Original article

Clinicopathological value of immunohistochemical detection of occult involvement in pT3N0 gastric cancer

JIANHUI CAI¹, MASAHIDE IKEGUCHI¹, MICHIO MAETA¹, NOBUAKI KAIBARA¹, and TAKASHI SAKATANI²

¹First Department of Surgery and First Department of ²Pathology, Tottori University Faculty of Medicine, 36-1 Nishichou, Yonago 683-8504, Japan

Abstract:

Background. In cases of pT3 gastric cancer, even when standard histological staining reveals no evidence of metastases in the regional lymph nodes, patients still may die of postoperative recurrence of the tumor. An attempt was made in the present study to explain the unfavorable outcome of such patients by investigating the presence of occult cancer cells in lymph nodes by immunostaining of cytokeratin.

Methods. We examined 2310 lymph nodes that had been removed from 83 patients with stage II gastric cancer (pT3, N0, M0). Two consecutive sections of 4 µm thick were prepared for simultaneous staining with hematoxylin and eosin and immunostaining with the CAM 5.2 monoclonal antibody against cytokeratin, respectively.

Results. Evidence of occult involvement was found in 299 of 2310 (13%) lymph nodes and in 54 of 83 (65%) patients with pT3 gastric cancer. An analysis of survival demonstrated the limited 5-year survival of patients with occult involvement in their resected lymph nodes, as compared with that of patients without involvement ($P < 0.01$). Moreover, the patients in whom group 2 lymph nodes had occult cancer cells had a significantly poorer prognosis than those in whom occult involvement was limited to group 1 lymph nodes ($P < 0.05$).

Conclusions. The accuracy of predictions of prognosis of patients with pT3 gastric cancer should be greatly enhanced if cytokeratin-specific immunostaining is performed in conjunction with routine histopathological examination of lymph nodes.

Key words: gastric neoplasm, microinvolvement, micro-metastasis, cytokeratin, immunohistochemistry

Introduction

Metastasis to regional lymph nodes is one of the most important predictive factors in establishment of the

prognosis of patients with primary gastric carcinoma [1–3]. In cases of advanced gastric cancer with invasion of the serosa, even when there is no clinicopathological evidence of metastasis to regional lymph nodes and to distant sites, many patients still die of recurrence of the tumor during follow-up. Recent studies have demonstrated that patients with stage II gastric cancer benefit significantly from D2 lymphadenectomy [4]. In theory, it seems unlikely that patients with no metastasis to lymph nodes would experience postoperative recurrence of the tumor and should benefit from surgical resection of large numbers of uninvolved lymph nodes. Recent advances in histochemical and molecular biological techniques now allow identification of so-called occult involvement of lymph nodes in cases of breast and colorectal cancers that is generally not detected after standard hematoxylin and eosin (H&E) staining [5–10]. In this study, we tried to determine the extent of such occult involvement in lymph nodes by immunostaining of cytokeratin with the CAM 5.2 monoclonal antibody. We then evaluated the clinicopathological significance of such immunostaining in improving the accuracy of diagnosis and the prediction of prognosis for patients with locally advanced gastric cancer.

Patients and methods

A total of 83 patients with stage II gastric cancer who had undergone surgery in our hospital from 1976 to 1992 were included in this study. Patients were subjected to curative total (29 cases) or subtotal (54 cases) gastrectomy with D2 lymphadenectomy, which included the resection of group 1 and group 2 lymph nodes. Group 1 consists of all perigastric nodes. Group 2 consists of the nodes along the left gastric artery, along the common hepatic artery, around the celiac artery, at the splenic hilum, and along the splenic artery. Clinico-

Offprint requests to: N. Kaibara

Received for publication on Dec. 24, 1998; accepted on March 31, 1999

pathological diagnosis had been made after standard H&E staining of primary tumors and regional lymph nodes, on the basis of the General Rules for the Gastric Cancer Study in Surgery and Pathology in Japan [11]. All the tumors were diagnosed as being associated with serosal invasion of the gastric wall without apparent metastasis to regional lymph nodes and to distant sites (pT3N0, M0), and all were classified as stage II. According to their clinicopathological characteristics, tumors were grouped by size and location, as determined by gross pathology; by histological type and the extent of lymphatic invasion, as determined by histopathological examination after H&E staining; and by the extent of vascular invasion, as determined after Weigert's fibrin staining.

