Diabetic Retinopathy and IGF-1 Gene Polymorphic Cytosine-Adenine Repeats in a Southern Indian Cohort


Key Words
Diabetic retinopathy · IGF-1 gene · Proliferative diabetic retinopathy · Genetic association studies

Abstract

Background/Aims: Growth factors have been implicated in the pathogenesis of diabetic retinopathy (DR). IGF-1 is known to trigger a critical cascade of molecular events that initiate retinal angiogenesis. Increased vitreous IGF-1 levels have been correlated with the severity of ischemia-associated diabetic retinal neovascularization. In the present study, a cytosine-adenine (CA)ₙ repeat in the promoter of the IGF-1 gene is studied for association with DR. Methods: A total of 127 patients with retinopathy (cases: DR+) and 81 patients without retinopathy (controls: DR–) who had type 2 diabetes were recruited for the study. Patients underwent detailed clinical examination and DR was graded based on stereoscopic digital fundus photographs. Frequencies of alleles and genotypes between the two groups were analyzed for significance using relevant statistical tests. (CA)₁₇ and (CA)₁₈ repeats were the more frequent alleles. Results: The frequency of the 18-repeat genotype was significantly higher in DR+ patients when compared to DR– patients and found to confer a 2.4 times (95% CI: 1.2–5.0) and 2.8 times (95% CI: 1.1–7.5) higher risk for developing DR and proliferative DR, respectively, when compared to <18-repeat genotypes.

Conclusions: Our study suggests that the 18-repeat genotype is a susceptibility genotype for DR and its clinical severity in a Southern Indian cohort.
is IGF-1 [1]. Studies on transgenic mouse models have shown the presence of retinal neovascularization associated with VEGF expression by increased induction of IGFl in retinal glial cells [2]. Simo et al. [3] observed a significant increase in vitreous IGFl levels in proliferative DR (PDR) patients when compared to controls. Increased serum IGFl levels leading to acceleration of the progression of DR to a sight-threatening status have been observed following insulin therapy in patients with diabetes; this is attributed to the insulin-induced activation of the growth hormone/IGFl axis [4]. Chantelau and Frystyk [5] have clearly observed that progression of DR in patients with good control of hyperglycemia through intensive insulin therapy could be treated by reduced insulin and somatostatin analogue management.

The IGFl gene is located on chromosome 12q22–23 [6]. It spans over 45 kb and has 5 exons. Two transcripts, IGFl-A (153 amino acids) and IGFl-B (190 amino acids) made up of exons 1, 2, 3, 5 and 1, 2, 3, 4, respectively, are produced due to alternate RNA splicing, as isoforms [6]. A cytosine-adenine (CA) repeat polymorphism, 1 kb upstream to the transcription site, has been studied for association of the CA repeat polymorphism in the IGFl-A (153 amino acids) and IGFl-B (190 amino acids) gene promoter with DR in a Southern Indian cohort.

**Materials and Methods**

A total of 127 unrelated subjects with DR (DR+) and 81 unrelated control subjects diagnosed with diabetes (DR–) were enrolled from a population-based study conducted in rural Tamil Nadu and urban Chennai city, Southern India, constituting an ethnically homogenous population [11, 12]. All patients had type 2 diabetes for more than 15 years. A history on details such as age, sex, duration of diabetes, BMI, systolic and diastolic blood pressures, and insulin use among others was documented. Glycosylated hemoglobin (HbA1c) and microalbuminuria were estimated by DIASTAT® autoanalyzer (Bio-Rad Laboratories, Inc., India) and Clinitek 50® urine chemistry analyzer (Bayer Diagnostics, India), respectively. Clinical examination was done by indirect ophthalmoscopy and 45-degree four-field stereoscopic digital photographs (posterior pole, nasal field, superior and inferior) were taken for all patients. Additional 30-degree seven-field digital stereo pairs were taken for patients with DR. Severity of DR was graded as per the modified classification used by Klein et al. [13] based on stereoscopic photographs of the fundus as described by Agarwal et al. [12]. The nature of the study was clearly explained and blood was collected from all subjects after obtaining informed consent. The study was approved by our Institutional Ethics Board and was in accordance with the Declaration of Helsinki as revised in 1996 [14].

