Yonago Acta medica 2003;46:125–131

Immunohistochemical Detection of Occult Serosal Microinvasion in Primary Lesions of Gastric Cancer with Subserosal Invasion

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In gastric cancer, the presence or absence of serosal invasion by cancer in the primary lesion is an important prognostic factor. Pathological findings are routinely determined by hematoxylin-eosin (H&E) staining, but it is well known that micrometastasis or microinvasion are easily overlooked by H&E staining. Cytokeratin (CK) proteins serve as reliable markers for cells from epithelial origins. The purpose of this study was to clarify the usefulness of CK immunohistochemical staining in the detection of serosal microinvasion in gastric cancer with subserosal invasion. We examined 50 primary lesions from 50 gastric cancer patients with subserosal invasion. Two consecutive sections were prepared for simultaneous staining with ordinary H&E and CK immunostaining with anticytokeratin antibody (CAM 5.2), respectively. Although there were no differences in the postoperative survival rates between patients with or without microinvasion, serosal microinvasion was detected in 8 (16%) of 50 patients by CK staining, including 1 patient whose invasion was detected by both H&E and CK stainings. CK immunostaining enabled us to make an accurate and detailed diagnosis which we believe to be useful for detecting serosal microinvasion in the primary lesion in gastric cancer with subserosal invasion.

Key words: cytokeratin; gastric cancer with subserosal invasion; hematoxylin-eosin staining; immunohistochemical staining; serosal microinvasion

The presence of serosal invasion by cancer within the gastric wall is the most important prognostic factor for patients with gastric cancer (Koga et al., 1984; Kaibara et al., 1986; Baba et al., 1989), as well as the extent of lymph node metastasis (Maruyama et al., 1989; Siewert et al., 1993; Matsushita et al., 1995). Even when curative gastrectomy is performed on patients with gastric cancer with serosal invasion, many patients die from recurrence of the cancer and the prognosis of such patients remains poor. The development of peritoneal metastasis accounts for more than half of all recurrences after curative resection for gastric cancer with serosal invasion (Kaibara et al., 1989). When cancer cells penetrate the tunica serosa of the stomach, exfoliation of these cells from the surface of the primary cancerous lesion into the peritoneal cavity is induced. Many investigators have reported that patients with gastric cancer with serosal invasion have free cancer cells in the peritoneal cavity and these cancer cells are viable by enzymological and autoradi-

Abbreviations: CK, cytokeratin; H&E, hematoxylin-eosin

ographycal examination (Nakajima et al., 1978; Iitsuka et al., 1979; Tanida et al., 1982). Additionally, these cancer cells may be able to implant themselves and proliferate in the peritoneum causing postoperative peritoneal metastasis. Therefore, precise diagnosis of the presence or absence of serosal invasion by the primary gastric lesion is very important for appropriate postoperative chemotherapy in order to prevent the development of peritoneal metastasis.

Clinicopathologic diagnosis relies on hematoxylin-eosin (H&E) staining in a respective section of lymph nodes and primary lesions to ascertain the presence of metastases and the depth of tumor invasion. However, it has been reported that discrete cancer cells, namely occult micrometastases or microinvasions are sometimes overlooked by routine examination with H&E staining (Trojani et al., 1987; Ishida et al., 1997; Sasaki et al., 1997).

Immunohistochemical techniques with antibodies against cytokeratin (CK) can identify lymph node micrometastasis overlooked by H&E staining in various solid malignancies (Sedmak et al., 1989; Greenson et al., 1994; Maehara et al., 1996), because CK proteins serve as reliable markers for cells of epithelial origin. In 1999, we reported that in gastric cancer with submucosal invasion, CK immunohistochemical analysis can detect not only micrometastases in lymph nodes but also microinvasion of the muscularis propria in primary lesions (Cai et al., 1999). Therefore, CK staining also seemed to be useful in the detection of the serosal microinvasion of gastric cancer with subserosal invasion. The purpose of the current study was to evaluate the usefulness of CK staining in the detection of microinvasion (serosal invasion) in gastric cancer with subserosal invasion in which serosal invasion by the cancer was undetectable by routine H&E staining.

