Differential expression of vascular endothelial growth factor-A, -C and -D for the diagnosis and prognosis of cancer patients with malignant effusions

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Abstract. Elevated levels of vascular endothelial growth factor (VEGF) contribute to angiogenesis and serous cavity effusions. The present study evaluated the diagnostic and prognostic values of VEGF-A, -C and -D proteins in the serum, supernatant fluid and exfoliated cells of cancer patients with malignant effusions compared with patients with benign effusions. An enzyme-linked immunosorbent assay was used to detect levels of VEGF-A, -C and -D proteins in the sera of 79 cases (30 lung cancer, 21 gastric cancer and 28 benign effusions) and the supernatant fluid of 96 cases (38 lung cancer, 30 gastric cancer, and 28 benign effusions). Immunocytochemistry detected the expression of VEGF-A, -C and -D proteins in effusion cells from 71 cases (34 lung cancer, 17 gastric cancer and 20 benign effusions). The data were further investigated to determine whether there was an association between VEGF subtype expression and clinicopathological characteristics and prognosis. The expression levels of VEGF-A in the supernatant fluid were increased in the lung and gastric cancer patient samples compared with the benign effusions (P<0.05). The VEGF-A level in the supernatant fluid was significantly increased compared with the corresponding sera of patients with malignant effusion (P<0.05). VEGF-A, -C and -D proteins in the exfoliated cells from primary lung or gastric cancer effusions were expressed at 52.94, 70.58 and 82.35%, respectively, whereas their expression was not detected in the exfoliated cells from benign effusion, with the exception of mesothelial cells. The levels of VEGF-A and VEGF-C in the supernatant fluid levels and the cell levels of VEGF-A were inversely associated with age; in addition, VEGF-A levels in the supernatant fluid were associated with malignant and bloody effusion, and only cavity metastasis (P<0.05). Survival analysis demonstrated a relatively reduced survival time for patients with VEGF-A levels of >406.19 pg/ml in the supernatant fluid compared with patients with VEGF-A levels of ≤406.19 pg/ml (P=0.066). Serum VEGF-A, -C and -D levels exhibited no evident clinical significance in the diagnosis and prognosis of serous cavity effusions. VEGF-A in the supernatant fluid merits further study as a tumor marker in the clinical setting to discriminate benign from malignant effusions, while cellular VEGF-C and -D may contribute to the formation of malignant effusions.

Introduction

The presence of malignant effusion is associated with significant morbidity and a poor quality of life. The most significant causes of malignant pleural effusion are lung or breast cancer, and malignant lymphoma. Once malignant pleural effusion has developed, the average survival time for the patient is often <6 months (1). Malignant ascites is most common in gastrointestinal and gynecological cancers, with a median survival time of <20 weeks. Patients with malignant ascites that result from gastrointestinal cancers have a particularly poor prognosis and the survival rate is only 12-20 weeks (2). Pericardial effusion is generally observed in dying patients, which worsens prognosis. The treatment of malignant effusions is often a challenge for physicians. Currently, the conventional treatment of malignant effusions is primarily composed of diuresis, salt restriction, serous cavity paracentesis, intracavitary chemotherapy, biological response modifiers, traditional Chinese medicine or thermotherapy, however, the therapies are not effective. Following treatment with these methods, there is no significant reduction in effusions and relapses often occur quickly. Furthermore, almost all of these treatment...
methods result in toxic side-effects of various degrees. Thus, it is important to understand the underlying molecular mechanisms associated with malignant effusion. Previous studies have demonstrated that elevated levels of vascular endothelial growth factor (VEGF), tumor angiogenesis and increased vascular permeability following tumor invasion or metastasis to the pleuroperitoneum are important mechanisms of serous cavity effusions. VEGF requires further study due to its presence in the pleural fluid and its potential use as a therapeutic target (3-5).

