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The Relapse of Epithelial Ovarian Cancer Under 2-Year Post-Treatment Observation Based on VEGF-A and VEGFR-2 Expression

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ABSTRACT

Background: Epithelial ovarian cancer is one of the most formidable lethal malignancies, with a 5-year survival rate of approximately 35% for advanced-stage ovarian carcinoma and a 10-year follow-up rate of about 18%. Although several regimens have been evaluated for improvement, relapse remains an inevitable challenge. Recently, specific prognostic factors related to “cell behavior”, including cell proliferation activity such as angiogenesis VEGF (vascular endothelial growth factors) have been recognized. Therefore, this study aims to predict relapse of epithelial ovarian cancer within two years of follow-up based on the expression of vascular endothelial growth factor A (VEGF-A) and vascular endothelial growth factor receptor-2 (VEGFR-2) at the mRNA and protein levels.

Methods: This study was conducted at “Dharmais” Cancer Hospital (DCH) with a retrospective cohort design in patients with advanced epithelial ovarian cancer who received complete treatment. The role of angiogenesis factors on relapse in epithelial ovarian cancer in 2 years of observation was evaluated. Semiquantitative expression analysis of VEGF-A and VEGFR-2 (protein) was conducted through the immunohistochemical method using the avidin-biotin-peroxidase complex (ABC) method. Meanwhile, the semiquantitative expression of VEGF-A and VEGF-R2 (mRNA level) was carried out using RT-PCR (Reverse Transcription- Polymerase Chain Reaction) - Zyber Green Bioline Kit - method, with specific primers.

Results: From 2008 to 2012, only 20 patients were tested for the role of VEGF-A and VEGFR-2 on the incidence of relapse within two years of observation. Among the 20 patients examined for VEGF-A and VEGFR-2 expression, 50% remained disease-free for 20 months, resulting in a cumulative disease-free survival probability of 48.1% for the patient. Those with positive VEGF-A expression at the mRNA level had a shorter median disease-free survival time (18 months), with a lower disease-free survival probability. Patients who showed positive VEGFR-2 expression (at the protein level) had a shorter median disease-free survival time, a lower cumulative probability of disease-free survival (DFS), and a greater risk of relapse (3 times) than patients who did not express (negative) VEGFR-2. In addition, the ROC-based analysis was also performed.

Conclusions: Expression of both VEGF-A and VEGFR-2 at mRNA and protein levels can be used as a predictor for relapse of epithelial ovarian cancer. This study showed that a positive VEGF-A expression at the mRNA level is associated with a twofold higher risk of relapse, while positive VEGF-R2 expression at the protein level is associated with a threefold shorter risk of relapse than negative expression.

INTRODUCTION

Epithelial ovarian cancer is one of the most lethal malignancies, with approximately 70% of women diagnosed at an advanced stage due to its asymptomatic nature until late stages [1]. A 5-year survival rate for advanced-stage ovarian carcinoma is about 35%, with a 10-year follow-up rate of approximately 18%. Survival

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is influenced by factors such as stage, size of residual tumor after cytoreductive surgery, histotype, and race-ethnicity. Despite optimal primary cytoreductive surgery and standard adjuvant chemotherapy, often with carboplatin/paclitaxel, most patients experience relapse within two years [1]. Although several regimens have been [evaluated to improve, relapse remains a persistent challenge [1,2].

The standard treatment for epithelial ovarian cancer involves cytoreduction surgery, aiming to maximize the removal of the tumor mass while minimizing residual tumor presence. The subsequent treatment is taxane-based first-line chemotherapy [3]. Although most cases of patients with advanced epithelial ovarian cancer respond to treatment after undergoing surgery and chemotherapy in 3 years of observation, about 60–70% experience relapse [3,4]. The median disease-free survival (DFS) time in Western European countries ranges from 15–17 months for stage III ovarian cancer and between 8–9 months for stage IV ovarian cancer [5].

