Prognostic Significance of Perirectal Lymph Node Micrometastases in Dukes' B Rectal Carcinoma: An Immunohistochemical Study by CAM5.2

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ABSTRACT

Lymph node metastasis is an important prognostic factor for rectal carcinoma, but only a few attempts at defining the relationship between lymph node micrometastases and prognosis have been made. The purpose of this study was to examine the correlation between the presence of micrometastases and prognosis in patients with rectal carcinoma. Six hundred forty-four lymph nodes were dissected from 42 patients with Dukes' B rectal carcinoma and stained immunohistochemically using a monoclonal antibody, CAM5.2, that binds cytokeratin. Clinicopathological factors, rate of recurrence, and prognosis were compared among patients with and without micrometastases. Micrometastases were detected in 19 lymph nodes (19 of 644 = 2.9%) from 9 patients (9 of 42 = 21.4%). The presence of micrometastases was not related to clinicopathological factors. There were significant differences in recurrence rates (5 of 9 *versus* 5 of 33, $P = 0.02$), relapse-free survival rates ($P = 0.04$), and 10-year survival rates ($P = 0.03$) between patients with and without micrometastases. Immunohistochemistry successfully identified micrometastatic foci in lymph nodes missed with conventional staining methods. The existence of micrometastases influenced the prognosis in patients with Dukes' B rectal carcinoma.

INTRODUCTION

Lymph node metastasis is one of the most important prognostic factors in rectal carcinoma (1, 2). In Japan, the 5-year survival rate of patients with tumor invading through the muscularis propria and no lymph node metastasis (Dukes' B carcinoma) is 79%, but the survival rate of those with lymph node metastasis (Dukes' C) is only 52% (3). In the United States, the 5-year survival rates are 55 and 41% for those without and with lymph node metastases, respectively (4). What is the difference between the groups with and without recurrence in Dukes' B patients? This difference may be explained by the existence of early metastasis in the lymph nodes, which cannot be detected with conventional staining techniques, *i.e.*, H&E staining.

Immunohistochemical staining (5–12) and PCR (13, 14) can be used to detect micrometastases in lymph nodes of patients with gastroenterological carcinomas that are determined to be free of metastases with conventional staining. In esophageal (11) and gastric carcinoma (12), micrometastases in the lymph nodes have been useful for prognosis. However, in colorectal carcinoma, the clinical significance of micrometastases in the lymph nodes is controversial (15). The purpose of this study was to examine the rate of micrometastases in lymph nodes in Dukes' B rectal carcinoma patients and to evaluate the prognostic significance of lymph nodes micrometastases in patients with Dukes' B rectal carcinoma.

MATERIALS AND METHODS

Forty-two patients diagnosed with Dukes' B rectal carcinoma and treated at the Tsukuba University Hospital from 1977 to 1994 were the subjects of this study. All patients underwent low anterior resection or abdominoperineal resection with adjacent lymph node dissection. Twenty-four of 42 patients had taken oral 5-fluorouracil, although none had received radiation or intensive chemotherapy. The follow-up period ranged from 97 to 6303 days, and the median follow-up time was 5.2 years.

The tissues were fixed in 10% phosphate-buffered formalin for 48 h and embedded in paraffin. Serial sections of 3-μm thickness were prepared from tissue blocks containing the main tumor and the lymph nodes. These sections were stained with H&E for morphological observation. They were also stained immunohistochemically with CAM5.2 (Becton Dickinson, San Jose, CA), a murine monoclonal antibody that is specific for human keratin polypeptides corresponding to component numbers 8 and 18 (16), by an immunohistochemical method (17) using avidin-biotin-peroxidase complex and visualized by diaminobenzidine. CAM5.2 (12.5 μg/ml) as primary antibody was applied to the sections at room temperature for 45 min. Negative control sections for immunohistochemical staining were stained without the primary antibody. Tissue sections from the primary lesion were used as a positive control. Antigen retrieval was not applied in this study because a preliminary study using Dukes' C rectal carcinoma specimens did not show any difference between the staining with and without antigen retrieval by 0.1% trypsin (Wako, Osaka, Japan) for 12 min before incubation with CAM5.2 (data not shown). CAM5.2 has been reported to have a staining reaction to fibroblastic reticulum cells of

