

# Is a Lymph Node Detected by the Dye-Guided Method a True Sentinel Node in Gastric Cancer?

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## ABSTRACT

**Purpose:** A sentinel node is defined as the initial lymph node, to which cancer cells metastasize from a primary tumor. Recently, sentinel node navigation surgery has been done using the dye-guided method. However, no study has shown that a lymph node detected by the dye-guided method is the true sentinel node from the viewpoint of micrometastasis. Micrometastases of lymph nodes, in which no metastasis was found by H&E staining, were examined to establish whether a lymph node detected by the dye-guided method is the true sentinel node.

**Experimental Design:** Isosulfan blue was injected endoscopically as the dye-guided method at a submucosal lesion of early gastric cancer. Total 345 lymph nodes, including 150 blue-dyed lymph nodes and 195 nondyed lymph nodes were collected from 57 patients and each was quartered. Two quarters were examined histologically by H&E staining and cytokeratin staining. The other specimens were used for quantitative reverse transcription-PCR of *CEA* and *CK20* mRNAs.

**Results:** Lymph node disease was not found in any of 345 lymph nodes from the 57 patients by routine H&E staining. By contrast, either *CEA* or *CK20* mRNA expression was detected in 21 of 345 lymph nodes obtained from the 10 (18%) of 57 patients by quantitative reverse transcription-PCR. Eight of the 21 micrometastasis-positive lymph nodes were confirmed to be positive for cytokeratin staining. Although micrometastasis of nondyed lymph nodes was found in three cases, these were included in the 10 cases with micrometastasis of blue-dyed nodes, such that there was no

patient who only had micrometastasis in nondyed nodes. Six of 10 cases were micrometastasis-positive in a single node; all six were blue-dyed nodes.

**Conclusion:** A lymph node detected by the dye-guided method should be a true sentinel node to which cancer cells metastasize initially.

## INTRODUCTION

Sentinel node is defined as the first lymph node to receive cancer cell drainage from the primary tumor, the lymph node to which cancer cells metastasize initially. In 1992, Morton *et al.* (1) reported that the sentinel node was detected successfully by dye injection into cutaneous melanoma. Since then, clinical applications of minimal operations based on the sentinel node concept have been practiced in melanoma (2) and breast cancer (3).

Lymph involvement is frequent in advanced gastric cancer, but most early gastric cancers do not show diseased lymph nodes. However, dissection of perigastric lymph nodes is done in operation for patients with either advanced or early gastric cancer, mostly because the lymph node status cannot be diagnosed before surgery. If node disease were known, minimal surgery with omission of lymph node dissection would be an option for patients with early gastric cancer. Recently, the sentinel node concept has been reported in gastric cancer (4–6). Some investigators have reported the usefulness of sentinel node biopsy for diagnosis of lymph node metastasis during the operation in gastric cancer (4–9); most have done sentinel node biopsy using the dye-guided method. If the lymph node detected by the dye-guided method is proved to be the true sentinel node to which cancer cells metastasize initially, appropriate surgery by sentinel node navigation could be done for early gastric cancer. It is important for clinical practice to clarify that dye flows into lymph in the same manner as cancer cells. Analysis of micrometastasis is important to detect the first lymph node to which cancer cells spread initially. However, no study has shown that lymph nodes detected by the dye-guided method are the true sentinel node from the viewpoint of micrometastasis. It has been reported that reverse transcriptase PCR (RT-PCR) is the most sensitive method for detection of micrometastasis (10), whereas Morton *et al.* (1) examined lymph node metastasis by routine H&E staining or immunohistochemical staining. This is the first study to investigate whether lymph nodes detected by dye-guided method are the true sentinel nodes from the viewpoint of micrometastasis.

## MATERIALS AND METHODS

**Patients.** Eighty patients diagnosed as early gastric cancer preoperatively, who underwent curative gastric resection in our department from 2000 to 2001, were enrolled in this study. Endoscopic examination and endoscopic ultrasound were done to choose patients with early gastric cancer before operation. Early gastric cancer is defined as a lesion in which the depth of invasion is limited to the submucosa, regardless of whether a regional lymph

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node metastasis is evident on histological examination. Informed consent to participate in this study was obtained from all patients before their surgery.

