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• BRIEF REPORTS •

# Detection of micrometastasis in peripheral blood by multi-sampling in patients with colorectal cancer

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## Abstract

**AIM:** To evaluate the reverse transcriptase-PCR assay and multiple sampling for detection of cytokeratin-positive cells in peripheral blood of colorectal carcinoma patients and to investigate the clinical significance of micrometastasis in peripheral blood.

**METHODS:** The expression of CK20 mRNA by RT-PCR was investigated in bone marrow, portal vein and peripheral blood in 58 colorectal cancer patients and 12 controls without known cancer. The peripheral blood was sampled twice at intervals of 3 d before operation. All the patients were followed up for one year.

**RESULTS:** There was no positive expression of CK20mRNA in 12 volunteers. The positive expression of CK20mRNA was 77.6% (45/58) in bone marrow, and that in portal vein was 74.1% (43/58) of colorectal carcinoma patients. The positive expression of CK20mRNA cells in peripheral blood rose from 44.8% (26/58) to 69.0% (40/58) ($P<0.01$). The total positivity of CK20mRNA expression in peripheral blood was similar to the positivity of CK20mRNA in bone marrow and portal vein. The positive rates became higher in later clinical stages than in early stages. The CK20mRNA positive patients had a higher relapse rate within one year than the CK20mRNA negative patients.

**CONCLUSION:** Multiple blood sampling can increase the detection of tumor cells in peripheral blood by RT-PCR for CK20mRNA in colorectal carcinoma patients and it is as sensitive and specific as that of bone marrow and portal vein. This technique may be reliable and convenient to diagnose micrometastasis of colorectal carcinoma and has an important significance in determining the prognosis of cancer patients.



**Key words:** Colorectal Cancer; CK20mRNA; Micrometastasis

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## INTRODUCTION

The incidence of colorectal carcinoma has become high in China. Despite radical excision of the primary tumor, almost half the patients with colorectal carcinoma would die from progression of a distant tumor[1,2]. In these patients, the principal cause of death is metastasis occurring early in tumor development, which leads to locoregional or distant tumor progression in later stages of the disease. How to identify a group of patients who have local diseases at high risk of developing metastases is a hot topic. Clonal or oligoclonal micrometastasis in blood is the earliest stage of metastasis. Diagnostic techniques currently available in clinical setting are not sufficiently sensitive to the detection of micrometastasis. The use of RT-PCR to detect tumor-associated mRNA is currently one of the most sensitive means to identify circulating tumor cells in cancer patients. Cytokeratin (CK) is an essential constituent of cytoskeleton in both normal and malignant epithelial cells, and can serve as a reliable marker for the epithelial origin of cells. Cytokeratin 20 expresses very strictly in epithelial cells of the stomach, intestine, colon, rectum, pancreas, and their tumors[3-6]. It can not be detected in circulation. RT-PCR mRNA assay is capable of identifying cultured colorectal carcinoma cells with a sensitivity of 1 in $10^7$ WBCs[5-8].

In this study, we used RT-PCR assays to examine the disseminated tumor cells in bone marrow, portal vein and peripheral blood in colorectal carcinoma patients to assess whether multiple blood sampling could increase the detection sensitivity of tumor cells. We compared the presence of CK20 mRNA-positive cells in peripheral blood with tumor development stages and survival rate of one year.

## MATERIALS AND METHODS

### *Patients*

A total of 58 colorectal carcinoma patients who underwent surgical treatment in our hospital between July 2001 to July 2002 were studied. The diagnosis of primary colorectal carcinoma was confirmed in all cases by endoscopic biopsy, and the primary tumor stage was confirmed by histological examination of the resected primary tumors. Twelve patients with no history of cancer who underwent abdominal operation were recruited as non-cancer controls. Forty-four male and 20 female patients were aged from 23 to 79 years. Informed consent was obtained from all the patients involved, and the ethic study was approved by the Ethics Committee of Nanjing Medical University.

