

# Association analysis of genetic variations of eNOS and $\alpha_2\beta_1$ integrin genes with type 2 diabetic retinopathy

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**Background:** Diabetic retinopathy (DR) is classically defined as a microvasculopathy that primarily affects the small blood vessels of the inner retina as a complication of diabetes mellitus. It has been suggested that nitric oxide (NO) and  $\alpha_2\beta_1$  integrin (a platelet receptor for collagen) play an important role in the pathogenesis of microvascular complications in DR.

**Aim:** The aim of this study was to investigate the association of two candidate genes involved in the regulation of retinal vasculature, endothelial nitric oxide synthase (*eNOS*) and  $\alpha_2\beta_1$  integrin (*ITGA2*) genes, with the development of DR in Egyptian patients with type 2 diabetes mellitus and to investigate whether genetic variants will affect the type of retinopathy (proliferative or nonproliferative).

**Methods:** In this study, 70 patients were enrolled and categorized into two groups: (1) a DR group consisting of 50 patients with DR, which was further subclassified into 25 patients with nonproliferative DR (NPDR group) and 25 patients with proliferative DR (PDR group) and (2) a diabetes without retinopathy (DWR) group, comprising 20 patients with type 2 diabetes of more than 10 years' duration who showed no signs of DR. Associations of the genetic polymorphisms of eNOS (G894T) and *ITGA2* (Bgl II) were studied. Polymerase chain reaction-restriction fragment length polymorphism analysis was performed for all samples to evaluate the genotypes and correlate with the phenotype of the disease.

**Results:** The allele frequencies of both polymorphisms showed considerable differences between patients with and without DR. The GG genotype of G894T polymorphism of eNOS was associated with a 9.75-fold increased risk of DR (95% confidence interval 1.7–55.4) and the genotype *ITGA2* Bgl II (+/+) was associated with a 10.1-fold increased risk of DR (95% confidence interval 1.8–57.9), while the  $\alpha_2\beta_1$  integrin gene polymorphism of genotype distribution of both eNOS and *ITGA2* polymorphisms did not differ significantly between the proliferative and nonproliferative DR groups.

**Conclusion:** A significant association between the G894T polymorphism of eNOS and Bgl II polymorphism of *ITGA2* genes and DR was observed, while there was no association between the genetic variants of those two polymorphisms and the type of retinopathy.

**Keywords:** polymorphism, retinopathy, diabetes, Egypt, retinal vasculature

## Introduction

Diabetic retinopathy (DR) is one of the most common microvascular complications of diabetes, affecting 80% of patients with a duration of diabetes of over 20 years.<sup>1</sup> Despite remarkable advances in the diagnosis and treatment of DR and its associated complications, DR remains the leading cause of blindness among working-age individuals in developed countries.<sup>2</sup>

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Most diabetic patients, especially those with poor glycemic control, develop DR. The Wisconsin Epidemiologic Study of Diabetic Retinopathy showed that 28.8% of diabetic patients develop retinopathy early, whereas 22.2% with a history of diabetes do not develop retinopathy, irrespective of glycemic exposure.<sup>3</sup> The authors suggested that genetic factors could promote the onset of retinopathy in diabetic patients. Over 30 candidate genes involved in different metabolic mechanisms and functional pathways have been reported to be associated with DR.<sup>4</sup>

eNOS is a constitutively expressed enzyme of 135 kDa in vascular endothelial cells. The *eNOS* gene, located on chromosome 7q35-36, is composed of 26 exons and spans 21 kb. The *eNOS* gene has several reported single-nucleotide polymorphisms, one of which is G894T. 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). Several studies have observed that this polymorphism has been associated with multiple disease outcomes, including essential hypertension, coronary artery disease, ischemic heart disease, myocardial infarction, and end-stage renal disease.<sup>5</sup> Therefore, the potential role of this polymorphism in the etiology of DR with type 2 diabetes (T2D) was investigated in this study.

DR is associated with disorders in the nitric oxide (NO) pathway including impaired NO-mediated vasodilatation, increased oxidative stress, dysregulation at NO synthase isoforms, and NO uncoupling.<sup>6</sup> This suggests that eNOS is involved in inflammation and ischemic process in the pathogenesis of DR and that the *eNOS* gene is a possible candidate gene in the pathophysiology of DR.<sup>7</sup>

Platelets are known to have a physiological role in maintaining homeostasis and platelet dysfunction associated with diabetes is known to contribute to the development of DR.<sup>8</sup> Wide variations in pathophysiological processes involved in the density of a platelet collagen receptor ( $\alpha_2\beta_1$  integrin or glycoprotein Ia/IIa) are reportedly associated with polymorphisms in the gene encoding the  $\alpha$  subunit at the receptor.<sup>9</sup> The aim of the study was to determine the relationship between the G894T polymorphism of the *eNOS* gene and the BgI II polymorphism of the *ITGA2* gene and susceptibility to DR and to determine whether their genetic variants will affect the type of retinopathy.

The gene encoding  $\alpha_2\beta_1$  integrin has at least eight polymorphisms and its genetic variations have been shown to affect the density of  $\alpha_2\beta_1$  receptors on the platelet surface.<sup>10</sup> One of these polymorphisms is the BgI II restriction fragment length polymorphism (BgI II, +/-) within intron 7.

