Immunohistochemical Assessment of Localization and Frequency of Micrometastases in Lymph Nodes of Colorectal Cancer[1]

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ABSTRACT

Purpose: **Micrometastases are often found in regional lymph nodes of colorectal cancer (CRC). The aim of this study is to examine the extent and distribution of such lymph nodes.**

Experimental Design: **We immunohistochemically assessed localization and frequency of micrometastases in 878 lymph nodes from 98 patients with CRC. The anatomical position of lymph nodes was defined as level 1 to level 3 according to distance from the main tumor.**

Results: **The frequency of micrometastasis increased through observation of the 4-μm-thick lymph node sections, from one to two to five slices. With five slices, micrometastasis was frequently and extensively present in 49.1, 35.7, and 53.3% patients of histologically node-negative patients, node-positive patients at level 1, and node-positive patients at level 2, respectively. We then assessed the value of the presence of micrometastasis in node-negative patients with regard to prognosis, but no significant impact was obtained. To examine the reproducibility of the results obtained with immunohistochemistry, serial sectioning (four consecutive slices at seven different levels) of lymph nodes was additionally performed in lymph nodes initially diagnosed as micrometastasis positive. Immunohistochemical detection revealed that the sectioning level highly affected the results.**

Conclusions: **Our results indicated frequent presence of micrometastasis in lymph nodes of CRC and that micrometastasis in node-negative CRC patients did not help in predicting the outcome, in part because of the limited reproducibility with immunohistochemistry.**

INTRODUCTION

Complete resection of cancer tissues is still the most important and effective treatment for patients with CRC,[3] and precise information on the expanse of cancer metastasis certainly helps clinicians in the overall management of cancer patients (1–3). Metastasis to regional LN is an important prognostic factor and used for staging of CRC and for clinical decision-making regarding the selection of the most appropriate treatment (4). Histological metastasis in regional LN is clinically identified through the examination of a few slices of H&E-stained sections as part of the routine pathological workup. Micrometastasis is defined as the presence of minimal cancer cells that pathological examination cannot detect (5). Occult cancer metastasis, represented by single cells or small clusters of tumor cells, has been examined by IHC, using CK or carcinoembryonic antigen, specific marker of epithelial cells (6–16). At present, it is believed that micrometastases exist in the LNs of patients with various types of human malignancies (6–29).

Although several studies showed the existence of micrometastases in LNs from CRC, little is known about their localization and frequency. Therefore, in the current study, we examined occult cancer cells in the LNs of 98 patients with CRC by IHC. This analysis was performed in relation to their anatomical position from the main tumor, *i.e.*, pericolic site (level 1), along intermediate named vascular trunks (level 2), and around the root of vascular trunks (level 3), for better analysis on cancer expansion (11, 16, 28).

Most of the early studies focused on node-negative patients, but micrometastasis is also frequently detected in patients with histopathologically node-positive CRC (25). In these advanced-stage cancers, little is known about the expansion of micrometastasis in relation to anatomical position, and the information is of interest from a surgical point of view. Therefore, we currently examined micrometastasis in both node-negative and node-positive patients with CRC.

Although micrometastasis is considered an important prognostic factor in N_0 gastric cancer (17–19), many immunohistochemical studies in N_0 CRC showed conflicting results and the conclusions were quite controversial (6–14). Because only one slice of LN section was examined to find occult cancer cells in the majority of the studies (6–10, 12, 13, 15), which might be

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[3] The abbreviations used are: CRC, colorectal cancer; CK, cytokeratin; IHC, immunohistochemistry; LN, lymph node.

one of the reasons for the controversial findings, we used five consecutive sections for 878 LNs in the current study; a total of 4390 sections were examined. A serial-sectioning study was additionally performed to investigate the reproducibility of the results of micrometastasis-positive LNs. This aspect has not been addressed in previous immunohistochemical studies.

Our current study is unique with regard to the above mentioned aspects. It provides detailed information on the distribution and frequency of micrometastases to LNs of CRC and may shed some light on the conflicting situation surrounding micrometastasis.

MATERIALS AND METHODS

Patients. Ninety eight patients with colorectal carcinoma were investigated. They underwent surgery at the Department of Surgery and Clinical Oncology, Graduate School of Medicine, Osaka University between 1989 and 1996; 55 were node-negative and 43 were node-positive patients. Both node-negative patients and node-positive patients were randomly selected from our database without other clinical and pathological information. The patients included 57 (58.2%) males and 41 (41.8%) females, with a mean age of 59.0 ± 8.8 years (mean ± SD; range, 33–80 years). Fifty-three tumors were located in the colon and 45 in the rectum, and they ranged in size from 0.8 to 12.0 cm (mean, 4.9 ± 2.2 cm). The majority (51.0%) of tumors were well-differentiated carcinomas, followed by moderately differentiated carcinomas (48.0%), and poorly differentiated carcinomas (1.0%). Clinical staging according to the tumor-node-metastasis (TNM) classification of UICC (Union International Contre le Cancer; Ref. 30) included 9 (9.2%) patients in Stage I, 46 (46.9%) patients in Stage II, and 43 (43.9%) patients in Stage III. The median postoperative follow-up period was 74.4 ± 40.9 months. None had preoperative chemotherapy or irradiation. Chemotherapy was applied after surgery in 11.1% of patients in Stage I, 37.0% in Stage II, and 65.1% in Stage III, using 5-fluorouracil or its derivatives, occasionally combined with mitomycin C.