### *Blood sampling*

Two 5 mL of bone marrow was aspirated from the anterior iliac crest or manubrium sterni under general anesthesis just before the operation was started. Before exploration, cannulae were utilized to aspirate blood (5-10 mL) from portal vein through right gastroepiploic vein. Five 10 mL of peripheral blood was aspirated from peripheral vein 3 d and 30 min respectively before operation. All blood samples were performed anti-coagulation with heparin, blood and bone marrow mononuclear cells (MNC) were isolated by density gradient centrifugation through lymphocytic separating medium. Then 1 mL Trizol reagent was

added, and stored at -20 ℃ until use.

### *RNA extraction*

Total RNA was extracted from the MNC pellets by Trizol reagent according to the manufacturer's instructions. Blood and bone marrow mononuclear cells were incubated in Trizol solution (1 mL/100 mg) for 15 min , and then an 1/5 volume of choloform was added. After vigorous agitation for 5 min, the inorganic phase was separated by centrifugation at 12 000 *g* for 20 min at 4 ℃. RNA was then precipitated in the presence of 1 volume of isopropanol and centrifuged at 10 000 *g* for 15 min at 4 ℃. RNA pellets were washed with 70% ice-cold ethanol and then dissolved in diethyl pyrocarbonate (DEPC) - treated $H_2O$. Total RNA concentration and quantity were assessed by absorbency at 260 nm using a nucleic acid and protein analyzer.

### *Two-step reverse transcription and polymerase chain reaction*

RNA was reverse transcribed in 20 μL RT buffer, and total RNA was prepared using a RNeasy kit. Five μg of total RNA was reverse-transcribed in a volume of 20 μL, at 42 ℃ for 60 min, and the reaction was terminated by heating at 95 ℃ for 5 min. The cDNA templates were subjected to PCR amplification, using primers: 5'-CAGACACACGGTGAACTATGG-3' (sense) and 5'-GATCAGCTTCCACTGTTAGACG-3' (antisense). The cycling protocol for CK20 (370 bp) consisted of: denaturation at 94 ℃ for 3 min, followed by 40 cycles, each for 45 s at 94 ℃, for 45 s at 60 ℃,for 60 s at 72 ℃, and a final extension at 72 ℃ for 5 min. All reactions were performed in a final volume of 50 μL PCR reaction mixture containing 5 μL cDNA, 3 μL $MgCl_2$, 5 μL 10× Buffer, 1 μL dNTP, 0.5 μL Taq polymerse, 1 μL primary. As a control of cDNA integrity, *$\beta_2$-microglobulin* expression was analyzed as well. The primer sets were 5'-CACTGTGTTGGCGTACAGGT-3' (forward) and 5'-TCATCACCATTGGCAATGAG-3' (reverse) for *$\beta_2$-microglobulin*. PCR products were analysed on a 2% agarose gel and visualised by ethidium bromide staining.

### *Statistical analysis*

Comparisons of data between groups were performed using $\chi^2$ test. $P<0.01$ was considered statistically significant.

## RESULTS

### *CK20mRNA expression in control patients*

There was no CK20 mRNA-positive expression in 12 control patients.

### *CK20mRNA-positive expression in bone marrow, portal vein in colorectal carcinoma patients*

Bone marrow and portal vein were RT-PCR positive for CK20mRNA in 45 colorectal carcinoma patients (45/58, 77.6%), and in 43 colorectal carcinoma patients (43/58, 74.1%). There were no significant differences in positive expression between bone marrow and portal vein.

### *CK20mRNA-positive expression in peripheral blood within single and two blood samples*

CK20mRNA-positive expression in peripheral blood within a single blood sample was 44.8% (26/58). It rose significantly to 69.0% (40/58) within two blood samples ($P<0.01$). There were no significant differences between two blood samples of peripheral blood, bone marrow and portal vein ($P>0.01$).

### *Relation between CK20mRNA-positive expression in peripheral blood within two blood samples and disease development stage*

CK20mRNA-positive expression in peripheral blood within two blood samples was significantly higher in Duke's C stage than in Duke's A and B stages ($P<0.01$), (Table 1).

