Quantitative Analysis of Carcinoembryonic Antigen Messenger RNA in Peripheral Venous Blood and Portal Blood of Patients with Pancreatic Ductal Adenocarcinoma

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ABSTRACT

One major therapeutic failure of pancreatic adenocarcinoma treatment is metastasis to the liver. To screen patients with high risk for such hematogenous dissemination, we previously developed a very sensitive system to detect carcinoembryonic antigen (CEA) in blood. For a more practical application, we improved this system by making it quantitative and capable of analyzing both preoperative peripheral blood and intraoperative portal blood for the presence of CEA mRNA. CEA mRNA was not detected in the peripheral venous blood of any of the three patients examined, but it was identified in the portal blood without fail. In addition, the quantities of CEA mRNA identified in the portal blood before and after pancreatectomy were different. This study suggests that analysis of the portal blood seems to be important for the precise evaluation of hematogenous dissemination and of the pathophysiology of pancreatic ductal adenocarcinoma.

INTRODUCTION

Despite recent advances in early diagnosis and surgery, the long-term prognosis of adenocarcinoma of the pancreas remains very poor. Two major therapeutic failures are hematogenous metastasis, mainly to the liver, and locoregional recurrence (1, 2). One possible way to improve prognosis for pancreatic adenocarcinoma patients is to screen patients with a high risk for hematogenous recurrence. We, therefore, previously developed a very sensitive system to identify the presence of pancreatic adenocarcinoma cells in the form of CEA3 in blood and used it to analyze the peripheral blood of eight patients with pancreatic adenocarcinoma (3). But despite half of the eight patients being in advanced stages, CEA mRNA was detected in only two patients. This apparent discrepancy from the clinical features led us to the conclusion that additional analysis of the portal blood was indispensable. Furthermore, because patients who are provided with a V-port for chemotherapy via the portal vein can be repetitively analyzed, the analysis could be made quantitative for the evaluation of therapeutic effect. Although there have been previous studies, including ours, on detecting cancer micrometastasis-targeting CEA mRNA, dealing with bone marrow aspirates (4), lymph nodes (5), and colorectal carcinoma (6), none was quantitative. Here, we describe the successful adaptation of our previous method for quantitative purposes and the results of its application to the measurement of CEA mRNA in both peripheral and portal blood, before and after pancreatectomy.

PATIENTS AND METHODS

Patients. Data for the three patients with resectable ductal adenocarcinoma of the pancreas are listed in Table 1. For controls, we examined the peripheral blood of three patients with chronic pancreatitis, one patient with acute pancreatitis, and four healthy volunteers.

Ethical Considerations. This study was performed after obtaining patients’ informed consent, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. For ethical reasons, analysis of the portal blood was performed only on patients who underwent pancreatectomy for ductal adenocarcinoma.

Sampling of the Portal Blood. Immediately after laparotomy, a catheter was inserted from the peripheral branch of the superior mesenteric vein into the portal vein. “Prepancreatectomy” blood was sampled right after the insertion of this catheter, and “postpancreatectomy” blood was collected directly after the pancreatectomy from the same catheter.

RNA Extraction and cDNA Synthesis. Total RNA was extracted from heparinized peripheral venous blood according to the acid guanidinium-phenol-chloroform method (7) with a slight modification (3, 8–11). Extracted RNA was reverse-transcribed, as described previously (3, 8–11), according to the manufacturer’s instructions.

Preparation of Positive Control Template. mRNA was extracted from a human colon adenocarcinoma cell line, Colo 205 (purchased from American Type Cell Culture; Ref. 12), as...
Quantitative PCR of CEA mRNA in Blood

Cob 205 cDNA as the template. The details of the procedure are described previously (3, 8–11). After reverse transcription, the Wizard mini prep kit (Promega, Madison, WI), after which the transformed clone (pCEAW) was purified with the aid of a SalI cutter. pCEAN was then extracted in the same manner as pCEAW. The obtained plasmid was then self-ligated and transformed again, and the desired plasmid was selected by 28 bp (Fig. 1), and this new insert was cloned into SalI-digested pCEAW2. The obtained plasmid was also sequenced for confirmation.

