word: Diabetic Retinopathy,Erthopoietn,Gene Polymorphism

Introduction

Diabetic Retinopathy (DR) is an ocular microvascular complication of diabetes mellitus (DM). Up to 40% of individuals with DM will develop DR at some stage of their illness, with approximately 8% becoming sight threatening (Kempen *et al*.,2004). The mechanisms underlying DR are still incompletely understood. Current models of DR suggest that damage to retinal blood vessels by long-standing hyperglycemia leads to retinal hypoxia, which stimulates the DNA-binding activity of hypoxia inducible factor. Hypoxia-inducible factor upregulates a large number of hypoxia inducible genes, including vascular endothelial growth factor and erythropoietin (*EPO*) (Wang *et al.,* 1993). Consequent increased expression of these cytokines may then contribute to DR progression.

EPO is a glycoprotein that plays a major role in stimulation of bone marrow stem cells and erythropoiesis. It has also been shown to stimulate proliferation, migration, and angiogenesis in vascular endothelial cells exposed to hypoxia (Yamaji *et al.,* 1996 and Anagnostou *et al.,* 1990) EPO messenger RNA is expressed in the human retina (Herna´ndez *et al.,* 2006 and Garcı´a-Ramı´rez *et al.,* 2008). Expression of EPO receptors in vascular endothelial cells (Anagnostou *et al.,* 1994) and the retina (Garcı´a-Ramı´rez *et al.,* 2008 and Grimm *et al.,* 2006) has also been demonstrated. Many studies have reported a higher concentration of EPO in the vitreous of patients with DM and proliferative DR (PDR) when compared with controls (Herna´ndez *et al.,* 2006 ; Katsura *et al.,* 2005 and Takagi *et al.,* 2007). Animal studies have shown increased EPO concentrations in ischemic retinas (Tong *et al.,* 2008 and Chen *et al.,* 2009) and EPO inhibitors preventing neovascularization, further supporting the role of EPO in the development of PDR (Chen *et al.,* 2009).

EPO expression is influenced by singlenucleotide polymorphisms (SNPs) in *EPO* (Tong *et al.,* 2008). Interestingly, there is no correlation between the vitreous and plasma levels of EPO, suggesting increased vitreous EPO is due to increased local production of EPO in the retina (Katsura *et al.,* 2005 and Inomata *et al.,* 2004) rather than due to increased systemic EPO production by the kidney.

The human *EPO* gene is located on chromosome 7q21.15 Recently, Tong *et al.,* (2008) genotyped 613 subjects with type 2 DM (T2DM) (374 with PDR and end-stage renal disease [ESRD] and 239 controls with complication free DM) for 19 SNPs in 11 genes involved in angiogenesis, including *EPO*. The only significant association with DR was found at SNP rs1617640 in the promoter of *EPO*, where the T allele was significantly associated with PDR and ESRD (*P*=.00191). This finding was replicated in 2 type 1DM(T1DM) cohorts (1244with PDR with or without ESRD and 715 controls with complication-free DM) with all subjects being

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European American (overall*P*=2.76_10−11). The TTA haplotype of SNPs rs1617640, rs507392, and rs551238 were also disease associated (*P*=.0005) (Tong *et al.,* 2008).

We aimed to determine whether the same *EPO* sequence variation was associated with DR development in Egyptian subjects with DM and without ESRD.

Materials and Methods

Subjects:

This was a multicenter study based on 100 subjects with long-standing diabetes mellitus (DM), 40 subjects with nonproliferative DR, 60 with proliferative DR. Subjects with end-stage renal disease were excluded. Study groups consisted of 50 control subjects with no DR .

Patients were recruited from the Ophthalmology Clinic of the Research Institute of Ophthalmology. Control subjects were 50 healthy volunteers with no history of diabetes, or any major clinical disorders and had normal fasting blood sugar and HbA1C

Methods:

Family history of DR for all patients and controls and the duration of diabetes for patients were registered. All patients underwent a complete ophthalmological examination, including best corrected visual acuity, slit-lamp examination, intraocular pressure measurement using Goldmann applanation tonometry, indirect ophthalmoscopy and biomicroscopy. Fundusg fluorescein angiography was done in needed cases using Topcon fundus camera TRC. 50 EX on image-net. Five ml of 10% sodium fluorescein was injected in the antecubital vein and photography was carried out. Retinopathy was diagnosed according to the Early Treatment

Diabetic Retinopathy Study (ETDRS) criteria: the presence of microaneurysms, hemorrhages, cotton wool spots, intraretinal microvascular abnormalities, hard exudates, venous beading and new vessels [15].

