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Cytokeratin-Positive Cells in Lymph Nodes in Which Metastases Are Undetectable by Conventional Histological Staining in Advanced Gastric Cancer

Michio Maeta, Jianhui Cai, Shinichi Oka, Hiroaki Saito, Akira Kondo, Shunichi Tsujitani, Masahide Ikeguchi and Nobuaki Kaibara

First Department of Surgery, Faculty of Medicine, Tottori University, Yonago 683-0826, Japan

Detection of occult metastases in lymph nodes by immunostaining is becoming of increasing interest as a way to improve the accuracy of predicting the prognosis for patients with gastric cancer. Immunohistochemical detection of cytokeratin (CK) is recognized as the most sensitive method for identification of cancerous epithelial cells. In this study, lymph nodes were stained for CK in an effort to detect micrometastases and the clinical implications of the results were examined. We immunostained sections from a total of 1,198 lymph nodes from 25 totally gastrectomized patients with T3 or T4 gastric cancer who had been diagnosed as having no nodal involvement by conventional hematoxylin-eosin (HE) staining. Eighty (6.7%) of 1,198 lymph nodes from 15 (60%) of the 25 patients were immunostained with a CK-specific monoclonal antibody. CK-positive cells were more frequent in patients with macroscopic types of 3,4 and 5 gastric cancer. Patients with nodes that were both HE-negative and CK-negative had the best postoperative survival, followed by patients with HE-negative and CK-positive nodes and, finally, by patients with HE-positive nodes. Our results indicate that the presence of micrometastases in lymph nodes is a reliable indicator of the prognosis of patients with advanced gastric cancer.

Key words: cytokeratin; gastric cancer; lymph nodes; micrometastases; prognosis

In patients with gastric cancer, metastasis to the lymph nodes is considered to be one of the most important predictive factors in the prognosis (Kaibara et al., 1988; Maruyama et al., 1989). Histological nodal metastasis is examined routinely by conventional hematoxylin-eosin (HE) staining. However, discrete cancer cells or small clusters of cancer cells within the nodes, namely, micrometastases, are easily overlooked after HE staining (Ishida et al., 1997; Sasaki et al., 1997). Immunostaining for cytokeratin (CK), using the monoclonal antibody CAM 5.2, is a very sensitive method for the detection of such micrometastases when cancers have developed from epithelial tissues (Makin and Bodmer, 1984; Sedmak et al., 1989; Maehara et al., 1996a, 1996b; Ishida et al., 1997; Sasaki et al., 1997).

Lymph nodes around the abdominal aorta are the furthest from the primary gastric lesion in the abdominal cavity. These paraaortic nodes are classified as most-distant group 4 (N4) in the Japanese system for classification of gastric cancer (Japanese Research Society for Gastric Cancer, 1993). We previously conducted a prospective study to compare morbidity, mortality and survival, from May 1990 to April 1997, between conventional extended D3 lymphadenectomy and super-extended D3 plus paraaortic lymphadenectomy (PAL; Yonemura et al., 1991). In the current study, we examined all lymph nodes by immunostaining for CK in patients who had been diagnosed as having no nodal metastasis by HE staining in our earlier prospective study. The purpose of this study was to evaluate the clinical implications of CK-

Abbreviations: CK, cytokeratin; HE, hematoxylin-eosin; PAL, paraaortic lymphadenectomy

positive cells in apparently metastasis-negative nodes.

Patients and Methods

Patients

Between May 1990 and April 1997, 70 patients were entered into a prospective study after preoperative informed consent had been obtained. The eligibility requirements for inclusion in the study were as follows. Patients had to be under 75 years of age without other malignancies in organs other than the stomach.

