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• GASTRIC CANCER •

Detection of micrometastasis of gastric carcinoma in peripheral blood circulation

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Abstract

AIM: To detect the micrometastasis of gastric carcinoma in peripheral blood circulation using immunomagnetic beads sorting technique and RT-PCR technique, and to discuss its significance and the difference between the two methods.

METHODS: Density gradient centrifugation was used to isolate mononuclear cells from peripheral blood, immunomagnetic beads sorting technique and RT-PCR technique were used to detect the disseminated carcinoma cells. HE, immunocytochemical and immunofluorescence staining were also used to identify the characteristics of the cells separated with immunomagnetic beads sorting technique.

RESULTS: Cells expressing cytokeratin were separated and enriched from the peripheral blood specimens of patients suffering from gastric carcinoma or chronic gastritis. After HE staining, two kinds of cells with little cytoplasm were found. Majority of these cells had small and round nuclei, even chromatins and the thickness of nuclear membrane was normal. Immunohistochemical staining indicated that there were CD34 and CD45 expression on the cell membrane of this kind of cells and these cells also showed expressed human telomerase reverse transcriptase by immunofluorescence staining, but the expression of carcinoembryonic antigen was absent. So, these cells might hematopoiesis precursors. Another kind of cells had larger and abnormal nuclei with thicker nuclear membranes. Massed chromatins and poly-nucleoli were found in the nuclei. These cells expressed human telomerase reverse transcriptase and carcinoembryonic antigen, but CD34 and CD45 were not found on the cell membrane. So, these cells were considered as gastric carcinoma cells escaping from the original focuses and existing in the peripheral blood circulation. Carcinoma cells were found in 25 of 60(41.7%) specimens of peripheral blood from patients with gastric carcinoma, while there were no such cells separated from the blood specimens of chronic gastritis patients. The difference of positive rates of disseminated carcinoma cells between two groups was markedly significant ($P<0.005$). The expressions of CK20 mRNA in peripheral blood specimens were examined with RT-PCR. CK20 mRNA was detected from 32 of 60(53.3%) peripheral blood specimens in the group of gastric carcinoma patients, while none of the specimens from patients suffering from chronic gastritis had CK20 mRNA. Significant difference was also found between two groups ($P<0.005$). Statistic analyses also showed that there was a significant difference between the positive rates of two methods in detecting the disseminated carcinoma cells from the peripheral blood circulation of gastric carcinoma patients ($P<0.05$).

CONCLUSION: The results demonstrated that there were disseminated carcinoma cells in the peripheral blood circulation of some patients with gastric carcinoma. Disseminated carcinoma cells can be detected from the peripheral blood samples with immunomagnetic beads sorting technique and RT-PCR technique. The positive rate of RT-PCR technique is higher than that of immunomagnetic beads sorting technique in detecting micrometastasis.

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INTRODUCTION

Gastric carcinoma is one of the most common causes of cancer mortality in China and is responsible for approximately 160 000 deaths annually. The disease is often advanced at first presentation, and only 30-40% patients undergoing surgery have a curative resection. Metastasis and relapse are the common reasons leading to death of patients[1-6]. During the development of malignant neoplasm of non-hematopoietic system, a few of tumor cells escape from the original focus and disseminated into the lymph system, blood circulation, bone marrow, liver, kidney and other organs, which is called micrometastasis. It cannot be detected by any routine biochemical and histopathological assays or any graphical methods such as X-ray, CT, MRI, *etc*.[7-11]. At the same time, the patients have not any obvious symptoms. Minute focus would grow rapidly and develop to metastasis and relapse. So, to develop an efficient diagnostic method for the detection of micrometastasis is of great clinical significance[12-14]. In this study, immunomagnetic beads sorting technique and RT-PCR technique were used to detect the micrometastatic carcinoma cells in peripheral blood circulation of patients suffering from gastric carcinoma; HE staining was utilized to observe the cells' morphology changes; immunocytochemical staining and immunofluorescence staining were also used to identify the characteristics of the cells separated by immunomagnetic beads sorting technique. The significance of the micrometastatic carcinoma cell detection and the difference between the two methods were also discussed.

MATERIALS AND METHODS

Specimens

Eighty specimens of peripheral blood from sixty advanced gastric cancer patients and twenty chronic gastritis patients were collected from the Department of Endoscope of the Tongji Hospital of Tongji University. The final diagnoses of these patients were determined pathologically. The blood specimens were anti-coagulated with heparin (5 U/mL).

