

# Correlations of serum VEGF and MMP-2 levels with CLM in CRC patients and effects of TACE on their expressions

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**Abstract.** – **OBJECTIVE:** To investigate the correlations of serum vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2 (MMP-2) levels with colorectal liver metastasis (CLM) in patients with colorectal cancer (CRC), and determine the effects of transhepatic arterial chemoembolization (TACE) on their expressions.

**PATIENTS AND METHODS:** A total of 110 CRC patients treated in the department of interventional radiology of our hospital from May 2013 to May 2016 were randomly enrolled. All patients received treatment of TACE after surgery. During surgery, 10 mL venous blood was taken, and the protein levels of VEGF and MMP-2 in serum were detected via enzyme-linked immunosorbent assay (ELISA). The correlations of VEGF and MMP-2 levels with clinicopathological features of patients were analyzed. The values of VEGF and MMP-2 in predicting CLM were analyzed using the receiver operating characteristic (ROC) curve. Moreover, changes in serum VEGF and MMP-2 levels in CRC patients were analyzed before TACE, and at 1 and 6 months after TACE.

**RESULTS:** The median VEGF and MMP-2 levels in serum of CRC patients were 64.8 ng/mL and 114.4 ng/mL, respectively, which were significantly higher than those in healthy control group (5.3 ng/mL and 6.8 ng/mL) ( $p < 0.05$ ). The expressions of VEGF and MMP-2 in serum of CRC patients were correlated with the depth of tumor infiltration, Dukes' staging, CLM and lymph node metastasis ( $p < 0.05$ ). Logistic regression analyses showed that the expression levels of VEGF and MMP-2 were positively correlated with CLM ( $p < 0.05$ ). The ROC curve revealed that the sensitivity, specificity and accuracy of serum VEGF in the diagnosis of CLM were 62.2%, 78.5% and 80.2%, respectively. The sensitivity, specificity and accuracy of serum MMP-2 in the diagnosis of CLM were 68.9%, 85.5% and 82.2%, respectively. The serum VEGF levels in CRC patients before TACE, and at 1 and 6 months after TACE were (64.8±58.8) ng/mL, (43.1±27.3) ng/mL and (24.8±12.3) ng/mL, respectively, show-

ing a downward trend ( $p < 0.05$ ). The serum MMP-2 levels in CRC patients before TACE, and at 1 and 6 months after TACE were (114.4±136.2) ng/mL, (73.4±58.5) ng/mL and (39.6±30.4) ng/mL, respectively, also indicating a reducing trend ( $p < 0.05$ ).

**CONCLUSIONS:** The levels of VEGF and MMP-2 in portal vein serum of CRC patients can be used as effective indexes for judging the prognosis of CRC and predicting CLM. Patients with high expression of VEGF and MMP-2 should be actively treated with TACE after surgery to improve the survival rate.

*Key Words:*

VEGF, MMP-2, CRC, CLM, TACE.

## Introduction

Colorectal cancer (CRC) ranks as the fourth most common malignant tumor in China. There are more than 130,000 new cases of CRC each year, accounting for 10% of cancer deaths in China<sup>1</sup>. Statistical data have shown that in 54% cases, tumor occurs in the colon (stage A and B of Dukes' staging), and it metastasizes to lymph nodes or distant organs in 46% cases (stage C and D of Dukes' staging)<sup>2</sup>. Although great progresses have been made in the surgical techniques, adjuvant radiotherapy and trans hepatic arterial chemoembolization (TACE) in the treatment of CRC, yet the major challenge in clinic still exists and impedes the prevention against metastatic spread<sup>3</sup>. According to statistics, some patients can be cured after isolated metastasis in a single organ, especially the liver, via removal, but most patients with progressive CRC eventually die of metastatic disease<sup>4</sup>. The 5-year survival rate of patients with colorectal liver metastasis (CLM) is only 25%. However, CRC relapses in two-thirds of pa-

tients, so it is significant that most patients fail to benefit from the surgery<sup>5</sup>. Therefore, it is of great clinical significance to classify the cases meeting the surgical requirement before surgery.

