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Correlation between serum vascular endothelial growth factor and endostatin levels in patients with breast cancer

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Abstract

Serum vascular endothelial growth factor (VEGF) and endostatin levels were detected in 59 patients with breast cancer before surgery and at 3 weeks after surgery. Pre-operatively, their levels were significantly elevated and correlated with each other. Post-operatively, VEGF level decreased significantly and endostatin remained at a high level. Patients with both normalized VEGF and elevated endostatin following surgery had a lower risk of relapse than patients whose VEGF failed to normalize. Univariate and multivariate analyses showed a correlation between elevated VEGF level and short free-relapse survival. These findings suggest a new angiogenesis balance is formed in the patients after surgery and such a resultant balance may be beneficial for the prognosis of breast cancer, which deserves more extensive study.

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1. Introduction

Angiogenesis is an acknowledged essential requirement for tumor growth, invasion and metastasis [1]. Solid tumors can produce antiangiogenic cytokines as well as proangiogenic cytokines [2–4]. It has been widely accepted that the angiogenic activation of a tumor is the result of a net balance between positive and negative regulators of neovasularization [3–5]. Vascular endothelial growth factor (VEGF) is thought to be a specific potent angiogenesis stimulator that may

be induced by the hypoxia of tumor [6,7], and endostatin (ES) is a recently discovered, specific, potent antiangiogenic factor that may be mediated in tumor by elastase or cathepsins [4,8,9]. The VEGF expression is related to the progression of disease in many types of tumors, while the ES expression has been discovered to be related with the prognosis of some types of tumors [9–17]. Interestingly, the circulating levels of both cytokines correlate well with each other in several types of tumors, such as colorectal cancer, hepatocellular carcinoma, and clear cell renal cancer [17–19]. Elucidation of the nature of this correlation and identification of the presence of VEGF and ES in humans with cancer may lead to insights into the regulation of tumor angiogenesis and

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Table 1

The serum ES and VEGF levels in health controls and pre-operative patients with primary breast cancers according to age groups

Age group	n	ES median (range) (ng/ml)		VEGF median (range) (pg/ml)	
		Healthy control	Breast cancer	Healthy control	Breast cancer
30s	5	13.5 (8.60–22.3)	55.0 (14.5–76.5)	88.5 (53.3–343.5)	194.7 (84.3–998.2)
40s	21	13.9 (3.9–25.2)	40.4 (12.8–98.6)	78.0 (34.0–350.0)	306.2 (68.1–1420)
50s	23	14.7 (3.9–30.8)	52.3 (12.4–110.6)	90.5 (47.9–453.0)	405.0 (49.6–1380)
60s	10	30.2 (15.2–68.0)*	64.4 (15.2–92.1)	104.2 (68.2–233.2)	327.1 (115–910.7)
Total	59	15.3 (3.9–68.0)	40.0 (12.4–110.6)	88.5 (34.1–453.0)	365.2 (49.6–1420)

* $P < 0.05$, compared with the other three age groups for ES level in healthy control (Kruskal–Wallis test).

serve as a promising tool in the antiangiogenic therapy and prognosis prediction.

As with most other tumors, breast cancer is also an angiogenesis-dependent tumor [20]. Several proangiogenic growth factors and endogenous inhibitors of angiogenesis have been identified in breast cancer, among which the most important angiogenic regulators are considered to be VEGF and ES [11–14, 20–22]. A number of studies have suggested that circulating VEGF level is significantly elevated in pre-operative primary breast cancer (PBC) patients and may be used as a strong angiogenesis marker [11–13]. Recently, Kuroi et al. [22] reported that circulating ES level was also elevated in pre-operative PBC patients and did not show any change after tumor removal. However, to our best knowledge, the correlation between VEGF and ES levels in breast cancer patients and their balance alternation after surgery remain unknown. To address this issue, we measured the circulating levels of VEGF and ES in series of breast cancer patients and examined the effect of tumor removal on their pre-operative levels and, a possible angiogenesis regulation manner was proposed.