The VEGF family of proteins is known to promote angiogenesis during embryonic development or wound healing, however, the altered expression of VEGF contributes to disease development, including tumorigenesis and tumor progression (6). The VEGF family contains a group of secreted proteins, including VEGF-A, -B, -C, -D and -E, and placental growth factor (7). Specifically, VEGF-A is a heparin-binding dimeric cytokine, which is important in vascular permeability and angiogenesis. VEGF-A is 50,000-fold more potent than histamine in the induction of vascular permeability (7). Increased VEGF-A levels produced by tumor, mesothelial and infiltrating immune cells may lead to increased vascular permeability, which is crucial for pleural or peritoneal fluid formation. By contrast, VEGF-C and -D are closely associated with lymph vessel neogenesis (8). Previous studies have demonstrated that VEGF-A protein is present in significant amounts in peritoneal and pleural effusions of varying etiologies (9,10). VEGF-A levels in malignant effusions were found to be significantly increased compared with those in non-malignant effusions, indicating that this difference may aid in the differentiation between malignant and non-malignant effusions (11,12). The altered expression of VEGF-A has been reported to be associated with the poor prognosis of various types of human cancer (13,14). Multiple clinical studies have also demonstrated the potential benefit of VEGF-A inhibition in patients with malignant effusions (15). Anti-angiogenic therapy (such as bevacizumab, a monoclonal antibody targeting VEGF-A) adjuvant to chemotherapy serves a potential function in the management of pleural effusions in advanced non-squamous non-small cell lung cancer (5,16).

VEGF-A is the most important regulatory factor in tumor angiogenesis, and VEGF-C and -D are the most important in tumor lymphangiogenesis (16). Previous studies of VEGF in serous cavity effusions have mainly focused on VEGF-A. However, suppressing lymphangiogenesis in malignant effusions formation may provide another therapeutic strategy for cancer patients with malignant effusions. The most effective and definitive technique to diagnose malignant effusion is cytological examination of the pleural or peritoneal fluid. The specificity of cytological examination is usually high, but the sensitivity has been reported to range between 30 and 90% (17). Thus, it is important to have molecular biomarkers available to aid in the diagnosis of malignant effusion. In the present study, the content and expression levels of VEGF-A, -C and -D were examined in the sera and supernatants of malignant effusion and exfoliated cells from patients with malignant serous cavity effusions that resulted from lung and gastric cancer. The association between the expression levels of the different VEGF subtypes and the clinicopathological characteristics and prognosis of the patients was also examined.

Materials and methods

Study population. In the present study, consecutive patients with pleural, peritoneal or pericardial fluid were recruited from The Fourth Hospital of Hebei Medical University (Shijiazhuang, China) between July 2012 and January 2013. All the cases were diagnosed pathologically using hematoxylin and eosin staining (H&E) and immunocytochemistry (ICC). All patients were followed up until January 2014. Supernatant fluids were collected from 96 patients (38 primary lung cancer, 30 primary gastric cancer and 28 benign effusions) and analyzed for levels of VEGF-A, -C and -D proteins. In addition, serum samples from 79 patients (30 primary lung cancer, 21 primary gastric cancer and 28 benign effusion) and cytological smears of the effusions from 71 patients (34 primary lung cancer, 17 primary gastric cancer and 20 benign effusion) were prepared for the assaying of VEGF-A, -C and -D levels. The characteristics of the patients are summarized in Table I. The study was approved by the Ethics Committee of The Fourth Hospital of Hebei Medical University, and all participants provided written informed consent to participate in this study.

Enzyme-linked immunosorbent assay (ELISA) detection of VEGF-A, -C and -D levels in serum and supernatant fluid. To assess the expression levels of VEGF-A, -C and -D proteins, 10 ml of fresh pleural, peritoneal or pericardial fluid was collected from each patient prior to treatment and then centrifuged at 200 x g for 10 min at 4°C to pellet the cellular elements. The supernatant was stored at -80°C until use. Serum was prepared from 5 ml of blood that was extracted from the cubital vein in the morning after fasting. The serum and supernatant fluid levels of the VEGF-A, -C and -D proteins were assayed using a double antibody sandwich ELISA with ELISA kits (VEGF-A kit, NeoBioscience, Shenzhen, China; VEGF-C kit, RayBiotec, Inc., Norcross, GA USA; and VEGF-D kit, ImmunoWay Biotechnology Company, Newark, DE, USA) according to the manufacturers' instructions. The values were read by a microplate reader (Anthos Labtec Instruments GmbH, Salzburg, Austria), and the data are presented as the median and interquartile range.

