18bp Fragment Insertion/Deletion Polymorphism of Vascular Endothelial Growth Factor (VEGF) Gene with Diabetes Mellitus Type 2 and Diabetic Retinopathy Patients of Quetta, Balochistan, Pakistan

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Abstract

**Background:** An important candidate gene for diabetic retinopathy (DR) is vascular endothelial growth factor (VEGF). The VEGF gene is extremely polymorphic. The 18 base pairs (bp) segment (I/D) polymorphism at -2549 position of the promoter region is of great importance.

**Aim:** The present study aimed to identify VEGF (I/D) polymorphism in DM and DR patients.

**Materials and Methods:** This cross section study involved 100 healthy control subjects, 100 diabetes mellitus (DM) subjects and 100 DR subjects. Blood samples were collected after informed consent of study subjects. DNA extraction was performed using inorganic method, VEGF gene promoter region that was confirmed using 2% agarose gel. Polymerase Chain Reaction (PCR) was used to identify (I/D) polymorphism of 18 bp fragment at position -2549, DNA Sequencing was done commercially to confirm the presence of 18bp I/D polymorphism. The allele (I/D) and genotypes (DD, I/D, II) frequencies of VEGF gene were compared among all the study subjects.

**Results:** The frequency of DD genotype in DR was 52% while in DM was 40% and in control was 2%. The significant differences (p<0.05) were observed when genotypes were compared among control and DM, DM and DR, Control and DR. The significant differences (p<0.05) were observed in Control and DM Control and DR, DM and DR at 95% CI.

**Conclusion:** These findings suggest that the DD genotype is possible risk factor for development and progression of retinopathy as compared to uncomplicated subject II genotype in local population.

**Keywords:** Diabetes mellitus; Diabetic retinopathy; Vascular endothelial growth factor gene; Insertion/Deletion; Polymorphism

**Abbreviations:** DM: Diabetes Mellitus; DR: Diabetic Retinopathy; VEGF: Vascular Endothelial Growth Factor Gene; I/D Polymorphism: Insertion/Deletion Polymorphism; DD: Deletion Genotype

Introduction

Uncontrolled glycemic level in DM patients is associated with certain microvascular complications [1,2]. Diabetic retinopathy (DR) is a microvascular complication of diabetes that is associated with the retina of eye. In DR either peripheral retina or macula is affected, or both are affected. It is most dreadful ocular complication that is associated with diabetes. DR usually results in complete loss of vision if not treated [1,3]. DR is becoming the chief cause of blindness around the globe in people of age group 20-60years thus accounts for 2.5 million blindness across the world [4,5].

DR is characterized via formation of irregular fragile new blood vessels that are liable to hemorrhage into vitreous thus can cause vascular permeability or hemorrhages, microaneurysms, intra retinal microvascular anomalies venous bleeding and cotton wool spots [6-8]. The VEGF gene of humans is located on chromosome 6 (6p21.1). It is a powerful angiogenic factor which is involved in several diseases that includes microvascular complications of diabetes and cancer. Raised serum and vitreous levels of VEGF have been strongly linked with proliferative diabetic retinopathy [9,10]. An important role played by growth factors to as response to tissue damage by hyperglycemia is the modification and acceleration of that damage thus contributing as risk factor in DM microvascular complications [11].

A potent multifunctional cytokine VEGF-A is involved in pathogenesis of microvascular complications of DM such as DR, DN and DPN [12-16]. This gene belongs to the family of growth factors that is involved in angiogenesis and vasculogenesis thus functioning as signaling proteins. This growth factors family has several members such as VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF and placental growth factor (PGF) [3]. The 18bp fragment (I/D) polymorphism at -2549 position of promoter region of VEGF gene is considered as possible genetic factor for development and progression of retinopathy in DM patients.

