Vascular Endothelial Growth Factor Gene Polymorphisms Are Associated with Prognosis in Ovarian Cancer

Lukas A. Hefler, Alexander Mustea, Dominique Köngen, Nicole Concin, Berno Tanner, Reiner Strick, Georg Heinze, Christoph Grimm, Eva Schuster, Clemens Tempfer, Alexander Reinthaller, and Robert Zeillinger

Abstract

Purpose: Vascular endothelial growth factor (VEGF), an important regulator of angiogenesis and vascular permeability, is involved in various steps of ovarian carcinogenesis. Gene polymorphisms within the gene encoding VEGF were shown to be independently associated with an adverse outcome in various malignancies. No data are available for ovarian cancer.

Experimental Design: In the present multicenter study, we examined three common polymorphisms within the VEGF gene (−634G/C, −1154G/A, and −2578C/A) known to be associated with an increased VEGF production in 563 Caucasian patients with ovarian cancer from Austria and Germany using pyrosequencing. Results were correlated with clinical data.

Results: The three investigated polymorphisms did not correlate with any of the investigated clinicopathologic variables. In univariate and multivariate models, no significant correlations between any polymorphism and patients’ overall survival were ascertained. Simultaneous carriage of the three homozygous genotypes (i.e., VEGF−634C/C, VEGF−1154G/G, VEGF−2578C/C) known to be associated with increased VEGF expression in an individual patient, however, was independently associated with a shortened overall survival (hazard ratio, 2.1; 95% confidence interval, 1.1–3.9; P = 0.02).

Conclusions: We present the first data on VEGF gene polymorphisms in ovarian cancer. Simultaneous carriage of the three investigated homozygous genotypes was shown to be an independent adverse prognosticator of overall survival.

Angiogenesis has been established as a crucial factor in carcinogenesis influencing tumor growth, invasion, and the formation of metastases (1, 2). Growth stress such as hypoxia induces connective tissue or tumor cells to secrete angiogenic molecules, thereby influencing the balance between proangiogenic and antiangiogenic factors (2, 3).

Vascular endothelial growth factor (VEGF), a dimeric glycoprotein with four splice variants consisting of 121, 165, 189, and 206 amino acid residues, is a proangiogenic molecule that elicits its effects by acting as an endothelial cell mitogen and a mediator of increased vascular permeability via binding to its receptors VEGF receptor I and kinase domain receptor (4).

In ovarian cancer, in vitro studies showed that VEGF is crucially involved in various steps of ovarian carcinogenesis (5–7). VEGF was shown to be associated with the promotion of angiogenesis in early stage ovarian cancer, suggesting that VEGF-driven angiogenesis might be an early event in ovarian carcinogenesis (8). Recently, immunohistochemically detected VEGF overexpression and elevated serum levels of VEGF were shown to be associated with an impaired prognosis (9, 10).

The VEGF gene is located on chromosome 6p21.3. At least 30 single-nucleotide polymorphisms in this gene have been described in the literature. Among these, the VEGF−634G/C, −1154G/A, and −2578C/A polymorphisms have been shown to be associated with an increased VEGF production (11–13). Recently, VEGF polymorphisms were evaluated in melanoma (14) and bladder (15), lung (16), prostate (17), and breast cancer (18, 19) patients. Various studies focused on the prognostic potential of VEGF polymorphisms (15, 18, 19). To the authors’ knowledge, no data are available on VEGF polymorphisms in ovarian cancer.

Materials and Methods

Patients. A total of 563 patients with ovarian cancer were included in the present study (Medical University of Vienna, Austria: n = 176; Charité, Campus Virchow-Klinikum, Berlin, Germany: n = 157; Medical
DNA extraction. DNA was isolated from centrifuged blood clots (Viena, Innsbruck) by a modified DNAzol procedure (20), from EDTA-blood (Berlin, Erlangen, Viena) using the QIAamp DNA Blood Midi Kit (Qiagen, Inc., Hilden, Germany), or from formalin-fixed paraffin-embedded tissue (Mainz; ref. 21). The extracted DNA was stored at 4°C until analyzed.