Tissue Specimens. The resected surgical specimens (CRCs and regional LNs) were fixed in formalin, processed through graded ethanol, and embedded in paraffin. LNs (878) from 98 patients were used for H&E staining and IHC. The locations of the LNs were divided into three groups according to the distance from the main tumor, as follows: level 1, pericolic nodes adjacent to the tumor; level 2, intermediate nodes along the course of the main blood vessel (*e.g.,* inferior mesenteric artery); level 3, central nodes around the root of the main blood vessel (11, 16, 28). On the basis of these criteria, 43 node-positive cases were subgrouped into those with histological metastasis at level 1 ($n = 28$) and at level 2 ($n = 15$).

H&E Staining and IHC. Six consecutive slices of 4-μm-thick sections were mounted on Capillary Gap Plus microscope slides (BioTek Solutions; Santa Barbara, CA). They were deparaffinized in xylene and rehydrated. One section was stained with H&E, and the other five were subjected to IHC. Immunostaining was performed on the TechMate Horizon automated staining system (DAKO, Glostrup, Denmark), as described previously (31). Briefly, sections were subjected to heat antigen retrieval in 10 mM citrate buffer (pH 6.0; Ref. 32). After blocking with 1% BSA, the sections were incubated with the pancytokeratin monoclonal antibody AE1/AE3 (DAKO, Carpinteria, CA) for 30 min at a concentration of 1.0 μg/ml, which has been previously used for the detection of micrometastases (6, 7, 9, 16). They were then incubated with antimouse secondary antibody conjugated with dextran polylinker (EnVision plus; DAKO) for 30 min (33). Visualization of the signals was achieved in brown color with DAB (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 PBS instead of the primary antibody, as substitute for primary antibody to exclude false positive responses from nonspecific binding to IgG or from the secondary antibody. Two investigators (S. N., and H. Y.) examined all of the sections under a two-head microscope. Only cells that were diagnosed as definite carcinoma cells based on morphological features were considered micrometastasis. N. M., a professor in the pathology department, Osaka University, double-checked the results, and no discrepant results were identified.

Multiple-Sectioning Study. Ten LNs containing micrometastases were randomly selected and were subjected further to the multiple-sectioning study. Four consecutive sections in 4-μm thickness were cut at seven different sectioning levels, and a total of 28 slices/LN were prepared. They were immunostained with anti-CK antibody, as described above.

Statistical Analysis. The postoperative period was measured from the date of surgery to the date of the last follow-up or death. Survival was censored if the patient was still alive or died from other causes. Statistical analysis was performed using the Statview J-5.0 program (Abacus Concepts, Inc. Berkeley, CA). The Kaplan-Meier method was used to estimate cancer-specific survival, and the log-rank test was used to examine statistical significance. A Cox proportional hazards model was used to assess the risk ratio under simultaneous contribution from several covariates. The associations between the discrete variables were assessed using Fisher's exact test. Mean values were compared using the Mann-Whitney test. Ps < 0.05 denoted the presence of a statistically significant difference.

RESULTS

Microscopic Survey of Micrometastasis. The mean numbers of LNs/patient were 12.0 in the metastasis-free group, 6.2 in node-positive patients in the group at level 1, and 2.9 in node-positive patients in the group at level 2. Five serial sections of 878 LNs (total 4390 sections) were stained and examined for occult cancer cells under a microscope at a magnification of ×100. When brown-colored objects were found, their morphological features were then carefully observed with a higher magnification (×200). Occult cancer cells were located in the subcapsular sinus or paracortical sinus. Immunoreactivity for CKs was found in the cytoplasm of occult carcinoma cells. They showed morphological features of malignant cells, such as large nucleus and clear nuclear small body (Fig. 1*A*). We occasionally found a weak immunoreactive signal in spindle-shaped reticular cells, which were easily distinguished from cancer cells, but other components of the LN were not stained. The majority of

