Detection of Cancer Micrometastases in Lymph Nodes by Reverse Transcriptase-Polymerase Chain Reaction

Masaki Mori, Koshi Mimori, Hiroshi Inoue, Graham F. Barnard, Koichi Tsuji, Shigeru Nanbara, Hiroaki Ueo, and Tsuyoshi Akiyoshi

INTRODUCTION

Lymph node metastasis is one of the most useful prognostic factors in carcinomas of the esophagus (1), stomach (2), colorectum (3), or breast (4). The detection of lymph node metastasis is performed routinely by histopathological analysis. Recent advances in immunohistochemical methods allow the assessment, in lymph nodes, of micrometastatic carcinoma cells that are missed by conventional histopathological examination (5-8). In addition, more recently, efforts have been made to detect micrometastases in lymph nodes at the molecular level (9-12).

With respect to gastrointestinal carcinomas, Hayashi et al. (9) reported that the mutant allele-specific amplification method could detect a few carcinoma cells with K-ras or p53 mutations in lymph nodes. The usefulness of this method, however, is restricted to tumors that express the K-ras or p53 gene. This is not the case for CEA-expressing carcinoma cells because CEA is expressed in the majority of cancers (16). Gerhard et al. (16) developed a sensitive assay for the specific detection of CEA-expressing carcinoma cells in bone marrow tissue. We modified their method and have applied it to detect micrometastases in lymph nodes of patients with gastrointestinal or breast carcinoma.

MATERIALS AND METHODS

Carcinoma Samples and Cell Lines. Fresh surgical specimens included 25 pairs of esophageal carcinomas and corresponding normal tissue, 25 pairs of gastric carcinomas and corresponding normal tissue, 25 pairs of colorectal carcinomas and corresponding normal tissue, and 25 breast carcinomas. The cells were immediately frozen in liquid nitrogen after surgical resection and kept at -70°C until the extraction of RNA. The cell lines used were as follows: TE-1 and TE-2, esophageal carcinoma; Kato III and MK 28, gastric carcinoma; Colo 201 and HT 29, colorectal carcinoma; and MCF 7, breast carcinoma.

Lymph Node Samples. A total of 117 lymph nodes were obtained from 2 patients with esophageal carcinoma, 6 patients with gastric carcinoma, 3 patients with rectal carcinoma, and 2 patients with breast carcinoma. Five lymph nodes were obtained from a patient with adenomatous poly of the colon. As a control, 15 lymph nodes obtained from patients with cholelithiasis, and with no evidence of epithelial cancer, were used. Each lymph node was cut into two pieces. One piece was formalin fixed and paraffin embedded for histological examination. The other piece was kept at -70°C until the extraction of RNA.

Serial 10-Fold Dilutions of Carcinoma Cells. Serial 10-fold dilutions of TE-2, Kato III, and Colo 201 cells were prepared in 2-5 x 10⁵ normal peripheral lymphocytes, obtained from a young healthy volunteer, to give a ratio ranging from 1:10² to 1:10⁵ carcinoma cells/lymphocytes.

RNA Extraction. For carcinoma samples, total cellular RNA was extracted according to the method described elsewhere (17). For cell lines and lymph node samples, the acid guanidinium thiocyanate-phenol-chloroform extraction procedure was used for extraction of total RNA (18).

RT-PCR. cDNA was synthesized from 2.5 µg total RNA in a 25-µl reaction mixture containing 5 µl 5X RT reaction buffer (BRL, Gaithersburg, MD), 200 µM dNTP, 100 µM solution of random hexadeoxynucleotide mixture, 50 units of RNasin (Promega, Madison, WI), 2 µl 0.1 M diethiothreitol, and 100 units of Molony leukemia virus RT (BRL). The mixture was incubated at 37°C for 60 min, heated to 95°C for 10 min, and then chilled on ice.

CEA-specific oligonucleotide primers used for nested PCR (19) were synthesized according to previous information (16, 20). The first PCR was performed using primers A and B followed by the second PCR using primers C and D. The first PCR product exhibited a 160-bp fragment and the second PCR product, a 131-bp fragment. The sequences were: A, 5'-CTGGGTTTCTTCCGCTGGG-3'; B, 5'-TGAGCTTTCAAGAAAAGCTTTAAGAGGAAGGAAGCC--C; and C, 5'-GGGCTTCCGGATGATGTC-3'. The nested PCR (19) was performed as follows: For the first PCR, 80 µl containing chelating buffer (Perkin Elmer/Cetus), 2.5 mM MgCl₂, primers A and B (0.5 µM each), and 200 µM dNTP were added to the tubes. Twenty rounds of amplification were performed in a thermocycler (Biometra, Gottingen, Germany) at 95°C (1 min) and 72°C annealing and extension (2 min), with a final extension step for 10 min. Five µl of the reaction were then

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confirmed that it was identical to the expected fragment of CEA. A band was seen in a few lymph node samples by 25 or 30 cycles. Consequently, no band was detectable in carcinoma samples by 20 or more cycles. Although no band was seen in all control lymph node samples at 15, 20, 25, or 30 cycles, a very faint band was seen in a few lymph node samples by 25 or 30 cycles. Consequently, we determined that 20 cycles were adequate for the second PCR. All samples were examined at 20 cycles of the first PCR followed by 20 cycles of the second PCR. We determined the nucleotide sequence of this PCR product and confirmed that it was identical to the expected fragment of cDNA of CEA. A GenBank-Update new sequences library nucleotide data base search demonstrated that the sequence is specific to CEA.