2. Materials and methods

2.1. Patients and sample collection

Fifty-nine female patients with PBC (median age, 48; range, 30–73) were included in this study. Patients who had received chemotherapy, radiotherapy or blood transfusion, or had the regional skin inflammation or ulcer before surgery were excluded. The 59 patients with PBC underwent standard or modified radical mastectomy with complete dissection

of axillary lymph nodes in our institute. In this series, there were neither post-operative septic complications nor abnormal blood routine testing result at the time of sampling. Tables 1 and 2 showed their age and clinical characteristics, respectively. Tumor staging was based on TNM of the International Union Against Cancer. The involvement of lymph nodes and the histological types were determined by the pathological examination.

Post-operative follow-up was performed for the 59 PBC patients. Because of expected risk factors for relapse, i.e. lymph node (+), tumor > 1 cm, age < 40 years or pathological grade > I, 57 patients received adjuvant therapy, in which two $T_3N_2M_0$ post-menopausal patients received six cycles of CAF (cyclophosphamide 400 mg/m², adriamycin 30 mg/m², 5FU 400 mg/m²) chemotherapy + radiotherapy + 2-year TAM (tamoxifen 20 mg/day), six $T_{1-3}N_2M_0$ pre- and post-menopausal patients received six cycles of CAF chemotherapy + radiotherapy, 11 $T_{1-3}N_1M_0$ and $T_3N_0M_0$ pre- and post-menopausal patients received six cycles of CAF chemotherapy, five $T_{1-3}N_{1-2}M_0$ and $T_3N_0M_0$ post-menopausal patients received six cycles of CAF chemotherapy + 2-year TAM, 21 $T_{1-2}N_0M_0$ pre- and post-menopausal patients received two cycles of CAF chemotherapy, and 12 $T_{1-2}N_0M_0$ post-menopausal patients received 2-year TAM. Physical examinations were regularly performed according to clinical protocols. The relapse-free survival (RFS) was calculated as the period from surgery until the date of the first relapse or the last follow-up if relapse did not appear. The median follow-up period of the patients was 48.6 (range, 6–60) months.

Blood samples from the 59 PBC patients on admission and after surgery for 3 weeks were collected without anticoagulant before breakfast.

Table 2
Clinical characteristics and serum ES and VEGF levels in 59 patients with primary breast cancer

Category	<i>n</i>	ES median (range) (ng/ml)	<i>P</i> value	VEGF median (range) (pg/ml)	<i>P</i> value
<i>Menopausal status (n = 59)</i>					
Pre	32	40.0 (12.8–98.6)		298.9 (49.6–1420)	
Post	27	42.8 (12.4–110.6)	<i>P</i> > 0.05*	287.5 (76.5–998.2)	<i>P</i> > 0.10*
<i>Tumor size (n = 59)</i>					
≤ 2 cm	18	45.7 (12.8–92.1)		220.2 (49.6–1380)	
2.1–5 cm	30	42.0 (12.4–110.6)		360.5 (76.5–850.3)	
> 5 cm	11	54.5 (23.4–88.3)	<i>P</i> > 0.05 [†]	265.8 (84.3–1420)	<i>P</i> > 0.05 [‡]
<i>Stage (n = 59)^a</i>					
I	14	42.3 (12.8–98.6)		305.0 (49.6–998.2)	
II	29	51.0 (12.4–110.6)		361.5 (58.7–1420)	
III	16	43.2 (17.4–88.3)	<i>P</i> > 0.05 [†]	323.1 (102.1–1380)	<i>P</i> > 0.05 [‡]
<i>Axillary nodal metastases (n = 59)</i>					
(+)	19	48.0 (16.9–88.3)		380.1 (49.6–1420)	
(–)	40	36.6 (12.4–110.6)	<i>P</i> > 0.10*	287.5 (76.5–910.7)	<i>P</i> > 0.05*
<i>Histology (n = 59)^b</i>					
IDC	52	48.7 (12.4–110.6)		380.1 (49.6–1420)	
Other	7	44.5 (12.8–73.9)	<i>P</i> > 0.10*	205.5 (110–998.2)	<i>P</i> > 0.05*
<i>Pre- and post-operation (n = 59)</i>					
Pre	59	40.0 (12.4–110.6)		365.2 (49.6–1420)	
Post	59	48.6 (14.5–91.8)	<i>P</i> > 0.05 [‡]	158.7 (54.3–823.5)	<i>P</i> < 0.01 [‡]