Twenty-three patients had died of recurrence of the cancer by the end of 1997, with an available follow-up of 4–21 years. Lymph node metastases and/or peritoneal dissemination were detected in 19 cases and hematogenic metastases were detected in 4 cases, according to clinical findings and, in some cases, an autopsy. Another 4 patients, who died within 30 days of surgery as a result of postoperative complications, were excluded from the statistical analysis of survival of the patients.

A total of 2310 lymph nodes were dissected from 83 gastrectomized patients with a median number of 28 nodes (range, 6–52) per patient. All samples were fixed in formalin and embedded in paraffin. Two consecutive sections of 4 μ m in thickness were prepared from each block of resected lymph node. One section was stained with H&E and one was subjected to cytokeratin-specific immunostaining so that we could compare the results obtained by the two methods in adjacent sections of each lymph node. Conventional H&E staining and immunohistochemical staining were limited to one section per lymph node, respectively, to simulate the condition of routine histological examination. All 83 primary tumors and 10 lymph nodes in which metastases had been found by H&E staining were used as positive controls. In addition, sections of 12 normal lymph nodes obtained from patients with benign diseases were used as negative controls.

Immunohistochemical staining was performed by the streptavidin-biotin (SAB) immunoperoxidase method with the murine monoclonal antibody CAM 5.2 (Becton Dickinson, San Jose, CA, USA), which specifically recognizes intracellular cytokeratin component numbers 8 and 18, an intermediate filament representing the intracellular network of the cytoskeleton that is expressed in simple epithelia and apparently nowhere else [10,13]. 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 the samples with 3% hydrogen peroxide in 100% methanol. The tissue sections were

then incubated with the primary murine monoclonal antibody CAM 5.2 at 25 μ g/ml overnight at 4°C. The second antibodies, biotinylated antibodies against mouse immunoglobulin, were applied next and were followed by peroxidase-labeled streptavidin. The reaction products were visualized with diaminobenzidine as the chromogen, and sections were counterstained with methyl green, which stains lymphocytes. Tris-buffered saline was used instead of the primary antibody for negative controls.

The H&E-stained slides were first assessed for the presence of metastases by an experienced pathologist at a magnification of $\times 200$ without the knowledge of sample groupings and previous diagnosis. Then, immunostained slides were examined and the results were compared with those obtained from H&E-stained slides. Occult involvement in lymph nodes was recognized when tumor cells were detected only by cytokeratin-specific immunostaining, having been overlooked after ordinary H&E staining. Occult involvement was expressed in terms of microinvolvement and micrometastases. Microinvolvement was defined as the presence of discrete or isolated tumor cells in the sinus or medulla of the lymph node. By contrast, the presence of one or more aggregates or clusters of more than two tumor cells was classified as micrometastasis. The patients were classified as having microinvolvement when microinvolvement, but no micrometastasis, was found in any of their lymph nodes. If only micrometastasis was detected in any of the lymph nodes, the patient was classified as having micrometastases.

Statistical analysis was performed by Student's *t*-test to examine the association between the number of lymph nodes with occult cancer cells and the clinicopathological characteristics of the primary tumor. The chi-square test was used to examine the relationships between microinvolvement and micrometastases in lymph nodes with occult cancer cells. The Kaplan–Meier method was used for construction of cumulative survival curves, and the generalized Wilcoxon test was used for tests of the statistical significance of differences. Multivariate survival analysis was performed using Cox's proportional hazards model. A *P* value less than 0.05 was considered an indicator of statistical significance.

Results

Characteristics of occult involvement

As shown in Table 1, 299 of 2310 (13%) lymph nodes contained cancer cells that were immunopositive for cytokeratin. Microinvolvement was recognized in 82 of these nodes (27%) and micrometastases in 217 (73%).

