Extensive Micrometastases to Lymph Nodes as a Marker for Rapid Recurrence of Colorectal Cancer: A Study of Lymphatic Mapping[1]

Yasuhiro Miyake, Hirofumi Yamamoto,[2] Yoshiyuki Fujiwara, Masayuki Ohue, Yurika Sugita, Naohiro Tomita, Mitsugu Sekimoto, Nariaki Matsuura, Hitoshi Shiozaki, and Morito Monden

Department of Surgery and Clinical Oncology, Graduate School of Medicine, Osaka University, Osaka 565-0871 [Y. M., H. Y., Y. F., M. O., Y. S., M. S., H. S., M. M.]; Department of Surgery, Kansai Rosai Hospital, Hyogo 660-0064, [N. T.]; and Department of Pathology, School of Allied Health Science, Faculty of Medicine, Osaka University, Osaka 565-0871 [N. M.], Japan

ABSTRACT

To provide a detailed assessment of micrometastases of colorectal cancer by anatomical mapping of regional lymph nodes (LNs), we analyzed 237 LNs from 11 patients with colorectal cancer by reverse transcription-PCR (RT-PCR) using carcinoembryonic antigen and cytokeratin 20 as genetic markers. All dissected LNs were mapped anatomically and subjected to detection assays for micrometastases. Immunohistochemical analysis was also performed using anti-pancytokeratin antibody AE1/AE3 to confirm the existence of occult cancer cells. By histological analysis, 20 of 237 LNs contained metastatic cells, and they were all positive by both immunohistochemistry and RT-PCR. Of the 217 histologically negative LNs, 14 (6.5%) harbored micrometastases by immunohistochemistry, and 57 (26.2%) were positive for at least one of the two genetic markers. Lymphatic mappings of all patients showed that micrometastases were distributed not only at the pericolic LNs but often at distant LNs. Clinical follow-up study showed that two patients developed recurrence within 1 year after surgery, and both of them had RT-PCR-positive micrometastases in not less than 70% of LNs examined. Moreover, both patients had frequent micrometastases at distant LNs, *i.e.*, those around the root or along the inferior mesenteric artery, when compared with patients with no recurrence. Our findings suggest that genetic diagnosis using the RT-PCR method may be clinically useful along with conventional pathological diagnosis, especially when micrometastases spread to distant LNs.

INTRODUCTION

Complete resection of cancer tissues is still the most important and effective treatment for patients with colorectal cancers, and precise information on the expanse of cancer metastasis certainly helps clinicians in the overall management of cancer patients (1, 2). Metastasis to regional LNs[3] is an important prognostic factor and is used for staging of colorectal cancer and for clinical decision-making with regard to the selection of the most appropriate treatment (3). Studies have shown that occult cancer metastasis (also designated as micrometastasis) exists in the LNs of patients with colorectal cancer. This type of metastasis is so minimal that it usually cannot be detected by examining one slide of a H&E-stained section. The clinical significance of micrometastasis to regional LNs has been studied intensively in node-negative colorectal cancer over the past several years, but conflicting results have been found, and the conclusions are still controversial (4–11).

In several types of human malignancy, including carcinoma of the breast and stomach, the concept of micrometastasis initially emerged from studies on reexamination of serial sections that had been previously diagnosed as pathologically negative (12–14). The rate of detection of metastases in LNs ranged from 9–33% of the cases. CK, a specific marker of epithelial cells, has been widely used for the immunohistochemical detection of micrometastasis of carcinomas of the colon (4–8). Immunohistochemistry provides direct evidence of the existence of occult cancer cells at a frequency of 17–39% of the cases with histologically negative LNs, but this method is limited for the detection of occult metastasis because it is impractical to examine great numbers of slides corresponding to the whole LN. Alternatively certain genes, *e.g.*, *CK19*, *CEA*, and *CK20*, whose mRNA expression is limited to specific organ tissues or cancer cells, have been recently used as targets for amplification by RT-PCR in patients with a variety of malignant tumors (15–19). This genetic method accomplishes high sensitivity by means of PCR but is known to occasionally produce false positive results. Although both methods are available, considering the above-mentioned benefits and deficiencies, we currently used both RT-PCR and immunohistochemistry for the detection of micrometastasis.

Most of the early studies focused on node-negative patients

Received 11/7/00; revised 1/29/01; accepted 1/29/01.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by Grant-in Aid for Cancer Research 12213078 (to H. Y.) from the Ministry of Education, Science, Sports and Culture, Japan.
[2] To whom requests for reprints should be addressed, at Department of Surgery and Clinical Oncology, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita-City, Osaka 565-0871, Japan. Phone: 81-6-6879-3251; Fax: 81-6-6879-3259; E-mail: kobunyam@surg2.med.osaka-u.ac.jp.

[3] The abbreviations used are: LN, lymph node; RT-PCR, reverse transcription-PCR; CEA, carcinoembryonic antigen; CK, cytokeratin; PBGD, porphobilinogen deaminase.

Table 1 Clinical and pathological characteristics of the patients with colorectal cancer

Case	Age (yrs)/gender	Location[a]	Tumor size (cm)	Histology[b]	Depth of tumor invasion	UICC stage[c]	Histological LN metastasis (H&E)	Micrometastases[d] IHC	Micrometastases[d] RT-PCR
1	56/F	S	2.0	Mod Ad	Submucosa	I	I	No	No
2	41/M	S	2.2	Mod Ad	Submucosa	I[e]	No	No	No
3	60/M	S	2.4	Mod Ad	Subserosa	II[e]	No	No	Yes
4	54/M	D	5.8	Well Ad	Subserosa	II	No	Yes	Yes
5	75/M	R	5.8	Well Ad	Adventitia	II	No	Yes	Yes
6	71/M	R	8.5	Mod Ad	Beyond the adventitia	II	No	Yes	Yes
7	57/F	Rs	2.9	Well Ad	Muscularis propria	III	Yes	Yes	Yes
8	61/M	R	7.2	Well Ad	Subserosa	III	Yes	Yes	Yes
9	84/M	R	4.4	Well Ad	Adventitia	III	Yes	No	Yes
10	74/M	R	6.5	Poor Ad	Beyond the adventitia	III	Yes	Yes	Yes
11	77/M	Rs	5.5	Mod Ad	Other organs	III	Yes	Yes	Yes

