The relationship of the angiogenesis regulators VEGF-A, VEGF-R1 and VEGF-R2 to p53 status and prognostic factors in epithelial ovarian carcinoma in FIGO-stages I-II

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Received October 21, 2015; Accepted November 27, 2015

DOI: 10.3892/ijo.2016.3333

Abstract. The aim of this study was to evaluate prognostic effect of the angiogenesis regulators VEGF-R1, VEGF-R2 and VEGF-A for recurrent disease and disease-free survival (DFS), and their relation to the apoptosis regulator p53, in 131 patients with FIGO-stages I-II with epithelial ovarian cancer. For the detection of positivity of the markers the techniques of tissue microarrays and immunohistochemistry (IHC) were used. In tumors the frequency of positive staining for VEGF-R1 was 19%, for VEGF-R2 and VEGF-A, it was 77 and 70%, respectively. Positivity for p53 was detected in 25% of tumors. The total number of recurrences in the complete series was 34 out of 131 (26%) and 5-year disease-free survival (DFS) was 68%. Positive staining for VEGF-A (P=0.030), VEGF-R2 (P=0.011) and p53 (P=0.015) was found more frequently in type II tumors than in type I tumors. Patients with VEGF-R1 negative tumors had worse (P=0.021) DFS compared to patients with VEGF-R1 positive tumors. In two multivariate Cox analyzes with DFS as endpoint, FIGO-stage (HR=3.8), VEGF-R2 status (HR=0.4) and p53 status (HR=2.3), all were significant and independent prognostic factors. When the variables VEGF-R2 and p53 were replaced with the new variable VEGF-R2^{+}p53$^{-/}$other three combinations in one group, it was found that patients from that subgroup had 86% reduced risk of dying in disease (HR=0.24). Findings above, confirmed relationship between VEGF-R2 and VEGF-A and p53, respectively, with regard to recurrent disease and survival. Some findings from the present study are different from results from previous studies on the regulation of angiogenesis. Despite many trials with anti-angiogenic agents in the front line of ovarian cancer have shown to be positive for progression-free survival, no one has demonstrated an impact on overall survival. Therefore, one of the greatest challenges in ovarian cancer research, is to discover predictive and prognostic biomarkers.

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Key words: angiogenesis, ovarian cancer, p53, prognostic markers, VEGF-A, VEGF-R1, VEGF-R2

Introduction

Tumor angiogenesis is essential for solid tumor growth and the key stages in the development of ovarian cancer are the passage of the carcinoma cells through the basic membrane and infiltration of adjacent tissues. Therefore, angiogenesis is an important process for creation of new blood and lymphatic vessels, which sustain the growth of the tumor (1). In neovascularization, it is known that VEGF-R1 and VEGF-R2 (vascular endothelial growth factor receptors) act as receptors for VEGF-A (vascular endothelial growth factor) (2). Although VEGF-A can bind both VEGF-R1 and VEGF-R2, most data suggest that binding of VEGF-A to VEGF-R2 accounts for the majority of the stimulatory signal for the angiogenesis observed *in vivo*. However, the discovery of dozens of other pro-antigenic cytokines and their cognate receptors have confirmed the complexity of the neo-vascular process (3). Thus, the p53 tumor suppressor gene has been implicated to play a central role in the regulation of angiogenesis, with genetic inactivation of p53 resulting in upregulation of pro-angiogenic factors and downregulation of angiogenic inhibitors (4). VEGF-A contains p53 response elements and is involved in formation and inhibition of new blood vessels (5).

Hypoxia is an important regulatory stimulus for tumor growth and for a variety of different biological processes, and tumor hypoxia also induces an 'antigenic switch', a change from an avascular to a vascular tumor, which is critical for the growth of solid tumors. Tumor hypoxia also plays an important role in malignant progression, radio-resistance, and chemotherapeutic drug resistance and can further lead to changes in cell cycle arrest, differentiation, angiogenesis, carcinogenesis and apoptosis (6). p53 is a central component of most cellular stress responses, including hypoxic stress. Despite potential cross-talk mechanisms between p53 and VEGF-A, it remains unclear how these two key cancer-signaling pathways could functionally interact (7). The p53 status (detected by IHC or mutational analysis) in a meta-regression analysis showed that the FIGO stage may influence the of outcome predictive value of p53 and the prognostic significance of p53 seems also to

be more restricted to low stage tumors (8,9). Studies on the importance of VEGF in epithelial ovarian cancer and its relationship to prognosis have shown inconsistent results (10,11). In the same manner, results from studies concerning the prognostic value of VEGF-R1 and VEGF-R2 for ovarian cancer are diverse (1,2).

The aim of the present study was to investigate the role of the angiogenesis regulator proteins VEGF-A, VEGF-R1 and VEGF-R2 and their relation to the tumor suppressor p53 in ovarian carcinoma in patients limited to FIGO-stages I-II.

Materials and methods

Study population. A total of 140 consecutive patients with FIGO-stage I-II epithelial ovarian cancer, who underwent primary surgery and post-surgical chemotherapy in the Uppsala-Örebro Medical Region during the 5-year period from January 1, 2000 to December 31, 2004, were entered into this study. All samples were collected with the patient's informed consent and were in compliance with the Helsinki Declaration (12) and used in accordance with the Swedish Biobank Legislation and Ethical Review Act [approval by Uppsala Ethical Review Board, decision ref. (UPS-03-477)].