Table 1. Frequency of microinvolvement and micrometastasis in lymph nodes of 83 patients with pT3 gastric cancer

Cancer cells in lymph nodes	No. of patients	No. of resected nodes	No. of involved nodes (%)	No. of involved nodes (%)			No. of patients (%)		
				G1 only	G2 only	G1 & G2	G1 only	G2 only	G1 & G2
Negative	29	742	0	0	0	0	0	0	0
Microinvolvement	18	579	82 (14)	37 (6)	0	45 (8)	10 (56)	0	8 (44)
Micrometastases	36	989	217 (22)*	39 (4)	7 (0.7)	178 (18)†	12 (33)	2 (6)	22 (61)‡
Total	83	2310	299 (13)	299			22 (26)	32 (39)	

G1 & G2, group 1 and group 2 lymph nodes

*, †, ‡ Difference between microinvolvement and micrometastasis: *, $P < 0.0005$; †, $P < 0.0001$; ‡, not significant**Table 2.** Characteristics of group 1 node involvement in 32 patients with group 2 lymph node involvement

Occult involvement in group 2 nodes	No. of patients	Occult involvement in group 1 nodes (%)			<i>P</i>
		Negative	Microinvolvement	Micrometastasis	
Microinvolvement	8	0	8 (100)	0	<0.001
Micrometastasis	24	2 (8)	2 (8)	20 (83)	

Micrometastases were more frequent when occult cancer cells were found not only in group 1 but also in group 2 lymph nodes.

Fifty-four of 83 patients (65%) had immunohistochemically detectable occult cancer cells in their resected lymph nodes that had been overlooked after routine staining with H&E. Microinvolvement was found in 18 (33%) of these patients, and micrometastases in 36 (67%). Twelve of 22 patients (33%) with occult cancer cells limited in group 1 lymph nodes and 24 of 32 patients (61%) with occult cancer cells in group 2 lymph nodes were determined to have micrometastases (Table 1). Moreover, 20 of 24 (83%) patients with micrometastases in group 2 lymph nodes were found to have micrometastases already in their group 1 nodes ($P < 0.001$; Table 2).

All 83 primary tumors were found to immunoreact consistently with the monoclonal antibody against cytokeratin. Moreover, all 10 sections of lymph nodes with metastases that were detectable by conventional H&E staining contained immunoreactive cancer cells. By contrast, no immunopositive cells were found in the 12 sections of normal lymph nodes.

Relationship between occult involvement and clinicopathological characteristics

Analysis of the relationships between the mean number of lymph nodes with occult cancer cells and the characteristics of the tumor revealed a statistically significant increase in the incidence of microinvolvement or micrometastases in cases of poorly differentiated

tumors as compared with well-differentiated tumors ($P < 0.05$) and in cases of larger tumors more than 7 cm in diameter as compared with smaller tumors 7 cm or less in diameter ($P < 0.001$; Table 3).

We found no significant relationship between the number of lymph nodes with occult involvement and other clinicopathological features of the primary tumor, such as patient's age, sex, location of the tumor, and lymphatic or vascular invasion.

Relationship between occult involvement and prognosis

The overall 5-year survival rate of the 79 patients who did not die of postoperative complications was 72%. None of the 27 patients without occult involvement died of recurrence of the tumor during follow-up. Survival analysis using the Kaplan–Meier method demonstrated the shorter survival of the patients with microinvolvement and the patients with micrometastases in less than 10% of resected nodes (5-year survival, 81%), as compared with the patients without occult cancer cells in lymph nodes ($P < 0.05$). However, the former group had significantly better survival than patients with micrometastases in 10% or more of their resected nodes (5-year survival, 42%; $P < 0.01$; Fig. 1). Our data also showed that patients with occult involvement in group 2 lymph nodes had a significantly poorer prognosis (5-year survival, 47%), than those with occult involvement limited to the group 1 lymph nodes (5-year survival, 75%; $P < 0.05$; Fig. 2). Sixteen of 23 (70%) patients in whom micrometastases were found in group 2 lymph nodes died of recurrence of the tumor, while only 2 of