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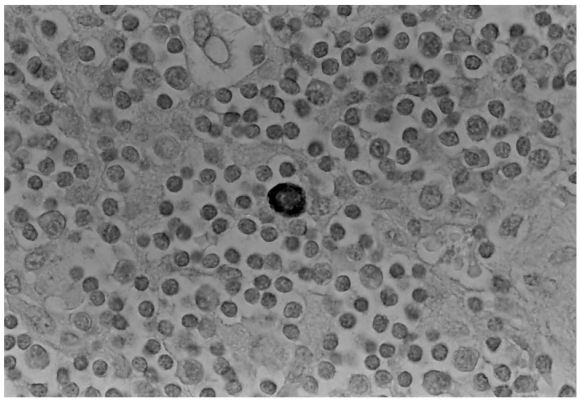

Fig. 1 A cytokeratin-positive tumor cell in a perirectal lymph node. Original magnification, ×200.

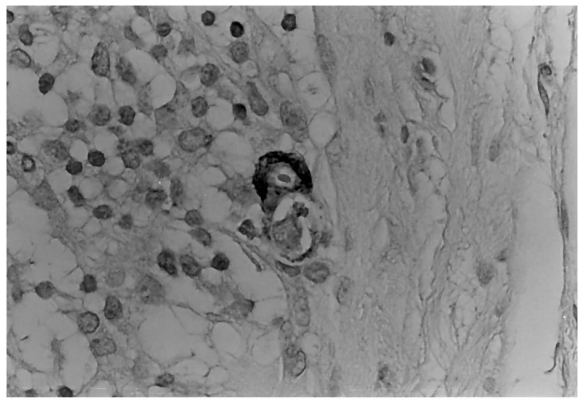

Fig. 2 A small cluster of cytokeratin-positive tumor cells in a perirectal lymph node. Original magnification, ×200.

human lymph nodes (18). Therefore, cell and nuclear features were observed closely in immunostained preparations so that misinterpretation would be avoided. Carcinoma cells positive for cytokeratin stained intensely in comparison with other components of the lymph nodes.

Statistical Analysis. Statistical correlation of categorical and parametric data were assessed by the χ^2 test, Fisher's exact test, and Wilcoxon rank-sum test. Kaplan-Meier actual statistics based on the life-table method were used to evaluate the relationship between micrometastases and disease outcome in 42 patients. Patients who died from other causes were excluded in the analysis of 10-year survival. Log-rank test was used for comparison of relapse-free survival rates and survival rates between the group with and without micrometastases. A P of <0.05 was accepted as statistically significant.

RESULTS

Micrometastases. Six hundred forty-four lymph nodes from 42 patients (mean, 15.3 ± 9.2 lymph nodes per case; range, 3–40 lymph nodes per case) with rectal carcinoma were examined after staining with the monoclonal antibody CAM5.2. Figs. 1 and 2 show a typical cytokeratin-positive tumor cell and cluster in the lymph node, respectively. Cytokeratin staining was positive in 19 lymph nodes (19 of 644 = 2.9%) from 9 patients (9 of 42 = 21.4%). Among the nine patients, clusters of tumor cells were detected in five patients, and a single tumor cell was identified in four patients. Among the 19 cytokeratin-positive lymph nodes, 18 lymph nodes (94.7%) were perirectal nodes, and 1 lymph node (5.2%) was a vascular trunk lymph node.

Clinicopathological Factors. Table 1 shows the clinicopathological features of 9 patients with cytokeratin-positive cells and 33 patients without those cells. The presence of micrometastases was not significantly related to age, sex, location of tumor, tumor invasion, histological differentiation, or surgical treatment (Table 1).

Recurrent Rate and Site. Ten of 42 patients (23.8%) had a recurrence. Five of 9 patients (55.6%) with micrometastases had a recurrence, whereas only 5 of 33 patients (15.2%) without micrometastases had a recurrence. This difference was statistically significant ($P = 0.02$, Table 2). With regard to the recurrent sites, there were no significant differences between the cytokeratin-positive and -negative groups (Table 2).

Regarding the relationship between recurrence and adjuvant chemotherapy, 5 of 10 patients (50.0%) with recurrence had taken oral 5-fluorouracil, whereas 19 of 32 patients (59.4%) without recurrence had taken it. There were no significant differences between the groups with or without recurrence.

Relapse-free Survival Rates. Fig. 3 shows Kaplan-Meier analysis of relapse-free survival in the micrometastasis-positive and -negative groups. There was a significant difference between the two groups ($P = 0.04$; Fig. 3).