After laparotomy, we also excluded patients with peritoneal seeding or liver metastasis, as well as those who had macroscopic metastases in the paraaortic lymph nodes. Patients who had T3 (serosal invasion) or T4 (invasion of adjacent structures) gastric cancer and who had undergone potentially curative total gastrectomy were entered into the study after extended dissection of group 1 (N1), group 2 (N2) and group 3 (N3) lymph nodes, namely, the D3 procedure. The extent of lymphadenectomy was expressed as follows: D1, complete removal of N1 nodes; D2, complete removal of N1 and N2 nodes; D3, complete removal of N1, N2 and N3 nodes; and D3 plus PAL, complete removal of N1, N2 and N3 nodes, as well as paraaortic lymphadenectomy. The 70 patients were divided, by alternate designation at the time of operation, into the D3 (n = 35) and D3 plus PAL (n = 35) groups.

Methods

All dissected lymph nodes were prepared for histologic analysis by one central cross-sectional passage through the hilus for microscopic examination of metastasis. Among 70 patients, 25 were diagnosed, after HE staining, as having no metastasis in all dissected nodes (12 of the 35 patients in the D3 group and 13 of the 35 patients in the D3 plus PAL group). In the present study, we examined a total of 1,198 nodes, consisting of 1,024 nodes (mean 41 nodes per patient) in groups N1, N2 and N3 and 174 nodes (mean 13 nodes per patient) in group N4 (paraaortic nodes) from these 25 node-negative patients.

Paraffin-embedded blocks of the 1,198 lymph nodes and the 25 primary gastric tumors were prepared from the above mentioned 25 patients. Serial sections of 4 μm in thickness were prepared from the blocks and subjected to HE staining and CK-specific immunostaining to allow comparison of results obtained by the 2 methods in adjacent sections of each lymph node and in the primary gastric tumor.

Immunohistochemical staining was performed by the streptavidin-biotin method with a murine monoclonal antibody, CAM 5.2 (Becton Dickinson, San Jose, CA) against low-molecular-weight CK. In brief, dewaxed and dehydrated sections were heated in a microwave oven (700 W) for 10 min for retrieval of antigens in the specimens. Endogenous peroxidase was blocked by incubation of samples with 3% hydrogen peroxide in 100% methanol. The tissue sections were then 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, and sections were then incubated with peroxidase-labeled streptavidin. Reaction products were visualized with diaminobenzidine as the chromogen, and sections were counterstained with methyl green for visualization of lymphocytes. Tris-buffered saline was used instead of the primary antibody for negative controls.

The lymph nodes were divided into 5 CK-groups according to the criteria defined by Ishida and colleagues (1997): CK-group 0 (CG 0), no cancer cells in the section of the node; CK-group 1 (CG 1), only 1 discrete cancer cell in the section of the node (Figs. 1a and b); CK-group 2 (CG 2), 2 or more discrete but not aggregated cancer cells in the section of the node; CK-group 3 (CG 3), in addition to discrete cancer cells, 1 or more aggregates of 2 or more cancer cells in the section; and CK-group 4 (CG 4), metastasized cells detectable by standard HE staining (Figs. 1c and 1d). The determination of the CK-group for each patient was based on the highest group recorded for any of that patient's lymph nodes.