*Mann–Whitney *U* test; [†]Kruskal–Wallis test; [‡]Wilcoxon signed rank test.

^a Stage: I (T₁N₀M₀), II (T₂N₀M₀, T_{1–2}N₁M₀), III (T₃N_{0–2}M₀, T_{1–2}N₂M₀).

^b Histology: IDC (invasive ductal carcinoma), other (2 invasive lobular carcinoma, 4 medullary carcinoma and 1 tubular carcinoma).

Serum was separated immediately by centrifugation at 3000 rpm for 10 min, then aliquoted and stored at –70 °C until assay. In addition, we obtained serum samples from 59 age- and sex-matched healthy volunteers (median age, 49; range, 30–75), and 30 female patients with benign breast diseases (BBD; median age, 37; range, 28–51). The regional research board approved the project and all subjects gave informed consent before their inclusion.

2.2. Enzyme immunoassay

Serum ES levels were measured with competitive enzyme immunoassay (Cat. No. CYT158, Chemikine™ Human Endostatin™ EIA Kit, Chemicon International, Inc.) according to the manufacturer's instruction. The detection limit for ES was 1.95 ng/ml. Serum VEGF levels were measured by ELISA (Cat. No FHK0043, Human VEGF ELISA Kit, Jingmei Biotech) with a detection limit lower

than 15 pg/ml. All analyses and calibrations were carried out in duplicate. Mean values were used as the final cytokine concentrations.

2.3. Statistical analysis

Data were tested for normality and were found to be non-normally distributed. So, all data were presented as median values (range) with non-parametric analyses being used to assess differences. The Kruskal–Wallis test, Mann–Whitney *U* test, Wilcoxon signed rank test or Fisher's exact test was used to evaluate differences among groups. Correlations were studied with the Spearman rank correlation. Relapse-free survival curves were drawn using the Kaplan–Meier method and compared using the log-rank test. Cox's proportional hazards model was used for the survival analyses, which were performed on the Statistical package, Stata 6.0. *P* < 0.05 was considered significant and all tests were two-sided.

ES and VEGF levels were divided into ‘elevated (>cut-off values)’ and ‘normal (\leq cut-off values)’ groups. The cut-off values were defined as the 95th percentile values in the healthy control group. Normalized serum VEGF levels referred to a decrease of the elevated pre-operative levels to normal levels after operation.

3. Results

3.1. Serum ES and VEGF levels in healthy controls

Serum ES and VEGF were detectable in all healthy controls. Their median values of serum ES and VEGF levels were 15.3 ng/ml (range, 3.9–68.0) and

88.5 pg/ml (range, 34.1–453.0), respectively. The relationships between serum ES or VEGF level and age are shown in Table 1. Only the ES levels in the control group of 60s were significantly higher than those of the other three age groups ($P < 0.05$).

