RESEARCH ARTICLE

# Association of vascular endothelial growth factor a gene (VEGFA) polymorphisms, rs699947 and rs1570360, with diabetic retinopathy and altered VEGF secretion in the Pakistani patients with type 2 diabetes mellitus: a case-control study

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## Abstract

**Objective:** To investigate the association of vascular endothelial growth factor A gene polymorphisms 2578C/A (rs699947) and 1154G/A (rs1570360) with type 2 diabetes mellitus, diabetic retinopathy and serum vascular endothelial growth factor levels in Pakistani patients.

**Method:** The case-control study was conducted from Jan 2017 to Dec 2018 after approval from the ethics review board of Riphah International University, Islamabad, Pakistan, and comprised type 2 diabetes mellitus patients of either gender with diabetic retinopathy in group A, and without diabetic retinopathy in group B. Non-diabetic healthy individuals were enrolled in control group C. Genotyping was done by amplification refractory mutation system-polymerase chain reaction and serum vascular endothelial growth factor levels were measured using enzyme-linked immunosorbent assay. Data was analysed using SPSS 22.

**Results:** Of the 450 subjects, 150(33.3%) were in each of the 3 groups. The mean age in group A was 58.16±9.42, in group B 56.25±8.5 years and in group C it was 55.90±10.90. The proportion of Punjabi ethnicity was significantly high in group B compared to other groups ($p<0.05$). There was no significant association of rs699947 and rs1570360 genotypic and allelic frequencies in group B compared to group A. Further, rs699947 AA genotype was significantly associated with proliferative diabetic retinopathy compared to group A ($p<0.05$). Minor allele A showed significant association in groups A and B compared to group C ($p<0.05$). Significantly raised serum vascular endothelial growth factor levels were found in group B compared to group A ($p<0.05$), and were associated with rs699947 and rs1570360 heterozygosity in group A ($p<0.05$). Also, rs699947 genotype showed significant association with groups A and B in Punjabi and Pathan ethnicities ($p<0.05$) and with Kashmiri ethnicity in group B ($p<0.05$).

**Conclusion:** There was a strong association of vascular endothelial growth factor 2578C/A (rs699947) gene polymorphism with proliferative diabetic retinopathy in type 2 diabetic Pakistani patients, suggesting its role in the pathogenesis of this condition.

**Key Words:** Diabetic retinopathy, Polymorphisms, Type 2 diabetes mellitus, Vascular endothelial growth factor A gene. (JPMA 73: 2348; 2023) **DOI: 10.47391/JPMA.6072**

## Introduction

The global prevalence of diabetes mellitus (DM) has increased drastically. According to the International Diabetes Federation, the worldwide prevalence of DM is projected to increase from 536.6 million in 2021 to 783.2 million by 2045.[1] Pakistan is currently ranked 3rd for diabetes prevalence in adults, and is expected to remain in this position up to the year 2045.[1] According to a recent nationwide study conducted in Pakistan, the prevalence of type 2 DM (T2DM) was 16.98%.[2] Diabetic patients have a greater risk of developing diabetic retinopathy (DR) with a global prevalence of 27% from 2015 to 2019[3] whereas in Pakistan it is 8.6%.[4]

Several diabetics show progression of DR despite good glycaemic control, implying potential role of genetics in its development.[5] Multiple candidate genes have been linked with DR till date among which vascular endothelial growth factor A (VEGFA) gene is of significance as VEGF is an important gene product contributing in the pathogenesis of T2DM. Its eminent role in ocular neovascularisation led to the use of anti-VEGF drugs for the management of DR. The targetted therapy of DR globally and in the local clinical context, largely emphasises on anti-VEGF drugs, but the patient response is unpredictable.[6,7] Genetic studies are imperative to

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assess this variable response. VEGFA gene, situated on chromosome 6p21.3, is greatly polymorphic and contains almost 30 functional single nucleotide polymorphisms (SNPs) in the promotor region, 3′ untranslated region (3′-UTR) and 5′-UTR. The polymorphisms located in the promoter region are considered important risk factors for DR development as most of the elements responding to hypoxia are in this region. Hypoxia-inducible factors bind these elements during retinal hypoxia observed in DR. Upregulation of VEGF enhances vascular permeability and breaks the blood-retinal barrier. Its serum and vitreous levels correlate with severity of disease.[8]

Therefore, serum VEGF is an important tool for risk evaluation in DR. Inflammation is a key component in diabetes-associated retinal damages. Increased expression of VEGF leads to release of various pro-inflammatory cytokines like tumour necrosis factor-alpha (TNF-$\alpha$), interleukin-1-beta (IL-1$\beta$) and IL-6. Molecular pathways, like oxidative stress, advanced glycation end-product (AGE) formation, growth factors and cytokines, add to the inflammatory response.[9] Due to these stimuli, several retinal cells produce VEGF, thus leading to the pericyte loss, endothelial cell proliferation and dysfunction of inner blood-retinal barrier.