It was reported that the platelet  $\alpha_2\beta_1$  density and the extent of platelet adhesion to collagen were higher in individuals with BgI II(+) homozygote than in individuals with the BgI II(-) homozygote.<sup>9</sup>

## Subjects and methods

The study included 70 unrelated participants with T2D. All study patients gave informed written consent before blood sampling. Approval was obtained from the Research Ethics Committee of the Menoufiya Faculty of Medicine. The patients were enrolled from the outpatient clinic of the Ophthalmology Department in Menoufiya University Hospital. They were divided into two groups:

1. DR group: 50 patients suffering from DR, further subclassified into two groups: 25 patients suffering from nonproliferative DR (NPDR group) and 25 patients suffering from proliferative DR (PDR group).
2. Diabetic without retinopathy (DWR) group: 20 patients with no signs of DR and with known diabetes mellitus (DM) duration  $\geq 10$  years (ie, controls).

Diagnosis of T2D was based on criteria established by the American Diabetes Association expert committee as follows: a fasting blood glucose level  $\geq 126$  mg/dL ( $\geq 7.0$  mmol/L) or 2-hour post-load plasma glucose  $\geq 200$  mg/dL ( $\geq 11.1$  mmol/L) or random plasma glucose  $\geq 200$  mg/dL, on more than one occasion. Alternatively, diagnosis of T2D was accepted if an individual was on pharmacological treatment and review of medical records indicated justification for treatment. Classification of diabetes was based on clinical features (age of onset) and laboratory data (serum c-peptide).<sup>11</sup>

A full medical history was taken for each subject, which included information on age, sex, age at onset of diabetes, duration of diabetes, family history, treatment protocols, history of hypertension, and smoking (a smoker was defined as someone who smoked at least five cigarettes daily for more than 1 year).<sup>6</sup> All patients underwent full ophthalmological examination, which included intraocular pressure measurement, slit-lamp examination, and fundus examination. Fundus fluorescein angiography was performed in patients with DR to differentiate between proliferative and nonproliferative retinopathy, then DR patients were classified into either the NPDR or PDR subtype according to Early Treatment Diabetic Retinopathy Study (ETDRS) criteria.<sup>12</sup> Finally, laboratory investigation was performed, including: fasting glucose level;<sup>13</sup> HbA<sub>1c</sub>;<sup>14</sup> lipid profile, including serum cholesterol;<sup>15</sup> triglycerides;<sup>16</sup> high-density lipoprotein (HDL)-cholesterol<sup>17</sup> and low-density lipoprotein (LDL)-cholesterol<sup>18</sup>

(by enzymatic colorimetric test); and genotyping for the G894T polymorphism of the *eNOS* gene and the Bgl II polymorphism of the *ITGA2* gene.

## Blood sampling

A total of 10 mL of fasting venous blood was withdrawn from the cubital vein of every subject: 4 mL of this was transferred slowly into a vacuum ethylenediaminetetraacetic acid (EDTA) tube for isolation of white blood cells for genotyping; 2 mL was transferred slowly into a vacuum EDTA tube for measuring hemoglobin A1c ( $HbA_{1c}$ ); and 2 mL was transferred slowly into a vacuum EDTA tube and centrifuged for 5 minutes at 4000 revolutions per minute. The plasma obtained for determination of plasma glucose was frozen at  $-20^\circ\text{C}$  till analysis. The remaining 2 mL was transferred slowly into a plain tube for determination of serum total cholesterol, triglycerides, and HDL-cholesterol; left for 30 minutes for clotting; and centrifuged for 10 minutes at 4000 revolutions per minute. The serum obtained was frozen at  $-20^\circ\text{C}$  till analysis. LDL-cholesterol was estimated by Friedewald's formula.<sup>18</sup>

## Peripheral blood mononuclear cells isolation

For separation of peripheral blood mononuclear cells, Lymphoflot solution (Biotest AG, Dreieich, Germany) was used.<sup>19</sup>

## DNA extraction

This was performed using QIAamp<sup>®</sup> DNA Blood Mini kits (QIAGEN, Hilden, Germany).

## Genotyping of *ITGA2* (Bgl II) polymorphism<sup>20</sup>

The amplification reaction was done in 25  $\mu\text{L}$  final volume (10  $\mu\text{L}$  DNA template + 15  $\mu\text{L}$  master mix containing 2.5  $\mu\text{L}$  10 $\times$  polymerase chain reaction [PCR] buffer, 1.5  $\mu\text{L}$  MgCl 25 mM, 0.5  $\mu\text{L}$  deoxyribonucleotide [dNTPs] 10 mM, 0.5  $\mu\text{L}$  Taq polymerase 5 U/ $\mu\text{L}$ , 1.0  $\mu\text{L}$  forward primer 50 mM {F 5'-GATTAACTTCCCGACTGCCTTCC-3'}, 1.0  $\mu\text{L}$  reverse primer 50 mM {R 5'-CATAGGTTTTGGGAACAGGTGG-3'}, and 8  $\mu\text{L}$  distilled water). PCR amplification was done using a PerkinElmer GeneAmp thermal cycler 2400 (Waltham, MA). PCR conditions were 34 cycles, with the first two cycles at  $94^\circ\text{C}$  for 1 minute,  $69^\circ\text{C}$  for 1 minute, and  $72^\circ\text{C}$  for 1 minute; the second two cycles at  $94^\circ\text{C}$  for 1 minute,  $67^\circ\text{C}$  for 1 minute, and  $72^\circ\text{C}$  for 1 minute; the remaining 30 cycles at  $94^\circ\text{C}$  for 1 minute,  $65^\circ\text{C}$  for 1 minute,

and  $72^\circ\text{C}$  for 1 minute; and the final extension step of  $72^\circ\text{C}$  for 1 minute.