In total, 131 patients out of 140 patients, who accepted to participate in the present study were included and there were 131 available tumors for analysis of p53. For VEGF-R1 there were 129 and for VEGF-R2 130 available tumors, respectively. For analysis of VEGF-A, there were 98 available tumors (lower numbers because of technical issues in the staining process). The primary surgery was performed at nine different surgical gynecological departments and the staging procedure was done at the time of primary surgery. Modified surgical staging according to the EORTC surgical staging categories in early ovarian cancer (13) was undertaken in 34 (26%) out of the 131 cases, and in the remaining 77 (74%) patients surgical staging was regarded as minimal or inadequate according to the same guidelines. All patients underwent adjuvant chemotherapy 4-6 weeks after primary surgery. In the total series 105 out of the 131 (80%) of patients received paclitaxel 175 mg/m^2 and carboplatin (AUC=5) at 3-week intervals usually in four courses. The remaining 26 patients were treated with single-drug carboplatin in 4-6 courses.

Patient characteristics, e.g. age, BMI in two groups, performance status (WHO), FIGO-stage and the type of tumor (type I/II) are demonstrated in Table I. No patients were lost from follow-up and the mean follow-up time was 65 months (range, 5-110 months). Survival was taken as date of confirmed histological diagnosis after primary surgery to date of recurrence or last visit.

Sampling and tissue microarray construction of ovarian cancer tissue. Tissue samples of the ovarian cancers were obtained at the primary surgery. The tissue microarrays were constructed as previously described (14). In brief, tumor tissues were embedded in paraffin and 5 μm sections stained with hematoxylin-eosin (H&E) were obtained to select representative areas for biopsies. Core tissue biopsy specimens (diameter 0.6 mm) were taken from these areas of individual donor paraffin blocks and precisely arrayed into a new recipient paraffin block with a custom-built instrument. Tissue core specimens from 131 ovarian carcinomas were arranged in three recipient paraffin blocks. Two core biopsies were obtained from each specimen. The presence of tumour tissue on the arrayed samples was verified by hematoxylin-eosin-stained section. The specimens were then reviewed, classified and graded by a single pathologist.

Table I. The patient characteristics.

Characteristics (n=131)	n (%)
Age (median) 59.0 years (range, 25-84)	
BMI	
BMI ≤25	69 (54.8)
BMI >25	57 (45.2)
WHO performance status	
0	37 (28.2)
1	66 (50.4)
2	21 (16.0)
3	6 (4.6)
FIGO-stage	
IA	39 (29.7)
IB	6 (4.6)
IC	66 (50.4)
II	20 (15.3)
Types of ovarian tumors[a]	
Type I	79 (65.8)
Low-grade (G1) serous	14
Mucinous (G1+G2+G3)	20
Low-grade endometrioid (G1+G2)	29
Clear cell	16
Type II	52 (34.2)
High-grade (G2+G3) serous	37
High-grade (G3) endometrioid	13
Anaplastic	2

[a]Tumors divided in type I and type II tumors according to combination of histological subtype and FIGO-grade.

Immunohistochemistry and interpretation. Sections (5 μm) were cut from each block on coated slides and dried overnight at 37˚C. The sections were pre-treated by heath-induced epitope retrieval in target-retrieval solution (Dako), pH 6.0 or EDTA buffer pH 9.0, for 7 + 7 min in microwave oven (99˚C). Blocking with peroxidase was performed for 5 min. The slides were counterstained with hematoxylin for 2 min. The procedure was performed in a TechMate 500 automated machine (LSAB detection kit; Dako ChemMate). The following monoclonal primary antibodies were used: DO-7, directed against p53 protein (dilution 1:1,000; Dako, Glostrup, Denmark), the IgG antibody (polyclonal rabbit) to the VEGF-A protein (Dako). For VEGF-R1 the polyclonal rabbit antibody Flt-1 was used as primary antibody and for VEGF-R2, the polyclonal mouse antibody Flk-1 was used as primary antibody (both from Santa Cruz Biotechnology). The work of tissue-microarray construction was undertaken at the Department of Pathology,

Table II. Status of protein expression in tumors of the VEGF-A, VEGF-R1 and VEGF-R2 vs. clinical and pathological features (N=131).