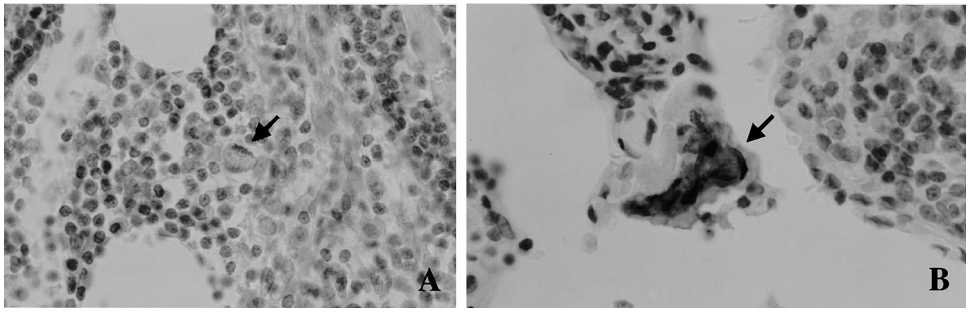

Fig. 1 Immunostaining with the pancytokeratin monoclonal antibody AE1/AE3; ×200. *A*, stained cells showed morphological features of malignancy, such as large nuclei and clear nuclear small body. The majority of occult carcinoma cells were single cells. *B*, however, they occasionally formed nests that consisted of several cancer cells.

Table 1 Detection rate of micrometastasis according to the section number

Section number	% of LN	% of cases
One slice	3.8% (33/878)	23.5% (23/98)
Two slices	6.3% (55/878)	36.7% (36/98)
Five slices	11.8% (104/878)	45.9% (45/98)

occult carcinoma cells were found as single cells (Fig. 1*A*), although they also occasionally formed a nest of several cancer cells (Fig. 1*B*).

We next compared the detection rate of micrometastasis by the section number (Table 1). Because the survey of five sections/LN gave superb detection rate for micrometastasis (11.8% of LNs and 45.9% of patients) compared with one- or two-slice sections, subsequent analysis was performed based on results obtained through examination of the five sections.

Localization and Frequency of Micrometastatic Cancer Cells. Profile of all patients with regard to distribution and frequency of LNs with micrometastasis is shown in Table 2. Micrometastasis was defined when it was found in LNs that were histologically negative for metastasis. LNs histologically positive for metastasis were not inspected for micrometastasis. The highest LN level with micrometastasis, number of occult cancer cells, existence type (single cell or cluster formation), and prognosis were also included. Table 3 summarizes the results with regard to patient number; Table 4 summarizes the results with regard to LN number. The analysis indicated that micrometastasis was found in 27 of 55 node-negative patients (49.1%), 10 of 28 patients with histological metastasis at level 1 (35.7%), and 8 of 15 patients with histological metastasis at level 2 (53.3%). In total, 45 (45.9%) of 98 patients proved to harbor micrometastasis in 11.8% of 878 LNs examined.

Relevance of Micrometastasis in Node-negative Patients. Clinical and pathological features were compared in node-negative patients between micrometastasis plus or minus groups. These parameters included age, gender, location of tumor (colon *versus* rectum), depth of invasion, tumor differentiation, lymphatic invasion, venous invasion, tumor size, and postoperative adjuvant chemotherapy. A significant correlation was found between the presence of micrometastasis and the depth of invasion ($P = 0.013$) or large tumor size ($P = 0.037$; Table 5). There was no significant correlation between the presence of micrometastasis and the other parameters (Table 5). Survival rates were then compared between the two categories, those with or without micrometastases. One case was excluded from this analysis because of death from another disease. As shown in Fig. 2, no significant difference was found in overall survival between the two groups.

In addition to micrometastases in LNs, various clinicopathological parameters were also evaluated for their impact on prognosis. None of the parameters were significantly associated with overall survival in node-negative CRC (Table 6). Multivariate Cox regression analysis was performed using the representative parameters from Table 6. In addition to micrometastasis in LNs, tumor differentiation, depth of invasion, tumor size, and venous invasion were selected as covariates. The results indicated that no significant covariates were found for overall survival (Table 7). When we analyzed Stage II CRC patients, no significant results were obtained by univariate or by multivariate analyses (data not shown).

Disease recurrence occurred within 5 years after surgery in 15 (27.3%) of 55 node-negative cases. These included systemic metastases to the liver, lung, bone, and brain. Local recurrences were also noted in the para-aortic LNs, pelvis, vagina, and peritoneum (Table 2A). When the incidence of disease recurrence was analyzed in relation to the presence of micrometastasis, no significant association was found (data not shown). Because the presence of micrometastasis itself did not seem to be of prognostic importance, we further explored other specific aspects associated with micrometastasis. The analyzed items included the number of LNs with micrometastasis, the percentage of LNs with micrometastasis, the anatomical site of LNs (levels 1, 2, or 3), the number of occult cancer cells, and the types of micrometastasis (none, single, or cluster; Table 2A). On the basis of these analyses, we found that the type of cluster formation was associated with a higher disease recurrence rate of 60.0% (3/5) than in the micrometastasis-free group (25.0%; 7/28) or single-cell type (22.7%; 5/22), but the number of patients displaying cluster formation was too small to allow proper statistical evaluation.