ICC detection of VEGF-A, -C and -D expression in exfoliated cells from effusion. To determine the expression of VEGF-A, -C, and -D proteins, 50-500 ml of fresh pleural, peritoneal or pericardial fluid samples were collected from patients using a disposable cell enrichment collector (diameter, 5-8 mm; Bejing Xincheng International Exhibit Trading Co., Ltd., Beijing, China). The exfoliated cells were prepared for cytological smear on glass slides and then fixed in 95% ethanol for 10 min, stained with H&E and viewed under a light microscope (BX51; Olympus Corporation, Tokyo, Japan). The well-distributed and qualified exfoliated cell smear slides were selected for immunocytochemical analysis. Briefly, the exfoliated cell smear slides were treated with H2O2 for 10 min, followed by microwave treatment for antigen retrieval. The slides were then incubated with a normal non-immunized serum at room temperature for 30 min and then with a primary rabbit anti-human VEGF-A, -C or -D antibody (all from BioWorld Technology, Inc., Dublin, OH, USA) at a dilution of 1:75, overnight at 4°C. The following day, the slides were washed with
phosphate-buffered saline (PBS) three times and then further incubated with an anti-rabbit immunoglobulin G antibody at a dilution of 1:100 (Wuhan Boster Biological Technology, Ltd., Wuhan, China). The color reaction was performed using DAB solution and counterstained with hematoxylin solution briefly.

**Evaluation of immunocytochemical stained slides.** A primary monoclonal mouse anti-human carcinoembryonic antigen antibody (Maixin-Bio, Fuzhou, China) at a dilution of 1:200 was used as the positive control and the primary antibody was replaced with PBS for the negative control. The stained slides were reviewed and scored independently by two investigators who did not have knowledge of the slide identification and clinical data. If there was a discrepancy, this was resolved by consensus. A semi-quantitative method was used to score the staining of each antibody. The percentage of immunopositive cells was assigned by a four-point system as follows: 0 points, no positive cells; 1 point, <25% positive cells; 2 points, 26-50% positive cells; 3 points, 51-75% positive cells; and 4 points, >75% positive cells. The staining intensity was scored similarly: 0 points, negative staining (colorless); 1 point, weak staining (light yellow); 2 points, moderate staining (brown); and 3 points, strong staining (dark brown). Immunoreactivity scores for each lesion were calculated by the summation of the two scores: Negative (-), 0 score; weakly positive (1), 1-2 scores; positive (2+), 3-4 scores; or strongly positive (3+), 5-7 scores.

**Statistical analysis.** Statistical analysis was performed using the SPSS software package, version 17.0 (SPSS, Inc., Chicago, IL, USA). Comparison of data between the groups was performed with the non-parametric Kruskal-Wallis test followed by the Mann-Whitney test. The association of VEGF-A, -C and -D levels with clinicopathological parameters was determined using multiple linear regression and multivariate logistic regression analysis. Survival curves were calculated by the Kaplan-Meier method and the significance of differences was estimated by the log-rank test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**VEGF-A**

**Expression of VEGF-A proteins in sera, supernatant fluid and cytological samples.** The expression of VEGF-A, -C and -D proteins in the serum (sVEGF-A, -C and -D) and in the pleural, peritoneal or pericardial supernatant fluid (pVEGF-A, -C and -D) is presented in Table II.
No statistically significant differences were observed in the level of sVEGF-A proteins in the patients with cancer compared with those with benign effusions (P>0.05). The cancer patients exhibited increased pVEGF-A expression levels compared with those with benign effusions (P<0.05). The upregulated level of pVEGF-A was not associated with tumor histological types (P>0.05). The pVEGF-A level was similar to the corresponding sVEGF-A level in the patients with benign effusions. Nevertheless, the pVEGF-A level in the malignant effusions were significantly increased compared with its corresponding sVEGF-A level (P<0.05).

