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Original Article

Correlation of VEGF expression with transvaginal color Doppler ultrasound blood flow parameters, angiogenesis, and cancer cell proliferation activity in patients with ovarian cancer

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Abstract: Objective: The objective of this study was to examine correlation of vascular endothelial growth factor (VEGF) expression with transvaginal color Doppler ultrasound blood flow parameters, including angiogenesis and cancer cell proliferation activity in ovarian cancer patients. Methods: Expression of VEGF, Ki67, and proliferating cell nuclear antigen (PCNA) was detected in 122 cases of ovarian cancer tissues and normal tissues via immunohistochemistry (IHC) streptavidin-peroxidase (SP) assay. The resistance index (RI), pulsatility index (PI) and blood flow signal grading of patients enrolled were measured via Doppler ultrasound. Statistical Product and Service Solutions (SPSS) 22.0 statistical software was used to analyze the results. Fisher exact probability was used to compare expression of VEGF in tumor tissues and normal tissues and its relationship with Doppler ultrasound blood flow parameters. The correlation of VEGF expression and microvessel density (MVD) with Doppler ultrasound blood flow parameters, and of VEGF with Ki67 and PCNA were analyzed by Spearman rank correlation. $P<0.05$ suggested that the difference was statistically significant. Results: Expression of VEGF in ovarian cancer was higher than that in the normal group, and the later the tumor stage, the higher the positive expression rate of VEGF. The values of RI and PI in ovarian cancer patients were lower than those in normal group, and the later the tumor stage, the lower the values of RI and PI. The expression level of VEGF was positively correlated with Doppler ultrasound blood flow grade. VEGF expression was related to RI and blood flow grade, but not to PI. MVD was correlated with RI, PI, and blood flow grade. The expression level of VEGF was positively correlated with Ki67 and PCNA. Conclusion: VEGF expression in ovarian cancer patients is correlated with the blood flow parameters of transvaginal color Doppler ultrasound, angiogenesis, and cancer cell proliferation activity. It can be used as an evaluation index for angiogenesis and malignancy of potential ovarian cancer tissues.

Keywords: Ovarian cancer, Doppler ultrasound, RI, PI, blood flow grade, VEGF, MVD, proliferation

Introduction

Ovarian cancer is one of the most common malignant tumors in women, with a high mortality rate. Since the early symptoms of ovarian cancer are rare, most patients are diagnosed at a late stage, and the 5-year survival rate is only 20-25% [1]. Although the specimen treatment of ovarian cancer surgery combined with chemotherapy is effective for some patients, the overall survival rate of ovarian cancer is also very low due to the high recurrence rate and the emergence of drug resistance [2]. Angiogenesis plays an important role in development, invasion, and metastasis of malignant tumors. Tumor cells regulate angiogenesis by synthesizing and secreting angiogenesis factors.

Vascular endothelial growth factor (VEGF), a vascular endothelial cell-specific heparin-binding growth factor, is widely expressed in endothelial cells and has a variety of functions including angiogenesis, stem cell implantation, tumor cell proliferation, and migration, etc. [3, 4]. Studies have suggested that in ovarian cancer, VEGF expression is associated with the

Table 1. Clinical data of patients enrolled

	Clinicopathological data	N	Proportion (%)
Age (years old)	>51	50	41.0
	≤51	72	59.0
Diameter of tumor	≥5 cm	62	50.8
	<5 cm	60	49.2
Differentiation degree	Well differentiated adenocarcinoma	48	39.3
	Moderately differentiated adenocarcinoma	39	32.0
	Poorly differentiated adenocarcinoma (including undifferentiated)	35	28.7
Clinical stage	Stage I~II	55	45.1
	Stage III~IV	67	54.9

International Federation of Gynecology and Obstetrics (FIGO) staging, pathological grade, and prognosis of the tumor [5]. It has been proven that [6-8] VEGF can inhibit proliferation and migration of ovarian cancer cells. Therefore, it is of great clinical value to study the correlation of VEGF expression with angiogenesis and tumor cell activity in ovarian cancer.