Various polymorphisms located at the VEGF gene promoter regions, 3′-UTR and 5'-UTR, have been assessed as risk alleles for the susceptibility of DR in diverse populations, including Caucasian, Chinese and Indian, with inconsistent results.[10–12] Pakistan is a multi-ethnic country with certain dominant regional groups, and genetic variations may be expected among various groups. Very limited relevant data is available in Pakistani population.[13–16] Understanding genomic markers associated with incomplete treatment response in DR is crucial for identifying potential causes. Advancements in pharmacogenetics and pharmacogenomics offer the potential for personalised management based on genotypic profiles.[17]

The current study was planned to investigate the association of VEGFA gene polymorphisms 2578C/A (rs699947) and 1154G>A (rs1570360), with T2DM, DR and serum VEGF levels, and to identify any difference in three major ethnic groups of the Pakistani population.

## Patients and Methods

The case-control study was conducted from Jan 2017 to Dec 2018 after approval from the ethics review board of Riphah International University (RIU), Islamabad, Pakistan, and comprised T2DM patients having a history of >5 years of either gender. Those with DR were in the DR group, and those without DR were placed in DWR group. Non-diabetic healthy individuals were enrolled in control group HC. The subjects were enrolled from the retina clinic at Al-Shifa Eye Hospital, Islamabad, the diabetic clinic at Pakistan Railway Hospital (PRH), Rawalpindi, and the Divisional Headquarters (DHQ) Hospital, Mirpur, using non-probability convenience sampling technique. The sample size was determined by Cochran's formula: n= Z2 (pq) ÷ e2 where n represented the required sample size, Z was the value of Z at 95% confidence interval (CI) which was 1.96, p represented the prevalence of disease, and q was the 1-p.[18]

Laboratory investigations were conducted at PRH RIU. Patients with T1DM or other chronic endocrine disorder, malignancy, acute/chronic systemic or ophthalmic disorders were excluded.

After taking informed consent from all the subjects, demographic and medical data was recorded. Fasting venous blood samples were collected for genomic deoxyribonucleic acid (DNA) extraction, serum VEGF estimation and biochemical analysis.

The simple and cost-effective technique of allele-specific amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) was used for genotyping of single nucleotide polymorphisms (SNPs) 1154G/A and 2578C/A. For 1154G/A SNP, allele-specific primers G and A were used along with a common primer. For 2578C/A SNP, allele-specific primers C and A were used along with a common primer. Internal control primers were also used for amplifying the internal control product. The primer sequences were obtained from literature.[19,20] The PCR was carried out using a thermal cycler and a commercially available PCR premix following the recommended instructions of the manufacturer (FIREPol® Master Mix Ready to Load). For the detection of SNPs, a total volume of 24µl containing 2µL template DNA (~100 ng/µL), 2µL primer mix with each 10pmol/ul, 14µL master mix and deoxyribonuclease (DNase)-free water up to 6µL were added into a 0.2 mL PCR tube. PCR was run in a thermal cycler with holding temperature at 95°C for 5min, followed by 30 cycles of denaturation at 95°C for 30s, annealing at 60°C for 30s, extension at 72°C for 30s and final extension at 72°C for 7min. The amplified PCR products were resolved onto 2% agarose gel containing 5ul ethidium bromide solution (0.5ug/ul) per 100ml agarose solution to visualise the DNA bands under ultraviolet (UV) light in gel documentation system (G-Box, Syngene, USA) (Figures 1-2).