This study aimed to identify the 18bp fragment I/D polymorphism at -2549 position of promoter region of VEGF gene in DM and DR patients of Balochistan, Pakistan.
Materials and Methods

Design and subjects

This cross sectional study involved 100 DM subjects without retinopathy, 100 DR and 100 uncomplicated healthy controls, the diagnosis criteria for DM was according to WHO diagnostic criteria. Patients of both sexes of DM type 2 were selected for the study that were registered, admitted diabetic wards in Bolan Medical Complex Hospital (BMC) Quetta and for DR from OPDs of Helpers Eyes Hospital Quetta. The patients history, their diabetic age and other complications were recorded using pre-structured questionnaires that were approved by Institutional Review Board (IRB) of BUITEMS, Quetta. All DR patients under gone complete ophthalmological examination in Out Patient Department of Helpers Eyes Hospital, Quetta.

Determination of VEGF genotypes

After obtaining written informs consent from patients, sample collection was done in EDTA tubes. 3ml of fasting blood was collected from patients and that was kept -20°C before extraction of DNA. Genomic DNA was extracted using inorganic method. The VEGF I/D polymorphism were analyzed using following primers: Forward 5'-GCTGAGAGTGGGGCTGACTAGGTA-3' and Reverse 5'-GT'TTCTGACCTGGCTATTTCCAGG-3'.

Standard PCR was used following conditions to find out VEGF I/D polymorphism using BIO-RAD Thermocycler (USA): Initial denaturation at 95°C for 6 minutes, 35 cycles each for 1 minute at 94°C, followed by 1.5 minutes at 58°C for primer annealing and 2 minutes at 72°C for extension and final extension for 10 min at 72°C. Agarose gel (2%) electrophoresis with ethidium bromide staining was used for analysis of results. Two bands of VEGF I/D variants were observed, band for D allele at 211bp and the band for I allele at 229bp. The 18bp fragment I/D was further confirmed via commercial sequencing (Macrogen South Korea) of heterozygous samples to confirm the size of the (I/D) polymorphism, its sequence and the region of the I/D polymorphism in Balochistan population.

Statistical analysis

SPSS Version 20 for windows 7 was used for identification of significance and its level between demographic features of different ethnic groups. The genotype and allele frequencies were also estimated and Chi-square test was used to test the significant level among the genotypes of Control, DM and DR.

VEGF I/D genotypes between Control and DM, Control and DR, DM and DR were compared using Pearson’s Chi-square test. The significant differences (p<0.05) were observed in Control and DM, DM and DR, Control and DR. The p-value <0.05 was considered to be statistically significant and to estimate high risk allele odds ratios (OR) with 95% confidence intervals (CI) were used.

Results

The clinical and biochemical variables are represented in Table 1 of all study groups. In all three study groups of each (n=100) the diabetic age, family history, hypertensive status, BMI (kg/m²), Glycemic status of patients via HbA1c profile was observed. The gender distribution was not similar in all the study subjects and age at sampling in all the study subjects was also not similar, there was significant difference (p<0.05) among the gender/age of the healthy control, DM and DR group. The diabetic patients without retinopathy DM group and with retinopathy DR group did not vary in diabetic complications as such as compared to control group, however greater number of hypertensive patients observed were those having longer duration of diabetes as in DR group (p<0.05).

Table 1: The clinical and biochemical parameters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control group</th>
<th>DM group</th>
<th>DR group</th>
</tr>
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<tbody>
<tr>
<td>N</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Gender(M/F)</td>
<td>54snl/46</td>
<td>38/62</td>
<td>34/66</td>
</tr>
<tr>
<td>Age at sampling (years)</td>
<td>44.08 ± 9</td>
<td>48.08 ± 10</td>
<td>54.58 ± 8.9</td>
</tr>
<tr>
<td>Duration of Diabetes (years)</td>
<td>N/A</td>
<td>10 ± 8.1</td>
<td>15 ± 7.2</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>N/A</td>
<td>7.4 ± 2.1</td>
<td>7.6 ± 2.1</td>
</tr>
<tr>
<td>BMI(kg/m²)</td>
<td>24.10 ± 2.7</td>
<td>27.12 ± 3.7</td>
<td>27.13 ± 5.7</td>
</tr>
<tr>
<td>Hypertensive (%)</td>
<td>40(40%)</td>
<td>60(60%)</td>
<td>64(64%)</td>
</tr>
<tr>
<td>Family history of diabetes</td>
<td>60(60%)</td>
<td>80(80%)</td>
<td>84(84%)</td>
</tr>
</tbody>
</table>

Table 2: VEGF genotypes and allele frequencies.

