

Analysis of MnSOD and VEGF genes polymorphisms in Diabetic Retinopathy

Sarah Farzadi Haghighi¹, Zivar Salehi^{1*}, Rasoul Sabouri Mohamad²

1. Department of Biology, Faculty of Sciences, University of Guilan, Rasht, 1914, Iran

2. Department of Ophthalmology, Guilan University of Medical Sciences (GUMS), Rasht, Iran

Original Submission: October 2013 Revised Submission: December 2013 Accepted: January 2014

Abstract

Background: Diabetic retinopathy (DR) is a sight-threatening microvascular complication of diabetes in which the vascular endothelium is damaged due to oxidative stress and inflammation, and vitreous VEGF concentration becomes elevated. The aim of the present study was to assess the association of DR with genetic variations of the MnSOD, a major antioxidant enzyme, and VEGF, an important mediator of neovascularisation, in northern Iran.

Methods: 70 patients with DR and 70 healthy control subjects matched for age and sex was recruited for this study. PCR-based RFLP assay was used to determine the genotypes of *MnSODA16V* and *VEGF+405 C/G* polymorphisms.

Results and Conclusions: A higher frequency of the AV genotype (71.43%) of the *MnSOD A16V* polymorphism was found in the patients compared with controls which had a 8.33-fold increase in risk of DR (OR= 8.33, 95% CI= 2.56-27.13, $P= 0.0004$). The frequency of GG, GC, and CC genotypes of *VEGF +405 C/G* polymorphism in controls were 42.86%, 45.71% and 11.43%, respectively, while in DR patients were 18.57%, 48.57%, and 32.86%, respectively. The +405C allele was considered as a high risk factor of DR (OR= 2.55, 95% CI= 1.57-4.14, $P= 0.0001$). In conclusion, It is suggested that the *MnSOD A16V* and the *VEGF+405 C/G* polymorphisms may be associated with the risk of DR in northern Iran.

Keywords: Diabetic retinopathy, gene polymorphism, VEGF and MnSOD.

Introduction

Diabetic retinopathy (DR) is a diabetes related sight-threatening microvascular complication of the retina. It is one of the main leading cause of blindness in industrialized countries and World Health Organization (WHO) has reported it is most common cause of blindness in the world,

accounting for 4.8% of global blindness [Resnikoff *et al.*, 2004]. DR is divided into two main stages: nonproliferative

DR (NPDR) and proliferative DR (PDR). NPDR is characterized by microaneurysm, hemorrhage, hard exudates, cotton wool spots and venous abnormality. PDR is identified by grow thing of abnormal new blood vessels (neovascularization) that frequently lead to preretinal and vitreous hemorrhage [Council, 2008]. About 20% of the patients with T2DM even with poor glycemic control do not develop DR [Cai and Boulton, 2002]. In contrast, others despite good glycemic control may develop PDR at a relatively early stage of diabetes. Therefore, DR is believed to be multifactorial disease with a complex interplay of environmental and genetic factors [Chun *et*

*Corresponding author. Dr. Zivar Salehi, Department of Biology, Faculty of Sciences, University of Guilan, Rasht, Iran. Tel., 0131-3233647. E-mail: geneticzs@yahoo.co.uk

al., 2010; Uthra *et al.*, 2008]. Glycemic control and the duration of diabetes have been identified as major risk factors for the development of DR [Jerneld and Algreve, 1986; Stratton *et al.*, 2000]. Oxidative stress (mediated by excessive reactive oxygen species (ROS)) and increased expression of growth factors like vascular endothelial growth factor (VEGF) are two important pathogenic mechanisms that potentially link hyperglycemia and DR [Tarr *et al.*, 2012].