Data was analysed using SPSS 22. Genotype and allelic frequencies were compared among all the subjects, and their association with DM, DR and ethnicity were assessed

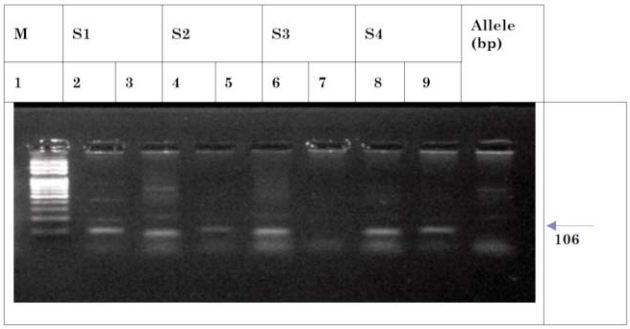


**Figure-1:** Electrophoretogram on 2% agarose gel showing amplified PCR products of VEGF gene polymorphism -2578C/A (rs699947) with DNA marker of 100bp.
Lane 1:M, DNA marker of 100 bp; lanes 2 & 3 sample 1, lanes 4 & 5, sample 2: heterozygous CA genotypes respectively; lanes 6 & 7 of sample 3: homozygous AA genotype and lanes 8 & 9 of sample 4: homozygous CC genotype. PCR: Polymerase chain reaction, VEGF: Vascular endothelial growth factor, DNA: Deoxyribonucleic acid.

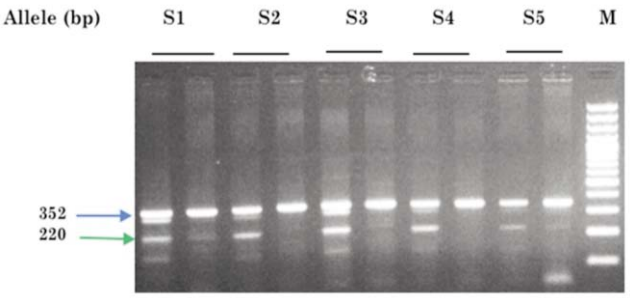


**Figure-2:** Electrophoretogram on 2% agarose gel showing amplified PCR products of -1154G/A (rs1570360) gene polymorphism with DNA marker of 100bp. S1 and S5 represent heterozygous GA, S2, S3 and S4 represent homozygous GG genotypes, and 352bp amplified product in all samples represent control bands, M represents DNA marker 100bp.
PCR: Polymerase chain reaction, DNA: Deoxyribonucleic acid.

using chi-square test or Fisher's exact test along with odds ratio (OR) and 95% CIs. Univariate and multiple logistic regression analysis were done to find the association of genotypic and allelic distribution and biochemical parameters with DM, DR and serum VEGF levels. The parameters found significant at univariate level were put in the multinomial regression model. The differences in biochemical variables were analysed using analysis of variance (ANOVA) followed by post-hoc Tukey's test. Data was checked for normality using Shapiro-Wilk test before ANOVA application. Both the dominant and recessive genetic models were used to find the association of different genotypes with DM and DR. The association analysis was controlled for confounding variables by excluding the potential confounders during sample selection and matching the groups for age and gender as already described by the authors16. P<0.05 was considered significant.

## Results

Of the 450 subjects, 150(33.3%) were in each of the 3 groups. The mean age in DWR group was 58.16±9.42, in the DR group it was 56.25±8.5 years and in the HC group it was 55.90±10.90. The proportion of Punjabi ethnicity was significantly high in DR group compared to DWR and HC (p<0.006 and p<0.007).

For SNP 2578C/A (rs699947), a significant association of heterozygous CA genotype was found with DWR compared to HC (p<0.05). Mutant homozygous AA genotype and mutant A allele were significantly associated with DM and DR compared to HC (p<0.05). No significant difference of genotypic and allelic distribution with DR was found between DWR and DR groups (p>0.05). In dominant and recessive models, the minor allele A showed increased association with DWR and DR compared to HC, and for SNP 1154G/A (rs1570360), no significant association with DM or DR was observed for any of the genotypes, alleles, dominant and recessive models among the groups (Table 1).

When DR subjects were split into non-proliferative DR (NPDR) and proliferative DR (PDR) sub-groups, a significant association of mutant homozygous AA genotype was found with PDR compared to DWR in both univariate (OR: 2.40, 95% CI: 1.01-5.69, p< 0.042) and multinomial regression analyses (OR: 1.55, 95% CI: 1.007-2.38, p< 0.046) (Table 2).