Essential prognostic factors for ovarian cancer include pathology-clinical prognostic factors, such as disease stage, postoperative tumor residue, histopathological type, degree of cell differentiation, and general condition of the patient [6]. In addition to the stage of ovarian cancer according to the classification made by FIGO (Federation of International Gynecologic and Obstetrics), postoperative tumor residue influences both the median total survival and disease-free survival [7]. There are also prognostic factors specifically related to “cell behavior” such as Ca 125 (tumor marker), cell proliferation activity including angiogenesis (formation of new blood vessels), tumor suppressor genes including p53, “growth factor” and CD 8 lymphocytes. These are promising for use as prognostic factors for ovarian cancer [8-11]. In cancer, VEGF (vascular endothelial growth factor) is a potent growth factor that plays a role in the process of angiogenesis, which results in the formation of new endothelial cells and blood vessels (capillaries) that are important in the process of expansion of a tumor, particularly solid tumors and also the process of metastasis [12]. Subsequently, an investigation on VEGF Expression conducted by O’Toole et al. [13], found a link between high VEGF-A expression and “progression-free survival” with tumor response to chemotherapy, with additional factors influencing chemotherapy sensitivity.

This study aims to determine whether the expression of VEGF-A and VEGFR-2 at the mRNA and protein levels can predict relapse of epithelial ovarian cancer within two years of follow-up.

METHODS

Study Population

This study was conducted at Dharmais Cancer Hospital using a retrospective cohort design focusing on 20 patients with advanced epithelial ovarian cancer. The objective is to assess the role of angiogenesis factors on recurrence in epithelial ovarian cancer in 2 years of observation. The limited number of samples was due to challenges in obtaining good preparation for molecular examination. There were no differences in the patient characteristics or relapse between these 20 participants and unstudied participants for angiogenesis (VEGF-A and VEGFR-2 expression).

Immunohistochemistry

Semiquantitative analysis of VEGF-A and VEGFR-2 (protein) expression was conducted using the immunohistochemical method, using the avidin-biotin-peroxidase (ABC) complex method. For VEGF-A expression, a primary monoclonal antibody of VEGF-A catalog no: SC-152 (Santa Cruz Biotech Inc) was used. Additionally, VEGF-A and VEGFR-2 expression measurements were performed by classifying negative results to weak expression as negative (0–1) and moderate to strong expression (2–4) as positive, which could be assessed visually directly by noting the color intensity and the extension (percentage) of the intensity into the examination field of view. Negative, weak, moderate, and strong expression was obtained through comparison with the control. Negative to weak results are classified as negative, while moderate to strong results are categorized as positive in this analysis. Assessment was carried out visually by looking at the color intensity and extension (percentage) intensity to the field of view of the examination

In addition to visual analysis in immunohistochemical, the scoring system was used to measure the expression of the protein level. They were classified based on the literature to obtain the intersection of positive and negative points, and the ROC method for relapse (as a gold standard). The results of the ROC calculation were consistent with the visual result in determining the positive or negative expression of VEGF-A (cut-off point score = 1). A negative visual is 0–1, the same as a negative based on the ROC score, which is <1.

Reverse transcription Polymerase Chain Reaction (RT-PCR)

The RT-PCR (Reverse Transcription-Polymerase Chain Reaction) - Zyber Green Bioline Kit method (using specific primers) was used for semiquantitative VEGF-A and VEGFR-2 expression at the mRNA level. VEGFR-2 assay uses a primary monoclonal antibody catalog no: SC-625. The forward primers for VEGF-A are 5’-TTGCAGATGTGACAAGCCGA-3’ the reverse primers are 5’-GAATGCTTCCGCCGGAGT-3’. On the other hand, for VEGFR-2, the forward primers are 5’-GCGACGAATTGACCAAAGCA-3’ and the reverse primers were 5’-GTAAGACCGCTTGCCAGCTA-3’. The assay involves 1) DNA amplification using DreamTaq Green PCR Master Mix 2) electrophoresis, and 3) expression assessment.

For each successive electrophoresis run, there is a positive control (a standard sample expressing the gene of interest) and a negative control (a standard sample lacking the gene expression). Comparison includes examining the DNA fragment array of the sample against the positive control, and searching for a visible “band.” This band manifests as a distinct white line characterized by consistent thickness and precise location equality. In cases where the anticipated “band” is not established, the same comparison procedure is repeated. Subsequently, the results are visually assessed and measured semi-quantitatively. In this study, the positive group were new patients with epithelial ovarian cancer who underwent complete treatment and expressed VEGF-A and/or VEGFR-2 receptors at concentrations exceeding normal levels.