Reexamination of Positivity by Multiple Sectioning. To what extent would the staining of five sections warrant reproducibility as to positivity for micrometastasis? To test this question, 10 LNs positive for micrometastasis were randomly selected from the study and reexamined by making four serial sections at seven different sectioning levels (total, 28 slices; Fig. 3). The results showed that four LNs had micrometastasis in all of the 28 sections (Fig. 3, *Type A*), two LNs had micrometastasis in >50% of sections (Fig. 3, *Type B*: 18 and 17 of 28 sections, respectively), four LNs

Table 2 Localization and frequency of micrometastasis

Case no.	Site[a]	Depth[b]	Stage[b]	Level 1	Level 2	Level 3	Highest level[c]	No. of occult cancer cells	Existence type	Prognosis	Recurrence[d]	Recurrent site
						A. Node-negative patients						
1	S	T_1	I	0/3	0/0	N.E.	0	0	None	Alive	No	
2	R	T_3	II	0/3	0/3	0/1	0	0	None	Alive	No	
3	S	T_3	II	0/4	0/4	0/0	0	0	None	Alive	No	
4	R	T_1	I	0/3	0/0	0/0	0	0	None	Alive	No	
5	R	T_3	II	0/2	0/2	0/4	0	0	None	Alive	No	
6	T	T_3	II	0/10	0/2	0/1	0	0	None	Alive	No	
7	R	T_3	II	0/4	0/3	0/1	0	0	None	Alive	No	
8	S	T_3	II	0/8	0/2	0/0	0	0	None	Alive	No	
9	S	T_3	II	0/24	0/3	0/5	0	0	None	Alive	No	
10	S	T_1	I	0/5	0/6	0/2	0	0	None	Alive	No	
11	S	T_3	II	0/8	0/3	N.E.	0	0	None	Alive	No	
12	S	T_3	II	0/3	0/4	N.E.	0	0	None	Alive	No	
13	S	T_3	II	0/8	0/10	0/0	0	0	None	Alive	No	
14	R	T_1	I	0/1	0/4	0/2	0	0	None	Alive	No	
15	R	T_1	I	0/5	0/1	0/1	0	0	None	Alive	No	
16	R	T_3	II	0/7	0/8	0/3	0	0	None	Alive	No	
17	R	T_3	II	0/8	0/12	0/2	0	0	None	Alive	No	
18	S	T_3	II	0/10	0/3	0/2	0	0	None	Alive	No	
19	R	T_3	II	0/10	0/2	0/6	0	0	None	Alive	No	
20	R	T_3	II	0/8	0/2	0/2	0	0	None	Alive	No	
21	S	T_3	II	0/8	0/1	0/2	0	0	None	Alive	No	
22	S	T_1	I	0/4	0/0	0/1	0	0	None	Alive	Yes	Liver
23	R	T_2	I	0/3	0/2	0/1	0	0	None	Alive	Yes	Liver, lung
24	A	T_4	II	0/3	0/3	0/1	0	0	None	Alive	Yes	Liver, lung
25	T	T_4	II	0/4	0/1	0/0	0	0	None	Dead	Yes	Peritoneal recurrence
26	S	T_3	II	0/1	0/2	0/3	0	0	None	Dead	Yes	Liver, lung
27	S	T_1	I	0/2	0/0	N.E.	0	0	None	Dead	Yes	Peritoneal recurrence, lung
28	R	T_3	II	0/3	0/10	0/1	0	0	None	Dead	Yes	Local recurrence in pelvis
29	R	T_1	I	1/3	0/7	0/2	1	1	Single	Alive	No	
30	S	T_3	II	2/18	0/4	0/4	1	2	Single	Alive	No	
31	R	T_3	II	1/12	0/7	0/5	1	1	Single	Alive	No	
32	R	T_3	II	1/11	0/3	0/3	1	2	Single	Alive	No	
33	A	T_3	II	3/10	0/5	0/0	1	3	Single	Alive	No	
34	A	T_3	II	3/12	0/3	0/1	1	6	Single	Alive	No	
35	R	T_3	II	1/12	0/8	0/0	1	6	Single + cluster	Alive	No	
36	D	T_3	II	5/11	0/2	0/2	1	8	Single	Alive	No	
37	R	T_3	II	5/14	0/1	0/1	1	10	Single	Alive	No	
38	R	T_3	II	6/17	0/3	0/2	1	11	Single	Alive	No	
39	S	T_3	II	1/3	0/1	N.E.	1	2	Single	Alive	Yes	Bone
40	R	T_3	II	1/2	0/3	0/1	1	4	Cluster	Alive	Yes	Liver
41	S	T_3	II	1/4	0/1	0/1	1	1	Single	Alive	Yes	Local recurrence in pelvis
42	R	T_3	II	2/5	0/3	0/0	1	4	Single	Dead	Yes	Liver
43	R	T_3	II	2/7	0/3	0/0	1	7	Single + cluster	Dead	Yes	Bone
44	T	T_3	II	1/3	1/2	N.E.	2	4	Single	Alive	No	
45	S	T_3	II	1/4	1/3	0/0	2	2	Single	Alive	No	
46	S	T_3	II	2/6	3/6	0/2	2	19	Single	Alive	No	
47	S	T_3	II	3/4	1/2	0/1	2	8	Single	Alive	No	
48	R	T_3	II	4/10	1/12	0/0	2	5	Single	Alive	No	
49	S	T_3	II	3/10	2/3	0/2	2	10	Single	Alive	No	
50	R	T_3	II	3/4	4/5	0/4	2	28	Single + cluster	Dead	Yes	Para-aortic lymph node, bone, lung
51	A	T_4	II	1/8	1/6	0/2	2	2	Single	Dead	Yes	Liver, lung
52	A	T_3	II	3/8	0/2	1/2	3	62	Single + cluster	Alive	No	
53	R	T_3	II	2/7	1/3	1/2	3	5	Single	Alive	No	
54	S	T_3	II	0/3	0/9	1/6	3	2	Single	Alive	Yes	Liver
55	R	T_3	II	1/3	1/3	1/2	3	4	Single	Dead[e]	No	
						B. Node-positive patients at level 1						
1	S	T_2	III	N.E.	0/4	0/2	1	0	None	Alive	No	
2	S	T_2	III	N.E.	0/10	0/2	1	0	None	Alive	No	
3	S	T_3	III	N.E.	0/8	0/1	1	0	None	Alive	No	
4	S	T_3	III	N.E.	0/5	0/7	1	0	None	Alive	No	