Separation of mononuclear cells

We carefully layered a maximum of diluted whole blood with Hank's solution over 10 mL Ficoll-Paque and centrifuged for 25 min at 2 400 r/min at room temperature, aspirated the mononuclear cells located at the plasm-Ficoll-Paque interface. We then washed the mononuclear cells once by 10 mL Hank's solution and centrifuged them at 600 r/min for 10 min.

Immunomagnetic beads separation

MACS carcinoma cell enrichment and detection kits were purchased from Miltenyi Biotech, the beads were coated with cytokeratin7 (CK7) and cytokeratin 8 (CK8). The mononuclear cells were diluted with 10 mL Dilution-Buffer and 1.3 mL MACS CellPerm Solution for 5 min. After incubated with 1.3 mL MACS CellFix solution for 30 min, the solution was centrifuged at 2 000 rpm for 10 min. Then, the sediment was mixed with 7.5 mL MACS CellStain solution and centrifuged, these two procedures were repeated again. The sediment acquired was incubated with 50 μL MACS CellStain solution, 50 μL FcR blocking regent and 50 μL MACS cytokeratin microbeads for 45 min, and after that, the mixture was centrifuged at 2 000 rpm for 5 min. The sediment was diluted with 500 μL dilution buffer and flew through the MACS column in magnetic field. The column was washed with 500 μL Dilution Buffer apart from magnetic field. The solution obtained included CK+ cells and was smeared on slides. The slides were fixed with acetone and stained with HE.

Immunocytochemical detection of CD34 and CD45 expressions in CK$^+$ cells

Immunohistochemical detection of CD34 and CD45 expressions was perpormed through a two-step procedure and the kits were purchased from Immunotech Co.

Immunofluorescence detection for hTERT and CEA expression in CK$^+$ cells

Immunofluorescence detection of human telomerase reverse transcriptase (hTERT) and carcinoembryonic antigen (CEA) expression was operated through two-step procedure and the kits were purchased from Sigma Co.

RT-PCR

Total RNA was extracted with Trizol (Gibco. Co) and resuspended in sterile RNase-free water for storage at -70 ℃. RT-PCR analysis was performed as follows. RNA was incubated at 60 ℃ for 10 min and chilled to 4 ℃ immediately before being reverse transcribed. Reverse transcription of 1 μg total RNA using antisense primers was performed in a volume of 20 μL for 40 min at 65 ℃, containing 200 U MMLV reverse transcriptase, 1×buffer RT, 1 MU/L RNasin, 2.0 mmol/L dNTP and 0.2 μmol/L oligo (dT) as a primer (All primers were synthesized by Life Technology, Shanghai). The primers of CK20 and β-actin were as follows: CK20 (485 bp) upstream primer: 5' AAG GCT CTG GGA GGT GCG TCT C3', downstream primer: 5' CAG TGT TGC CCA GAT GCT TGT G3'; β-actin (317 bp) upstream primer: 5' ATC ATG TTT GAG ACC A3', downstream primer: 5' CAT CTC TTG CTC GAA GTC CA3' [15]. The samples were heated to 94 ℃ for 5 min to terminate the reverse transcription reaction. The amplification reaction mixture consisted of 10×buffer 5 μL, 0.2 mmol/L dATP, dGTP, dCTP and dTTP respectively, 2.5 U TaqDNA polymerase, and 0.2 μmol/L each of sense and antisense primers including CK20 and β-actin respectively in a final volume of 50 μL. The reaction mixture was first heated at 94 ℃ for 5 min and amplification was carried out for 35 cycles at 94 ℃ for 1 min, at 58 ℃ for 1 min, at 72 ℃ for 1 min, followed by an incubation at 72 ℃ for 10 min. A volume of 10 μL RT-PCR products was added in 20 g/L agarose gel containing 0.5 mg/L EB and visualized with UV illumination after electrophoresis.

Statistic analysis

Experimental results were analyzed with Chi-square tests and $P<0.05$ was accepted as the level of significance.

RESULTS

Detection of micrometastasis by immunomagnetic sorting technique

After immunomagnetic separation, CK$^+$ cells were separated in all of the peripheral blood specimens from gastric carcinoma and chronic gastritis patients. There were two kinds of cells on the slides after HE staining. Because of the application of CellPerm solution, the cytoplasm of these cells decreased obviously. One kind of these cells had small and round nuclei, even chromatins and the thickness of nuclear membrane was normal. The other kind of cells had larger nuclei, which had abnormal forms and thicker membrane. Massed chromatins and more than one nuclei could also be found in this kind of cells.

