

PROGNOSTIC VALUE OF IMMUNOHISTOCHEMICALLY IDENTIFIABLE TUMOR CELLS IN LYMPH NODES OF PATIENTS WITH COMPLETELY RESECTED ESOPHAGEAL CANCER

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ABSTRACT

Background Current methods of disease staging often fail to detect small numbers of tumor cells in lymph nodes. Metastatic relapse may arise from these few cells.

Methods We studied 1308 lymph nodes from 68 patients with esophageal cancer without overt metastases who had undergone radical en bloc esophagectomy. A total of 399 lymph nodes obtained from 68 patients were found to be free of tumor by routine histopathological analysis and were studied further for isolated tumor cells by immunohistochemical analysis with the monoclonal anti-epithelial-cell antibody Ber-EP4. This antibody did not stain lymph nodes from 24 control patients without carcinoma.

Results Of the 399 "tumor free" lymph nodes, 67 (17 percent), obtained from 42 of the 68 patients, contained Ber-EP4-positive tumor cells. Fifteen of 30 patients who were considered free of lymph-node metastases by histopathological analysis had such cells in their lymph nodes, and 5 of the 15 had small primary tumors. Ber-EP4-positive cells found in "tumor free" nodes were independently predictive of significantly reduced relapse-free survival ($P=0.008$) and overall survival ($P=0.03$). They predicted relapse both in patients without nodal metastases ($P=0.01$) and in those with regional lymph-node involvement ($P=0.007$). All 12 patients whose lymph nodes were negative on both histopathological and immunohistochemical analysis and who were available for follow-up survived without recurrence. The presence of micrometastatic tumor cells in bone marrow had no additional prognostic value.

Conclusions Immunohistochemical examination of lymph nodes may improve the pathological staging of esophageal cancer. (N Engl J Med 1997;337:1188-94.)

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ESOPHAGEAL cancer is an aggressive carcinoma with a poor prognosis. Although postoperative mortality has declined and rates of complete resection have improved considerably, reported rates of survival five years after surgery range from 20 to 36 percent.¹⁻⁵ Early metastatic relapse after the complete resection of an apparently localized primary lesion⁶⁻⁸ indicates that disseminated tumor cells, undetectable by current methods, may already have been present at the time of surgery.

Monoclonal antibodies against tumor-associated antigens or epithelial-cell proteins can be used to detect individual epithelial tumor cells in lymph nodes that are free of metastases on routine histopathological examination.⁹⁻¹² The clinical significance of these immunohistochemical assays is controversial, however,¹³⁻²¹ and they have not been used in patients with esophageal cancer. In view of the critical role of lymph-node metastases in such patients,^{2,6,11,22-24} we prospectively studied the clinical implication of immunohistochemically identifiable tumor cells in lymph nodes of patients with completely resected esophageal cancer.

METHODS

Patients and Study Design

This study was approved by the ethics committee of the chamber of physicians in Hamburg. Informed consent was obtained from all the patients before their inclusion in the study. Tumor samples, lymph nodes, and bone marrow aspirates of the upper iliac crest were collected from 71 consecutive patients with resectable esophageal cancer who had undergone radical en bloc esophagectomy between April 1992 and July 1995 and had tumor-free resection margins on microscopical examination of the surgical specimen. In each patient reconstruction was performed with a gastric tube with a cervical esophagogastric anastomosis. Tumor stage and grade were classified according to the fourth edition of the tumor-node-metastasis classification of the International Union against Cancer¹⁶ by investigators unaware of the immunohistochemical findings in the lymph nodes and bone marrow. Three patients were excluded from the study because they had evidence of liver metastases at surgery; thus, 68 patients were included. None received neoadjuvant or adjuvant therapy.

The patients were examined again on an outpatient basis every three months after esophagectomy for two years and at six-month intervals thereafter by physicians who had no knowledge of the immunohistochemical findings. These evaluations included a physical examination, plain chest radiography, endoscopy, endosonography, computed tomography of the chest and abdomen, abdominal ultrasonography, studies of tumor markers (squamous-cell carcinoma antigen, carcinoembryonic antigen, and CA 19-9), and bone scanning. All 68 study patients were available for follow-up. Five patients (three patients with no nodal involvement [pN₀]

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and two patients with regional nodal involvement [pN₁]) were excluded from the analyses of survival because they died in the hospital within 90 days after surgery (mortality rate, 7 percent).