A significant association of rs699947 genotype with DM and DR in Punjabi and Pathan ethnic groups (p<0.013 and p<0.042, respectively) and with DR in Kashmiri ethnic group (p<0.021) was noted compared to HC. A significant association of rs1570360 genotype was found with DR in

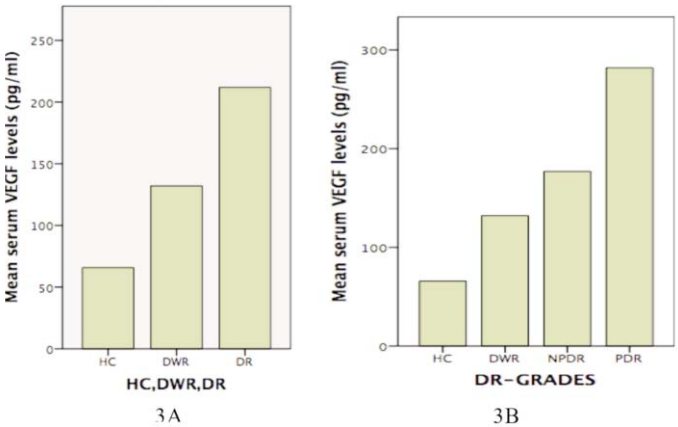


**Figure-3(A,B):** Comparison of mean serum VEGF levels among HC, DWR and DR groups. Figure 3B: Comparison of mean serum VEGF levels among HC, DWR, NPDR and PDR groups.
VEGF: Vascular endothelial growth factor, HC: Healthy controls, DWR: Diabetic without retinopathy, DR: Diabetic retinopathy, NPDR: Non-proliferative diabetic retinopathy, PDR: Proliferative diabetic retinopathy.

Kashmiri ethnic group compared to DWR (p=0.031) (Table 3).

Serum VEGF levels were significantly raised in DR and DWR groups compared to HC (211.8±85.2, 132.05±59.06 and 65.3±27.8 pg/ml, respectively, p<0.001). A significant variance was found in VEGF levels in DR and DWR patients (p< 0.001). Serum VEGF levels were raised in PDR and NPDR patients compared to DWR (283.8±93.4, 177.0±53.6, 132.05± 59.06pg/ml, respectively, p<0.001) (Figure 3A-B).

Serum VEGF levels showed a significant positive correlation with fasting plasma glucose (FPG), cholesterol and low-density lipoprotein (LDL-C) (r: 0.616, p< 0.001, r: 0.221, p< 0.007, r: 0.202, p< 0.013) in DWR group. In DR group, duration of DM and DR, FPG, cholesterol, LDL-C, triglycerides and creatinine were positively correlated

**Table-1:** Comparison of genotype and allele distribution of VEGF 2578C/A and 1154G/A gene polymorphisms among HC, DWR and DR groups.

| | Genotype/Allele | HCn=150 (%) | DWRn=150 (%) | DRn=150 (%) | Odds ratio (95% CI) | P Value | Multinomia lRegression |
|---|---|---|---|---|---|---|---|
| **2578C/A polymorphism** | CC | 140 (93.3) | 118(78.7) | 116 (77.3) | Ref 1 | | |
| | CA | 7 (4.7) | 17(11.3) | 14 (9.3) | 2.88 (1.15-7.18) a<br>2.41 (0.94-6.17) b<br>0.83 (0.39-1.77) c | 0.011 a<br>0.067 b<br>0.612 c | 0.023 a |
| | AA | 3 (2) | 15 (10) | 20 (13.3) | 5.93 (1.67-20.98) a<br>8.04 (2.33-27.75) b<br>1.35 (0.66-2.77) c | 0.002 a<br>0.0001 b<br>0.400 c | 0.006 a<br>0.001 b |
| | C | 287(95.7) | 253(84.3) | 246(82) | 4.10 (2.16-7.75) a<br>4.84(2.58-9.09) b<br>1.18 (0.76-1.81) c | 0.0001 a<br>0.0001 b<br>0.435 c | < 0.001 a<br>< 0.001 b |
| | A | 13 (4.3) | 47(15.7) | 54(18) | | | |
| **Dominant Model** | CCCA+AA | 140 (93.3)<br>10 (6.7) | 118 (78.7)<br>32 (21.3) | 116 (77.3)<br>34 (22.7) | 33.79 (1.79-8.04) a<br>4.10 a (1.94-8.65) b<br>1.08 (0.62-1.86) c | 0.0002 a<br>0.0001 b<br>0.728 c | < 0.001 a<br>< 0.001 b |
| **Recessive Model** | CC+CAAA | 47(98)<br>3 (2) | 135(90)<br>15 (10) | 130(86.7)<br>20 (13.3) | 5.44 (1.54-19.22) a<br>7.53 (2.18-25.95) b<br>1.38 (0.67-2.82) c | 0.003 a<br>0.0002 b<br>0.389 c | 0.008 a<br>0.001 b |
| **1154G/A polymorphism** | GG | 98 (65) | 100 (66.6) | 93(62) | Ref 1 | | |
| | GA | 30 (20) | 32 (21.3) | 34(22.7) | 1.04 (0.59-1.84) a<br>1.19 (0.67-2.10) b<br>1.14(0.65-1.99) c | 0.881<br>0.533<br>0.639 | |
| | AA | 22 (15) | 18 (12) | 23 (15.3) | 0.80(0.40-1.58) a<br>1.10(0.57-2.10) b<br>1.37(0.69-2.70) c | 0.522<br>0.765<br>0.358 | |
| | G | 226 (75.3) | 232 (73.3) | 220 (73.33) | Ref | | |
| | A | 74(24.7) | 68 (22.6) | 80 (26.6) | 0.89 (0.61- 1.30) a<br>1.11 (0.76-1.60) b<br>1.24 (0.85-1.80) c | 0.563<br>0.575<br>0.251 | |
| **Dominant Model** | GGGA+AA | 98 (65)<br>52 (35) | 100 (66.6)<br>50 (33.4) | 93(62)<br>57(38) | 0.94(0.58-1.51) a<br>1.15(0.72-1.84) b<br>1.22 (0.76-1.96) c | 0.808a<br>0.540b<br>0.391 c | |
| **Recessive Model** | GG+GAAA | 128(85.3)<br>22 (16.6) | 132(88)<br>18 (12) | 127 (84.6)<br>23 (15.3) | 0.79(0.40-1.54) a<br>1.05(0.55-1.98) b<br>1.32(0.68-2.57) c | 0.492 a<br>0.865 b<br>0.398 c | |