Manganese superoxide dismutase (SOD2 or MnSOD) is a key enzyme in cell defense against mitochondrial ROS. It catalyzes the dismutation of two superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2) [Baker *et al.*, 1997]. The human MnSOD gene is a single-copy gene consisting of five exons interrupted by four introns and located in the chromosome 6q25.3 region [Wan *et al.*, 1994]. The most common polymorphism of MnSOD is A16V (C47T) single nucleotide polymorphism (SNP) in codon 16 of 24-amino acid mitochondrial targeting sequence (MTS domain) which changes (C) alanine to (T) valine amino acid leading a conformational change of the secondary structure from α -helix to β -sheet [Rosenblum *et al.*, 1996]. Import of the valine protein was partially arrested in the mitochondrial inner membrane resulting in 30–40% less active MnSOD protein in the mitochondrial matrix [Sutton *et al.*, 2003]. Vascular Endothelial Growth Factor (VEGF) is a heparin-binding glycoprotein and an endothelial cell-specific mitogen which plays a fundamental role in regulating angiogenesis and vascular permeability in both physiological and pathological states [Ikuhashi *et al.*, 2007].

The VEGF gene is located on chromosome 6p12 and includes eight exons and seven introns [Vincenti *et al.*, 1996]. One of the common polymorphisms in the 5'-untranslated region (UTR) is +405 C/G (rs2010963) polymorphism which has been shown to significantly increase VEGF production [Watson *et al.*, 2000]. The +405 C/G polymorphism alters the activity of the

internal ribosomal entry site B enhancing initiation of translation at the AUG start codon. Also regulates the production of the large VEGF isoform translated at an alternative CUG codon. So it is believed the +405 C/G polymorphism likely affects expression at the post-transcriptional level [Huez *et al.*, 2001]. Based on the significant roles of MnSOD A16V polymorphism in enzyme activity and VEGF+405 C/G polymorphism in protein expression, we investigated the association of these polymorphisms with diabetic retinopathy in north Iranian population.

Materials and Methods

Subjects: Blood samples were obtained from 70 T2DM patients with DR (40 women, 30 men) and 70 age and sex matched control subjects of northern Iranian population. The participants underwent on ophthalmological examination including visual acuity, slit lamp examination and funduscopy for the absence or presence of DR. Control subjects were unrelated healthy individuals with no family history of T2DM and with normal result of glycated hemoglobin (HbA1c) test. All individuals provided informed consent for their participation in the study.

The study was approved by ethics committee of Guilan University and was conducted in accordance with the Declaration of Helsinki regarding the use of human samples.

Genotyping: The total DNA was isolated from a 1 ml blood sample using the Gpp Solution kit (Gene Pajoohan Pouya, Iran) according to the manufacturer's recommendations. Genotyping of both polymorphisms was carried out by PCR–RFLP analysis. The relevant fragments including the VEGF+405 C/G variation and the MnSODA16V variation were amplified using pair of primers (Forward: 5'-AGCTCCAGAGAGAAGTCGAG-3', Reverse: 5'-GAACAGCCCAGAAGTTGGAC-3') and (Forward: 5'-CGGGCTGTGCTTTCTCGTC-

3', Reverse: 5'-TCAGCCTGGAACCTACCCT T-3'), respectively. Each reaction mixture (25 µl) comprised 30 ng of genomic DNA, 1X PCR buffer, 0.2 µM dNTP, 1.5 µM of MgCl₂, 0.5 µM of each primer (forward and reverse) and 1.5 U of Taq DNA polymerase (Bioflux, Japan). Following an initial denaturation step (5 min at 95°C), samples were subjected to 35 rounds of PCR at 95°C for 45s, 58°C (A16V) or 57°C (+405 C/G) for 1 min, and 72°C for 45 s with a final extension time of 5 min at 72°C followed by a 4°C hold cycle. The PCR products were digested with restriction enzyme *Bsa*WI (New England Biolabs, USA) at 60°C for 1 h for the A16V polymorphism or with *Bsm*FI (Fermentase, USA) at 37°C overnight for the +405 C/G polymorphism. The 16V allele was cut into two fragments of 196 and 47 bp, while the 16A allele remained uncut (243 bp). The +405G allele was cut into two fragments of 265, 85 bp, while the +405C allele remained uncut (350 bp). The PCR products and the restriction fragments were separated in 2% agarose gel stained with ethidium bromide, and were visualized by gel documentation system (Bio Rad, USA).