Expression	VEGF-A$^+$ n (%)	VEGF-A$^-$ n (%)	VEGF-R1$^+$ n (%)	VEGF-R1$^-$ n (%)	VEGF-R2$^+$ n (%)	VEGF-R2$^-$ n (%)
Total	60 (61)	38 (39)	18 (19)	111 (86)	100 (77)	30 (23)
Histopathology						
Serous	28 (47)	13 (34)	6 (33)	46 (41)	43 (43)	9 (30)
Non-serous	32 (53)	25 (66)	12 (67)	65 (59)	57 (57)	21 (70)
P-value (χ^2)	0.223		0.515		0.202	
Tumor grade						
G1+G2	34 (57)	28 (74)	7 (39)	67 (60)	56 (56)	19 (63)
G3	26 (43)	10 (26)	11 (61)	44 (40)	44 (44)	11 (37)
P-value (χ^2)	0.088		0.087		0.476	
Type of tumors						
Type I	31 (52)	28 (74)	13 (72)	64 (58)	54 (54)	24 (80)
Type II	29 (48)	10 (26)	5 (28)	47 (42)	46 (46)	6 (20)
P-value (χ^2)	0.030		0.242		0.011	
FIGO-stage						
IA-IB	20 (33)	16 (42)	8 (44)	37 (33)	34 (34)	11 (37)
IC	31 (52)	19 (50)	8 (44)	57 (52)	49 (49)	16 (53)
II	9 (15)	3 (8)	2 (12)	17 (15)	17 (17)	3 (10)
P-value (χ^2)	0.482		0.644		0.647	
Recurrent disease						
Without	44 (73)	28 (74)	17 (94)	78 (70)	78 (78)	18 (60)
With	16 (27)	10 (26)	1 (56)	33 (30)	22 (22)	12 (40)
P-value (χ^2)	0.969		0.030		0.049	

the University Hospital MAS in Malmö in South-Sweden, but the immunohistochemical analyses and interpretation were performed at the Department of Pathology, Halmstad Medical Central Hospital. The IHC stains were interpreted by two of the authors (I.S. and T.S.). At the time of evaluation no information was available on the specific diagnosis and prognosis for the individual cases. A semi-quantitative analysis (15) was used and the stains were graded as negative, +, ++ and +++ for p53, VEGF-A, VEGF-R1 and VEGF-R2, and all those markers were dichotomized into negative and positive (+, ++ and +++) cases (16). The staining for p53 was considered to be positive when there was a strong, granular staining of the nuclei of the majority of tumor cells. Positive staining for VEGF-A, VEGF-R1 and VEGF-R2 were confined to the membrane and cytoplasm of the tumor cells.

Statistical analyses. The Pearson's χ^2 was used for testing proportional differences in univariate analyses. The survival curves were generated by using the Kaplan-Meier technique and differences between these curves were tested by the log-rank test. All tests were two-sided and the level of statistical significance was $P \leq 0.05$. The Statistica 12.5 (StatSoft^{TM}) statistical package for personal computers was used for the analyses. For multivariate analyses the logistic regression model was used with recurrence as the endpoint and Cox regression model was used with disease-free survival (DFS) as the endpoint.

Results

Background characteristics. Patient characteristics, including age, BMI (dichotomized), performance status of the patients (WHO), FIGO-stage and types of ovarian tumors (type I and type II) are demonstrated in Table I. Primary cure was achieved in all 131 patients. The total number of recurrences in the complete series was 34 out of 131 (26%), and 22 of these patients (67%) died due to disease. In the complete series, recurrent disease was significantly associated with FIGO substages (P=0.0005), FIGO-grade (P=0.030), adequate surgical staging (P=0.033) and residual disease (P=0.001). In the complete series the 5-year disease-free survival rate was 68%, the disease-specific survival rate 76% and the overall survival rate was 71%.

Expression of angiogenesis regulator proteins and relation to clinicopathological factors and survival. In the present study, the status of protein expression (positivity/negativity) for the angiogenesis regulators, VEGF-A, VEGF-R1 and VEGF-R2 (Table II) are compared with the clinical and pathological factors.

VEGF-A staining was confined to the membrane and cytoplasm. Positivity for VEGF-A was observed in 60 (61%) out of 98 available tumors for this marker. There were no differences in mean age between the groups of patients with

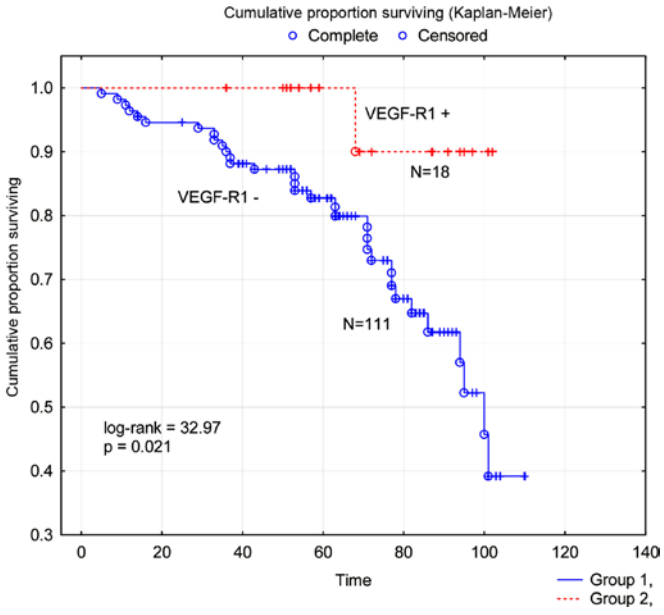

Figure 1. Survival analysis showed significantly worse (P=0.021) disease-free survival for the subgroup of patients with VEGF-R1 negative tumors compared to the subgroup with VEGF-R1 positive tumors.