Survival Rates. Four of nine patients with cytokeratin-positive lymph nodes died of rectal cancer recurrence, and one died as a result of a traffic accident. Four of 33 patients with cytokeratin-negative lymph nodes died of rectal carcinoma recurrence, and another four patients died of other diseases. Excluding the deaths due to unrelated causes, there was a significant difference in 10-year survival rate between the two groups ($P = 0.03$; Fig. 4).

DISCUSSION

This study suggests that anticytokeratin antibody can be used to identify micrometastases in rectal carcinomas that are missed with conventional staining techniques, *i.e.*, H&E staining. The advantage of immunohistochemistry for identifying micrometastases in perirectal lymph nodes has recently been reported in several colorectal carcinoma studies (5–8). Jeffers *et al.* (8) detected micrometastases in 5 of 25 rectal cancer patients (20%) by immunohistochemical staining for cytokeratin AE1:AE3. Broll *et al.* (7) detected micrometastases in 3 of 13 patients (23%) with rectal carcinoma and 10 of 36 (28%) patients with colon carcinoma. There was no difference in rates of micrometastasis between colon and rectal carcinoma. Our data showed a positive rate (21.4%) that was similar to that reported previously.

Some of the previous immunohistochemical studies showed that immunohistochemistry does not provide additional prognostic information in patients with colorectal carcinoma (5, 6, 8, 9). However, Broll *et al*. (7) showed that the presence of micrometastases detected by immunohistochemical techniques might increase the risk of recurrence but does not influence the prognosis. On the other hand, Greenson *et al*. (10) found a relationship between the presence of micrometastases in colorectal carcinomas and poor prognosis. The reason for these differences in results is unclear.

Table 1 Clinicopathological factors of 42 patients with Dukes' B rectal carcinoma

	No. of patients (%)		
	Micrometastases (n = 9)	No micrometastases (n = 33)	P
Median follow-up period (days)	1801	2015	NS[a]
Range	503–3065	97–6303	
Mean age (yr)	61.3	59.9	NS
Sex			NS
Male	7	23	
Female	2	10	
Location of tumor			NS
Rectosigmoid	4 (44.4)	6 (18.2)	
Upper rectum	4 (44.4)	16 (48.5)	
Lower rectum	1 (11.1)	11 (33.3)	
Tumor size[b]			NS
T_3	4 (44.4)	19 (57.6)	
T_4	5 (55.6)	14 (42.4)	
Tumor differentiation			NS
Well-differentiated	8 (88.9)	31 (93.9)	
Moderately differentiated	1 (11.1)	1 (3.0)	
Poorly differentiated	0 (0.0)	1 (3.0)	
Operation			NS
Anterior resection	7 (77.8)	16 (48.5)	
Abdominoperineal resection	2 (22.2)	17 (51.5)	

[a] NS, not significant.
[b] Tumor-node-metastasis classification.

Table 2 Location of recurrence after resection for Dukes' B rectal carcinoma

	No. of patients (%)		
	Micrometastases (n = 9)	No micrometastases (n = 33)	P
No recurrence	4 (44.4)	28 (84.8)	0.02
Recurrence	5 (55.6)	5 (15.1)	
Recurrent site			
Lung	2 (22.2)	3 (9.1)	NS[a]
Liver	0 (0.0)	3 (9.1)	NS
Local	1 (11.1)	1 (3.0)	NS
Dissemination	2 (22.2)	1 (3.0)	NS

[a] NS, not significant.

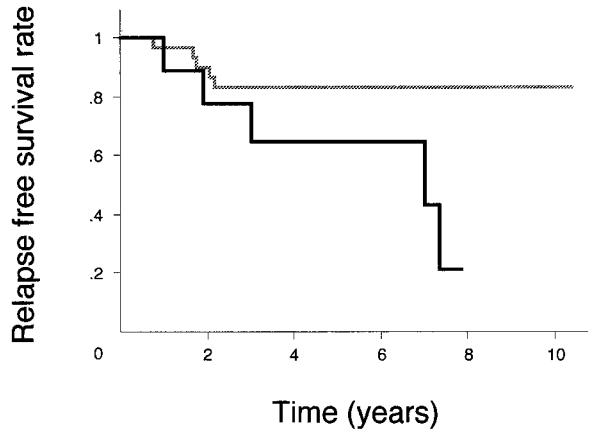

Fig. 3 Relapse-free survival curves of the patients with (*black line*) or without (*gray line*) perirectal lymph node micrometastases (Kaplan-Meier curves, log-rank test, P = 0.04).