Tissue Preparation and Immunohistochemical Analysis

Of 1308 resected lymph nodes, 680 were judged to be tumor-free by the surgeons. These nodes were systematically sampled during lymphadenectomy from lymph nodes in five locations (regional mediastinal lymph nodes both in the vicinity of the tumor and distant from it, perigastric lymph nodes, common hepatic lymph nodes, and lymph nodes at the celiac trunk). No tumor cells were found on routine histopathological examination in 399 of these 680 lymph nodes. All the nodes were mapped by the surgeon according to the scheme of the American Thoracic Society¹⁷ as modified by Casson et al.¹⁸

Each of the lymph nodes sampled was divided into two parts. One part was embedded in paraffin for routine histopathological staging and stained with hematoxylin and eosin; the other part and a representative sample of the primary tumor were snap-frozen in liquid nitrogen within three hours after their removal and stored at -80°C until use. Lymph nodes from patients in whom there was no evidence of nodal metastasis on routine histopathological examination were screened by immunohistochemical analysis with the anti-epithelial-cell monoclonal antibody Ber-EP4 (IgG1; Dako, Hamburg, Germany), which can be used on snap-frozen or paraffin-embedded material to detect isolated tumor cells, as described previously.¹⁰ Ber-EP4 is an antibody against two glycopolypeptides of 34 and 49 kd on the surface and in the cytoplasm of all epithelial cells (except parietal cells, hepatocytes, and the superficial layers of squamous epithelium). The antibody does not react with mesenchymal tissue, including lymphoid tissue.^{12,19} Cryostat sections 6 to 8 μm thick were cut at three different levels in each node and transferred onto glass slides treated with 3-triethoxysilylpropylamin (Merck, Darmstadt, Germany). One section of the sample obtained at each level was stained by the alkaline phosphatase-antialkalin phosphatase technique.¹⁰ We have found that lymph nodes from 24 control patients with nonepithelial tumors or inflammatory diseases consistently stained negative.¹⁰ Sections of normal colonic mucosa served as positive staining controls, and isotype-matched, irrelevant murine monoclonal antibodies served as negative controls (purified immunoglobulin mouse myeloma protein for IgG1; Sigma, Deisenhofen, Germany).

Aspirates of 4 to 8 ml of bone marrow from the iliac crest were obtained from all the patients before surgery and were processed as previously described.²⁰ The specimens were collected in heparin, and mononuclear cells, isolated by density-gradient centrifugation through Ficoll-Hypaque (Pharmacia, Freiburg, Germany) at 400×g for 30 minutes, were deposited onto glass slides by cytocentrifugation at 150×g for 3 minutes. To detect tumor cells in bone marrow, we used the monoclonal antibody A45-B/B3 (IgG1; Micromet, Munich, Germany), which detects an epitope on a variety of cytokeratin components, including cytokeratins 8, 18, and 19.²¹ The antibody reaction was developed with the alkaline phosphatase-antialkalin phosphatase technique combined with the new fuchsin stain (Sena, Heidelberg, Germany) for the visualization of alkaline phosphatase bound to the antibody.²⁰

The slides stained with hematoxylin and eosin and the immunostained slides were evaluated in a blinded fashion by two observers working independently. For 90 percent of the slides, the observers' evaluations were identical; the remaining slides were re-evaluated, and consensus decisions were made. Minimal tumor-cell involvement in a lymph node that was considered to be tumor-free by routine histopathological staining was defined as the presence of one to three Ber-EP4-positive cells in the body of the node (Fig. 1). In the 42 patients who had Ber-EP4-positive cells in histopathologically "tumor free" lymph nodes, two lymph-node sections adjacent to the one that contained the immunostained cells were prepared, stained with hematoxylin and eosin, and evaluated by a pathologist with no knowledge of the initial results.