During the metastasis of malignant tumor cells, a large number of cancer cells are continuously released from the primary tumor into the circulation, so it can be believed that a single cancer cell can rarely establish a metastasis focus. Matrix metalloproteinases (MMPs) have been extensively studied in CRC, which are considered to be essential for invasion during the metastasis<sup>6</sup>. Some MMPs are also involved in the transformation process of adenomatous polyps into invasive colon cancer and metastasis. MMPs can jointly degrade almost all extracellular matrix (ECM) components, namely collagen, laminin, fibronectin, actin and proteoglycan<sup>7</sup>. In cancer, special attention has been paid to the degradation of type IV collagen, which is the main protein component of MMP-2 and MMP-9 in the basement membrane<sup>8</sup>. An important process of promoting metastasis is angiogenesis. Angiogenesis is controlled by a variety of angiogenic factors, among which vascular endothelial growth factor (VEGF), also known as VEGF- $\alpha$ , is considered as one of the most significant angiogenic factors. Importantly, it is reported that VEGF is closely related to CLM, and its expression level can serve as not only an index of judging CLM, but also as prognostic marker<sup>9</sup>.

In this study, a total of 110 patients with CRC were enrolled to understand the correlations of VEGF and MMP-2 concentrations in portal vein blood in CRC patients with CLM and TACE, and to evaluate whether they can be used as effective indexes to predict the occurrence of CLM in CRC patients.

## Patients and Methods

### Patients

A total of 110 CRC patients treated in the Oncology Department of our hospital from May 2013 to May 2016 were randomly enrolled. All patients underwent surgical treatment and TACE after surgery. There were 53 males and 57 females with a median age of 56 years old (34-77 years old). In terms of the Dukes' staging (clinicopathological staging program of the National Colorectal Cancer Assistant Group in 1982), there were 17 cases in stage A, 20 cases in stage B, 33 cases in stage C, and 40 cases in stage D. None of the patients received radiotherapy or chemotherapy before surgery. In terms of postoperative pathological

grading, there were 35 cases of high differentiation, 48 cases of moderate differentiation, and 27 cases of low differentiation. All patients enrolled received no anti-cancer therapy before surgery. Another 50 healthy subjects receiving physical examination were enrolled as control group, including 11 males and 9 females with an average age of (50.3 $\pm$ 12.9) years old. All subjects signed the informed consent, and this clinical trial was approved by the Ethics Committee of hospital.