Immunocytochemical and immunofluorescence staining

Immunohistochemical staining showed that there were CD34 (Figure 1) and CD45 (Figure 2) expressions on the cell membranes of the first kind of cells and these cells also had positive expressions of hTERT (Figure 3) after immunofluorescence staining, but the expression of CEA was negative. So this kind of cells might be hematopoiesis precursors (Figure 4)[17-20]. The other kind of cells had positive hTERT and CEA (Figure 5) expressions after immunohistochemical staining, but expression of CD34 and CD45 was absent. According to the morphology and the outcome of immunohistochemical and immunofluorescence staining, these cells were considered as the disseminated carcinoma cells escaped from the primary neoplasm focus (Figure 6)[17,21-23]. The first kind of cells was found on all slides, while the other kind of cells was only found on the slides from some gastric carcinoma patients (25/60), the positive rate was about 41.7%(Table 1). There was a significant difference between two groups ($P<0.005$).

Detection of micrometastasis by RT-PCR technique

CK20 mRNA was detected in 32 blood specimens from gastric carcinoma patients; the positive rate was 53.3% (Figure 7). While no CK20 mRNA was detected from the blood specimens of patients with chronic gastritis. There was a significant difference between these positive rates ($P<0.005$,Table 2).

Table 1 Comparison of tumor cells detected with immunomagnetic beads technique

Patients	+	-	Total	%
Gastric carcinoma	25	35	60	41.7
Chronic gastritis	0	20	20	0.0
Total	25	55	80	41.7

$\chi^2=10.26$, $P<0.005$.

Table 2 Comparison of tumor cells detected with RT-PCR

Patients	+	-	Total	%
Gastric carcinoma	32	28	60	53.3
Chronic gastritis	0	20	20	0.0
Total	32	48	80	53.3

$\chi^2=15.63$, $P<0.005$.

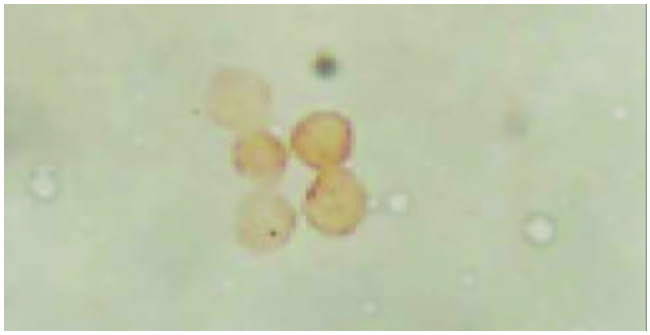

Figure 1 Immunohistochemical staining showed that there was CD34 expression on the membranes of the cells, these cells had small and round nuclei, even chromatins and the thickness of nuclear membrane was normal (amplification ×1 000).

Figure 2 Immunohistochemical staining showed that there was CD45 expression on the membranes of the cells, these cells had small and round nuclei, even chromatins and the thickness of nuclear membrane was normal (amplification ×1 000).

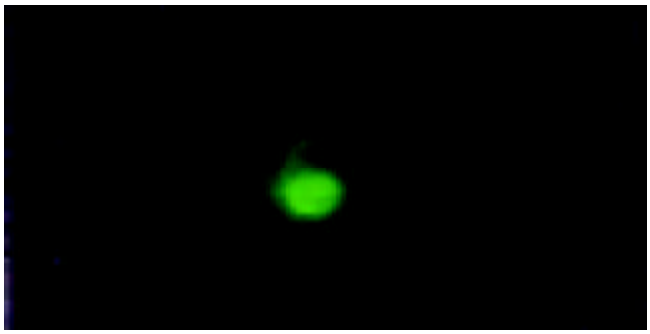

Figure 3 Immunofluorescence staining the cells, which had small and round nuclei, even chromatins and the thickness of nuclear membrane was normal (amplification ×200), had positive expressions of hTERT.

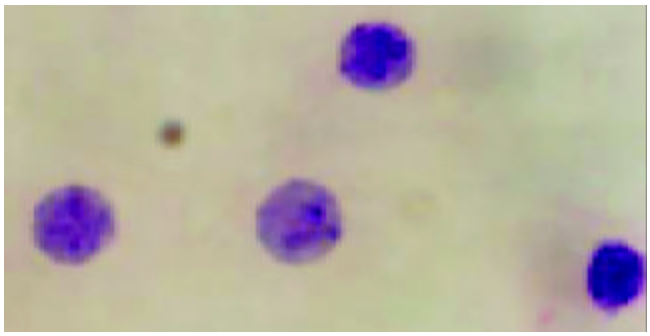

Figure 4 The cells, which had small and round nuclei, even chromatins and the thickness of nuclear membrane was normal (HE staining, amplification×1 000), might be hematopoiesis precursors.