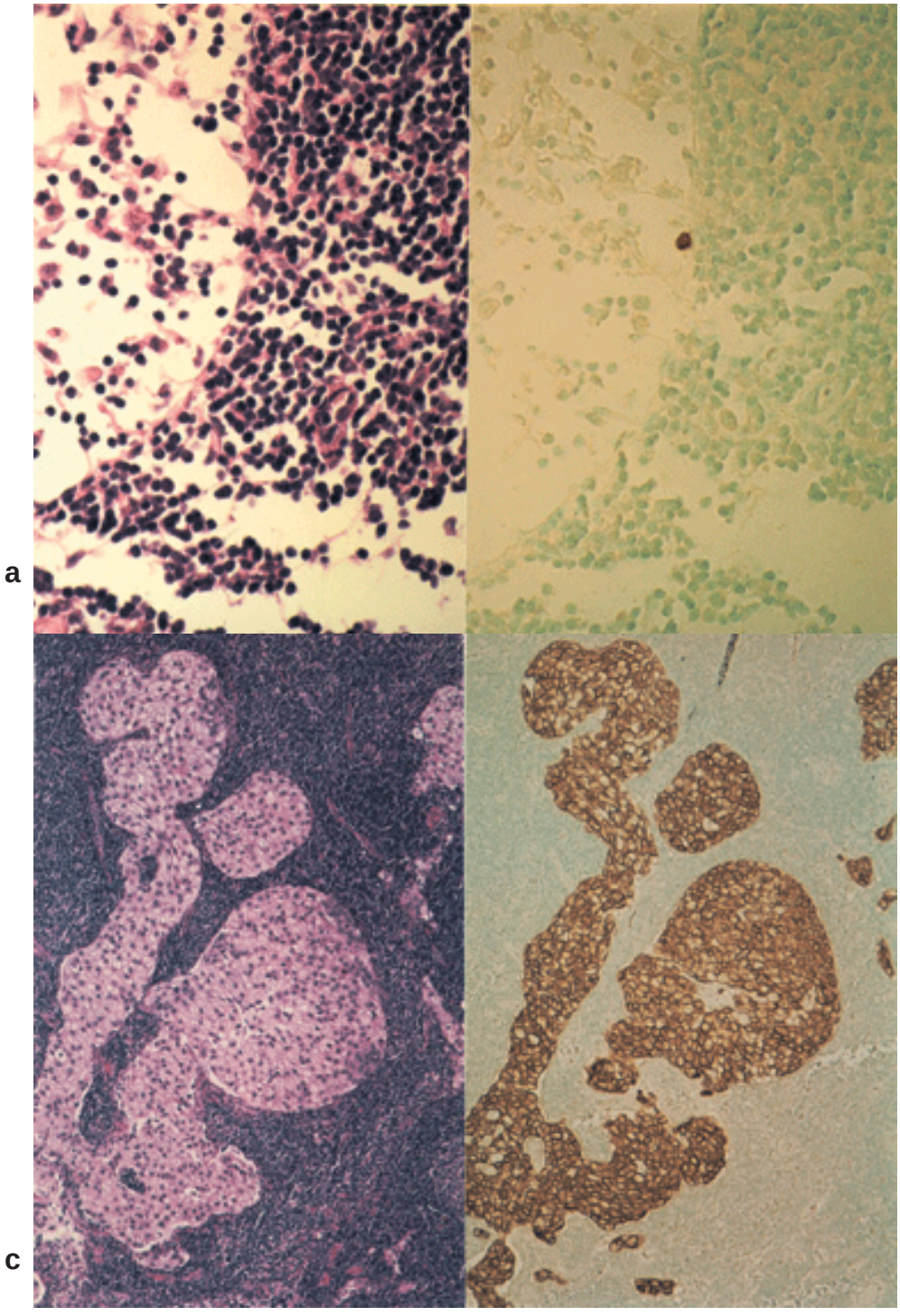

Fig. 1. Examples of stained lymph nodes after staining with hematoxylin-eosin (HE) and after immunostaining specific for cytokeratin (CK). **a and b:** a discrete cancer cell is hard to identify after HE staining (**a**) but is readily identifiable after CK-specific immunostaining (**b**) (× 95). **c and d:** aggregated cancer cells are recognized after both HE staining (**c**) and immunostaining with CK (**d**) (× 48).

Table 1. Incidence of CK-positive lymph nodes in 25 patients with nodes in which metastases were undetectable after HE staining

Number of patients	CK-negative	CK-positive			
	CG 0	CG 1	CG 2	CG 3	CG4
25 (100)	10 (40)	2 (8)	4 (16)	3 (12)	6 (24)
		15 (60)			

( ), percentage.
CG 0, no cancer cells in the sections of lymph nodes; CG 1, only 1 discrete cancer cell; CG 2, 2 or more discrete but not aggregated cancer cells; CG 3, in addition to discrete cancer cells, 1 or more aggregates of 2 or more cancer cells; CG 4, metastasized cells detectable by standard HE staining; CK, cytokeratin; HE, hematoxylin-eosin.