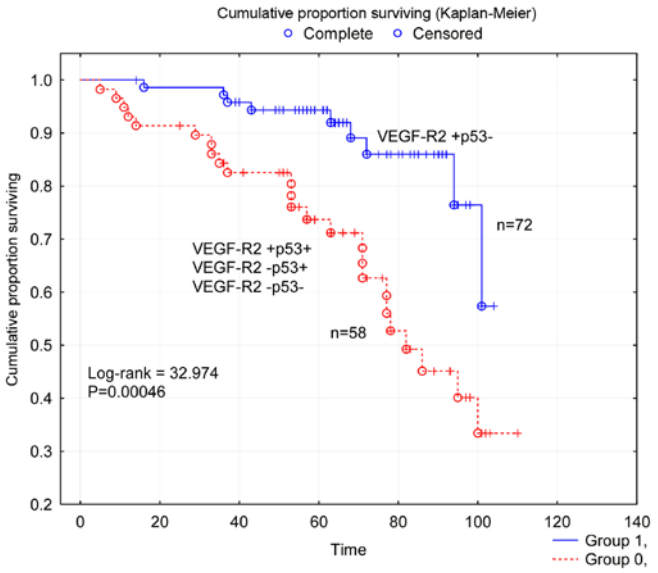

Figure 3. Survival analysis showed significantly better (P=0.00046) disease-free survival for the subgroup of patients with concomitant positivity for VEGF-R2 and negativity for p53 (VEGF-R2^{+}p53^{-}) of tumors compared to other subgroups in group (VEGF-R2^{+}p53^{+}, VEGF-R2^{-}p53^{+}, and VEGF-R2^{-}p53^{-}).

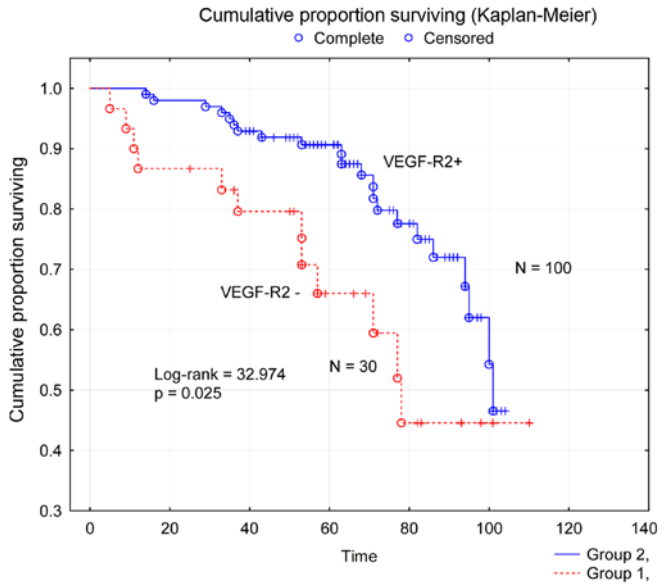

Figure 2. Survival analysis showed significantly worse (P=0.025) disease-free survival for the subgroup of patients with VEGF-R2 negative tumors compared to the subgroup with VEGF-R2 positive tumors.

VEGF-A positive and VEGF-A negative tumors (61 vs. 59 years; P=0.333). As demonstrated in Table II, the VEGF-A status was not related to serous/non-serous histological subtype, tumor grade, FIGO-sub-stages or recurrent disease. However, the status of VEGF-A was statistically significantly (P=0.030) related to the type of tumor. Thus, positive staining for VEGF-A was seen more frequently in type II tumors than in type I tumors (P=0.030). The VEGF-A status alone was not related to survival.

VEGF-R1 staining was confined to the membrane and cytoplasm and positivity for VEGF-R1 was observed in 18 (19%) out of 129 available tumors. There were no differences in mean age between the groups of patients with VEGF-R1 positive and VEGF-R1 negative tumors (59 years for both of the groups). As shown in Table II, the VEGF-R1 status was not associated with serous/non-serous tumors, tumor grade, tumor type or FIGO-stage. However, the VEGF-R1 status was significantly (P=0.030) associated with recurrent disease in univariate analysis. Thus, negativity of VEGF-R1 was detected in tumors by 33 (97%) out of the 34 patients with recurrent disease in the present study. Survival analysis (Fig. 1) showed significantly worse (P=0.021) disease-free survival for the subgroup of patients with VEGF-R1 negative tumors compared to the subgroup with VEGF-R1 positive tumors.

VEGF-R2 staining was confined to the membrane and cytoplasm, and positivity for VEGF-R2 was observed in 100 (77%) out of the 130 tumors. There were no differences in mean age between the groups of patients with VEGF-R2-positive and VEGF-R2-negative tumors (59 vs. 58 years; P=0.620). The VEGF-R2 status (Table II) was not associated with serous/non-serous tumors, tumor grade or FIGO-stage. However, the VEGF-R2 status was significantly (P=0.011) associated with type II tumors. Furthermore, recurrent disease occurred more frequently (P=0.049) in the subgroup of patients with VEGF-R2 negative tumors. The survival analysis (Fig. 2) showed significant worse (P=0.025) DFS for the subgroup of patients with VEGF-R2 negative tumors compared to the subgroup with VEGF-R2 positive tumors. Thus, patients from the subgroup with VEGF-R2 negative tumors had a 5-year disease-free survival rate of 66% compared to 90% in the other subgroup.

VEGF-A was strongly (P=0.001) related to VEGF-R2, but 76 (70%) out of the 98 patients available for statistical analysis had concomitant positivity for both VEGF-A and VEGF-R2.