**Table 1** CK20mRNA expression in peripheral blood within two blood samples in different stages of colorectal carcinoma

| | CK20 (+) | CK20 (-) |
|---|---|---|
| Duke'A stage | 3 | 5 |
| Duke'B stage | 22 | 13 |
| Duke'C stage | 15 | 0 |

### *Relation between CK20mRNA-positive expression in peripheral blood within two blood samples and one year survival*

Patients with CK20mRNA-positive expression in peripheral blood within two blood samples had a significantly lower one year survival rate (18/40, 45.0%) than patients with negative expression (12/18, 66.7%) ($P<0.01$).

## DISCUSSION

The prognosis of patients with colorectal carcinoma remains poor. Approximately half the patients undergoing curative resection would die within 5 years because of recurrent diseases, mostly of liver metastases[1,2]. A highly sensitive method is needed to predict the metastastic potential and clinical outcome and to design pertinent treatments. Colorectal carcinoma markers might provide the prognostic information independent of and complementary to conventional parameters, including growth potential, oncogenes, tumour-suppressor genes and DNA flow cytometry, as well as other growth factors[9-12]. The common feature of these prognostic factors is that they correlate the nature of primary tumours with the subsequent outcome.

Detection of tumor cells in circulation by RT-PCR relates to the actual behaviours of the tumour. Cytokeratin proteins are essential constituents of the cytoskeleton of both normal and malignant epithelial cells. They are absent in haematopoietic and lymphatic cells. So, cytokeratin expression can serve as reliable markers for the epithelial origin of cells. Among cytokeratin proteins the expression of cytokeratin 20 is very strictly in gastric and intestinal epithelial cells. So, cytokeratin 20 is suitable for the detection of micrometastasis present in circulation in colorectal carcinoma patients[3-6]. Micrometastasis has been reported in the bone marrow of patients with colorectal carcinoma patients[7,11,12]. The evidence of micrometastasis means that an early relapse and the clinical outcome in these patients could be predicted. In the present study, no CK20mRNA-positive expression was found in all 6 non-cancer control patients. The CK20mRNA-positive expression in bone marrow and portal vein of colorectal carcinoma patients was high even in early disease stage, and also correlated with the depth of invasion. It is suggested that CK20mRNA expression can be a special and sensitive marker of micrometastasis of colorectal carcinoma. Micrometastasis can occur in early stage of cancer and correlate with the depth of invasion.

Many authors have focused on the micrometastasis in bone marrow. Cancer cells can be intensified in bone marrow because of its special structure. The detection of cancer cells in bone marrow was higher than that in any other tissues[11,12]. In our previous study, we reported the micrometastasis in portal vein was similar to that in bone marrow and it could be detected in peripheral blood[13]. Peripheral blood sample could be repeatedly aspired and is easy to be accepted by patients, but its expression is lower than bone marrow and portal vein samples. In the present study, we compared two blood samples with one blood sample. Depending on the mRNA assessed, the positivity for

circulating cancer cells increased 24.2%. It is suggested that circulating cancer cells are aggregated in clumps with varied sizes. Sufficient cancer cells for a positive test were not present in all blood samples from patients in which circulating cells were identified within some blood samples. It is also possible that despite precautions, variations in detection sensitivity occurred between samples[14-16]. Studies involving repeated blood sampling in patients would help solve this problem.

The capacity of cancer cells to proliferate in circulation and bone marrow to metastasize depends on the microenvironment. Whether micrometastasis has clinical significance is controversial. Some authors have reported that micrometastasis has no significant impact on prognosis, but other authors have found that micrometastasis in lymph nodes is a significant indicator of poorer prognosis after esophagectomy in patients with esophageal cancer. Our results revealed that CK20mRNA expression had a close relationship with disease stage and patients with CK20mRNA-positive expression in peripheral blood within two blood samples had a significantly lower one-year survival rate than patients with negative expression. It is suggested that patients with CK20mRNA-positive expression in peripheral blood are at a higher risk of recurrent cancer, and they therefore should receive postoperative chemotherapy.

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