[a] S, sigmoid colon; D, descending colon; R, rectum; Rs, rectosigmoid colon.
[b] Mod, moderately differentiated; Poor, poorly differentiated; Well, well differentiated; Ad, adenocarcinoma.
[c] UICC, International Union Against Cancer.
[d] Micrometastases were defined when immunohistochemistry (IHC) or RT-PCR showed positive results in LNs devoid of histological metastasis.
[e] The number of regional LNs of these cases could not be found under 12 LNs.

because of the simplicity of comparing the clinical course between micrometastasis-positive cases and micrometastasis-negative cases within the category. On the other hand, micrometastasis is also frequently detectable in patients with node-positive colorectal cancer (18). Although pathological LN metastasis is important, there might be a further advantage of the search for micrometastasis in this advanced-stage group. This possibility has not yet been examined. Therefore, in this study, we examined the clinical relevance of micrometastasis in both node-negative and node-positive patients with colorectal cancer. We also examined the extent of micrometastasis in each case by constructing a map of involved LNs because, to our knowledge, there are only a few studies that have examined micrometastasis according to anatomical position. Thus, all dissected LNs (n = 237) were examined by both RT-PCR and immunohistochemistry, and they were mapped in relation to the anatomical positions, *i.e.*, pericolic site, along intermediate named vascular trunks, and around the root of vascular trunks. Our detailed analyses had the advantage of providing genetically detected micrometastases in reference to their anatomical position.

MATERIALS AND METHODS

Patients. Eleven patients with colorectal cancer who underwent curative operation at the Department of Surgery and Clinical Oncology (Osaka University Hospital, Osaka, Japan) were studied. Colonic resection was done with prophylactic LN resection, and a total of 237 LNs were collected. The follow-up period was 22–28 months (mean, 24 months). The number of LNs/patient ranged from 9–42, with a mean number of 19.6 ± 11.7 (SD). Six patients had histologically negative LNs (node-negative patients), and the remaining five patients had histologically positive LNs (node-positive patients). Clinical and pathological characteristics of these patients are shown in Table 1, including age, gender, location and size of the tumors, histological grade, depth of invasion, and status of pathological LN metastasis. The patients were nine men and two women with a mean age of 64.5 years (range, 41–84 years), and tumor size ranged from 2.0–8.5 cm (mean ± SD, 4.8 ± 2.2 cm). According to the tumor-node-metastasis (TNM) classification of UICC (20), patients were categorized into three stages: (*a*) stage I (n = 2); (*b*) stage II (n = 4); and (*c*) stage III (n = 5). As a negative control, 15 LNs were obtained from 11 patients without malignant disease who had surgery because of splenomegaly (n = 3), gall bladder stones (n = 4), acute appendicitis (n = 1), abdominal aneurysm (n = 1), adrenal adenoma (n = 1), and sigma elongation (n = 1).

Tissue Preparations. Each LN was cut into halves under sterile conditions to prevent RNA cross-contamination between specimens (21). One half of the node was fixed with 10% buffered formalin and embedded in paraffin for H&E staining and immunohistochemistry. The other half was stored in Trizol reagent (Molecular Research Center, Cincinnati, OH) at −80°C until RNA extraction. A blade sterilized with ethanol was used in each step that involved slicing the LN. The colorectal cancer tissues were also prepared for RT-PCR assays.

Immunohistochemistry. Two sets of two serial 4-μm-thick sections were prepared for analyses of different levels of LNs. The cut sections were placed on Capillary Gap Plus Microscope slides (BioTek Solutions, Santa Barbara, CA) and stained with H&E and pan-CK monoclonal antibody AE1/AE3 for immunohistochemistry (DAKO, Carpinteria, CA), which has been used previously for the detection of micrometastases (4–7). Immunostaining was performed on the TechMate Horizon automated staining system (DAKO) as described previously (22). Briefly, sections were deparaffinized, dehydrated, and subjected to heat antigen retrieval in 10 mM citrate buffer (pH 6.0; Ref.23). In the primary antibody reaction step, the sections were incubated with anti-CK antibody for 30 min at a concentration of 1.0 μg/ml, followed by reaction with antimouse secondary antibody conjugated with a dextran polylinker (EnVision+) for 30 min (24). Visualization of the signals was achieved with 3,3′-diaminobenzidine plus H_2O_2. Sections of CK-positive colon cancer tissue served as positive controls in each staining procedure. For negative controls, sections were incubated with nonimmunized mouse IgG (Vector Laboratories, Burlingame, CA) or TBS instead of the primary antibody as a

substitute for primary antibody to exclude false positive responses from nonspecific binding to IgG or from the secondary antibody. Two investigators (Y. M. and H. Y.) examined all sections independently. A final consensus was reached under a two-head microscope, under the direction of N. M. who is a professor in the Department of Pathology, Osaka University (Osaka, Japan).