Table 3. Number of lymph nodes with occult cancer cells and clinicopathological characteristics of the primary tumor

Variable	No. of patients (<i>n</i> = 83)	No. of lymph nodes with occult cancer cells (mean ± SD)	<i>P</i>
Gender			
Male	46	3.59 ± 4.42	NS
Female	37	3.62 ± 3.89	
Age (years)			
<60	44	3.65 ± 3.80	NS
≥60	39	3.54 ± 4.60	
Tumor location			
Upper	14	3.36 ± 3.78	NS
Middle	47	3.74 ± 4.74	
Lower	22	3.50 ± 3.13	
Maximum diameter (cm)			
≤7	43	1.91 ± 2.59	<0.001
>7	40	5.42 ± 4.77	
Histology			
Differentiated	19	2.05 ± 2.48	<0.05
Undifferentiated	64	4.06 ± 4.46	
Lymphatic invasion			
Negative	44	3.27 ± 3.55	NS
Positive	39	3.97 ± 4.79	
Vascular invasion			
Negative	39	2.92 ± 3.67	NS
Positive	44	4.21 ± 4.52	

NS, not significant

Fig. 1. Kaplan-Meier postoperative survival curves for 79 patients with pT3 gastric cancer. *a* The 5-year survival of 27 patients with no occult cancer cells in lymph nodes (100%). *b* The 5-year survival of 17 patients with microinvolvement and 4 patients with micrometastases in less than 10% of resected lymph nodes (81%). *c* The 5-year survival of 31 patients with micrometastases in 10% or more of resected lymph nodes (42%). Generalized Wilcoxon test: *a* vs. *b*, $P < 0.05$; *b* vs. *c*, $P < 0.01$ **Fig. 2.** Kaplan-Meier postoperative survival curves for 79 patients with pT3 gastric cancer. *a* The 5-year survival of 27 patients with no occult cancer cells in lymph nodes (100%). *b* The 5-year survival of 20 patients with occult cancer cells limited to the group 1 lymph nodes (75%). *c* The 5-year survival of 32 patients with occult cancer cells in group 2 lymph nodes (47%). Generalized Wilcoxon test: *a* vs. *b*, $P < 0.05$; *b* vs. *c*, $P < 0.05$

Table 4. Multivariate analysis of independent prognostic factors in 79 patients with pT3 gastric cancer

Variable	Relative risk	<i>P</i>
Gender	0.96	0.93
Age	1.88	0.21
Maximum diameter of the primary tumor	3.47	0.02
Histological type	0.50	0.30
Lymphatic invasion	1.62	0.37
Vascular invasion	0.86	0.77
Occult cancer cells in lymph nodes	4.09	0.001

12 (17%) patients with micrometastases limited to group 1 lymph nodes died of recurrence of the tumor ($P < 0.005$).

The independent prognostic factors that were identified by multivariate survival analysis are given in Table 4. Survival was independently correlated with only two factors: occult cancer cells in lymph nodes and the size of the primary tumor.

Discussion

Microinvolvement and micrometastases in regional lymph nodes of patients with gastric cancer have been the focus of increasing interest in recent years because of their significance in determination of the extent of lymphadenectomy and in prediction of the prognosis of patients.

Recently published studies [4,12,13] have demonstrated the presence of isolated tumor cells or small clusters of such cells in lymph nodes of patients with early and advanced gastric cancer. These cells probably affected the prognosis of the patients. However, only a few cases of locally advanced cancers (pT3) were included among the cases studied. We examined sufficiently large numbers of 83 patients with a subgroup of pT3N0 gastric cancer and 2310 resected lymph nodes in the present study, using cytokeratin-specific immunostaining with the CAM 5.2 monoclonal antibody. We found that a large percentage of patients (65%) who had been diagnosed as being free of lymph node metastases by routine H&E staining had in fact immunoreactive cancer cells in their regional lymph nodes.

