

Detection of Cancer Micrometastases in Lymph Nodes by Reverse Transcriptase-Polymerase Chain Reaction¹

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ABSTRACT

There are few DNA-based studies that detect cancer micrometastases in lymph nodes. We have assayed for the specific detection of carcinoembryonic antigen (CEA)-expressing carcinoma cells in the lymph nodes of patients with gastrointestinal or breast carcinomas. A CEA-specific nested reverse transcriptase (RT)-PCR assay was optimized using limiting dilutions of a CEA-positive cancer cell line mixed with normal lymphocytes. The expression of CEA mRNA was studied in 100 carcinoma tissues, 75 normal mucosal tissues, and 15 lymph nodes from patients with cholelithiasis. Each of 117 lymph nodes from 13 patients with carcinoma was divided into two pieces: one was used for histological examination and the other for RT-PCR, and the results were compared. The sensitivity ratio was one CEA-expressing cancer cell detected in 1×10^5 normal lymphocytes. All carcinoma tissues and normal mucosal tissues expressed CEA mRNA, while no amplification was detected in any control lymph nodes. Thirty of 117 lymph nodes were histologically involved by carcinoma cells, and all of these yielded the expected product by RT-PCR. Of the remaining 87 histologically negative nodes, CEA mRNA was detected in 47 lymph nodes by RT-PCR. The positive rate increased from 26% by histological examination to 66% by RT-PCR. The assay by CEA-specific nested RT-PCR is not only sensitive but widely applicable for the detection of cancer micrometastases in lymph nodes. This method may lead to an earlier diagnosis and treatment of patients with subclinical lymph node metastasis.

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 with *K-ras* or *p53* mutations. *p53* mutations are seen in about one-half of the cases of esophageal, gastric, or colorectal carcinomas, and *K-ras* mutations are also seen in about one-half of the cases of colorectal carcinoma. The frequency of *K-ras* mutation, however, is recognized in less than 10% of cases of esophageal or gastric carcinomas (13-15). Thus, a method that can be used in almost all tumors

would be desirable. CEA³ is a widely accepted tumor marker and 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 samples 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 polyp 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 $2-5 \times 10^7$ normal peripheral lymphocytes, obtained from a young healthy volunteer, to give a ratio ranging from $1:10^2$ to $1:10^5$ 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 guanidium 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 dithiothreitol, 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 B. The first PCR product exhibited a 160-bp fragment and the second PCR product, a 131-bp fragment. The sequences were: A, 5'-TCTGGAAC-TCTCCT-GGTCTCTCAGCTGG-3'; B, 5'-TGTAGCTGTTGCAAAATGCT-TTAAGGAAGAAGC-3'; and C, 5'-GGGCCACTGTCGGCATCATGAT-TGG-3'. The nested PCR (19) was performed as follows: For the first PCR, 80 μl containing chelating buffer (Perkin Elmer/Cetus), 2.5 mM MgCl_2 , 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, Göttingen, 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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³ The abbreviations used are: CEA, carcinoembryonic antigen; RT, reverse transcriptase; dNTP, deoxynucleotide triphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

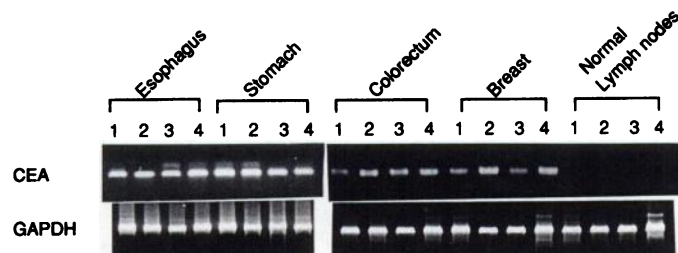


Fig. 1. The first lane of each esophagus, stomach, colorectum, or breast site shows cell lines of TE-1, MK 28, HT 29, or MCF 7, respectively. The second, third, and fourth lanes for each site show the representative primary tumors. Note that no band is detected in samples from lymph nodes obtained from patients with benign diseases.

transferred into a second tube containing 200 μ M dNTP, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.001% gelatin, 0.4 μ M 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 at all in any control lymph node samples by 15 or 20 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 thus 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'-AGTCTTCTGGGTGGCAGTGAT-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 a truly metastasis-positive lymph node under the same conditions but with no reverse transcription step.

