Role of VEGF and CD44v6 in differentiating benign from malignant ascites

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AIM: To detect the vascular endothelial growth factor (VEGF) and soluble splice variant 6 of CD44 (sCD44v6) levels in ascites and to explore their role in differentiating benign from malignant ascites.

METHODS: Cirrhotic ascites (n=36), tuberculosis ascites (n=8) and malignant ascites (n=23) were collected and studied. Concentrations of soluble VEGF and sCD44v6 in various kinds of ascites (n=67) were measured using a sandwich enzyme-linked immunosorbent assay.

RESULTS: VEGF and sCD44v6 levels in malignant ascites were 640.74±264.81 pg/ml and 89.22±38.20 ng/ml, respectively, both of which were significantly higher than those in cirrhotic ascites and tuberculosis ascites (p=18.98, 11.89 and q=8.92, 5.09; P<0.01). However, the levels of VEGF and sCD44v6 in cirrhotic and tuberculosis ascites had no significant difference (q=0.48, 0.75; P>0.05). Furthermore, VEGF levels in malignant ascites in patients with ovarian cancer were higher than those with gastric and colon cancer (q=5.03, 6.79; P<0.01, respectively). But differences of VEGF levels between gastric and colon cancer were not significant (q=1.90, P>0.05). Whereas, sCD44v6 levels in malignant ascites from patients with ovarian, gastric and colon cancer had no significant difference (q=0.06, 0.91, 0.35; P>0.05, respectively). In comparison with cirrhotic and tuberculosis ascites, when the upper limit of its VEGF mean levels 119.44 pg/ml (70.90±48.54) and sCD44v6 mean levels 63.59 ng/ml (48.54±19.17) was taken as the minimum cutoff limit, the sensitivity and specificity of VEGF and sCD44v6 of this assay to the diagnosis of malignant ascites were 91.3 %, 90.9 % and 73.9 %, 88.7 % respectively.

CONCLUSION: Elevated levels of VEGF and sCD44v6 may be useful in differential diagnosis of benign and malignant ascites.

INTRODUCTION
Angiogenesis is an absolute requirement for neoplastic growth of solid tumors after tumors reach a critical size of 1-2 mm[11], and is also essential for tumor invasion and metastasis, facilitates the shedding of tumor cells into surrounding blood vessels. Tumor cells have been shown to secrete a variety of angiogenic factors and thereby induce local formation of new blood capillaries. Among these factors, vascular endothelial growth factor (VEGF), also called vascular permeability factor (VPF), is a bifunctional cytokine and has the role in enhancing vascular permeability and stimulating endothelial growth[2-5], and is recognized as one of the most important molecules in the growth, invasion, metastasis and recurrence of human tumors[6-9].

However, tumor invasion and metastasis are considered to be a complex and multi-step process. Since the initial observation that a splice variant of CD44 (CD44v) could endow non-metastasizing cells with metastasis potential[10]. Many studies have demonstrated that CD44v, especially splice variant 6 of CD44 (CD44v6), probably promoting cancer cells to adhere to vascular endothelium and base membranes and enhancing moving ability of cancer cells, is most likely responsible for the invasion and metastasis of several tumor systems[11-13].

Malignant ascites is the direct and prominent manifestation of advanced carcinoma metastasized to the peritoneum[14]. Thus it is reasonable to hypothesize that VEGF and CD44v6 can be detected in malignant ascites. In the present study, we measured the concentration of VEGF and soluble CD44v6 (sCD44v6) using an enzyme-linked immunosorbent assay (ELISA) in various kinds of ascites in order to assess the value of VEGF and CD44v6 in identifying benign and malignant ascites.

MATERIALS AND METHODS

Patients
A total of 67 inpatients with ascites were collected at Renmin Hospital of Wuhan University, Zhongnan Hospital of Wuhan University and Tumor Hospital in Hubei Province from July 2002 to March 2003(Table 1). Informed consent of the patient and approval of the hospital were provided prior to collection of samples and medical records. All the cases were confirmed by cytologic examination of ascites, pathological examination, B-ultrasound and CT scan, etc.

Table 1 Patient characteristics

| Diagnosis               | No. of Patients | Mean age (range) | Female/ Male |
|------------------------|-----------------|------------------|--------------|
| Ascite                  | 67              | 47(19-96)        | 26/41        |
| Cirrhotic ascites       | 36              | 48(30-96)        | 10/26        |
| Tuberculous ascites     | 8               | 28(19-33)        | 4/4          |
| Carcinoma ascites       | 23              | 66(35-76)        | 12/11        |
| Ovarian cancer          | 8               | 60(35-70)        | 8/0          |
| Gastric cancer          | 6               | 68(38-74)        | 1/5          |
| Colon cancer            | 5               | 64(46-71)        | 2/3          |
| Hepatocellular cancer   | 2               | -                | 0/2          |
| Pancreatic cancer       | 1               | -                | 0/1          |
| Primary peritoneal cancer| 1              | -                | 1/0          |

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| Primary peritoneal cancer| 1              | -                | 1/0          |
Sample processing
Ascites samples were collected during therapeutic or diagnostic paracentesis and centrifuged at 3,000 rpm for 15 minutes at 4 °C. Cell-free supernatants were collected and aliquots were stored at -70 °C before determination.