RT-PCR Assay. Tissue specimens were minced with disposable homogenizer (IEDATM, Tokyo, Japan) in Trizol reagent to avoid RNA degradation. RNA extraction was carried out according to the protocol recommended by the manufacturer, as we described previously (25). Purified RNA was quantified and assessed for purity by UV spectrophotometry. cDNA was generated with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) according to the protocol provided by the manufacturer. Briefly, 1 μg of RNA was incubated at 70°C for 5 min and then placed on ice before the addition of RT reaction reagents with oligo$(dT)_{15}$ priming. The RT reaction was performed in total 20 μl at 42°C for 90 min, followed by heating at 95°C for 5 min. PCR was performed in a 25-μl reaction mixture containing 2 μl of cDNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphatase, 0.2 μM each primer, and 1 unit of Taq DNA polymerase (AmpliTaq Gold; Roche Molecular Systems, Inc.). PCR was set up using the following protocol: (*a*) one cycle of denaturing at 95°C for 12 min, followed by 35 cycles of 95°C for 1 min and 72°C for 1.5 min before a final extension at 72°C for 10 min for CEA; and (*b*) one cycle of denaturing at 95°C for 12 min, followed by 35 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min before a final extension at 72°C for 10 min for CK20. These PCR conditions were set up in a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT). The integrity of all RNA samples was verified by amplification of PBGD mRNA by RT-PCR. The primer sequences used for CEA detection were 5′-TCT-GGA-ACT-TCT-CCT-GGT-CTC-TCA-GCT-GG-3′ and 5′-TGT-AGC-TGT-TGC-AAA-TGC-TTT-AAG-GAA-GAA-GC-3′ (17). Primer sequences were newly designed for specific CK20 detection using OLIGO Primer Analysis Software Version 5.1 (NBI/Genovus, Inc., Plymouth, MN; 5′-GGT-CGC-GAC-TAC-AGT-GCA-TAT-TAC-A-3′ and 5′-CCT-CAG-CAG-CCA-GTT-TAG-CAT-TAT-C-3′). PCR primers used for PBGD cDNA detection were synthesized as reported previously (26). PCR products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide. The resultant cDNA products of CEA, CK20, and PBGD were 160, 120, and 127 bp, respectively.

RESULTS

Determination of Optimal Condition of RT-PCR

In colorectal cancers, CEA and CK20 transcripts have been used as sensitive markers to detect micrometastases to LNs (17, 18). Because false positivity is the major issue with regard to the RT-PCR assay, we initially determined the optimal condition under which CEA and CK20 cDNAs prepared from 15 control LNs of 11 noncancer patients were never amplified. Under 35 cycles of PCR, these control samples expressed neither CEA nor CK20 transcripts (Fig. 1, *Lanes 1–3*), but at 40 cycles, bands for CEA mRNA appeared in 1 of 15 LNs (6.7%), and bands for CK20 mRNA appeared in 3 of 15 LNs (20%). These experiments were reproducible. Based on these results, PCR was performed at 35 cycles throughout the subsequent experiments. CEA and CK20 mRNAs were expressed in all of the colorectal cancer tissues tested ($n = 11$; Fig. 1, *Lanes 4–6*).

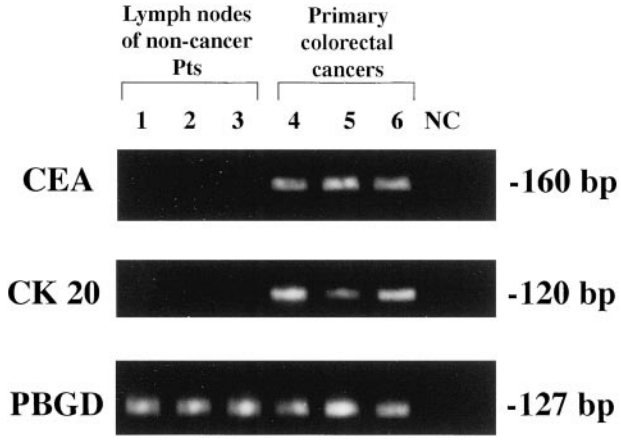

Fig. 1 Results of RT-PCR assays with CEA, CK20, and PBGD gene markers. PCR was performed at 35 cycles, a condition under which CEA and CK20 cDNAs prepared from 15 control LNs of 11 noncancer patients were not amplified. In contrast, CEA and CK20 mRNAs were expressed in all colorectal cancer tissues tested ($n = 11$). Representative results are shown here. *Lanes 1–3*, histologically positive LNs; *Lanes 4–6*, primary colorectal cancers; *NC*, negative control for PCR performed with all reagents except RNA. The estimated size of each of the amplified products is 160, 120, and 127 bp for CEA, CK20, and PBGD, respectively.

LN Specimens from Colorectal Cancer Patients

A total of 237 LNs from 11 patients with colorectal cancer were subjected to immunohistochemical analysis and RT-PCR.

Immunohistochemistry. Occult carcinoma cells were strongly stained with anti-CK antibody and showed morphological features of malignant cells, such as a large nucleus and condensed nuclear small body (Fig. 2). Other components of the LN were not stained. Only cells that were diagnosed as definite carcinoma cells based on morphological features were considered positive. Most occult carcinoma cells were found singly, but they occasionally formed small clusters. They were located in the subcapsular sinus (Fig. 2*A*) or paracortical sinus (Fig. 2*B*). H&E staining indicated that 20 of 237 (8.4%) LNs contained metastatic cells. In these metastatic nests, CKs were expressed exclusively by the carcinoma cells. Of 217 histologically negative LNs, 14 (6.5%) contained micrometastatic carcinoma cells detected by immunohistochemistry.

RT-PCR. CEA and CK20 mRNAs were detected in 19 (95%) and 20 (100%) of 20 histologically positive LNs, respectively. Of 217 histologically negative LNs, 49 (22.5%) were positive for CEA, and 41 (18.9%) were positive for CK20. Overall, 57 (26.2%) LNs were found to be positive for at least one of the markers. These results were reproducible. Table 2 summarizes the relationship between results obtained with RT-PCR assay and histological examination.