Statistical analysis: The statistical significance of differences between groups was calculated by the Chi-square test. Odds ratios and 95% confidence intervals were also calculated. A *P* value less than 0.05 was considered statistically significant. All

statistical analyses were conducted using the MedCalc (version 12.1, Belgium).

Results

Clinical features of the study subjects are shown in Table 1. The PCR products of *MnSODA16V* and *VEGF+405 C/G* polymorphisms are shown in Figure 1. The genotyping of both polymorphisms was performed according to the results of enzyme digestion (Figure 2). Genotype distributions and allele frequencies of *MnSODA16V* and *VEGF+405 C/G* polymorphisms are shown in Table 2. According to the genotype distributions, the heterozygous AV of *MnSOD* A16V polymorphism had a significant frequency (71.43%) in patients. It was also observed the heterozygous AV had a 8.33-fold increase in risk of DR (OR=8.33, 95% CI=2.56-27.13, *P*=0.0004). However, it was not found a significant increase in the V allele frequency among patients when compared with controls (*P*=0.63). The homozygous carrier state of *VEGF +405C* allele presented a high risk factor for DR (*P*= 0.0003). Also assuming a dominant model (CC+CG vs. GG), there was significant association between CC+CG genotypes and DR (OR=3.29, 95% CI=1.53-7.07, *P*= 0.002). The prevalence of *VEGF+405C* allele was higher in patients than controls (*p*< 0.05) and the statistically significant association was found between C allele and DR (OR=2.55, 95% CI=1.57-4.14, *P*= 0.0001).

Table 1 Characteristics of patients with DR and controls

Clinical characteristics	Patients n (%)	Controls n (%)
Sex {		
female	40 (57.14)	40 (57.14)
male	30 (42.86)	30 (42.86)
Age (years)	52.5±22.5	52.5±22.5
Duration of diabetes (years)	22±12	-
Family history of DR	68 (97.14)	-
HbA/C	7.83±1.1	4.5±1

Table 2 Genotype and allele frequencies of *MnSOD* A16V and *VEGF* +405C/G polymorphisms.

Genotype	Patients n (%)	Controls n (%)	OR (95% CI)	P Value
MnSOD				
AA	4 (5.71)	18 (25.71)	1.00 (Ref)	-
AV	50 (71.43)	27 (38.57)	8.33 (2.56-27.13)	0.0004
VV	16 (22.86)	25 (35.71)	2.88 (0.82-10.07)	0.09
A	58 (41.43)	63 (45)	-	-
V	82 (58.57)	77 (55)	-	0.63
VEGF				
GG	13 (18.57)	30 (42.86)	1.00 (Ref)	-
CG	34 (48.57)	32 (45.71)	2.17 (0.97-4.88)	0.06
CC	23 (32/86)	8 (11.43)	6.63 (2.36-18.67)	0.0003
CG+CC	57 (81/43)	40 (57.14)	3.29 (1.53-7.07)	0.002
G	60 (42.86)	92 (65.71)	1.00 (Ref)	-
C	80 (57.14)	48 (34.29)	2.55 (1.57-4.14)	0.0001