Table 1 Patient data and PCR results

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Operation performed</th>
<th>Tumor size (cm)</th>
<th>Chronic pancreatitis</th>
<th>Preoperative CEA protein level (ng/ml)</th>
<th>Postoperative CEA protein level (ng/ml)</th>
<th>Copies of CEA mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>56</td>
<td>F</td>
<td>Subtotal distal pancreatectomy</td>
<td>4.0 × 2.8 × 3.5</td>
<td>Yes</td>
<td>10.3</td>
<td>&lt;1.0</td>
<td>Not found 1,090</td>
</tr>
<tr>
<td>2</td>
<td>71</td>
<td>F</td>
<td>Pancreaticoduodenectomy</td>
<td>3.0 × 2.5 × 2.1</td>
<td>No</td>
<td>3.8</td>
<td>2.5</td>
<td>Not found 545</td>
</tr>
<tr>
<td>3</td>
<td>79</td>
<td>M</td>
<td>Pancreaticoduodenectomy</td>
<td>4.0 × 3.0 × 2.5</td>
<td>No</td>
<td>2.3</td>
<td>2.3</td>
<td>Not found 545</td>
</tr>
</tbody>
</table>

a Presence of chronic pancreatitis in noncancerous portion.
b Portal venous blood.
c Portal venous blood before pancreatectomy.
d Portal venous blood after pancreatectomy.

Fig. 1 Schematic illustration of the original sequence (A) and the competitor (B). Arrows 1, 2, and 3, primer sites; ····, SalI and SpeI sites; ⌧, synthetic insert.

Quantification for Control Experiment. Basic analysis to investigate the amplification profile for certain cycles and the manner of coamplification was performed with a specified quantity of either the original sequence or the competitor, solubilized in 2 µl of TE (pH 8.0). Using this TE-solubilized cDNA as the template, three-step PCR was performed as described previously (3), with some modification. The first-step PCR was performed by using primers 3 and 3, which amplify a 105-bp fragment of the inserted CEA sequence at only one site: 105 bp from exon B2 (the two 3' bases C and A) to exon A3 (3).

PCR Primers. The primers for CEA gene (13, 14) detection were sense primer 1 (5'-AGGGCTGGGCAGCTCCGCA-3') and sense primer 2 (5'-TTAAGGCCATTTC-CCC-3'), both within exon B2, and common antisense primer 3 (5'-AGGGCATTGGCCAGCTCCGCA-3'), which extends from exon B2 (the two 3' bases C and A) to exon A3 (3).

Purification of Original Sequence. The first-step PCR was performed as described previously (3), by using 0.1 µg of Colo 205 cDNA as the template. The details of the procedure are described under "Quantification for Control Experiment." The 265-bp first-step PCR product was extracted from SeaKem GTG agarose gel (FMC BioProducts, Rockland, ME), cloned into the EcoRV site of pT7 blue plasmid (Takara Shuzo, Shiga, Japan), and transformed into Escherichia coli. The plasmid from the transformed clone (pCEAW) was purified with the aid of a Wizard mini prep kit (Promega, Madison, WI), after which inclusion of the expected sequence was confirmed by sequencing. Next, pCEAW was digested with BamHI and EcoRI to exclude the SalI site in the vector for the subsequent SalI digestion of the insert and was blunted. The blunt pCEAW was then self-ligated and transformed again, and the desired cloned pCEAW2 was obtained in the same manner with pCEAW. The final product was also sequenced for confirmation.

Preparation of Competitor. Two 5'-phosphorylated complimentary oligonucleotides OLI-1 (5'-TTACCTCACAGTGACACCGACTCTAAAGCT-3') and OLI-2 (5'-TGGATCGTCTGTAGTCTGAGTGGTTAAAGCT-3') were designed so that annealing of these two oligonucleotides would result in the formation of a small DNA fragment with the protruding SalI site (4-mer) on both 3' ends and a SpeI site within the sequence. This DNA fragment was purified and cloned into SalI-digested pCEAW2. The obtained plasmid pHAN was then extracted in the same manner as pCEAW. SalI cuts the inserted CEA sequence at only one site: 105 bp upstream, toward the 5' end of primer 3. Because this site is located between the pair of second primers, this ligation elongated the original insert by 28 bp (Fig. 1), and this new insert was assumed to be amplified in two steps by using the same pair of primers as for the amplification of the original CEA sequence. The insertion check was successfully performed both by PCR of the plasmid with the first-step pair of primers and subsequent digestion of the PCR product with SpeI and by sequencing.