**Dye-Guided Method.** Isosulfan blue (1% Lymphazurin 5 ml; Ben Venue Laboratories, Bedford, OH) was used for the dye-guided method (11). Isosulfan blue was injected into submucosal lesions around the primary gastric cancer using endoscope just after laparotomy. Ten minutes after blue dye injection, blue-dyed nodes were detected macroscopically; blue-dyed nodes and surrounding nondyed nodes were collected. Each collected lymph node was cut in quarters. Two quarter specimens were examined histologically by H&E staining and immunohistochemical staining, and the other specimens were examined by RT-PCR (Fig. 1). Dye-guided method was done in all 80 patients. Blue-dyed nodes were detected in 73 of 80 patients. There were 51 patients with early gastric cancer and six patients with advanced cancer in which all lymph nodes were negative by routine H&E staining. A total of 345 lymph nodes, including 150 blue-dyed nodes and 195 nondyed nodes, were collected

from the 57 patients. Clinicopathological features of the 57 patients are summarized in Table 1. The mean age of patients was 59.8 years (range 33–80). Eleven tumors were located in the upper stomach, 36 in the middle stomach, and 10 in the lower stomach (Table 1).

**RNA Extraction and cDNA Synthesis.** Two quarter specimens of lymph nodes cut for RT-PCR were minced with homogenizer in Trizol Regent (Gibco, Gaithersburg, MD). The mixture and 0.5 ml of chloroform in a 1.5 ml tube was centrifuged at 12,000 rpm for 15 minutes. The supernatant was transferred to a fresh tube, and 0.2 ml propanol was added. RNA was precipitated after centrifugation at 12,000 rpm for 10 minutes, washed with 75% ethanol, and diluted with diethyl pyrocarbonate-treated water. Purified RNA was quantified and assessed for purity by UV spectrophotometry. After RNA isolation, cDNA was prepared from each sample using oligo-(dT)<sub>15</sub> primer and M-MLV Reverse Transcriptase (Life Technologies, Inc. Rockville, MD). RT reaction was done at 37°C for 50 minutes, followed by heating at 70°C for 10 minutes.

*Fig. 1* Lymphazurin-dye method; sampling of blue-dyed nodes and surrounding nondyed nodes. *A*, blue-dyed nodes were detected by the Lymphazurin-dye method, and blue-dyed nodes and surrounding nondyed nodes were then collected. Each lymph node was cut into quarters. Two quarter specimens were examined histologically by H&E and immunostaining by cytokeratin, and the other specimens were examined by RT-PCR for *CEA* and *CK20*. *B*, example of blue-dyed nodes and surrounding nondyed nodes by the Lymphazurin-dye method. *LGA*, left gastric artery; *CHA*, common hepatic artery.