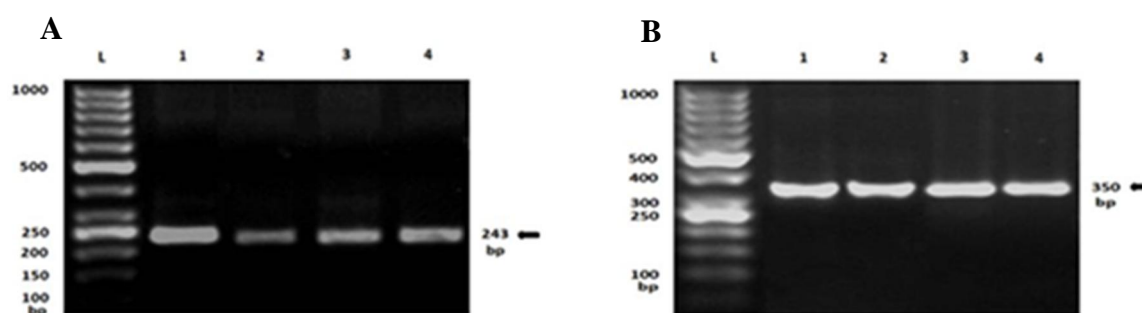


Figure 1 Agarose gel electrophoresis after PCR amplification of *MnSOD* (A) and *VEGF* (B). Lanes: L, DNA Ladder; 1-4, amplified fragment.

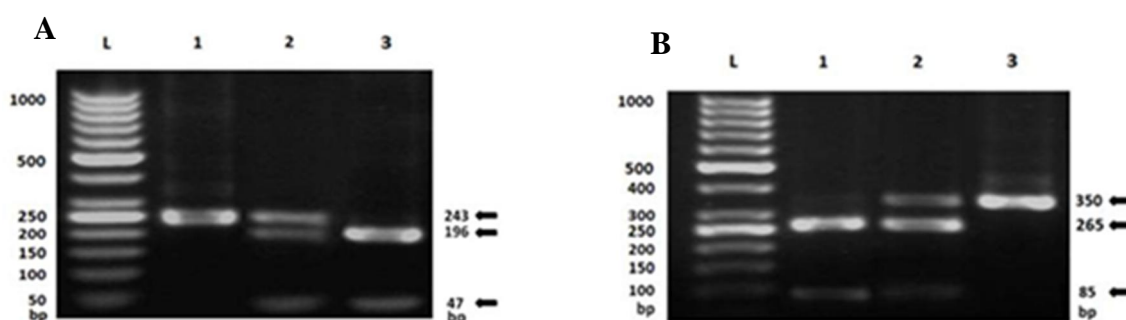


Figure 2 RFLP analyses of the polymorphisms. (A) *MnSOD* A16V polymorphism; L: DNA ladder, 1: AA homozygote, 2: AV heterozygote and 3: VV homozygote. The 47 bp fragment isn't clear because of being small. (B) *VEGF* +405C/G; L: DNA ladder, 1: GG homozygote, 2: CG heterozygote and 3: CC homozygote. The 85 bp fragment isn't clear because of being small.

Discussion

In the current study the association of genetic variations of *MnSOD* A16V and *VEGF* +405 C/G with development of DR in north Iranian population with type 2 diabetes has been evaluated. The statistical analysis has shown the individuals heterozygous for the variant AV genotype in the codon 16 of MTS domain of MnSOD have higher risks of developing DR compared with AA genotype (OR=8.33, 95% CI=2.56-27.13, $P=0.0004$). Also, the combined genotypes of *VEGF* +405 C/G (CC+CG) showed a significant association with DR (OR=3.29, 95% CI=1.53-7.07, $P=0.002$). The +405C allele was considered as a high risk factor of DR (OR=2.55, 95% CI=1.57-4.14, $P=0.0001$). The *MnSOD* A16V and *VEGF* +405 C/G are two main polymorphisms that many studies have investigated the association of them with various diseases, including type II diabetes, prostate cancer, colorectal cancer and endometriosis [Arsova-Sarafinovska *et al.*, 2008; Kim *et al.*, 2005; Maltese *et al.*, 2009; Nakanishi *et al.*, 2008]. A variant of MnSOD enzyme is 30–40% more active and has more efficient import into the mitochondrial matrix than the V variant, therefore the AA genotype may have higher enzyme activity than VV genotype [Sutton *et al.*, 2003]. The decrease of importing of MnSOD results in decrease the enzyme concentration in the matrix leading to overproduction of superoxide which links hyperglycemia and metabolic pathways involved in vascular complications of diabetes [Brownlee, 2005]. The association of the *MnSOD*A16V polymorphism with DR have been investigated in a few studies which two ones had consistent results. In one study was shown that VV genotype of the A16V polymorphism of the *MnSOD* was associated with DR in Caucasians with T2DM [Petrovič *et al.*, 2008]. In other study by Hovnik *et al.* in patients with T1DM in Ljubljana, Slovenia was shown the patients with *MnSOD* VV genotype were in a 2.49-fold higher risk of

development of DR compared with AA genotype (OR=2.49, 95% CI=1.00–6.16, $P=0.045$) [Hovnik *et al.*, 2009].