LN metastases detected by H&E staining, immunohisto-

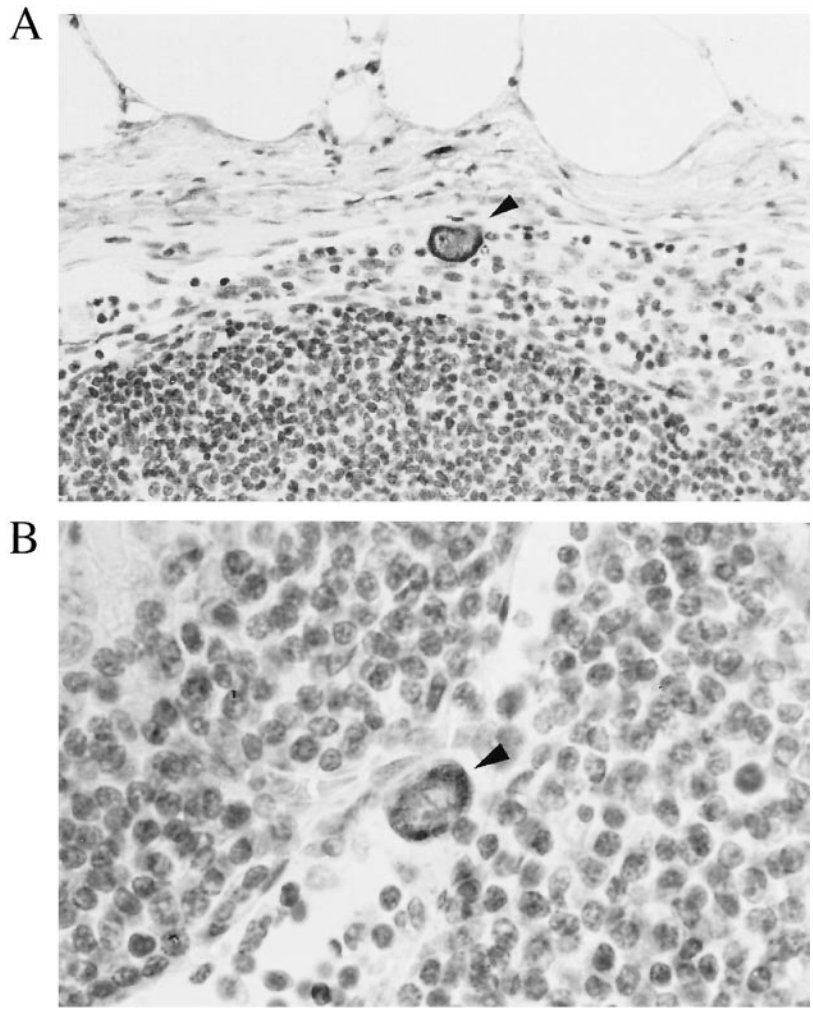

Fig. 2 Representative photographs of micrometastases detected by immunohistochemistry using anti-CK antibody. *A*, *arrowhead* indicates a carcinoma cell located in the subcapsular sinus showing morphological characteristics of malignant cells including a large nucleus and condensed nuclear small body. *B*, *arrowhead* points to another carcinoma cell located in the paracortical sinus. Original magnification: *A*, ×100; *B*, ×200.

Table 2 LN metastases detected by RT-PCR and histological examination

		Histological examination	
	RT-PCR	Positive (n = 20)	Negative (n = 217)
CEA	Positive	19	49
	Negative	1	168
CK20	Positive	20	41
	Negative	0	176
CEA and/or CK20	Positive	20 (100%)	57 (26.3%)

chemistry, and RT-PCR assay were also included in Table 1. Of six patients with stage N_0 disease by histological examination (cases 1–6), two patients with submucosal tumors displayed no positive results with both immunohistochemistry and RT-PCR assay (cases 1 and 2). One patient with subserosa showed positive LNs by the RT-PCR assay alone (case 3). Furthermore, three patients with tumors invading the subserosa, the adventitia, or beyond the adventitia (cases 4–6) showed positive results by both methods. In patients with histological LN metastasis (cases 7–11), micrometastases were found in four patients by immunohistochemistry and in five patients by RT-PCR, respectively.

Anatomical Mapping of Micrometastases in Regional LNs of Colorectal Cancer

We then constructed a detailed map of the regional LNs, using the 237 analyzed LNs. The main tumors included in this study were all located in the left colon or rectum. The location of the LNs was divided into three groups, according to the distance from the main tumor, as follows: (*a*) level 1, pericolic nodes adjacent to the tumor; (*b*) level 2, intermediate nodes along the course of the main blood vessel (for example, inferior mesenteric artery); and (*c*) level 3, central nodes around the root of the main blood vessel. The prophylactic LN dissection was done up to level 2 in cases 1, 2, and 7 and up to level 3 in the remaining eight patients. The constructed maps of four representative cases are shown in Fig. 3. In case 1, 22 regional LNs from levels 1–3 were examined, and no positive metastases were found by H&E staining, immunohistochemistry, and RT-PCR assay (Fig. 3*a*). Case 3 had 10 LNs, of which 7 were positive by RT-PCR from level 1 to level 3 (◎), but none were positive