Statistical Analysis

Associations between categorical variables were assessed by Fisher's exact test. The Kaplan-Meier method was used to estimate overall survival and survival free of local recurrence, distant metastases, and relapse. Point and interval estimates of the survival rates at 24 months were calculated. For comparison purposes, log-rank tests and exact stratified log-rank tests were performed. Cox proportional-hazards models were fitted for multivariate

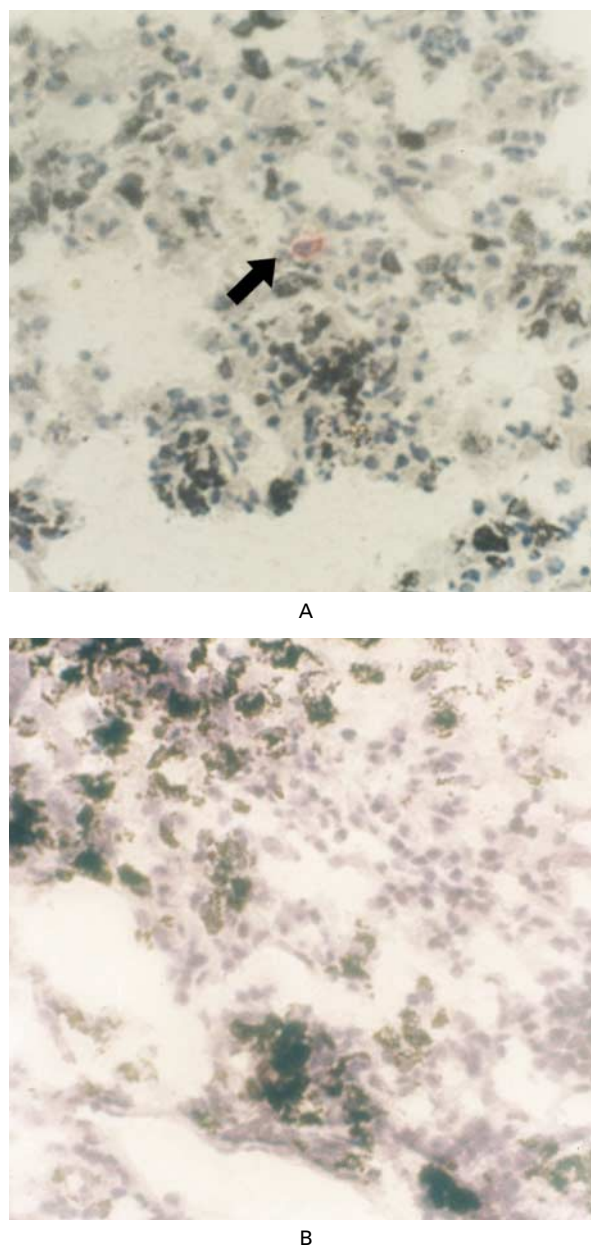


Figure 1. Detection of Ber-EP4-Positive Tumor Cells by Immunohistochemical Analysis.

Panel A shows a lymph-node section containing an isolated Ber-EP4-positive cell (arrow) (×400). Panel B shows an adjacent lymph-node section stained only by the standard hematoxylin-eosin method (×400).

analysis.²⁵ Differences between groups were considered statistically significant if the P values were less than 0.05 in a two-tailed test. The follow-up times were calculated according to the method proposed by Schemper and Smith.²⁶

RESULTS

The material used in the study was obtained from 68 patients with esophageal cancer (Table 1). The mean age was 57 years (range, 34 to 76). There were 14 women and 54 men. The location of the primary tumor was supracarinal in 30 patients (44 percent) and infracarinal in 38 (56 percent). Forty-nine tumors (72 percent) were classified as squamous-cell carcinoma, and 19 (28 percent) as adenocarcinoma (Table 1); in 12 of these 19 patients, the adenocarcinoma arose from Barrett's mucosa. The prevalence of squamous-cell carcinomas was slightly higher in our study than in others, in which such carcinomas accounted for about 60 percent of all tumors.²⁷⁻³⁰

The immunohistochemical assay was used to screen cryostat sections of all the primary tumors and 399 lymph nodes obtained from the 68 patients. All these nodes were "tumor free" on conventional histopath-

ological analysis. Sixty-five of the 68 primary tumors (96 percent) showed homogeneous staining with the Ber-EP4 antibody, and the remaining 3 (4 percent) had heterogeneous staining patterns. To study whether the Ber-EP4 antigen was lost from metastatic cells, lymph nodes from the first 25 consecutive patients who had regional nodal involvement that was classified as metastatic on routine staining with hematoxylin and eosin were stained with the Ber-EP4 antibody. A homogeneous staining pattern was found in all these specimens, indicating that metastatic cells retain the antigen bound by Ber-EP4.