3.2. Pre-operative serum ES and VEGF levels in patients with PBC

Median value of serum ES levels in patients with PBC was 40.0 ng/ml (range, 12.4–110.6), and significantly higher than that in patients with BBD (median, 22.6; range, 5.1–92.3 ng/ml; Mann–Whitney U test, $P < 0.01$) or healthy controls ($P < 0.005$, Fig. 1A). There was no significant difference in the ES levels between BBD patients and healthy controls

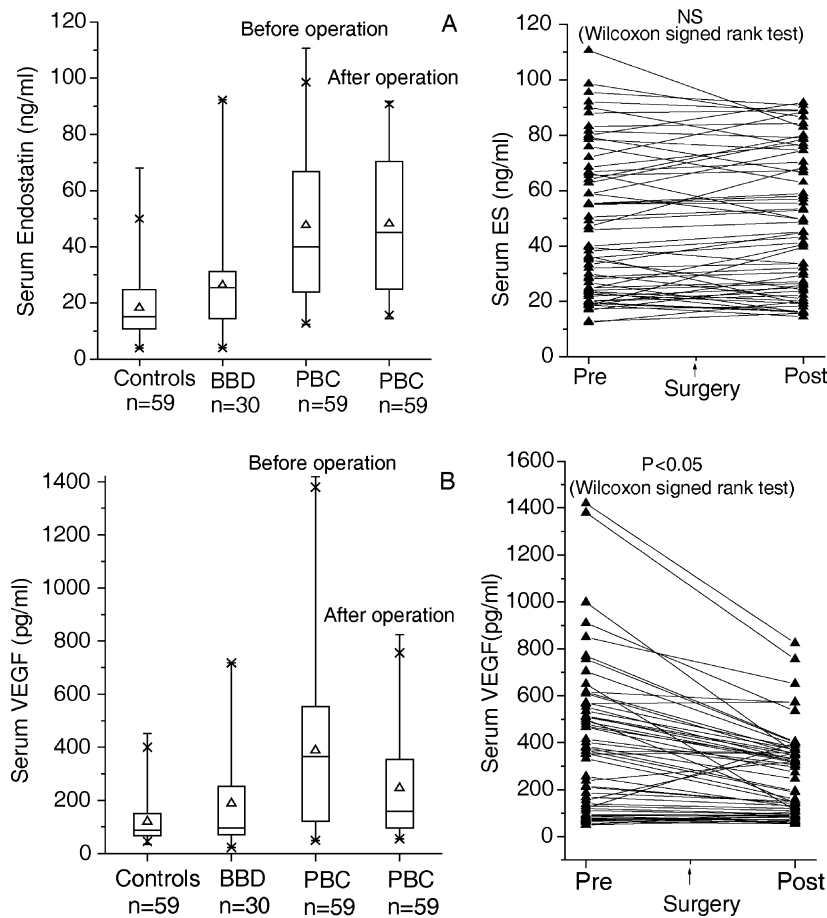


Fig. 1. Serum levels of ES (A) and VEGF (B) in healthy controls, BBD patients, and PBC patients before and after surgery. (—) median value; (Δ) mean; (\square) 25–75%; (I) min–max; (\times) 99 and 1%. Right, individual cancer patient (\blacktriangle).

($P > 0.10$). Median value of serum VEGF levels in patients with PBC was 365.2 pg/ml (range, 49.6–1420), and significantly higher than that in patients with BBD (median, 95.5; range, 43.1–718.3 pg/ml; $P < 0.005$) or healthy controls ($P < 0.005$, Fig. 1B). There was no significant difference in the VEGF levels between BBD patients and healthy controls ($P > 0.05$).

According to the above definition, the cut-off values for ES and VEGF were 49.0 ng/ml and 402.5 pg/ml, respectively. Using these cut-off values, serum ES levels were elevated in 27 of 59 (45.8%) patients with PBC and four of 30 (13.3%) patients with BBD, and serum VEGF levels were elevated in 25 of 59 (42.3%) patients with PBC and six of 30 (23.3%) patients with BBD.

3.3. Correlation between pre-operative serum ES and VEGF levels

There was a significant correlation between pre-operative serum ES and VEGF levels in the cancer patients ($r = 0.55$, $P < 0.01$, Fig. 2), while this correlation was absent in healthy controls ($r = 0.11$, $P > 0.20$). After tumor removal for 3 weeks, a weak correlation between serum ES and VEGF levels still existed ($r = 0.41$, $P < 0.05$). Both pre-operative ES and VEGF levels were not associated with patient age at diagnosis, menopausal status, tumor size, stage, axillary nodal status or histological type ($P > 0.05$ for all these comparisons, Tables 1 and 2).