Quantification for Control Experiment. Basic analysis to investigate the amplification profile for certain cycles and the manner of coamplification was performed with a specified quantity of either the original sequence or the competitor, solubilized in 2 µl of TE (pH 8.0). Using this TE-solubilized cDNA as the template, three-step PCR was performed as described previously (3), with some modification. The first-step PCR was performed by using primers 1 and 3, which amplify a 105-bp fragment. The 50 µl of individual PCR mixture were composed of TE-solubilized template cDNA and 5 µl of 10× PCR buffer (Perkin-Elmer Corp., Norwalk, CT), 50 pm each primer in 1 µl of TE, 1 µg of BSA, 0.2 mm dNTP, and diethyl pyrocarbonate-treated water. The reaction mixture was overlaid with mineral oil, heat-denatured at 93°C for 3 min, and then cooled to 80°C before the addition of 2.5 units of Taq polymerase (AmpliTaq, Perkin-Elmer Corp.). Each amplification cycle consisted of 3-s denaturing at 94°C, followed by a 20-s annealing (62°C) using a DNA Thermal Cycler (Perkin-Elmer Corp.). Because the PCR product was small, the chain extension reaction was performed during the heating step from the annealing temperature to the denaturing temperature. This shuttle-type

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**Note:** The image contains a table and a figure (Fig. 1) which are not part of the natural text representation. The table lists patient data and PCR results, while the figure illustrates the original sequence and the competitor.
The annealing step for the third PCR diluted to 60 μl with TB.

transcribed cDNA sample corresponding to 1 ml of blood was used as the template. X10, X15, X20, X25, X30, and X35, numbers of PCR cycles performed. The original sequence produced a 260-bp band, and the competitor produced a 288-bp band, as expected. A single heterodimeric band between the original and the competitor sequences was also visible.

PCR was effective in further excluding nonspecific amplification in addition to the nested PCR and in shortening the duration without affecting the PCR yield in our preliminary experiment. Different from our previous protocol, a 10-s extension at 72°C was performed only in the 35th cycle. After 35 cycles, 1/50 of the first PCR product was used as the template for the second PCR. The second set of primers consisted of primers 2 and 3, which amplify 190-bp fragments. These primers were added individually at 50 pmol. Other components of the second PCR mixture were the same as those of the first PCR mixture, except for the different quantity of diethyl pyrocarbonate-treated water. The annealing step for the third PCR was performed at 60°C for 20 s. The rest of the amplification program for the second PCR was the same as the first.

Ten μl of the PCR product were subjected to electrophoresis on a 3.5% NuSieve:SeaKem GTG (3:1) agarose gels (FMC BioProducts, Rockland, ME). After staining with ethidium bromide, densitometric analysis was performed, and the quantity of the competitor at a density equivalent to that of the original sequence was determined.

Quantitative Analysis of Patients' Samples. A reverse-transcribed cDNA sample corresponding to 1 ml of blood was diluted to 60 μl with TE. PCR was performed with 10 μl of this diluted per reaction, with or without various serially diluted, known quantities of the competitor. At first, the experiment was performed for a rough estimate of the amount of CEA mRNA present in the sample, and the respective points of equivalence could be determined even with this first experiment. Next, to confirm this result, the two-step PCR was performed again with more finely divided serial dilutions of the competitor within the limited, focused range estimated from the first experiment.

Electrophoresis was performed in the same manner as for the control experiment. After the point of equivalence was decided, the quantity of the competitor at that point was divided by 0.44 to correct for the 44% efficiency of the reverse transcription, which used the Moloney murine leukemia virus reverse transcriptase according to the manufacturer's instructions (15, 16). The resulting value was then multiplied by 2 to correct for the differential amplification during the first-step PCR with the single-stranded original cDNA (16). Because the original sequence is smaller than the competitor, the obtained value was multiplied by a correction factor of 1.10 (293 bp versus 265 bp and 218 bp versus 190 bp). Finally, this value was multiplied by the figure representing the dilution of the cDNA obtained from 1 ml of the blood sample.

To test the reliability of RNA extraction, a 319-bp β-actin cDNA fragment was amplified by using the 50-pmol primer pair reported by Fuqua et al. (17), in a previously described manner (3, 8–11).

