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Original Article

Circulating tumor cell count and serum CEA mRNA level predict postoperative recurrence of digestive tract cancer

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Abstract: Objective: To evaluate the predictive value of circulating tumor cell count (CTC) and serum carcinoembryonic antigen (CEA) mRNA levels in postoperative recurrence of digestive tract cancer. Methods: A total of 52 patients with digestive tract cancer who underwent radical resection in our hospital were divided into a recurrent group (n=18) and a non-recurrent group (n=34). The CTC and CEA mRNA levels of the two groups were compared one day before the operation. Pearson correlation analysis was used to analyze the correlation between CTC and CEA mRNA levels. The curve of subjects' working characteristics was used to analyze the predictive value of the serum CTC and the level of CEA mRNA in postoperative recurrence of digestive tract cancer. The correlation of CTC and CEA mRNA expression with different age, sex, cancer type and TNM stage was further analyzed. Results: CTC and CEA mRNA levels in the recurrent group were higher than those in the non-recurrent group (P<0.05). CTC was positively correlated with the CEA mRNA level by Pearson correlation (r=0.609, P=0.000). ROC curve showed that the area under the curve of CEA mRNA, CTC and combined test in predicting postoperative recurrence of digestive tract cancer were 0.912, 0.831 and 0.965 respectively. The area under the curve of the combined test and CEA mRNA level was significantly higher than that of CTC (Z=3.794, P=0.000; Z=2.001, P=0.045). There was no significant difference in the area under the curve between combined detection and CEA mRNA (Z=1.437 and P=0.151). There was no significant difference in CTC and CEA mRNA levels among patients with different sex, age, body weight, differentiation degree and histological type (P>0.05). The CTC and CEA mRNA levels of patients in stage III and IV were higher than those in stage I and II and the difference was statistically significant (P<0.05). By Spearman rank correlation analysis, it was found that CTC and CEA mRNA levels were positively correlated with the TNM stage (r=0.532, 0.712, P<0.05). Conclusion: levels of CTC and serum CEA mRNA have a certain value in predicting postoperative recurrence of digestive tract cancer and their expression is closely related to TNM stage. Clinical diagnosis of both of them can improve the diagnostic efficiency of predicting postoperative recurrence.

Keywords: Circulating tumor cell count, serum carcinoembryonic antigen, mRNA, digestive tract cancer, recurrence

Introduction

Digestive tract cancer refers to malignant tumors that occur in the large intestine, small intestine, stomach, esophagus, pharynx, oral cavity and other parts, among which esophageal, gastric and colorectal cancer are more common [1-3]. The incidence of the three accounted for 30-35% of the total incidence of cancer and 31% of all cancer deaths, seriously endangering human health. In recent years, with the continuous improvement of medical level, the curative effect of digestive cancer treated by surgery, radiotherapy and chemotherapy has been significantly improved and the 5-year survival rate of patients has been significantly improved, but some patients still have a recurrence after radical operation. Postoperative recurrence is one of the important causes of death [4]. If the postoperative recurrence of digestive tract cancer patients can be accurately predicted and effective prevention and treatment measures can be taken, the mortality rate can be effectively reduced.

Circulating tumor cell count (CTC) refers to malignant tumor cells with the characteristics of tumor antigen or primary tumor gene in blood circulation after falling off from primary tumor or metastatic tumor, which is a direct risk factor in the formation of tumor metastasis [5, 6]. As the focus of clinical research, CTC has shown unique advantages in judging the prognosis of many kinds of malignant tumors, such as breast cancer, rectal cancer, lung cancer and so on [7-9]. Detection of CEA mRNA in peripheral blood and resected lymph nodes by reverse transcriptase-polymerase chain reaction (RT-PCR) has high sensitivity and specificity, which helps the detection of micro-metastasis of the tumor [10]. However, at present, there are few clinical studies on the relationship between the expression of CTC and CEA mRNA and digestive tract tumor recurrence, and there are few reports on the diagnostic efficacy of the combination of the two. Based on this, this study preliminarily evaluated the predictive value of CTC, serum CEA mRNA levels and their combination in postoperative recurrence of digestive tract cancer to improve the accuracy of postoperative recurrence prediction and individual treatment.