Inclusion Criteria: 1. Patients diagnosed with stage II–III/IV epithelial ovarian cancer who have undergone surgery and received complete treatment according to the established protocol at Dharmais Cancer Hospital. 2. Had paraffin-embedded tumor tissue stored in the Anatomic Pathology Installation. 3. Capable of being observed for 2 years after undergoing complete treatment. Exclusion criteria: 1. results of VEGF-A and or VEGFR-2 examination.

Statistical Analysis

All statistical analyses were performed using the SPSS, version 20. DFS is defined as the duration from the date of surgery until the date of recurrence detection, based on data retrieved from the medical record. The role of VEGF-A and VEGF-R2 was assessed using Cox regression analysis on ovarian epithelial cancer relapse by the size of the crude HR (hazard ratio) and the 95% confidence interval. Univariate analyses were carried out using the Kaplan-Meier method. Multivariate analysis could not be conducted due to the constraints posed by the limited sample size (N=20).

RESULTS

Characteristics study of patients

There were 225 epithelial ovarian cancer cases at Dharmais Cancer Hospital from 2008 to 2012, but only 20 patients were assessed for the study of the role of VEGF-A and VEGFR-2 on relapse cases within two years of observation since the patient completed the course of treatment.

Among the 20 patients, the majority were more than 45 years old (70%), more than two parity (25%), junior and senior high school education (60%), stage III-IV (65%), CA-125 level greater than 200 (80%), pre-operative Hb level greater than ten g/dl (75%), preoperative albumin level less than 3.5 gram% (45%), serosal PA type (45%), taxane chemotherapy type (95%), and residual tumor less than 1 cm (60%). There was considerable missing data in the parity variable (55%), payment method (30%), as well as albumin and CA-125 (both 15%). The characteristics data of patients are shown in **Table 1** below.

Table 1. Demographic and Clinicopathologic characteristics of Epithelial Ovarian Cancer Patients at Dharmais Cancer Hospital from 2008–2012

	Characteristics	IHC examination N (%)
Age at diagnosis (years)	≤ 45	6 (30)
	> 45	14 (70)
Parity	≥ 2	5 (25)
	0–1	4 (20)
	Missing	11 (55)
Education status	Junior High School	6 (30)
	Senior High School	12 (60)
	Missing	2 (10)
Preoperative Albumin	> 3,5 g/dL	8 (42,1)
	≤ 3,5 g/dL	9 (42,1)
	Missing	3 (15,8)
Preoperative Hemoglobin	> 10 g/dL	8 (40)
	≤ 3,5 g/dL	9 (45)
	Missing	3 (15)
CA-125	≤ 35 U/mL	1 (5)
	35,1–200 U/mL	0 (0)
	> 200	16 (80)
	Missing	3 (15)
Stage	II	7 (35)
	III-IV	13 (65)
Histology	Serous Adenocarcinoma	9 (45)
	Mucinous adenocarcinoma	1 (5)
	Endometrioid adenocarcinoma	4 (20)
	Adenocarcinoma	5 (25)
	Clear cell adenocarcinoma	1 (5)
Chemotherapy	Cisplatin	1 (5)
	Taxane	19 (95)
Residual tumor	≤ 1 cm	12 (60)
	> 1 cm	8 (40)

Relapse

In general, both the median and cumulative DFS probability were lower in patients examined for VEGF-A and VEGFR-2 expression compared to the overall patients cohort (**Table 2**). Despite the slight differences, the demographic and clinicopathologic characteristics of the

Table 2. The Median Relapse-free Time and the Cumulative Relapse-free Survival Probability of the 20 patients examined for VEGF-A, and VEGFR-2 Expression versus total patients

	Median Disease Free Time (month)	Cumulative Disease-free Survival Probability (%)
Patients with Immunohistological examination (20)	20,0	48,1
All the patients (65)	*	50,7

Note: * Means that the median relapse-free time was greater than 24 months.