Contrary to the hypothesis that the V allele might be a high-risk allele, Kangas-Kontio *et al.* reported a higher frequency of the AA genotype ($P=0.03$) and A allele ($P=0.04$) of the *MnSOD* in the diabetic (type 1 or type 2) patients with DR versus the diabetic controls without DR in Finland [Kangas-Kontio *et al.*, 2009]. Also, some studies in cancers affirm the role of AA genotype in increasing of cancer risk, such as higher risk of prostate cancer of male heavy smokers in the Finnish study [Woodson *et al.*, 2003] or greater risk of ovarian carcinoma [Olson *et al.*, 2004]. These conflicts results even on the same diseases may be due to variability of sample features and disease stages [Li *et al.*, 2005; Woodson *et al.*, 2003].

The +405 C/G is most probably a functional polymorphism, because it enhances *VEGF* expression at both transcriptional and translational levels [Huez *et al.*, 2001]. Many studies have done about *VEGF* +405 C/G polymorphism and its association with DR that had conflict results. In many studies have been reported no significant association between the +405 C/G and DR, regardless of ethnicity [Nakamura *et al.*, 2009; Petrovič *et al.*, 2008; Ray *et al.*, 2004; Uthra *et al.*, 2008]. However, based on study in Japanese population, the +405C allele was significantly increased in the NPDR group ($P=0.0026$) versus patients T2DM without retinopathy (DWR). It was also shown that the GG genotype was significantly decreased in the NPDR group ($P=0.0009$), while CC genotype was significantly increased in the PDR group ($P=0.021$) [Awata *et al.*, 2002]. In a study of Brazilian population of European ancestry, +405CC genotype is an high risk factor for the development of PDR in T2DM patients (OR= 1.85, 95% CI= 1.2–2.8, $P=0.003$) [Errera IV *et al.*, 2007]. Suganthalakshmi *et al.* has found significant difference of the genotype

distribution of the +405 C/G polymorphism between patients T2DM with diabetic retinopathy (DR) and without retinopathy (DWR) ($P=0.021$) in Indian population and found heterozygous genotype is significantly higher in the DR group against DWR group (OR=2.33, 95% CI= 1.24-4.36, $P=0.008$) [Suganthalakshmi *et al.*, 2006].

Another study by Watson *et al.* was shown that the highest VEGF production was observed for the GG genotype, the intermediate production for the CG genotype, and the lowest production was observed for the CC genotype. This conflict result maybe due to the methodological differences, the measurement of lipopolysaccharide (LPS)-stimulated VEGF production in peripheral blood mononuclear cells (PBMCs) of 21 healthy subjects in the culture medium in this study [Watson *et al.*, 2000].

In conclusion, our study implicates that the heterozygous AV in *MnSOD* A16V may be considered as a risk factor for DR. Also, the combined genotypes of *VEGF* +405 C/G (CC+CG) have significant association with DR and the C allele has a 2.55-fold increase in risk of DR. However, larger population and different ethnicities – based studies are required to achieve a definitive conclusion.

Acknowledgements

The authors would like to appreciate all participants who contribute to this project. This research was supported by University of Guilan.