Table 2 Continued

Case no.	Site[a]	Depth[b]	Stage[b]	Level 1	Level 2	Level 3	Highest level[c]	No. of occult cancer cells	Existence type	Prognosis	Recurrence[d]	Recurrent site
5	S	T_3	III	N.E.	0/4	0/1	1	0	None	Alive	No	
6	A	T_3	III	N.E.	0/2	0/2	1	0	None	Alive	No	
7	S	T_3	III	N.E.	0/10	0/1	1	0	None	Alive	No	
8	A	T_3	III	N.E.	0/0	0/2	1	0	None	Alive	No	
9	R	T_3	III	N.E.	0/1	0/2	1	0	None	Alive	No	
10	R	T_3	III	N.E.	0/1	0/2	1	0	None	Alive	No	
11	R	T_2	III	N.E.	0/0	0/1	1	0	None	Alive	No	
12	S	T_3	III	N.E.	0/4	0/2	1	0	None	Alive	Yes	Liver
13	R	T_3	III	N.E.	0/2	0/2	1	0	None	Death	Yes	Vagina
14	R	T_3	III	N.E.	0/1	0/4	1	0	None	Death	Yes	Lung, brain
15	R	T_3	III	N.E.	0/2	0/11	1	0	None	Death	Yes	Brain
16	R	T_3	III	N.E.	0/4	0/3	1	0	None	Death	Yes	Local recurrence in pelvis
17	S	T_3	III	N.E.	0/1	0/1	1	0	None	Death	Yes	Lung
18	S	T_2	III	N.E.	0/2	0/2	1	0	None	Death	Yes	Lung, liver
19	R	T_2	III	N.E.	1/5	0/2	2	2	Cluster	Alive	No	
20	S	T_2	III	N.E.	1/3	0/1	2	2	Single	Alive	No	
21	S	T_3	III	N.E.	1/7	0/2	2	1	Single	Alive	No	
22	S	T_3	III	N.E.	1/5	0/2	2	20	Single	Alive	No	
23	R	T_3	III	N.E.	2/6	0/1	2	2	Single	Alive	No	
24	R	T_4	III	N.E.	1/6	0/1	2	1	Single	Death	Yes	Local recurrence in pelvis
25	R	T_3	III	N.E.	1/3	0/1	2	4	Cluster	Death	Yes	Brain
26	C	T_3	III	N.E.	2/3	0/3	2	2	Single	Death[e]	No	
27	S	T_3	III	N.E.	1/3	1/2	3	6	Single + cluster	Alive	No	
28	T	T_3	III	N.E.	4/5	1/3	3	6	Single	Alive	No	
						C.	Node-positive patients at level 2					
1	R	T_2	III	N.E.	N.E.	0/5	2	0	None	Alive	No	
2	R	T_3	III	N.E.	N.E.	0/5	2	0	None	Alive	No	
3	S	T_3	III	N.E.	N.E.	0/3	2	0	None	Death	No	
4	D	T_3	III	N.E.	N.E.	0/1	2	0	None	Death	Yes	Liver
5	R	T_3	III	N.E.	N.E.	0/6	2	0	None	Death	Yes	Local recurrence in pelvis
6	S	T_3	III	N.E.	N.E.	0/2	2	0	None	Death	Yes	Lung, bowel anastomosis
7	R	T_3	III	N.E.	N.E.	0/2	2	0	None	Death[e]	No	
8	R	T_2	III	N.E.	N.E.	1/2	3	2	Single	Alive	No	
9	S	T_3	III	N.E.	N.E.	1/1	3	1	Single	Alive	No	
10	R	T_3	III	N.E.	N.E.	1/3	3	2	Single	Alive	No	
11	S	T_3	III	N.E.	N.E.	1/6	3	1	Single	Alive	No	
12	S	T_3	III	N.E.	N.E.	1/3	3	4	Cluster	Alive	No	
13	R	T_3	III	N.E.	N.E.	1/1	3	1	Single	Alive	No	
14	R	T_3	III	N.E.	N.E.	1/2	3	2	Single	Death	Yes	Local recurrence in pelvis, lung
15	R	T_3	III	N.E.	N.E.	1/1	3	2	Single	Death	Yes	Lung