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DNA was extracted by the conventional phenol-chloroform method [15]. The promoter region spanning the CA repeat was amplified using forward (6-FAM 5'-GCTAGCAGCTGTGTTATATT-3') and reverse (5'-ACCACTCTGGGAAGGGTA-3') primers [8]. The PCR protocol consisted of initial denaturation at 94°C for 5 min followed by 25 cycles, each comprising a 30-second denaturation step at 94°C, a 45-second primer annealing step at 55°C and a 1-min extension at 72°C followed by final extension at 72°C for 7 min. The products were run on an ABI PRISM 3100 Avant genetic analyzer with GeneScan™ 500 LIZ™ size standard (Perkin-Elmer, Foster City, Calif., USA) to compare the sizes of the amplified products. The run was analyzed using GeneMapper software version 3.7 (ABI Applied Biosystems, Foster City, Calif., USA).

**Statistical Analyses**

The χ² and Fisher’s exact tests were used to compare genotype frequencies between DR+ and DR– groups and the t test was used for the continuous variables. The Wilcoxon two-sample test was used to assess the differences in allele length between DR+ and DR– groups. The odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using a multivariate logistic regression model adjusting for age, duration of diabetes, HbA1c level, use of insulin and BMI. The alleles were built into genotypes by longer CA repeats. They were categorized into ≤17-, 18- and ≥19-repeat genotypes [8]. Subjects carrying genotypes 10/10, 14/14, 15/15, 16/16 and 17/17 were considered as homozygotes in the ≤17 category. Heterozygotes in the ≥17 group were those who carried an allele along with a longer allele (e.g.: 10/14…20, 14/15…20, 15/16…20, 16/17…20, and so on). Likewise, genotypes were built in 18-repet and ≥19-repet genotype categories. The association of the 18-repeat genotype was assessed using a multivariate logistic regression model adjusting for age. The gene dosage effect for the 18-repeat genotype was tested by the Mantel-Haenszel χ² test. A χ² test for proportions with a two-sided significance level of 0.05 was used to calculate the power of the analysis. Two-sided p values of less than 0.05 were considered to indicate statistical significance. SAS 9.1 software (SAS Institute, Cary, N.C., USA) was used for all statistical analyses.

**Results**

Of the 127 DR+ subjects, 85 (66.9%) had nonproliferative DR (NPDR) and 42 (33.1%) had PDR. Forty-one (48.2%) of the 85 subjects with NPDR had clinically significant macular edema compared to 13 (31.0%) out of 42 subjects with PDR.

The baseline characteristics of the DR+ and DR– patients are presented in table 1. The mean age of the DR+ subjects was 59.9 years (SD = 7.4) and of the DR– subjects it was 65.2 years (SD = 8.9). This difference was statistically significant (p < 0.001). The average BMI (p = 0.04) and systolic blood pressure (p = 0.05) were slightly high for DR– subjects. Use of insulin (p = 0.005) and abnormal microalbuminuria (p < 0.001) were high in DR+ subjects.
Gender, duration of diabetes, HbA1c level, and diastolic blood pressure level were not statistically significant between DR+ and DR− subjects.

Eight alleles of CA repeats of the IGF-1 gene were identified in the study cohort ranging from 10 to 20 repeats in the DR+ and DR− subjects. There was no significant difference in mean CA repeats between DR+ and DR− patients (17.4 ± 1.1 vs. 17.3 ± 0.9, p = 0.42). The alleles 17 and 18 were more frequently observed in the DR+ and DR− subjects compared to the other alleles (table 2). The allele frequency of the (CA)18 repeat was high in DR+ patients (25.2%) compared to DR− subjects (20.4%). However, this difference was not statistically significant (p = 0.26). The allele frequency of the 17-repeat allele between DR+ and DR− patients (50.0 vs. 52.5%) was not statistically significant (p = 0.63) either.