Patients and Methods

Patients

Between 1985 and 1995, 64 patients with gastric cancer with subserosal invasion underwent gastrectomy in the First Department of Surgery, Tottori University Hospital. We excluded 14 patients who had metachronous or simultaneous primary malignancies in organs other than the stomach and/or patients whose primary lesions were limited to the muscularis propria but classified as gastric cancer with subserosal invasion by the presence of lymphatic or venous invasions by the cancer in the tela subserosa apart from the primary lesion. The subjects of this study were 50 patients whose depth of cancer invasion was limited to the tela subserosa of the gastric wall. There were 30 men and 20 women, ranging in age from 31 to 80 years, with a mean age of 63 years. Clinicopathologic data were evaluated according to the General Rules for Gastric Cancer Study in Surgery and Pathology (Japanese Research Society for Gastric Cancer, 1981).

CK-specific immunostaining of the primary lesions

One to 4 paraffin-embedded blocks of the primary lesions were prepared in each patient where the subserosal invasion by the cancer had been identified by the previous H&E-stained sections. Serial sections of 4 μm in thickness from each block were subjected to conventional H&E staining and CK-specific immunostaining to allow comparison of results by the 2 methods in adjacent sections of each primary lesion. For controls, sections from 5 patients with mucosal cancer and 5 patients with serosal invasion in the primary lesion were also stained with the 2 methods in the same manner.

Immunohistochemical staining was performed by the streptavidin-biotin immunoperoxidase method with a murine monoclonal antibody, CAM 5.2 (Becton Dickinson, San Jose, CA) against low-molecular weight CK. CAM 5.2 especially recognizes intracellular CK component numbers 8 and 18, an intermediate filament representing the intracellular network of the cytoskeleton that is expressed in simple epithelia. In brief, dewaxed and dehydrated sections were heated in a microwave oven (700 W) for 10 min for retrieval of antigens in the specimens. Endogeneous peroxidase was blocked by incubation of samples with 3% hydrogen peroxi-

dase in 100% methanol. The tissue sections were then incubated with primary antibody CAM 5.2 at 25 μg/mL overnight at 4˚C. The 2nd set of antibodies, biotinylated against mouse immunoglobulin, were applied with subsequent application of peroxidase-labeled streptavidin. 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 for negative controls.

The H&E-stained slides were first assessed for the determination of the depth of invasion in the primary lesion, followed by the assessment of immunostained slides. Microinvasion in the primary lesions was defined as the presence of isolated or clustered cancer cells exposed to the tunica serosa of the stomach wall.

Statistical analysis

Statistical analysis was performed by the chi-square test to examine the relationships between microinvasion and the clinicopathologic characteristics of the primary lesion. For analysis of survival after gastrectomy, we used the Kaplan-Meier method and the generalized Wilcoxon test for the statistical significance. A *P* value less than 0.05 was considered to be significant. None of the patients was lost during follow-up.

Results

The controls used were 5 CK-stained specimens of mucosal cancer whose cancerous lesions were confirmed to be limited to the mucosal layer without microinvasion of the submucosal layer. Moreover, the non-cancerous normal epithelium was also CK-positive in all specimens. Five specimens stained with CK of serosally exposed cancer confirmed complete exposure of cancer cells on the tunica serosa of the stomach. Further, some mesothelial cells and/or smooth muscle were stained with CK unexpectedly in some specimens.

Table 1 summarizes the results of the current study in 50 gastric cancer patients with subserosal invasion in which serosal invasion was undetectable by H&E staining. Eight patients (16%) were determined to have serosal microinvasion, including 1 patient (Patient 1) whose serosal invasion was detected by both H&E and CK stainings (Figs. 1a and b). In 7 patients (Patients 2 to 8), serosal microinvasion was detected by CK staining, but H&E staining failed to detect it, as shown in Figs. 1c and d.