PCR. The primers VEGF–634 SE 5′-GGAAAGAGAGACGCCCCCATC-3′, VEGF–634 AS 5′-GGAACTCCCCCAAGAACG-3′, VEGF–1154 SE 5′-CTGGCTGGGCTGGAGC-3′, VEGF–1154 AS 5′-GGACAGGCAGGCTC-3′, VEGF–2578 SE 5′-AGAGCTATGACCCTGGATAG-3′, VEGF–2578 AS 5′-AACAAAGTTGGGGCTCTGAG-3′ were used to amplify fragments of the VEGF gene. Antisense primers were biotinylated. PCR was carried out in a total volume of 25 μL including 25 ng template, 5 μmol of each sense and antisense primers and puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Little Chalfont, United Kingdom), which contain 2.5 units of puReTaq DNA polymerase, 10 mmol/L Tris-HCl (pH 9.0 at room temperature), 250 mmol/L KCl, 1.5 mmol/L MgCl2, 200 μmol/L dATP, dCTP, dGTP, and dTTP, and stabilizers, including bovine serum albumin. PCR was done on a Perkin-Elmer GeneAmp PCR system 9600 with 40 cycles at 94°C for 30 s, at 58°C (VEGF–2578, VEGF–1154) or 57°C (VEGF–634) for 30 s, and 72°C for 30 s. The reaction was preceded by a primary denaturation step at 94°C for 1 min and incubated at 72°C for 7 min.

Detection of polymorphisms by pyrosequencing. Three common functional single nucleotide polymorphisms (−634 G/C, −1154 G/A, −2578 C/A) within the VEGF gene were detected using Pyrosequencer PSQ 96 and the PSQ 96 SNP Reagent Kit (Pyrosequencing AB, Uppsala, Sweden). Twenty-five microliters of PCR product were used for pyrosequencing according to the instruction of the manufacturer. Five picomoles of the sequencing primers VEGF–634 SE 5′-GTGGCATGAGCAGCAGAAA-3′, VEGF–1154 SE 5′-CGACCCGGTTCGTA-3′, and VEGF–2578 SE 5′-AGGCCAGACCTGGCA-3′ were applied to detect the polymorphisms.

Statististics. After testing for normality using Kolmogorov-Smirnov test, the following values were found to be normally distributed (P > 0.05) and therefore were given as means (SD): age of patients, time to recurrent disease, and time of follow-up. χ² tests were used to compare frequencies of VEGF genotypes between groups defined by clinopathologic variables. Presence of all three of the homozygous genotypes (i.e., VEGF–634G/C, VEGF–1154G/A, VEGF–2578C/C) in an individual was shown to be associated with an increased VEGF production (11–13). Therefore, further statistical analysis focused at comparing the end point of overall survival of the group of patients with simultaneous carriage of these three genotypes (n = 29) with all other patients. Survival times of patients with no evidence of disease, with stable disease, and patients having died of non-cancer-related events were censored with the last follow-up date. Survival times of patients who died of disease and of patients with progressive disease at the time of last follow-up were not censored. Survival probabilities were calculated by the product limit method of Kaplan and Meier and resulting survival curves were compared using the Breslow test.

To evaluate the simultaneous carriage of these three genotypes as an independent predictor of survival, a multivariate Cox regression model was estimated comprising the clinically established Federation Internationale des Gynecologistes et Obstetristes (FIGO) stage (II-IV versus I), tumor grade (2-3 versus 1), age of patients (>70 versus ≤70 years), and the newly investigated variable simultaneous carriage of these three genotypes as independent variables. Interactions between the simultaneous carriage of these three genotypes and FIGO stage, tumor grade, and age of patient were tested for statistical significance. The proportional hazards assumption of the Cox model was visually checked by plotting Schoenfeld residuals of each variable against time and tested by evaluating the statistical significance of the interaction of each variable with log time.

As our study was designed as a multicenter study to generate as many DNA samples as possible, we did not carry out any power analysis before the study. Therefore, we cannot report on any original study power. However, we did a post hoc study power, as follows. The power to detect a hazard ratio of a given magnitude for a polymorphism depends on the distribution of the genotypes of that polymorphism, on the distribution of survival time, and on the distribution of follow-up time. Using the distributions as they present in our data, we calculated a post hoc power of 80% for detecting a hazard ratio of 1.55 for VEGF–634G/C, 1.95 for VEGF–1154G/A, and 1.7 for VEGF–2578C/A. In comparison, the observed hazard ratios for these polymorphisms were 1.2, 1.25, and 0.92, respectively.

The SAS System (version 9.1, SAS Institute, Inc., Cary, NC) and the SPSS statistical software system (SPSS 11.0, SPSS, Inc., Chicago, IL) were used for statistical computations. We used the SAS/Genetics software (version 9.1, SAS Institute) to test for the presence of any linkage disequilibrium. Two-sided P < 0.05 was considered statistically significant.