Immunocytochemical Detection of CEA Protein-positive Cells in Patients' Blood. This analysis was performed by the method we described previously (9), with several important modifications. After addition of one-fourth (v/v) of 6% dextran sulfate in saline, 2 ml of the heparinized patient's blood were left to rest for 30 min for the sedimentation of red cells. The white cell suspension was then collected and centrifuged, and the collected cells were smeared onto a glass slide and air-dried for more than 2 h. The cellular fixation was performed by cold acetone (4°C) for 3 min. Blocking of endogenous alkaline phosphatase was performed with the aid of levamizole (DAKO A/S, Glostrup, Denmark). For the blockade of nonspecific binding, 10% normal rabbit serum (DAKO A/S) was used because the secondary antibody was obtained from rabbit. The cells on the slide were stained by monoclonal mouse antibody to CEA (Dako-CEA, 2–7; DAKO) and then by the alkaline phosphatase anti-alkaline phosphatase method using the DAKO APAAP kit system (DAKO) according to the manufacturer's instructions. The counterstaining was performed with Meyer's hematoxylin. Colo 205 cells either stained in the same manner or stained

**Fig. 2** PCR performed with the first primer pair. Either 1 × 10^10 or 1 × 10^7 double-stranded molecules of both the original sequence and competitor were used as the template. X10, X15, X20, X25, X30, and X35, numbers of PCR cycles performed. The original sequence produced a 260-bp band, and the competitor produced a 288-bp band, as expected. A single heterodimeric band between the original and the competitor sequences was also visible.

**Fig. 3** PCR performed with the second primer pair. Either 1 × 10^10 or 1 × 10^7 double-stranded molecules of both the original sequence and competitor were used as the template. X10, X15, X20, X25, X30, and X35, numbers of PCR cycles performed. The original sequence produced a 190-bp band, and the competitor produced a 218-bp band, as expected. A single heterodimeric band between the original and the competitor sequences was also visible.
RESULTS

Simultaneous One-Step Amplification of the Same Quantities of the Original Sequence and the Competitor. Figs. 2 and 3 show the respective results of PCR performed with the first and second primer pair as a single-step PCR. The PCR protocol was the same for each step of the PCR. Either 1 × 10^10 or 1 × 10^7 double-stranded molecules of both the original sequence and the competitor were used as the template. It is evident from Figs. 2 and 3 that, regardless of the initial quantity of molecules, the original sequence and the 28-bp-longer competitor were both amplified in parallel with the increase in the number of cycles. This was true for both steps.

The single additional band moving a little more slowly than the competitor on the gel proved to be the heterodimer of the original sequence and the competitor, which was the result of both sequencing and the electrophoresis of the repetitively heat-denatured and then annealed mixture of the purified original sequence and the purified competitor (data not shown). Thus, this additional band consists of an equal mixture of both sequences, making its formation unrelated to the quantitative evaluation of either homodimeric band.

Simultaneous One-Step Amplification of Different Quantities of the Original Sequence and the Competitor with Individual Primer Pairs. With 1 × 10^9 molecules as the basal quantity, either the original sequence or the competitor was added in 2, 4, 8, or 16 times larger quantities than its counterpart. Figs. 4 and 5 show the results of PCR performed with the first and second primer pair, respectively, as a single-step PCR. As is evident from the figures, the serial dilution ratio of the two sequences present in the mixture was not affected by any amplification. The difference in the quantities of the two amplified products was detectable even in 2:1 mixtures at both steps.

Quantitative Detection of CEA mRNA in Patients’ Samples. Fig. 6 shows an example of the analysis of an actual patient’s sample. Prior to the first-step PCR, 1000, 100, 20, 10, or no molecules of the competitor were added to the template cDNA, that is, one-sixth of the cDNA obtained from 1 ml of blood sample. The second through sixth lanes (counting from the left) represent the portal blood samples, and the last two lanes on the right are the peripheral blood samples. Only in the portal blood was CEA mRNA recognizable, and for this sample, 20 molecules of the competitor were determined as the equivalent point. Sequencing of the final PCR products revealed that the amplified products were, in all cases, identical to the expected cDNA sequence.

The calculated CEA mRNA copy number in 1 ml of individual blood is indicated in Table 1. The peripheral blood was obtained 1 or 2 days before surgery. As is evident from Table 1, even if a patient’s peripheral blood was negative for CEA mRNA, the portal blood was already positive, even before the surgical maneuver. This was true for all three patients analyzed.

Peripheral blood from all control patients was negative for CEA mRNA. In the samples of all patients, on the other hand, the PCR product of β-actin mRNA was clearly detectable without notable individual differences (data not shown).

Immunocytochemical Detection of CEA Protein-positive Cell in CEA mRNA-positive Patients’ Blood. Immunohistochemical analysis identified red-stained, CEA-positive cells (Fig. 8) in the CEA mRNA-positive portal blood samples but not in the blood samples of CEA mRNA-negative portal or peripheral blood.
DISCUSSION

We previously developed a very sensitive system to identify gastric and pancreatic carcinoma cells in blood in the form of CEA mRNA (3), colorectal carcinoma cells in blood in the form of cytokeratin 20 mRNA (10, 11), and hepatocellular carcinoma cells in blood in the form of α-fetoprotein mRNA (8, 9). As a subsequent development, here we describe a competitive reverse transcription-PCR system designed to identify the quantity of CEA mRNA present in blood and its application to the analysis of both peripheral blood and portal blood in the same patient.