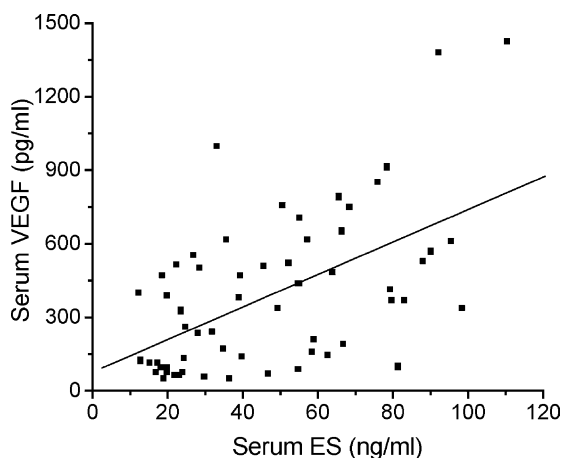


Fig. 2. The significant correlation between pre-operative serum ES and VEGF levels in 59 PBC patients ($r = 0.549$, $P < 0.01$, Spearman rank correlation).

3.4. Changes in serum ES and VEGF levels after surgical treatment

The effect of surgical resection of the tumor was evaluated by measuring serum ES and VEGF levels in the patients before surgery and after surgery for 3 weeks. There was no significant difference between pre- and post-operative serum ES levels (median, 40.0 and 48.6 ng/ml, respectively; $P > 0.50$; Table 2), and serum VEGF levels decreased significantly after tumor removal (median, 365.2 vs 158.7 pg/ml; $P < 0.01$). Among the 25 patients with pre-operatively elevated serum VEGF levels, a prompt decrease of VEGF with return to normal levels was observed in 19 patients (76%), while six patients (24%) either remained persistently at elevated serum VEGF levels or showed a decrease of the VEGF level but failed to normalize. These patients had an increased risk of relapse (66.7%, four of six patients) during follow-up when compared with those patients with normalized post-operative serum VEGF (21%, three of 19 patients, Fisher's exact test, $P = 0.09$), particularly with those patients with both normalized serum VEGF and elevated ES following surgery (8.3%, one of 12 patients, $P < 0.05$).

3.5. Correlations between serum ES or VEGF levels and patient relapse-free survival

During the follow-up, 10 of 59 PBC patients (17%) developed recurrences. Univariate Cox regression analysis showed tumor size, pre- and post-operative VEGF levels to be significant factors affecting RFS (Table 3). Log-rank analysis showed that elevated pre-operative VEGF level was associated with poor RFS ($P = 0.02$, Fig. 3), while ES did not display this association ($P = 0.09$). When the 59 patients were stratified by nodal status, the association between VEGF and RFS above was also seen in both node-negative and node-positive subgroups (log-rank test, $P = 0.018$ and 0.046).

In the case of multivariate analysis for tumor size, stage, lymph nodal status, pre-operative serum ES, pre- and post-operative serum VEGF, serum VEGF levels in both pre- and post-operative patients were still related to RFS in some degree ($P = 0.052$ and 0.066, respectively, Table 3). Lymph nodal status and TNM stage were not of prognostic significance for

Table 3
Cox regression analysis for relapse-free survival in 59 patients with primary breast cancer