Transvaginal color Doppler ultrasound is a commonly used method to evaluate the blood flow state of ovarian cancer tissues in clinic. Through quantitative measurement of blood flow parameters, it can quantitatively reflect the blood flow volume in local tissues. Microvessel density (MVD) is a quantitative index reflecting angiogenesis, which is related to the prognosis of tumors. In recent years, attenuation constants of Doppler waveforms have become an additional index of malignant tumors, characterized by exponential attenuation from contraction to diastole. A study found that [9] ultrasonic blood flow parameters, resistance index (RI), and pulsatility index (PI) are decreased significantly. However, there has been little research on the correlation of VEGF expression in ovarian cancer with RI, PI, and blood flow grade. This study aimed to explain the relationship between VEGF expression in ovarian cancer and color Doppler ultrasound and the relationship between VEGF and tumor cell proliferation activity.

Materials and methods

Clinical data

Tissue specimens and clinical data of 122 ovarian cancer patients in our hospital from January 2014 to January 2017 were randomly enrolled in the study (**Table 1**). At the same time, 34 cases of ovarian benign lesions were collected as control group. The specimens were used for making frozen sections which were stained with hematoxylin and eosin (H&E). Paraffin sections were made for immunohistochemistry (IHC) analysis.

Inclusion criteria: (1) Patients pathologically diagnosed with ovarian cancer. (2) Patients whose clinical and pathological data were true and complete, and whose histopathological specimens were available. (3) Patients not receiving radiotherapy, chemotherapy, and targeted therapy before enrollment. (4) Patients without family history of genetic disease, heart, lung, liver, kidney, and other severe fatal organ diseases.

IHC detection

Ovarian cancer tissues and benign control tissues were fixed with formaldehyde and embedded in paraffin. VEGF polyclonal antibody (purchased from Abcam) was diluted at 1:1000, cluster of differentiation 34 (CD34) monoclonal antibody (purchased from CST) was diluted at 1:2000, Ki67 monoclonal antibody (purchased from Abcam) was diluted at 1:5000, and proliferating cell nuclear antigen (PCNA) monoclonal antibody (purchased from CST) was diluted at 1:2000. IHC streptavidin-peroxidase (SP) assay was used for staining. Positive control: In reference to Abcam and CST's official websites, a strong positive expression could be produced by detecting known tissues containing test antigen. Negative control: The results were negative when the first antibody was replaced by phosphate buffered solution (PBS). All positive signals were yellow, brown yellow, or tan signals. Five high-power fields (10×40) were randomly observed under an electron microscope.

Table 2. Expression of VEGF in ovarian cancer and normal group

Group	N	Positive	Negative	Positive (%)	*P*
Normal group	34	9	25	26.5	*P*<0.001
Stage I~II ovarian cancer	55	37	18	67.3	
Stage III~IV ovarian cancer	67	54	13	80.6	

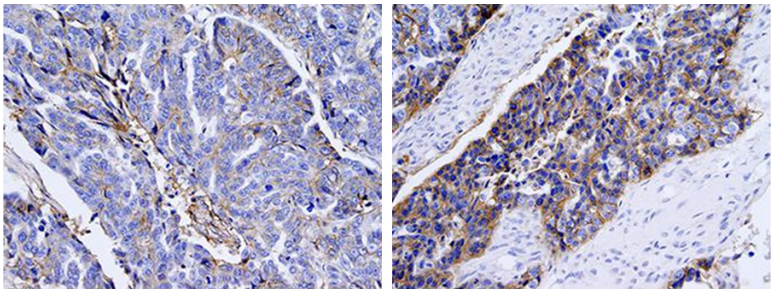

Figure 1. Positive expression of VEGF in ovarian cancer.

The proportion of positive cells with yellow, brown yellow, or tan signals and the intensity of the signals were taken as determination standard.

MVD counting method: The whole tissue section was scanned in low-power field, and visual fields with clear staining of endothelial cells and dense number of microvessels in tumor infiltrating area were selected to count the number of all the stained microvessels at a 400 magnification. In each case, the number of microvessels in 5 different microvascular dense areas was counted, and the mean value was taken as MVD.