Results from statistical analysis of the combination of VEGF-A and VEGF-R2 in four groups were undertaken for

Table III. Status of protein expression in tumors of the p53, p53$^-$VEGF-R2$^+$/other[a] (p53$^+$VEGF-R2$^+$, p53$^+$VEGF-R2$^-$ and p53$^-$VEGF-R2$^-$) and p53$^+$VEGF-A$^-$/other (p53$^+$VEGF-A$^+$, p53$^-$VEGF-A$^+$ and p53$^-$VEGF-A$^-$) vs. clinical and pathological features (N=131).

Expression p53$^+$	p53$^+$ n (%)	p53$^-$ n (%)	VEGF-R2$^+$ and p53$^-$ n (%)	Other[a] n (%)	VEGF-A and p53$^+$ n (%)	Other n (%)
Total	33 (25)	98 (75)	72 (56)	58 (44)	11 (12)	87 (88)
Histopathology						
Serous	16 (48)	37 (38)	31 (43)	32 (55)	5 (45)	36 (41)
Non-serous	17 (52)	61 (62)	41 (57)	26 (45)	6 (55)	51 (59)
P-value (χ^2)	0.277		0.428		0.796	
Tumor grade						
G1+G2	16 (48)	60 (61)	42 (58)	32 (55)	8 (73)	54 (62)
G3	17 (52)	38 (39)	30 (42)	26 (45)	3 (27)	33 (38)
P-value (χ^2)	0.199		0.602		0.489	
Type of tumors						
Type I	14 (42)	65 (66)	42 (58)	36 (62)	7 (64)	52 (60)
Type II	19 (58)	33 (34)	30 (42)	22 (38)	4 (36)	35 (40)
P-value (χ^2)	0.015		0.665		0.805	
FIGO-stage						
IA-IB	9 (27)	36 (37)	25 (35)	20 (35)	3 (27)	33 (38)
IC	16 (48)	50 (51)	38 (53)	27 (46)	7 (64)	43 (49)
II	8 (24)	12 (12)	9 (12)	11 (19)	1 (9)	11 (13)
P-value (χ^2)	0.223		0.570		0.674	
Recurrent disease						
Without	17 (52)	80 (82)	63 (87)	33 (57)	5 (45)	67 (77)
With	16 (48)	18 (18)	9 (13)	25 (43)	6 (55)	20 (40)
P-value (χ^2)	0.0006		0.00008		0.025	

the same variables as shown in Table II, but no statistical significance was detected. Furthermore, no relation between the status of VEGF-R1 and VEGF-A was found (P=0.475). In separate analysis of patients with type I tumors (n=79) and type II tumors (n=52), respectively, no statistically significant difference for VEGF-A, VEGF-R1 and VEGF-R2 after age, serous/non-serous histology, tumor grade or FIGO-sub-stages was found. However, for the patients with type I tumors (n=78), recurrent disease was strongly (P=0.008) related to VEGF-R2 negativity of tumors. In the same manner, survival analysis for the patients with type II tumors (n=52), showed significantly worse (log-rank=14.498; P=0.0003) DFS for the subgroup of patients with VEGF-R2 negative tumors compared to the subgroup with VEGF-R2 positive tumors. For the subgroup of patients with type I tumors and type II tumors, respectively, no relation was found between the VEGF-A status of tumors and recurrent disease.

The relationship of p53 status to angiogenesis regulators and association to clinicopathological factors and survival. In a previous study (17) including the total series of patients (n=131) results from IHC and interpretation for p53 have already been presented. Positivity for p53 was found more frequently in type II tumors (37%) compared to type I tumors (17%), (P=0.015). Furthermore, recurrent disease (Table III) occurred more often (48%) in the group of patients with p53 positive tumors compared with the group of patients with p53 negative tumors (18%), (P=0.0006). However, the p53 status alone was not related to VEGF-A (P=0.805), VEGF-R1 (P=0.724) or VEGF-R2 (P=0.210) in the present study. Survival analysis of the concomitant VEGF-R2 status and p53 status in four groups was undertaken (χ^2=14.360; P=0.00246) and it was found that the subgroup of patients with concomitant positivity for VEGF-R2 and negativity for p53 and (VEGF-R2$^+$p53$^-$) of tumors had a favorable survival compared to the other three subgroups. Therefore, disease-free survival for patients in the first subgroup was compared to survival for the other three collective subgroups a further survival analysis (Fig. 3) where the difference (log-rank=32.974; P=0.00046) was highly significant.

Concomitant VEGF-R2$^+$p53$^-$ vs. other combinations in one group were compared after the same clinicopathological factors as before (Table III) without detection of any significance with the exception of recurrent disease (P=0.00008). It was found, that only 9 (15%) out of the 34 patients with recurrent disease in this subgroup compared with 25 (85%) out of the remaining 34 patients, who belonged to the other subgroup of patients.

Table IV. Cox analysis.