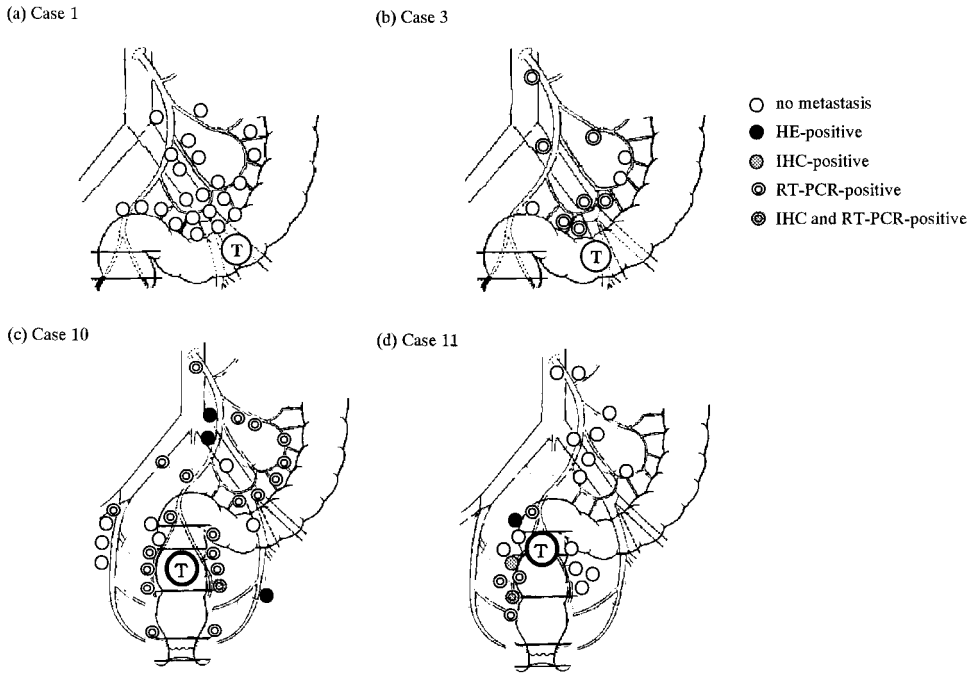

Fig. 3 Anatomical mapping of micrometastases in regional LNs detected by RT-PCR, immunohistochemistry, and histological examination in four representative cases. *a*, case 1 had no positive metastases by H&E staining, immunohistochemistry, and RT-PCR assay. *b*, case 3 had extensive micrometastases from level 1 to level 3 LNs, and 70% of LNs were positive by RT-PCR (◎). *c*, case 10 had three histological metastases at level 2 (●), and the RT-PCR assay revealed several other positive LNs (77.4%) from level 1 to level 3. *d*, case 11 had one histological LN metastasis at level 1 and five RT-PCR-positive and two immunohistochemistry-positive LNs near the tumor. Cases 3 and 10 developed recurrence shortly after surgery. ○, no metastasis; *shaded* ◎, positive by both immunohistochemistry and RT-PCR; ◎, p, positive by RT-PCR only; *gray circle*, positive by immunohistochemistry only; ●, histologically confirmed metastasis. *T*, primary colorectal tumor.

Table 3 Results of pathological diagnosis, immunohistochemistry, and RT-PCR

	Case 1			Case 2			Case 3			Case 4			Case 5			Case 6			Case 7			Case 8			Case 9			Case 10			Case 11		
	L1[a]	L2	L3	L1	L2	L3	L1	L2	L3	L1	L2	L3	L1	L2	L3	L1	L2	L3	L1	L2	L3	L1	L2	L3	L1	L2	L3	L1	L2	L3	L1	L2	L3
Number of LNs	16	6	0	7	2	0	7	2	1	9	6	3	14	8	20	12	8	3	9	4	0	15	9	7	10	3	4	11	6	14	14	5	2
Pathological Dx	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	7	0	0	5	1	0	0	3	0	1	0	0
IHC Dx	0	0	0	0	0	0	0	0	0	2	0	0	1	0	0	3	2	0	4	2	0	8	4	3	5	1	0	1	3	0	3	0	0
RT-PCR Dx	0	0	0	0	0	0	4	2	1	0	0	1	0	3	0	2	0	0	5	2	0	8	4	3	8	3	1	9	5	10	6	0	0

[a] L1 (level 1), pericolic nodes adjacent to the tumor; L2 (level 2), intermediate nodes along the course of the main vessel (for example, inferior mesenteric artery); L3 (level 3), central nodes around the root of the main vessel; Dx, diagnosis; IHC, immunohistochemistry.

immunohistochemically or by H&E staining (Fig. 3*b*). In case 10, there were three histological metastases at level 2 (●) of 31 LNs (Fig. 3*c*). The RT-PCR assay revealed 21 positive LNs from levels 1–3, and immunohistochemistry showed only one positive LN at level 1 (*gray circle*). In case 11, there was one histological LN metastasis at level 1 of 21 LNs (Fig. 3*d*). There were five RT-PCR-positive and two immunohistochemically positive LNs near the tumor. These results are summarized in Table 3, together with those of other patients.

Spread of Micrometastasis and Disease Recurrence

We then analyzed the frequency of LN metastases at adjacent LNs (level 1) *versus* distant LNs (levels 2 and 3; Fig. 4, *A* and *B*). By histological diagnosis, cases 7–11 were node positive, and cases 9 and 10 had LN metastases at a distant level at a frequency of approximately 20% (Fig. 4*A*). On the other hand, the percentage of RT-PCR-positive LNs ranged from 0–77.4% in total (Fig. 4*B*). In particular, cases 3 and 10 had RT-PCR-positive LNs at no less than 70% of regional LNs. The results also showed that RT-PCR-positive LNs often spread out to the distant level (cases 3–5 and 7–10). There was more apparent difference in the positive results at the distant level between cases 3 and 10 and the other cases. Thus, cases 3 and 10 had >75% RT-PCR-positive LNs at levels 2 and 3, whereas the other cases had no more than 50% RT-PCR-positive LNs at levels 2 and 3. The follow-up study indicated that cases 3 and 10 had a rapid recurrence within 1 year, whereas the others did not. Case 3 developed peritoneal recurrence 4 months after surgery, and case 10 had lung metastasis and local recurrence at inguinal LNs 9 months after surgery.

