Vascular Endothelial Growth Factor and Its Receptor VEGFR-2 Are Highly Expressed in Ovarian Granulosa Cell Tumors

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Short running title: VEGF and VEGFR-2 in ovarian GCTs

Word count: 3074
Number of Tables and Figures: 6
Abstract

Objective Ovarian granulosa cell tumors (GCTs) are hormonally active sex cord stromal tumors accounting for 3-5% of all ovarian cancers. These tumors are generally diagnosed at an early stage but there is a high risk of recurrence, associated with high mortality. Treatment of recurrent GCTs is difficult, and biologically targeted treatment modalities are lacking. GCTs are highly vascularized, and angiogenic factors most probably play a role in their pathology. Vascular endothelial growth factor (VEGF) is a key regulator of tumor angiogenesis, but in GCTs the role of VEGF and its receptors VEGFR-1 and VEGFR-2 remains largely unknown. Our objective was to study the expression of VEGF and its receptors in human GCTs.

Methods We analyzed GCTs from 106 patients for the expressions of VEGF and its receptors utilizing tumor tissue microarray, tumor mRNA and patient serum samples.

Results We found that VEGF and its main biologically active receptor VEGFR-2 were highly expressed in primary and recurrent GCTs, when compared to normal granulosa-lutein cells. The expression of VEGF correlated positively to tumor microvessel density and to VEGFR-2 expression at a protein (p<0.05) and mRNA level (p<0.05). In contrast to VEGFR-2, the expression of VEGFR-1 was weak. Tumor VEGF protein expression was not prognostic for recurrence, however we found high levels of circulating VEGF in the serum of patients with primary GCT.

Conclusions The results suggest an important role for VEGF and VEGFR-2 in GCT pathology and support the possibility of applying novel VEGF or VEGFR-2 targeted treatments to patients with GCT.
Introduction

Ovarian granulosa cell tumors (GCT) are sex cord stromal tumors that account for 3-5% of all ovarian cancer (Reviewed in 1). GCTs are hormonally active tumors, producing estrogens and inhibins, causing the characteristic hormonal signs and symptoms of the disease 2. GCTs are also highly vascularized and hemorrhagic, and may present as large abdominal masses with acute abdominal pain 1. The median age of diagnosis in the adult subtype of GCT is 50-54 years, while the more uncommon juvenile subtype (5%) is diagnosed in children and adolescents 3, 4. Generally GCTs are diagnosed at an early stage, and are considered to have relatively good prognosis with over 90% 5-year survival rate 1. These tumors have, however, a high risk of recurrence associated with high mortality 5, 6.

Vascular endothelial growth factor (VEGF) is a key regulator of physiological and pathological angiogenesis (reviewed in 7), and acts by binding to its two tyrosine kinase receptors VEGFR-1 (Fms like kinase-1, Flt-1) and VEGFR-2 (Fetal liver kinase-1 Flk-1 or kinase-insert domain receptor, KDR) expressed primarily in endothelial cells 7. In the human ovary, VEGF is crucial for reproductive function, regulating follicular development, angiogenesis and the development and maintenance of the corpus luteum 8, 9. VEGF is expressed in the granulosa cells of preovulatory and ovulatory follicles, and most abundantly in the granulosa-lutein cells of the highly vascularized corpus luteum 10, 11. Both VEGFR-1 and VEGFR-2 are expressed in the granulosa-lutein cells in the corpus luteum 11, 12, and VEGFR-1 is also in the granulosa cells of preovulatory follicles 11. The expression of the VEGFRs in malignant granulosa cells is undocumented.

VEGF regulates tumor angiogenesis, and it is expressed in the vast majority of human tumors, including those of the ovary 13. Its expression correlates with tumor malignancy, and encouraging results have been obtained with anti-VEGF therapy in a wide range of neoplastic diseases 14, 15. A
Humanized anti-VEGF antibody (bevacizumab) is now indicated as a first-line treatment for several metastatic cancers, and has shown promise in the treatment of recurrent epithelial ovarian cancer\textsuperscript{16}. VEGFR targeting tyrosine kinase inhibitors are also being evaluated in phase I-III clinical trials\textsuperscript{17,18}.