Transvaginal color Doppler ultrasound

After the bladder was emptied prior to examination, the patient was placed in lithotomy position and then the ultrasound probe was inserted into the vagina with the probe tightly close to the vaginal vault to scan ovarian tissues at a frequency of 7.5 MHZ. The angle between the pulse wave (PW) sampling line and the blood flow was less than 30°, and the sampling volume was 2 mm. A clear blood flow spectrum was obtained. The pulsatile blood flow in the tumor was searched and RI was measured for 5 times. The maximum peak systolic velocity (PSV) and its corresponding RI value that were measured and recorded. Blood supply in tumor area was observed via Color Doppler Energy (CDE). According to the vascular morphology and the degree of blood flow abundance, the mass blood flow was graded by Adler grading method: Those with a small amount of blood flow, and visible punctate blood flow at 1~2 points were classified as grade I, those with moderate blood flow, and a visible major blood vessel with the length longer than the radius of the lesion or several small vessels were classified as grade II, those with rich blood flow, and more than 4 visible blood vessels, or blood vessels intertwined into a network were classified as grade III, and those with color blood flow signals difficult to clearly show due to false image interference or patient's inability to cooperate were classified as grade 0.

Statistical analysis

Statistical Product and Service Solutions (SPSS) 22.0 was used for statistical analysis. The positive rate of VEGF expression was analyzed by Wlicoxon rank sum test. *t* test was used for comparison of measurement data between two groups. Analysis of variance was adopted for multigroup comparison. All the test methods were bilateral distributed, and $P<0.05$ suggested that there was statistical significance.

Results

Expression of VEGF in ovarian cancer

The protein expression of VEGF in different stages of ovarian cancer and the normal group was detected via IHC. Positive expression of VEGF was found in cytoplasm, and brown yellow or tan staining was observed in those with positive expression via IHC. The expression rate of VEGF in ovarian cancer was higher than that in the normal group, and the later the tumor stage, the higher the positive expression rate of VEGF ($P<0.001$) (**Table 2**; **Figure 1**).

Doppler ultrasound blood flow parameters in ovarian cancer

Transvaginal color Doppler ultrasound analysis of ovarian cancer patients showed that the RI

Table 3. Comparison of Doppler ultrasound blood flow parameters between ovarian cancer and normal group

Group	N	RI	PI	*P*
Normal group	34	0.77al gr	1.71al gr	*P*<0.05
Stage I~II ovarian cancer	55	0.57e I~I	0.87e I~I	
Stage III~IV ovarian cancer	67	0.32e III	0.53e III	

Table 4. Comparison of Doppler ultrasound blood flow grades between ovarian cancer and the normal group

Group	N	Grade I-II	Grade III	*P*
Stage I~II ovarian cancer	55	26	29	*P*<0.05
Stage III~IV ovarian cancer	67	21	46	

Table 5. Correlation between VEGF expression and color Doppler ultrasound blood flow parameters

		VEGF		*P*
		Positive	Negative	
Blood flow grade	Grade I-II	28	19	*P*<0.001
	Grade III	63	12	

Table 6. Correlation of VEGF expression and MVD with Doppler ultrasound blood flow parameters

Parameter	VEGF	MVD
RI	0.37*	0.49*
PI	0.11	0.47*
Blood flow grade	0.41*	0.72*

*P<0.05.

Table 7. Correlation of VEGF expression with Ki67 and PCNA

		VEGF		*P*
		Positive	Negative	
Ki67	High expression	65	13	*P*<0.05
	Low expression	26	18	
PCNA	High expression	59	15	*P*<0.05
	Low expression	32	16	

and PI values of ovarian cancer patients were lower than those in the normal group, and the later the tumor stage, the lower the RI and PI values. The differences were statistically significant (P<0.05). The later the stage of ovarian cancer, the higher the Doppler ultrasound blood flow grade (P<0.05) (**Tables 3**, **4**).

Correlation between VEGF expression and color Doppler ultrasound blood flow parameters in ovarian cancer

The patients included were divided into a positive group and a negative group according to VEGF expression. Correlation between VEGF expression and blood flow parameters of Doppler ultrasound was analyzed. The expression level of VEGF was positively correlated with Doppler ultrasound blood flow grade (P<0.001) (**Table 5**).