Cellular levels of VEGF-A, -C and -D were also assessed using ICC (cVEGF-A, -C and -D). The expression levels of VEGF-A, -C and -D were detected in 20 cytological smears from benign effusions. No positive immunoreactivity was observed for VEGF-A, -C and -D in the 20 benign cases, with the exception of partial staining in the mesothelial cells. The VEGF-A expression rate was 52.94% and was mainly expressed in the cytoplasm of positively-expressed tumor cells (Fig. 1).

Association between VEGF-A level and patient clinico-pathological parameters. Using multiple linear regression analysis, the data demonstrated that pVEGF-A was negatively associated with age, and positively associated with malignant and bloody effusion, and cavity only metastasis (P<0.05; Table III). Using multiple logistic regression analysis, it was demonstrated that cVEGF-A was inversely associated with patient age.

Association between VEGF-A level and overall survival. With the median VEGF-A level as the cut-off value, patients were divided into high and low supernatant fluid parameter groups. Survival time analysis demonstrated a relatively shorter survival time for patients with pVEGF-A levels of >406.19 pg/ml compared with those presenting with pVEGF-A levels of ≤406.19 pg/ml, although this effect was not statistically significant (P=0.066; Fig. 2). sVEGF-A expression similarly did not exhibit statistical significance in predicting survival time for patients with malignant effusions (P>0.05).

VEGF-C and -D

Expression of VEGF-C and -D proteins in sera, supernatant fluid and cytological samples. No statistically significant differences were observed in the expression levels of sVEGF-C and -D proteins or pVEGF-C and -D proteins compared with those with benign effusions (P>0.05). The cancer patients exhibited increased pVEGF-A expression levels compared with those with benign effusions (P<0.05). The upregulated level of pVEGF-A was not associated with tumor histological types (P>0.05). The pVEGF-A level was similar to the corresponding sVEGF-A level in the patients with benign effusions. Nevertheless, the pVEGF-A level in the malignant effusions were significantly increased compared with its corresponding sVEGF-A level (P<0.05).

Cellular levels of VEGF-A, -C and -D were also assessed using ICC (cVEGF-A, -C and -D). The expression levels of VEGF-A, -C and -D were detected in 20 cytological smears from benign effusions. No positive immunoreactivity was observed for VEGF-A, -C and -D in the 20 benign cases, with the exception of partial staining in the mesothelial cells. The VEGF-A expression rate was 52.94% and was mainly expressed in the cytoplasm of positively-expressed tumor cells (Fig. 1).

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VEGF-C and -D

Expression of VEGF-C and -D proteins in sera, supernatant fluid and cytological samples. No statistically significant differences were observed in the expression levels of sVEGF-C and -D proteins or pVEGF-C and -D proteins compared with those with benign effusions (P>0.05). The cancer patients exhibited increased pVEGF-A expression levels compared with those with benign effusions (P<0.05). The upregulated level of pVEGF-A was not associated with tumor histological types (P>0.05). The pVEGF-A level was similar to the corresponding sVEGF-A level in the patients with benign effusions. Nevertheless, the pVEGF-A level in the malignant effusions were significantly increased compared with its corresponding sVEGF-A level (P<0.05).

Cellular levels of VEGF-A, -C and -D were also assessed using ICC (cVEGF-A, -C and -D). The expression levels of VEGF-A, -C and -D were detected in 20 cytological smears from benign effusions. No positive immunoreactivity was observed for VEGF-A, -C and -D in the 20 benign cases, with the exception of partial staining in the mesothelial cells. The VEGF-A expression rate was 52.94% and was mainly expressed in the cytoplasm of positively-expressed tumor cells (Fig. 1).

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between cancer and benign patients (P>0.05). cVEGF-C and -D expression was 70.58 and 82.35%, respectively, in the 51 patients with lung or gastric cancer (Figs. 3 and 4). These proteins were predominantly expressed in the cytoplasm of positively-expressed tumor cells. However, the levels of cVEGF-D in the cancer patients were significantly increased compared with cVEGF-A and -C (P<0.05). No statistically significant differences were observed in the cVEGF-A, -C and -D expression levels between lung and gastric cancer patients (P>0.05; Fig. 5).