Statistical analysis was performed by the chi-squared test. The Kaplan-Meier method was used to calculate cumulative survival curves and the generalized Wilcoxon test was used for examination of the statistical significance of differences. A *P* value of less than 0.05 was considered to be statistically significant.

Results

A total of 1,198 lymph nodes from 25 patients with advanced gastric cancer were examined in this study. All 25 primary tumors were consistently immunostained with the monoclonal antibody against CK. No CK-positive nodes were found in 10 of the 25 patients, while 15 patients (60%) had CK-positive nodes. After the results of immunostaining had been recorded, all HE-stained samples were reexamined, and 6 cases (seven lymph nodes) in which metastasized cancer cells had been recognized on HE-stained slides were classified as CG 4. It was difficult to identify metastatic cells on other HE-stained slides from 19 patients (a total of 73 nodes), even when CK-positive cells had been detected by immunostaining. We divided our cases into 5 CK-groups, CG 0, CG 1, CG 2, CG 3 and CG 4, and there were 10, 2, 4, 3 and 6 patients in each group, respectively (Table 1). Among 1,198 lymph nodes, 80 lymph nodes (6.7%) from a total of 15 patients were immunopositive for CK, as shown in Table 2.

The cytoplasm of immunopositive cells was diffusely stained and products of the immunoreaction were visualized as brown circles that were due to staining by diaminobenzidine. The nuclei of such cells were barely stained.

Relationships between the presence of CK-positive nodes and the clinicopathological features of primary tumors are shown in Table 3.

Table 2. Number and classification of CK-positive lymph nodes among 1,198 nodes from 25 patients

Number of lymph nodes	CG 0	CG 1	CG 2	CG 3	CG4
1,198 (100)	1118 (93.3)	40 (3.3)	28 (2.3)	5 (0.4)	7 (0.6)
		80 (6.7)			

( ), percentage.
CG 0–4, see footnote to Table 1; CK, cytokeratin.

Table 3. Relationships between immunoreactivity in lymph nodes and clinicopathological features

Variable	CK-negative [10]	CK-positive [15]	*P* value
Age (years; mean ± SD)	58.9 ± 9.8	53.8 ± 10.1	NS
Gender			
Male	5	10	NS
Female	5	5	
Tumor size			
<7 cm	5	8	NS
≥7 cm	5	7	
Histology			
Differentiated	4	3	NS
Undifferentiated	6	12	
Depth of cancer invasion			
Muscularis propria	0	4	NS
Subserosa	1	0	
Serosa	9	11	
Macroscopic type			
1, 2	5	1	$P < 0.05$
3, 4	5	10	
5	0	4	
Lymphatic vessel invasion			
Negative	7	12	NS
Positive	3	3	
Blood vessel invasion			
Negative	2	8	NS
Positive	8	7	

[ ], number of patients.
CK, cytokeratin; NS, not significant.

We found no significant relationships between the presence of CK-positive nodes and the size of the tumor, the histological type, the depth of tumor invasion, and lymphatic or blood-vessel invasion by the primary lesion. However, the incidence of CK-positive nodes was significantly higher in patients with macroscopic types of 3, 4 and 5 gastric cancer (Table 3).

Table 4 shows the numbers and CG-groups of CK-positive nodes in terms of the sites of immunopositive lymph nodes. CK-positive nodes were found most frequently among lymph nodes along the greater curvature (18 nodes), followed by lymph nodes along the lesser curvature (17 nodes), infrapyloric nodes (12 nodes), and right cardial nodes (10 nodes). Lymph nodes in group N1 included the largest number of CK-positive nodes followed by group N2. No CK-positive nodes were found in lymph nodes in groups N3 and N4.