## Recognition site Bgl II

5' ... A▼GATCT ... 3'

3' ... TCTAG▲A ... 5'

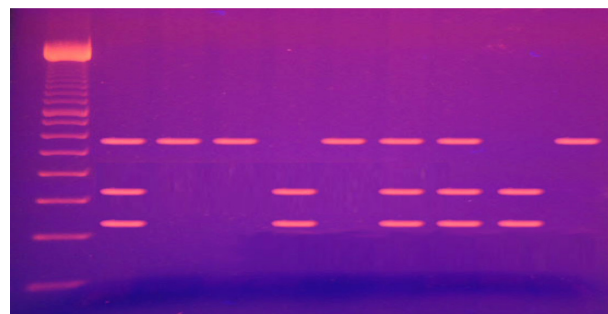
Amplified DNA was digested with Bgl II restriction enzyme (Bgl II) at  $37^\circ\text{C}$  for 3 hours (2  $\mu\text{L}$  10 $\times$  buffer, 1.0  $\mu\text{L}$  Bgl II, 7  $\mu\text{L}$  distilled water, and 10  $\mu\text{L}$  PCR product).

The Bgl II digestive products were run by 3% agarose gel electrophoresis for 30 minutes and stained with ethidium bromide. The bands were visualized under ultraviolet light. Digested PCR products yielded visible fragments of 240 and 340 bp of the PCR product containing Bgl II(+), whereas those containing Bgl II(-) would not cut with a single band of 580 bp (Figure 1).

## Genotyping of G894T polymorphism of *eNOS* gene<sup>21</sup>

The amplification reaction was done in 25  $\mu\text{L}$  final volume (10  $\mu\text{L}$  DNA template + 15  $\mu\text{L}$  master mix containing 2.5  $\mu\text{L}$  10 $\times$  PCR buffer, 2.0  $\mu\text{L}$  Mgcl 25 mM, 1.0  $\mu\text{L}$  dNTPs 10 mM, 0.5  $\mu\text{L}$  Taq polymerase 5 U/ $\mu\text{L}$ , 1.0  $\mu\text{L}$  forward primer 20 mM {F 5'-AAGGCAGGAGACAGTGGATGGA-3'}, 1.0  $\mu\text{L}$  reverse primer 20 mM {R 5'-CCCAGTCAATCCCTTTG-GTGCTCA-3'}, and 7  $\mu\text{L}$  distilled water).

PCR amplification was done under the following conditions: one cycle at  $94^\circ\text{C}$  for 5 minutes; five cycles at  $95^\circ\text{C}$  for 20 seconds,  $65^\circ\text{C}$  for 20 seconds, and  $74^\circ\text{C}$  for 20 seconds; five cycles at  $95^\circ\text{C}$  for 20 seconds,  $60^\circ\text{C}$  for 20 seconds, and  $74^\circ\text{C}$  for 20 seconds; five cycles at  $95^\circ\text{C}$  for 20 seconds,  $55^\circ\text{C}$  for 20 seconds, and  $74^\circ\text{C}$  for 20 seconds; five cycles at  $95^\circ\text{C}$  for 20 seconds,  $50^\circ\text{C}$  for 20 seconds, and  $74^\circ\text{C}$  for 20 seconds; and a final extension step of  $74^\circ\text{C}$  for 7 minutes.



**Figure 1** Electrophoresis of digested *ITGA2* (Bgl II) polymerase chain reaction products.

**Notes:** Lane 1: 100 bp ladder; Lanes 2, 7, 8: Bgl II +/- genotype; Lanes 3, 4, 6, 10: Bgl II +/- genotype; Lanes 5, 9: Bgl II +/- genotype.

## Recognition site Ban II

5' ... GRGCY▼C ... 3'

3' ... C▲YCGRG ... 5'

The G894T polymorphism was genotyped using PCR-RFLP using the Ban II digestive enzyme (New England Biolabs, Ipswich, MA). A guanine at nucleotide position 894 results in a glutamic acid at amino acid position 298, while a thymine at nucleotide position 894 results in aspartic acid at amino acid position 298. The PCR product was incubated with the restriction enzyme at 37°C for 4 hours (2 uL 10× buffer, 2.5 uL Ban II, 10 uL PCR product, and 5.5 uL DW). Ban II restriction enzyme produces two fragments of 163 bp and 85 bp in length (Figure 2).

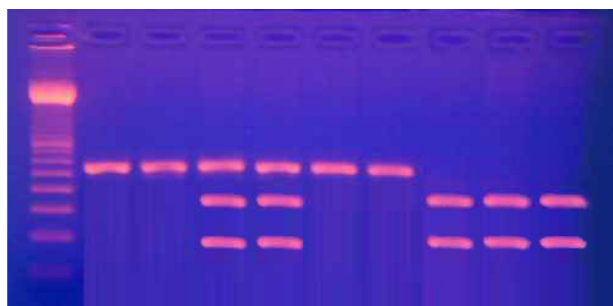
## Statistical analysis

Statistical analysis was made by IBM computer with the aid of Microsoft® Excel (Redmond, WA) and other statistical software programs including SPSS (v 11.5; IBM Corp, Armonk, NY), EpiCalc 2000 (Brixton Health, UK), and Epi Info™ 2000 (Centers for Disease Control and Prevention, Druid Hills, GA).