There are two possible explanations for these differences. (*a*) Previous studies analyzed data from colon and rectal cancer although prognosis, recurrence patterns, and the clinical importance of lymph node metastasis are different between colon and rectal cancer (1, 2). Therefore, we gathered data from patients with rectal cancer but not from patients with colon cancer. Our data suggests that future large and prospective studies using immunohistochemical technique should distinguish between data from rectal cancer patients and from colon cancer patients. (*b*) The number of lymph nodes harvested from each patient appears to influence the results of the studies. Previous studies have shown that the proportion of lymph node metastasis-positive cases increased with the number of lymph nodes harvested but plateaued when the number of lymph nodes harvested exceeded 13 (19). A few immunohistochemical studies for micrometastases in patients with colorectal carcinomas harvested fewer than 10 lymph nodes per case, on average. Adell *et al.* (5) examined 4.7 nodes per case, and Jeffers *et al.* (8) investigated 7.3 nodes per case. The colorectal study by Greenson *et al.* (10) examined 11.3 nodes per case, and the data revealed poor prognosis for patients with lymph node micrometastasis. Our data were derived from 15.3 nodes per case and the data revealed poor prognosis for patients with lymph node micrometastasis. In addition, Haboubi *et al.* (20) reported similar results as he examined 41 cases of colorectal carcinoma and, on average, 55.1 lymph nodes per case using a combination of a clearing method and immunohistochemical staining technique with CAM5.2 (20). He reported 55% of patients with Dukes' B classification changed to Dukes' C. However, this report did not mention recurrence or survival rates. Liefers *et al.* (13) used PCR techniques to detect the mRNA of carcinoembryonic antigen, and the results were similar to those of Haboubi *et al.* (20) with immunohistochemical staining. Haboubi *et al.* (20) examined 7.4 lymph nodes

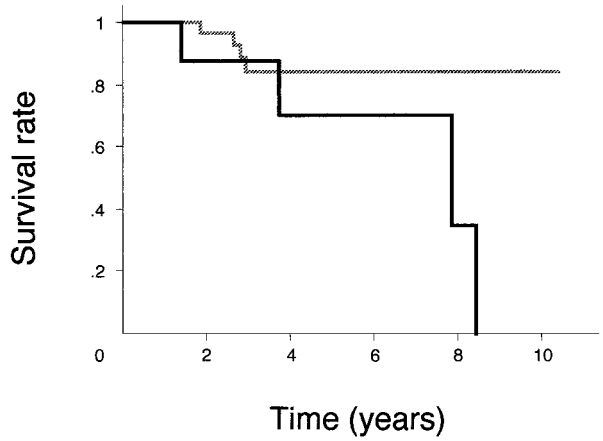

Fig. 4 Survival curves of the patients with (*black line*) or without (*gray line*) perirectal lymph node micrometastases (Kaplan-Meier curves, log-rank test, $P = 0.03$).

per case of patients with stage II colorectal carcinoma. Micrometastasis was detected in 14 of 26 cases (54%), and there was an association with prognosis. The sensitivity of this method, which was reported as one cell per 10^5 cells, was higher than the sensitivity of the immunohistochemical staining technique (21). This suggests that the greater the number of lymph nodes examined, the more micrometastases are detected. Only under this condition would a correlation between micrometastasis and recurrent or survival rate be found.

The survival data from our study did not show any significant differences at 5 years after operation but showed significant differences at 10 years after operation. According to the survival curves, both micrometastasis-positive and -negative groups showed a decrease in survival 3 years after operation. After three years, no recurrent death was observed until 7 years after operation when it appeared only in micrometastasis-positive group. There was a significant difference in survival between the two groups. This late recurrence may have been caused by micrometastasis because it takes a long time for a micrometastatic lesion to become life-threatening. Therefore, we believe that >5 years of follow-up would be necessary for appreciating the prognostic value of micrometastasis.

In conclusion, our data showed that the immunohistochemical technique using anticytokeratin antibody could identify micrometastases in perirectal lymph nodes. The presence of micrometastases increased the possibility of recurrence and led to poorer prognosis in patients with rectal cancer. Therefore, patients presenting cytokeratin-positive cells in lymph nodes must be closely monitored and treated appropriately.

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