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Original article

Significant correlation between micrometastasis in the lymph nodes and reduced expression of E-cadherin in early gastric cancer

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Abstract

Background. **E-cadherin has been recognized as an important factor associated with tumor metastasis. However, the relationship between micrometastasis in the lymph nodes and the expression of E-cadherin in the primary tumor in gastric cancer remains unclear.**

Methods. **Two consecutive sections of 4522 lymph nodes from 162 patients with early gastric cancer were prepared for simultaneous hematoxylin and eosin (H&E) and cytokeratin (CK) staining. Sections of primary tumors from 135 of these patients were prepared for E-cadherin immunostaining.**

Results. **The incidence of lymph node involvement was significantly increased, from 6.8% (11/162 patients) by H&E staining, to 27% (43/162 patients) by CK immunostaining ($P < 0.0001$). Micrometastasis in the lymph node was found in 32 of 151 (21%) patients who had no lymph node metastasis evidenced by H&E staining. Micro-lymph node metastasis was frequently found in tumors with a diameter more than 1.0 cm, of those that were poorly differentiated, deeply invaded, showed lymphatic on vascular invasion, and in those that showed reduced expression of E-cadherin. Loss of expression of E-cadherin in the primary tumor was closely correlated with micro-lymph node metastasis. Patients with tumors with micro-lymph node metastasis detected by CK immunostaining had a significantly lower 5-year survival rate ($P < 0.01$) than those without such metastases.**

Conclusion. **Tumors more than 1.0 cm in diameter and those that exhibit poor differentiation, deep invasion (i.e., to the submucosa), lymphatic or vascular invasion, and reduced expression of E-cadherin are risk factors for lymph node metastasis in early gastric cancer. Thus, it is recommended that cancers confined to the mucosa (m-cancers) that are more than 1.0 cm in diameter should not be treated with limited surgery without lymphadenectomy.**

Key words Early gastric cancer · Micrometastasis · Cytokeratin · E-cadherin · Immunohistochemistry

Introduction

Early gastric cancer (EGC) is defined as cancer that is confined to the mucosa (m-cancer) or submucosa (sm-cancer), regardless of the presence or absence of regional lymph node metastasis [1]. Recent improvements in endoscopic techniques and the widespread use of mass screening have made it possible to detect gastric cancer at a very early stage, and have led to excellent outcomes for patients after surgical treatment [2–4]. Although gastrectomy with D2 lymphadenectomy has been the standard treatment for early gastric cancer [5–7], a less invasive surgerical prodecure without lymphadenectomy has recently been used to treat these patients and has achieved not only complete cure of the disease but also a better postoperative quality of life [8,9].

It is well known that lymph node metastasis is the most important prognostic factor for patients with early gastric cancer [10,11]. Moreover, micrometastasis in the lymph node has been detected by cytokeratin (CK) immunostaining [12–14]. The clinicopathological significance of these so-called micrometastases in the lymph nodes and the correlation of these micrometastases with the reduced expression of E-cadherin (E-cad), which has been recognized as an important factor in tumor metastasis [15], have not been extensively discussed. Because it is still sometimes difficult to predict lymph node involvement preoperatively, a better understanding of the clinicopathological characteristics of micrometastasis in the lymph node is important for the establishment of a therapy for early gastric cancer. Accordingly, in the present study, we examined the relationship between micrometastasis in lymph nodes and the expression of E-cad, in patients with EGC.

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Patients and methods

Patients

A total of 162 patients with EGC who underwent curative gastrectomy at Tottori University Hospital from 1986 to 1990 were investigated. There were 98 men and 64 women, ranging in age from 37 to 82 years, with a mean age of 63 years. Total gastrectomy was performed in 19 patients (12%), distal subtotal gastrectomy in 126 patients (78%), proximal subtotal gastrectomy in 15 patients (9.3%), and limited resection in 2 patients (1.2%). Two patients underwent D1 lymphadenectomy (removal of perigastric nodes; group 1), 115 patients underwent D2 lymphadenectomy (D1 plus removal of the nodes along the left gastric, common hepatic, celiac, and splenic arteries; group 2), and 45 patients underwent D2 plus a part of group 3 lymphadenectomy (lymph nodes in the hepatoduodenal ligament, behind the pancreas head, and at the root of the mesentery). All patients were followed for at least 5 years after surgery.