Qualitative data were expressed in the form of number and percentage and quantitative data were expressed as mean and standard deviation. The following statistical tests were used: chi-square test, a two-way and multiway test of significance to determine the association between two or more qualitative variables; the *t*-test, which assesses whether the means of two groups are statistically different from each other; and multivariate logistic regression analysis for the association of ITGA2 genotype and eNOS genotype with DR and the type of DR.

## Results

In this study, there was no significant statistical difference between the DR group and the DWR group regarding age and sex distribution ( $P > 0.05$ ) (Table 1).



**Figure 2** Electrophoresis of digested G894T polymorphism of eNOS gene polymerase chain reaction products.

**Notes:** Lane 1: 50 bp ladder; Lanes 2, 3, 6, 7: GG genotype; Lanes 8, 9, 10: TT genotype; Lanes 4, 5: CT genotype.

The age of patients ranged from 50 to 64 in the DR group and 54 to 64 in the DWR group. The mean value for DM duration was  $15.4 \pm 7.23$  in the DR group and  $14.4 \pm 3.02$  in the DWR group, so there was no significant statistical difference between the two groups ( $P > 0.05$ ) (Table 1).

Concerning the medical history of the patients, there was no statistical difference between the DR group and DWR group in terms of family history of DM, the presence of hypertension, or smoking ( $P > 0.05$ ), but there was significant statistical difference between the two groups regarding insulin therapy ( $P < 0.001$ ) (Table 2).

The biochemical laboratory profile showed that fasting blood sugar and HbA<sub>1c</sub> levels were significantly higher in the DR group compared with the DWR group ( $P < 0.0001$ ). Concerning the lipid profile of the patients, only serum cholesterol and serum triglyceride levels were significantly higher in the DR group compared with the DWR group, while there was no significant statistical difference between the two groups regarding HDL and LDL levels ( $P > 0.05$ ) (Table 3).

For the BgI II polymorphism of the *ITGA2* gene, in the DR group 18 patients (36%) were ++ genotype, 24 patients (48%) were +/- genotype, and eight patients (16%) were -- genotype; in the DWR group, two patients (10%) were ++ genotype, nine patients (45%) were +/- genotype, and nine patients (45%) were -- genotype. There was significant statistical difference between the two groups concerning the distribution of genetic alleles, with a significantly higher frequency of the ++ genotype in patients with DR. Increased risk for DR was observed in the ++ genotype (odds ratio [OR] -10.1, 95% confidence interval [CI] 1.8–57.9) (Table 4).

For the G894T polymorphism of the *eNOS* gene, in the DR group 13 patients (26%) were GG genotype, 29 patients (58%) were GT genotype, and eight patients (16%) were TT genotype; in the DWR group, two patients (10%) were GG genotype, six patients (30%) were GT genotype, and twelve patients (60%) were TT genotype. There was significant statistical difference between the two groups regarding allele distribution, with a predominance of the G-allele carried in the DR group compared with the DWR group ( $P < 0.001$ ). Increased risk for DR was observed in the GG genotype (OR 9.75, 95% CI 1.7–55.4) (Table 4).

Multivariable logistic regression analysis for the relationship between DR and *ITGA2* polymorphism and *eNOS* polymorphism (with adjustment for factors such as age at onset of diabetes, sex, duration of DM, presence of hypertension, family history of DM, and insulin therapy) showed that

**Table 1** Differences in the sex distribution, age of patients at the onset of diabetes mellitus (DM), and duration of DM between diabetic retinopathy (DR) and diabetic without retinopathy (DWR) patients

Parameters	Studied diabetic patients (n = 70)				Test of significance	P value
	DR group (n = 50)		DWR group (n = 20)			
	n	%	n	%		
Sex						
Male	32	64	13	65	$\chi^2 = 0.006$	>0.05
Female	18	36	7	35	OR (95% CI) = 0.96 (0.32–2.83)	
Age of onset of DM in years (SD)	57.0 $\pm$ 6.52		59.2 $\pm$ 4.28		t = 1.37	>0.05
Duration of DM (SD)	15.4 $\pm$ 7.23		14.4 $\pm$ 3.02		t = 0.61	>0.05

**Abbreviations:**  $\chi^2$ , chi-squared test; CI, confidence interval; OR, odds ratio; SD, standard deviation; t, Student's t-test.

ITGA2 and eNOS polymorphisms have independent effects on the development of DR even after adjustment for all the previously mentioned factors (Table 5).

Based on the type of retinopathy, the DR group was subclassified into an NPDR group and a PDR group. Concerning patient characteristics, there was significant statistical difference regarding the age of onset of DM, with a younger age of onset in the PDR group ( $P < 0.005$ ), while there was no significant statistical difference between the two groups regarding sex distribution. There were no significant statistical difference regarding duration of DM or the interval between the onset of DM and development of retinopathy ( $P > 0.05$ ) (Table 6).

There was also no significant statistical difference between the NPDR group and the PDR group in terms of family history of DM, presence of hypertension, smoking, and insulin therapy (Table 7).

The biochemical laboratory profile showed that fasting blood sugar and HbA<sub>1c</sub> levels did not differ significantly between the two groups ( $P > 0.05$ ), while the lipid profile – including serum cholesterol, serum triglycerides, serum HDL-cholesterol, and serum LDL-cholesterol – was significantly higher in the PDR group ( $P < 0.0001$ ) (Table 8).

