Carcinoembryonic Antigen Messenger RNA Expression Using Nested Reverse Transcription-PCR in the Peripheral Blood During Follow-up Period of Patients Who Underwent Curative Surgery for Biliary-Pancreatic Cancer: Longitudinal Analyses

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ABSTRACT

Purpose: Outcome for patients with biliary-pancreatic cancer is still poor, despite curative operation. We investigated the clinical significance of molecular detection of circulating cancer cells in the blood as an early indicator of relapse during follow-up of patients who underwent a curative operation for biliary-pancreatic cancer.

Patients and Methods: We followed 53 patients who underwent a curative operation for biliary-pancreatic cancer between 1996 and 2001. We used reverse transcription-PCR in the peripheral blood to evaluate carcinoembryonic antigen (CEA) mRNA expression for molecular detection of circulating cancer cells. Follow-up examinations every 3 months after surgery included CEA mRNA expression in the blood, serum CEA, serum carbohydrate antigen 19-9 (CA19-9), and computed tomography or magnetic resonance imaging.

Results: Sixteen of 53 patients (30.2%) were diagnosed with a recurrence by imaging studies. The CEA mRNA detection rate in the peripheral blood of these 16 patients was 75% compared with 5.4% in the 37 patients without relapse (P < 0.001). Sensitivity of CEA mRNA, CEA, and CA19-9 serum levels was 75.0%, 50.0%, and 68.8%, respectively. Similarly, specificity was 94.6%, 64.9%, and 81.1%, respectively. CEA mRNA was expressed in the blood, even though tumor markers CEA and CA19-9 were within the normal range in patients with relapse. CEA mRNA expression in the blood, as well as the serum level of CA19-9, tended to be detected a few months earlier than detection by imaging modalities.

Conclusions: During the follow-up of patients who undergo a curative operation for biliary-pancreatic cancer, CEA mRNA expression in the peripheral blood might be a useful and early indicator of relapse.

INTRODUCTION

Although curative resection is considered to be the best treatment for biliary-pancreatic cancer (1, 2), the prognosis remains extremely poor because treatment for relapse cannot be efficiently performed without early detection of recurrence or metastases or both. Current methods for the detection of recurrence or metastasis in the postoperative period rely on serum tumor markers (3–7) and imaging modalities, which include radiography, computed tomography, magnetic resonance imaging, and sonography (8–10).

The reverse transcription-PCR (RT-PCR) assay has been used frequently in recent years for the detection of occult cancer cells or micrometastasis in lymph nodes, bone marrow, and the peritoneal cavity (11–15). There have been several studies on the molecular detection of circulating cancer cells in the blood by RT-PCR targeted to carcinoembryonic antigen [CEA (16–18)]. We have shown previously, by using RT-PCR in patients with biliary-pancreatic cancer and gastric cancer treated with surgery, that surgical procedures provoke hematogenous dissemination of cancer cells. Furthermore, we have shown that intraoperative molecular detection of circulating cancer cells correlated to a high risk of hematogenous metastasis (19–21). We suggested that early detection of micrometastases, which are undetectable with current imaging methods, may induce subsequent treatment and improve outcomes for patients with relapse after a curative operation.

Therefore, our longitudinal studies were conducted with RT-PCR to examine CEA expression in circulating cancer cells in the peripheral blood. We also used two different serum markers in the follow-up of patients who underwent a curative operation for biliary-pancreatic cancer.

MATERIALS AND METHODS

Patients. We evaluated 53 patients with biliary-pancreatic cancer who underwent a curative operation (33 males and 20 females; mean age, 67 ± 8.8 years; age range, 44–84 years) at Kagoshima University Hospital between 1996 and 2001. None of the patients received preoperative chemotherapy or radiotherapy. Histopathological diagnoses revealed 11 ampol-
CEA mRNA Expression in Blood of Pancreatic Cancer Patients

lary cancers, 22 bile duct cancers, and 20 pancreatic cancers. Tumors were classified as follows according to the tumor-node-metastasis classification (TNM) classification: stage 0, n = 5; stage I, n = 9; stage II, n = 15; stage III, n = 14; and stage IVa, n = 10. Curative operations were performed in all patients. This study also included 15 patients with benign disease [gallbladder stones (n = 5), intraductal papillary tumors (n = 4), pancreatitis (n = 2), serous cyst adenoma (n = 1), pseudocyst of the pancreas (n = 1), Mirizzi syndrome (n = 1), and common bile duct stone (n = 1)]. Fifteen healthy volunteers served as controls.