Clinicopathological data

Clinicopathological parameters were evaluated according to the *General rules for gastric cancer study in surgery and pathology* proposed by the Japanese Research Society for Gastric Cancer [1]. Tumor size was determined on the basis of the superficial maximum diameter of the primary lesions. Macroscopic types were described as 0-I, protruded; 0-IIa, superficial elevated; 0-IIb, flat; 0-IIc, superficial depressed; and 0-III, excavated. Histologically, 107 tumors were classified as differentiated carcinomas (51 well differentiated and 57 moderately differentiated carcinomas). Fifty-five tumors were classified as poorly differentiated carcinomas. The depth of invasion of the tumors, as determined by H&E staining, was classified into four subgroups: intramucosal cancer (tumor invasion limited to mucosa; m-cancer; $n = 84$), submucosal 1 cancer (slight invasion, limited to the upper two-thirds of the submucosa; sm1; $n = 49$), and submucosal 2 cancer (deep submucosal invasion close to the muscularis properia; sm2; $n = 29$). Lymphatic invasion was detected in 15 patients, and vascular invasion in 14 patients.

Cytokeratin immunostaining of lymph nodes

A total of 4522 lymph nodes from the 162 patients with EGC were sampled, with a median number of 28 nodes (range, 10–61 nodes) per patient. Two consecutive sections, each 4-μm-thick, were prepared from the lymph nodes for simultaneous staining with ordinary H&E and CK immunostaining. Forty perigastric lymph nodes, obtained from 20 patients with benign gastric ulcers, were used as normal controls, and 135 main tumors were used as positive controls. Mouse monoclonal immunoglobulin, CAM 5.2 (Becton Dickinson, San Jose, CA, USA), was used as a primary antibody. A standard streptavidin-biotin (SAB) method was used for CK immunostaining, as previously described [16]. Briefly, the sections were dewaxed and dehydrated. Endogenous peroxidase was blocked by incubation of the samples with 3% hydrogen peroxide in 100% methanol. The tissue sections were incubated with the primary antibody, CAM 5.2, at 25 μg per ml, overnight at 4 °C. The second antibodies, biotinylated antibodies against mouse immunoglobulin, were applied, followed by the application of peroxidase-labeled streptavidin. The reaction products were visualized with diaminobenzidine as the chromogen, and sections were counterstained with methyl green. Tris-buffered saline was used instead of the primary antibody as a negative control.

E-cadherin immunostaining of primary tumors

Sections were prepared from 135 primary tumors of the 162 patients with EGC; 27 primary tumors were excluded because the samples were too small to be cut into sections again. Mouse anti-human E-cadherin monoclonal antibody, HECD-1 (Diluted 1:400; Takara, Otsu, Japan), was used as the primary antibody. A standard avidin biotin penoxidase complex (ABC) technique was performed, using a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA), as described previously [17,18]. Phosphate-buffered saline (PBS) was used instead of the primary antibody as a negative control. Adjacent noninvolved gastric mucosa was used as an internal positive control.

Interpretation of the immunostaining

The H&E-stained slides, at a magnification of 200, were first assessed by an experienced pathologist (M.M.) for the presence of a metastasis in the lymph node. The CK-immunostained slides were then examined, and the results were compared with those obtained from the H&E slides. Micrometastasis in the lymph node was recognized when tumor cells were detected only by CK immunostaining, and were not detected by H&E staining. E-cad immunostaining was scored in a semiquantitative fashion, from 0 to 3 [19], with 0 denoting absent staining (normal staining less than 10% of the tumor areas), 1 denoting cytoplasmic distribution, 2 denoting heterogeneous staining (normal staining in 10% to 90% of the tumor area), and 3 denoting a normal membranous staining pattern (normal staining in more than 90% of the tumor area). For the purpose of data

analysis, the absent, cytoplasmic, and heterogeneous staining patterns (i.e., scores 0, 1, and 2) were classified as a loss of or reduced expression of E-cad. Tumors with a normal membranous pattern that was similar to that in the adjacent normal mucosa (i.e., score of 3) were classified as having preserved expression of E-cad. All the staining results for CK and E-cad were examined in relation to the clinicopathological parameters of the tumors.