### Sample Collection

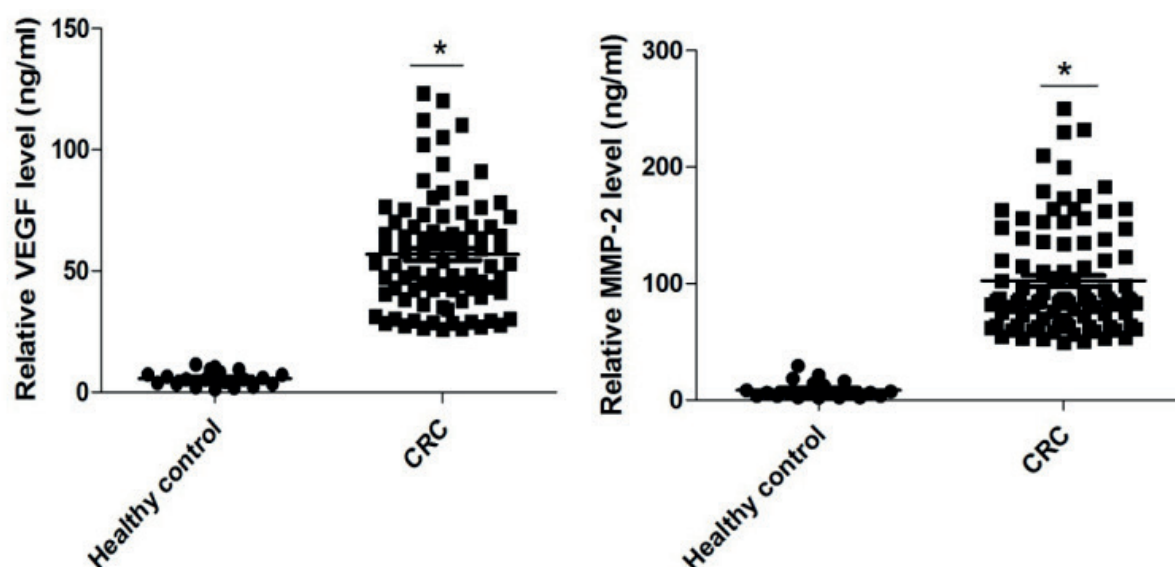
After the abdomen was opened, 2.5 mL portal vein blood were collected from CRC patients, and the blood sample was centrifuged at 1,000 g for 10 min to obtain the serum. Next, the serum was stored in a freezer at -80°C to be detected. CRC surgical specimens were made into paraffin-embedded sections and stored for hematoxylin-eosin (HE) staining.

### Detection Methods

The serum VEGF level (ng/mL) was detected using the Human VEGF ELISA Kit (Invitrogen Corporation, Art No.: D2583, Carlsbad, CA, USA), and the serum MMP-2 level (ng/mL) was measured using the MMP-2 ELISA Kit (Multi-Sciences, Art No.: F0186, Hangzhou, Zhejiang, China). The test steps were performed according to the kit test procedures. Finally, the optical density (OD) value was read at 450 nm using the Anthos 2010 microplate reader (Anthos Labtec Instruments GmbH, Salzburg, Austria), and the measured values were converted into VEGF and MMP-2 content based on the standard curve.

### TACE Program

TACE was performed using FOLFOX regimen, and the specific operation method was as follows: the right femoral artery was punctured using the modified Seldinger's method, and the 5F femoral artery sheath was implanted. Under the guidance of digital subtraction angiography (DSA), the location of tumor and the tumor-feeding artery were determined via angiography. The tumor-feeding artery in the liver was infused with 30 mg Endostar, 1 g 5-fluorouracil (5-FU) and 85 mg/m<sup>2</sup> oxaliplatin via the microcatheter, and epirubicin (20-30 mg) and iodized oil (5-10 mL) were prepared into suspension emulsion for chemoembolization. After that, the catheter was washed with normal saline, and angiography was performed to confirm no tumor vascular staining shadow in the liver. Under the fluoroscopy, the catheter was withdrawn, followed



**Figure 1.** Comparisons of VEGF and MMP-2 levels between healthy control group and CRC group. \*:  $p < 0.05$  vs. healthy control group.

by hemostasis by local compression, and pressure dressing. After patients returned to the ward, they received intravenous infusion, hydration, anti-nausea, acid suppression, liver protection, diuresis and other symptomatic and supportive therapies. After interventional therapy, Endostar was given intravenously for 2 consecutive days (15 mg/d). After drug administration, dynamic-contrast enhanced magnetic resonance imaging (DCE-MRI) was performed immediately. Patients were treated using this program for 2 consecutive courses at an interval of 1 month. Changes in tumor morphology and DCE-MRI parameters were measured before and after treatment, and adverse reactions of patients were observed closely.

#### HE Staining

CRC and CLM tissues were cut into 1-2 mm-thick blocks, and immersed in 4% formalin buffer overnight, followed by dehydration with gradient ethanol, transparency with n-butyl alcohol, immersion into wax and embedding into paraffin blocks. The paraffin blocks were sliced into 4  $\mu$ m-thick sections, and baked in an oven at 60 °C for 3 h, followed by dewaxing with xylene, rehydration with ethanol, and HE staining. Finally, sections were observed under a microscope (Philips, EM-300, Amsterdam, The Netherlands).