**Blood Samples.** Samples included peripheral venous blood (5 ml) that was drawn from each patient and healthy volunteer. The initial 10 ml of blood obtained by transcatheter needle venipuncture were discarded to prevent contamination with epithelial cells. The 5-ml sample was withdrawn into Vacutainers that contained EDTA. Blood samples were obtained every 3 months after surgery.

**Determination of CEA and Carbohydrate Antigen 19-9 (CA19-9) in Serum Samples.** Serum samples were used for assay of tumor markers every 3 months after surgery. Serum CEA and CA19-9 levels were determined with an immunoenzymatic assay test kit (Tosoh Co., Yamaguchi, Japan). A serum CEA level of >5 ng/ml and a serum CA19-9 level of >37 ng/ml were judged as positive.

**RNA Extraction.** Five ml of blood samples including EDTA were diluted with the addition of 5 ml of 0.05 M PBS (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Blood cells were isolated as peripheral blood mononuclear cells with monoply resolving medium (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) and subjected to density gradient centrifugation at 420 °C until RNA extraction. The layer of cells was collected and washed with 40 ml of sterile PBS. After centrifugation at 1350 × g at 4°C for 15 min, cell pellets were suspended in 1 ml of Isogen (Nippon Gene, Toyama, Japan) and stored at −80°C until RNA extraction. Total RNA was extracted according to the manufacturer’s protocol. Total RNA was dissolved in 10 μl of diethylpyrocarbonate-treated water. The concentration, purity, and amount of total RNA were determined by absorption measurements at optical densities of 260 and 280 nm with the UV-visible spectrophotometer Bio-Spec-1600 (Shimazu Co., Kyoto, Japan).

**Primers.** CEA and glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) primers were designed and synthesized as described previously (21). These primers were as follows: GAPDH forward-primer, 5′-TCCCCATCACTCTTCTTTAAAAA-3′; GAPDH reverse-primer, 5′-CATCAAGCTAGGGTTTTC-3′; CEA-A (outer sense) primer, 5′-TCTGGAATCTCTCCGTACCCCT-3′; CEA-B (inner sense) primer, 5′-GGCCGTGTGTCAGCTGG-3′; CEA-C (antisense) primer, 5′-GTAGCTTGTTGCAAATGCTTTAAAGGAAGC-3′; and CEA-D (antisense) primer, 5′-GGCCGTGTGTCAGCTGG-3′.

**cDNA Synthesis.** Before the synthesis of cDNA, 0.5 unit of DNase I (Stratagene, La Jolla, CA) and 1 μl of 10× PCR buffer were added to 5 μg of total RNA in a total volume of 9.5 μl. The reaction mixture was incubated at 37°C for 5 min. Next, 1.5 μl of 50 mM EDTA were added. An 11-μl aliquot of reaction mixture was incubated at 70°C for 20 min and rapidly quenched on ice. After 50 ng of random hexamer were added, 5 μg of total RNA in a volume of 12 μl were incubated at 72°C for 10 min. Two μl of 25 mM MgCl2, 1 μl of 10× PCR buffer, 1 μl of 0.1 mM DTT, 1 μl of 10 mM deoxynucleotide triphosphate mixture, and 2 μl of diethylpyrocarbonate-treated water were added to the reaction solution. Nineteen μl of reaction mixture were incubated at 25°C for 10 min and rapidly quenched on ice. One hundred units of SuperScript II reverse transcriptase were added to the reaction mixture, which was then incubated at 25°C for 10 min, at 42°C for 90 min, and at 72°C for 15 min. It was then rapidly quenched on ice, and 1 unit of Escherichia coli RNase H (Invitrogen, Carlsbad, CA) was added to the reaction mixture. A 20-μl aliquot of reaction mixture was incubated at 37°C for 40 min. Next, 5 μl of Tris-EDTA were added to cDNA, which was then stored at −20°C until use.