References

1. Arsova-Sarafinovska Z, Matevska N, Petrovski D, Banev S, Dzikova S, et al. 2008. Manganese superoxide dismutase (*MnSOD*) genetic polymorphism is associated with risk of early-onset prostate cancer. *Cell Biochem Funct* 26(7): 771-777.
2. Awata T, Inoue K, Kurihara S, Ohkubo T, Watanabe M, et al. 2002. A common polymorphism in the 5'-untranslated region of the *VEGF* gene is associated with diabetic retinopathy in type 2 diabetes. *Diabetes* 51(5): 1635-1639.
3. Baker AM, Oberley LW, Cohen MB. 1997. Expression of antioxidant enzymes in human prostatic adenocarcinoma. *The Prostate* 32(4): 229-233.
4. Brownlee M. 2005. The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* 54(6): 1615-1625.
5. Cai J, Boulton M. 2002. The pathogenesis of diabetic retinopathy: old concepts and new questions. *Eye* 16(3): 242-260.
6. Chun MY, Hwang HS, Cho HY, Chun HJ, Woo JT, et al. 2010. Association of vascular endothelial growth factor polymorphisms with nonproliferative and proliferative diabetic retinopathy. *J Clin Endocrinol Metab* 95(7): 3547-3551.
7. Council NHaMR. 2008. Guidelines for the Management of Diabetic Retinopathy. NHMRC.
8. Errera IV F, Canani LH, Silva MER, Yeh E, Takahashi W, et al. 2007. Functional vascular endothelial growth factor-634G>C SNP is associated with proliferative diabetic retinopathy: a case-control study in a Brazilian population of European ancestry. *Diabetes care* 30(2): 275-279.
9. Hovnik T, Dolžan V, Bratina NU, Podkrajšek KT, Battelino T. 2009. Genetic polymorphisms in genes encoding antioxidant enzymes are associated with diabetic retinopathy in type 1 diabetes. *Diabetes care* 32(12): 2258-2262.
10. Huez I, Bornes S, Bresson D, Créancier L, Prats H. 2001. New vascular endothelial growth factor isoform generated by internal ribosome entry site-driven CUG translation initiation. *Mol Endocrinol* 15(12): 2197-2210.
11. Ikuhashi Y, Yoshida S, Kennedy S, Zondervan K, Takemura N, et al. 2007. Vascular endothelial growth factor +936 C/T polymorphism is associated with an increased risk of endometriosis in a Japanese population. *Acta Obstet Gynecol Scand* 86 (11): 1352-1358.
12. Jerneld B, Algvere P. 1986. Relationship of duration and onset of diabetes to prevalence of diabetic retinopathy. *Am J Ophthalmol* 102(4): 431-437.
13. Kangas-Kontio T, Vavuli S, Kakko S, Penna J, Savolainen E-R, et al. 2009. Polymorphism of the manganese superoxide dismutase gene but not of vascular endothelial growth factor