Table 1. Clinicopathologic findings of 8 patients with serosal microinvasion

Patient		Depth of invasion		Gross finding	Tumor size (cm)	Histologic type	Nodal metastasis	Vascular invasion	
Number	Age (year)/ gender	H&E	CK					Lymphatic	Venous
1	78/F	se	se	Type 2	3.9	Undiff	+	+	–
2	73/M	ss	se	Type 2	8.6	Diff	+	+	+
3	70/F	ss	se	Type 2	8.0	Undiff	+	+	+
4	76/M	ss	se	Type 2	7.0	Undiff	–	+	+
5	37/F	ss	se	Type 3	2.2	Diff	–	–	–
6	80/M	ss	se	Type 3	11.0	Undiff	+	+	+
7	42/M	ss	se	Type 2	3.5	Undiff	+	+	–
8	75/F	ss	se	Type 5	4.6	Undiff	–	+	+

CK, cytokeratin; Diff, differentiated adenocarcinoma; F, female; H&E, hematoxylin and eosin; M, male; se, tunica serosa; ss, tela subserosa; Undiff, undifferentiated adenocarcinoma.
Type 2, ulcerated carcinomas with sharply demarcated and raised margins; Type 3, ulcerated carcinomas without definite limits, infiltrating into the surrounding wall; Type 5, non-classifiable carcinomas that cannot be classified into any of the other types.