Because we were unable to find natural sequences amplified in an appropriate manner, we created a competitor sequence that could be amplified successively by the same primers and in the same manner as CEA mRNA. As for the single-step quantitative PCR, previous investigators used an internal standard with the same sequence as the original template, except for the presence of either a small intron (18, 19), a small deletion (20), or a mutated restriction site (21). For our purposes, we inserted into the original sequence a small synthetic DNA fragment that did not interfere with the amplification by the original primers. Because the fragment was inserted between the second-step primers, the amplification of this competitor was successful through two PCRs. Furthermore, this competitor is 28 bp longer than the original sequence (or 28 nucleotides, in the case of a single strand); this difference made it possible to distinguish between the two sequences by simple electrophoresis. This type of competitive PCR has two significant merits: first, it is independent of the number of cycles and does not require the exponential phase of amplification; and second, even if any other nonspecific amplification products are present, they do not have any influence on the competitor:unknown ratio because these nonspecific products can be considered competitors for both species of template (18, 19). This is also true for the heterodimer, which consists of one original sequence and one competitor.

From the facts that we were able to detect the presence of CEA mRNA from a five-cell sample (3) and that β-actin mRNA was detected in all cases without notable individual differences, the efficiency of reverse transcription appears to be as high as reported previously (15, 16). Another consideration is that the long-term preservation of the mRNA until needed for patients, repetitive freezing and thawing, and multiple dilution can cause its contamination or destruction. In terms of the clinical application of this system, it seems, therefore, more convenient to deal with a competitor in the form of DNA than in the form of mRNA because the competitor itself does not need to be checked for each analysis. For these reasons, we adopted a DNA competitor as was done by Gilliland et al. (18), Cottrez et al. (22), and Lanzillo et al. (16).

We succeeded in making our previous system into quantitative, and by using this new system we could focus on (a) the difference between the peripheral blood and the portal blood and (b) the changes before and after pancreatectomy. For ethical reasons, we investigated both the portal and peripheral blood only of patients to whom pancreatectomy could be performed. Therefore, the number of patients investigated thus far stands at only three. Yet, by the application of this new system, we were able to obtain some very important findings. The first is that, even if the peripheral venous blood is negative for CEA mRNA, it does not mean that there is no risk of hematogenous dissemination because the portal blood of all three patients was positive for CEA mRNA despite negative results for their peripheral blood. The second is that the portal blood of all three patients before pancreatectomy contained more CEA mRNA and, thus, more carcinoma cells estimated by the quantity of CEA mRNA than did their portal blood after pancreatectomy. Because of the small number of patients investigated, it is not sure if this difference is true for all patients undergoing pancreatectomy. As for the results for the three patients, it seems that there are at least two interpretations of this second result. One is that the operative maneuver is not the most powerful promoter of carcinoma cell migration into the portal vein. The motility of the carcinoma cells themselves or another factor is, thus, likely to be a more critical determinant. Another possibility is that, by the end of the operation, the carcinoma cells floating in the portal vein had already been prompted to move into the liver as a result of the surgical maneuver. Analysis of the results for more patients undergoing pancreatectomy should provide additional information, which is needed to determine which interpretation is correct.

Although we did not perform the follow up analysis in
these patients because they had just undergone operation, repetitive analyses after surgery would give some clinically important information.

Finally, we succeeded in detecting actual CEA-producing cells in the CEA mRNA-positive portal blood. This would strongly support the validity of our genetic diagnosis. Even the loss of one cell or of an aggregate of cells during any step of cell collection, fixing, or staining would have a very serious effect on cytological diagnosis, although the loss of the cells might not be recognized. In contrast to cytological diagnosis, therefore, mRNA-based diagnosis can be expected to be more accurate.

In conclusion, the quantitative analysis of CEA mRNA in portal blood is thought to yield clinically very important information on the hematogenous dissemination of pancreatic ductal adenocarcinoma.

REFERENCES
Quantitative analysis of carcinoembryonic antigen messenger RNA in peripheral venous blood and portal blood of patients with pancreatic ductal adenocarcinoma.

N O Funaki, J Tanaka, R Hosotani, et al.


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