Table 1 shows that in 42 of the 68 patients Ber-EP4-positive cells were found in lymph nodes that were considered to be tumor-free according to standard criteria. These Ber-EP4-positive cells or cell clusters (containing up to three cells) were found in the sinuses, the lymphoid interstitium, or both in 67 lymph nodes from the 42 patients. Immunostaining revealed tumor cells at one or two lymph-node levels in 24 patients and at more than two levels in 16. Four patients with histologically negative but Ber-EP4-positive lymph nodes had small primary tumors (Table 1), and one patient in this group had a carcinoma in situ. Table 1 indicates the tumor-staging nomenclature.

To compare immunostaining with conventional staining, two sections adjacent to the Ber-EP4-positive sections were stained with hematoxylin and eosin. In none of these sections were tumor cells detected by staining with hematoxylin and eosin, suggesting that greater sensitivity of the analysis rather than sampling error accounted for the higher rate of detection of tumor cells by the immunohistochemical method.

There was no correlation between the detection of Ber-EP4-positive cells in lymph nodes and other factors we studied, such as primary-tumor stage (T stage), pathological stage, tumor type, presence or absence of lymphovascular invasion, or histopathological grade (Table 1). All 25 patients who had isolated tumor cells in their bone marrow, as revealed by immunostaining with monoclonal antibody A45-B/B3 against cytokeratins, also had Ber-EP4-positive tumor cells in their lymph nodes (Table 1). However, lymph-node involvement was found with the Ber-EP4 antibody in 17 of the 43 patients who had negative bone marrow findings (Table 1).

The mode of spread of the Ber-EP4-positive cells into lymph nodes was erratic. Spatial progression of micrometastasis throughout the regional node levels was seen in only 50 percent of patients with Ber-EP4-positive cells. In the remaining patients, the micrometastases appeared to skip one or more lymph-node levels (data not shown).

After a median observation period of 21 months (range, 2 to 51) the presence of Ber-EP4-positive

TABLE 1. CHARACTERISTICS OF THE PATIENTS AND TUMORS.

VARIABLE	NO. OF PATIENTS	NO. (%) WITH BER-EP4-POSITIVE TUMOR CELLS IN LYMPH NODES
All patients*	68	42 (62)
Male	54	33 (61)
Female	14	9 (64)
Primary tumor		
Carcinoma in situ (pT _{is})	2	1 (50)
Invading the submucosa (pT ₁)	9	4 (44)
Invading the muscularis propria (pT ₂)	19	9 (47)
Invading the adventitia (pT ₃)	35	25 (71)
Invading contiguous structures (pT ₄)	3	3 (100)
Lymph nodes		
No nodal involvement (pN ₀)	30	15 (50)
Regional nodal involvement (pN ₁)	32	22 (69)
Distant nodal involvement (pM _(lymph))	6	5 (83)
Lymphovascular invasion	10	8 (80)
Tumor type		
Squamous-cell carcinoma	49	31 (63)
Adenocarcinoma	19	11 (58)
Tumor grade		
Well differentiated (G I)	1	1 (100)
Moderately differentiated (G II)	51	30 (59)
Poorly differentiated (G III)	16	11 (69)
Tumor localization		
Supracarinal	30	20 (67)
Infracarinal	38	22 (58)
Tumor cells in bone marrow†		
Yes	25	25 (100)
No	43	17 (40)‡

*A total of 399 histopathologically "tumor free" lymph nodes from 68 patients were screened with the monoclonal antibody Ber-EP4.

†These cells were detected immunocytochemically with monoclonal antibody A45-B/B3.