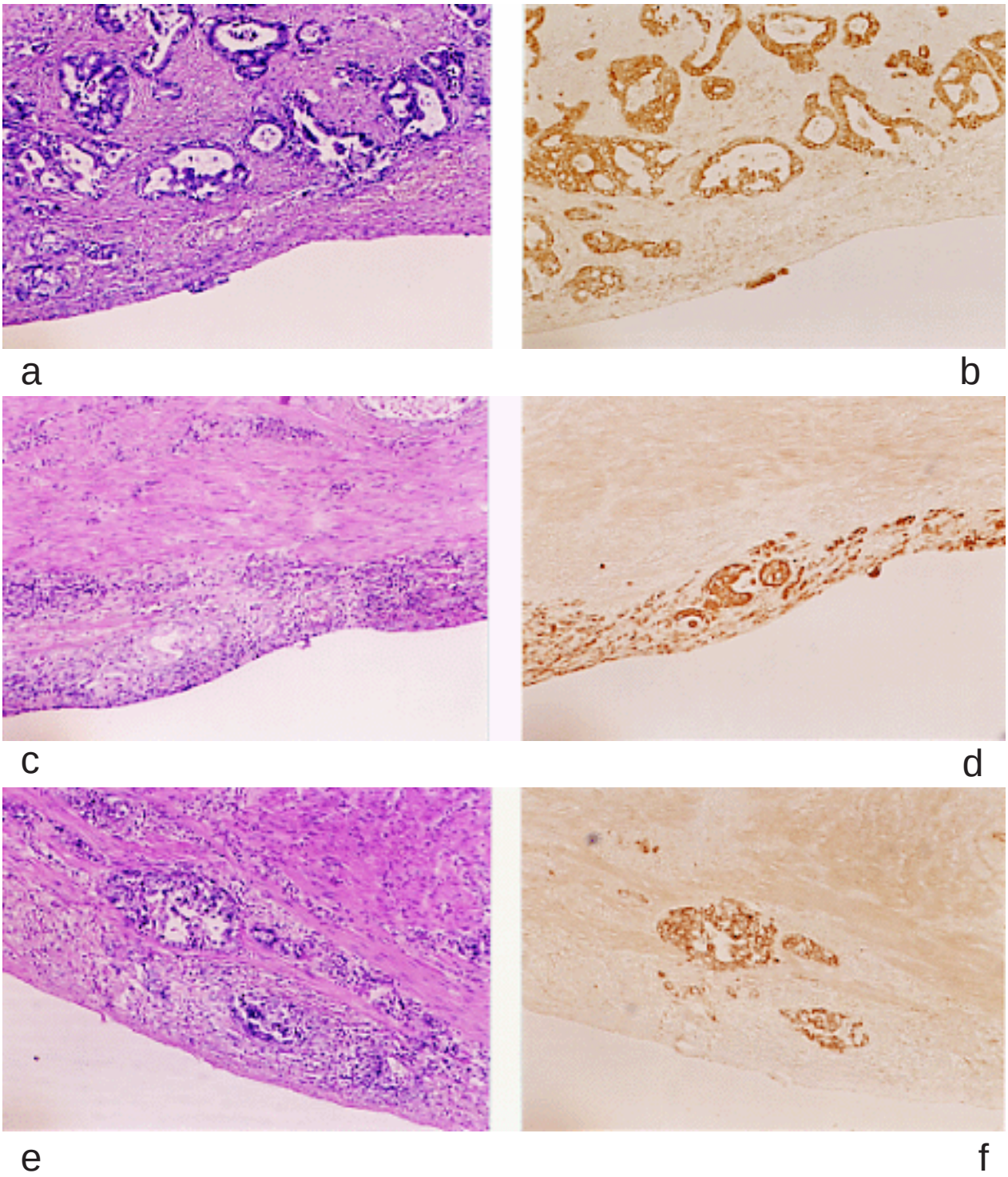

Fig. 1. Comparison of the results between H&E and CK stainings.
a and **b:** Example of stained primary lesion after staining with H&E (**a**) and CK (**b**) (2.5 × 10 each). Serosal microinvasion was detected by both H&E and CK stainings (Patient 1).
c and **d:** Example of stained primary lesion after staining with H&E (**c**) and CK (**d**) (2.5 × 10 each). Serosal microinvasion was not detected by H&E staining but by CK immunostaining.
e and **f:** Example of stained primary lesion after staining with H&E (**e**) and after staining with CK (**f**) (2.5 × 10 each). Serosal microinvasion was not detected by either H&E or CK staining.

Table 2. Serosal microinvasion in the primary lesion and clinicopathologic characteristics

Variable		Number of patients	Microinvasion of the serosa		*P* value
			Positive (%) [n = 7]	Negative (%) [n = 42]	
Age (year)		49	64.7 ± 17.5	62.2 ± 10.9	
Gender (male/female)		30/19	4/3	26/16	
Gross finding	Type 0, 1	8	0	8	
	Type 2	24	4 (17)	20 (83)	
	Type 3	13	2 (15)	11 (85)	
	Type 4	1	0	1	
	Type 5	3	1 (33)	2 (67)	0.6423
Histologic type	Differentiated	19	2 (11)	17 (89)	
	Undifferentiated	30	5 (17)	25 (83)	0.5495
Depth of invasion*	ssα, β	26	3 (12)	23 (88)	
	ssγ	23	4 (17)	19 (83)	0.5590
Nodal metastasis	Negative	19	3 (16)	16 (84)	
	Positive	30	4 (13)	26 (87)	0.8108
Lymphatic invasion	Negative	15	1 (7)	14 (93)	
	Positive	34	6 (18)	28 (82)	0.3114
Venous invasion	Negative	17	2 (12)	15 (88)	
	Positive	32	5 (16)	27 (84)	0.7132

Types 0 and 1, superficial, flat tumors with or without minimal elevation or depression and polypoid tumors, sharply demarcated from the surrounding mucosa, usually attached on a wide base, respectively; Type 4, diffusely infiltrating carcinomas in which ulceration is usually not a marked feature; Type 2, 3 and 5, see footnote to Table 1.

* When cancerous invasion extends to the tela subserosa (ss), the growth patterns can be divided into 3 categories; ssα, expansive growth pattern with a distinct border; ssβ, intermediate pattern between ssα and ssγ; ssγ, infiltrative growth pattern with an ill-defined border.

Six of these 8 patients with serosal microinvasion had undifferentiated adenocarcinoma as a histologic type of gastric cancer. The remaining 42 patients were determined to have no serosal microinvasion by either H&E or CK-staining (Figs. 1e and f).

Clinicopathologic characteristics of the 7 patients with serosal microinvasion in the primary lesions (Patient 1, whose serosal invasion was detected by H&E and CK stainings, was excluded) were compared with those of 42 patients without it (Table 2). Microinvasion was found to be more frequent in undifferentiated types of cancer and in patients with lymphatic invasion by cancer. However, there were no statistical differences between the 2 groups among any variables.

Figure 2 compares the 5-year Kaplan-Meier survival curves for 7 patients with and 42 patients without microinvasion. There was no difference in survivals between the 2 groups.