Results

The frequencies of the genotypes were 45.1% (GG), 46.8% (GC), and 8.1% (CC) for VEGF–634G/C; 41.4% (GG), 46.1% (GA), and 12.5% (AA) for VEGF–1154G/A; and 25.5% (CC), 24.7% (AC), and 49.8% (AA) for VEGF–2578C/A.
50.5% (CA), and 24.0% (AA) for VEGF –2578C/A; all were in Hardy-Weinberg equilibrium (P = 0.08, P = 0.8, and P = 0.9, respectively). All three VEGF polymorphisms were in linkage disequilibrium [VEGF –634G/C and VEGF –1154G/A; Lewontin’s D’, D” = −0.74 (P < 0.001); VEGF –634G/C and VEGF –2578C/A; D’ = −0.83 (P < 0.001); and VEGF –1154G/A and VEGF –2578C/A; D’ = 0.79 (P < 0.001)] within the study population. No significant associations between the three investigated VEGF polymorphisms as well as the simultaneous carriage of the three homozygous genotypes and the clinicopathologic variables FIGO stage, tumor grade, and age of patients at diagnosis were ascertained.

In a univariate analysis, FIGO stage, tumor grade, age of patients at diagnosis, and the simultaneous carriage of the three homozygous genotypes, VEGF –634C/C, VEGF –1154G/G, and VEGF –2578C/C (n = 29), were associated with overall survival (Table 2; Fig. 1). When genotypes were ascertained independently, no significant association was found. In a multivariate Cox regression model, these results remained unchanged (Table 2). No interactions between the simultaneous carriage of the three homozygous genotypes, patients at diagnosis, and the simultaneous carriage of the three homozygous genotypes and the clinico-pathologic variables FIGO stage, tumor grade, and age of patients at diagnosis were ascertained.

**Discussion**

Various studies have investigated gene polymorphisms in patients with ovarian cancer. Relatively few data have been published on their prognostic effect (22, 23). The present multicenter study was set up to evaluate polymorphisms within candidate genes as prognostic markers in a large series of Caucasian patients with ovarian cancer. Based on the important role of VEGF-driven angiogenesis in ovarian carcinogenesis and based on previously published promising data on VEGF polymorphisms in other malignancies (15, 18, 19), we ascertained the prognostic effect of three common functional polymorphisms in patients with ovarian cancer. In our series, VEGF genotypes were not associated with any investigated clinicopathologic variable. With respect to patients’ prognosis, none of the VEGF genotypes alone showed any statistical significance. The VEGF –634C/C, –1154G/G, and –2578C/C genotypes have been shown to be associated with an increased VEGF production (11–13). Therefore, it can be reasonably speculated that simultaneous carriage of these three homozygous genotypes would lead to the highest circulating VEGF value. In our series, these patients had a significantly impaired overall survival. This finding is biologically plausible. Furthermore, we and others have previously shown that patients with elevated serum VEGF levels have a shortened survival (10).

We found a critical combination of VEGF genotypes to be independently associated with survival in a large series of patients with ovarian cancer. These data are new and biologically plausible. The clinical value of these results in the beginning era of bevacizumab (24–28) and other even newer VEGF inhibitors, such as sorafenib (29) and sunitinib (30), remains to be proved.

**Table 2. Univariate Kaplan-Meier analysis and multivariate Cox regression model of prognostic covariates in patients with ovarian cancer**

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Univariate P</th>
<th>HR (95% CI)</th>
<th>Multivariate P</th>
<th>HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGO stage (II-IV vs I)</td>
<td>&lt;0.001</td>
<td>8.2 (3.8-17.5)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Tumor grade (G2-3 vs G1)</td>
<td>0.002</td>
<td>1.8 (1.1-3.2)</td>
<td>0.027</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis (&gt;70 vs ≤70 y)</td>
<td>0.044</td>
<td>1.7 (1.2-2.6)</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>VEGF –634G/C (G/G, G/C vs C/C)</td>
<td>0.08</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>VEGF –1154G/A (G/G vs A/A)</td>
<td>0.24</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>VEGF –2578C/A (C/C vs C/A, A/A)</td>
<td>0.75</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Simultaneous carriage of the</td>
<td>0.005</td>
<td>2.3 (1.3-4.2)</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>three homozygous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>genotypes† vs others</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Multivariate test.
1 Multivariate Cox regression analysis.
2 Hazard ratio and 95% confidence interval.

**Acknowledgments**

We thank Dan Casire Castillo-Tong for selecting PCR and pyrosequencing primer sequences.
References