VEGF: Vascular endothelial growth factor, HC: Healthy controls, DWR: Diabetic without retinopathy, DR: Diabetic retinopathy, CI: Confidence interval, a: HC compared with DWR, b: HC compared with DR, c: DWR compared with DR; p<0.05 significant".

**Table-2:** Comparison of genotype distribution of VEGF 2578C/A and 1154G/A gene polymorphisms among DWR, NPDR and PDR groups.

| Geno type | DWR n=150 (%) | NPDR n=100 (%) | PDR n=50 (%) | OR (95% CI) | *p* - value | Multinomial Regression *p* value |
|---|---|---|---|---|---|---|
| 2578C/A | | | | | | |
| CC | 118 (78.7) | 80 (80) | 36 (72) | ref | | |
| CA | 17 (11.3) | 11 (11) | 3 (6) | 0.95 (0.42-2.14) [a]<br>0.57 (0.16 -2.08) [b] | 0.921[a]<br>0.576 [b] | |
| AA | 15 (10) | 9 (9) | 11(22) | 0.88 (0.36-2. 12) [a]<br>2.40 (1.01-5.69) [b] | 0.770 [a]<br>0.042 [b] | 0.046 [b] |
| 1154G/A | | | | | | |
| GG | 100 (66.6) | 63 (63) | 30 (60) | Ref 1 | | |
| GA | 32 (21.3) | 26 (26) | 8 (16) | 1.28(0.70 –2.36) [a]<br>0.83 (0.34-2.00) [b] | 0.521 [a]<br>0.680 [b] | |
| AA | 18 (12) | 11(11) | 12 (24) | 0.97 (0.42-2.18) [a]<br>2.22(0.96 - 5.13) [b] | 0.920 [a]<br>0.057 [b] | |

VEGF: Vascular endothelial growth factor, DWR: Diabetic without retinopathy, NPDR: Non-proliferative diabetic retinopathy, PDR: Proliferative diabetic retinopathy, OR: Odds ratio, CI: Confidence interval, a: DWR compared with NPDR, b: DWR compared with PDR,p<0.05 significant.

**Table-3:** Association analysis of VEGF gene polymorphisms 2578C/A and 1154 G/A genotypes with ethnicities for HC, DWR and DR groups.