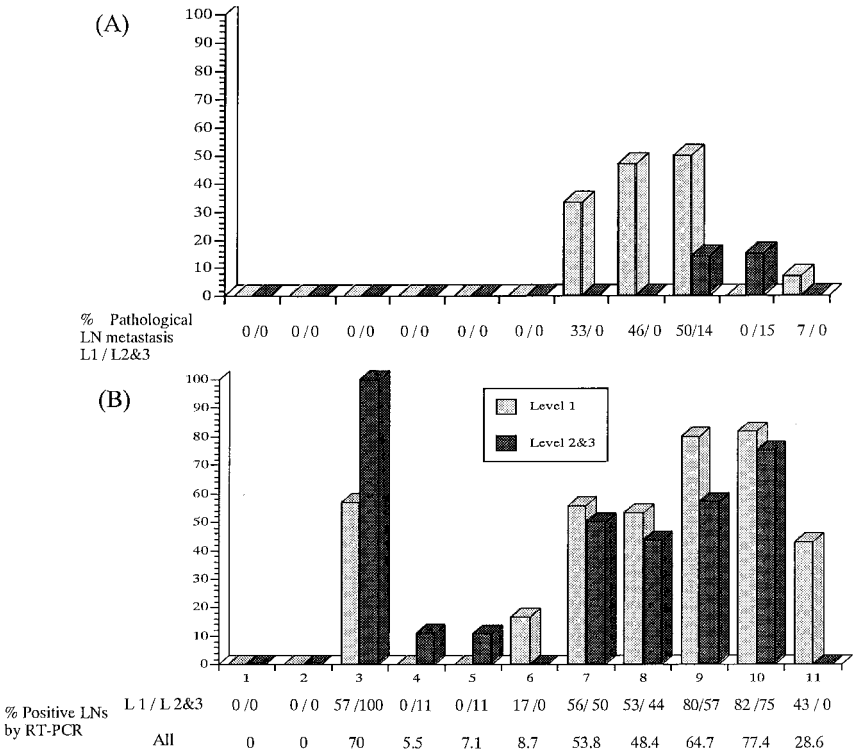

Fig. 4 Frequency of LN metastases at adjacent LNs (level 1) *versus* at distant LNs (levels 2 and 3) by pathological diagnosis (*A*) and RT-PCR assays (*B*). *A*, cases 1–6 were node-negative patients, and cases 7–11 were node-positive patients. Cases 9 and 10 had LN metastases at distant level at a frequency of approximately 20%. *B*, percentage of genetically positive LNs ranging from 0–77.4%. Cases 3 and 10 had genetically positive LNs at no less than 70% of the total regional LNs examined and had RT-PCR positive LNs at >75% at levels 2 and 3.

DISCUSSION

It is known that even in node-negative patients, about 10–20% of patients suffer from relapse in less than 5 years (27). Although micrometastasis is one candidate to be the reason for this phenomenon, the value of micrometastasis in node-negative colorectal cancer remains controversial. In Dukes' A and B stage tumors, immunohistochemical studies by several investigators showed that no relationship between the existence of micrometastasis and unfavorable prognosis (4, 6–8), although Greenson *et al.* (5) reported a significant correlation.

On the other hand, using the techniques of molecular genetics, only two groups have thus far suggested a positive correlation between the existence of micrometastases and poor prognosis in patients with histologically node-negative colorectal cancer. The first study targeted alterations of DNA within regional LNs, *i.e.*, mutations of the K-*ras* and *p53* genes. This method is certainly a useful strategy in selecting patients with high risk for disease recurrence. However, we and other investigators have revealed that fragmented DNA derived from the main tumor can be detected even in the serum of patients with various types of cancer (28, 29). Therefore, there is concern that mutated DNA found in the regional LNs might be a fraction of free tumor DNA rather than being derived from viable cancer cells within the LNs. Another limitation of DNA-based methods is that mutations of K-ras or p53 are found in subsets of colorectal carcinomas, but not in all cases. To avoid these problems, RT-PCR assays have subsequently been applied because mRNA is extremely unstable, thereby warranting that tumor cells detected by RT-PCR are very likely to be viable. Liefers *et al.* (11) reported that micrometastasis to LNs is a sensitive marker for poor prognosis in 26 patients with stage II node-negative colorectal cancers by using RT-PCR with CEA as a genetic marker. To confirm their results, many prospective studies with larger population samples are currently underway worldwide, but it will still take several years before any firm conclusion can be made.

The best advantage of the RT-PCR assay is that it allows examination of a large bulk of LNs compared to H&E staining and immunohistochemistry. Indeed, the number of LNs showing micrometastases diagnosed by RT-PCR was about four times that detected by immunohistochemistry (57 *versus* 14 of 217 node-negative LNs). To ensure true positive results by RT-PCR assay, we paid great attention during experiments and sampling. CEA and CK20 transcripts used as targets for amplification of PCR are expressed in both normal epithelial cells and colorectal cancer cells. This fact necessitated a strict protocol that avoided contamination by epithelial cells. Every time an individual LN was cut from the fat tissues and cut into halves, the knife was sterilized with ethanol to avoid contamination with cancer cells, as recommended by our previous study on sampling conditions (19). Amplification cycles of PCR for CEA and CK20 transcripts were restricted to <40 by the appearance of a few false positive samples in normal control LNs. From the data shown in Table 2, it is calculated that the number (and percentage) of positive LNs by both CEA and CK20, CEA alone, and CK20 alone is 33 (57.9%), 16 (28.1%), and 8 (14%), respectively. These results suggest that although approximately 60% of the nodes were judged as positive by both CEA and CK20, CEA may be a more sensitive marker to reveal micrometastasis than CK20. In Table 3, the RT-PCR assay tended to show a higher

sensitivity in the majority of LNs when compared to immunohistochemistry. However, there was a contrary situation in six LNs of cases 4–6. Thus, immunohistochemistry showed positive results, although RT-PCR showed negativity. This could be attributable to an uneven distribution of cancer cells, which has been pointed out in other works (14). Thus, there is a possibility that cancer nests may exist in only half of the LN and not in the other half.

The major feature of the present study was the analysis of the clinical significance of RT-PCR-based diagnosis by anatomical mapping of regional LNs. This strategy should help in revealing the extent of spread of carcinoma cells. We use "prophylactic" LN dissection during surgery for colorectal cancer. Thus, we usually dissect a distant level of LNs that look free from metastasis to keep a safe surgical margin. The present mapping study showed that occult carcinoma cells spread to the distant LNs much more than we had expected through pathological diagnosis (Fig. 4). From a surgical point of view, these findings are of importance and support the significance of prophylactic LN dissection in curative operation on colorectal cancer.