Experimental groups
Cirrhotic, tuberculous and malignant ascites were defined as groups 1, 2 and 3, respectively. Malignant ascites from patients with ovarian, gastric and colon cancer were grouped as groups A, B and C, respectively.

Immunooassay for human VEGF
Concentrations of VEGF in ascites were determined with an ELISA kit (R & D Systems) following the manufacturer’s guidelines. All samples were analyzed in the laboratory of the Department of Gastroenterology, Renmin Hospital, Wuhan University. For determination of VEGF, samples were analyzed in duplicate, human recombinant VEGF	extsubscript{HSA} was diluted in series and used as a standard. VEGF concentrations were measured according to the standard curve. Samples with VEGF values beyond the standard curve were diluted and reanalyzed.

ELISA for human sCD44v6
Levels of sCD44v6 in ascites were measured with a sCD44v6 ELISA kit (Bender MedSystems, Austria). Briefly, monoclonal antibody against CD44v6, VFF-7, was absorbed by microwells in 96-well microtiter plates. sCD44v6 in the sample or in the standard bound to antibodies was adsorbed by each microwell. Horseradish peroxidase-conjugated monoclonal antibody against CD44v6 was then added and bound to the sCD44v6 that had been captured by the first antibody. After incubation, unbound enzyme conjugated antibodies were removed by washing and a substrate solution was added to each well. A colorful reactive product was formed, the reaction was terminated by addition of acid, and absorbance was measured at 450 nanometers. A standard curve was prepared from six standard dilutions of sCD44v6, which allowed determination of the levels of sCD44v6 in our samples.

Statistical analysis
The data were presented as mean ± S.D. One-way analysis of variance was used for statistical analysis. Differences were considered significant when P value was less than 0.05.

RESULTS
Concentrations of VEGF in ascites
Figure 1 shows VEGF levels in malignant ascites (640.74±264.81 pg/ml), which were significantly higher than those in cirrhotic ascites (67.05±51.91 pg/ml), tuberculous ascites (88.25±24.12 pg/ml) (P<0.01). However, there was no significant difference of VEGF levels between cirrhotic and tuberculous ascites (P>0.05).

Levels of sCD44v6 in ascites
sCD44v6 levels in malignant ascites (89.22±38.20 ng/ml) were higher than those in cirrhotic ascites (44.79±18.02 ng/ml), tuberculous ascites (50.25±12.57 ng/ml) (P<0.01). But the difference of sCD44v6 levels in cirrhotic and tuberculous ascites was not statistically significant (P>0.05) (Figure 2). We found both VEGF and sCD44v6 levels were increased in malignant ascites.

Comparison of VEGF and sCD44v6 levels in different kinds of malignant ascites
Statistical comparison of VEGF and sCD44v6 levels in these kinds of malignant ascites was not performed due to the limited number of hepatocellular cancer (n=2), pancreatic cancer (n=1) and primary peritoneal carcinoma (n=1).

Figure 1 Comparison of VEGF concentrations in different kinds of ascites. Group 1: cirrhotic ascites, Group 2: tuberculous ascites, Group 3: malignant ascites.

Figure 2 Comparison of sCD44v6 concentrations in different kinds of ascites. Group 1: cirrhotic ascites, Group 2: tuberculous ascites, Group 3: malignant ascites.

Figure 3 Concentrations of VEGF and sCD44v6 in different kinds of malignant ascites. Group A: ovarian cancer, Group B: gastric cancer, Group C: colon cancer. Concentrations of VEGF in group A were higher than those in groups B and C (P<0.01), while the difference of CD44v6 levels among groups A, B and C was not statistically significant (P>0.05).

Figure 3 shows VEGF levels in ascites from patients with ovarian cancer (866.25±208.46 pg/ml), which were higher than those with gastric cancer (541.30±123.17 pg/ml) and colon cancer (402.80±140.10 pg/ml), respectively (P<0.01). There was no significant difference of VEGF levels between gastric and colon cancer (P>0.05). Whereas, no statistical difference of sCD44v6 levels in ascites of patients with ovarian cancer.
ascites, which remains a knotty problem all the time.

Moreover, detecting VEGF levels may contribute to the diagnosis of malignant ascites. Meanwhile, we also found that VEGF was significantly increased in patients with advanced carcinoma. To our knowledge, however, concentration of sCD44v6 has not been examined in malignant ascites, this might be the first study to document sCD44v6 in malignant ascites.

We found sCD44v6 levels were high in malignant ascites, and relatively low in nonmalignant ascites. It implies that elevated CD44v6 appears to be correlated to the invasion and metastasis of cancer cells into peritoneal cavity. But it is unclear why CD44v6 is closely associated with malignant ascites. The ability of CD44v6 to bind peritoneal mesothelial surfaces of abdominal cavity, and a subsequent cancer cell implantation may contribute to it. At the same time, our results showed a higher sensitivity and specificity of sCD44v6 to the diagnosis of malignant ascites. However, no evidence is available to show that detection of sCD44v6 could contribute to the determination of a potential primary cancer causing malignant ascites. It is reasonable to consider sCD44v6 may be a diagnostic index of malignant ascites.

In summary, VEGF and sCD44v6 are detectable in ascites and are significantly elevated in malignant ascites. Prospective monitoring of VEGF and sCD44v6 levels in ascites would be helpful in differential diagnosis of benign and malignant ascites.

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