**Association between VEGF-C and -D levels and clinicopathological patient parameters.** pVEGF-C expression was inversely associated with patient age. pVEGF-D expression was associated with age, and inversely associated with malignant effusion and only cavity metastasis (P<0.05; Table III). However, no association was observed between the sVEGF-C and -D proteins and the examined clinicopathological factors (P>0.05).

**Discussion**

Angiogenesis has a critical effect on cancer growth and metastasis, and VEGF is a potent angiogenic and lymphangiogenic mediator. Previous studies demonstrated that an increased level of pleural VEGF-A was associated with malignancy...
and that VEGF-A was considered to be a marker for the diagnosis of malignant effusion (9,11). VEGF-C and -D are the ligands of VEGF receptor-3 (VEGFR-3) and the latter is localized on lymph-endothelial cells; when VEGFR-3 is activated through the binding of VEGF-C and/or -D, it may to be sufficient to promote the metastasis of cancer cells (18). In solid tumors, experimental and clinical evidence has indicated that the expression of VEGF-C or -D proteins can contribute to increased lymphatic vessel density and tumor lymphatic metastasis, and that the expression of VEGF-C or -D is an independent prognostic factor for patients with oral squamous cell carcinoma (19). Cancer cell lymphatic spread induced by VEGF-D may be blocked with an antibody against VEGF-D (20). Limited animal studies have been conducted on effusions, but have demonstrated that VEGF-C and -D may be important in producing pleural dissemination (21,22). In the present study, the protein expression levels of VEGF-A, -C and -D in the supernatant fluid, exfoliated cells and sera from patients with benign and malignant diseases were determined. The analysis demonstrated that pVEGF-A levels in the supernatant fluid from pleural, peritoneal or pericardial effusion were significantly upregulated compared with that in the benign effusions, indicating that the detection of pVEGF-A levels may have potential diagnostic value for malignant effusions.

To analyze whether these markers were associated with tumor histological types, patients were recruited with malignant effusions from two types of malignancies, primary lung and gastric cancer, which most often lead to pleural and peritoneal effusion. No significant differences were observed in pVEGF-A levels between lung and gastric cancer patients, which is consistent with other studies that observed no significant difference in supernatant fluid VEGF-A levels in patients with different histological types or clinical stages of lung cancers (12,23). Multiple linear regression analysis was used to demonstrate that the levels of VEGF-A in the supernatant fluid of the patients with bloody effusion were increased compared with the patients with non-bloody effusion, which is consistent with other previous studies that demonstrated that the pleural VEGF-A level is associated with the number of red blood cells (14,24,25). In the present study, the levels of pVEGF-A protein were inversely associated with age, indicating that age is a protective factor and reflecting the reduced angiogenesis capacity in aging individuals. In addition, the supernatant fluid levels of VEGF-A in the patients with only cavity metastasis were increased compared with the patients with metastases other than only cavity metastasis.

In the current study, statistically significant differences in the levels of pVEGF-C and -D were not observed between cancerous and benign disease, which is in accordance with the results reported by Croghan et al (26). Using multiple linear regression analysis, it was demonstrated that supernatant fluid levels of pVEGF-C proteins were inversely associated with age, and that levels of pVEGF-A and -D were positively associated with a number of clinicopathological parameters, including malignant effusion and only cavity metastasis. However, the underlying mechanisms remain to be determined.

The prognostic significance of pVEGF-A has been estimated in several previous studies (13,14). Hirayama et al followed 28 malignant pleural mesothelioma patients closely for up to 600 days (13) and demonstrated that a VEGF level of >2,000 pg/ml was a significant predictor of patient survival (13). Hsu et al (14) retrospectively studied 97 NSCLC patients with only malignant pleural effusion and observed that a VEGF level of >1,350 ng/ml was a significant negative predictor of patient survival. In the present prospective study that followed 66 patients with malignant serous cavity effusions from lung and gastric cancer closely for up to 550 days, pVEGF-A levels with a median level of 406.19 pg/ml as a cut-off value did not reach statistical significance as a potential predictor of poor clinical outcome (P=0.066). The Kaplan-Meier method demonstrated that pVEGF-C and -D did not exhibit statistical significance in predicting survival for patients with malignant effusions. Prospective studies with long-term follow-up of malignant effusion patients are required.