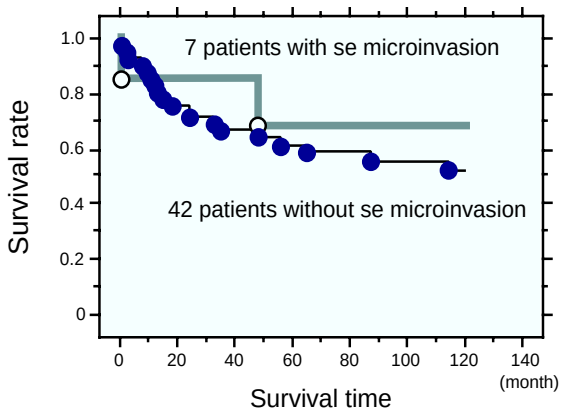

Fig. 2. Postoperative survivals (Kaplan-Meier method). se, tunica serosa.

Discussion

Identification of lymph node metastasis is an important factor in predicting postoperative survival of patients with gastric cancer, and H&E staining has been widely used for this purpose. However, immunohistochemical techiques with antibodies against CK can identify micrometastases in lymph nodes missed during routine H&E staining from gastric cancer (Maehara et al., 1996; Ishida et al., 1997; Maeta et al., 1998; Cai et al., 1999), colo-rectal cancer (Greenson et al., 1994; Adell et al., 1996; Sasaki et al., 1997) and breast cancer (Sedmak et al., 1989; Dowlatshahi et al., 1997). Because CK proteins are essential constituents of the cytoskeleton of both normal and malignant epithelial cells in lymph nodes, they serve as reliable markers for the epithelial origin of cells. Patients with these CK-positive cells in lymph nodes have a significantly decreased postoperative survival time.

Since it is known that in patients with gastric cancer, the depth of cancer invasion within the stomach wall is also an important prognostic factor, as well as nodal metastases, we reported previously that immunohistochemical staining with CK was useful for the detection of microinvasion of the muscularis propria in gastric cancer with submucosal invasion (Cai et al., 1999). In gastric cancer patients with serosal invasion, serosally exposed cancer cells are easily isolated in the peritoneal cavity, and these cells proliferate as a peritoneal metastasis. On cytologic examination of peritoneal lavage fluid immediately after laparotomy at the time of surgery, free intraperitoneal cancer cells are frequently found in such patients. Kaibara et al. (1986) reported that there were close relationships both between the rate of detection of intraperitoneal free cancer cells and the area of serosal invasion, and between the area of serosal invasion and prognosis in patients with gastric cancer with serosal invasion. Therefore, in patients with gastric cancer, the presence of serosal invasion implies a very high risk for the development of postoperative peritoneal metastasis.

In the current study, the usefulness of CK staining was examined in order to find out whether it can detect serosal microinvasion in gastric cancer with subserosal invasion, because serosal microinvasion may be overlooked by routine H&E staining. As a result, microinvasion was detected in 8 patients, including 1 whose microinvasion was detected by both CK and H&E stainings. However, serosal microinvasion was not a significant prognostic factor, and there were no differences in postoperative survivals between patients with or without microinvasion. The area of microinvasion detected by CK staining in this series may be too narrow to both introduce the isolation of free cancer cells into the peritoneal cavity and affect postoperative survival.

Based on our findings above, it may be suggested that there is no clinical implication in detecting serosal microinvasion using CK staining. However, the important point is how to make an effort to diagnose the presence or absence of serosal invasion and/or microinvasion accurately and precisely. In our series, the number of patients was too small to answer the question of whether the detection of serosal microinvasion by CK staining is important or not, and thus a multi-institutional study using a large number of patients is necessary. Nevertheless, induction of the CK staining as in this series in the pathohistological diagnosis of serosal microinvasion may be the 1st trial ever for gastric cancer. We believe that H&E and CK stainings should be seriously considered for simultaneous use in the accurate determination of both nodal metastasis and depth of cancer invasion.

Although CK is a reliable marker of cells from epithelial origins, Makin et al. (1984) reported that there were some exceptions to negative staining, such as normal stratified squamous esophageal epithelia and of positive staining of cells from non-epithelial origins, such as smooth muscle and mesothelium. As indicated in this series, when CK immunostaining is used for detecting serosal microinvasion, careful attention should be paid to the unexpected exceptional positive staining of mesothelial one-layered cells.

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Received October 15, 2003; accepted November 17, 2003

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