#### Statistical Analysis

Statistical Product and Service Solutions (SPSS Inc., Chicago, IL, USA) 12.0 statistical software

were used for data analysis. Measurement data were presented as mean  $\pm$  standard deviation, and t-test was used for the comparison of indexes between two groups. Continuous data from multiple groups were analyzed by using one-way ANOVA, with the Tukey's post-hoc test.  $\chi^2$ -test was used to analyze the differences in VEGF and MMP-2 levels between healthy group and CRC group, and the correlations of VEGF and MMP-2 levels with clinicopathological indexes of CRC. Correlations of VEGF and MMP-2 expression levels with CLM were studied via Logistic regression analysis.  $p < 0.05$  suggested that the difference was statistically significant.

## Results

#### VEGF and MMP-2 Levels in Healthy Group and CRC Group

The median VEGF and MMP-2 levels in serum in CRC group were 64.8 ng/mL and 114.4 ng/mL, respectively, which were significantly higher than those in healthy control group (5.3 ng/mL and 6.8 ng/mL, respectively) ( $p < 0.05$ ) (Table I, Figure 1).

#### Correlations of Serum VEGF and MMP-2 Levels with Clinicopathological Indexes of CRC

The expressions of VEGF and MMP-2 in serum of CRC patients were correlated with the depth of tumor infiltration, Dukes' staging, CLM and lym-

**Table I.** Comparisons of serum VEGF and MMP-2 levels ( $\bar{x} \pm s$ ).

Group	n	VEGF (ng/mL)			MMP-2 (ng/mL)				
		Min	Max	Med	Mean	Min	Max	Med	Mean
Control group	20	1.2	11.6	5.3 (3.7, 12.6)	6.4	2.4	21.3	6.8 (5.8, 14.6)	8.3
CRC group	110	20.6	123.6	64.8 (57.3, 84.2)	51.3	49.5	250.4	114.4 (93.5, 285.7)	108.4
$\chi^2$				13.3				25.5	
<i>p</i>				<0.001				<0.001	

Note: Min: minimum, Max: maximum, Med: median, Mean: mean.

ph node metastasis ( $p < 0.05$ ). With the progression of disease, their expressions were significantly increased. In particular, the expressions of VEGF and MMP-2 in serum of patients with CLM were significantly higher than those in patients without metastasis (Table II).

**Pathological Examination Results of CLM in CRC**

The pathological examination for 42 patients with CLM and 68 CRC patients without CLM confirmed that CLM tumor was often accompanied with the formation of cancerous nodes. Pathological results are shown in Figure 2.

**Logistic Regression Analyses of Two Indexes**

Logistic regression analyses showed that the expression levels of VEGF and MMP-2 were positively correlated with CLM ( $p < 0.05$ ). Under  $X_1 = \text{VEGF}$  and  $X_2 = \text{MMP-2}$ , the regression equation of prediction probability value of CLM was obtained:  $Y = 1/[1 + \text{EXP}(2.119 - 0.139X_1 - 0.336X_2)]$ , and a new variable Y was obtained (Table III).

**ROC curve analyses of VEGF and MMP-2 in Prediction of CLM**

As the area under the receiver operating characteristic (ROC) curve (AUC) enlarged, the dia-