For the BgI II polymorphism of the *ITGA2* gene, in the NPDR group nine patients (36%) were ++ genotype, eleven patients (44%) were +/- genotype, and five patients (20%) were -- genotype. In the PDR group, nine patients (36%) were ++ genotype, 13 patients (52%) were +/- genotype, and three patients (12%) were -- genotype. There was no significant statistical difference for BgI II polymorphism between the two groups (Table 9).

For the G894T polymorphism of the *eNOS* gene, in the NPDR group five patients (20%) were GG genotype, 15 patients (60%) were GT genotype, and five patients (20%)

**Table 2** Distribution of present, family, and medical histories between diabetic retinopathy (DR) and diabetic without retinopathy (DWR) patients

Parameters	Studied diabetic patients (n = 70)				$\chi^2$	OR (95% CI)	P value
	DR group (n = 50)		DWR group (n = 20)				
	n	%	n	%			
Family history of DM							
Present	18	36	6	30	0.23	1.31 (0.43–4.01)	>0.05
Absent	32	64	14	70			
Hypertension							
Present	27	54	8	40	1.12	1.76 (0.61–5.05)	>0.05
Absent	23	46	12	60			
Smoking							
Smoker	18	36	4	20	1.70	2.25 (0.65–7.76)	>0.05
Nonsmoker	32	64	16	80			
Insulin therapy							
Present	31	62	4	20	10.08	6.53 (1.90–22.45)	<0.001
Absent	19	38	16	80			

**Abbreviations:**  $\chi^2$ , chi-squared test; CI, confidence interval; OR, odds ratio; SD, standard deviation.

**Table 3** Differences in the biochemical laboratory profile between diabetic retinopathy (DR) and diabetic without retinopathy (DWR) groups

Parameters	Studied diabetic patients (n = 70)		Student's t-test	P value
	DR group (n = 50) (SD)	DWR group (n = 20) (SD)		
Fasting blood sugar level (mg/dL)	156.9 ± 16.42	124.4 ± 7.09	8.52	<0.0001
HbA <sub>1c</sub> (%)	7.8 ± 1.15	6.3 ± 0.79	5.32	<0.0001
Serum cholesterol (mg/dL)	242.7 ± 40.8	215.8 ± 20.6	2.80	<0.01
Serum triglycerides (mg/dL)	169.6 ± 17.89	147.8 ± 23.31	4.21	<0.0001
Serum HDL (mg/dL)	33.7 ± 2.05	33.0 ± 1.00	1.56	>0.05
Serum LDL (mg/dL)	174.9 ± 37.91	158.3 ± 26.27	1.80	>0.05

**Abbreviations:** HbA<sub>1c</sub>, hemoglobin A<sub>1c</sub>; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SD, standard deviation.

were TT genotype. In the PDR group, eight patients (32%) were GG genotype, 14 patients (56%) were GT genotype, and three patients (12%) were TT genotype. There was no statistical difference concerning G894T polymorphism between the two groups (Table 9).

Multivariable logistic regression analysis for the relationship between the type of retinopathy, whether proliferative or nonproliferative, and ITGA2 and eNOS polymorphism after adjustment for age at onset of DM, sex, duration of DM, gap between onset of DM and development at retinopathy, the presence of hypertension, family history of DM, and insulin therapy, showed that ITGA2 and eNOS polymorphisms still have no effect on the type of retinopathy (Table 10).

## Discussion

DM is a worldwide medical problem and is a significant cause of morbidity. Microvascular and macrovascular complications are highly prevalent among diabetic patients.<sup>22</sup>

DR, one of the most significant complications of diabetes, has become the leading cause of vision loss and blindness in both developed and developing countries.<sup>23</sup> Visual loss results

mainly from central macular edema and less frequently from PDR. The development of these pathological changes is strongly related to hyperglycemia.<sup>24</sup>

Although the Diabetes Control and Complications Trial showed that tight control of hyperglycemia can reduce the incidence of retinopathy, it is clear that hyperglycemia alone does not explain the development of this complication, as it may be absent in some patients with poor glycemic control even over a long period, while others may develop retinopathy in a relatively short period despite good glycemic control.<sup>25</sup> These observations suggest the presence of hereditary factors. The strongest evidence for the genetic predisposition towards DR has been derived from twin, family, and transracial studies.<sup>26</sup>

The search for DR genes has predominantly been undertaken using the candidate gene approach. Several pathways and processes have been strongly implicated, including the renin-angiotensin system, the polyol pathway, nonenzymatic glycation, endothelial dysfunction, vascular tone maintenance, extracellular matrix remodeling, and angiogenesis, which is dysregulated in diabetes leading to proliferation of new fragile capillaries that culminate in PDR.<sup>27</sup>

**Table 4** Association of ITGA2 genotype and eNos genotype with diabetic retinopathy

Parameters	Studied diabetic patients (n = 70)				$\chi^2$	OR (95% CI)	P value
	DR (n = 50)		DWR (n = 20)				
	n	%	n	%			
ITGA2 genotype							
+/-	18	36	2	10	8.35	10.1 (1.8–57.9)	<0.01
+/-	24	48	9	45			
--	8	16	9	45			
eNOS genotype							
GG	13	26	2	10	13.63	9.75 (1.7–55.4)	<0.001
GI	29	58	6	30			
TT	8	16	12	60			