VEGF expression has recently been reported in small series of GCT patients\textsuperscript{19,20}, and a few case reports show beneficial effects of bevacizumab on recurrent GCT patients\textsuperscript{20,21}. The expression of VEGF has, however, not been thoroughly evaluated in primary or recurrent GCTs, and the expression patterns of the VEGF receptors in GCTs remain unknown. We addressed these issues in our series of 106 GCT patients to elucidate the role of VEGF and its receptors in the biology of these ovarian tumors.

\textit{Materials and methods}

\textbf{Patient characteristics} The ethical committee of Helsinki University Central Hospital and National Supervisory Authority for Welfare and Health approved this study. We identified 106 GCT patients with available tumor tissue samples diagnosed at Helsinki University Central Hospital from 1965 to 2009 and collected the clinicopathological data of the patients. The diagnoses were re-evaluated by an experienced pathologist (R.B.) using immunohistochemical (IHC) markers to confirm the adult GCT diagnoses\textsuperscript{22}. All available freshly frozen samples (n=35) were tested for the C134W mutation in FOXL2\textsuperscript{23-25} and 97\% of the tumors were mutation-positive. Tumor subtype, mitotic index and nuclear atypia were defined by the pathologist (R.B.), as previously described\textsuperscript{22}. For controls, normal human ovaries were retrieved from 3 patients undergoing hystero-oophorectomy for benign indications.

The mean age at diagnosis was 51.5 years (range 19-87 years), and 60 patients (57\%) were postmenopausal and 46 (43\%) premenopausal at the time of diagnosis. Of all patients 30.2\% (n = 32) had a recurrent disease, with a mean follow-up time of 13.7 years (range 0.1-37.8 years). The
Kaplan-Meier analysis of recurrence was performed on 76 patients with available tissue samples, with a mean follow up time of 14.7 years (range 0.7-33.5 years). GCT patient and tumor sample characteristics are described in detail in Table 1.

**Tumor tissue microarray** We constructed a tumor tissue microarray containing 4 cores from each tumor in duplicates as described previously. Seventy-nine primary and 12 recurrent GCT samples were available for this tissue array and for consecutive immunohistochemical analysis.

**Immunohistochemistry** Paraffin-embedded sections of the microarray and normal human ovaries were subjected to immunohistochemistry for the expressions of VEGF (A-20, sc-152, Santa Cruz Biotechnology, Santa Cruz, California, USA), VEGFR-1 (C17, sc-316, Santa Cruz Biotechnology) and VEGFR-2 (A-3, sc-6251, Santa Cruz Biotechnology) as previously described. In control experiments, nonimmune serum replaced the primary antibody. The intensity of staining was analysed from 4 cores of each tumor as a consensus of two researchers (A.F. and M.A). The staining patterns of VEGF, VEGFR-1 and VEGFR-2 were homogenous and the immunoreactivities of the tumor cells were compared to those of the granulosa-lutein cells of the normal ovary. The tumors were further divided into two groups with staining intensities being either “high” or “low”, the latter group including also the tumors that remained negative. The blood vessels were visualized by staining for blood endothelial marker CD34 (mouse anti-human CD34, M7165, DAKO, Glostrup, Denmark), and counted in duplicates per visual field from 4 cores of each tumor. Tumor microvessel density (MVD) was graded as “high” with ≥60 vessels per visual field and as “low” if <60 per visual field.