Table 3. The Median time, Cumulative Probability, and Hazard Ratio of DFS Based on VEGF-A and VEGFR-2 Expression.

Variable	Median (Month)	Cumulative Probability	Log-Rank (p-value)	HR crude (95% CI)	p-value
The VEGF-A expression at mRNA levels					
Negative N = 4	*	57,1	0,754	1	0,402
Positive N = 5	18,0	30		2,0 (0,39–10,17)	
VEGFR-2 Expression at mRNA Level					
Negative N = 5	*	60,0	0,264	1	0,286
Positive N = 4	20,0	0		2,44 (0,47–12,55)	
VEGF-A Expression at protein level					
Negative N = 4	20,0	26,7	0,673	1	0,678
Positive N = 5	*	67,5		0,71 (0,14–3,55)	
VEGFR-2 Expression at protein level					
Negative N = 5	*	53,3	0,158	1	0,185
Positive N = 4	10,0	41,7		3,02 (0,59–15,48)	

Table 4. Median Time and Cumulative Probability of DFS by Characteristics According to the Expression of VEGF-A, VEGFR-2 using Immunohistochemical method in Epithelial Ovarian Cancer Patients during 24-month observation at Dharmais Cancer Hospital from 2008 to 2012.

Variables	Median	Cumulative Probability	Log-rank
The VEGF-A expression, visual analysis			
Negative N = 4	20,0	26,7	0,673
Positive N = 5	*	67,5	
The VEGF-A expression, literature score			
0–4	20,0	46,7	0,555
5–15	all censored	-	
The VEGF-A expression, ROC analysis Score			
< 1	20,0	26,7	0,673
≥ 1	*	67,5	
The VEGFR-2 expression, visual analysis			
Negative N = 5	20,0	0,0	0,701
Positive N = 4	*	57,0	
The VEGFR-2 expression, literature score			
0–4	*	53,3	0,158
5–15	10,0	41,7	
The VEGFR-2 expression, ROC analysis score			
< 4,5	*	53,3	0,158
≥ 4,5	10,0	41,7	

patients undergoing molecular analysis were not significantly different from those in the main study (data not shown).

The median DFS in the group assessed for VEGF-A and VEGFR-2 expression was found to be lower than that of the overall patients in the main study. The results showed that half of the molecularly screened patients were disease-free for 20 months within two years of follow-up, while 50% of all patients in the main study (both molecularly screened and non-molecularly screened) were disease-free for more than 24 months. The cumulative DFS probability in the 20 patients with molecular data examined was 48.1%, slightly less than the overall probability of being disease-free in the main study, 50.7% (N=65 people).

Analysis of VEGF-A and VEGF-R Expression

VEGF-A and VEGFR-2 expression measurement was performed by categorizing negative to weak results as negative, and moderate to strong results as positive. Immunohistochemical Assessment involves visual examination of color intensity and extension (percentage) within the field of view, as shown below **(Figures 1 and 2).**

Analysis of VEGF-A and VEGFR-2 Gene Expression (based on immunohistochemical method) on relapse of disease

Patients with positive VEGF-A expression at the mRNA level had short median disease-free survival time and a low cumulative probability of disease-free survival, resulting in a high risk of relapse compared to patients with negative expression. However, there are different results for VEGF-A expression at the protein level, as positive expression had a high median disease-free survival time and high cumulative probability of disease-free survival, resulting in a lower risk of relapse compared to those with negative expression.

VEGFR-2 expression can only be examined at the protein level due to optimization problems at the mRNA level. The results showed that patients with positive expression had shorter median disease-free survival time and lower cumulative probability of DFS, resulting in a greater risk of relapse (3 times) compared to patients with negative (non-expressing) VEGFR-2.

Use of scoring as an alternative to determining expression occurrence

The determination of VEGF-A and VEGFR-2 expression presence or absence was established through scoring using ROC analysis, followed by a comparison with the outcomes derived from the immunohistochemical method. This assessment was further in line with the recurrence of epithelial ovarian cancer (**Table 4**).