Patients were classified into 2 groups: group (1): 43 diabetic patients without retinopathy, group (2): 44 diabetic patients with diabetic retinopathy (DR) and 44 healthy control group. Informed consent was obtained from participants after a clear explanation of potential risk of the study.

HbA1C was measured with a cation exchange chromatography method to assess glycaemic control. The procedure is a microchromatographic methodology for the quantitation of glycosylated haemoglobin (nondiabetic reference 5.5 % - 7.7%) (GLYCO Hb Quick column procedure). Serum total cholesterol andtriglycerides were measured using enzymatic methods.

DNA was extracted from peripheral blood samples using the QiaAmp Blood Maxi Kit (Qiagen, Valencia, California). Single-nucleotide polymorphism genotyping was checked for compliance with Hardy-Weinberg equilibrium (HWE) using a _2 test. Linkage disequilibrium between markers and allelic association tests were calculated in Haploview 4.0 (h.broadinstitute.org/mpg/haploview). Genotypic associations were assessed in SNPStats.17 Dominant and recessive models were considered with respect to the minor allele. Odds ratios were calculated in SPSS (version 15.0; SPSS Inc, Chicago, Illinois). Haplotypic associations were undertaken in Haplo Stats (version 1.2.1)18 in 1 block of linkage disequilibrium.

Results:

Table 1: Clinical Characteristics of Participants with No DR Compared with DR Cases in Diabetic Patients.

Clinical Characteristic	No DR (n=40)	DR (n=60)	p
Female, No. (%)	23(56)	25(37)	<.001
Age, y, mean (SD)	64.5 (15.1)	64.2 (11.1)	.82
Disease duration, y, mean (SD)	12.6 (8.9)	17.3 (8.4)	<.001
HbA1c level, %, mean (SD)	6.5 (3.1)	8.5 (8.6)	.005
BMI, mean (SD)	32.5 (9.1)	29.7 (10.9)	.01
Hypercholesterolemia, No. (%)	106 (64)	113 (61)	.90
Nephropathy, No. (%)	37 (22)	53 (30)	.12
Smoker, No. (%)	86 (52)	99 (55)	.52
Hypertension, No. (%)	135 (81)	150 (84)	.54

Table 2: Genotype Frequencies for Each SNP in Participants with No DR and DR Cases

SNP	No DR (n=40)	DR (n=60)
rs1617640		
TT	24 (37)	40 (39)
TG	30 (46)	44 (43)
GG	11(17)	18(18)
rs507392		

TT	24 (37)	40 (39)
TC	30 (46)	44 (43)
CC	11 (17)	18(18)
rs551238		
AA	24 (37)	40 (39)
AC	30 (46)	44 (43)
CC	11 (17)	18(18)

Abbreviations : DR, diabetic retinopathy: SNP, single – nucleotide polymorphism

Table 3: P Values for Association of EPO SNPs with DR

		Unadjusted P Value		Adjusted P Value[a]	
SNP	Minor Allele	Dominant	Recessive	Dominant	Recessive
rs1617640	G	.770	.900	.760	.130
rs507392	C	.770	.900	.760	.130
rs551238	C	.770	.900	.760	.130

Table 4: Association of Haplotypes with Blinding DR in 11 DM and I2DMa

				T1DM			
Haplotype	Allelers			Haplotype Frequency	No DR Frequency	DR Frequency	P Value
	1	2	3				
1	G	T	A	0.602	0.600	0.604	.758
2	C	C	C	0.398	0.400	0.396	.103

Discussion:

EPO is an important cytokine that stimulates proliferation, migration, and angiogenesis in vascular endothelial cells (Yamaji *et al.,* 1996 and Anagnostou *et al.,* 1990). EPO expression has been shown to be influenced by SNPs in *EPO*12 and is elevated in the vitreous of subjects with PDR. (Herna´ndez *et al.,* 2006 ; Katsura *et al.,* 2005 and Takagi *et al.,* 2007) *EPO* is thus a biologically plausible candidate gene with potential to influence susceptibility to develop DR and this study has investigated the association of *EPO* gene variation with DR development.