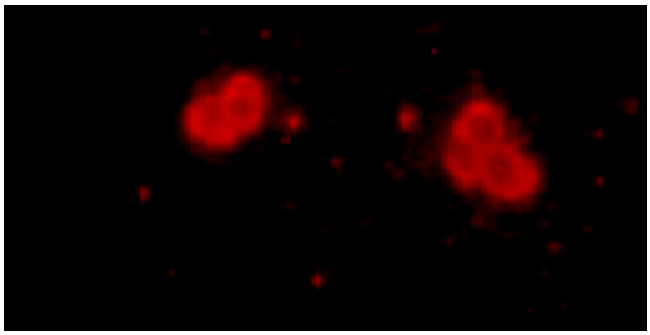

Figure 5 The cells, which had larger nuclei, abnormal forms and thicker membrane, and massed chromatins and more than one nuclei could also be found in this kind of cells, had positive expression of CEA (Immunofluorescence staining, amplification ×200).

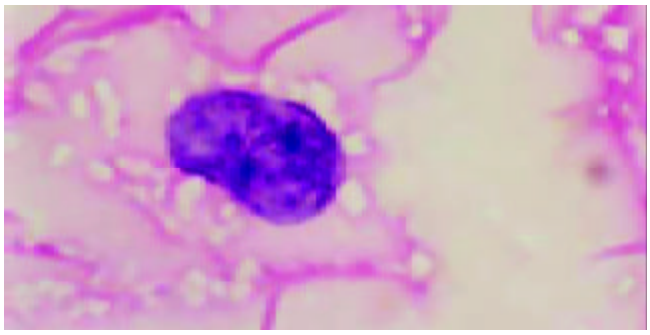

Figure 6 The cells, which had larger nuclei, abnormal forms and thicker membrane, and massed chromatins and more than one nuclei could also be found in this kind of cells, had positive expressions of CEA and hTERT, and negative expressions of CD34 and CD45, were considered as the disseminated carcinoma cells escaped from the primary neoplasm focus (HE staining, amplification ×1 000).

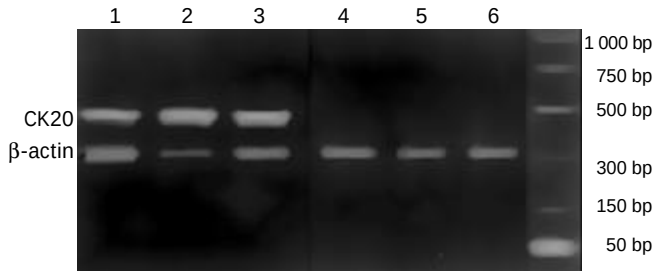

Figure 7 RT-PCR for CK20 and β-actin. M: Marker; Lanes1-4 blood from gastric cancer; Lanes5-6 blood from chronic gastritis.

Difference between positive rates of these two methods

Statistic analysis indicated that the difference between the positive rates of these two methods which were used to detect the disseminated carcinoma cells was significant ($P<0.05$, Table 3), that is to say the positive rate of RT-PCR was higher than that of immunomagnetic bead separation technique.

Table 3 Comparison of positive rates between two methods

RT-PCR	Immunomagnetic bead separation		Total
	+	-	
+	25	7	32
-	0	28	28
Total	25	35	60

$\chi^2=5.14$, $P<0.05$.

DISCUSSION

The first reporter of micrometastasis is Ashworth who claimed that he had found cells similar to tumor cells from the peripheral blood circulation of a carcinoma patient in 1869. After that more than twenty methods have been used to detect micrometastasis on about 5 000 patients. But the latest researches indicated that those so called tumor cells were components of hematopoietic tissues, especially prokaryotic cells[24,25].