**Quantitative Real-Time PCR** The RNA was isolated from freshly frozen tumor samples of 30 primary and 5 recurrent GCTs according to manufacturer’s instructions (Nucleospin RNA/Protein kit, catalog no. 740 933.250, Macherey-Nagel, Düren, Germany), and further purified with RNA purification kit (Nucleospin RNA Clean up kit, catalog no. 740 948.50). First-strand cDNA synthesis was performed according to the manufacturer’s instructions from 0.8 µg total RNA using SYBR GREEN RT-PCR reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). The following primers were used for Real Time PCR: for VEGF; forward 5’-TGCAGATTATGCGGATCAAACC, reverse 5’-TGCATTCACATTTGTTGTGCTGTAG, for VEGFR-2 forward 5’- GGAAGCTCCTGAAGATCTGT, reverse 5’- GAGGATATTTCGTGCCGC,
for GAPDH control forward 5’- TCATTTCCTGGTATGACAACG, reverse 5’- TTAICTCCTTGGAGGCCATGT. Standard curve method was applied using purified mRNA from an established human GCT cell line (KGN) as standard. All analysis were performed in triplicate with an ABI PRISM 7700 sequence detection system (Applied Biosystems) according to the manufacturer’s instructions. Quantitative Real-Time PCR of VEGFR-1 was not conducted due to the minimal expression in semi-quantitative PCR (data not shown).

**Serum analyses** Serum samples were obtained from 12 patients at time of diagnosis of the primary (n=7) or recurrent (n=5) GCT. Serum samples were prepared and stored at -80°C until analysis, and analyzed in duplicates with Enzyme Linked Immunosorbent Assay (ELISA) (R&D Systems, Minneapolis, Minneapolis, USA) according to the manufacturer’s instructions. According to the manufacturer the mean serum VEGF in healthy subjects is 220 pg/ml and range 62-707 pg/ml.

**Statistical analysis** Statistical analysis was performed with JMP® software (JMP 7.0.1) and possible correlations were tested with students t-test, x² and Fischer’s exact test or simple regression when appropriate. Kaplan-Meier analysis was performed according to the methodology, using the time from diagnosis to first recurrence as the end point. Log-rank test was applied to compare the differences between the groups. P value less than 0.05 was considered statistically significant.

**Results**

**VEGF and its receptors VEGFR-1 and VEGFR-2 are expressed in human granulosa cell tumors**

Immunohistochemical staining for VEGF, VEGFR-1, VEGFR-2 and CD34 are illustrated in Figure 1. The staining for VEGF was observed in the cytoplasm of tumor cells with even distribution across the tumor cells, and also in the endothelial lining of tumor blood vessels (Figure 1 A, higher inset in A). Staining for VEGFR-1 was less pronounced in the tumor cell cytoplasm and membranes, and expression was also detected in the tumor blood vessel walls (Figure 1 B, higher inset in B). VEGFR-2, however, was strongly expressed in GCT tumor cells, mostly on the tumor cell
membranes (Figure 1 C), and also in the tumor blood vessel walls (Figure 1 C, higher inset in C). For comparison, normal human ovaries were stained for VEGF, VEGFR-1 and VEGFR-2 (lower insets in Figure 1 A-C). In accordance with previous findings \(^{11,12}\), VEGF and VEGFR-2 were expressed in the granulosa-lutein cells of the corpus luteum (lower insets in Figure 1 A, C). VEGFR-1 expression in the granulosa-lutein cells was, however, negligible (lower inset in Figure 1 B).

**VEGF protein expression correlates with VEGFR-1 and VEGFR-2 protein expression, and tumor microvessel density**

The intensities of staining in the tumor cells were evaluated in 79 primary and 12 recurrent GCTs, and the tumors were divided into high or low staining groups (Table 2 A). The intensity of staining in normal granulosa-lutein cells represented the low expression group for VEGF, VEGFR-1 and VEGFR-2. Of all tumors 65 (74%) stained high for VEGF, whereas VEGF staining was low in 23 (26%) of the tumors. Only 6 (7%) tumors (5 primary and 1 recurrent) were negative for VEGF. Staining for VEGFR-1 was generally weak (Table 2 A) with 82% of all tumors exhibiting low staining, and 44 (48%) tumors (40 primary and 4 recurrent) remained negative. The staining for VEGFR-2 was more intense, being high in 82 (93%) of the tumors and low in 6 (7%) of all tumors, respectively (Table 2 A). Only 1 (1%) primary tumor was negative for VEGFR-2. The number of microvessels varied from 6-171 (mean 45) per visual field (Table 2 A). There were no statistically significant differences in the staining patterns of primary and recurrent tumors. In all GCTs, the expression of VEGFR-1 and VEGFR-2 protein correlated positively to that of VEGF (p<0.05) (Table 2 B). The correlation between VEGFR-1 and VEGFR-2 expressions did not reach statistical significance probably due to small numbers in the different groups (Table 2 B). We also found that tumor MVD correlated positively to VEGF expression as analyzed in all tumors (Table 2 B).