**Table II.** Correlations of serum VEGF and MMP-2 levels with clinicopathological indexes of CRC ( $\bar{x} \pm s$ ).

Item		N (110)	VEGF (ng/mL)	MMP-2 (ng/mL)
Gender	Male	53	62.2±37.4	112.4±103.4
	Female	57	64.7±35.9	115.6±97.3
Age	<50 years old	45	65.2±32.4	115.3±98.3
	>50 years old	65	63.8±30.5	118.2±102.4
Tumor location	Colon cancer	62	60.6±38.2	109.4±103.5
	Rectal cancer	58	64.2±32.4	114.8±95.3
Depth of infiltration	T1, T2	50	57.4±25.4	94.4±98.2
	T3, T4	60	69.3±35.8*	128.8±105.2
Dukes' staging	A & B	37	58.6±29.5	97.4±104.2
	C & D	73	69.3±34.2*	119.8±92.3*
CLM	Yes	42	75.6±32.2	142.4±89.2
	No	68	40.28±27.4**	74.4±94.6***
Lymph node metastasis	Yes	51	69.7±31.4	128.3±107.7
	No	59	56.6±29.5*	98.3±104.3**

Note: In the comparison between two groups, \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ .

**Table III.** Logistic regression analyses of VEGF and MMP-2.

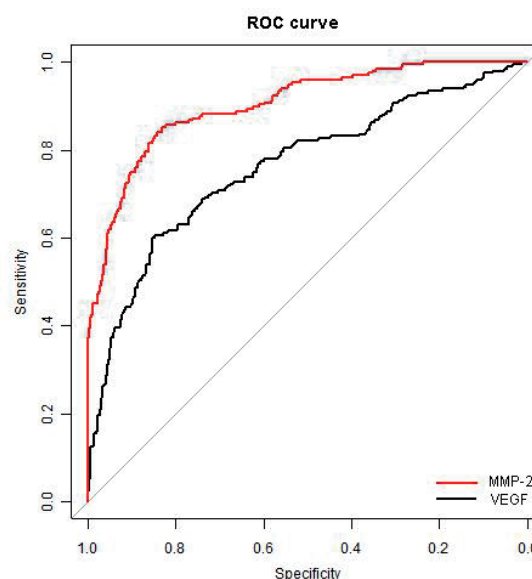
Index	CR	Sx	Wald value	p
VEGF	0.476	0.052	7.98	0.024
MMP-2	0.238	0.041	12.43	0.009

Note: CR: coefficient of regression, Sx: standard error.

gnostic accuracy of index for CLM increased. The AUC values of VEGF and MMP-2 were  $0.732\pm 0.044$  and  $0.835\pm 0.070$ , respectively. The AUC value of variable Y was  $0.906\pm 0.050$ , and the difference was statistically significant ( $p < 0.05$ ). Based on the results of pathological examination, the sensitivity, specificity and accuracy of VEGF in the diagnosis of CLM were 62.2%, 78.5% and 80.2%, respectively, and those of MMP-2 in the diagnosis of CLM were 68.9%, 85.5% and 82.2%, correspondingly (Figure 3).

**Changes in VEGF and MMP-2 Levels Before and After TACE**

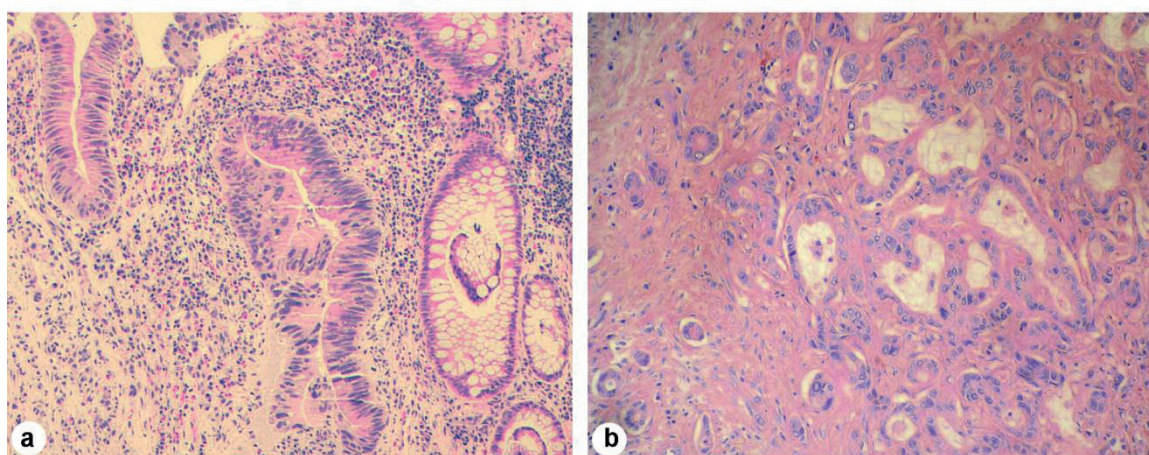
The serum VEGF levels in CRC patients before TACE, and at 1 and 6 months after TACE, were  $(64.8\pm 58.8)$  ng/mL,  $(43.1\pm 27.3)$  ng/mL and  $(24.8\pm 12.3)$  ng/mL, respectively, which were gradually decreased as the treatment time extended ( $p < 0.05$ ). Similarly, the serum MMP-2 levels in CRC patients before TACE, and at 1 and