Correlations of VEGF expression and MVD with Doppler ultrasound blood flow parameters in ovarian cancer

Spearman rank correlation analysis of the correlation between two variable indexes showed that VEGF expression was correlated with RI (r=0.37), blood flow grade (r=0.41) (P<0.05), but not with PI (P>0.05) (r=0.49), and MVD was correlated with RI (r=0.49), PI (r=0.47) and blood flow grade (r=0.72) (P<0.05) (**Table 6**).

Correlation of VEGF expression with Ki67 and PCNA in ovarian cancer

The correlation of VEGF expression with Ki67 and PCNA in ovarian cancer were analyzed. The results revealed that VEGF expression was positively correlated with the expressions of Ki67 and PCNA (P<0.05), and the expressions of Ki67 and PCNA in VEGF positive patients were also increased (**Table 7**).

Discussion

In 1971, Folkman suggested that tumor growth and metastasis depend on tumor angiogenesis [10]. There are two different stages of angiogenesis: slow and fast. Primary tumors will not grow without angiogenesis. Tumor blood vessels provide necessary oxygen and nutrition for tumor tissues to grow rapidly, and also promote distant metastasis [11]. Inhibition of angiogenesis is a new anti-tumor strategy. VEGF plays an important role in vascular formation under physiological and pathological conditions. VEGF promotes endothelial cell proliferation and migration, increases vascular permeability, inhib-

its endothelial cell apoptosis, and increases angiogenesis [12]. Doppler ultrasound is of great value in the differential diagnosis of benign and malignant tumors. In particular, the flow index obtained from Doppler ultrasound, RI and PI has a high clinical value in the evaluation of malignant tumors [13]. In this study, color Doppler ultrasound blood flow parameters of different stages of ovarian cancer focus were analyzed. It was found that RI, PI, and blood flow grade were correlated with the clinical staging of ovarian cancer, and the later the tumor stage, the lower the RI and PI values, but the higher the blood flow grade. Tumor angiogenesis is the pathological basis for the decrease of blood flow resistance and the increase of blood perfusion. The resistance of blood flow in ovarian cancer focus is decreased significantly, and with the increase of tumor staging and progression of tumor, the resistance of blood flow in the cancer nest will be further decreased, which is related to the special structure of angiogenesis in tumor focus, whose aim is to ensure adequate blood perfusion for the growth of ovarian cancer focus. The results of this study indicate that tumor angiogenesis reflects the malignancy and progression of tumors to a certain extent. Further analysis manifested that the blood flow parameters of Doppler ultrasound were correlated with VEGF, indicating that the expression level of VEGF and Doppler ultrasound parameters could jointly reflect the tumor angiogenesis of ovarian cancer.

PCNA is a process factor of deoxyribonucleic acid (DNA) polymerase, which acts as a loading scaffold for replication mechanism through association with various replication-related factors. PCNA is named for its presence in normal proliferative cells and tumor cells. It has been found that PCNA is closely related to cell DNA synthesis and plays an important role in the initiation of cell proliferation. It is a good index to reflect the state of cell proliferation [14, 15]. Ki67 is a nuclear localization protein closely related to cell proliferation, which exists at all active stages of the cell cycle, but not in stationary cells [16]. Ki67 is also a mature prognostic biomarker in several tumor entities, such as breast cancer, lymphoma, and neuroendocrine tumors [17-19]. It has been proven that VEGF 165, an important member of the VEGF family, can bind to NPR-1, a receptor on endothelial cell and tumor cell membranes, thus promoting formation of the tubular structure of endothelial cells and promoting growth of tumor cells [20]. In this study, the expression level of VEGF was positively correlated with the expressions of PCNA and Ki67, indicating that the proliferation of tumor cells was active in ovarian cancer with a high expression level of VEGF. The mechanism may be that the increased angiogenesis and increased blood flow create a favorable local environment for cancer cell proliferation. However, the specific mechanism of VEGF in promoting proliferation of ovarian cancer cells needs to be further studied and elaborated.

In summary, this study found that expression of VEGF in ovarian cancer is correlated with tumor cell proliferation activity and Doppler ultrasound parameters, which provides a new idea for the clinical diagnosis and prognosis prediction of ovarian cancer.

Disclosure of conflict of interest

None.

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