In addition to visual analysis in the immunohistochemical method, a scoring system was used to measure expression. They were classified based on the literature to obtain the intersection of positive and negative points, and the ROC method for relapse (as a gold standard). The results of the ROC calculation were consistent with the visual result in determining the positive or negative expression of VEGF-A (cut-off point score = 1). A negative visual result (0–1) corresponds to a negative based on the ROC score (< 1), and both measurements yield the same median relapse-free period. Specifically, for those without VEGF-A expression,

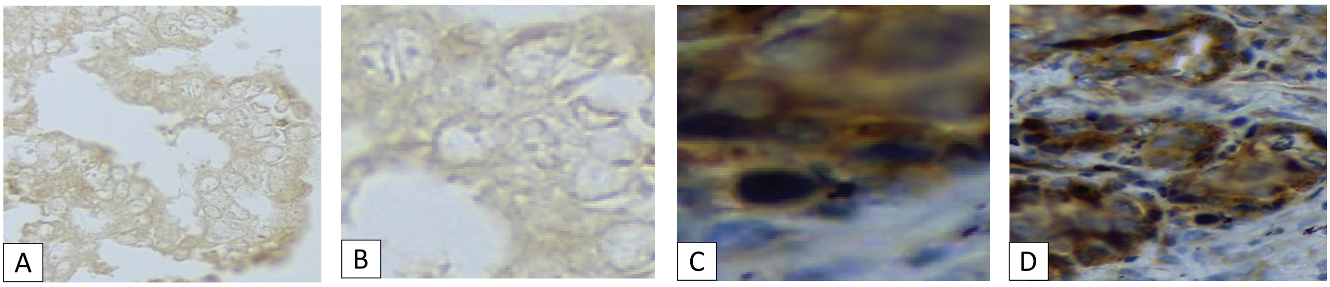

Figure 1. Immunohistochemical VEGF-A Expression (A and B, negative), (C and D, positive)

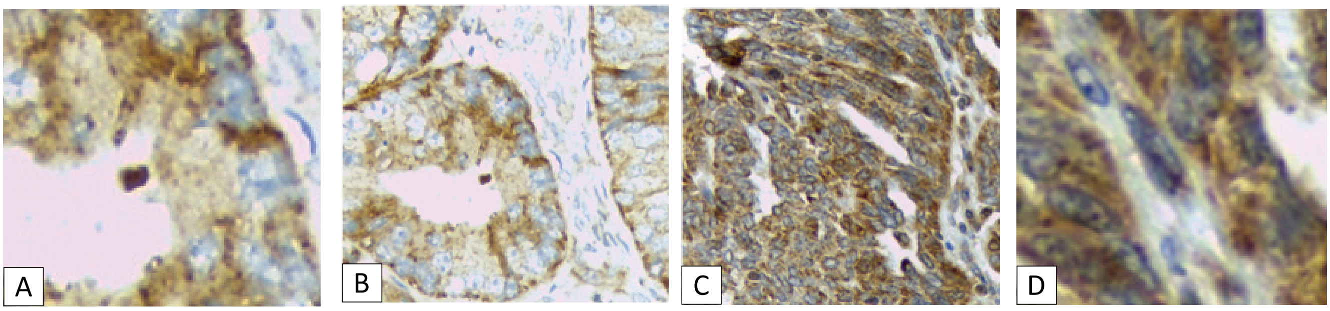

Figure 2. Immunohistochemical VEGFR-2 Expression (A and B, negative), (C and D, positive)

the median relapse-free period was 20 months, while individuals with positive VEGF-A expression exhibited a median relapse-free period surpassing 24 months. The cumulative probability of relapse also differed, with 67.5% in those expressing VEGF-A compared to 26.7% in those without expression. In addition, those with VEGF-A expression had a cumulative probability of relapse of 67.5% versus 26.7% in those without expression. Scores based on the literature were different from the results of the visual analysis or based on ROC scores.

VEGFR-2 results exhibited variation between visual scoring and scoring derived from literature and ROC assessment. Despite this, consistency was observed between literature and ROC scores, both using negative expression when the score fell below 4.5 according to ROC or was less than or equal to 4 based on the literature. The results were also followed by the median relapse-free and cumulative relapse-free probability of the two measurement systems (in those who did not express VEGFR-2, the median relapse-free for 24 months, and the cumulative relapse-free probability was 53.3%).