**VEGF protein expression is not prognostic for recurrence**

In the primary tumors we could not find any correlations between the expressions of VEGF, VEGFR-1 or VEGFR-2 and primary tumor characteristics (tumor stage, subtype, tumor size, nuclear atypia, mitotic index) or patient characteristics (age at diagnosis, menopausal status). Neither were
there any correlations between VEGF, VEGFR-1 or VEGFR-2 expressions and tumor characteristics (tumor size, subtype, nuclear atypia, mitotic index) in the recurrent tumors. When all GCTs were analyzed, there was, however, a positive correlation between tumor MVD and tumor mitotic index (p<0.05), but not between MVD and other tumor characteristics. We also analyzed whether VEGF protein expression in the primary tumor could be prognostic for recurrence in patients with GCT. The time to first recurrence is shown by Kaplan-Meier analysis as to primary tumor VEGF expression in 76 patients (Figure 2). There were 12 recurrences in the high VEGF group (n=55) and 6 in the low VEGF expressing group (n=21) and the recurrence probability was similar in these groups.

**VEGF and VEGFR-2 mRNA expressions correlate positively in primary and recurrent GCTs**

We next analyzed the mRNA levels of VEGF and VEGFR-2 with quantitative Real Time PCR in 35 GCTs (30 primary and 5 recurrent). We found that VEGF and VEGFR-2 mRNA were expressed in primary and recurrent GCTs (Figure 3 A and B), without any statistical difference between primary and recurrent GCTs. The mRNA expression of VEGF correlated positively to that of VEGFR-2 (Figure 3 C). The mRNA expression of VEGF or VEGFR-2 in primary or recurrent tumors did not show significant correlations to tumor or patient characteristics.

**Circulating VEGF is present in high quantities in the serum of patients with primary GCT**

We evaluated serum levels of VEGF from 7 primary and 5 recurrent GCT patients. At the time of diagnosis mean serum VEGF levels tended to be higher in primary GCT patients (mean 557 pg/ml, median 411 pg/ml, range 107-1020 pg/ml) than in recurrent GCT patients (mean 219 pg/ml, median 149 pg/ml, range 40-539 pg/ml) (Figure 4), although there was no statistical difference between these groups (p= 0.097). Serum VEGF levels did not correlate to tumor size or stage, and there was no statistical difference in tumor size between primary and recurrent tumors.
Discussion

Tumor angiogenesis is a critical step in cancer progression, and anti-angiogenic cancer treatments have been extensively studied over the past decade. VEGF, being one of the main pro-angiogenic growth factors in many cancers, was the first to be targeted and anti-VEGF treatments are now in wide clinical use \(^{15}\). Recently, also the VEGF receptors have shown promise as targets for anticancer drugs \(^{28, 29}\). So far, little has been known about the expression of VEGF and its receptors in GCTs. We show that VEGF and its receptors are expressed in both primary and recurrent GCTs, and these findings provide the biological basis for the development and implementation of biologically targeted treatments to patients with GCT.

In accordance with previous findings \(^{19, 20}\), we found that VEGF was abundantly expressed in GCTs, with almost all tumors (93%) staining positive for VEGF. VEGF was expressed homogenously in the cytoplasm of the tumor cells and also in the blood vessel endothelium. The expression of VEGF protein was higher in GCTs than in the non-malignant granulosa cells, indicating a role for VEGF in these tumors. The expression levels were similar in primary and recurrent GCTs, both at protein and mRNA level. Although we found no correlations between VEGF expression and tumor aggressiveness, the fact that both primary and recurrent tumors express VEGF at high levels, suggests an important role for VEGF in the GCT pathology.