Variable	Univariate		Multivariate ^a	
	P	HR (95% CI)	P	HR (95% CI)
Age	0.144	0.97 (0.94–1.01)	–	–
Tumor size (≤ 2.0 vs 2.1–5.0 vs > 5.0)	0.043	1.95 (1.05–3.84)	0.127	1.47 (0.93–2.68)
TNM stage (I vs II vs III)	0.186	1.30 (0.88–1.93)	0.208	1.59 (0.77–3.33)
Nodal metastases (– vs +)	0.413	1.33 (0.67–2.64)	0.640	0.81 (0.33–1.97)
Preoperative serum ES (elevated vs normal ^b)	0.088	1.52 (0.91–3.14)	0.289	0.99 (0.63–2.09)
Preoperative serum VEGF (elevated vs normal ^b)	0.021	2.13 (1.12–4.04)	0.052	1.95 (0.99–5.00)
Postoperative serum VEGF (elevated vs normal ^b)	0.039	2.15 (1.08–4.25)	0.066	1.86 (1.01–4.16)

HR, hazard ratio; CI, confidence interval.

^a Multivariate analysis was performed for all variables with HR > 1.0 in univariate analysis.

^b Patients were divided into elevated and normal groups of cytokine using the cut-off value (i.e. 49.0 ng/ml for ES; 402.5 pg/ml for VEGF).

RFS in the study. This might reflect the routine use of nodal status and TNM stage as a selection of the intensity of adjuvant treatment in our institute.

4. Discussion

The assessment of angiogenesis in breast cancer is important as a crucial indicator of metastasis and prognosis [21,23]. It has been demonstrated that VEGF expression in tumor tissue correlates directly with intratumoral microvessel density and contributes to the promotion of tumor angiogenesis. So it is used as a tumor marker or prognostic factor for breast cancer [14,21]. However, evaluation of tissue VEGF has the disadvantages of being invasive, inconvenient and considerably related to observer and to the site of

tumor tissue examined, and it cannot provide the information on the prediction and monitor of response to therapy and follow-up surveillance for relapse. More recently, investigation into the implications of circulating levels of this cytokine is progressing at an exponential rate, and it has been demonstrated that the circulating level of VEGF is of clinical value as a promising surrogate marker [7,10–13].

In this report, circulating VEGF is represented by serum rather than plasma VEGF. The serum VEGF level is contributed from plasma and platelet VEGF [24]. The platelets function as VEGF scavengers confining angiogenesis to sites such as wound healing and tumor neovasculature [25]. Recently, several investigations believed that serum VEGF level also reflects biology of tumor, and serum would be the more useful specimen for measurement of circulating

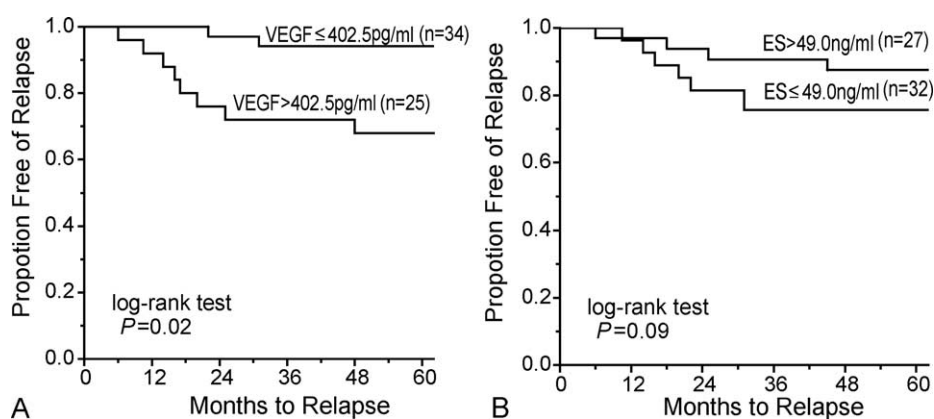


Fig. 3. Kaplan–Meier survival curves in relation to serum VEGF (A) and ES levels (B) in 59 PBC patients.

VEGF in cancer patients [26–29]. On the other hand, in our additional experiment, the plasma VEGF levels in 40 cancer patients were detected. The plasma VEGF values were in the range of < 15–43.1 pg/ml, while the corresponding serum VEGF levels were from 49.6 to 829.3 pg/ml. The plasma VEGF level was very low, similar to those reported previously [29, 30]. Thus, it was difficult to compare the cases with plasma VEGF values less than 15 pg/ml.