Another aspect of the LN of colorectal cancer is its role as an indirect indicator of disease progression and poor prognosis. However, it is not known whether micrometastasis would provide additional value on the pathological diagnosis. Among the node-negative patients, case 3 showed positive results with the RT-PCR assay (Fig. 4*B*, 70% positive), and he developed a rapid recurrence 4 months after surgery. Lymphatic mapping showed that micrometastasis spread out from adjacent LNs to those around the root of the inferior mesenteric artery (Fig. 3*b*). It was noteworthy that the main tumor in this patient showed no specific features. Thus, tumor size was 2.4 cm in diameter, histological type was moderately differentiated adenocarcinoma, and depth of invasion was limited to within the subserosa. Therefore, we were very surprised to find extensive peritoneal dissemination at the reoperation performed for sustained ileus. In this case, it is apparent that genetic diagnosis was the best tool in predicting poor prognosis, and such an unfavorable event could not be predicted based on conventional clinical and pathological information.

Among the node-positive patients, RT-PCR-based LN metastasis also showed some value in predicting a rapid recurrence in case 10. This patient had highly extended micrometastasis that was 77.4% positive by RT-PCR, and, notably, a more evident difference between case 10 and other node-positive patients was found in the incidence of micrometastasis at distant LNs (Fig. 4*B*, see levels 2 and 3; 75% *versus* 50%, 44%, 57%, and 0%) rather than adjacent LNs. High incidence of micrometastasis at a distant level was also noted in case 3. By contrast, although a high frequency of micrometastasis at level 1 was similarly noted in cases 9 and 10, case 9 did not have recurrence. These findings suggest that a high frequency of micrometastasis at distant LNs may be a better marker of poor prognosis than assessment using adjacent LNs.

It is probable that cases with LN metastasis other than case 10 will develop recurrence in the future because node positivity is a powerful prognostic parameter for colorectal cancer. However, RT-PCR is still useful because it could predict a shorter disease-free period in case 10. One of the reasons for the rapid recurrence in this case is probably the histological type of poorly differentiated adenocarcinoma. This type of colon cancer is known to be extremely aggressive, thus carcinoma cells may rapidly invade the surrounding tissues and spread easily to LNs. It is probable that micrometer-sized poorly differentiated carcinoma cells of case 10 spread out from the primary tumor to distant-level LNs and were effectively detected by RT-PCR, but not by H&E staining. It is therefore suggested that RT-PCR is useful to detect promptly expanding cancer cells, even before they form large nests enough to be detectable by histological examination.

In conclusion, the results of the present study suggest that genetic diagnosis using the RT-PCR method may provide additive value to histopathological examination in predicting rapid recurrence, especially when micrometastasis spreads out extensively from the main tumor to distant LNs.

REFERENCES

1. Lopez, M. J., and Monafo, W. W. Role of extended resection in the initial treatment of locally advanced colorectal carcinoma. Surgery (St. Louis), *113:* 365–372, 1993.

2. Taat, C. W., Laschot, J. J., Gouma, D. J., and Obertop, H. Role of extended lymph node dissection in the treatment of gastrointestinal tumors: a review of the literature. Scand. J. Gastroenterol., *30* (Suppl. 212*):* 109–116, 1995.

3. Adachi, Y., Mori, M., Matsushima, T., Kido, A., Shimono, R., Inoue, T., and Sugimachi, K. The distribution of lymph node metastases in right-sided colon cancer. J. Clin. Gastroenterol., *19:* 210–243, 1994.

4. Cutait, R., Alves, V. A., Lopes, L. C., Cutait, D. E., Borges, J. L., Singer, J., da Silva, J. H., and Goffi, F. S. Restaging of colorectal cancer based on the identification of lymph node micrometastases through immunoperoxidase staining of CEA and cytokeratins. Dis. Colon Rectum, *34:* 917–920, 1991.

5. Greenson, J. K., Isenhart, C. E., Rice, R., Mojsisik, C., Houchens, D., and Martin, E. W. Identification of occult micrometastases in pericolic lymph nodes of Duke's B colorectal cancer patients using monoclonal antibodies against cytokeratin and CC49. Correction with long-term survival. Cancer (Phila.), *73:* 563–569, 1994.

6. Jeffers, M. D., O'Dowd, G. M., Mulcahy, H., Stagg, M., O'Donighue, D. P., and Toner, M. The prognostic significance of immunohistochemically detected lymph node micrometastases in colorectal carcinoma. J. Pathol., *172:* 183–187, 1994.

7. Adell, G., Boeryd, B., Franlund, B., Sjodahl, R., and Hakansson, L. Occurrence and prognostic importance of micrometastases in regional lymph nodes in Duke's B colorectal carcinoma: am immunohistochemical study. Eur. J. Surg., *162:* 637–642, 1996.

8. Oberg, A., Stenling, R., Tavelin, B., and Lindmark, G. Are lymph node micrometastases of any clinical significance in Dukes stages A and B colorectal cancer. Dis. Colon Rectum, *41:* 1244–1249, 1998.

9. Hayashi, N., Ito, I., Yanagisawa, A., Kato, Y., Nakamori, S., Imaoka, S., Watanabe, H., Ogawa, M., and Nakamura, Y. Genetic diagnosis of lymph-node metastasis in colorectal cancer. Lancet, *345:* 1257–1259, 1995.

10. Nakamori, S., Kameyama, M., Furukawa, H., Takeda, O., Sugai, S., Imaoka, S., and Nakamura, Y. Genetic detection of colorectal cancer cells in circulation and lymph nodes. Dis. Colon Rectum, *40* (Suppl)*:* S29–S36, 1997.

11. Liefers, G. J., Cleton-Jansen, A. M., van de Velde, C. J., Hermans, J., van Krieken, J. H., Cornelisse, C. J., and Tollenaar, R. A. Micrometastases and survival in stage II colorectal cancer. N. Engl. J. Med., *339:* 223–228, 1998.