To monitor the synthesis of cDNA, GAPDH RT-PCR was performed by using the GAPDH primer. cDNA (0.5 μl), 5 μl of 10× PCR buffer, 200 μM deoxynucleotide triphosphate mixture, 0.2 μM forward-primer, 0.2 μM reverse-primer, and 1.25 units of Taq DNA polymerase (Takara Shuzo Co., Ltd., Otsu, Japan) were added to a 40-μl sample of the PCR mixture. First, the GAPDH sample was denatured at 95°C for 4 min; subjected to 40 cycles of denaturation at 95°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 45 s; followed by a final extension at 72°C for 9 min using the GeneAmp PCR system 9700 (Applied Biosystems, Foster, CA). The GAPDH PCR product was resolved by electrophoresis on 2% agarose in Tris-acetate-EDTA buffer and visualized with ethidium bromide staining of the gel. The RT-PCR product was detected as a 390-bp fragment.

**CEA Nested RT-PCR.** The nested RT-PCR was performed as described previously (21), with modifications. For the first round of PCR, 0.5 μl of cDNA, 2 μl of 10× PCR buffer, 200 μM deoxynucleotide triphosphate mixture, 0.2 μM CEA primer A and B, and 0.5 unit of Taq DNA polymerase (Takara Shuzo Co., Ltd.) were added to a 20-μl aliquot of reaction mixture. CEA amplification was performed by heating at 95°C for 4 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 64°C for 1 min, and extension at 72°C for 30 s; and a final extension step at 72°C for 6 min by using the GeneAmp PCR system 9700 (Applied Biosystems). In nested RT-PCR, 1 μl of the first PCR product, 4 μl of 10× PCR buffer, 200 μM deoxynucleotide triphosphate mixture, 0.2 μM CEA primer B and C, and 1 unit of Taq DNA polymerase (Takara Shuzo Co., Ltd.) were added to a 40-μl aliquot of reaction mixture. The samples were heated to 95°C for 4 min to activate the Taq DNA polymerase; followed by 32 cycles of denaturation at 95°C for 30 s, annealing at 64°C for 1 min, and extension at 72°C for 30 s; and a final extension step at 72°C for 6 min. The CEA RT-PCR product was identified by electrophoresis on 2% agarose in Tris-acetate-EDTA buffer and visualized with ethidium bromide staining of the gel. The PCR product was detected as a 131-bp fragment. Each RT-PCR run included a positive control synthesized from the MCF-7 cell line, a negative control from a healthy volunteer, and water.

**Sensitivity of the Nested RT-PCR Assay for CEA mRNA.** To evaluate the sensitivity of the CEA-specific RT-PCR assay, we used two cell lines, MCF-7 (breast cancer cell line) and MKN-45 (gastric cancer cell line). They were sequentially diluted 10-fold (106, 105, 104, 103, 102, and 101) and mixed with 107 peripheral blood mononuclear cells obtained.
from healthy volunteers. Total RNA was extracted using the method described above and then subjected to the nested RT-PCR assay.

**Imaging Diagnosis.** All patients were followed-up with computed tomography, magnetic resonance imaging, and/or sonography every 3 months after surgery. If recurrent disease was detected by any imaging modality, it was confirmed by another type of imaging and/or needle biopsy. Follow-up data were obtained for all patients with a median follow-up period of 49 months (range, 24–89 months).

**Statistical analyses.** Continuous variables were expressed as mean ± SD. The correlation between the presence of CEA mRNA in the blood and various clinical parameters was evaluated by means of the χ² test, applying the Yates correction when needed. Student’s t test for paired data and one-way ANOVA were used to compare CEA mRNA expression levels. Differences with Ps of <0.05 were considered to be significant.