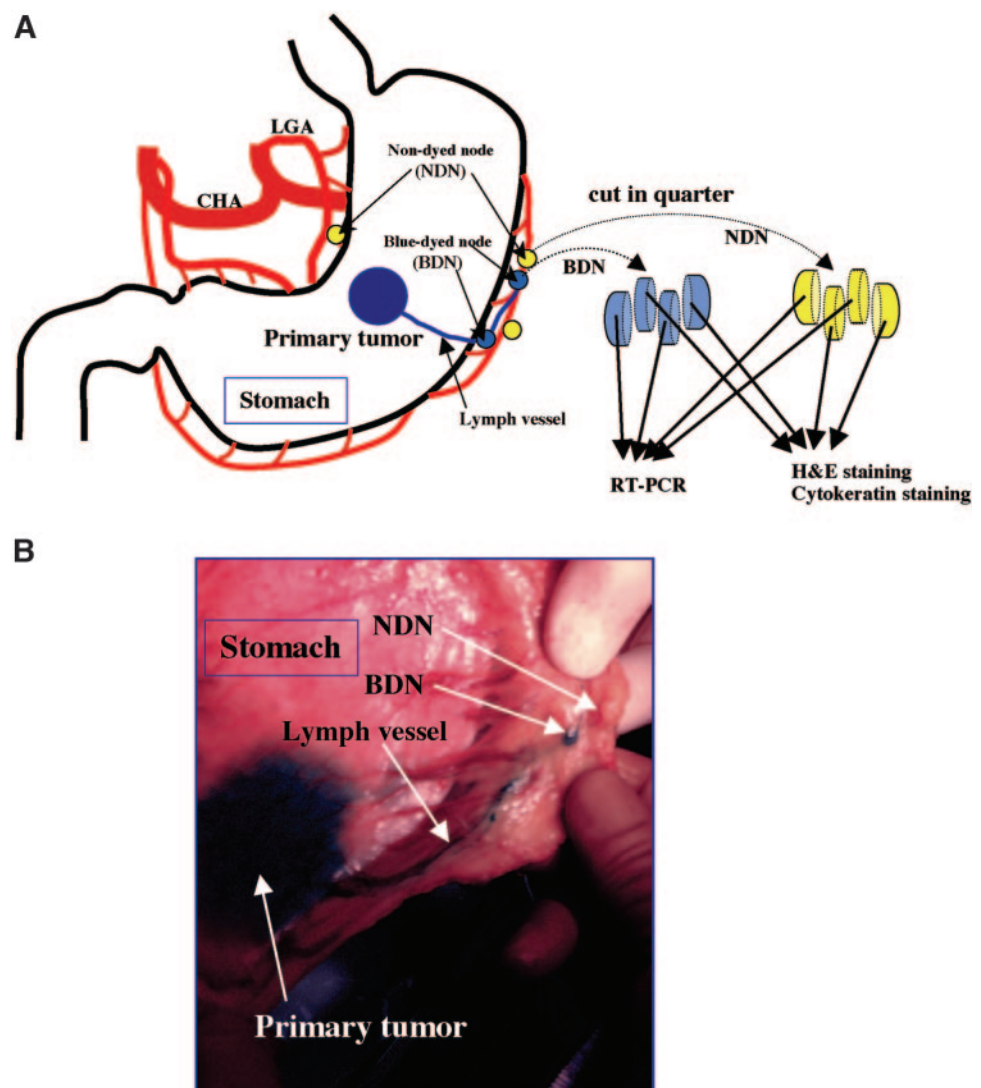


Table 1 Clinicopathologic findings of 57 patients

	<i>n</i> = 57
Mean age	59.8 (33–80)
Male:female	35:22
Tumor location in stomach	
Upper third	11
Middle third	36
Lower third	10
Macroscopic shape*	
IIa	3
IIb	2
IIa+IIc	5
IIc	41
IIc+III	6
Invasion depth	
Mucosa	27
Tela submucosa	24
Tunica muscularis propria	3
Tela subserosa	2
Serosa exposed	1
Histology	
Differentiated	33
Undifferentiated	24

NOTE. H&E staining showed no histologically positive lymph nodes in 57 patients.

\* IIa: flat elevated type, IIb: flat type, IIc: flat ulcerative type, III: ulcerative type.

**Quantitative RT-PCR.** Primer Express software (Applied Biosystems, Foster City, CA) was used to design primers and probes. The primer and probe sequences were as follows. For *cytokeratin20*: forward 5'-CTCTCCTCAAAAAGGAGCA-TCAG-3'; reverse 5'-CAACCTCCACATTGACAGTGTG-3'; Taqman probe FAM-CAGATGCTTGTGTAGGCCATCGACT-TCTT-TAMRA. For *CEA*: forward 5'-CAATAGGACCACAGT-CACGACGAT-3', reverse 5'-GGT TGGAGTTGTTGCTGGT-GAT-3'; Taqman probe FAM-ACAGTCTATGCAGAGCCAC-CCAAACCCTT-TAMRA. For *GAPDH*: forward 5'-GAAGGT-GAAGGTCGGAGTC-3', reverse 5'-GAAGATGGTGATGGGA-TTTC-3', Taqman probe VIC-CAAGCTTCCCGTTCTCAGCC-TAMRA. RT-PCR was carried out with the ABI PRISM 7700 Sequence Detection system (Applied Biosystems). The total volume of PCR reaction was 50  $\mu$ L, containing 2  $\mu$ L of cDNA template, 25  $\mu$ L TaqMan Universal PCR Master Mix (Applied Biosystems), 0.1  $\mu$ mol/L probe, and 0.3  $\mu$ mol/L of each primer. PCR conditions were as follows: after incubation at 50°C for 2 minutes and denaturing at 95°C for 10 minutes, 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C.