**Abbreviations:** DWR, diabetics without retinopathy; OR, odds ratio; SD, standard deviation.

**Table 5** Multivariate logistic regression analysis for the association of ITGA2 genotype and eNOS genotype with diabetic retinopathy (DR)

Parameters	B-coefficient	SE	Odds ratio	P value
Age of onset of diabetes	0.08	0.06	1.08	0.22
Sex	-0.04	0.69	0.96	0.95
Duration of DM	-0.04	0.06	0.96	0.51
Hypertension	0.88	0.68	2.41	0.20
Family history of DM	0.04	0.71	1.04	0.96
Insulin therapy	1.18	0.73	3.26	0.001
(ITGA2) genotype	0.77	0.51	2.16	0.004
eNOS genotype	0.66	0.54	1.93	0.001
Constant	-11.24	4.58	-	-

**Abbreviations:** DM, diabetes mellitus; SE, standard error.

Correspondingly, host genes involved in these pathway processes have been treated as potential candidate genes. These genes include the angiotensin-converting enzyme, vascular endothelial growth factor (VEGF), aldose reductase, the receptor for advanced glycation end products, glucose transporter-1, inducible and constitutive NO synthases (NOS2A, NOSB), transforming growth factor beta, and endothelin isoforms and their cellular receptors.<sup>26</sup>

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 signaling pathway.<sup>7</sup> It also inhibits platelet as well as leukocyte adhesion to vascular endothelium and inhibits proliferation of smooth muscle cells via guanylate cyclase-independent mechanisms.<sup>27</sup> It has been demonstrated, in vivo and in vitro, that overproduction of NO may induce oxidative stress in retinal, endothelial, and glomerular cells.<sup>7</sup> Also, it has been experimentally shown that NO contributes to angiogenic properties of VEGF, a

molecule implicated in the development of proliferative retinopathy.<sup>3</sup>

Since platelets are essential for hemostasis, and abnormalities of platelet function may cause vascular abnormalities, platelets are thought to be involved in the pathogenesis of microvascular complications in diabetes.<sup>8</sup> Platelets from diabetic patients are hyperreactive to aggregating agents such as collagen, thrombin, and adenosine diphosphate. The platelet membrane glycoprotein Ia/IIa,  $\alpha_2\beta_1$  integrin, serves as a platelet receptor for collagen.<sup>20</sup> It mediates platelet primary adhesion to subendothelial tissues, which is an essential step in thrombus formation.

To examine the hypothesis that genetic variation of  $\alpha_2\beta_1$  integrin is associated with the development of diabetic microangiopathy, the association between the Bgl II polymorphism and development of DR among T2D patients was also investigated in this study. There was no significant statistical difference between the DR group and the DWR group regarding the duration of DM and that duration did not statistically affect the type of retinopathy. These findings are in contrast to Roy's<sup>28</sup> and Longo-Mbenza et al's,<sup>29</sup> who found that the frequency and severity of DR were significantly associated with longer duration of diabetes.

Results of the present study showed that there is significant statistical difference between the NPDR and PDR groups concerning the age of onset of DM, with younger age of onset ( $54.52 \pm 6.48$  years) for PDR compared with NPDR ( $59.7 \pm 5.41$  years). This is in agreement with Li et al,<sup>6</sup> who showed that subjects who were younger at DM onset had a higher risk of PDR than subjects with later onset of DM.

Further, the present study showed that fasting blood sugar and HbA<sub>1c</sub> levels were significantly higher in the DR group

**Table 6** Differences in the sex distribution, age of patients at onset of diabetes mellitus (DM), duration of DM, and the interval between onset of DM and onset of retinopathy, between patients with proliferative and nonproliferative diabetic retinopathy (PDR and NPDR, respectively) groups

Parameters	DR patients (n = 50)				Test of significance	P value
	NPDR group (n = 25)		PDR group (n = 25)			
	(SD)		(SD)			
	n	%	n	%		
Sex						
Male	15	60	17	68	$\chi^2 = 0.35$	>0.05
Female	10	40	8	32	OR (95% CI) = 0.71 (0.22–2.25)	
Age at onset of DM in years	59.7 $\pm$ 5.41		54.2 $\pm$ 6.48		t = 3.25	<0.005
Duration of DM	15.5 $\pm$ 8.31		15.4 $\pm$ 6.14		t = 0.06	>0.05
Interval between onset of DM and onset of retinopathy	5.9 $\pm$ 2.42		5.5 $\pm$ 1.92		t = 0.58	>0.05

**Abbreviations:**  $\chi^2$ , chi-squared test; CI, confidence interval; OR, odds ratio; SD, standard deviation; t, Student's t-test.

**Table 7** Distribution of present, family, and medical histories between patients with proliferative and nonproliferative diabetic retinopathy (PDR and NPDR, respectively)

Parameters	DR patients (n = 50)				$\chi^2$	OR (95% CI)	P value
	NPDR (n = 25)		PDR (n = 25)				
	n	%	n	%			
Family history of DM							
Present	10	40	8	32	0.35	1.42	>0.05
Absent	15	60	17	68		(0.44–4.52)	
Hypertension							
Present	15	60	12	48	0.73	1.63	>0.05
Absent	10	40	13	52		(0.53–4.98)	
Smoking							
Smoker	7	28	11	44	1.39	0.49	>0.05
Nonsmoker	18	72	14	56		(0.15–1.61)	
Insulin therapy							
Present	13	52	18	72	2.12	0.42	>0.05
Absent	12	48	7	28		(0.13–1.36)	
Macular edema							
Present	12	48	12	48	0.08	1.00	>0.05
Absent	13	52	13	52		(0.33–3.03)	

**Abbreviations:**  $\chi^2$ , chi-squared test; CI, confidence interval; DM, diabetes mellitus; DR, diabetic retinopathy; OR, odds ratio; SD, standard deviation.

compared with the DWR group; however, those levels did not differ between NPDR group PDR group, which is in agreement with the findings of Lecaire et al,<sup>30</sup> Axer-Siegel et al,<sup>31</sup> Higgins et al,<sup>32</sup> and Longo-Mbenza et al,<sup>29</sup> who all observed higher levels of blood glucose and HbA<sub>1c</sub> in diabetic patients with DR compared with those without DR.