- gene is a risk factor for diabetic retinopathy. *BJO* 93(10): 1401-1406.
14. Kim SH, Choi YM, Choung SH, Jun JK, Kim JG, et al. 2005. Vascular endothelial growth factor gene +405 C/G polymorphism is associated with susceptibility to advanced stage endometriosis. *Hum Reprod* 20(10): 2904-2908.
 15. Li H, Kantoff PW, Giovannucci E, Leitzmann MF, Gaziano JM, et al. 2005. Manganese superoxide dismutase polymorphism, prediagnostic antioxidant status, and risk of clinical significant prostate cancer. *Cancer Res* 65(6): 2498-2504.
 16. Maltese P, Canestrari E, Ruzzo A, Graziano F, Falcone A, et al. 2009. VEGF gene polymorphisms and susceptibility to colorectal cancer disease in Italian population. *Int J Colorectal Dis* 24(2): 165-170.
 17. Nakamura S, Iwasaki N, Funatsu H, Kitano S, Iwamoto Y. 2009. Impact of variants in the VEGF gene on progression of proliferative diabetic retinopathy. *Graefes Arch Clin Exp Ophthalmol* 247(1): 21-26.
 18. Nakanishi S, Yamane K, Ohishi W, Nakashima R, Yoneda M, et al. 2008. Manganese superoxide dismutase Ala16Val polymorphism is associated with the development of type 2 diabetes in Japanese-Americans. *Diabetes Res Clin Pract* 81(3): 381-385.
 19. Olson S, Carlson M, Ostrer H, Harlap S, Stone A, et al. 2004. Genetic variants in SOD2, MPO, and NQO1, and risk of ovarian cancer. *Gynecol Oncol* 93(3): 615-620.
 20. Petrovič MG, Cilenšek I, Petrovič D. 2008. Manganese superoxide dismutase gene polymorphism (V16A) is associated with diabetic retinopathy in Slovene (Caucasians) type 2 diabetes patients. *Dis markers* 24(1): 59-64.
 21. Petrovič MG, Korošec P, Košnik M, Osredkar J, Hawlina M, et al. 2008. Local and genetic determinants of vascular endothelial growth factor expression in advanced proliferative diabetic retinopathy. *Mol vis* 14(1): 1382-1387.
 22. Ray D, Mishra M, Ralph S, Read I, Davies R, et al. 2004. Association of the VEGF gene with proliferative diabetic retinopathy but not proteinuria in diabetes. *Diabetes* 53(3): 861-864.
 23. Resnikoff S, Pascolini D, Etya'ale D, Kocur I, Pararajasegaram R, et al. 2004. Global data on visual impairment in the year 2002. *Bull World Health Organ* 82(11): 844-851.
 24. Rosenblum JS, Gilula NB, Lerner RA. 1996. On signal sequence polymorphisms and diseases of distribution. *Proc Natl Acad Sci U S A* 93(9): 4471-4473.
 25. Stratton IM, Adler AI, Neil HA, Matthews DR, Manley SE, et al. 2000. Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. *BMJ* 321(7258): 405-412.
 26. Suganthalakshmi B, Anand R, Kim R, Mahalakshmi R, Karthikprakash S, et al. 2006. Association of VEGF and eNOS gene polymorphisms in type 2 diabetic retinopathy. *Mol Vis* 12(1): 336-341.
 27. Sutton A, Khoury H, Prip-Buus C, Cepanec C, Pessayre D, et al. 2003. The Ala16Val genetic dimorphism modulates the import of human manganese superoxide dismutase into rat liver mitochondria. *Pharmacogenet Genomics* 13(3): 145-157.
 28. Tarr JM, Kaul K, Wolanska K, Kohner EM, Chibber R. 2012. Retinopathy in diabetes. In: Ahmed SI, editor. *Diabetes: an old disease, a new insight*. Landes Bioscience, p 19.
 29. Uthra S, Raman R, Mukesh BN, Kumari R, Sharma T, et al. 2008. Genetics of diabetic retinopathy. *Int J Hum Genet* 8(1/2): 155-159.
 30. Uthra S, Raman R, Mukesh BN, Rajkumar SA, Padmaja KR, et al. 2008. Association of VEGF gene polymorphisms with diabetic retinopathy in a south Indian cohort. *Ophthalmic Genet* 29(1): 11-15.
 31. Vincenti V, Cassano C, Rocchi M, Persico G. 1996. Assignment of the vascular endothelial growth factor gene to human chromosome 6p21.3. *Circulation* 93(8): 1493-1495.
 32. Wan XS, Devalaraja MN, ST. Clair DK. 1994. Molecular structure and organization of the human manganese superoxide dismutase gene. *DNA Cell Biol* 13(11): 1127-1136.
 33. Watson CJ, Webb NJ, Bottomley MJ, Brenchley PE. 2000. Identification of polymorphisms within the vascular endothelial growth factor (VEGF) gene: correlation with variation in VEGF protein production. *Cytokine* 12(8): 1232-1235.

34. Woodson K, Tangrea JA, Lehman TA, Modali R, Taylor KM, et al. 2003. Manganese superoxide dismutase (MnSOD) polymorphism, α -tocopherol supplementation and prostate cancer risk in the Alpha-

Tocopherol, Beta-Carotene Cancer Prevention Study (Finland). *Cancer Causes Control* 14(6): 513-518.