When genotypes were categorized by the longer CA repeat alleles, the genotype frequency of the 18-repeat in DR+ subjects was significantly higher when compared to DR− subjects (table 3). When adjusted for age, duration of diabetes, HbA1c level, use of insulin, BMI and blood pressure, subjects with the 18-repeat genotype seemed to have a 2.4 times increased risk (95% CI: 1.2–5.0) of having DR when compared to <18-repeat genotypes. Based on the observed risk (OR = 2.4) for the development of DR for subjects with the presence of the 18-repeat genotype, this study was found to have 76% power to detect the above OR. We also analyzed the distribution of the 18-repeat genotype between DR+ and DR− subjects with the presence of the 18-repeat genotype, this study was found to have 76% power to detect the above OR. We also analyzed the distribution of the 18-repeat genotype between DR+ and DR− patients among the insulin-treated and similarly among untreated patients, but found no significant results (OR = 2.9; 95% CI: 0.4–21.6 and OR = 2.0; 95% CI: 0.9–4.3, respectively). To assess the association of the 18-repeat genotype with the progression of the disease, we further classified DR+ subjects into NPDR and PDR. Subjects with the 18-repeat genotype were found to have a 2.8 times (95% CI: 1.1–7.5) higher risk for the development of PDR when compared with ≤17-repeat genotypes (table 3). However, there was no statistical difference in the distribution of 18-repeat genotypes amongst patients with and without clinically significant macular edema.

When we looked at the homozygous and heterozygous state of 18-repeat genotypes, we found that there was a 2.5 times (95% CI: 0.5–11.4) higher risk of having DR for the homozygous group and a 1.5 times (95% CI: 0.8–2.7) higher risk for the heterozygous group when compared to noncarriers of this genotype; however, this difference was not statistically significant (table 4). A nonsignificant similar risk was also obtained in subjects with the 18-repeat genotype when compared with noncarriers (OR = 1.5, 95% CI: 0.8–2.8). The gene dosage effect for the 18-repeat genotype was not statistically significant (p = 0.24).

Discussion

IGF-1 has been shown to have a vital role in the pathogenesis of DR. Kitamei et al. [16] reported characteristic retinal neovascularization in a 12-year-old girl with the severe insulin resistance syndrome, leprechaunism,
treated with recombinant human IGF-1. Poulaki et al. [1] showed through cell culture and animal studies that IGF-1 induced VEGF expression primarily through PI-3K/Akt, HIF-1α/HIF-2α, and NF-κB and secondarily through JNK/AP-1 induction, thereby contributing to DR pathogenesis. IGF-1 receptor knockout studies on vascular endothelial cells have shown protection against neovascularization of the retina suggesting a pathogenic role of IGF-1[17]. Investigations on the effect of neutralizing antibodies to IGF-1 receptor in in vivo models showed a significant reduction in blood-retina barrier breakdown, a process thought to be an important feature of the retinal pathology in diabetes that starts with integrin/ICAM-1-dependent adhesion of leukocytes to retinal endothelium [1]. Contrary to these findings, Laron and Weinberger [18] reported DR in 2 patients with homozygous mutations in the growth hormone receptor leading to congenital IGF-1 deficiency (Laron syndrome), which is marked by failure to produce IGF-1 due to defective growth hormone receptor. Whether IGF-1 has contrasting roles in retinal neovascularization in the extremes of its production spectrum, however, needs further investigation.

The (CA)₁₉ repeat allele has been significantly associated with breast cancer, prostate cancer and benign prostatic hyperplasia [8, 19]. However, in another study, non-carriers of the 19- and 20-repeat genotypes were found to have higher concentrations of low-density lipoprotein levels, a risk factor for cardiovascular disease and type 2 diabetes mellitus [10]. Vaessen et al. [9] observed significantly lower serum levels of IGF-1 in patients with type 2 diabetes mellitus and myocardial infarction who were non-carriers of 19-repeat alleles when compared to those who were homozygous for the repeat. We investigated in this study the association of CA repeat polymorphism with DR in a Southern Indian cohort.

