THE C936T POLYMORPHISM OF THE VEGF GENE IN ROMANIANS – DISTRIBUTION ANALYSIS IN HEALTHY VOLUNTEERS AND IN RECURRENT SPONTANEOUS ABORTION PATIENTS

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Summary

The Vascular Endothelial Growth Factor (VEGF) family of proteins exerts an essential role in vascular development. Variances in the expression of the VEGF gene have been associated with cancer, inflammatory diseases, pregnancy disorders. A single nucleotide polymorphism (SNP) in the 3'-untranslated region (C936T) is responsible for the under-expression of the gene. Our aim was to determine for the first time in our country the frequency of the C936T SNP in a Romanian population group and to compare its general distribution with the data obtained in a group of recurrent spontaneous abortion (RSA) cases. Using the molecular genetics technique PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism), we genotyped 163 Romanian unrelated healthy subjects who consented on participating in the screening for the VEGF C936T polymorphism and an additional number of 113 patients with history of RSA. The screening revealed a genotype distribution of 3.1% TT homozygotes, 23.9% heterozygotes and 73% CC homozygotes reflecting a frequency of 15.0% of the mutant allele T and 85.0% of the wild-type allele C. In comparison with the general population a slight higher frequency of 15.9% for the mutant allele was found in the RSA group. Comparing our findings with the ones obtained in other ethnic groups, no specific pattern can be drawn for the variance of this polymorphism, a fact explained by the still dispersed studies on the epidemiological aspects of this SNP. Although there is little difference between the screening results and those concerning the RSA group, a future prospective of this approach is the effectuation of a case-control study in order to obtain specific information.

Key words: vascular endothelial growth factor, single nucleotide polymorphism, frequency, recurrent spontaneous abortions

Introduction

The Vascular Endothelial Growth Factors (VEGF) are a family of glycoproteins with a very important role in the formation and maintenance of the organism’s normal vascular network. Among the members of this family we mention VEGF-A, VEGF-B, VEGF-C, VEGF-D or PIGF (Platelet Growth Factor) which exert their function by activating specific tyrosine-kinase receptors (VEGFR1, VEGFR2, VEGFR3) situated on endothelial cells of arteries, veins and lymphatics. The signal transduction pathways end in the proliferation, migration as well as increased survival rate of endothelial cells together with pronounced vascular permeability (Ferrara, 2001; Kowanetz and Ferrara, 2006).

The most important of the above mentioned factors is VEGF-A (hereafter referred to as simply VEGF) which was first purified and demonstrated as potent endothelial mitogen by Ferrara and Henzel, 1989. Multiple isoforms (VEGF189,
VEGF_{165}, VEGF_{121}, VEGF_{206}) are obtained through alternate splicing of the 8 exons that constitute the VEGF gene (6q12) (Tischer et al., 1991).

On the one hand, the crucial role of VEGF in the proper formation of the vascular tree and optimal blood supply is demonstrated by the fact that the knockout of a single allele leads to lethality between days 11-12 of gestation in the mouse embryo through impairment of blood island formation and angiogenesis and serious developmental anomalies (Ferrara et al., 1996). In addition, the role of VEGF in normal vasculogenesis and both branching and non-branching angiogenesis has been observed throughout all three semesters of pregnancy in humans (Geva et al., 2002).

On the other hand, pathologic vascularization is also VEGF-dependent, situation apparent in the stimulation of VEGF functions under hypoxic conditions (Nilsson et al., 2004) or, most strikingly, in tumour angiogenesis where VEGF is recognized as a key regulator (Hiklin and Ellis, 2005). Consequently, multiple approaches have been taken in order to achieve beneficial results in cancer therapy using VEGF inhibitors (Kowanetz, and Ferrara, 2006).

Considering its remarkable implications, VEGF has been studied over the past years from the genetics’ perspective through numerous studies aiming at the assessment of polymorphisms affecting the VEGF gene in correlation with its expression as well as with pathologies that might be influenced by altered VEGF functions – examples start with different types of cancer (colorectal cancer - Ungerbäck et al., 2009; breast cancer - Aydan Eroglu et al., 2008, Kataoka et al., 2006; lung cancer - Heist et al., 2008; thyroid cancer - Bunone et al., 1999), familial mediterranean fever (Gunesacar et al., 2007), rheumatoid arthritis (Han et al., 2004), retinopathy in diabetic patients (Awata et al., 2002), psoriasis (Young et al., 2004), preeclampsia (Shim et al., 2007; Papazoglou et al., 2004), recurrent miscarriage (Papazoglou et al., 2005; Vuorela et al., 2000).

Up to this point, no information on the distribution of VEGF polymorphisms in our population has been revealed. In our study we pursued to obtain the first epidemiological data in Romanians on a single nucleotide polymorphism (SNP) in the 3'UTR (untranslated region) consisting in the substitution of a cytosine with tymine at position 936 (C936T - rs3025039) of the VEGF gene. Knowing the high impact of VEGF in terms of pregnancy, together with analyzing the C936T SNP in a general Romanian group, we sought to comparatively evaluate it’s frequency in patients with recurrent spontaneous abortions (RSA).