**Standard Curve.** The gastric cancer cell line, OCUM-2M, was used for constructing a standard curve of *CEA* and *CK20* expression. cDNA from 1  $\mu$ g of RNA from OCUM-2M cells (12) was diluted with normal monocytes at various ratios. Standard curves for *CEA*, *CK20*, and *GAPDH* were then constructed. Three gastric cancer cell lines (MKN-45, OCUM-2M, OCUM-2M-D3), 29 primary gastric cancers, and 13 histologically positive lymph nodes were obtained as positive controls. As a negative control, blood samples from six healthy volunteers were obtained.

**Immunohistochemistry.** Sample specimens were formalin-fixed and paraffin-embedded. We stained one additional section from lymph node immunohistochemically by cytokeratin, using the

AE1/AE3 (20:1 mixture of AE1 to AE3; Boehringer, Mannheim, Germany), a monoclonal antibody mixture that is reactive with a broad spectrum of human cytokeratins (13). Immunohistochemical staining was done by the streptavidin-biotin method.

## RESULTS

**Determination of Cutoff Values.** *CK20* and *CEA* mRNA were detectable by RT-PCR at a concentration as low as 1 cancer cell/10<sup>6</sup> normal lymphocytes. Standard curves of *GAPDH*, *CEA*, and *CK20* are shown in Fig. 2. Based on the standard curves, *CEA* mRNA and *CK20* mRNA of primary tumors (*n* = 29), diseased lymph nodes (*n* = 13), histologically

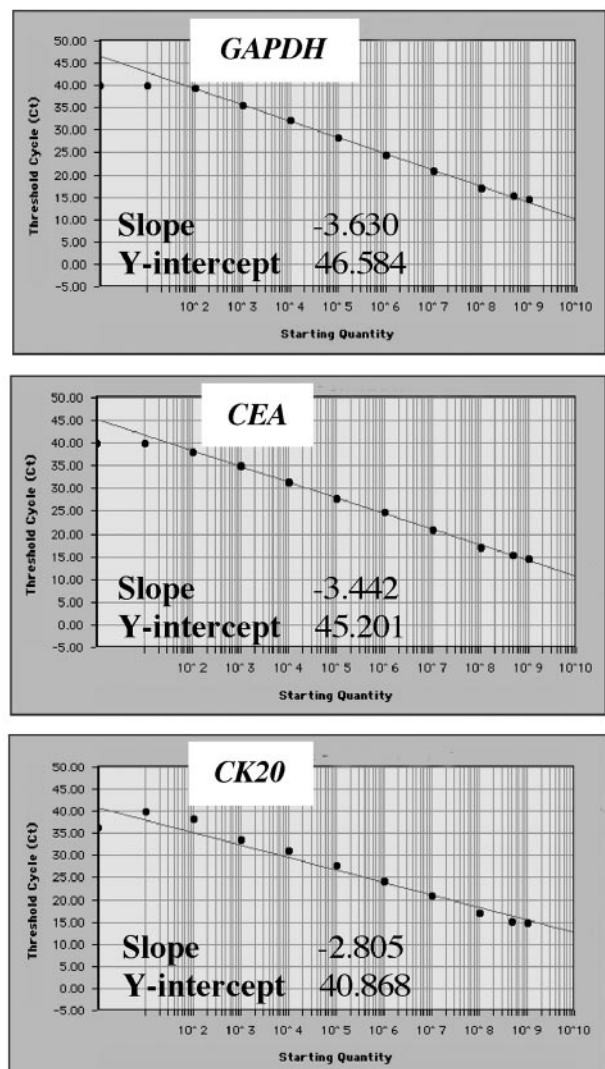


Fig. 2 Standard curves of RT-PCR amplification of *GAPDH*, *CEA*, and *CK20*. Samples containing 10 different dilutions (1:1, 10, 10<sup>2</sup>, 10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup>, 10<sup>8</sup>, 10<sup>9</sup>) of OCUM-2M in normal monocytes were subjected to real-time RT-PCR. The threshold cycle Ct represents the fractional cycle number at which a substantial increase above the baseline signal was first detected. The log starting copy number is plotted against the threshold cycle Ct. According to the standard curve, *CEA* and *CK20* mRNA levels in lymph nodes were calculated.