Statistical analysis

The χ^2 test was used for correlating lymph node involvement and antigen expression with clinicopathologic indices. Survival curves were constructed according to the method of Kaplan-Meier; for differences between curves, the *P* value was calculated using the generalized Wilcoxon test. A multivariate Cox regression model was used to examine the risk factors associated with nodal involvement and patients' survival. A *P* value of less than 0.05 was considered an indication of statistical significance.

Results

Lymph node involvement in EGC

The incidence of lymph node involvement was found to be 6.8% (11/162 patients) according to H&E staining, with 1 (1.2%) of the 84 patients with m-cancer and 10 (13%) of the 78 patients with sm-cancer. However, the incidence of lymph node involvement was found to be 27% (43/162 patients) according to CK-immunostaining ($P < 0.0001$), with 16 (19%) of the 84 patients with m-cancer and 27 (35%) of the 78 patients with sm-cancer. With regard to the total number of dissected lymph nodes, the frequency of lymph node involvement was significantly increased, from 0.5% (22/4522 nodes) detected by H&E staining, to 2.5% (112/4522 nodes) detected by CK immunostaining ($P < 0.0001$). The incidence of micrometastasis in lymph nodes with no evidence of metastasis according to H&E staining was 21% (32/151 patients). Twelve (75%) of the 16 m-cancers that were node-positive were found to consist of only single or scattered cancer cells in the lymph nodes. On the other hand, metastases, in the form of scattered or clustered cancer cells in the lymph nodes, were detected in 19 (70%) of the 27 sm-cancers that were node-positive (Fig. 1, a, b, c). Nineteen (44%) of the 43 patients with node-positive cancer were found to have involvement of the extra-perigastric nodes; 16 patients with involvement in group 2 nodes and 3 patients with involvement in group 3 nodes. No CK-positive cells were detected in any of the 40 nodes from patients with benign gastric ulcers.

Clinicopathological significance of lymph node involvement

We analyzed clinicopathologic factors in the 43 patients with lymph node involvement according to CK immunostaining (Table 1). Lymph node involvement was detected more frequently in tumors with a diameter of more than 2.0 cm compared with tumors of less than 1-cm diameter ($P < 0.05$). Twenty-three (85%) of the 27 sm-cancers with lymph node metastasis detected by CK immunostaining were found to be more than 2.0 cm in diameter, while 4 (15%) of these sm-cancers were found to be 2.0 cm or less in diameter and 3 of these 4 had metastasized to the extra-perigastric (group 2) lymph nodes (Table 2). Six (38%) of the 16 m-cancers that were CK-positive in the lymph nodes were found to be 2.0 cm or less in diameter, with 5 of them being 1.1–2.0 cm in diameter, although all of these micrometastases were only single cancer cells in the perigastric (group 1) lymph nodes. In all 43 patients with CK-positive lymph nodes, metastases, in the form of scattered or clustered cancer cells in the lymph node, occurred more frequently in tumors with a diameter of more than 2 cm. As shown in Table 1, poorly differentiated tumors (shown as "undifferentiated" in Table 1), deeply invaded tumors (i.e., to the submucosa), and tumors with lymphatic or vascular invasion were strongly associated with lymph node involvement. In addition, 16 (62%) of 26 tumors with discrete or clustered cancer cells in the lymph node were poorly differentiated carcinomas. Moreover, 12 (60%) of 20 undifferentiated tumors were found to have extraperigastric lymph node metastases (group 2 and/or 3).