VEGFR-2, the main mediator of VEGF function, is usually expressed only in blood vessel endothelial cells \(^{30}\). We found that compared to normal granulosa-lutein cells, VEGFR-2 protein is highly expressed in the granulosa tumor cells. In normal endothelial cells VEGF exposure leads to downregulation of the VEGFRs \(^{31}\). This regulatory function is, however, lost in VEGFR-2 expressing cancer cells, \(^{32}\) and previous findings from ovarian \(^{33}\) and breast \(^{34}\) cancer cells suggest a survival-promoting VEGF-VEGFR-2 autoloop, that can be inhibited with anti-VEGF treatment (bevacizumab) \(^{35}\). In our analysis, the expression of VEGFR-2 protein and mRNA correlated with those of VEGF,
implicating an autocrine role of VEGF in also GCTs. Furthermore, VEGFR-2 expression did not correlate to tumor MVD, reflecting the strong expression in the tumor cells, and further indicating that the role of VEGFR-2 in GCTs may be independent of the tumor vasculature. Although VEGFR-2 expression did not correlate to tumor aggressiveness, the remarkably high expression suggests a role for VEGF-VEGFR-2 signaling in GCTs that is worth further studies.

In contrast to VEGFR-2, the expression of VEGFR-1 in GCTs was low. Further, VEGFR-1 is usually considered to function as a decoy receptor for VEGF, and it is not markedly phosphorylated upon VEGF binding. The prognostic role of VEGFR-1 in tumors has remained controversial. In the search for novel anti-cancer treatments, some of the new small-molecule tyrosine kinase inhibitors are, however, designed also to inhibit VEGFR-1. We found high VEGFR-1 protein expression in a minority of primary GCTs, and there was no correlation between VEGFR-1 expression and tumor aggressiveness.

As an indirect indicator of tumor angiogenesis, MVD has been shown to be of prognostic significance in ovarian cancer, but its role in GCTs is still unknown. Previous data suggest that mitotic index is a prognostic marker in GCTs, but this has not been found in all studies. We found that VEGF protein expression correlated positively to tumor MVD, independent of tumor size or stage. High tumor MVD also correlated to high tumor mitotic index, implicating enhanced angiogenesis in the tumors with active growth. These results implicate a role for VEGF driven angiogenesis in the growing GCT, and offer a possibility to implement biological adjuvant treatments on the actively growing CCTs with high mitotic index, even regardless of tumor size or stage.

It was of interest to evaluate if VEGF and its receptors can be used in delineating the prognosis of GCT patients. We found no correlations between primary tumor VEGF, VEGFR-1 and VEGFR-2 protein or mRNA expressions and recurrence, and neither did primary tumor VEGF expression predict recurrence free survival. Thus, based on this study, these parameters are likely not to be of value in the prognostic evaluation of GCT patients. In our analysis circulating mean VEGF levels in
primary tumor patients tended to be higher than in patients with recurrent disease. This may be due to increased VEGF producing tumor burden in primary compared to recurrent GCTs. The mean circulating VEGF in primary GCT patients is similar to that observed in epithelial ovarian cancer patients\textsuperscript{44,45}, but the range of circulating VEGF is wide, even in healthy subjects\textsuperscript{46}. A larger series of GCT patients and healthy control subjects needs to be studied to evaluate the clinical value of serum VEGF in these patients.

Surgery is the first line treatment in primary GCTs, but the treatment of recurrent GCTs consists of a combination of surgery and chemotherapy. Recurrences are rather common (reaching 30% in our series) and often present in locations where surgical removal is not feasible\textsuperscript{5,47}. Despite the developments in conventional chemotherapeutic combination treatments, mortality in recurrent GCT is high\textsuperscript{5,6}, and thus the most challenges rise in the treatment of recurrent GCTs. Recently, anti-VEGF (bevacizumab) monotherapy was shown to be highly effective in a retrospective study of eight patients with recurrent GCTs\textsuperscript{20}. In addition, markedly reduced ascites formation was reported with bevacizumab treatment in a patient with recurrent GCT\textsuperscript{21}, reflecting the role of tumor derived VEGF in the formation of cancer related ascites\textsuperscript{48,49}. Considering our findings on the high expression of VEGF and VEGFR-2 also in recurrent GCTs, these patients could benefit from the introduction of VEGF or VEGFR-2 targeted anti-cancer drugs, in combination with traditional treatments. To test this, however, large multicenter clinical trials are required.