A, Cox analysis (univariate and multivariate) with DFS as endpoint

	Univariate analysis		Multivariate analysis		
Variables	HR	95% CI	HR	95% CI	P-value
Age	1.016	0.986-1.046	1.018	0.987-1.051	0.245
Stage (I/II)	3.318	1.655-6.654	3.834	1.847-7.954	0.0003
VEGF-R1	0.148	0.019-1.116	0.206	0.027-1.568	0.127
VEGF-R2	0.436	0.215-0.883	0.292	0.126-0.868	0.004
VEGF-A	0.943	0427-2.083	1.404	0.597-3.303	0.436
p53	2.318	1.173-4.582	2.634	1.278-5.390	0.008

B, Cox analysis (univariate and multivariate) with DFS as endpoint

	Univariate analysis		Multivariate analysis		
Variables	HR	95% CI	HR	95% CI	P-value
Age	1.016	0.986-1.046	1.029	0.994-1.066	0.100
Stage (I/II)	3.318	1.655-6.654	2.934	1.236-6.962	0.0145
VEGF-R1	0.148	0.019-1.116	0.429	0.055-3.311	0.417
VEGF-A	0.943	0427-2.083	1.151	0.503-2.634	0.738
VEGF-R2$^+$p53$^-$/ the collective group[a]	0.278	0.130-0.599	0.237	0.094-0.600	0.002

[a]The collective group (p53$^+$VEGF-R2$^+$, p53$^+$VEGF-R2$^-$, p53$^-$VEGF-R2$^-$).

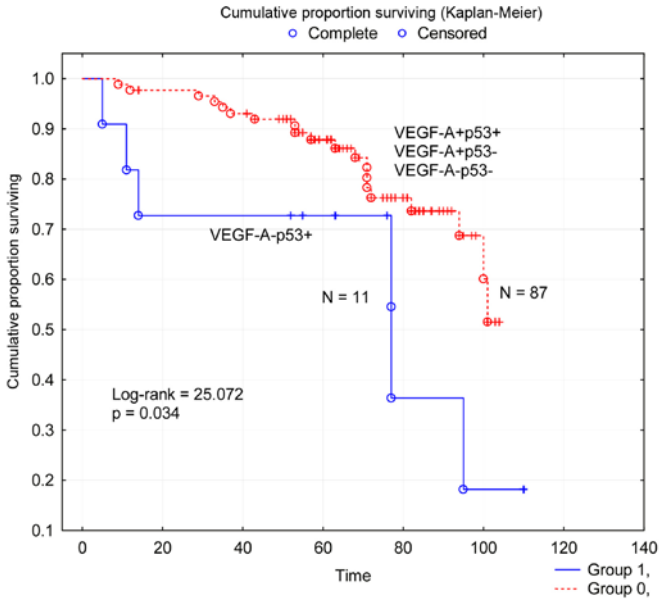

Figure 4. Survival analysis showed significantly worse (P=0.034) disease-free survival for the subgroup of patients with concomitant negativity for VEGF-A and positivity for p53 (VEGF-A$^-$p53$^+$) of tumors compared to the other collective group (VEGF-A$^+$p53$^+$, VEGF-A$^+$p53$^-$ and VEGF-A$^-$p53$^-$).

Furthermore, survival analysis according to concomitant VEGF-A status and p53 status of tumors for available patients (n=98) in four subgroups was undertaken and the results reported (χ^2, 8.240; P=0.0413). The subgroup of patients with tumors of concomitant negativity for VEGF-A and positivity for p53 (VEGF-A$^-$p53$^+$) (n=11) was the only one of the four subgroups of patients, which had statistically different survival (log-rank=25.072; P=0.034) from the other collective subgroup (Fig. 4). The variable VEGF-A$^-$p53$^+$ vs. other combinations in one group was related to recurrent disease (P=0.025), but not to any other of the clinicopathological factors (Table III).

Multivariate analysis. Results are shown for univariate and multivariate Cox analysis with disease-free survival (DFS) as endpoint in Table IVA and B. Only 98 out of 131 patients in the study could be included because of the limited number of tumors with available information on the VEGF-A status. In the first analysis (Table IVA), FIGO-stage, VEGF-R2 status and p53 status, all were significant and independent prognostic factors. For VEGF-A with a HR=1.40, there was a trend for decreased survival for the subgroup of patients with VEGF-A positive tumors (P=0.436). In Table IVB the variables VEGF-R2 and p53 were replaced with the new variable VEGF-R2$^+$p53$^-$/three collectively in one group. The value HR=0.237 (P=0.002) indicates that patients from that subgroup had 86% reduced risk of dying of the disease. The variable type (I/II) of tumor (a combination of histological subtype and tumor grade shown in Table I), was related to the

Table V. Predictive factors for recurrent disease.

A, Predictive factors for recurrent disease (univariate and multivariate logistic regression analysis)

	Univariate analysis		Multivariate analysis		
Variables	OR	95% CI	OR	95% CI	P-value
Age	1.013	0.981-1.047	1.017	0.978-1.057	0.395
Stage (I/II)	7.959	2.801-22.617	11.799	3.282-42.427	0.0001
VEGF-R1	0.139	0.017-1.110	0.141	0.016-1.243	0.074
VEGF-R2	0.423	0.175-1.018	0.229	0.078-0.668	0.006
p53	4.179	1.477-12.071	4.709	1.662-13.339	0.003

B, Predictive factors for recurrent disease (univariate and multivariate logistic regression analysis)

	Univariate analysis		Multivariate analysis		
Variables	OR	95% CI	OR	95% CI	P-value
Age	1.013	0.981-1.047	1.014	0.975-1.055	0.468
Stage (I/II)	7.959	2.801-22.617	9.786	2.663-35.963	0.0005
VEGF-R1	0.139	0.017-1.110	0.187	0.022-1.600	0.122
Type (I/II)	2.456	1.099-5.490	2.217	0.827-5.940	0.109
$VEGF\text{-}R2^{+}p53^{-}$/ the collective group[a]	0.278	0.130-0.599	0.169	0.061-0.468	0.005

[a]The collective group ($p53^{+}VEGF\text{-}R2^{+}$, $p53^{+}VEGF\text{-}R2^{-}$, $p53^{-}VEGF\text{-}R2^{-}$).

variables VEGF-A, VEGF-R2 and p53 and therefore could not be included in the multivariate analyses.