**RESULTS**

CEA mRNA was detectable at a concentration as low as 1 tumor cell/10⁷ normal lymphocytes in MKN-45 and MCF-7 cancer cell lines (Fig. 1). CEA mRNA was not detected in peripheral blood obtained from 15 patients with benign disease or the 15 healthy volunteers. We then examined the RT-PCR of CEA mRNA in the peripheral blood during the follow-up of 53 patients who underwent a curative operation for biliary-pancreatic cancer.

CEA mRNA in the peripheral blood of patients with different stages of biliary-pancreatic cancer was analyzed by RT-PCR and compared with the serum levels of tumor markers and imaging diagnoses during the follow-up period after curative surgery (Table 1). Metastases were detected by imaging modalities in 16 of the 53 patients (30.2%) followed. The positive rate of CEA mRNA (75%) in the peripheral blood of those 16 patients with relapse was higher than the positive rate in the 37 patients without recurrence (5.4%) (Table 1). According to the site of metastasis, positive RT-PCR results were seen in more than six patients (50%) with liver metastasis and six of seven patients (86%) with local recurrence (Table 1).

Changes in the levels of serum CEA were followed in the 37 patients without tumor recurrence. Twenty-seven of those 37 patients showed nearly flat curves that were less than the cutoff level. The remaining 10 patients, however, had curves that fluctuated above and below the cutoff level of serum CEA. Two of those 10 patients were also positive for CEA mRNA in the blood (Fig. 2A). However, on examination of the changes of serum CEA levels in the 16 patients with relapse, 11 patients showed continuous elevations above the cutoff level. Seven of those patients were positive for CEA mRNA. Five patients showed flat curves below the cutoff level, and these five patients were positive for CEA mRNA (Fig. 2B).

The changes in serum CA19-9 levels in the 37 patients without relapse revealed 34 patients with nearly flat curves below the cutoff level. The remaining three patients, who included one patient who was positive for CEA mRNA in the blood, showed fluctuating curves above the cutoff level (Fig. 3A). However, the changes of serum CA19-9 levels in the 16 patients with relapse showed 10 patients with continuous elevations above the cutoff level. Six of those patients were positive for CEA mRNA. Six patients showed flat curves below the cutoff level, including three patients who were positive for CEA mRNA (Fig. 3B).

The initial elevations above the cutoff level of CEA and CA19-9 proteins were detected in 8 of these 16 patients (50%) and 11 of these 16 patients (68.8%), respectively. On the other hand, imaging modalities did not detect any site of metastasis in 37 of the 53 patients (69.8%), although the first elevations above the cutoff level of CEA and CA19-9 were determined in 13 of these 37 patients (35.1%) and 7 of these 37 patients (18.9%). CEA mRNA in the blood was detected in only two of these patients (5.4%). Sensitivity by using CEA mRNA, CEA, and CA19-9 serum levels was 75.0%, 50.0%, and 68.8%, respectively. Similarly, specificity was 94.6%, 64.9%, and 81.1%, respectively (Table 2). Both sensitivity and specificity were highest for CEA mRNA. No significant association was found

<table>
<thead>
<tr>
<th>No. of cells</th>
<th>CEA</th>
<th>GAPDH</th>
</tr>
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<tbody>
<tr>
<td>10⁶</td>
<td></td>
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<td>10⁵</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>390bp</td>
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</tr>
</tbody>
</table>

**Fig. 1** The top panel shows reverse transcription-PCR (RT-PCR) analysis of carcinoembryonic antigen (CEA) mRNA expression in 10⁶, 10⁵, 10⁴, 10³, 10², 10¹, and 10⁰ MCF-7 cancer cells mixed with 10⁷ peripheral blood mononuclear cells obtained from healthy volunteers. CEA mRNA of 1 cancer cell/10⁷ peripheral blood mononuclear cells could be detected. M, molecular weight marker.
between CEA mRNA expression and clinicopathological characteristics (Table 3).

According to the TNM classification, sensitivity of CEA mRNA in stage I and II and specificity of CEA mRNA in stage 0, 1, and III were 100%, so sensitivity and specificity of CEA mRNA tended to be higher in lower stages. In the five stage 0 patients, CEA serum marker had only 20% specificity (Table 4). In the nine stage I patients, CA19-9 serum marker had 0% specificity.
sensitivity, but RT-PCR detected two recurrences that were not detected by the CA19-9 serum marker (Tables 1 and 4).