Materials and methods

General data

This study has been approved by the Medical Ethics Committee of Ji'nan Sixth People's Hospital. Patients (n=52) with digestive tract cancer who underwent radical resection Ji'nan Sixth People's Hospital from December 2017 to November 2019 were selected and divided into a recurrent group (n=18) and non-recurrent group (n=34) according to whether they recurred 6 months after the operation. In the recurrent group, there were 11 males and 7 females with the age ranged from 42-74 years old with an average of 55.6±3.6 years, and the bodyweight ranged from 41-79 kg with an average of 62.75±5.12 kg. In the non-recurrent group, there were 21 males and 13 females. The age was 40-78 years old with an average of 56.2±3.9 years and the bodyweight ranged from 44-81 kg, with an average of 63.86±5.59 kg.

Inclusion criteria: Digestive tract cancer was confirmed by a pathology of tissues acquired from operation; all patients received surgical treatment and completed radical resection; the expected survival time after the operation was more than 3 months; the age was between 18 and 80 years old; patents need to know the content of this study and sign the informed consent form.

Exclusion criteria: Patients have benign tumors; patients have received radiotherapy and chemotherapy before operation and patients have other malignant tumors that affect the level of CEA.

Methods

Methods of grouping: All patients were followed up for 6 months. Postoperative recurrence was judged according to tumor markers, imaging, gastroscopy and enteroscopy. Patients with postoperative recurrence were included in the recurrence group. Patients without recurrence were included in the non-recurrence group.

Sample collection: About 7.5 mL of fasting peripheral blood was taken from patients with digestive tract cancer in the morning one day before operation and the initial 1 mL blood was discarded each time to prevent the skin epidermis at the tip of the needle from contaminating the blood samples and causing false-positive results. Blood was centrifugated at 3000 r/min for 10 minutes and the cell precipitation was stored in the refrigerator at 70°C.

The detection method: The blood sample was diluted when a 6.5 mL buffer solution was added. Then the sample was centrifuged at 4000 r/min for 20 minutes. The supernatant was sucked out and magnetic beads coupled with specific antibodies against epithelial cell adhesion molecules provided by Beijing Kairuiji Biotechnology Co., Ltd. were added and incubated for 15 minutes. The liquid and unbound magnetic particles were sucked out. Then the magnetic field was removed and the enriched cells were re-suspended in the buffer. The stained cells were transferred to the sample box for CTC and interpretation. The cells were separated from the lymphocyte separation solution provided by Beijing Baiolaibo Technology Co., Ltd., and the total RNA was extracted by Trizol reagent provided by Shanghai Yuanye Biotechnology Co., Ltd. The CEA mRNA was detected by RT-PCR technology and the detection kit is provided by Chongqing Prico

Biotechnology Co., Ltd. The reaction conditions were as follows: 94°C 120 s, 92°C 10 s, 50°C 25 s, 72°C 2 s for 35 cycles, and finally 72°C 300 s to 4°C. The primer sequences were synthesized by Dalian Baocheng Biological Co., Ltd. Primer sequence: upstream primer 5'-AGTGAGGGCAAACCGCAGTGACAC-3', downstream primer 5'-TT-GAGGTCGCTCCCGAAAAAAA-AAAAAAAAAAAAAAGMAG-3'. CEA-cDNA PCR amplification products were taken for 2% agarose gel electrophoresis and the results were observed and imaged under ultraviolet digital imager. The CT values of each sample were corrected with GAPDH mRNA as the internal reference. Upstream primers: 5'-GACAACTTT-GGTATCGTGGAAGG-3'; downstream primers: 5'-CCAGTAGAGCAGGGGATGATGT-3'; probe: fam 5'-CTCATGACCACAGTCCATGCCATCACT-3' Tamara. CT values were obtained and the $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression of CEA mRNA.

Outcome measures

Primary observation indicators: CTC and CEA mRNA level were compared between the two groups; the correlation between CTC and CEA mRNA level were analyzed by Pearson correlation; postoperative recurrence was taken as state variables and CTC and CEA mRNA level as test variables; curve of subjects' working characteristics was drawn and the value of serum CTC, CEA mRNA level and their combination in predicting postoperative recurrence of digestive tract cancer were analyzed.

The secondary observation index: The correlation between the positive rate of CTC and CEA mRNA and different age, sex, cancer type and TNM stage was further analyzed.