| SNP and Genotypes | Ethnicity | HC N=150n (%) | DWR N=150n (%) | DR N=150n (%) | Odds Ratio (95% CI) | *p* value |
|---|---|---|---|---|---|---|
| 2578C/ACCCA+AA | Punjabi | 59 (39.3) | 60 (40) | 86 (57.3) | | |
| | | 53 (35.3) | 43 (28.6) | 66 (44) | 3.49 (1.26-9.62) [a] | 0.01 [a] |
| | | 6 (4) | 17 (11.3) | 20 (13.3) | 2.67 (1.00- 7.14) [b] | 0.04 [b] |
| | | | | | 0.76 (0.36 -1.62) [c] | 0.481 [c] |
| | Kashmiri | 52 (34.6) | 56 (37.3) | 34 (22.6) | | |
| | | 49 (32.6) | 47 (31.3) | 26 (17.3) | 3.12(0.79-12.26) [a] | 0.085 [a] |
| | | 3 (2) | 9(6) | 8 (5.3) | 5.02 (1.22 - 20.57) [b] | 0.021 [b] |
| | | | | | 1.60 (0.55 - 4.66) [c] | 0.383 [c] |
| | Pathan | 39 (26.0) | 34 (22.6) | 30 (20) | | |
| | | 38 (25.3) | 28 (18.6)6(4) | 24 (16) | 8.14(0.92-71.50) [a] | 0.041 [a] |
| | | 1 (0.6) | | 6 (4) | 9.5 (1.07 -83.85) [b] | 0.037 [b] |
| | | | | | 1.16 (0.33 - 4.09) [c] | 0.800 [c] |
| 1154 G/AGGGA+AA | Punjabi | 59 (39.3) | 60 (40) | 86 (57.3) | | |
| | | 33 (22) | 38 (25.3) | 56 (37.3) | 0.73 (0.35-1.53) [a] | 0.409 [a] |
| | | 26 (17.3) | 22 (14.6) | 30 (20) | 0.67 (0.34-1.34) [b] | 0.263 [b] |
| | | | | | 0.92 (0.46- 1.83) [c] | 0.823 [c] |
| | Kashmiri | 52 (34.6) | 56 (37.3) | 34 (22.6) | | |
| | | 35 (23.3) | 42 (28) | 18 (12) | 0.68(0.29- 1.58) [a] | 0.372 [a] |
| | | 17 (11.3) | 14 (9.3) | 16 (10.6) | 1.83(0.75- 4.44) [b] | 0.281 [b] |
| | | | | | 2.66 (1.07- 6.59) [c] | 0.031 [c] |
| | Pathan | 39 (26.0) | 34 (22.6) | 30 (20) | | |
| | | 30 (20) | 20 (13.3) | 19 (12.6) | 2.33(1.07-6.59) [a] | 0.09 [a] |
| | | 9 (6) | 14 (9.3) | 11 (7.3) | 1.92(0.67- 5.52) [b] | 0.217[b] |
| | | | | | 0.82(0.30-2.26) [c] | 0.708 [c] |

VEGF: Vascular endothelial growth factor, HC: Healthy controls, DWR: Diabetic without retinopathy, DR: Diabetic retinopathy, CI, Confidence interval; a: HC compared with DWR, b: HC compared with DR, c: DWR compared with DR, p<0.05 significant.

**Table-4:** Comparison of biochemical characteristics of HC, DWR and DR groups.

| Parameters | HC n = 150 | DWR n = 150 | DR n = 150 | p value |
|---|---|---|---|---|
| FPG (mg/dl)[L/SEP] | 88.06 ± 8.58 | 165.34 ± 51.93 | 190.87 ± 55.33 | <0.001 [a, b]<br><0.001 [c] |
| Total cholesterol (mg/dl)[L/SEP] | 148.86± 21.535 | 206.54 ± 42.89 | 220.67± 42 | <0.001 [a, b]<br>0.003 [c] |
| Triglyceride (mg/dl)[L/SEP] | 137.09 ± 28.25 | 180.71± 62.53 | 175.15± 43.53 | <0.001 [a, b]<br>0.560 [c] |
| HDL-C (mg/dl)[L/SEP] | 47.66 ± 7.12 | 44.0± 8.31 | 45.0± 8.02 | <0.001 [a, b]<br>0.509 [c] |
| LDL-C (mg/dl)[L/SEP] | 90.85± 16.95 | 128.36± 42.13 | 143.31± 37.65 | <0.001 [a, b]<br><0.001 [c] |
| Urea (mg/dl)[L/SEP] | 25.91± 9.34 | 30.89± 9.38 | 33.22± 9.57 | <0.001 [a, b]<br>0.083 [c] |
| Creatinine (mg/dl)[L/SEP] | 0.91± 0.33 | 1.09± 0.38 | 1.19± 0.38 | <0.001 [a, b]<br>0.066 [c] |

HC: Healthy controls, DWR: Diabetic without retinopathy, DR: Diabetic retinopathy, FPG:Fasting plasma glucose, HDL-C: High-density lipoprotein-cholesterol, LDL-C: Low-density lipoprotein-cholesterol, a: HC compared with DWR, b: HC compared with DR, c: DWR compared with DR; p value < 0.05 significant.

with serum VEGF levels (r: 0.217, p = 0.007, r: 0.310, p< 0.001, r: 0.561, p< 0.000, r: 0.199, p< 0.015, r: 0.165, p = 0.04, r: 0.266, p < 0.001, r: 0.223, p< 0.006).