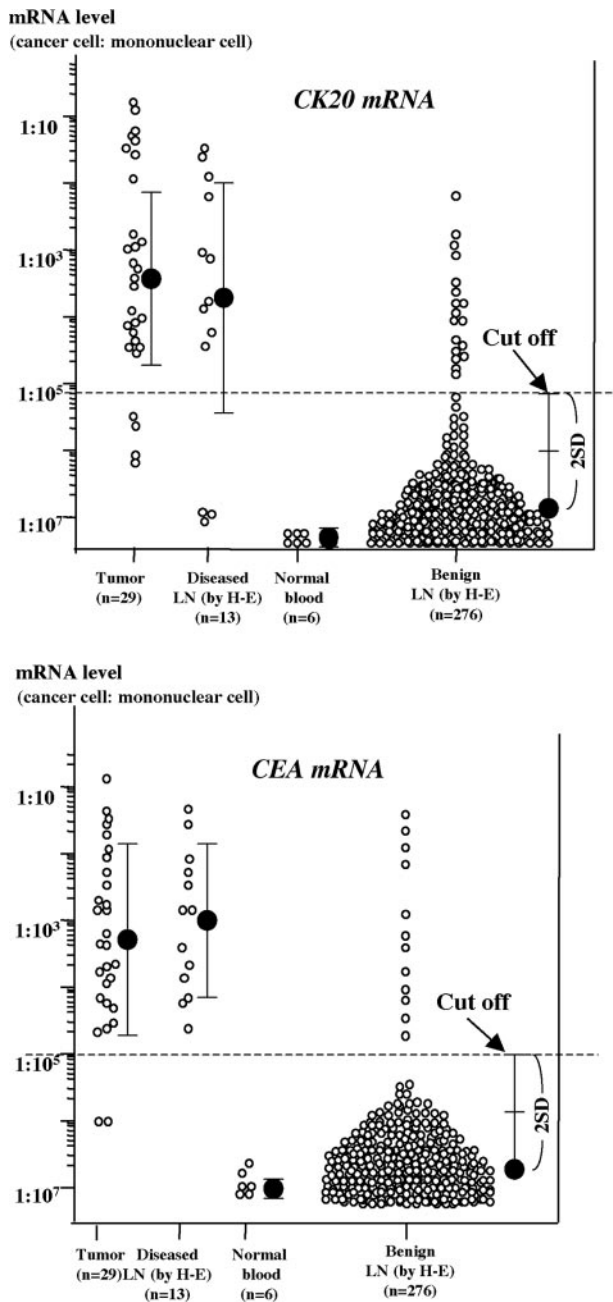


Fig. 3 Expression levels of *CK20* and *CEA* mRNA. On the basis of standard curves, *CEA* and *CK20* mRNA in primary tumors, diseased lymph nodes (H&E staining), noninvolved lymph nodes, and normal blood of healthy volunteers were quantified by RT-PCR. Each quantified value was plotted. Each cutoff value was decided based on the mean plus 2 SD of *CK20* and *CEA* level in noninvolved lymph nodes.

benign lymph nodes ( $n = 345$ ), and normal blood of healthy volunteers ( $n = 6$ ) were quantified by RT-PCR. Each quantified value was plotted in Fig. 3. Each cutoff value was determined as the mean plus 2 SDs based on the quantified values of non-involved lymph nodes. The cutoff value of both *CK20* and *CEA* mRNA levels were equal to  $1:10^5$  cancer cell/

Table 2 *CEA* and *CK20* mRNA expression of tumors by quantitative RT-PCR

Tumor	<i>CEA</i> (%)	<i>CK20</i> (%)	<i>CEA</i> and/or <i>CK20</i> (%)
Gastric cancer cell lines*	3/3† (100)	3/3 (100)	3/3 (100)
Primary gastric tumors	27/29 (93)	25/29 (86)	28/29 (97)
Metastatic lymph nodes	13/13 (100)	10/13 (77)	13/13 (100)

NOTE. Twenty-nine primary tumor samples and 13 histologically positive lymph nodes by H&E staining were obtained from patients with advanced gastric cancer.

\* Three gastric cancer cell lines (MKN-45, OCUM-2M, OCUM-2M-D3) were used.

† Number of *CEA* and *CK20* positive tumor/total number of tumor.

