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

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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.

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Key words: Colorectal Cancer; CK20mRNA; Micrometastasis

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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 chloroform was added. After vigorous agitation for 5 min, the inorganic phase was separated by centrifugation at 12 000 g for 20 min at 4 °C. RNA was then precipitated in the presence of 1 volume of isopropanol and centrifuged at 10 000 g for 15 min at 4 °C. RNA pellets were washed with 70% ice-cold ethanol and then dissolved in diethyl pyrocarbonate (DEPC) - treated H2O. 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 °C for 60 min, and the reaction was terminated by heating at 95 °C for 5 min. The cDNA templates were subjected to PCR amplification, using primers: 5'-CAGACACACGGTAATACCTGAG-3' (sense) and 5'-GATGCACTCTCCACTGTTAGACG-3' (antisense). The cycling protocol for CK20 (370 bp) consisted of: denaturation at 94 °C for 3 min, followed by 40 cycles, each for 45 s at 94 °C, for 45 s at 60 °C, for 60 s at 72 °C, and a final extension at 72 °C for 5 min. All reactions were performed in a final volume of 50 µL PCR reaction mixture containing 5 µL cDNA, 3 µL MgCl2, 5 µL 10× Buffer, 1 µL dNTP, 0.5 µL Taq polymerase, 1 µL primary. As a control of cDNA integrity, δ-β-microglobulin expression was analyzed as well. The primer sets were 5'-CACCTGTTGCGTACAAGTG-3' (forward) and 5'-TCATCCATTGCGCAATGAG-3' (reverse) for δ-β-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 χ2 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

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

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[7,11,12]. 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[8,4]. Micrometastasis has been reported in the bone marrow of patients with colorectal carcinoma patients[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 aspirated 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 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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