The time period from surgery to first detection of CEA mRNA by RT-PCR was 270 ± 188 days in 12 of 16 cases with relapse. The time period from surgery to first elevation of serum CEA was 303 ± 169 days in 8 of 16 cases with relapse. The time period from surgery to the first elevation of serum CA19-9 was 237 ± 172 days in 11 of 16 cases with relapse. However, the time period from surgery to first detection of relapse by imaging modalities in 16 patients was the longest (375 ± 268 days; Table 1).

**DISCUSSION**

The prognosis for patients who undergo even a curative operation for biliary-pancreatic cancer is still poor (1, 2). Supposedly, recurrence of this type of cancer is progressive, and its status is advanced by the time of detection. If relapse can be detected earlier, the appropriate anticancer treatment might improve the prognoses of these patients.

We reported recently that surgical procedures provoke hematogenous dissemination of cancer cells. We have also shown that perioperative molecular detection of these circulating cancer cells by RT-PCR for CEA mRNA correlates to a higher risk of hematogenous metastasis (19–21). Therefore, recognition of CEA mRNA expression in the blood with RT-PCR is supposedly the appropriate method for detecting circulating cancer cells because CEA mRNA is present in all epithelial cells including carcinoma cells (16, 17). These results strongly suggest that CEA mRNA expression in the blood can predict recurrences earlier than other available options.

Guadagni et al. (22) suggested that preoperative detection of blood-borne cells for CEA mRNA with RT-PCR is potentially different from serum levels of tumor markers in the management of colorectal cancer patients. In the present study, we performed RT-PCR to identify circulating cancer cells by detecting CEA mRNA expression. This method preceded the diagnosis of recurrence by other examinations in the postoperative period in patients who underwent a curative operation for biliary-pancreatic cancer. We then investigated longitudinal analyses of patients using CEA-specific RT-PCR. Furthermore, we performed a comprehensive analysis comparing RT-PCR values for CEA with those of serum CEA and CA19-9 levels to monitor biliary-pancreatic cancer patients for relapse after surgery.

**Table 2** Relationship between recurrence and CEA mRNA expression in the blood and first elevations of CEA or CA19-9 serum levels in 53 follow-up patients who underwent curative operation for biliary-pancreatic cancer

<table>
<thead>
<tr>
<th></th>
<th>CEA mRNA</th>
<th>CEA</th>
<th>CA19-9</th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Over</td>
<td>Less</td>
</tr>
<tr>
<td>Recurrence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>12</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>No</td>
<td>2</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td></td>
<td>75.0</td>
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</tr>
<tr>
<td>Specificity (%)</td>
<td></td>
<td>94.6</td>
<td>64.9</td>
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*CEA, carcinoembryonic antigen.*

![Fig. 2](image-url) The change in serum carcinoembryonic antigen levels and reverse transcription-PCR results. Two of 37 patients (5.4%) who did not have relapse (A) and 12 of 16 patients (75%) who had relapse by imaging modalities (B) showed reverse transcription-PCR detection of carcinoembryonic antigen mRNA. Closed circles indicate the time period of the detection of carcinoembryonic antigen mRNA expression. M, month.
In this study, 12 of 16 patients (75%) with disease recurrence had molecular detection of circulating cancer cells during the follow-up period. The incidence of relapse was significantly higher in patients with molecular detection compared with those without molecular detection. Interestingly, the time period from surgery to molecular detection of circulating cancer cells tended to be shorter than the time period from surgery to imaging diagnosis. These results suggest that cancer cells from a recurrent tumor recirculate into the blood before detection by imaging modalities.