Expression of E-cadherin

E-cad was strongly expressed at the cell-cell boundaries of normal gastric mucosa adjacent to the tumor. Loss of expression of E-cad was found in 77 (57%) of the 135 patients with EGC examined for E-cad (Fig. 2a,b). Expression of E-cad was significantly reduced in tumors with a diameter of more than 1.0 cm ($P < 0.05$ vs $\leq$ 1.0 cm), undifferentiated tumors ($P < 0.0001$), deeply invaded tumors ($P < 0.005$), and tumors with lymphatic invasion and vascular invasion ($P < 0.05$ vs no invasion; Table 3). As shown in Table 4, 30 (81%) of the 37 cases with CK-positive lymph nodes had loss of E-cad expression ($P < 0.0005$). A high incidence of loss of E-cad expression was found not only in the tumors with clustered cancer cells in the lymph nodes but also in those with single or scattered cancer cells in the lymph nodes. Moreover, the tumors with loss of E-cad expression had a large number of metastatic lymph nodes and a large number of extra-perigastric metastatic lymph nodes (Table 4).

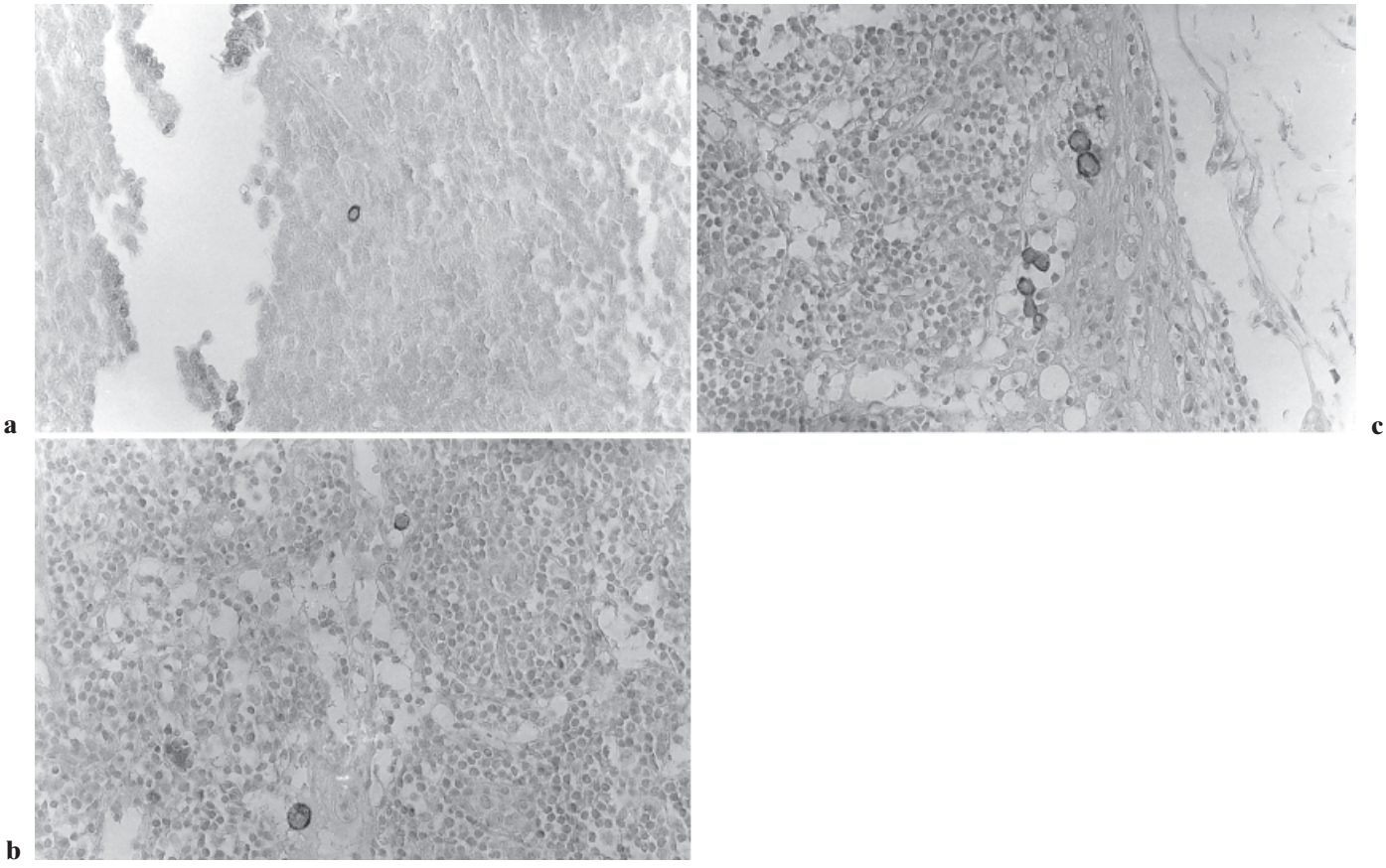

Fig. 1a–c. Micrometastases in lymph nodes, detected by cytokeratin (CK) immunostaining, that had been overlooked by ordinary H&E staining. ×200. **a** A single cancer cell in the lymph node **b** Scattered cancer cells in the lymph node **c** Clustered cancer cells in the lymph node

Prognosis of patients with EGC

The overall 5-year survival rate of our 162 patients with EGC was 98%. Four patients died of a postoperative recurrence of the tumor (1 of liver metastasis and 3 of peritoneal and lymph node metastasis). Two of these patients had already been diagnosed with lymph node metastases by H&E staining immediately after the operation. In another 2 patients, the lymph nodes were diagnosed as micrometastasis-positive by CK immunostaining, although this condition had not been detected by H&E staining. In comparison with the 119 patients without lymph node involvement (5-year survival rate of 100%), the 32 patients with CK-positive lymph nodes who were H&E-negative (5-year survival rate of 93.6%) and the 11 patients with H&E-positive lymph nodes (5-year survival rate of 81.8%) had a significantly less favorable outcome ($P < 0.01$ respectively; Fig. 3).