Our results clearly suggest that the 18-repeat genotype is a risk marker for DR in our population. It was also found to have a significant association with clinical severity of the disease, like PDR. Mean age and BMI were significantly high in control subjects and did not predict risk for DR. Not surprisingly, the DR+ cohort in our study had a higher percentage of abnormal/highly abnormal microalbuminuria, as it has been shown to be associated with DR [20]. Despite this, regression analyses evidently showed that the genotype of an individual was independently associated with a higher risk for developing DR. When we further categorized DR+ into clinical phenotypes such as NPDR and PDR, we found a predisposition to develop the proliferative status in those carrying the 18-repeat genotype (OR = 2.8; 95% CI: 1.1–7.5). The CA repeat polymorphism of the IGF-1 gene has recently been studied by the Rotterdam study group [21]. They analyzed the distribution of the repeat polymorphism at baseline in patients with impaired glucose tolerance and diabetes. They took 19 and 20 repeats as wild type and other repeats as variants and found a modest association of variant genotypes with susceptibility and progression of DR. These findings were correlated with DR at a mean follow-up of 6.5 years from baseline. In contrast, all our

### Table 3. ORs with respect to IGF-1 genotype for DR+ and DR− patients adjusting for age, duration of diabetes, glycosylated Hb level, use of insulin, BMI and blood pressure

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DR+ (n = 27)</th>
<th>DR− (n = 81)</th>
<th>OR; 95% CI</th>
<th>NPDR (n = 85)</th>
<th>PDR (n = 42)</th>
<th>OR; 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 17</td>
<td>46 (36.2)</td>
<td>41 (50.6)</td>
<td>1.0</td>
<td>36 (42.4)</td>
<td>10 (23.8)</td>
<td>1.0</td>
</tr>
<tr>
<td>18</td>
<td>48 (37.8)</td>
<td>23 (28.4)</td>
<td>2.4; 1.2–5.0</td>
<td>29 (34.1)</td>
<td>19 (45.2)</td>
<td>2.8; 1.1–7.5</td>
</tr>
<tr>
<td>≥ 19</td>
<td>33 (26.0)</td>
<td>17 (21.0)</td>
<td>2.0; 0.9–4.5</td>
<td>20 (12.5)</td>
<td>13 (31.0)</td>
<td>2.6; 0.9–7.5</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate percentages.

1 The genotypes were categorized by the longer CA repeat allele.

### Table 4. ORs (95% CI) for the 18-repeat genotype in DR+ and DR− patients adjusting for age

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DR+ (n = 127)</th>
<th>DR− (n = 81)</th>
<th>OR; 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noncarrier</td>
<td>69 (54.3)</td>
<td>51 (63.0)</td>
<td>1.0</td>
</tr>
<tr>
<td>Homozygous</td>
<td>6 (4.7)</td>
<td>3 (3.7)</td>
<td>2.5; 0.5–11.4</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>52 (40.9)</td>
<td>27 (33.3)</td>
<td>1.5; 0.8–2.7</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate percentages.

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study subjects had been diabetic for more than 15 years and therefore the likelihood of patients in the control group developing retinopathy was far less and the association found in the present study was with the 18-repeat genotype. This is also the first report, to the best of our knowledge, to associate the longer repeats of CA with risk for developing DR. However, dividing the DR+ group into NPDR and PDR made the sample size quite small and therefore, we suggest that genotyping a larger sample size is essential to validate the association of the 18-repeat genotype with PDR.

By and large, there does not seem to be a uniformity in the association of the promoter polymorphisms with various diseases such as breast and prostate cancers, myocardial infarction, type 2 diabetes mellitus, or DR. We attempted a bioinformatics analysis using P-match software (version 1.0) to explore the polymorphic repeat region for the presence of any putative transcription factor binding sites [22] and found that this region does not harbor a binding site for any transcription factor. Functional studies explaining the precise effect of these polymorphic repeats on the gene expression have not yet been reported. Therefore, the role that the CA repeat number plays in the disease pathology is not clearly understood. The present study is preliminary and does not explain the functional aspects of the variation with respect to its impact on the gene expression. Moreover, the 18-repeat genotype may not be the true disease variant and may probably be in linkage disequilibrium with the true disease-causing variant. Therefore, studies that delineate the mechanism by which the promoter polymorphism possibly influences gene expression and screening of this gene for other known and unknown genetic markers would be the future scope of the present study. This would help us understand the underlying disease mechanism and gain an insight into the possibility of developing genotype-specific management of the disease.

In conclusion, this is the first report of an association of the IGF-1 promoter (CA)18 repeat genotype with high risk for developing DR and PDR in a Southern Indian sample cohort. Since our study cohort represented the Southern Indian population, our results could be considered for further investigations from multiple centers across the country to understand the involvement of this polymorphism as a possible genetic marker for the development of DR in India.

Acknowledgements

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