**Figure 3.** ROC curve analyses of VEGF and MMP-2 in prediction of CLM.

**CRC**

**CLM**



**Figure 2.** Pathological section images of CRC and CLM. a) Pathological section image of CRC: Cancer cells are distributed around the glandular cavity, there is abundant cytoplasm, nuclei are large and irregular, nucleoli are clear, and multi-nuclear tumor giant cells can be seen. b) Pathological section image of CLM in CRC: Cancer cells gather into masses with small glandular cavity structure, the differentiation degree of cancer cells is high with significant atypia, and cells are in fusiform shape with little cytoplasm, large and deeply-stained nuclei, irregular shape, significant nucleoli and visible mitotic figure ( $\times 400$ ).

**Table IV.** Comparisons of serum VEGF and MMP-2 levels in CRC patients before and after TACE ( $\bar{x} \pm s$  ng/mL).

Gene	N	Before treatment	1 month after treatment	6 months after treatment
VEGF	110	64.8±58.8	43.1±27.3*	24.8±12.3*#
MMP-2	110	114.4±136.2	73.4±58.5**	39.6±30.4*#

Note: CR: coefficient of regression, Sx: standard error.

6 months after TACE were (114.4±136.2) ng/mL, (73.4±58.5) ng/mL and (39.6±30.4) ng/mL, respectively, presenting a downward trend following the treatment time ( $p < 0.05$ ) (Table IV).

## Discussion

The liver is one of the most common organs of malignant tumor metastasis, and it has a special anatomical structure with very abundant blood supply from both the portal vein (70-75%) and the hepatic artery (20-25%)<sup>10</sup>. Hepatic sinusoid is a junction of multiple arteries and veins with strong permeability, and there are defects (0.1 mm) not covered with basement membrane among endothelial cells, providing an important anatomical basis for the occurrence of liver metastatic tumor. Besides, the portal vein flow provides a kinetic basis for liver metastasis of malignant intestinal tumors, which is conducive to the tumor cell retention and growth, and formation of metastatic tumor<sup>11</sup>. Therefore, the detection of portal vein blood has more important clinical significance in the prediction of CLM.

More than 600,000 people die of CRC every year in the world. Many scholars in China and foreign countries have actively explored the factors influencing its prognosis, and the surgical treatment effect has been confirmed. However, only 10-20% patients can actually receive operation due to various reasons<sup>12</sup>. Currently, TACE is commonly used in clinic for the palliative therapy of CLM in unresectable CRC and treatment after resection of liver metastatic tumor. TACE closely combines the chemotherapy and embolization technique mainly to increase the drug concentration in the lesion area without increasing the drug concentration in surrounding normal tissues, so that the concentration of drug reaching the tumor tissues is increased by 10-30 times compared with that in intravenous systemic chemotherapy<sup>13</sup>. Therefore, the number of cancer cells that have been destroyed will be significantly increased. When iodized oil is mixed and emulsified with anti-cancer

drugs, anti-cancer drugs can be taken into the tumor tissues due to the guiding role of iodized oil, and then slowly released, so that anti-cancer drugs in a high concentration can act on cancer cells for a long time<sup>14</sup>.