Statistical methods

Statistical software SPSS 21.0 was used to process the data. The measurement data were expressed by mean ± standard deviation ($\overline{x}$ ± sd) and the counting data were measured by x^2 test. Z test was used for normal distribution. Single-factor analysis of variance was used for comparison between groups and the Bonferroni test was used for pairwise comparison at different time points in the group. The predictive value of serum CTC and CEA mRNA in predicting postoperative recurrence of digestive tract cancer was detected by drawing the ROC curve area (AUC). AUC<0.5 has no value. 0.5-0.7 is of low diagnostic value while 0.7-0.9 is of moderate diagnostic value. And AUC>0.9 is of high diagnostic value. Delong et al. method is used in AUC comparison. The correlation between CTC and CEA mRNA level was analyzed by Pearson analysis. The correlation between CTC, CEA mRNA level and TNM stage was analyzed by Spearman rank correlation analysis. The difference was statistically significant (P<0.05).

Results

Comparison of general data

There was no significant difference between the two groups in sex, age, body weight, degree of differentiation, histological type, TNM stage and other general data (P>0.05). See **Table 1**.

Comparison of CTC and CEA mRNA level

CTC and CEA mRNA level in the recurrence group were significantly higher than those in the non-recurrence group (P<0.05). See **Table 2**.

Correlation

CTC was positively correlated with CEA mRNA level according to Pearson correlation analysis (r=0.609, P=0.000). See **Figure 1**.

The predictive value of CTC, CEA mRNA level and their combination in recurrence of digestive tract cancer

The results of ROC curve showed that the AUC of CEA mRNA for predicting postoperative recurrence of digestive tract cancer was 0.912 and the best cutoff value was 3816.20 copies/ml. The corresponding sensitivity, specificity and Yoden index were 0.889, 0.706 and 0.595 respectively. The AUC of predicting postoperative recurrence of digestive tract cancer by CTC was 0.831 and the best cut-off value was 6.87 per 7.5 mL. The corresponding sensitivity, specificity and Jordan index were 0.833, 0.735, 0.568, respectively. The AUC of CEA mRNA combined with CTC in predicting postoperative recurrence of digestive tract cancer was 0.965 and the best cut-off value was 3727.44. The corresponding sensitivity, specificity and Yoden index were 0.944, 0.824 and 0.768 respectively.

Table 1. Comparison of general data

Parameter	Recurrent group (n=18)	Non-recurrence group (n=34)	χ^2/Z	P
Gender (male/female)	11/7	21/13	χ^2=0.002	0.963
Age (years)			χ^2=0.569	0.451
≥60	12	19		
<60	6	15		
Weight (kg)			χ^2=0.416	0.519
≥60	8	12		
<60	10	22		
Degree of differentiation			Z=0.187	0.852
Low degree of differentiation	8	16		
Medium degree of differentiation	6	11		
High degree of differentiation	4	7		
Histological type			χ^2=0.036	0.982
Gastric cancer	7	13		
Colorectal cancer	5	13		
Esophageal carcinoma	6	8		
Tumor-node-metastasis stage			χ^2=0.032	0.857
I, II stage	8	16		
III, IV stage	10	18		

Table 2. Comparison of CTC and CEA mRNA level ($\bar{x} \pm sd$)

Group	CTC (/7.5 mL)	CEA mRNA level (copies/mL)
Recurrent group (n=18)	8.46±3.15	4278.12±571.24
Non-recurrence group (n=34)	6.06±2.37	3359.41±268.06
t	3.094	7.920
P	0.003	<0.001

Note: CTC: circulating tumor cell count; CEA: carcino-embryonic antigen.

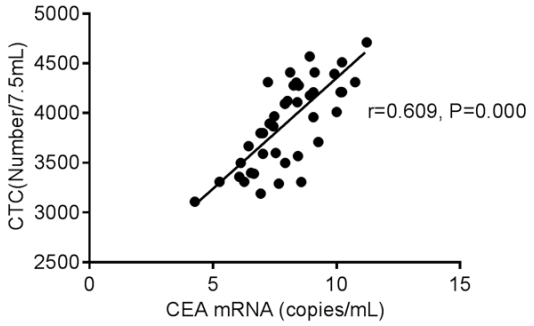

Figure 1. Scatter plot of horizontal correlation between CTC and CEA mRNA level. CTC: circulating tumor cell count; CEA: carcino-embryonic antigen.