We observed an elevation of serum VEGF level in PBC patients, which correlated with early relapse and might also be used as a tumor marker, in accordance with previous reports [14,21]. Additionally, a significantly decreased serum VEGF level was observed after tumor removal. These facts supported further the hypothesis that the high level of VEGF in circulation was attributed to the secretion of VEGF from tumor [11–13]. On the other hand, a significant increase of VEGF level in renal cancer patients after surgery has been reported [31]. Therefore, we suggest that the alternative VEGF level in circulation may vary with different types of tumors. Kuroi et al. [21] reported that the circulating ES level in PBC patients was higher than that in healthy controls, but not different from that in BBD patients. In our report, the pre-operative serum ES level in patients with PBC is significantly higher than that in both BBD patients and healthy controls (Table 2), which is in agreement with the results of Kuroi et al. except in BBD patients. Such a slight difference might result from the methodological or histological variation. In addition, both our data and the results of Kuroi et al. indicated the surgery did not change circulating ES level, suggesting that the high level of ES in circulation was attributed to the systemic expression in human body rather than tumor tissue. Therefore, even after the tumor was removed and serum VEGF decreased significantly, the circulating ES was still maintained at a high level. The resultant antiangiogenesis balance should be beneficial for prognosis.

Based on an observation on circulating VEGF and ES levels in pre-operative patients with clear cell renal cancers, Feldman et al. [19] found a direct correlation between the two cytokines and proposed two hypotheses to explain such correlation: (1) In addition to VEGF, invasive tumors can secrete multiple collagenases that cleave ES from collagen XVIII; (2) elevated VEGF, in addition to promoting

endothelial cells proliferation, also induces the release of multiple collagenases and other proteases from endothelial cells for ES production. Our work also showed a good correlation between serum VEGF and ES levels in pre-operative patients with PBC. However, after the radical removal of tumor, serum VEGF level decreased dramatically, while ES level remained at the same level as pre-operation. Thus the above hypotheses might be unsuitable for the case of breast cancer, and in patients with breast cancer, the production of ES was independent of either tumor or VEGF secreted from tumor. ES is originally identified in the conditioned media of the murine hemangioendothelioma cell line, EOMA, however, several other murine and human tumor cell lines could not produce measurable ES levels in their supernatants [32]. It has been reported that collagen XVIII is a precursor protein of ES, which localizes in vessel walls and several basement membranes, particularly in the liver [18,22,33]. The proteolytic activity that accompanies tumor growth may mobilize ES from collagen XVIII [22]. The exact source of circulating ES is still unclear.

Interestingly, some reports observed both VEGF and ES levels in renal cancer increased significantly after primary tumor was removed [31], both levels in endometrial cancer decreased significantly [34], and VEGF level in hepatocellular carcinoma did not change with an decreasing ES [17]. Thus, we hypothesize that both VEGF and ES in circulation are regulated in tumor tissue-specific manner. Furthermore, investigating the angiogenesis balance of each specific tumor would be beneficial for prognosis prediction of performing operation and antiangiogenesis therapy.

In conclusion, this work demonstrates that in pre-operative PBC patients the serum VEGF and ES levels are elevated and show a significant correlation, after surgery serum VEGF decreases promptly and ES remains at a high level, implying a new angiogenesis balance is formed. Considering the fact that the functional status of circulating endogenous endostatin in humans is not known, the balance between endostatin and VEGF is speculative. In these studied patients, there are still 24% patients whose serum VEGF levels fail to normalize after surgery. They seem to have an increased risk of relapse during the follow-up when compared with the patients with a new

angiogenesis balance of both normalized VEGF and elevated ES following surgery. Additionally, univariate and multivariate analyses show that patients with elevated VEGF levels before and after surgery are related to a short free-relapse survival. Because of a small subgroup, further studies with larger patient cohorts are required to confirm this prognosis value.

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