In this study, immunomagnetic beads sorting technique and RT-PCR technique were used to detect the micrometastatic gastric carcinoma cells from peripheral blood circulation and the difference of these two methods was also discussed. There are many components in peripheral blood. It has been found that tumor cells had the similar density with mononuclear cells and they located in the same layer after density gradient centrifugation[26,27]. So we removed about 90% hemocytes from blood specimens with Ficoll' s solution and mononuclear cells mixed with carcinoma cells were enriched. Because gastric carcinoma is derived from epithelial tissue, so carcinoma cells could express CK, a particular component of epithelial tissue[28,29]. To separate micrometastatic gastric carcinoma cells expressing CK, immunomagnetic beads coated with anti-CK7 and anti-CK8 antibodies were used to incubate or those mononuclear cells that had been reacted with MACS CellPerm. Anti-CK7 and anti-CK8 antibodies combined with CK in the plasma of carcinoma cells by antigen-antibody reaction. So, the cells expressing CK combined with magnetic beads and detained in the magnetic field. To define the origin and characteristics of these cells, HE, immunocytochemical and immunofluorescence staining were performed. Two kinds of cells were separated after staining. Majority of these cells had small and round nucleusi, and their chromatins were even. These cells' nuclear membranes had normal thickness and expressed CD34 and CD45. They also expressed hTERT, but no CEA. Normally, CD34 and CD45 in hematopoiesis precursors, could also express hTERT[30-32]. So, these cells might be hemaptooietic precursors. Minority of these cells had larger and abnormal forms of nuclei that had thicker nuclear membrane. Massive chromatins could be observed in these cells. These cells expressed hTERT and CEA, but the expressions of CD34 and CD45 were absent on the membrane of cells. Usually, hTERT and CEA could be found in tumor cells and CEA was a marker of carcinoma derived from digestive tract[33-35], so these cells were gastric carcinoma cells escaped from the primary focuses and existed in peripheral blood circulation. This kind of cells was detected from 25 of 60 patients suffering from gastric carcinoma, while no cells were acquired from chronic gastritis patients.

CK is a component of cell skeleton and distributes in cells deriving from ectoderm. CK20, a member of this family has been found to have a stricter selectivity to epithelial tissue[28,36]. CK8 and CK9 mRNA could be detected in blood circulation with nested RT-PCR in healthy subjects, but CK20 mRNA could not be detected[37]. In this study, anti-CK7 and anti-CK8 but not anti-CK20 antibodies were coated on the beads, this should take responsibility for the separation of those hematopoietic precursors in the process of immunomagnetic beads sorting. If the immunomagnetic beads were coated with CK20, cells derived from epithelial tissue would be separated from hemocytes. These cells should be gastric carcinoma cells escaped from primary gastric carcinoma focus. Traditional methods for detecting disseminated tumor cells such as cell morphology, flowcytometer and cytogenetics were not so sensitive[38-40]. It has been found that immunomagnetic beads sorting technique has a high sensitivity to $1/10^5$[41], but it has a complicated procedure and a high cost and is not so practical in clinical application. In recent years, convenient RT-PCR was invented and it has a high efficiency in detecting the micrometastasis of peripheral blood circulation, bone marrow, lymph tract and peritoneal cavity. It could detect one tumor cell diluted with one-milliliter body fluid or one gram tissue and it has been presently regarded as the most effective method to detect the disseminated carcinoma cells[42].

In this study, RT-PCR was also used to detect micrometastatic gastric carcinoma cells. According to the researches of Soeth and Burchill[15,37], CK20 was selected as the target gene for amplification. Because of the RNase existing in blood circulation, mRNA free to live cells would be decomposed rapidly. This means that there were live cells expressing CK20 mRNA existing in blood specimens when CK20 mRNA was detected from the peripheral blood circulation of patients. On the other hand, hemocytes came from mesoderm and there were no epithelial cells in blood circulation. So, when CK20 was amplified from blood circulation, there must be tumor cells in the specimen. If the specimen was extracted from a gastric carcinoma patient, the tumor cells might be gastric carcinoma cells. In this study, CK20 mRNA was detected from 32 of 60 specimens extracted from gastric carcinoma patients, but not from patients with chronic gastritis. This also confirmed that there were carcinoma cells in the peripheral blood circulation of gastric carcinoma patients.

Statistical analysis also indicated that the positive rate of detection of disseminated carcinoma cells with RT-PCR technique was higher than that with immunomagnetic beads sorting technique. This might be related with the high sensitivity of RT-PCR, which cell morphology and immunocytochemistry could not possess[43,44]. On the other hand, it is cheaper in detecting disseminated carcinoma cells with RT-PCR than immunomagnetic beads sorting technique, so it is more worthy of clinical practice. Immunomagnetic beads sorting technique could directly separate micrometastatic carcinoma cells with high behaviors of metastasis. Those tumor cells separated with immunomagnetic beads sorting technique could be cultured and the characteristics of the cells could be determined, so it is more valuable for further research[45].

In conclusion, our results indicate that there are micrometastatic carcinoma cells in the peripheral blood circulation of patients suffering from gastric carcinoma. Immunomagnetic beads sorting technique and RT-PCR technique can detect the disseminated carcinoma cells in the peripheral blood circulation. RT-PCR technique has a higher positive rate and a lower cost than immunomagnetic beads sorting technique, so it is worthy of clinical practice. Immunomagnetic beads sorting technique can directly separate carcinoma cells, so this method is more valuable for research work.

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