In a pairwise comparison of each index of AUC, the AUC of combined test and CEA mRNA was significantly higher than that of CTC count (Z=3.794, P=0.000; Z=2.001, P=0.045). The difference was statistically significant. There was no significant difference in AUC between the combined test and CEA mRNA (Z=1.437, P=0.151). See **Table 3** and **Figure 2**.

The expression of CTC and CEA mRNA in patients with different characteristics

There was no significant difference in CTC and CEA mRNA levels among patients with different sex, age, body weight, differentiation degree and histological type (P> 0.05). But CTC and CEA mRNA level in stage III and IV were significantly higher than those in stage I and II (P<0.05). By Spearman rank correlation analysis, it was found that CTC and CEA mRNA levels were positively correlated with the TNM stage (r=0.532, 0.712, P<0.05). See **Table 4**.

Discussion

Digestive tract cancer is one of the most common malignant tumors in clinics, which brings a heavy burden to the family and society. It is clinically found that postoperative recurrence is not only the main cause of treatment failure in patients with digestive tract cancer, but also a risk factor for death. With the combined sur-

Table 3. Results of drawing ROC curve

Indicators	CTC	CEA mRNA	Combination
AUC	0.831	0.912	0.965
Standard error	0.040	0.048	0.018
P	0.000	0.000	0.000
95% CI	0.746-0.905	0.894-0.987	0.905-0.992
Optimal truncation value	6.87 (/7.5 mL)	3816.20 copies/mL	3727.44
sensitivity	0.833	0.889	0.944
specificity	0.735	0.706	0.824
Yoden index	0.568	0.595	0.768

Note: CTC: circulating tumor cell count; CEA: carcino-embryonic antigen; AUC: area under the curve; CI: confidencial interval.

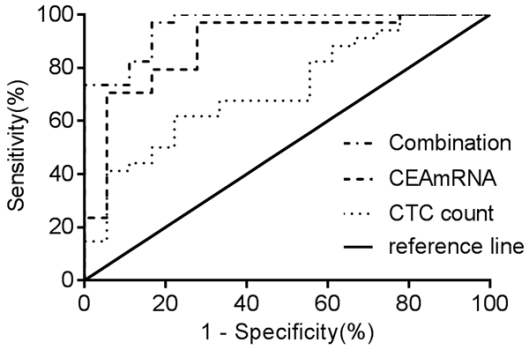

Figure 2. ROC chart of CTC, CEA mRNA level and their combination for prediction of recurrence. CTC: circulating tumor cell count; CEA: carcino-embryonic antigen.

gery, radiotherapy and chemotherapy, and targeted therapy etc., the mortality rate of digestive tract cancer patients decreased significantly, but some patients still have the risk of recurrence [11]. Related studies have shown that the main cause of the postoperative recurrence of cancer patients is micro-metastasis. And micro-metastasis mainly exists in the blood, so monitoring the micro-metastasis in the blood of cancer patients can effectively locate those with a high risk of relapse and give effective prevention and treatment to reduce recurrence and mortality [12].

CTC is a tumor cell in the blood circulation, which can be colonized in the target organ, and is one of the indispensable conditions for tumor metastasis and recurrence [13, 14]. Compared with imaging examination, CTC detection can detect micro-metastasis in the blood earlier. Because CTC comes from tumor tissue, it can reflect tumor load to some extent. One of the main causes of tumor blood metastasis in CTC [15, 16]; the relationship with lymph node metastasis is likely to be similar to the mechanism of tumor angiogenesis and lymphangiogenesis, both related to the regulation of vascular endothelial growth factor (VEGF) and its receptor. Recent studies have shown that CTC plays an important role in monitoring the condition and evaluating the prognosis of many kinds of digestive tract cancers such as colorectal cancer [17, 18]. Monitoring CTC can predict the risk of postoperative recurrence and is of great significance to improve the prognosis of patients. The results of this study showed that the CTC in the recurrent group was higher than that in the non-recurrent group and the AUC of CTC in predicting postoperative recurrence of digestive tract cancer was 0.831, which was of moderate diagnostic value. The expression was positively correlated with the TNM stage of gastrointestinal cancer patients. It can be seen that monitoring CTC expression has a certain value in predicting postoperative recurrence and can effectively reflect the clinical stage of patients with digestive tract cancer.