[a] C, cecum; A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum; N.E., not examined.
[b] Grade/stage per UICC, International Union Against Cancer.
[c] The farthest level of micrometastasis.
[d] Recurrence within 5 years after surgery.
[e] Death from other diseases.

showed micrometastasis in <50% of the sections examined (Fig. 3, *Type C*: 12, 9, 6, and 5, of 28 sections, respectively). Fig. 3 shows a schematic distribution of occult cancer cells in each representative type. These findings strongly suggested that the positive results for micrometastasis could be negative in a subset of LNs according to the sectioning level.

DISCUSSION

It is important to know the distribution of micrometastasis in the regional LNs of CRC. Although it is believed at present that micrometastasis exists in LNs of CRC, little is known about localization and frequency of LNs with micrometastasis. This issue is important because surgeons should know the extent of invasion of cancer cells during surgery, even when such cells are few. In fact, in the past 120 years' history of our Surgery Department, we have performed LN dissection in patients with CRC without this information.

Table 3 summarizes the number of patients with micrometastasis. The percentage of patients positive for micrometastasis ranged from 35.7 to 53.3%. These results were far beyond what

Table 3 Relationship between histological LN metastasis and micrometastasis with regard to patient number

Status of histological LN metastasis (No. of patients)	No. of patients harboring micrometastasis			% micrometastasis
	Level 1	Level 2	Level 3	
Metastasis-free (n = 55)	15 (27.3%)	8 (14.5%)	4 (7.3%)	27 (49.1%)
Positive at level 1 (n = 28)	N.E.[a]	8 (28.6%)	2 (7.1%)	10 (35.7%)
Positive at level 2 (n = 15)	N.E.	N.E.	8 (53.3%)	8 (53.3%)
Total = 98				45 (45.9%)

[a] N.E., not examined.

Table 4 Relationship between histological LN metastasis and micrometastasis with regard to LN number

Status of histological LN metastasis (No. of LNs)	No. of micrometastasis-positive LNs/LNs examined			% micrometastasis
	Level 1	Level 2	Level 3	
Metastasis-free (n = 662)	59/373 (15.8%)	16/203 (7.9%)	4/86 (4.7%)	79/662 (11.9%)
Positive at level 1 (n = 173)	N.E.[a]	15/107 (14.0%)	2/66 (3.0%)	17/173 (9.8%)
Positive at level 2 (n = 43)	N.E.	N.E.	8/43 (18.6%)	8/43 (18.6%)
Total = 878				104/878 (11.8%)

[a] N.E., not examined.

Table 5 Relationship between micrometastasis in LNs and clinicopathological factors in node-negative patients

	Micrometastasis in LNs		
	Positive (n = 27)	Negative (n = 28)	P
Age (yr)	60.6 ± 8.5	59.0 ± 8.7	0.517
Gender			
Male	17 (63.0%)	19 (67.9%)	0.703
Female	10 (37.0%)	9 (32.1%)	
Tumor site			
Colon	14 (51.9%)	16 (57.1%)	0.694
Rectum	13 (48.1%)	12 (42.9%)	
Histological grade			
Well differentiated	14 (51.9%)	17 (60.7%)	0.508
Moderately differentiated	13 (48.1%)	11 (39.3%)	
Depth of invasion[a]			
~mp	1 (3.7%)	8 (28.6%)	0.013[b]
ss~	26 (96.3%)	20 (71.4%)	
Lymphatic invasion			
Absent	15 (55.6%)	17 (60.7%)	0.698
Present	12 (44.4%)	11 (39.3%)	
Venous invasion			
Absent	20 (74.1%)	25 (89.3%)	0.144
Present	7 (25.9%)	3 (10.7%)	
Tumor size (cm)	5.7 ± 1.7	4.3 ± 2.8	0.037[b]
Adjuvant chemotherapy			
Yes	10 (37.0%)	8 (28.6%)	0.504
No	17 (63.0%)	20 (71.4%)	

[a] mp, muscularis propria; ss, subserosa.
[b] Statistically significant.