12. Gusterson, B. A., and Ott, R. Occult axillary lymph-node micrometastases in breast cancer. Lancet, *336:* 434–435, 1990.

13. International (Ludwig) Breast Cancer Study Group. Prognostic importance of occult axillary lymph node micrometastases from breast cancers. Lancet, *335:* 1565–1568, 1990.

14. Isozaki, H., Okajima, K., and Fujii, K. Histological evaluation of lymph node metastasis on serial sectioning in gastric cancer with radical lymphadenectomy. Hepatogastroenterology, *44:* 1133–1136, 1997.

15. Noguchi, S., Hiratsuka, M., Furukawa, H., Aihara, T., Kasugai, T., Tamura, S., Imaoka, S., Koyama, H., and Iwanaga, T. Detection of gastric cancer micrometastases in lymph nodes by amplification of keratin 19 mRNA with reverse transcriptase-polymerase chain reaction. Jpn. J. Cancer Res., *87:* 650–654, 1996.

16. Noguchi, S., Aihara, T., Motomura, K., Inaji, H., Imaoka, S., and Koyama, H. Detection of breast cancer micrometastases in axillary lymph nodes by means of reverse transcriptase-polymerase chain reaction. Am. J. Pathol., *148:* 649–656, 1996.

17. Matsumura, M., Niwa, Y., Kato, N., Komatsu, Y., Shiina, S., Kawabe, T., Kawase, T., Toyoshima, H., Ihori, M., Shiratori, Y., and Omata, M. Detection of α-fetoprotein mRNA, an indicator of hematogenous spreading hepatocellular carcinoma, in the circulation: a possible predictor of metastatic hepatocellular carcinoma. Hepatology, *20:* 1418–1425, 1994.

18. Mori, M., Mimori, K., Inoue, H., Barnard, G. F., Tsuji, K., Nanbara, S., Ueo, H., and Akiyoshi, T. Detection of cancer micrometastases in lymph nodes by reverse transcriptase-polymerase chain reaction. Cancer Res., *55:* 3417–3420, 1995.

19. Futamura, M., Takagi, Y., Koumura, H., Kida, H., Tanemura, H., Shimokawa, K., and Saji, S. Spread of colorectal cancer micrometastases in regional lymph nodes by reverse transcriptase-polymerase chain reactions for carcinoembryonic antigen and cytokeratin 20. J. Surg. Oncol., *68:* 34–40, 1998.

20. Sobin, L. H., and Witedkind, C. H. UICC TNM Classification of Malignant Tumours, 5th ed. New York: Wiley-Liss, Inc., 1997.

21. Fujiwara, Y., Ooka, M., Sugita, Y., Sakita, I., Tamaki, Y., and Monden, M. Prevention of cross-contamination during sampling procedure in molecular detection for cancer micrometastasis. Cancer Lett., *153:* 109–111, 2000.

22. Takemasa, I., Yamamoto, H., Sekimoto, M., Ohue, M., Noura, S., Miyake, Y., Matsumoto, T., Aihara, T., Tomita, N., Tamaki, Y., Sakita, I., Kikkawa, N., Matsuura, N., Shiozaki, H., and Monden, M. Overexpression of CDC25B phosphatase as a novel marker of poor prognosis of human colorectal carcinoma. Cancer Res., *60:* 3043–3050, 2000.

23. Ciaparrone, M., Yamamoto, H., Yao, Y., Sgambato, A., Cattoretti, G., Tomita, N., Monden, T., Rotterdam, H., and Weinstein, I. B. Localization and expression of $p27^{KIP1}$ in multistage colorectal carcinogenesis. Cancer Res., *58:* 114–122, 1998.

24. Sabatini, E., Bisgaard, K., and Ascani, S. The EnVision™+ system: a new immunohistochemical method for diagnostics and research. Critical comparison with the APAAP, ChemMate™, CSA, LABC, and SABC techniques. J. Clin. Pathol. (Lond.), *51:* 506–511, 1998.

25. Miyake, Y., Fujiwara, Y., Ohue, M., Yamamoto, H., Sugita, Y., Tomita, N., Sekimoto, M., Shiozaki, H., and Monden, M. Quantification of micrometastases in lymph nodes of colorectal cancer using real-time fluorescence polymerase chain reaction. Int. J. Oncol., *16:* 289–293, 2000.

26. Finke, J., Fritzen, R., Ternes, P., Lange, W., and Dolken, G. An improved strategy and a useful housekeeping gene for RNA analysis for formalin-fixed, paraffin-embedded tissues by PCR. Biotechniques, *14:* 448–453, 1993.

27. Ratto, C., Sofo, L., Ippoliti, M., Merico, M., Bossola, M., Vecchio, F. M., Doglietto, G. B., and Crucitti, F. Accurate lymph-node detection in colorectal specimens resected for cancer is of prognostic significance. Dis. Colon Rectum, *42:* 143–154, 1999.

28. Fujiwara, Y., Chi, D. D., Wang, H., Keleman, P., Morton, D. L., Turner, R., and Hoon, D. S. Plasma DNA microsatellites as tumor-specific markers and indicators of tumor progression in melanoma patients. Cancer Res., *59:* 1567–1571, 1999.

29. Chen, X., Bonnefoi, H., Diebold, B. S., Lyautey, J., Lederrey, C., Faltin, T. E., Stroun, M., and Anker, P. Detecting tumor-related alterations in plasma or serum DNA of patients diagnosed with breast cancer. Clin. Cancer Res., *5:* 2297–2303, 1999.

Clinical Cancer Research

Extensive Micrometastases to Lymph Nodes as a Marker for Rapid Recurrence of Colorectal Cancer: A Study of Lymphatic Mapping

Yasuhiro Miyake, Hirofumi Yamamoto, Yoshiyuki Fujiwara, et al.

Clin Cancer Res 2001;7:1350-1357.