<table>
<thead>
<tr>
<th>Study Groups</th>
<th>Genotype Frequency</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>II</td>
<td>ID</td>
</tr>
<tr>
<td>Control (n=100)</td>
<td>2(2%)</td>
<td>96(96%)</td>
</tr>
<tr>
<td>DM (n=100)</td>
<td>26(26%)</td>
<td>34(34%)</td>
</tr>
<tr>
<td>DR (n=100)</td>
<td>12(12%)</td>
<td>36(36%)</td>
</tr>
</tbody>
</table>

Table 3: Comparison of (I/D) VEGF genotypes using Chi-square.

The VEGF I/D polymorphism are shown in figure 1. The band for DD genotype is observed at 211bp and the band for II genotype was confirmed 229bp.
observed at 229bp. This 18bp fragment I/D polymorphism of VEGF gene was further confirmed via sequence analysis of heterozygous sample for I/D polymorphism figure 2. The sequence of 18bp fragment was the same as already reported in other populations, its site in Balochistan population was also the same as -2549 position of promoter region and its sequence is also the same when confirmed in SNP database and the SNP-ID for this 18bp I/D polymorphism was dbSNP: rs36991894.

Figure 1: PCR amplification results of VEGF 18bp fragment I/D polymorphism at -2549 position of promoter region. Lane: 3, 7, 9, 11, 12, 13 I/D genotypes (211bp & 229bp). Lane: 1, 14, 15 DD genotypes (229bp). Lane: 2, 4, 5, 6, 8, 10 II genotypes (229bp).

Figure 2: Sequencing chromatogram of I/D heterozygous sample showing 18bp fragment I/D polymorphism at -2549 position.

Discussion

An important potent multifunctional cytokine gene involved in many diseases that are based on angiogenesis is the human VEGF gene which cytogenic location is short arm p of chromosome 6 that is 6(6p21.1). This gene is highly polymorphic and one of the polymorphism 18bp fragment insertion/deletion (I/D) of promoter region at position -2549 is of great significance [17-19].

The depiction of DR is given usually by increased neovascularization, tissue ischemia and vascular permeability. The human VEGF gene plays key role in the diabetic microvascular complications pathlogy. The stimulation of angiogenesis by VEGF is triggered when in hypoxia condition hypoxia inducible factors get bound to the hypoxia response elements and thus prompt VEGF expression which in turn stimulates the angiogenesis. The VEGF along with angiogenesis increases the microvasculature permeability [13,20-22].

In the present study the potential association of the 18bp fragment (I/D) polymorphism of promoter region at position -2549 has been identified in the Balochistan population. The study was carried out for the first time in the Pakistan as so in Balochistan, so no literature is available for this population. However in this study the positive association of the DD genotype or D allele was found out as independent possibility factor for the expansion of retinopathy in DM patients as the study was carried out in three study groups viz. Control, DM (Diabetes non-retinopathy) and DR (Diabetes with retinopathy).

Buraczynska et al. had presented that the DD genotype or the D allele in (I/D) polymorphism of the VEGF gene may be related to predisposition of DR but not with DN in patients with type 2 DM which may reveal some effects that are cell-specific effects of VEGF polymorphism [17].

The connotation of the D allele with DR predisposition can be explained to some extent by the boosted level of transcription of VEGF gene comparatively to I allele this would in turn result in increased level of VEGF in the patients having DD genotype as compared to uncomplicated patients having I allele [23].