In a multivariate logistic regression analysis with recurrent disease as endpoint, FIGO-stage, VEGF-R2 status and p53 status all were independent predictive factors (Table VA). In a further logistic regression analysis with recurrent disease as endpoint, only FIGO-stage and status of $VEGF\text{-}R2^{+}p53^{-}$ were independent predictive factors (Table VB).

Discussion

In the present study, positive staining for the angiogenesis regulators VEGF-A and VEGF-R2 and the apoptosis regulator p53 was found more frequently in type II tumors than in type I tumors. Furthermore, positivity for VEGF-R1, VEGF-R2 and concomitant positivity for VEGF-R2 and negativity for p53 vs. the other three groups collectively, all were associated with decreased risk of recurrent disease and better survival compared with other alternatives. Differently, positive staining for p53 alone or concomitant negativity for VEGF-A and positivity for p53 vs. the collective group was related to increased risk of recurrent disease and worse survival compared with other alternatives.

High expression for VEGF-R1 was detected in 33 (49%) out of the 67 ovarian tumors in a study concerning the prognostic value of antigenic markers including patients with ovarian cancer in FIGO-stages III-IV, but VEGF-R1 was not associated with progressions-free or overall survival (4). This could be compared with positivity for VEGF-R1 in only 18 (19%) out of 129 available tumors from patients in FIGO-stages I-II in the present study. The subgroup of patients with VEGF-R1 positivity in the present study had significantly better 5-year disease-free survival rate than patients with VEGF-R1 negative tumors. Positivity for VEGF-R2 was observed in 100 (77%) out of the 130 available tumors in the present study. This is in line with results from as study by Nishida *et al* (2), where positivity for VEGF-R2 was detected by IHC in 60 (75%) out of the 80 ovarian tumors from patients in FIGO-stages I-IV. Patients, who had high expression of VEGF-R2 in tumors had worse disease-free survival compared to tumors with negative or low expression of VEGF-R2 in that study. In a further study conducted by western blot analysis, higher concentration of VEGF-R2 was found in ovarian tumors in FIGO-stages I-II compared with FIGO-stages III-IV. However, high expression of VEGF-R2 did not show any effect on progressions-free or overall survival (1).

The VEGF-A status alone was not related to survival in the present study, but positivity for VEGF-A was observed in 60 (61%) out of 98 available tumors. Patients, who had tumors with high expression of VEGF-A tended to have longer disease-free survival in a study by Nishida *et al* (2). Differently, findings in other study including 36 patients in FIGO-stages I-II showed shorter disease-free survival with increased expression of VEGF-A in tumors detected by IHC in 10 (28%) out of the 36 tumors (18). High expression of VEGF-A (using mRNA) was detected in 48% of tumors

to be predictive of poor prognosis by Shen *et al* (19). On the contrary, Engels *et al* (20) illustrated in the subgroup of 54 patients with macroscopic complete tumor resection among 112 patients with primary serous cancer in FIGO-stages I-IV, that high expression of VEGF-A had improved progression-free survival for this group of patients and this was sustained in multivariate analysis as independent factor. Despite large numbers of studies reporting the role of VEGF in ovarian cancer, the mechanisms by which VEGF mediates these effects remain unclear (21). In randomized trials for primary disease (GOG 218 and ICON7) and for recurrent platinum sensitive disease (OCEANS and GOG213), significant improvement of progression free-survival was observed by the addition of bevacizumab to conventional chemotherapy. Overall survival has not, however, been affected and bevacizumab, and >50% of patients do not show benefit from treatment. Therefore, biomarkers that accurately predict resistance to the drug would find immediate clinical use (22). The development of highly specific inhibitors of both the VEGF ligand (bevacizumab and VEGF-Trap, ranibizumab) as well as the VEGF receptor (cediranib, pazopanib and sorafenib) relates to the central role that this pathway plays in ovarian cancer (3,23). There are four positive trials with different anti-angiogenic agents in the front line of ovarian cancer, which have different strategies, drugs, schedules, ways of administration, and toxicity profiles why several questions have been raised and there is room for maximizing the effect of this therapy (24,25). Although all trials have shown to be positive for progression-free survival, no one has demonstrated an impact on overall survival. For instance in a pre-planned test for interaction in predefined groups of the ICON7 trial (24), there was a different magnitude of benefit of anti-angiogenic agents after subgroup. For patients defined as high-risk (stage IV, stage III not operated, and stage III with residual disease after surgery >1 cm), there was benefit in progression-free survival (PFS) with HR of 0.73 and OS with HR of 0.78. While such analyses have limitations, they can provide direction for future research (25). In the results from a newly published meta-analysis (26) the interaction between three different VEGF polymorphism variants and decreased risk for ovarian cancer was explored. It was concluded that one of these (VEGF +936>T) may be a protective factor for epithelial ovarian cancer among the white ethnicity.