‡P<0.001 by Fisher's exact test for the comparison with patients with tumor cells in the bone marrow.

tumor cells in lymph nodes was associated with significantly reduced disease-free survival. The rate of relapse-free survival at two years was 68 percent in patients without Ber-EP4-positive cells and 25 percent in patients with such cells ($P < 0.001$) (Table 2 and Fig. 2A). Stratification according to pathological stage showed that the presence of Ber-EP4-positive lymph nodes predicted early relapse in both patients with no nodal involvement ($P = 0.01$) and those with regional nodal involvement ($P = 0.007$) (Table 2). A subgroup analysis of the prognostic influence of Ber-EP4-positive cells in patients with no nodal involvement, adjusted for tumor stage and grade by exact stratified log-rank tests for relapse-free survival, yielded results identical to those of the unstratified analyses ($P = 0.01$), indicating that Ber-EP4 status is an independent prognostic variable in patients without nodal involvement. Nevertheless, the small number of patients in this sub-

group analysis is an argument for cautious interpretation.

Ber-EP4-positive cells also had significant predictive value with regard to overall survival. The rate of overall survival was 68 percent in patients without Ber-EP4-positive tumor cells in their lymph nodes, as compared with 25 percent in patients with such cells ($P < 0.001$) (Table 2 and Fig. 2B).

There was a significant relation between the presence of Ber-EP4-positive tumor cells in lymph nodes and relapse with distant metastases, but not between the presence of such cells and local recurrence. Distant metastases occurred in 25 of 41 patients with Ber-EP4-positive tumor cells and 4 of 22 without these cells ($P < 0.001$) (Table 2). The additional presence of A45-B/B3-positive tumor cells in bone marrow had no further influence on relapse-free survival ($P = 0.66$ by the log-rank test) or overall survival ($P = 0.28$) (data not shown).

TABLE 2. PROGNOSTIC VALUE OF BER-EP4-POSITIVE TUMOR CELLS IN LYMPH NODES.*

VARIABLE	NO BER-EP4-POSITIVE CELLS	BER-EP4-POSITIVE CELLS	P VALUE†
Months of follow-up			
Median	25	19	
Range	2–51	2–49	
Overall survival			
All patients — no. of events/no. of patients (%)	6/22 (27)	26/41 (63)	
2-Yr survival rate (95% CI)	0.68 (0.46–0.89)	0.25 (0.09–0.41)	<0.001
pN ₀ tumors — no. of events/no. of patients (%)	0/12	3/14 (21)	0.06
pN ₁ tumors — no. of events/no. of patients (%)	6/10 (60)	18/22 (82)	0.03
Relapse-free survival			
All patients — no. of events/no. of patients (%)	6/22 (27)	29/41 (71)	
2-Yr relapse-free survival rate (95% CI)	0.68 (0.47–0.90)	0.25 (0.09–0.41)	<0.001
pN ₀ tumors — no. of events/no. of patients (%)	0/12	5/14 (36)	0.01
pN ₁ tumors — no. of events/no. of patients (%)	6/10 (60)	19/22 (86)	0.007
Local-recurrence-free survival			
All patients — no. of events/no. of patients (%)	4/22 (18)	11/41 (27)	
2-Yr event-free survival rate (95% CI)	0.78 (0.59–0.97)	0.58 (0.37–0.79)	0.08
pN ₀ tumors — no. of events/no. of patients (%)	0/12	2/14 (14)	0.16
pN ₁ tumors — no. of events/no. of patients (%)	4/10 (40)	7/22 (32)	0.49
Distant-metastasis-free survival			
All patients — no. of events/no. of patients (%)	4/22 (18)	25/41 (61)	
2-Yr event-free survival rate (95% CI)	0.78 (0.59–0.97)	0.23 (0.06–0.41)	<0.001
pN ₀ tumors — no. of events/no. of patients (%)	0/12	5/14 (36)	0.01
pN ₁ tumors — no. of events/no. of patients (%)	4/10 (40)	15/22 (68)	0.02

*The disease stages are defined in Table 1. CI denotes confidence interval.

†The log-rank test was used.

Cox regression analysis showed that Ber-EP4-positive cells in lymph nodes had independent prognostic importance for relapse-free survival (relative risk, 3.76; 95 percent confidence interval, 1.40 to 10.07; $P=0.008$) and overall survival (relative risk, 3.0; 95 percent confidence interval, 1.04 to 8.67; $P=0.03$). As expected, histopathological lymph-node stage was a strong independent prognostic factor for both clinical end points ($P<0.001$). Histologic tumor grade and lymphovascular invasion were also independent predictors of relapse-free survival ($P=0.01$) and overall survival ($P=0.04$) (Table 3). Primary-tumor stage and tumor type had no independent prognostic influence (Table 3).