In conclusion, our findings implicate a role for VEGF and its receptor VEGFR-2 in GCT pathogenesis and support the targeting of VEGF and VEGFR-2 in the validation of new treatments for GCT patients.

\textit{Conflict of Interest statement}

The authors have no conflicts of interests
**Funding**

This work was supported by Academy of Finland, Finnish Cancer Organizations, Helsinki University Central Hospital Research Funds, Sigrid Juselius Foundation and National Clinical Graduate School.

**Acknowledgements**

We thank Ms Taru Jokinen, Ms Gynel Arifdshan and Ms Teija Karkkulainen for excellent technical assistance, and B.Sci Marjut Kauppinen for critical reading of the manuscript.

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Table and Figure legends

Table 1

Patient and tumor sample characteristics. Mean age, menopause (MP) status, tumor size and stage are reported at the time of primary GCT diagnosis i.e. the time of the primary tumor operation. P = primary tumors, R = recurrent tumors, IHC = immunohistochemistry.

Figure 1

VEGF and its receptors VEGFR-1 and VEGFR-2 are expressed in GCTs. The staining patterns of GCTs are shown for VEGF (A), VEGFR-1 (B), VEGFR-2 (C) and CD-34 (D). Note the expression of VEGF and VEGFR-2 in the tumor cells (A, C) and in the blood vessels (higher insets in A, C). Arrows indicate blood vessels in higher insets in A-D. GL indicates granulosa-lutein cells in the corpus luteum of the normal human ovary (lower insets in A-C). Original magnification 20x, scale bar 100µm.

Table 2

VEGF protein expression correlates positively to the expressions of VEGFR-1, VEGFR-2 and to tumor MVD. The intensity of staining and MVD were divided into groups with high or low expression. Note that high expression was seen in 74% of the tumors for VEGF, 82% for VEGFR-2 and only 16% for VEGFR-1. There were no statistical differences between the expression levels of primary and recurrent tumors. Correlations of all GCT samples are shown in B. Note the positive correlation of VEGF expression to the expressions of VEGFR-1 and VEGFR-2, and to MVD.

Figure 2

VEGF protein expression is not prognostic for recurrence. Kaplan-Meier curves for recurrence in the high (red line, n=55) and low (blue line, n=21) VEGF expression groups. The recurrence probability was similar between the groups.
Figure 3

**VEGF mRNA expression correlates positively to VEGFR-2 mRNA expression.** Quantitative Real-Time PCR was performed on the mRNA isolated from 30 primary (P in A, B) and 5 recurrent (R in A, B) GCTs. Note the similar expression of VEGF and VEGFR-2 in primary and recurrent GCTs, and the positive correlation of VEGF mRNA expression to that of VEGFR-2 in primary and recurrent tumors (C).

Figure 4

**High level of VEGF is present in the serum of patients with primary GCT.** VEGF was measured at time of diagnosis from the serum of patients with primary (n=7) and recurrent (n=5) GCT. The mean VEGF tended to be higher in the primary GCT samples than in the recurrent GCT samples, although the difference was not statistically significant (p=0.097).
## Table 1

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Table 2

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P = primary, R = recurrent, Tot = all tumors,
+ = positive correlation with p<0.05, ns = not significant.
Figure 1
Figure 2

![Graph showing recurrence free proportion over follow-up time (years) for two groups: VEGF protein expression: High (n=55) and Low (n=21).](image)
Figure 3
Figure 4

![Graph showing S-VEGF pg/ml levels for Primary and Recurrent samples with sample points, median, and mean indicated.](image)