For both SNPs, none of the study parameters were found inconsistent among three genotypes in HC (Table 4).

Serum VEGF levels were significantly high in CA compared to CC genotype for rs699947 in both univariate (184.4 ± 77.1 vs 124.1 ± 52.8, p <0.001) and multinomial regression analysis (OR: 1.01, 95% CI: 1.00 - 1.02, p <0.001) in DWR group. In DR group, serum cholesterol and urea were significantly high in CA compared to CC genotype at univariate level (p< 0.031 and 0.042) but these parameters could not retain their significance at multivariate level (OR: 0.98 , 95% CI: 0.97 – 1.00, p< 0.101, OR: 0.93, 95% CI: 0.87- 1.00, p< 0.061).

For SNP rs1570360, serum VEGF and FPG levels were significantly high in GA compared to GG genotype (p< 0.001 and 0.006) at univariate level in DWR group. In the multinomial regression model, FPG could not retain significance (p<0.230). In DR group, serum total cholesterol levels were significantly raised in AA compared to GA genotype at univariate (p<0.042) and multivariate levels (OR:1.01, CI:1.00-1. 2, p< 0.011).

## Discussion

The current study explored the association of SNPs rs699947 and rs1570360 of VEGFA gene, with DR and DWR Pakistani patients. A significant association of rs699947 heterozygous CA genotype was found with DWR whereas mutant homozygous AA genotype and minor allele were significantly associated with both DWR and DR compared to HC (Table 1). No significant association of rs1570360 genotypic and allelic frequencies was found between DWR and DR. In comparison to the wild type genotype of rs699947, the homozygosity of the minor alleles showed an increased association with PDR (Table 2). According to a previous study, rs699947 influenced the expression of messenger ribonucleic acid (mRNA) and VEGF promoter activity. [21]

The promoter region polymorphisms of VEGF are significant genetic risk factors for DR through their vaso-permeable and pro-angiogenic effects. During retinal hypoxia in DR, hypoxia-inducible factors bind to the hypoxia-responsive elements in the promoter region. This upregulates VEGFA transcription and generates multiple isoforms through alternative mRNA splicing. The effects of VEGF are exerted through its binding to two receptors, VEGFR1 and VEGFR2. VEGFR2 is the active receptor whereas VEGFR1 acts as a decoy receptor by preventing the binding of VEGF to its active receptor.[22] This activates downstream signalling pathways, including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 kinase (PI3K)/protein kinase B (PI3K/Akt), promoting cell proliferation and neovascularisation.[22] The increased serum VEGF levels result in elevated vascular permeability and disruption of tight junctions in endothelial cells, further increasing vascular permeability.[9] The release of VEGF is regulated by growth factors, oxidative stress, and pro-inflammatory/inflammatory cytokines.

Within the specific regional context, only one study was found from Pakistani where four VEGFA SNPs, including rs833061, rs1570360, rs2010963 and rs13207351, were genotyped in T2DM and DR patients. Consistent with the current findings, no association was seen between – rs1570360 and DR risk, but, in contrast, serum VEGF levels were not significantly different in DWR vs HC and DWR vs DR sub-groups.[13] Global studies conducted in T2DM patients in Chinese, Japanese and Spanish populations also found a significant association of DR with rs699947[10,11,23] In a study in South Korean population, rs699947, rs2010963 and rs1570360 polymorphisms were studied in type 2 diabetics with and without retinopathy. The rs699947 A allele showed a significant association with DR in comparison to DWR, whereas, consistent with the current results, no association was seen between rs1570360 and DR.[24] According to a meta-analysis that comprised 8 studies related to European and Asian populations, a marginal association of rs699947 was seen with DR in a dominant and homozygous co-dominant model ($p< 0.040$ each).[23]

However, several studies have reported opposing results regarding genotypic frequencies and association of rs699947 and rs1570360 with T2DM and DR. In two recent studies conducted in India and Iraq, the association between rs699947 polymorphism and type 2 diabetic retinopathy was examined, and both the studies found no significant association between the rs699947 polymorphism and DR, indicating that it may not have a significant impact on the development of DR in the studied populations.[25,26] Other studies found no association of rs699947 polymorphism with DR in Chinese and Egyptian populations.[27,28]

In a meta-analysis, rs699947 showed no significant association with DR in Asians and Caucasians.[12] A meta-analysis including studies till 2015 attempted to determine the relationship of VEGF SNPs - rs3025039, rs833061, rs699947, rs1570360 and rs2010963 with DR susceptibility. No significant associations of rs699947 and rs1570360 with increased DR risk was detected in European and East Asians.[11] Incongruences among studies may be due to differences in inclusion criteria of the subjects, sampling bias, difference in ethnicity, population heterogeneity, phenotypic variations, gene-environment interactions, methodological differences and publication bias.