None of the 12 patients without nodal involvement, as determined by both histopathological and immunohistochemical analysis, had tumor recurrences during the study period (Table 2). Four of the 12 patients had invasion of the adventitia, 3 had invasion of the muscularis propria, 4 had invasion of the submucosa, and 1 had a carcinoma in situ. The median observation period for these 12 patients was 21 months (range, 2 to 51), as compared with 19 months (range, 2 to 49) in the patients with immunohistochemical involvement and 26 months (range, 2 to 26) in those who were positive by both methods (P not significant).

DISCUSSION

Lymph-node metastasis identified on histopathological examination is the most important prognostic factor in patients with esophageal cancer.^{2,6,11,22-24} Our study provides evidence that isolated tumor cells or small clusters of cells, which can be detected in lymph nodes by immunohistochemical analysis, are independent prognostic factors in esophageal

TABLE 3. MULTIVARIATE COX REGRESSION ANALYSES OF THE 63 PATIENTS WHO SURVIVED MORE THAN 90 DAYS AFTER SURGERY.*

RISK FACTOR	RELATIVE RISK (95% CONFIDENCE INTERVAL)	P VALUE
Relapse-free survival†		
Lymph-node micrometastases — positive (41) vs. negative (22)	3.76 (1.40–10.07)	0.008
pN ₁ (37) vs. pN ₀ (26)	7.99 (2.79–22.90)	<0.001
Grade III (15) vs. grades I and II (48)	2.77 (1.27–6.04)	0.01
pT ₃ and pT ₄ (35) vs. pT ₁ and pT ₂ (28)	1.23 (0.53–2.82)	0.62
Lymphovascular invasion — positive (8) vs. negative (55)	2.52 (0.95–6.70)	0.06
Tumor type — adenocarcinoma (18) vs. squamous-cell carcinoma (45)	0.91 (0.42–1.93)	0.81
Overall survival		
Lymph-node micrometastases (positive vs. negative)	3.00 (1.04–8.67)	0.03
pN ₁ vs. pN ₀	12.21 (3.29–45.22)	<0.001
Grade III vs. grades I and II	1.33 (0.59–2.95)	0.48
pT ₃ and pT ₄ vs. pT ₁ and pT ₂	0.81 (0.32–2.02)	0.65
Lymphovascular invasion (positive vs. negative)	2.87 (1.05–7.82)	0.04
Tumor type (adenocarcinoma vs. squamous-cell carcinoma)	1.01 (0.45–2.24)	0.97

*The disease stages are defined in Table 1.

†The numbers of patients are shown in parentheses.

cancer. Remarkably, all the patients who were found to be free of nodal involvement by both histopathological and immunohistochemical analysis survived the median observation period of 21 months without recurrence. In contrast, patients in whom no lymph-node metastases were found by conventional means but who had immunostained tumor cells in their lymph nodes had outcomes similar to those of patients with histopathologically proved