By multivariate regression analysis, lymphatic invasion and abnormal expression of E-cad were found to be independent risk factors for lymph node metastasis as detected by CK immunostaining ($P < 0.05$, respectively; Table 5). Multivariate survival analysis indicated that macroscopic type and ulceration of the tumor, but not lymph node involvement, were of independent prognostic significance ($P < 0.05$, respectively).

Discussion

It has been widely recognized that large tumor size, lymphatic invasion, and deep invasion (to the submucosa) are important risk factors for lymph node metastasis [2,3]. In the present CK-immunohistochemical study, tumor size, deep invasion to the submucosa, lymphatic invasion, vascular invasion, and poor differentiation of the tumor were five important risk factors correlated with lymph node involvement in EGC detected by CK-immunostaining. Histologically, metastasis to the lymph node was extremely rare in tumors with a diameter of less than 2 cm [2,7]. We also found that tumors with a diameter of more than 2 cm more frequently metastasized to the lymph nodes, and most of these involvements were in the form of clusters of cancer cells in the lymph nodes. However, in five m-cancers

Table 1. Lymph node involvement and clinicopathological characteristics of the primary tumor in 162 patients with EGC

Variables[a]	Total no. of cases	Number of CK-positive cases (%)	*P*
Superficial diameter (cm)			
≤1.0	18	1 (6)	
1.0–2.0	41	9 (22)	0.0472
>2.0	103	33 (32)	
Macroscopic type			
I, IIa, IIa+I	23	4 (17)	
IIa+IIc, IIc+IIa	27	10 (37)	0.1294
IIc, III, IIc+III	112	29 (26)	
Ulcer formation			
Negative	143	36 (25)	0.2792
Positive	19	7 (37)	
Histopathology			
Differentiated	107	23 (21)	0.0424
Undifferentiated	55	20 (36)	
Depth of invasion			
m	84	16 (19)	
sm1	49	11 (22)	0.0005
sm2	29	16 (55)	
Lymphatic invasion			
Negative	147	33 (22)	0.0002
Positive	15	10 (67)	
Vascular invasion			
Negative	148	36 (24)	0.0376
Positive	14	7 (50)	