In the current study, rs699947 and rs1570360 genotypic and allelic frequency distribution were reported for the first time in three major ethnic groups of Pakistani population; Punjabi, Kashmiri and Pathan. In patients with Punjabi and Pathan ethnicities, a significant association of rs699947 genotype was found in both DWR and DR groups compared to HC, whereas the Kashmiri ethnic group showed a significant association with DR compared to HC. A significant association of rs1570360 genotype was found with DR compared to DWR in the Kashmiri ethnic group. To authenticate these findings, future studies are required with larger sample sizes from all ethnic groups within the Pakistani population.

Serum VEGF levels are an important regulator of DR, and provide a possible tool for risk assessment in diabetic patients. The current study found significantly raised levels of serum VEGF in DWR and DR patients compared to HC ($p<0.001$). These levels were also raised in DR and its sub-groups compared to DWR ($p<0.001$each). PDR is a microvascular disease where relative retinal ischaemia produces a pro-angiogenic environment. Persistent hyperglycaemia may provoke inflammatory cytokines and hypoxia-inducible factors in target organs. The VEGF genetic polymorphisms are linked with inflammatory cytokine expression. A 2015 study in India investigated rs1570360, rs2010963, rs2071559 and rs3025039 VEGF gene polymorphisms and serum VEGF and nuclear factor kappa beta (NFkB) levels for their association with DR. Consistent with the current findings, no significant difference was seen in genotypic and allele frequencies of rs1570360 among PDR, DWR and HC subjects. Serum VEGF and NFkB were found significantly elevated among NPDR and PDR patients. Increased activation of NFkB in DR subjects may cause upregulation of VEGF by binding DNA at kB-binding site, leading to increased inflammation, cell proliferation and angiogenesis.[29] In another study in India, plasma and vitreous concentrations of VEGF were found significantly raised in PDR patients ($p<0.001$) compared to subjects who had vitrectomy for other causes.[30]

The current study found a positive correlation of serum VEGF levels with FPG, cholesterol and LDL-C in both DR and DWR groups, and rs699947 and rs1570360 heterozygosity was significantly associated with raised serum VEGF in diabetics. Hyperglycaemia stimulates increased secretion of VEGF which plays an important role in the development and progress of DR.[31,32] In a prospective study in Turkish population, serum VEGF levels were found significantly raised in the DR group compared to HC and DWR groups ($p<0.05$).[33] A meta-analysis revealed a significant association between raised VEGF levels and severity of DR, and serum VEGF correlated more to the severity of disease compared to its plasma levels in Asian subgroups compared to European groups.[34] Serum VEGF levels are an important regulator of DR and provide a possible tool for risk assessment in

diabetic patients.

The highly polymorphic VEGF gene, with multiple functional SNPs in different regions, leads to the varied disease patterns and severity observed in different populations. These polymorphisms are of interest in DR due to the emphasis on anti-VEGF therapy. However, <50% of patients respond to this treatment, highlighting the need for genomic biomarkers associated with treatment response. Screening susceptible patients based on a group of implicated SNPs may help identify those who are more likely to benefit from therapy. Targetting resistant alleles and developing new drugs can improve outcomes. Pharmacogenetics and personalised medication are essential in achieving sustained treatment response, identifying high-risk patients, and understanding disease pathways in DR.[17]

Further research is needed to evaluate serum and vitreous levels of VEGF in different stages of DR for better understanding of the interaction between local and systemic angiogenic and inflammatory factors.

The current study has limitations as, because of resources and financial constraints, it had a relatively smaller sample size and used only two SNPs in the VEGFA gene promotor region. The chance that other SNPs in the region might be involved in the progression of DWR and DR could not be ruled out.

## Conclusion

There was a strong association of VEGF 2578C/A (rs699947) gene polymorphism with PDR in T2DM patients, suggesting its role in the pathogenesis of the condition. Raised serum VEGF levels in these patients may give a clue to severity of the disease.

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