The relationship between CK-positive nodes and postoperative survival of patients is shown in Fig. 2. To indicate the survival of control patients, we have included in Fig. 2 the survival of 45 of the 70 patients in our prospective study. These 45 patients had been diagnosed as having HE-detectable nodal involvement (therefore, lymph nodes from these 45 patients were not immunostained in this series). The 5-year survival rates for 9 patients (excluding 1 patient who died of complications) with both HE-negative and CK-negative nodes [HE(–), CK(–)], for 15 patients with HE-negative but CK-positive nodes [HE(–), CK(+)], and for 44 patients with HE-positive nodes [HE(+)] were 85.7%, 52.4% and 30.8%, respectively. Although there was no significant difference in survival between patients with HE(–) plus CK(–) nodes and patients with HE(–) plus CK(+) nodes, there was a statistically significant dif-

Table 4. Number and classification of CK-positive nodes analyzed in terms of sites of lymph nodes

Site of lymph nodes	CG 1	CG 2	CG 3	CG 4	Total
Group N1 (peri-gastric nodes)					
Right cardial	6	2	1	1	10
Left cardial	0	2	0	0	2
Along the lesser curvature	8	4	2	3	17
Along the greater curvature	9	7	1	1	18
Suprapyloric	2	1	0	0	3
Infrapyloric	3	7	1	1	12
Group N2					
Along the left gastric artery	4	1	0	0	5
Along the common hepatic artery (anterosuperior group)	4	0	0	0	4
Around the celiac artery	1	0	0	0	1
At the splenic hilum	2	2	0	1	5
Around the splenic artery	1	2	0	0	3
Group N3*	0	0	0	0	0
Group N4 (paraaortic lymph nodes)	0	0	0	0	0
Total	40	28	5	7	80

* Lymph nodes along the common hepatic artery(posterior group), in the hepato-duodenal ligament, on the posterior surface of the pancreas head, and along the superior mesenteric vein.
CG 0–4, see footnote to Table 1; CK, cytokeratin.

ference in survival between patients with HE(–) and CK(–) nodes and those with HE(+) nodes ($P < 0.05$).

Discussion

In the current study, we immunostained sections of 1,198 lymph nodes from 25 patients who had been diagnosed previously as having no nodal metastasis by HE staining. Although these 25 patients were selected from our prospective study, our purpose in the present study was to evaluate clinical implications of CK-positive nodes.

Micrometastases in malignant tumors have been a focus of increasing interest in recent years because of their significance in predicting

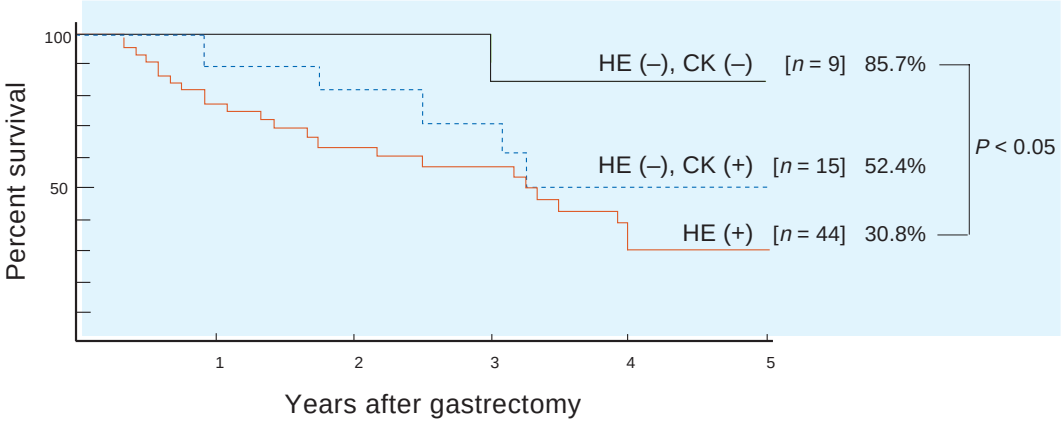

Fig. 2. Postoperative survival of totally gastrectomized patients with T3 or T4 gastric cancer. HE(–), CK(–): patients with both HE-negative and CK-negative nodes. HE(–), CK(+): patients with HE-negative but CK-positive nodes. HE (+): patients with HE-positive nodes.