To ensure that the RNA was not degraded, a PCR assay with primers specific for the gene glyceraldehyde-3-phosphate dehydrogenase cDNA (21) was carried out in each case, except that only 24 cycles were performed with the cycling conditions: 1 min at 94°C, 2 min at 56°C, and 2 min at 72°C. The primer sequences for glyceraldehyde-3-phosphate dehydrogenase were as follows: 5'-GTCAACGGATTTGGTCTGTATT-3' and 5'-AGTCTTCTGGGTG- CAGTGAT-3'.

To check for possible artifacts based on the possible contamination of RNA by genomic DNA during RT-PCR, a few RT-PCR reactions were done using carcinoma samples and control lymph node samples, 15, 20, 25, or 30 more cycles were run at 95°C (1 min), 69°C (1 min), and 72°C (1 min), with the final extension step for 10 min. A strong band of CEA cDNA was transferred into a second tube containing 200 μM dNTP, 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 0.001% gelatin, 0.4 μM each of the two primers (C and B), and 2.5 units Taq polymerase (Perkin Elmer/Cetus). When using carcinoma samples and control lymph node samples, 15, 20, 25, or 30 more cycles were run at 95°C (1 min), 69°C (1 min), and 72°C (1 min), with the final extension step for 10 min. A strong band of CEA cDNA was detectable in carcinoma samples by 20 or more cycles. Although no band was seen in all control lymph node samples at 15, 20, 25, or 30 cycles, a very faint band was seen in a few lymph node samples by 25 or 30 cycles. Consequently, we determined that 20 cycles were adequate for the second PCR. All samples were examined at 20 cycles of the first PCR followed by 20 cycles of the second PCR. We determined the nucleotide sequence of this PCR product and confirmed that it was identical to the expected fragment of cDNA of CEA. A GenBank-Update new sequences library nucleotide data base search demonstrated that the sequence is specific to CEA.

RESULTS

Carcinoma Samples and Cell Lines. All specimens of 25 esophageal carcinomas, 25 gastric carcinomas, 25 colorectal carcinomas, and 25 breast carcinomas showed a positive band of the 131-bp DNA fragment (Fig. 1). All corresponding normal tissue of the esophagus, stomach, or colorectum also expressed CEA. All seven carcinoma cell lines (two esophageal, two gastric, two colonic, and one breast) demonstrated the same band in the agarose gel (Fig. 1). These findings disclose that gastrointestinal carcinomas, breast carcinomas, and normal gastrointestinal mucosal tissues exhibit CEA mRNA. The RT-PCR with the omission of the RT enzyme eliminated a signal from a metastasis-positive lymph node, indicating no possibility of contamination of genomic DNAs causing the positive bands.

Lymph Node Samples. Fifteen lymph nodes from 5 patients with cholelithiasis showed no band of the 131-bp DNA fragment under the chosen amplification conditions, and were therefore the appropriate control tissue (Fig. 1). The results of the detection of CEA using lymph nodes from 13 patients with carcinoma and 1 patient with a colon polyp are shown in Table 1. The colon polyp was histologically an adenoma; all five lymph nodes from this patient with a colon polyp demonstrated no evidence of epithelial cells by histological diagnosis and no band of CEA by RT-PCR (Case 14). There were seven patients whose regional lymph nodes were negative for metastasis by histological diagnosis. Of these, four (Cases 2, 5, 8, and 11) disclosed lymph node metastases and the other three (Cases 6, 7, and 13) no lymph node metastasis by RT-PCR (Table 1 and Fig. 2). In five patients (Cases 1, 3, 4, 9, and 10), the number of positive lymph nodes was increased by RT-PCR compared with that of the histological diagnosis (Table 1 and Fig. 2). In the remaining one patient (Case 12), all six lymph nodes showed metastases by both methods. In no case was the RT-PCR negative when the histological diagnosis was positive. One patient with esophageal carcinoma (Case 2) who showed no metastasis by histological examination, but showed metastasis by RT-PCR, demonstrated an increase of serum squamous cell carcinoma antigen and CEA 6 months after the operation. A computed tomographic study demonstrated probable metastatic lymph nodes in the mediastinum.

Sensitivity of DNA-based Diagnosis. CEA mRNA could be detected at a concentration of as low as 10² tumor cells/10⁷ normal lymphocytes in cancer cell lines of the colon (Colo 201), but not in the esophagus (TE-2) or stomach (Kato III). It needed 10⁵ TE-2 or Kato III cells/10⁷ lymphocytes to detect CEA mRNA. The degree of amplification was not quantitative in any of the cell lines (Fig. 3).