Serum CEA has been well documented as a tumor marker for cancers of the gastrointestinal tract. Serum CA19-9 has a high sensitivity and specificity for pancreatic cancer (23, 24). In the present study, both sensitivity and specificity of CEA mRNA were higher than those of serum CEA and CA19-9. In two patients with recurrent disease (3.8%), serum levels of tumor markers were not persistently elevated during the follow-up period, despite expression of CEA mRNA. According to the report of Guadagni et al. (22) on longitudinal analysis of CEA mRNA expression and three tumor markers, CEA mRNA was expressed in two recurrent cases without tumor marker elevation. Those findings suggested the independent use of this technique for diagnosis (22). However, 4 of 39 patients (10.3%) who were without molecular detection eventually suffered from tumor recurrence. Among them, three cases had elevation of serum CA19-9, and one case had elevation of serum CEA. Therefore, the determination of CEA mRNA in the blood has high sensitivity and specificity, but it may be more useful for the detection of tumor relapse when used in combination with tumor markers.

We reported previously that surgical maneuvers provoke circulating cancer cells to detach from primary tumors (19–21). These circulating cancer cells disappear early after surgical resection but reappear during the follow-up period. Our hypothesis is that recirculating cancer cells are a part of the second phase that is derived from the recurrent tumor and appear during the follow-up period. The first phase includes cells that are derived from the primary tumor to form the metastatic tumor. Released cancer cells of the second phase tend to circulate at the same time or just after detection of high levels of CEA or CA19-9. These cells then spread another metastatic foci when a recurrent tumor has been clinically detected by imaging modalities. Interestingly, the positive rate (50%) of RT-PCR in six patients with liver metastasis was relatively lower compared with the rate (86%) in seven patients with local recurrence in this series. However, CEA mRNA expression in the blood during operation was related to blood-borne metastasis (21). According to the relation between tumor microvessels and liver metastasis, microvessels with lumens of >100 μm in diameter were not detected in liver metastatic lesions (25). Moreover,
microvessel densities of primary tumors in colorectal cancer were significantly greater than those of metastases (26). The smaller number of microvessels in metastatic lesions may cause difficulty in the release of cancer cells from metastatic foci into the blood. These results suggest that the number of circulating cancer cells from hematogenous metastatic tumor is lower than the number of circulating cancer cells from local recurrent tumor. Therefore, the detection of circulating cancer cells by RT-PCR may be appropriate for examination during the time of invasiveness surrounding tissue and hematogenous metastasis phase from the local recurrence.

Thus, RT-PCR for CEA mRNA may be useful not only to detect a metastatic foci earlier to direct treatment but also as a monitoring tool for experimental treatments to suppress the release of cancer cells from metastatic foci or local relapses. After detection of CEA mRNA in the blood with RT-PCR, recurrence tends to be diagnosed with imaging modalities in 3 months. This time period might be an important interval for appropriate antimetastatic treatment against recurrence.

In conclusion, the molecular detection of circulating cancer cells in the peripheral blood by RT-PCR for CEA mRNA during the follow-up period of patients who underwent a curative operation for biliary-pancreatic cancer might be potentially useful for early prediction of recurrence. A randomized prospective study will be required to clearly identify the relationship between circulating cancer cells and recurrence in the management of biliary-pancreatic cancer. These results might provide us with an appropriate treatment against relapse as “a strategy for micrometastasis” after curative surgery.

REFERENCES


Table 4 Sensitivity and specificity for CEA* mRNA versus serum tumor markers in the blood according to the TNM classification

<table>
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<tr>
<th>Stage</th>
<th>No. of patients</th>
<th>Relapse by image diagnosis</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
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<tr>
<td></td>
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<td>CEA mRNA</td>
<td>Serum CEA</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>0 (0.0%)</td>
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</tr>
<tr>
<td>I</td>
<td>9</td>
<td>2 (22.2%)</td>
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</tr>
<tr>
<td>II</td>
<td>15</td>
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</tr>
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<td>53</td>
<td>16 (30.2%)</td>
<td>94.6</td>
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* CEA, carcinoembryonic antigen; TNM, tumor-node-metastasis.
detection of circulating tumor cells by reverse transcription-polymerase
chain reaction in patients with biliary-pancreatic cancer is associated
in colorectal cancer patients by nested reverse transcription-polymerase
chain reaction for carcinoembryonic antigen messenger RNA: longitudi-
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