the prognosis of patients. Many human tumors, such as breast (Sedmak et al., 1989), colorectal (Sasaki et al., 1997) and gastric (Maehara et al., 1996a; Ishida et al., 1997) cancers, are associated with such metastases in the lymph nodes or bone marrow (Maehara et al., 1996b), which probably affect survival. Various studies have explored the possible application of epithelial cell markers in the detection of micrometastases (Trojani et al., 1987; Davison et al., 1990; Siewert et al., 1996; Broll et al., 1997). In addition, the predictive value of molecular or genetic methods for detection of occult metastases, which exploit polymerase chain reaction or mutant allele-specific amplification, has been suggested (Hayashi et al., 1994; Nakamori et al., 1997; Nakanishi et al., 1997; Dorudi et al., 1998). However, it is hard to avoid false-positive results in many cases, even though these methods reveal occult metastases with so-called supersensitivity. Many investigators have reported the results of immunostaining for CK in bone marrow or lymph nodes and have stressed the usefulness of this marker in the detection of micrometastases from gastric cancer (Maehara et al., 1996a, 1996b; Ishida et al., 1997). In the current study, we used monoclonal antibody CAM 5.2, which binds specifically to CK peptide 8.

In our series, all sections of primary tumors were CK-positive. Moreover, 80 lymph nodes (6.7%) among 1,198 nodes dissected from 15 (60%) of 25 patients, who had been diagnosed as having no metastases by conventional HE staining, were also CK-positive. However, the CK-positive nodes were confined to groups N1 or N2 and no CK-positive nodes were detected in groups N3 and N4. The frequency (60%) of CK-positive nodes in our series of patients with T3 or T4 gastric cancer was higher than the frequency (40%) reported by Ishida and colleagues (1997) for patients with gastric cancer that included T1, T2, T3 and T4 cancers, and than the frequency (23.5%) reported by Maehara and coworkers (1996a) for patients with early gastric cancer. The deep invasion of the serosa by the tumor in all of our patients with T3 or T4 gastric cancer might explain the high frequency of CK-positive nodes in our study.

As shown in this study, discrete and scattered cancer cells or small clusters of such cells are easily overlooked after standard HE staining. Establishment of an easy method for detecting such small numbers of cancer cells is clinically important. In our study, histopathological examination of lymph nodes was made after only a single central cross-sectional passage. The sensitivity of detection of metastatic deposits might be expected to improve significantly by multiple sections of each node (Isozaki et al., 1997). In fact, second sections cut from the lymph nodes examined in the present study revealed metastatic deposits not only after immunostaining but also after conventional HE staining in 7 of the 1,198 lymph nodes. In these cases, metastatic cells had been undetectable on the first section that had been stained with HE. However, this 2-section procedure would not be practical in routine histopathology. Our results suggest that immunostaining with CK-specific antibody, in addition to conventional HE staining, would permit a more accurate diagnosis of lymph node metastases.

Metastasis to the lymph nodes is likely to occur together with the progression of the main tumor. The major risk factors for lymph node metastasis are generally considered to be the large size of the primary tumor, infiltrative growth, diffuse-type gastric cancer, poor differentiation and deep invasion by the primary tumor (Kaibara et al., 1988; Maruyama et al., 1989). In our series, we found that a gross finding of diffuse-type gastric cancer was associated with a high frequency of CK-positive nodes.

In our patients, the 5-year survival rate for patients without nodal involvement [HE(–), CK(–); 85.7%] was the highest, followed by rates for patients with HE(–) plus CK(+) nodes (52.4%) and for patients with HE(+) nodes (30.8%) (Fig. 2). Although there was no significant difference in survival between patients with or without CK-positive nodes, perhaps because of the small number of patients, there was a significant difference between patients

with HE(–) plus CK(–) nodes and patients with HE(+) nodes. Our results indicate that the accuracy of predictions of prognosis for patients with advanced gastric cancer will be enhanced when CK-immunostaining is performed in combination with conventional histopathologic analysis.

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