Material and methods

Study groups

A total of 163 healthy, unrelated volunteers of Romanian ethnicity originating from different parts of the country were recruited for the screening of the VEGF C936T SNP. Simultaneously, a group of RSA patients was formed of 113 patients with a history of at least two consecutive spontaneous pregnancy losses before the 20th week of gestation – miscarriages had been declared idiopathic after regular investigations: ultrasonography, karyotype analysis, hormonal dosage, autoantibody testing (for anticardiolipin and anti-β2-microglobulin antibodies), TORCH serologic tests. The study was led in compliance with the Helsinki Declaration and all participants gave their written and informed consent on inclusion in the study and genetic testing.
Molecular analysis
Genomic DNA was extracted out of white blood cells from 300µl peripheral blood using a commercial kit (Wizzard Genomic DNA Purification Kit, Promega®). For genotype identification the PCR-RFLP (Polymerase Chain Reaction–Restriction Fragment Length Polymorphism) technique was used as previously described (Papazoglou et al., 2004), with minor modifications. Briefly, the PCR reaction was performed in a final volume of 25µl containing: 12.5µl PCR Mix – Taq DNA-polymerase 0.05U/µl, MgCl₂ 4mM, dNTPmix 0.4mM each (Fermentas MBI, Vilnius, Lithuania®); 1µl BSA (Bovine Serum Albumine, Fermentas MBI, Vilnius, Lithuania®) 2mg/ml solution; 8pmoles of each primer (Eurogentec, Seraing, Belgium®); approximately 100ng genomic DNA; nuclease-free water. The reaction was set up on a MastercyclerGradient thermal cycler (Eppendorf®, Hamburg, Germany) and the program consisted of initial denaturation of 5 min at 94°C followed by 35 cycles of denaturation 40 sec at 94°C, annealing 1 min at 64°C and elongation 40 sec at 72°C, with a final elongation of 5 min at 72°C. A fragment of 208bp was amplified and was then subjected to 12h of digestion at 37°C using the restriction endonuclease NlaIII for which the studied polymorphism induces a restriction site. Digested fragments were separated through ethidium bromide stained agarose gel electrophoresis (Fig.1).

Results and discussion
The results reflecting VEGF C936T status in healthy volunteers and RSA patients can be viewed in Table1. Although there is a slightly higher prevalence of the VEGF 936T allele in the RSA group, no apparent association can be mentioned at the moment. The observed and expected frequencies did not significantly differ; therefore the obtained distribution complies with the Hardy-Weinberg equilibrium (data not shown).
Table 1. Genotype and allele frequencies for the VEGF C936T SNP in healthy volunteers and in RSA patients.

<table>
<thead>
<tr>
<th>VEGF C936T</th>
<th>Genotypes</th>
<th>Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT</td>
<td>CT</td>
</tr>
<tr>
<td>Healthy volunteers</td>
<td>3%</td>
<td>24%</td>
</tr>
<tr>
<td>%, (n)</td>
<td>(5)</td>
<td>(39)</td>
</tr>
<tr>
<td>RSA patients</td>
<td>2.65%</td>
<td>26.5%</td>
</tr>
<tr>
<td>%, (n)</td>
<td>(3)</td>
<td>(30)</td>
</tr>
</tbody>
</table>

Comparing our results with the ones obtained in studies on other populations, a similar distribution is evident in Austrians. However, among Europeans, in a Greek population a significantly lower frequency of the 936T allele has been seen, and, in a study performed on the Turkish population, a higher frequency of this allele made a very significant difference in relation to our findings. Concerning Asian populations, this polymorphism tends to be more frequent but only in relation to the Chinese has significance been observed. These examples are illustrated in Table 2.

Table 2. VEGF 936T in Romanians versus other populations.

<table>
<thead>
<tr>
<th>Country</th>
<th>Group size</th>
<th>VEGF 936T</th>
<th>p-value (2-sided)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Romania</td>
<td>163</td>
<td>0.15</td>
<td>-</td>
<td>Present study</td>
</tr>
<tr>
<td>Austria</td>
<td>123</td>
<td>0.16</td>
<td>&gt;0.05</td>
<td>Renner et al, 2000</td>
</tr>
<tr>
<td>Greece</td>
<td>164</td>
<td>0.116</td>
<td>0.02</td>
<td>Papazoglou et al, 2005</td>
</tr>
<tr>
<td>Turkey</td>
<td>122</td>
<td>0.197</td>
<td>0.006</td>
<td>Gunesacar et al, 2007</td>
</tr>
<tr>
<td>China</td>
<td>1,233</td>
<td>0.19</td>
<td>0.02</td>
<td>Kataoka et al, 2006</td>
</tr>
<tr>
<td>Korea</td>
<td>207</td>
<td>0.18</td>
<td>&gt;0.05</td>
<td>Kim et al, 2003</td>
</tr>
</tbody>
</table>

The functional consequences of the C936T polymorphism upon the role of VEGF have been demonstrated in 2 studies (Krippel et al, 2003; Renner et al, 2000). This SNP has been proven to correlate with the under-expression of the gene, significantly lowering the plasma VEGF levels in carriers of at least one VEGF 936T allele. It has been previously hypothesized that this effect might be due to the abolishment of a specific interaction site between VEGF and Angiopoetin 4 (Renner et al, 2000) or to the alteration of the recognition sites situated in the 3'UTR of posttranscriptional regulators which prolong the mRNA half-life (Scandurro et al, 1998).

Several studies were driven to seek the putative association between this polymorphism and the pathological processes associated to pregnancy. Some correlations have been found with pre-eclampsia (Papazoglou et al, 2004; Shim et al, 2007) but no evidence has been observed in relation to RSA. A future prospective of our work would be the effectuation of a case-control study in order to specifically assess the potential influence of this genetic polymorphism in the occurrence of RSA.
Conclusion

In summary, VEGF has been given a lot of attention due to its undoubted importance in the development of the vascular system and also for the therapeutic perspective that it brings, especially related to cancer. Knowledge of the genetic variants of VEGF and their population-based distribution is useful before starting association studies with pathologies that might be influenced by variances of the VEGF function. Our study represents a first step in knowing the status of the VEGF genetic polymorphisms in our population and also a preliminary information on VEGF and recurrent spontaneous abortions.

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