The results of this study also showed that only serum cholesterol and triglyceride levels were significantly higher in the DR group compared with the DWR group; there were no significant statistical differences between the groups regarding HDL and LDL levels. Regarding type of retinopathy, all elements of the lipid profile, including serum cholesterol, triglycerides, HDL, and LDL, were significantly higher in the PDR group. Lyons et al<sup>33</sup> found that there was no significant association between plasma triglyceride and cholesterol

levels between their DR group and patients without retinopathy, while there was a significant positive association with LDL and significant negative association with HDL. Rianta et al<sup>34</sup> found significant association between lipid profile and DR but no significant association between lipid profile and the severity of the disease.

In the present study, for the G894T polymorphism of the *eNOS* gene, there was a significant statistical difference between the DR and DWR groups regarding the genotype distribution, with a predominance of G-allele carriers in the DR group. Increased risk for DR was observed in patients with GG genotype (OR 9.75, 95% CI 1.7–55.4). However, there was no statistical difference concerning the genotypes of the polymorphism between the PDR and NPDR groups. This is in agreement with Chen et al,<sup>7</sup> who found that the GG genotype is

**Table 8** Differences in the biochemical laboratory profile between patients with proliferative and nonproliferative diabetic retinopathy (PDR and NPDR, respectively)

Parameters	DR patients (n = 50)		Student's t-test	P value
	NPDR (n = 25) (SD)	PDR (n = 25) (SD)		
Fasting blood sugar level (mg/dL)	161.2 ± 16.38	152.6 ± 15.62	1.89	>0.05
HbA <sub>1c</sub> (%)	7.9 ± 1.39	7.6 ± 0.82	1.23	>0.05
Serum cholesterol (mg/dL)	213.4 ± 18.85	272.0 ± 35.47	7.30	<0.0001
Serum triglycerides (mg/dL)	158.7 ± 15.86	180.5 ± 12.41	5.41	<0.0001
Serum HDL (mg/dL)	34.6 ± 2.16	32.8 ± 1.45	3.61	<0.001
Serum LDL (mg/dL)	149.9 ± 22.63	199.9 ± 33.46	6.19	<0.0001

**Abbreviations:** DM, diabetes mellitus; DR, diabetic retinopathy; HbA<sub>1c</sub>, hemoglobin A<sub>1c</sub>; SD, standard deviation.



**Table 9** Association of integrin  $\alpha_2\beta_1$  genotype (ITGA2) and eNOS genotype with the type of diabetic retinopathy (DR)

Parameters	DR patients (n = 50)				$\chi^2$	OR (95% CI)	P value
	NPDR (n = 25)		PDR (n = 25)				
	n	%	n	%			
ITGA2 genotype							
+/+	9	36.0	9	36.0	0.67	0.60 (0.11–3.30)	>0.05
+/-	11	44.0	13	52.0		0.51 (0.10–2.62)	
-/-	5	20.0	3	12.0		–	
eNOS genotype							
GG	5	20.0	8	32.0	1.23	0.38 (0.06–2.30)	>0.05
GT	15	60.0	14	56.0		0.64 (0.13–3.20)	
TT	5	20.0	3	12.0		–	

**Abbreviations:**  $\chi^2$ , chi-squared test; CI, confidence interval; NPDR, nonproliferative diabetic retinopathy; OR, odds ratio; PDR, proliferative diabetic retinopathy.

associated with increased risk for DR. Suganthalakshmi et al<sup>3</sup> found that the genotypes of eNOS did not differ significantly between their DR and DWR study groups. However, it was significantly associated with the severity of DR in Caucasian populations. Awata et al<sup>10</sup> found that the eNOS polymorphism was not associated with the presence of DR nor the type of DR, whether PDR or NPDR. Rippin et al<sup>35</sup> found no association between the eNOS polymorphism and the development of diabetic microvascular complications in UK Caucasian populations. Li et al<sup>6</sup> showed no association between the eNOS polymorphism and the occurrence or the type of DR. Miyamoto et al<sup>21</sup> found limited information about whether this missense mutation gives rise to functional alteration of eNOS enzymatic activity or a genetic marker associated with some causal loci. Thus, they investigated the Glu298Asp variants and did not find them to be located in any functional consensus sequence. However, computer analysis revealed that the Glu298Asp mutation results in a conformational change in the eNOS protein from helix to tight turn.