It is still unclear whether these occult cancer cells can proliferate and cause recurrence of the tumor or whether some of them are removed by the host's immune response [12]. However, it is of interest that the patients with only microinvolvement in the form of discrete or isolated cancer cells in lymph nodes had better survival, while patients with micrometastases in the form of small clusters of cancer cells in their lymph

nodes had a significantly poorer prognosis. These findings are comparable with Siewert's results [4]. Moreover, we found that the group 2 lymph nodes were more likely to contain occult cancer cells when group 1 lymph nodes contained micrometastases. Patients with occult involvement in group 2 nodes had a significantly poorer prognosis. The lymphatic system thus plays a crucial role in the spread of primary gastric cancer. These results appear to explain why patients in whom no metastases have been found in lymph nodes after routine H&E staining still tend to die of recurrence of the tumor. If these findings, that occult involvement in lymph nodes detected by cytokeratin-immunostaining can strongly affect the prognosis of the patients, could be confirmed in a large population of patients in further studies it might contribute to a more individual tailoring of future multimodal therapies.

The major risk factors for metastasis to lymph nodes are generally considered to be large size, poor differentiation, and deep invasion of the primary tumor [14–16]. In the present study, we also found that occult cancer cells were more frequent in lymph nodes of patients with large tumors more than 7cm in diameter and with undifferentiated tumors. Moreover, the frequency (65%) of occult involvement in our patients with pT3 gastric cancer was significantly higher than the frequency (40%) reported by Ishida et al. for a group of patients with advanced gastric cancer [12] and than the frequency (23.5%) reported by Maehara et al. for a group of patients with early gastric cancer [13]. The deep invasion by pT3 gastric cancer in our group of patients might explain the high frequency of occult involvement that we detected.

The failure to detect microinvolvement and micrometastases in lymph nodes is clearly a drawback of conventional H&E staining. The accuracy of such staining might well be improved by examination of a large numbers of lymph nodes from each patient and of multiple sections of each node, as well as by using a panel of monoclonal antibodies against tumor-associated antigens. However, such procedure would not be practical for a routine histopathology service [9]. A sensitive supplementary tool, for use in addition to standard H&E staining, would be most useful for improvement of clinical diagnoses. The predictive value of current molecular genetic methods for detecting occult involvement, which exploit the polymerase chain reaction or mutant allele-specific amplification, has been suggested [7,8]. However, it is hard to avoid false-positive results in many cases, even though these methods reveal occult involvement with so-called supersensitivity. Immunostaining with the cytokeratin-specific monoclonal antibody CAM 5.2 has been suggested to be the most useful method for detecting discrete or small clusters of tumor

cells in lymph nodes [12,17]. In this study, we found that all primary tumors and all lymph nodes with metastases that has been detected by H&E staining were immunoreactive. By contrast, none of the normal lymph nodes removed from patients with benign diseases was immunostained. Among resected lymph nodes with no evidence of metastasis by H&E staining, 13% were found to contain occult cancer cells when only one section of each node was immunostained. The results indicate that immunostaining with the cytokeratin-specific monoclonal antibody CAM 5.2 is an accurate and sensitive method for detecting occult cancer cells in lymph nodes. Such immunostaining would seem to be a useful supplement to standard H&E staining especially in cases of patients with high-risk factors, such as large, poorly differentiated, and deeply invasive tumors.

It has been reported that patients with stage II gastric cancer benefit from D2 lymph node dissection [1,4]. In the subgroup of patients with pT3N0 gastric cancer, it would seem surprising that removal of large numbers of apparently uninvolved regional lymph nodes should have an impact on prognosis. Our results provide a persuasive explanation for this phenomenon. Moreover, we also found that when group 1 lymph nodes contained micrometastases, group 2 lymph nodes were more likely to contain cancer cells. Patients in whom with group 2 lymph nodes contain occult cancer cells had a 5-year survival of only 47%. Removal of these nodes might have improved the prognosis of these patients. Thus, systematic radical D2 lymphadenectomy should always be included in the treatment of these patients with pT3 gastric cancer.