At the present stage, the main clinical methods for detecting micro-metastasis in the blood of patients with digestive tract cancer are to detect tumor markers such as carbohydrate antigen 19-9 (CA19-9) and CEA, but the levels of CEA and CA19-9 in peripheral blood are low. Conventional detection methods have some missed diagnoses. Besides, micro-metastasis has occurred in the blood of most patients with digestive tract cancer, but the clinical manifestation is not obvious. There are also missed diagnoses in pathological examination and imaging examination. In recent years, with the continuous progress of medical technology, RT-PCR has been widely used in the clinical detection of tumor micro-metastasis. Its principle is to amplify the target genes in order to detect them with low content [19]. CEA is a sugar chain protein with a molecular weight of 180 kb, which is usually found in the liver, pancreas and digestive tract of embryo and fetus, and its expression is significantly decreased in

Table 4. Expression of CTC and CEA mRNA in patients with different characteristics ($\bar{x} \pm sd$)

Characteristic	CTC (/7.5 mL)	CEA mRNA level (copies/mL)	t_1 or F_1/P_1	t_2 or F_2/P_2
Gender			0.397/0.697	0.585/0.561
Male (n=32)	7.16±3.15	4154.57±684.49		
Female (n=20)	6.89±2.76	4037.74±725.51		
Age (years)			0.379/0.710	0.971/0.142
≥60 (n=31)	6.01±3.81	4368.24±712.46		
<60 (n=21)	6.97±2.97	4298.18±702.79		
Weight (kg)			0.519/0.606	0.565/0.575
≥60 (n=20)	6.54±2.84	3864.68±625.84		
<60 (n=32)	6.98±3.05	3968.75±658.71		
Degree of differentiation			0.661/0.521	0.425/0.656
Low differentiation (n=24)	6.35±2.12	3889.74±611.74		
Medium differentiation (n=17)	7.06±2.25	3967.67±637.98		
High differentiation (n=11)	7.11±2.67	4100.31±651.32		
Histological type			0.833/0.418	0.132/0.897
Gastric cancer (n=20)	6.97±2.84	4365.46±701.86		
Colorectal cancer (n=18)	6.99±2.97	4261.17±721.07		
Esophageal cancer (n=14)	7.05±3.05	4204.16±687.75		
TNM staging			2.949/0.010	4.128/0.000
I, II stage (n=24)	4.24±1.37	3445.46±571.12		
III, IV stage (n=28)	10.12±4.37	4134.87±624.34		

Note: CTC: circulating tumor cell count; CEA: serum embryonic antigen. 1 is CTC, 2 is CEA mRNA.

adults [20]. At first, CEA was found to be abnormally expressed in the serum of patients with gastric cancer, and then gradually detected in pancreatic cancer, lung cancer and other malignant tumors. It is clinically found that CEA mRNA can be detected in cancer cells and RNA is easy to be decomposed and destroyed outside the cells, so the existence of cancer cells can be judged by detecting CEA mRNA [21]. For patients with digestive tract cancer, if CEA mRNA is detected in peripheral blood, it means that the cancer cells have entered the blood circulation, increasing the risk of postoperative recurrence. In this study, the CEA mRNA in the recurrent group was higher than that in the non-recurrent group, and the AUC for predicting postoperative recurrence was 0.912. It was positively correlated with the TNM stage of digestive tract cancer patients, indicating that level of CEA mRNA has a higher predictive value. However, when a tumor marker gene is detected alone, there are still some problems such as low specificity or low positive rate. So, this study further analyzed the predictive value of the combined detection of levels of CTC and CEA mRNA. The results showed that the AUC of combined detection and CEA mRNA was significantly higher than that of CTC, but there was no significant difference in AUC between combined detection and CEA mRNA, indicating that the combined detection of CTC and CEA mRNA or simple CEA mRNA can effectively predict the prognosis of patients with postoperative recurrence of digestive tract cancer. The purpose of this study is to provide a basis for guiding treatment and judging the disease. However, this study speculated that the predictive value of combined detection of CTC and CEA mRNA should be higher than that of simple CEA mRNA. We did not get statistically significant results, which may be related to the low sample size of this study and the failure to analyze the levels of indicators at different time points after the operation. Therefore, a multicenter, large sample and prospective study are needed in the future to obtain confirmative conclusion.

To sum up, levels of CTC and serum CEA mRNA have a certain value in the prediction of postoperative recurrence of digestive tract cancer and their expression is closely related to TNM staging. Clinical diagnosis can be combined to

improve the diagnostic efficiency of predicting postoperative recurrence.

Disclosure of conflict of interest

None.

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