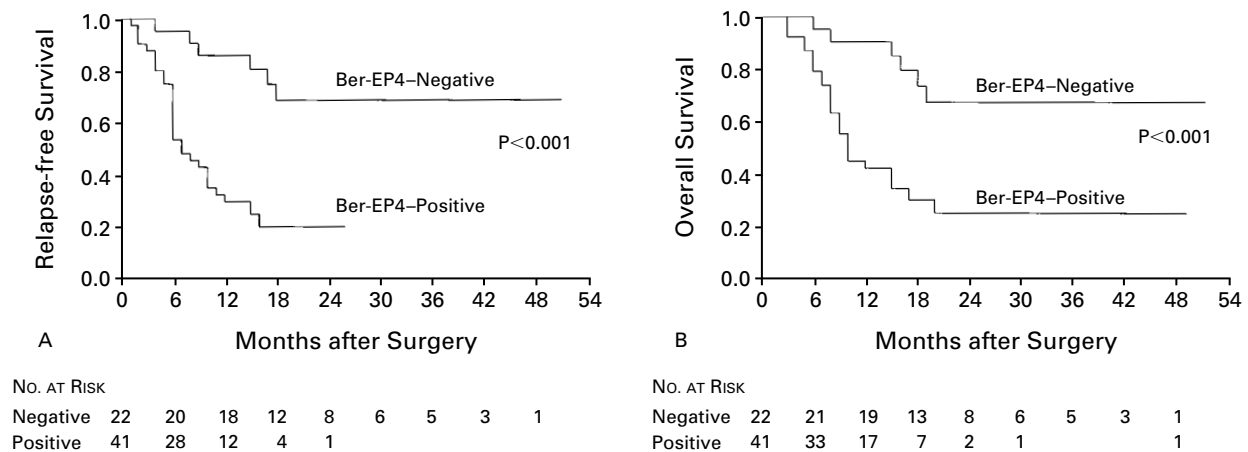


Figure 2. Kaplan-Meier Survival Curves for Patients with and without Ber-EP4-Positive Tumor Cells in Their Lymph Nodes. Panel A shows relapse-free survival, and Panel B overall survival.

lymph-node metastases. Moreover, patients with regional nodal involvement who had immunostained tumor cells in other lymph nodes that were found to be tumor-free by standard analyses had shorter relapse-free and overall survival than similar patients without such cells. This surprising finding may be explained by the total load of residual tumor cells, a potential source of subsequent metastatic relapse.

The predisposition to have distant metastases among patients with Ber-EP4-positive tumor cells in their lymph nodes suggests that these cells are the consequence of advanced tumors, rather than indicators of sites of subsequent relapse. According to Paget's seed-and-soil hypothesis, the growth of disseminated tumor cells into overt metastases is influenced by the environment into which the cells have been seeded. The presence of growth factors in the local environment and the capacity of isolated tumor cells to respond to them may determine the pattern of relapse.³¹ In this context, it is interesting that lymphovascular infiltration did not correlate with the detection of Ber-EP4-positive tumor cells in the lymph nodes, which suggests that not all tumor cells that gain access to lymphatic vessels become established in the draining lymph nodes. Squamous-cell carcinoma and adenocarcinoma have different biologic features, but both have a similar potential for dissemination into secondary organs.¹⁰

Lymph-node micrometastasis has been assessed in breast cancer by the histopathological examination of numerous consecutive sections.³² This time-consuming method is not practical as a routine procedure. Recently, our group showed that immunohistochemical analysis with monoclonal antibody Ber-EP4 is a sensitive and specific method for detecting isolated lung-cancer cells or clusters of cells in lymph nodes.¹⁰ In the present study, sections were cut from only three levels of the lymph node, which could introduce a sampling error. Analyzing more than three sections, however, would not be routinely feasible. The positive correlation between the result of our assay and the prognosis of patients indicates that examining three levels is sufficient.

Immunohistochemical assessment of tumor-cell dissemination into bone marrow³³ may be useful before preoperative chemotherapy or radiotherapy. Immunocytochemical assays with the monoclonal and anti-cytokeratin antibody A45-B/B3 are more sensitive than flow-cytometric analysis with monoclonal antibody CK2 against cytokeratin 18, which is not frequently expressed in esophageal-cancer cells.^{33,34} Our data suggest that there is dissemination of tumor cells into lymph nodes before blood-borne spread, because only 60 percent of the patients we studied who had Ber-EP4-positive tumor cells in their lymph nodes also had immunohistochemically identifiable tumor cells in their bone marrow. The lack of a significant difference in relapse-free survival

between patients with only Ber-EP4-positive lymph nodes and those with involvement of both lymph nodes and bone marrow suggests that the main prognostic factor in esophageal cancer is the spread of tumor to the lymph nodes; hematogenous spread may be a secondary event.

Our data indicate that immunohistochemical assessment of lymph nodes can be used to refine the staging system for esophageal cancer and help identify patients who will not be cured by surgery alone. The value of adjuvant therapy in patients with small primary tumors who are thought to have no nodal involvement is unknown, but patients with a minimal amount of residual tumor may respond better to such therapy than patients with more advanced disease.

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