DISCUSSION

Among the patients tested for VEGF-A and VEGFR-2, comprising 20 out of the total sample, their characteristics are in line with the broader demographic and clinicopathological features of the entire study cohort. Therefore, expression results for VEGF-A and VEGFR-2 in these 20 patients are reasonably applicable to the overall sample.

In the 2-year follow-up, the median overall DFS rate was 20 months for the 20 cases, meaning that 50% would experience relapse within this timeframe. The median disease-free survival in stages III-IV was 18 months, with a DFS rate = of 48.1%. The median disease-free survival period in this study is similar to the results of other studies that ranged from 18 months [14] to 19 months [15], even better than the results obtained by Inciura et al. [5], where the median disease-free survival period in stage III was 15–17 months and in stage IV was 8–9 months in Lithuania.

From all patients eligible for inclusion in the main study, it was found that patients with stage II had a median disease-free survival period of more than 24 months. However, this may be because these patients were still in good health and had not undergone further investigations.

In groups expressing VEGF-A mRNA, the median disease-free survival was shorter than those without expression (18 months versus more than 24 months). Similarly, the DFS rate in patients who expressed VEGF-A mRNA was lower than those who did not (30% versus 57.1%). The risk of relapse in those who expressed compared to those who did not express VEGF-A mRNA was twice (HR = 2.003, 95% CI: 0.39–10.15 and p-value = 0.401). Skirnizdottir et al. [16] showed relapse in 16 patients with positive VEGF-A expression and 73 with negative VEGF-A expression.

At the protein level, patients who expressed VEGF-A had a longer median disease-free survival than those who did not express it, as well as the DFS rate. However, this is contrary to the study hypothesis. In addition to the 20 patients, three individuals expressing VEGF-A were identified but lost to follow-up after one year, leaving their relapse status unknown. In a worst-case scenario, assuming that the three people above experienced relapse, the results obtained will not contradict the hypothesis of this study (median disease-free survival VEGF-A (+) is shorter than median VEGF-A (-), (18 months versus 20 months), DFS rate of VEGF-A (+) is not different (26.7% versus 27%), and HR is 1.27, 95% CI: 0.31-5.10), although these results are influenced by chance. Based on the above results, it can be concluded that VEGF-A at both the mRNA and protein levels increases the risk of relapse in ovarian cancer patients. The results are in line with the results found by previous researchers [17–23].

In this study, patients lacking VEGFR-2 receptor expression at the protein level showed a median disease-free survival of approximately 24 months, while those expressing the receptor had a shorter median DFS of about 10 months. The DFS rate for the negative group was 53.3%, while the positive group was 41.5% (p = 0.158). The result had big differences between the two groups compared to studies by Nishida N et al. [24]. The DFS rates of the positive and negative groups were 85% and 28% respectively.

This study found a tendency to increase the risk of relapse by 3 times which was associated with VEGFR-2 receptor expression at the protein level (HR 3.02, 95% CI 0.59–15.49, p = 0.185). No other studies have found VEGFR-2 expression as a prognostic factor for relapse in epithelial ovarian cancer. The results showed the effect of VEGFR-2 on survival in epithelial ovarian cancer with HR = 1.85, 95% CI 0.79–4.27 [25]. Assessing VEGFR-2 expression at the mRNA level is possible, but conducting further measurements proves challenging due to suboptimal conditions encountered during both the isolation and optimization processes.

CONCLUSIONS

Both expression of VEGF-A and VEGFR-2 at mRNA and protein levels can be used as a predictor for relapse of epithelial ovarian cancer. Subsequently, this study showed that positive VEGF-A expression at the mRNA level was associated with twice a higher risk of relapse, while positive VEGF-R2 expression at the protein level had a shorter three times higher risk of relapse than negative expression.

DECLARATIONS

Competing interest

No potential conflict of interest relevant to this article was reported.

Ethics Approval

This research was approved by the Ethics Commission of the Faculty of Public Health, Indonesia University No. 124/H2.F10/PPM.00.02/2015.

Acknowledgment

Not applicable

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