In addition, the present study also assessed the expression levels of VEGF-A, -C and -D proteins in exfoliated cells from the effusion, and observed that these proteins were most...
highly expressed in the cytoplasm of tumor and mesothelial cells, which is consistent with the literature on VEGF-A in effusion wax blocks (10,14). Multiple logistic regression analysis demonstrated that the expression of cVEGF-A proteins was inversely associated with age, indicating that age is a protective factor and reflects the reduced angiogenesis capacity in aging individuals. Since the number of cytological samples was limited in the present study, the association between cVEGF-A, -C and -D expression and overall survival was not examined. The present study also demonstrated that cVEGF-D expression was increased compared with cVEGF-A and -C expression in exfoliated cells from malignant effusion, particularly in strongly positive cells. This result indicates that VEGF-D-mediated lymphangiogenesis may be important in the formation of malignant effusion and may provide a novel targeted therapy for cancer patients. No statistically significant differences were observed in pVEGF-C and -D expression between benign and malignant effusions, however, the proteins were highly expressed at the cellular level. Additional clinical samples are required to further study the diagnostic value of pVEGF-C and -D following disease stratification.

In the present study, serum VEGF-A, -C and -D levels exhibited no marked clinical significance in the diagnosis and prognosis of serous cavity effusions, and were also not significantly associated with the examined clinicopathological parameters. Certain previous studies have demonstrated that supernatant fluid VEGF-A levels in malignant effusions are consistently increased compared with serum levels, while other studies have observed no correlation between the levels of supernatant fluid VEGF-A in malignant effusions and plasma (27-30). In the present study, the pVEGF-A levels were similar to the corresponding sVEGF-A levels in the patients with benign effusions. However, the pVEGF-A levels in the patients with malignant effusions were significantly increased compared with their corresponding sVEGF-A levels. Analysis of this phenomenon demonstrated that serous cavity markers do not easily enter the blood circulation inactivated by the liver, however, serological markers are easily affected by numerous factors, such as body metabolism, which results in concentration of the serological markers being reduced compared with the serous cavity markers. It has previously been indicated that serological markers may not be as effective for the diagnosis and prognostic values of serous cavity effusions compared with local effusion markers.

The present study applied ELISA to detect local effusion markers from the supernatant and ICC to the cells. Using ELISA for the detection was convenient due to the small amount of effusion required and the quantitative analysis provided, but the method is easily affected by the whole body disease and reagent instruments. The ICC analysis may be performed on wax blocks and fresh exfoliated cells. The former has been used in previous studies, with the advantage of long sample storage and the disadvantage of a long, complex production process that is easily affected by impurities. The latter was used in the present study and has the advantage of using fresh cells, not being easily affected by impurities and possibly providing a rapid clinical diagnosis. However, the method has the disadvantage of requiring at least 50 ml of sample and has no long-term sample preservation. Notably, the wax block method is suitable for retrospective studies and fresh exfoliated cells are suitable for prospective studies, with timely clinical diagnosis and treatment. The methods of using ELISA and ICC to identify local effusion markers require improvement by multicenter, large sample, randomized, prospective clinical trials and adequate follow-up, and this may be used to determine which detected form is active and suitable for VEGF-A, -C, and -D in clinical to timely diagnosis and target therapy.

In conclusion, the present study demonstrated that the pVEGF-A expression level may be useful in the differential diagnosis of malignant effusion from lung or gastric cancer samples. cVEGF-C and -D may be important in the formation of malignant effusion. The levels of VEGF-A and -C protein in the supernatant fluid and VEGF-A protein in the cells were negatively associated with age, while supernatant fluid VEGF-A was positively associated with malignant and bloody effusion, and only cavity metastasis. Serum VEGF-A, -C and -D levels exhibited no marked clinical significance in the diagnosis or prognosis of serous cavity effusions. Therefore, future studies with a larger sample size and long-term follow-up are required to establish the role of VEGF-A, -C and -D for the diagnosis, prognosis and targeted therapy of malignant effusions.

References