This study found an association between 3 *EPO* SNPs and DR in a cohort of subjects with DM, with the GCC haplotype having an increased frequency in patients with T2DMwith DR. In contrast to our study, Tong *et al.,* (2008) identified the T allele of rs1617640 to be the risk-associated allele with PDR. It was also reported that the same allele in the EPO promoter region had a major effect on EPO messenger RNA transcription levels in an in vitro model.

The opposite haplotype (TAA) was reported as the risk allele for PDR in their study. (Tong *et al.,* 2008). Similar overall allele frequencies (across combined cases and controls) were observed in the 2 studies. However, a major and important difference between the 2 cohorts is the complete lack of ESRD in the current study, compared with the majority of cases having ESRD in the Tong *et al.,* (2008) study. As opposed to Tong et al, this study did not find an association of the *EPO* SNPs with DR in T1DM. The substantially larger T1DM cohort of Tong *et al.,* (2008) had additional power, which may explain the lack of association observed in the current study. End-stage renal disease leads to anemia through reduced EPO production. Although similar vascular processes may be involved in the pathogenesis of both diabetic nephropathy and DR, it is possible that different genetic factors play a role in their susceptibility. The development of DR (Hallman *et al.,* 2005 ; Arar *et al.,* 2008 ; Monti *et al.,* 2007 ; Hietala *et al.,* 2008 ; Rema *et al.,* 2002 ; Imperatore *et al.,* 1998 and Looker *et al.,* 2007) and nephropathy (Rogus *et al.,* 2008 ; . Osterholm *et al.,* 2007; Chistiakov *et al.,* 2004 Imperatore *et al.,* 2001; Placha *et al.,* 2005 and . Iyengar *et al.,* 2007) have each been shown to have a strong genetic component. However, the majority of the chromosomes believed to be involved in the inheritance of nephropathy and retinopathy susceptibility are not shared. (Hallman *et al.,* 2005 ; Imperatore *et al.,* 1998 ; Looker *et al.,* 2007; Imperatore *et al.,* 2001 and Iyengar *et al.,* 2007). It is therefore possible that different variations within *EPO* play a role in DR and ESRD development and therefore possible that Tong et al have identified variations in *EPO* responsible only for ESRD, accounting for the differences between our and their findings. Previous studies have also suggested no association of plasma EPO levels with increased vitreous EPO, but rather increased vitreous levels to be due to local production of EPO in the retina (Katsura *et al.,* 2005 and Watanabe *et al.,* 2005). Further studies investigating factors influencing local and systemic production of EPO and tissue-specific effects of *EPO* gene regulation are also required to further understand the role of EPO in DR and ESRD pathogenesis.

This study is not the first to report an opposite allele/ genotype of the same SNP to be associated with a disease. For example, the G and C allele of SNP rs3741916 of the glyceraldehyde-3-phosphate dehydrogenase gene have been reported by different studies to be significantly associated with Alzheimer disease in white subjects. (Li *et al.,* 2004 and Lin *et al.,* 2006). This "flip flop" phenomenon may occur when a single locus association is found in the presence of multilocus effects and this single locus association may be confounded by

other loci. Another reason can include sampling variation among the studies, whereby the magnitude of an association between an allele and risk of disease varies across different (especially ethnic) populations because of the presence of different linkage disequilibrium patterns. Finally, associations of opposite alleles of the same SNP may occur because of differences in its relationship with other causal variants, including environmental factors (Lin *et al.,* 2007).

Another important consideration is the deviation from HWE observed in the T2DM no DR group. This group was as highly selected as the controls, and thus, the deviation is not unexpected in the presence of a true association. However, there is a chance that this deviation is due to population stratification. The T1DM groups conformed to HWE as did the T2DM with DR group, indicating that recruitment bias and genotyping errors are likely not the cause. However, the reported association does depend on the group that does not conform to HWE.

Subjects with no DR in our study had shorter duration of DM and fewer associated vasculopathic risk factors when compared with those with DR. We accept this as a limitation of our study; however, an attempt to over come these influences on the outcome of results has been made by adjusting for these factors in the multivariate analyses.

In conclusion, our results show that in Egyptians variation in *EPO* predicts the risk of developing DR, independent of duration of DM. There is clearly a need for further independent association studies to further explore the role of EPO sequence variation in DR susceptibility. Also, further functional characterization is required to better elucidate the role of *EPO* in DR.

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