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Analysis of MnSOD and VEGF genes polymorphisms in Diabetic Retinopathy

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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 MnSODA16V 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 MnSODA16V 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
Glycemic control and the duration of diabetes have been identified as major risk factors for the development of DR [Jerneld and Algvere, 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$_2$O$_2$) 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 $\alpha$-helix to $\beta$-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 isofrm 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 MnSOD A16V variation were amplified using pair of primers (Forward: 5'-AGCTCCAGAGAGAAGTCGAG-3', Reverse: 5'-GAACAGCCCAGAAGTTGGAC-3') and (Forward: 5'-CGGGCTGTGCTTTTCTCGTC-3', Reverse: 5'-CGCGTTGAGAAGTTGGAC-3').
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 MgCl$_2$, 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 BsawI (New England Biolabs, USA) at 60°C for 1 h for the A16V polymorphism or with BsmFI (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 MnSOD A16V 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 MnSOD A16V 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 an 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>
<thead>
<tr>
<th>Table 1 Characteristics of patients with DR and controls</th>
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<tbody>
<tr>
<td>Clinical characteristics</td>
</tr>
<tr>
<td>Sex</td>
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<tr>
<td>female</td>
</tr>
<tr>
<td>male</td>
</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>Duration of diabetes (years)</td>
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<tr>
<td>Family history of DR</td>
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<td>HbA/C</td>
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Table 2 Genotype and allele frequencies of MnSOD A16V and VEGF +405C/G polymorphisms.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients n (%)</th>
<th>Controls n (%)</th>
<th>OR (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnSOD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>4 (5.71)</td>
<td>18 (25.71)</td>
<td>1.00 (Ref)</td>
<td>-</td>
</tr>
<tr>
<td>AV</td>
<td>50 (71.43)</td>
<td>27 (38.57)</td>
<td>8.33 (2.56-27.13)</td>
<td>0.0004</td>
</tr>
<tr>
<td>VV</td>
<td>16 (22.86)</td>
<td>25 (35.71)</td>
<td>2.88 (0.82-10.07)</td>
<td>0.09</td>
</tr>
<tr>
<td>A</td>
<td>58 (41.43)</td>
<td>63 (45)</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>82 (58.57)</td>
<td>77 (55)</td>
<td></td>
<td>0.63</td>
</tr>
<tr>
<td>VEGF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>13 (18.57)</td>
<td>30 (42.86)</td>
<td>1.00 (Ref)</td>
<td>-</td>
</tr>
<tr>
<td>CG</td>
<td>34 (48.57)</td>
<td>32 (45.71)</td>
<td>2.17 (0.97-4.88)</td>
<td>0.06</td>
</tr>
<tr>
<td>CC</td>
<td>23 (32/66)</td>
<td>8 (11.43)</td>
<td>6.63 (2.36-18.67)</td>
<td>0.0003</td>
</tr>
<tr>
<td>CG+CC</td>
<td>57 (81/43)</td>
<td>40 (57.14)</td>
<td>3.29 (1.53-7.07)</td>
<td>0.002</td>
</tr>
<tr>
<td>G</td>
<td>60 (42.86)</td>
<td>92 (65.71)</td>
<td>1.00 (Ref)</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>80 (57.14)</td>
<td>48 (34.29)</td>
<td>2.55 (1.57-4.14)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

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 +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; Petrović 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 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; Petrović 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 this study [Watson et al., 2000].

In conclusion, our study implicates that the heterozygous AV in \(\text{MnSOD A16V}\) may be considered as a risk factor for DR. Also, the combined genotypes of \(\text{VEGF +405 C/G (CC+CG)}\) 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.

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References
gene is a risk factor for diabetic retinopathy. BJO 93(10): 1401-1406.