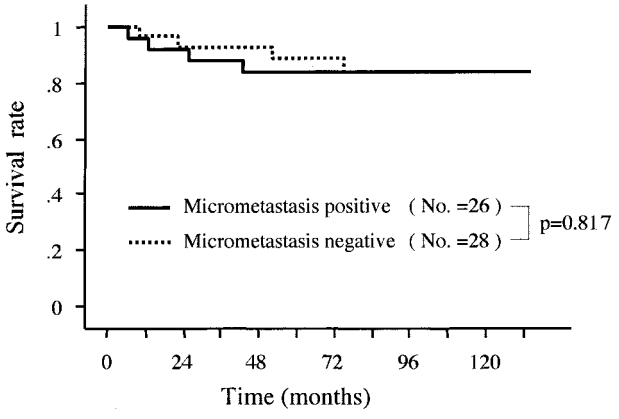

Fig. 2 Comparison of survival rates in patients with or without LN micrometastasis, in histopathologically node-negative patients. One case was excluded from 55 patients because of death of another disease. No significant difference in overall survival was found between patients with and without LN micrometastasis.

we had expected before the study, and they indicate that micrometastasis to regional LNs is a common event in CRC. Table 4 shows the distribution and frequency of micrometastases with regard to LN number. The results showed that even node-negative patients had micrometastasis not only in level 1 LNs, but also in LNs at levels 2 and 3, and the expansion to the more distant LNs was gradually reduced (from 15.8 to 7.9 to 4.7%; Table 4). These findings suggest that cancer cells invade LNs from the proximal LNs (relative to CRC) to distant LNs.

Prophylactic LN dissection up to level 2 or 3 is applied during surgery for CRC. The aim of this strategy is to ensure a safe surgical margin against cancer cells present in LNs. Thus, even when apparent node metastasis is not noted, we usually dissect LNs up to level 2 or mostly level 3, if the tumor is no longer a localized disease. According to the data shown in Table 2, prophylactic LN dissection appears to be effective in removing micrometastatic cancer cells. Thus, 27 of 55 node-negative patients, 10 of 28 patients with node metastasis at level 1, and 8 of 15 patients with node metastasis at level 2 (total 45 of 98 patients) eventually proved to harbor micrometastases within LNs resected as prophylactic dissection. At present, we are not certain whether these minimal cancer cells would have matured

Table 6 Univariate analysis of clinicopathological factors for overall survival in node-negative patients

Factors	*P*
Age (<60:≥60)	0.919
Gender (male:female)	0.301
Tumor site (colon:rectum)	0.664
Depth of invasion[a] (~mp:ss~)	0.831
Tumor differentiation[b] (well:mod)	0.050
Lymphatic invasion (yes:no)	0.156
Venous invasion (yes:no)	0.083
Tumor size (<5.0 cm:≥5.0 cm)	0.532
Adjuvant chemotherapy (yes:no)	0.557
Micrometastasis in LNs (positive:negative)	0.817

[a] mp, muscularis propria; ss, subserosa.
[b] well, well differentiated; mod, moderately differentiated.

Table 7 Multivariate Cox regression analysis of survival in node-negative patients

Factors	RR[a]	95% CI	*P*
Tumor differentiation[b] (well:mod)	4.073	0.792–20.957	0.093
Venous invasion (yes:no)	2.751	0.503–15.057	0.243
Depth of invasion[c] (~mp:ss~)	0.630	0.055–7.170	0.709
Tumor size (<5.0 cm:≥5.0 cm)	1.226	0.208–7.225	0.822
Micrometastasis in LNs (positive: negative)	1.031	0.226–4.716	0.968

[a] RR, risk ratio; CI, confidence interval.
[b] well, well differentiated; mod, moderately differentiated.
[c] mp, muscularis propria; ss, subserosa.

and formed cancer nests if the LN had not been excised. However, from a surgical point of view, it would be safe to perform prophylactic LN dissection, to remove such minimal cancer cells.

The current data showed a negative impact of micrometastasis on prognosis in node-negative patients. This is consistent with the reports by other investigators (6, 9, 10, 12), but different from those by Greenson *et al.* (7) and Isaka *et al.* (13). The reasons that the results have been mutually contradictive may be attributable to differences in study design, patient number, and statistical method. In addition, the IHC itself may confer additional reasons. IHC is a sensitive technique because it enables the detection of a single cancer cell among more than 1,000,000 lymphoid cells. However, it is difficult to apply IHC to clinical use because of the unstable results as suggested in the serial sectioning experiments (Fig. 3). When micrometastatic cells are found in a representative one slice, there are two possibilities; one is that micrometastasis would spread out all over the LN, thereby other slices should also display micrometastasis. Another is that micrometastasis is found on a slice by chance, and the other slices may not display occult cancer cells. The current data on reproducibility tests support the latter probability and suggest that prepared sections could lead to discrepant results as long as only a few slices are examined. This inherent feature of IHC may in part account for the past controversial conclusions.