CLM of CRC represents a complex process involving many aspects, such as angiogenesis, adhesion, migration and invasion, the interaction of metastatic tumor cells with liver microenvironment, and the synergistic or antagonistic effect among multiple genes and proteins<sup>15</sup>. Angiogenesis provides abundant nutrients and oxygen for tumor tissues, which is an important basis for tumor cell growth and migration. If there is no neovascularization, the volume of tumor tissues cultured *in vitro* is unable to exceed 4 mm<sup>3</sup>, and that of tumor *in vivo* is limited smaller than 1-2 mm<sup>3</sup><sup>16</sup>. As one of the strongest proangiogenic factors, VEGF is closely related to the occurrence, development, invasion and metastasis of tumor. Soumaoro et al<sup>17</sup> studied and found that the expression of VEGF in normal tissues around the tumor is lower than that in tumor tissues, and the VEGF mRNA expressions in primary tumor and metastatic focus of patients with multiple liver metastases are significantly higher than those in patients with single liver metastasis. The high expression of VEGF in tumor tissues promotes not only tumor angiogenesis but also tumor invasion and metastasis, which facilitate the occurrence, metastasis and prognosis of CRC<sup>18</sup>.

Previous evidence<sup>19</sup> showed that MMPs were associated with all aspects of cancer progression. Although serine, cysteine, aspartate and MMPs are involved in the invasion process, MMPs play dominant roles seemingly. In CRC, tumor cells are responsible for producing a large amount of MMP-2, while the tumor-infiltrating inflammatory cells produce a higher level of MMP-9, jointly promoting the tumor cell invasion and metastasis<sup>20</sup>. Parsons et al<sup>21</sup> detected the expressions of MMP-2 and pro-MMP-2 in 45 cases of CRC tissues via gelatin zymography, and results showed that the levels of MMP-2 and pro-MMP-2 in CRC tissues were 20 times higher than those in

normal colorectal epithelial tissues. Barozzi et al<sup>22</sup> evaluated the role of biomarkers in predicting the clinical prognosis of CRC patients via fluorescence in situ hybridization (FISH) and immunohistochemistry, and found that the prediction rate of elevated level of MMP-2, insulin-like growth factor-II (IGF-II) or transforming growth factor- $\beta$  (TGF- $\beta$ ) for liver metastasis reaches 99%.

Under the clinical scenario, new liver tumor often relapses in patients within a short period after liver metastatic tumor is removed, indicating the possibility of differentiation of CRC patients with and without metastasis through detecting the expressions of VEGF and MMP-2, in order to avoid unnecessary surgery and provide a better treatment strategy for CLM.

In this study, we found that the expressions of VEGF and MMP-2 in serum of CRC patients were correlated with the depth of tumor infiltration, Dukes' staging, CLM and lymph node metastasis. With the progression of disease, their expressions were significantly increased, which is consistent with previous study<sup>23</sup>. The ROC curve revealed higher efficiency for the diagnosis and prediction of CLM by detecting serum VEGF and MMP-2. The serum VEGF and MMP-2 levels in CRC patients before TACE, and at 1 and 6 months after TACE gradually decreased, which provides alternative evaluation for prognosis and metastasis characteristics of colorectal cancer<sup>24</sup>.

## Conclusions

We demonstrated that the expressions of VEGF and MMP-2 in portal vein serum of CRC patients can be used as effective indexes for judging the prognosis of CRC and predicting CLM. The treatment of TACE after surgery provides a promoting way for the survival rate particularly in CRC patients with high level of VEGF and MMP-2.

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