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 10^2 tumor cells/ 10^7 normal lymphocytes in cancer cell lines of the colon (Colo 201), but not in the esophagus (TE-2) or stomach (Kato III). It needed 10^3 TE-2 or Kato III cells/ 10^7 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 1 Detection of metastatic carcinoma cells or CEA mRNA in lymph nodes

Case	Age/sex	Diagnosis	Histology	Depth of tumor invasion	No. of metastatic lymph nodes	
					Histological Dx ^a	DNA-based Dx
1	61/M	Esophageal carcinoma	Mod. SCC	Adventitia	4/19	15/19
2	46/M	Esophageal carcinoma	Mod. SCC	Submucosa	0/9	5/9
3	80/F	Gastric carcinoma	Mucinous	Beyond the serosa	10/20	18/20
4	64/F	Gastric carcinoma	Mod. Ad	Subserosa	6/9	7/9
5	62/M	Gastric carcinoma	Signet	Mucosa	0/12	5/12
6	67/M	Gastric carcinoma	Well Ad	Submucosa	0/4	0/4
7	75/F	Gastric carcinoma	Signet	Mucosa	0/5	0/5
8	38/F	Gastric carcinoma	Poor. Ad	Subserosa	0/5	2/5
9	69/M	Rectal carcinoma	Mod. Ad	Adventitia	3/11	8/11
10	88/F	Rectal carcinoma	Well Ad	Adventitia	1/7	7/7
11	69/M	Rectal carcinoma	Mod. Ad	Adventitia	0/5	4/5
12	66/F	Breast carcinoma	Invasive ductal carcinoma		6/6	6/6
13	65/F	Breast carcinoma	Invasive ductal carcinoma		0/5	0/5
14	69/M	Colon polyp	Adenoma		0/5	0/5

^a 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 methylcholanthrene for breast carcinoma (6), Ber-Ep 4, which is directed against two epithelial cell surface glycoproteins, 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 immunohis-

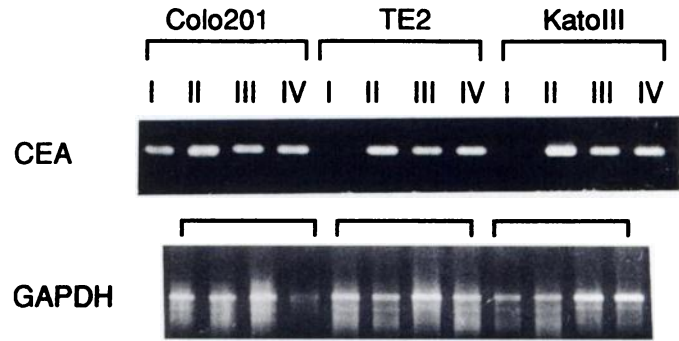


Fig. 3. Different numbers of carcinoma cells were added to 10^7 normal lymphocytes. The number of tumor cells is 10^2 , 10^3 , 10^4 , and 10^5 in Lanes I, II, III, and IV, respectively.

tochemical 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 the 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.

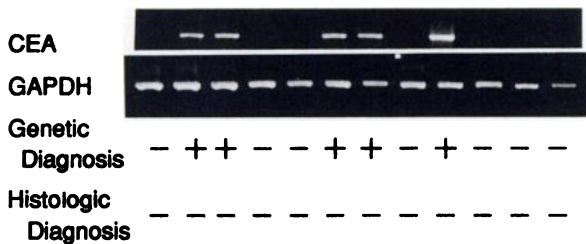
As suggested by several investigators (10-12, 16) the following factors should be kept in mind: (a) RNA preparation must be carefully controlled with protection against RNase activity, (b) cross-contamination between samples must be avoided during the procedures, and (c) contamination of genomic DNA 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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Case 5



Case 9



Fig. 2. Two representative cases. Case 5 was a gastric carcinoma. Although the histological diagnosis showed no metastasis in any of 12 examined lymph nodes, the genetic diagnosis disclosed the 131-bp band of CEA in five lymph nodes. Case 9 was a rectal carcinoma. The number of involved lymph nodes was three by the histological diagnosis and eight by the genetic diagnosis.

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