EGC, Early gastric cancer; CK, cytokeratin (immunostaining)
[a] Evaluated according to the *General rules for gastric cancer study in surgery and pathology* [1]

Table 2. Correlation between lymph node metastases detected by CK immunostaining and tumor size

Depth of invasion	*n*	≤1.0 cm		1.1–2.0 cm		>2.0 cm	
		Perigastric	Extraperi.[a]	Perigastric	Extraperi.[a]	Perigastric	Extraperi.[a]
m-cancer	16	1	0	5	0	5	5
sm-cancer	27	0	0	1	3	12	11

[a] Extra-perigastric lymph nodes

and four sm-cancers, micrometastases were found with tumors 1 to 2 cm in diameter, although all of these micrometastases were of only single or scattered cancer cells in the lymph nodes.

It is interesting to note that all five of the m-cancers had only perigastric lymph node involvement, but three of the four sm-cancers had an extra-perigastric lymph node metastasis. This finding strongly agrees with a recent report by Yasuda et al. [20], which also suggested the histological possibility of metastasis in a tumor with a diameter of 1 to 2 cm. These findings indicate that an EGC with a diameter of more than 1.0 cm seems to be a risk for lymph node metastasis.

It is widely accepted that tumors with deep invasion or lymphatic invasion are more likely to involve lymph node metastasis [21–23]. We found that the incidence of lymph node metastasis detected by CK immunostaining was 19% in m-cancers but 35% in sm-cancers. Moreover, most of the metastases detected from m-cancers were only single or scattered cancer cells in the lymph nodes, while, in comparison, most of those detected from sm-cancers were scattered or clustered cancer cells in the lymph nodes. It seems that deeper invasion indicates a greater number of cancer cells metastasizing to the lymph nodes. Moreover, in the current study, 67% of the patients with lymphatic invasion were found to have cancer-positive lymph nodes. Multivariate analysis showed that lymphatic invasion was an independent risk factor for lymph node metastasis. The existence and abundance of lymphatic vessels in the submucosal layer of the gastric wall may facilitate tumor metastasis [22,24]. These findings indicate that tumors that have

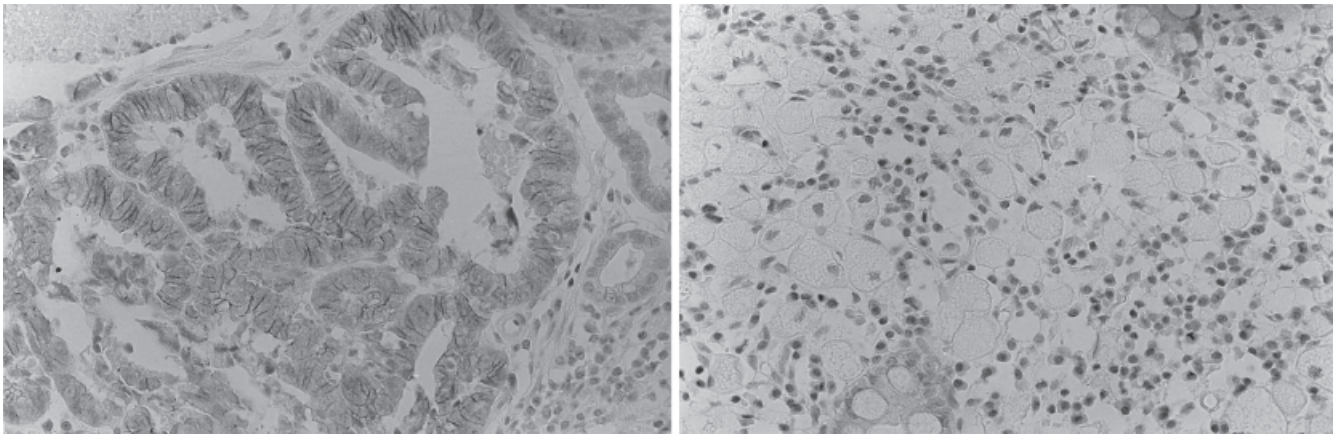

Fig. 2a,b. Expression of E-cadherin in cell-cell boundaries of early gastric cancer cells. ×200. **a** Preserved expression **b** Loss of expression