References

1. Siewert JR, Bottcher K, Roder JD, Busch R, Hermanek P, Meyer HJ, et al. Prognostic relevance of systematic lymph node dissection in gastric carcinoma. *Br J Surg* 1993;80:1015–8.
2. Adachi Y, Kamakura T, Mori M, Baba H, Maehara Y, Sugimachi K. Prognostic significance of the number of positive lymph nodes in gastric carcinoma. *Br J Surg* 1994;81:414–6.
3. Roder JD, Bottcher K, Siewert JR, Busch R, Hermanek P, Meyer HJ, et al. Prognostic factors in gastric carcinoma: results of the German Gastric Carcinoma Study 1992. *Cancer (Phila)* 1993;72(7):2089–97.
4. Siewert JR, Kestlmeier R, Busch R, Bottcher K, Roder JD, Muller J, et al. Benefits of D2 lymph node dissection for patients with gastric cancer and pN0 and pN1 lymph node metastases. *Br J Surg* 1996;83:1144–7.
5. Trojani M, Mascare I, Bonichon F, Coindre JM, Delsol G. Micrometastases to axillary lymph nodes from carcinoma of breast: detection by immunohistochemistry and prognostic significance. *Br J Cancer* 1987;55:303–6.
6. Sedmak DD, Meineke TA, Knechtges DS. Detection of metastatic breast carcinoma with monoclonal antibodies to cytokeratins. *Arch Pathol Med* 1989;113:786–9.
7. Yamamoto N, Kato Y, Yanagisawa A, Ohta H, Takahashi T, Kitagawa T. Predictive value of genetic diagnosis for cancer micrometastasis: histologic and experimental appraisal. *Cancer (Phila)* 1997;80:1393–8.
8. Hayashi N, Arakawa H, Nagase H, Yanagisawa A, Kato Y, Ohta H, et al. Genetic diagnosis identifies occult lymph node metastases undetectable by the histopathological method. *Cancer Res* 1994;54:3853–6.
9. Davidson BR, Sams VR, Styles J, Deane C, Boulos PB. Detection of occult nodal metastases in patients with colorectal carcinoma. *Cancer (Phila)* 1990;65:967–70.
10. Sasaki M, Watanabe H, Jass JR, Ajioka Y, Kobayashi M, Matsuda K, et al. Occult lymph node metastases detected by cytokeratin immunohistochemistry predict recurrence in “node-negative” colorectal cancer. *J Gastroenterol* 1997;32:758–64.
11. Japanese Research Society for Gastric Cancer. The general rules for the gastric cancer study in surgery and pathology. Part. Clinical classification. *Jpn J Surg* 1981;11:127–39. Part. Histological classification of gastric cancer. *Jpn J Surg* 1981;11:140–5.
12. Ishida K, Katsuyama T, Sugiyama A, Kawasaki S. Immunohistochemical evaluation of lymph node micrometastases from gastric carcinomas. *Cancer (Phila)* 1997;79:1069–76.
13. Maehara Y, Oshiro T, Endo K, Baba H, Oda S, Ichiyoshi Y, et al. Clinical significance of occult micrometastasis in lymph nodes from patients with early gastric cancer who died of recurrence. *Surgery (St. Louis)* 1996;119:397–402.
14. Maruyama K, Gunven P, Okabayashi K, Sasako M, Kinoshita T. Lymph node metastases of gastric cancer: general pattern in 1931 patients. *Ann Surg* 1989;210:596–602.
15. Maehara Y, Orita H, Okuyama T, Moriguchi S, Tsujitani S, Korenaga D, et al. Predictors of lymph node metastasis in early gastric cancer. *Br J Surg* 1992;79:245–7.
16. Murakami T. Early cancer of the stomach. *World J Surg* 1979;3:685–92.
17. Makin C, Borow LG, Bodmer W. Monoclonal antibody to cytokeratin for use in routine histopathology. *J Clin Pathol* 1984;37:975–83.