Findings from the present study confirmed that the angiogenesis regulators VEGF-R2 and VEGF-A correlate to the p53 status with regard to recurrent disease and survival. Thus, the subgroup of patients with concomitant positivity for VEGF-R2 and negativity for p53 (VEGF-R2$^+$p53$^-$) of tumors had a favorable survival compared to the other collective subgroup. In multivariate analysis the variable (VEGF-R2$^+$p53$^-$) was independent predictive and prognostic factor with recurrent disease and disease-free survival as endpoint, respectively. Negativity of p53 (p53$^-$) detected by IHC means sustained function of the tumor suppressor p53. Differently, p53 positivity (p53$^+$) means a defect function of p53 (mutant p53) in the present study. Disease-free survival for patients with tumors of positivity for VEGF-R2 alone was unchanged (95% disease-free survival after 5 years) after addition of p53 with sustained function in the present study. The question is whether patients with concomitant positivity of VEGF-R2 and negativity of p53 of tumors exhibit higher rate of clinical benefits because of unknown tumor-biological properties or could it be explained with effect of the given post-surgical chemotherapy.

The subgroup of patients with tumors of concomitant negativity for VEGF-A and positivity for p53 (VEGF-A$^-$p53$^+$) vs. the collective group had increased risk for recurrent disease and worse survival. The variable (VEGF-A$^-$p53$^+$) was not an independent prognostic or predictive factor in a multivariate analysis. However, Secord *et al* (4) demonstrated that p53 overexpression (p53 positivity) was associated with low expression of VGEF-A (or VEGF-A negativity) and their findings could partly confirm results from the present study study. However, there are conflicting reports in the literature regarding associations between p53 and VEGF protein expression in tumor specimens. Thus, a study (7) with *in vitro* experiments was performed to understand cross-talk between p53 and VEGF-A regulation under hypoxic conditions better. The authors concluded, that there is growing evidence that the p53 tumor suppressor (wild-type) down-regulates VEGF-A expression under hypoxia, and according to a further *in vitro* study (6) the p53 tumor suppressor (mutant type) showed decreased apoptosis in response to sustained chronic relative hypoxia and increased release of VEGF-A. The development of targeted agents has accelerated in ovarian cancer during recent years. However, selecting drugs for testing needs greater knowledge of the biology of ovarian cancer, and new predictive and prognostic biomarkers so that novel therapeutics can be introduced more rationally and effectively (11). The initial aim of anti-angiogenic therapy by using the VEGF/VEGF-receptor axis was to inhibit growth of new vessel of tumors, usually in combination with chemotherapy. Studies with various anti-VEGF/VEGF-receptor therapies have shown that these agents, when used in combination with chemotherapy, significantly improve survival and response rates in patients (23,27). A number of studies have shown that blockade of VEGF or its receptor VEGF-R2 have normalized tumor vasculature and increased oxygen tension or improved drug penetration. The increased neo-vascular damage might explain improved anti-tumor activity when combining VEGF-targeted agents with chemotherapy (28). This hypothesis could not explain improved survival rate for patients in the subgroup with VEGF-R2 positivity alone or with concomitant negativity for p53 in tumors in the present study. However, in studies by Adham *et al* (29,30) on chemoresistance, it was explored whether a more complete blockade of VEGF signaling would be an effective strategy for ovarian cancer control by knocking down VEGF-R2 expression in chemo-resistant OVCAR-3 EOC cells. Thus, cells with VEGF-R2 knockdown demonstrated more aggressive subcutaneous growth *in vivo*. NRP-1 (Neuropilin-1), a co-receptor for VEGF-R2 is also a receptor for VEGF-A, and enhances the antigenic signal for VEGF-R2, but the cells lacking VEGF-R2 showed increased NRP-1 expression. Evaluation of 80 clinical cases of EOC for NRP-1 versus VEGF-R2 expression showed a significantly higher NRP-1:VEGF-R2 ratio with cancer progression, which means higher NRP-1 expression and/or decreased VEGF-R2 expression. Therefore, it could be concluded that overexpression of VEGF-R2 (positivity) in the tumor cells could protect cancer progression and this findings could partly explain improved survival rate for patients in the subgroup with VEGF-R2 positivity in the present study.

Limitations of the present study corresponds to the relatively limited number of patients included, the tissue microarray technology and the method of semi-quantitative analysis was used for the interpretation, where all markers were dichotomized into negative and positive groups. Findings from the present study confirmed that the angiogenesis regulators VEGF-R2 and VEGF-A have association with the p53 status with regard to recurrent disease and survival. However, some findings from the present study are not in line with results from different previous studies on the regulation of angiogenesis. Although many trials with anti-angiogenic agents in the front line of ovarian cancer treatment have shown positive results for progression-free survival, none has demonstrated an impact on overall survival. Therefore, one of the greatest challenges in ovarian cancer research, is to discover predictive and prognostic biomarkers.

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