Table 3. Relationship between clinicopathological parameters and reduced expression of E-cadherin in 135 patients with EGC tested for E-cadherin expression

Clinicopathological parameters	No. of cases	Loss of expression of E-cadherin (%)	*P*
Tumor size (cm)			
≤1.0	14	2 (14)	
1.1–2.0	33	16 (48)	0.0005
>2.0	88	59 (67)	
Macroscopic type			
I, IIa, IIa+I	20	8 (40)	
IIa+IIc, IIc+IIa	25	15 (60)	0.2489
IIc, III, IIc+III	90	54 (60)	
Ulcer formation			
Negative	118	64 (54)	
Positive	17	13 (76)	0.0834
Histopathology			
Differentiated	90	37 (41)	
Undifferentiated	45	40 (89)	< 0.0001
Depth of invasion			
m	75	36 (48)	
sm1	35	19 (54)	0.0020
sm2	25	22 (88)	
Lymphatic invasion			
Negative	123	66 (54)	0.0111
Positive	12	11 (92)	
Vascular invasion			
Negative	122	66 (54)	0.0346
Positive	13	11 (85)	
CK staining in lymph nodes			
Negative	98	47 (48)	0.0005
Positive	37	30 (81)	

invaded to the submucosa may easily intrude into the lymphatic system and further metastasize to the lymph nodes. Because deeply invaded sm-cancer has a high risk of lymph node metastasis, lymphadenectomy should be recommended for these tumors.

The E-cadherin gene has generally been recognized as an invasion-suppressor gene [15,25]. E-cadherin (E-cad) plays a key role in the establishment and maintenance of epithelial tissue structure, and its down-regulation is potentially important in the formation of metastases from carcinomas [26,27]. To elucidate the correlation between the expression of E-cad and the behavior of micrometastases in EGC, we examined the E-cad tissue status immunohistochemically. We found

Table 4. Association between characteristics of lymph node metastases and loss of expression of E-cadherin in the 135 patients with EGC examined for E-cadherin expression

Lymph node involvement	No. of cases	Loss of expression of E-cadherin (%)	*P*
Features of node involvement			
Negative	98	47 (48)	
Single or scattered cells	26	19 (73)	0.0008
Clusters of cells	11	11 (100)	
Location of node involvement			
Negative	98	47 (48)	
Group 1 nodes only	22	16 (73)	0.0011
Group 2 or 3 nodes	15	14 (93)	
No. of CK-positive nodes			
Negative	98	47 (48)	
One node	13	9 (53)	0.0032
Two-three nodes	17	14 (82)	
Four or more nodes	7	7 (100)	

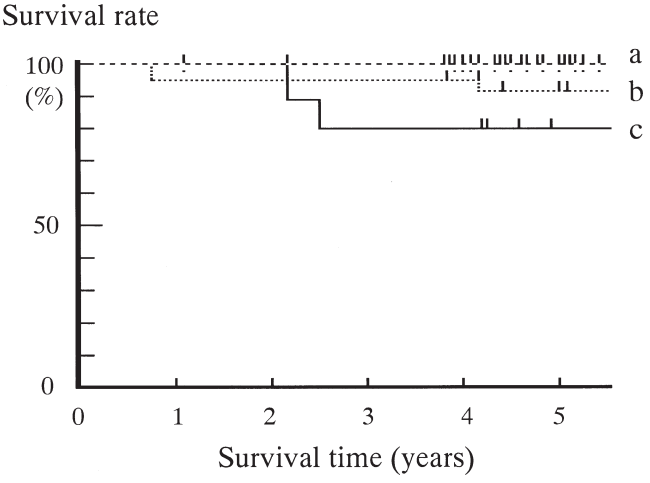

Fig. 3. Five-year survival curves of 162 patients with early gastric cancer (EGC). *a* The 5-year survival of the 119 patients with no lymph node metastases (100%) *b* The 5-year survival of the 32 patients with micrometastases in lymph nodes as detected by CK immunostaining (93.6%) *c* The 5-year survival of the 11 patients with metastases in lymph nodes as detected by H&E staining (81.8%). Generalized Wilcoxon test: *a* vs *b*, $P < 0.01$; *a* vs *c*, $P < 0.01$