Most of the previous studies used only one slice for searching for micrometastases in node-negative CRC, and the detection rate in the examined patients ranged from 21.4 to 39.0% (6–10, 12, 13). Here we used five serial sections/one LN. As shown in Table 1, apparently a higher rate of detection was obtained as the slice number increased from one to two to five. Our results showed that it was difficult to identify cancer cells using one slice only. Because the diameter of colon cancer cell is ~10–15 μm, part of cancer cells would often be cut into one 4-μm thick section. In such a case, although a brown-colored stain was present on the section, a definite diagnosis was very difficult to make because of the lack of the entire cell. Careful observation of adjacent slices at either side occasionally showed large stained cells with malignant morphology, convincingly enough that they were judged to be cancer cells and led to an increased detection rate. Because we obtained this high rate of patients with micrometastasis (~45%) through examination of 5 slices (a total of 20 μm in thickness), it is possible that the real rate of micrometastasis is much higher than thus far anticipated. Indeed, Yasuda *et al.* (14) examined five sections and obtained a high positive value of 32 (76.2%) of 42 Dukes' B stage patients. Furthermore, Sasaki *et al.* (11) examined 10 sections and obtained 100% positivity (19 of 19 Dukes' A and B stage patients).

Similar findings have been reported in cancers of the esophagus. The rate of micrometastasis is reported to be 36.6, 38.0, 40.0, and 50.0, in node-negative esophageal cancer (20–23). There is also a debate on whether micrometastasis could be a prognostic factor in this cancer type. It is probable that the clinical significance of micrometastasis differs among cancer types. Yet, we consider that micrometastasis may be so frequent in carcinomas of the colon and esophagus that discrete detection in abundance of occult cancer cells (*i.e.*, many or few) might be of greater importance than simply a presence or an absence. To support this hypothesis, we previously found that the number of cancer cells in LN samples devoid of histological metastasis from CRC varied widely, when measured by a real-time PCR method using carcinoembryonic antigen mRNA as a genetic marker (29).

Although our results failed to show any prognostic significance for micrometastasis in LNs of node-negative CRC, we believe that this phenomenon would be clinically important under such conditions that ensure strict reproducibility and that render quantification possible. Recently, we found that extensive micrometastases to LNs is indicative of rapid recurrence of CRC, when examined by reverse transcription-PCR, in a small set of prospective study (16). Using the techniques of molecular genetics, two groups of investigators have also suggested a positive correlation between the presence of micrometastases and poor prognosis in patients with node-negative CRC (26, 27). The best advantage of these molecular genetic techniques is that they allow examination of a large quantity of LNs; thereby, unstable features of IHC caused by the use of only a few slices can be avoided.

We also analyzed whether the distance from the main tumor to micrometastasis-containing LNs might be indicative of a poor prognosis of CRC patients including both node-negative and positive patients, but no clear correlation was obtained (data not shown). Although the distance from the main tumor to histological LN metastasis is definitely important as reported by Hermanek *et al.* (34), it is unlikely that micrometastasis in LNs would be a powerful indicator for poor prognosis, especially in node-positive CRC.

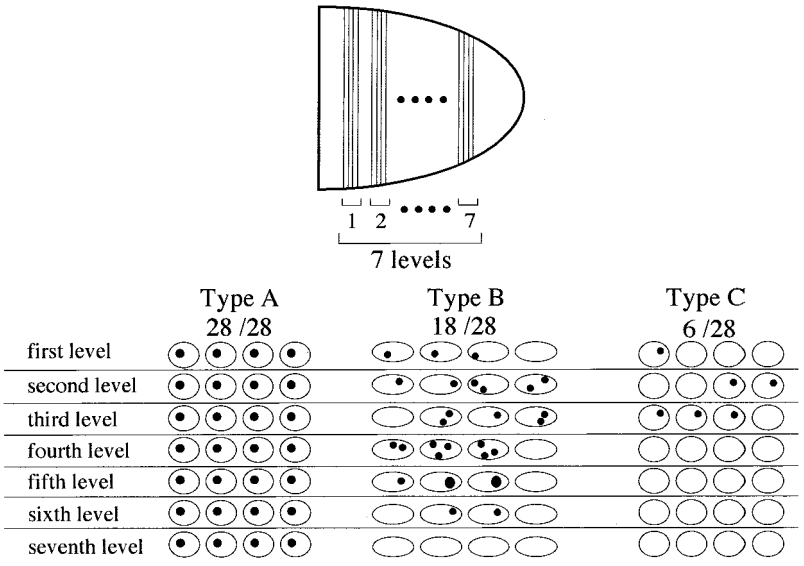

Fig. 3 Ten LNs positive for micrometastasis were randomly selected from the study and reexamined by making four serial sections at seven different sectioning levels (total, 28 slices). Four LNs contained micrometastatic cells in all of the 28 sections (*Type A*), two LNs had micrometastasis in >50% sections (*Type B*: 18 and 17 of 28 sections, respectively), four LNs showed micrometastasis at <50% sections examined (*Type C*: 12, 9, 6, and 5 of 28 sections, respectively).

In conclusion, we have demonstrated in the current study that micrometastases are frequently present in regional LNs of CRC. Although IHC is a useful method to find occult cancer cells, it may not be the best tool for clinical decision-making in patients with CRC.

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