DISCUSSION

Among many prognostic factors, lymph node metastasis is one of the more useful indicators for patients with carcinomas including esophageal, gastric, colorectal, and breast carcinomas (1-4). Even when determined by experienced pathologists, however, metastasis sometimes may be barely detected by routine diagnostic evaluation. Gusterson (22) described that about one-fifth of the cases diagnosed as lymph node negative had micrometastasis on reexamination by

<table>
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<th>Case</th>
<th>Age/sex</th>
<th>Diagnosis</th>
<th>Histology</th>
<th>Depth of tumor invasion</th>
<th>Histological Dx</th>
<th>DNA-based Dx</th>
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<tr>
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* SCC, squamous cell carcinoma; Mod., moderately differentiated; Ad, adenocarcinoma; Dx, diagnosis.
serial sectioning. Recently, immunohistochemical assays for epithelial-specific proteins have been used to overcome this problem. For example, CEA and cytokeratins were used for colorectal carcinoma (8), CA 15-3 and methylicholanthrene for breast carcinoma (6), Ber-Ep 4, which is directed against two epithelial cell surface glyco-peptides, for non-small cell lung carcinoma (5), and S-100 protein for melanoma (23). Serial sectioning and immunohistochemical staining certainly increases the yield of occult metastasis (5, 6, 8, 23); however, it seems to be time consuming and labor intensive. This method, therefore, has not been performed routinely in most hospitals.

More recently, diagnostic procedures for the detection of micrometastasis at the genetic level have developed rapidly. These methods aim to detect genes that are exclusively expressed in carcinoma cells but not in normal lymph node or bone marrow cells by means of RT-PCR (10–12, 16). Wang et al. (10) and Smith et al. (24) made use of tyrosinase, which is a key enzyme during melanin synthesis, for the detection of micrometastases of melanoma cells in lymph nodes and peripheral blood, respectively. With respect to breast carcinoma, Noguchi et al. (11) and Schoenfeld et al. (12) used muc 1 and cytokeratin 19 genes, respectively, for detection of micrometastases in axillary lymph nodes. Gerhard et al. (16) developed a sensitive assay for the detection of CEA-expressing carcinoma cells in the bone marrow of patients with colorectal, pancreatic, or gastric carcinoma.

Concerning the DNA-based diagnosis of micrometastases of gastrointestinal carcinomas in lymph nodes, Hayashi et al. (9) used a mutant allele-specific amplification method which can detect one tumor cell containing K-ras or p53 mutations in a background of thousands of normal cells. The limitation of this method is that the usage is restricted to carcinomas with these specific genetic mutations. We thus used the modified method of Gerhard et al. (16) for the detection of micrometastases in lymph nodes. Although immunohistochemical expression of CEA varies widely from case to case (25), CEA mRNA is certainly expressed at some level not only in all specimens of esophageal, gastric, colorectal, or breast carcinomas but in all corresponding normal gastrointestinal tissue. This method could detect 10^2 tumor cells in a background of 10^7 normal lymphocytes, giving a sensitivity ratio of 1:10^5. In contrast, all lymph nodes obtained from patients with benign diseases such as cholelithiasis or a colon polyp did not show any CEA mRNA at all under the chosen amplification conditions. This illustrates that the RT-PCR method is not falsely positive because of the lymph node composition when the nested PCR cycles we used are applied.

This present study demonstrated that 30 of 117 examined lymph nodes obtained from 13 carcinoma patients showed positive lymph node metastases by routine histological examination. The number of positive lymph nodes increased to 77 by the RT-PCR method. Four of seven patients who had no lymph node metastases by histological examination were revealed to have lymph node metastasis by RT-PCR. One patient with esophageal carcinoma (Case 2), who showed metastasis by RT-PCR but not by histological examination, demonstrated probable lymph node metastases in the mediastinum 6 months after the operation. The results indicate that CEA mRNA detection by RT-PCR for the detection of micrometastasis in lymph nodes was superior to routine histological examination. RT-PCR is a highly sensitive and specific method if a cancer cell-specific gene is applied. The method can save time and expense because many samples can be tested at the same time, with the entire procedure taking a couple of days. We thus consider that the diagnosis using CEA-specific RT-PCR may be specific and useful for almost all cases of gastrointestinal or breast carcinomas. This promising application will need further validation with a larger number of cases. A prospective study of in situ hybridization for CEA in the lymph nodes is also feasible to confirm the results of RT-PCR.

As suggested by several investigators (10–12, 16) the following factors should be kept in mind: (a) RNA preparation must be carefully controlled with protection against RNase activity, (b) cross-contamination between samples must be avoided during the procedures, and (c) contamination of genomic DNA should be avoided because of the possible artifact accompanying RT-PCR. The first and the second points can be addressed by careful preparation. To address the third point, we selected primers located in different exons according to Gerhard et al. (16) and carried out the RT-PCR omitting the RT enzyme for a control.

RT-PCR is powerful for the detection of micrometastases in lymph nodes and is valuable in selecting high-risk patients.


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