that E-cad was strongly expressed, without exception, in the cell-cell boundaries of normal gastric mucosa adjacent to the primary tumor. Reduced expression of E-cad was found in 57% of our patients with EGC, a finding that strongly agrees with that in a report by Bolk et al. [28]. There was a tendency towards loss of expression of E-cad in tumors that were poorly differentiated, deeply invaded, or more than 1.0 cm in diameter, and in tumors with lymphatic or vascular invasion, and all of these factors were found to be strongly associated with lymph node metastasis as detected by CK immunostaining. Markedly reduced expression of E-cad was found in 40 of 45 patients with poorly differentiated cancers. Poorly differentiated cancers and tumors with loss of expression of E-cad were also found to be more likely to metastasize to the extra-perigastric lymph nodes with a large number of clustered cancer cells. Moreover, 81% of the patients with lymph node involvement detected by CK immunostaining were found to have loss of expression of E-cad. Thus, reduced expression of E-cad would appear to lead to loss of its invasion-suppressor function, and subsequent loss of tumor differentiation, promoting cancer metastasis to the lymph nodes [25,27]. These findings, together with our results, suggest that poorly differentiated tumors with loss of expression of E-cadherin seem to constitute a very important risk factor for predicting lymph node involvement in EGC.

It has been reported that abnormal expression of E-cad contributes to gastric cancer metastases [29,30]. In the present study, only a single cancer cell or scattered cancer cells in the lymph nodes were detected in 65% of the 43 patients with CK-positive lymph nodes. It is still unclear whether these single or scattered cancer cells remain alive in the lymph node, or if they are destroyed or removed by the immune response of the host [12]. It was interesting to note, however, that 73% of these patients with only single or scattered cancer cells in the lymph node showed a marked loss of expression of E-cad in their primary tumor. Multivariate analysis showed that loss of expression of E-cad was an independent risk factor for these micrometastases with only single or scattered cancer cells in the lymph nodes. Survival analysis indicated that the outcome of these patients with micrometastases in the lymph nodes was significantly less favorable than outcome in patients

Table 5. Logistic regression multivariate analysis of risk factors associated with lymph node metastasis detected by CK immunostaining

Variables	Regression coefficient	*P*
Tumor size	0.005	0.7597
Macroscopic type (elevated, depressed)	0.061	0.4887
Ulceration (negative, positive)	0.123	0.2843
Histological type (differentiated, undifferentiated)	0.107	0.2144
Depth of invasion (mucosal, submucosal)	0.100	0.2242
Lymphatic invasion (negative, positive)	0.394	0.0098
Vascular invasion (negative, positive)	0.041	0.7746
Expression of E-cadherin (normal, abnormal)[a]	0.222	0.0061

[a] See text for definition

with no such micrometatases. Therefore, it can be concluded that tumors with micrometatases of only a single or a few scattered cancer cells in the lymph node may already have potential metastatic activity, and these micrometastases may have the same consequence as clustered cancer cells in the lymph node, i.e., a reduced patient survival rate.

In recent years, limited surgery has been performed for the treatment of EGC. Based on our observations, we suggest the following possibilities: an endoscopic mucosal resection (EMR) or laparoscopic wedge resection without lymphadenectomy may be feasible for an m-cancer of less than 1.0-cm superficial diameter, because lymph node involvement is extremely rare in these tumors; a limited perigastric lymph node dissection can be performed for an m-cancer that is 1.0 to 2.0 cm in diameter, because these tumors rarely metastasize to the extra-perigastric lymph nodes; a radical D2 lymphadenectomy is needed for an m-cancer that is more than 2.0 cm in diameter, and for all sm-cancers, because these tumors have the potential for extra-perigastric lymph node involvement. Preoperative E-cadherin immunostaining and determination of histology from an endoscopic biopsy sample may be helpful for the prediction of lymph node involvement.

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