In vitro functional studies results proposed that at the -2549 position of promoter region of VEGF gene the existence of D allele leads the aggravated expression of the gene [6]. Previous data or studies had presented that the promoter region polymorphism as well as polymorphism in 3’-UTR region of the VEGF gene mark the VEGF production [24].

Fouda et al. suggested that in Egyptian subjects the increased frequency of the DD genotype of VEGF (I/D) polymorphism in DR patients as compared to the non-retinopathy DM subjects and in controls they have not found the significant difference in their genotype distribution such as (II, DD and I/D). Conversely they found out the increased risk of DD genotype in DR patients by more than two folds and the increased risk of more than one and half fold with D allele in DR patients as compare to control [5].

Amle et al. suggested that in North Indian population DD genotype polymorphism of VEGF (I/D) at -2549 position of promoter region is expressly associated with DN [25]. The significant increase in increase in frequency of DD genotype in DN group in comparison to DM group was observed. However in their findings they have not found any significant increase in frequency of DD genotype in DM group when compared with control group. They have suggested that the increased frequency of DD genotype and D allele was observed in DN group as compared to both DM and Control group hence DD genotype is related with greater than before risk of DN in north Indian population.

Stoian et al. for the first time in Romania studied the (I/D) polymorphism of the VEGF gene in type 2 DM, DPN and control...
group. His study suggested that the significant association of DD genotype with DPN while in their study II genotype was more frequent in control group suggesting the protective role of this genotype in development of DM and DPN [1]. In the Romanian population the presence of (I/D) and DD genotype was the risk factor for development DNP. Whereas I allele was more common in control group as compared to D allele. Their study also supported that the D allele is associated with micro vascular complication of DM such as retinopathy, nephropathy and Neuropathy.

In another study conducted by Kapahi et al. in north Indian population of Amritsar the (I/D) polymorphism of VEGF gene has been identified in sporadic breast cancer patients and interestingly they have found the significant association of II genotype and I allele in breast cancer patients as compared to control group which gives clear indication of reduced transcriptional activity of VEGF in patients [6].

In present study there was significant increase in frequency of DD genotype in DR patients as compared to DM group. However when the frequency of DD genotype was compared in DM and control group significant increase was observed in DM patients as compared to control group. Hence it can be conferred DD genotype is the risk factor for development of retinopathy in Balochistan population also it can be interpreted that DD genotype is more significant in DR group as compared to DM and it is more significant in DM group as Present study evaluated the significant increase in DD genotype in DR patients as compared to DM group. However when the frequency of DD genotype was compared in DM and control group significant increase was observed in DM patients as compared to control group. Hence it can be conferred DD genotype is the risk factor for development of retinopathy in Balochistan population also it can be interpreted that DD genotype compared to Control group, so it is the risk factor for development of retinopathy in those DM patients carrying DD genotype. Also the significant difference among the groups in terms of genotypes was found out using Chi-square test and it was found that the (p<0.05) that was significant. Due lack of data or research about this gene polymorphism in Pakistan it can be not be further elaborated.

Furthermore the 18bp I/D of VEGF gene was confirmed via sequence analysis and 18bp fragment sequence is 5’-tccaccttcttcccagg-3’ that supports the reported deletion sequence was confirmed using UCSC Genome Browser.

Conclusion

The VEGF (I/D) polymorphism has been carried for first time in Balochistan so in Pakistan. This study suggest that the DD genotype and D allele is independent risk factor for development of retinopathy in DM patients also other factors such as Diabetic age and Family history of DM play key role in the development of retinopathy in DM patients. However the significant association of II genotype or I allele in DM patients as compared to control group. Further productive results can be obtained by increasing the sample size and the same (I/D) polymorphism of -2549 position of promoter region of VEGF gene can be studied in other diabetic subjects having other micro-vascular complications of diabetes.

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References