**Table 10** Multivariate logistic regression analysis for the association of ITGA2 genotype and eNOS genotype with the type of diabetic retinopathy

Parameters	B-coefficient	SE	Odds ratio	P value
Age of onset of diabetes	-0.16	0.06	0.86	0.01
Sex	-0.33	0.74	0.72	0.66
Duration of DM	0.01	0.07	1.01	0.93
Gap between onset of DM and retinopathy	-0.08	0.21	0.92	0.70
Hypertension	0.56	0.83	1.75	0.50
Family history of DM	-0.07	0.74	0.94	0.93
Insulin therapy	-0.27	0.75	0.77	0.72
(ITGA2) genotype	-0.09	0.53	0.92	0.87
eNOS genotype	-0.11	0.56	0.90	0.85

**Abbreviations:** DM, diabetes mellitus; SE, standard error.

The eNOS protein is a member of cytochrome P<sub>450</sub> reductase and is composed of a heme-binding region, a Ca/calmodulin binding region, and nicotinamide adenine dinucleotide phosphate-cytochrome P<sub>450</sub> oxidoreductase. The Glu298Asp variant in exon 7 is located in the intermediate portion of the heme-binding and Ca/calmodulin binding sites, thus alters the activity of the enzyme.<sup>36</sup>

Du et al<sup>37</sup> have demonstrated that in patients with DR, the levels of NO in plasma, vitreal fluid, and aqueous fluid are increased. However, although eNOS was localized in Müller cells and in the vascular endothelium of the retina, both in vivo and in vitro studies revealed that the level of the constitutively expressed eNOS was reduced in retinal vascular endothelial cells when diabetes was present.<sup>22</sup> It was suggested that both high glucose levels and osmotic stress in the retinal endothelial cells of diabetics may increase inducible NOS activity and generate an uncoupled eNOS, resulting in the overproduction of NO and, subsequently, its derivatives as peroxynitrite.<sup>7</sup> Excess amounts of peroxynitrite result in oxidative stress and generate cytotoxic effects by increasing DNA damage, stimulating lipid peroxidation, and depleting glutathione levels.<sup>38</sup> Furthermore, NO is known to increase vascular permeability and angiogenesis, a major feature of advanced DR.<sup>39</sup> Thus, it was thought that in diabetics, high glucose levels and osmotic stress may directly cause downregulated expression of eNOS in subjects with the GG genotype, which in turn activates an uncoupled overproduction of NO and its high reactive oxidants. However, in subjects with the AA genotype, NO is produced mainly from the constitutively expressed eNOS with uncoupled expression relatively suppressed.<sup>7</sup>

Several pathological pathways could contribute to the development of DR. Decreased activity of eNOS will lead to deficiency of endothelial-derived NO, coupled

with concomitant activation of inducible NOS, which can produce a large amount of NO. This NO, when encountering reactive oxygen species, can generate highly reactive oxygen species, leading to oxidative stress in retinal tissues.<sup>40</sup> A second pathway is that decreased activity of eNOS increases expression of glial fibrillary acidic protein in Müller cells in diabetic retina. Such upregulation of glial fibrillary acidic protein is an early event in the pathogenesis of retinopathy and precedes the onset of vascular changes in the disease.<sup>41</sup> A third pathway involves the observation that the G894T polymorphism is associated with the development of essential hypertension, an important independent risk factor for both initial development and subsequent progression of DR.<sup>42</sup> Lastly, hypoxia-induced angiogenesis is an important pathology in DR. A number of factors are expressed in hypoxic conditions and have been shown to trigger the hypoxia response elements of the *VEGF* gene, bringing about overexpression of VEGF that leads to neovascularization. eNOS is one such important part of hypoxia events. Knockout animal models of eNOS have shown poor angiogenesis, suggesting the contribution of eNOS to DR pathology.<sup>43</sup>

In this study, regarding the BgI II polymorphism of the *ITGA2* gene, there was a significant statistical difference between the DR and DWR groups in the distribution of genetic alleles, with a significantly higher frequency of the genotype *+/+* in patients with DR. Increased risk of DR was observed in BgI II+ allele carriers (OR 10.1, 95% CI 1.8–57.9), while in terms of the type of retinopathy, there was no significant statistical difference between the NPDR and PDR groups regarding genotype distribution. Matsubara et al<sup>44</sup> stated that the BgI II polymorphism is associated with the prevalence of retinopathy among patients with T2D and suggested that BgI II(+)-containing platelets can more easily interact with nonenzymatically glycosylated collagen, which is higher in diabetic patients, enhancing microthrombus formation and small vessel occlusion and thus accelerating the occurrence of retinopathy. Therefore, BgI II(+) allele carriers might benefit more from antiplatelet therapies. Petrovic et al<sup>20</sup> also provided evidence for the association of BgI II(*+/+*) genotype and risk for DR in a group of Caucasians with T2D; this was in contrast to Li et al,<sup>6</sup> who found no significant difference between the DR and DWR groups regarding genotype or allele distribution of *ITGA2* polymorphism.

## Conclusion

This study observed a significant association between the G894T polymorphism of eNOS gene and the BgI II polymorphism of *ITGA2* gene and DR, while there was no association

between the genetic variants of those two polymorphisms and the type of retinopathy.

Identification of genetic variations contributing to DR disease pathogenesis would have tremendous implications in terms of clinical intervention and management of the disease. A number of inhibitors to key molecules that are involved in detrimental pathways of DR, such as anti-VEGF, antiplatelet, anti-protein kinase C, and anti-insulin-like growth factor, are under research, so specific management of the disease will depend upon each patient's genetic and environmental profile rather than generalized treatment of laser photocoagulation.<sup>38</sup>

## Disclosure

The authors report no conflicts of interest in this work.

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