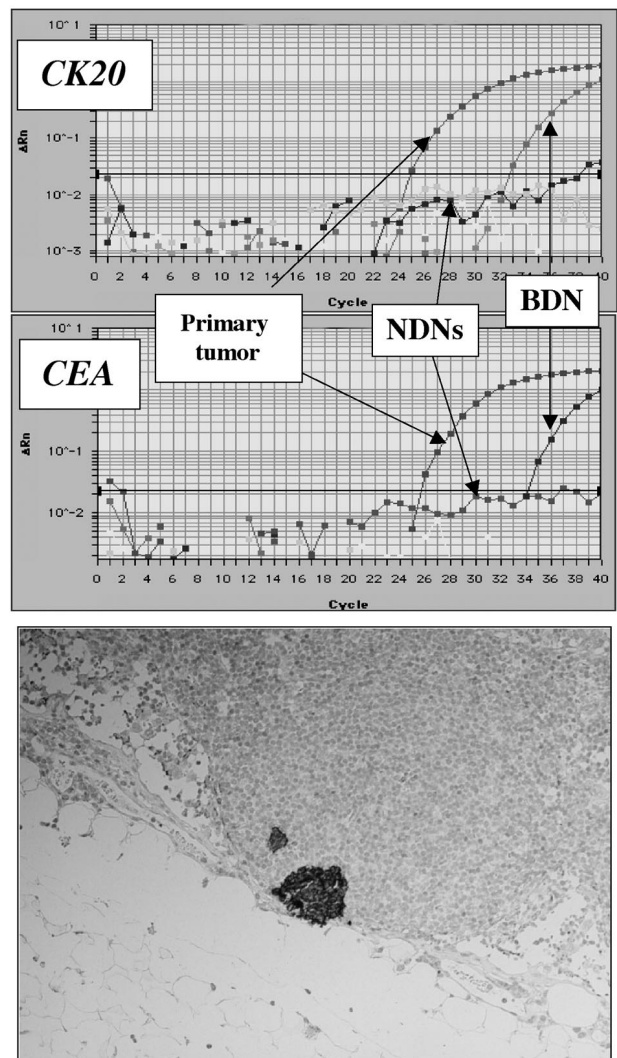


Fig. 4 Positive case of micrometastasis in lymph nodes. A, both *CK20* and *CEA* mRNA were positive in a blue-dyed node, whereas *CK20* and *CEA* expression were not found in a nondyed lymph node. Primary tumor also expressed *CK20* and *CEA* mRNA (case 1). B, micrometastasis in the blue-dyed node detected by cytokeratin staining. An isolated tumor cell cluster was found in a blue-dyed node.

Table 3 Individual cases determined to be micrometastasis positive in lymph node

Case	Blue-dyed node				Nondyed lymph node				Invasion depth	Histologic findings		
	CK20 (RT-PCR)	CEA (RT-PCR)	Micro-metastasis†	CK (IHC)‡	CK20 (RT-PCR)	CEA (RT-PCR)	Micro-metastasis	CK (IHC)		Lymphatic invasion	Vascular invasion	Histology
1	1/2*	1/2	+	1/2	0/3	0/3	–	0/3	sm	–	–	tub
2	0/2	1/2	+	0/2	0/3	0/3	–	0/3	m	–	–	tub
3	1/4	1/4	+	0/4	0/5	0/5	–	0/5	ss	+	+	por
4	1/6	1/6	+	0/6	0/6	0/6	–	0/6	sm	–	–	sig
5	1/3	0/3	+	1/3	0/3	0/3	–	0/3	sm	–	–	tub
6	0/2	1/2	+	0/2	0/4	0/4	–	0/4	m	–	–	tub
7	3/5	3/5	+	1/5	0/5	0/5	–	0/5	sm	–	–	por
8	2/2	0/2	+	0/2	2/4	0/4	+	0/4	ss	+	–	tub
9	2/4	0/4	+	2/4	1/4	0/4	+	1/4	sm	–	–	tub
10	1/6	2/6	+	1/6	0/3	3/3	+	1/3	m	–	–	por

NOTE. +, positive; –, negative.

Abbreviations: m, mucosa; sm, submucosa; ss, subserosa; tub, tubular adenocarcinoma; por, poorly differentiated adenocarcinoma; sig, signet ring cell carcinoma.

\* Number of positive lymph node/total lymph node number.

† Micrometastasis was determined to be positive when a lymph node expressed either CK20 or CEA.

‡ Immunohistochemistry by cyokeratin staining.

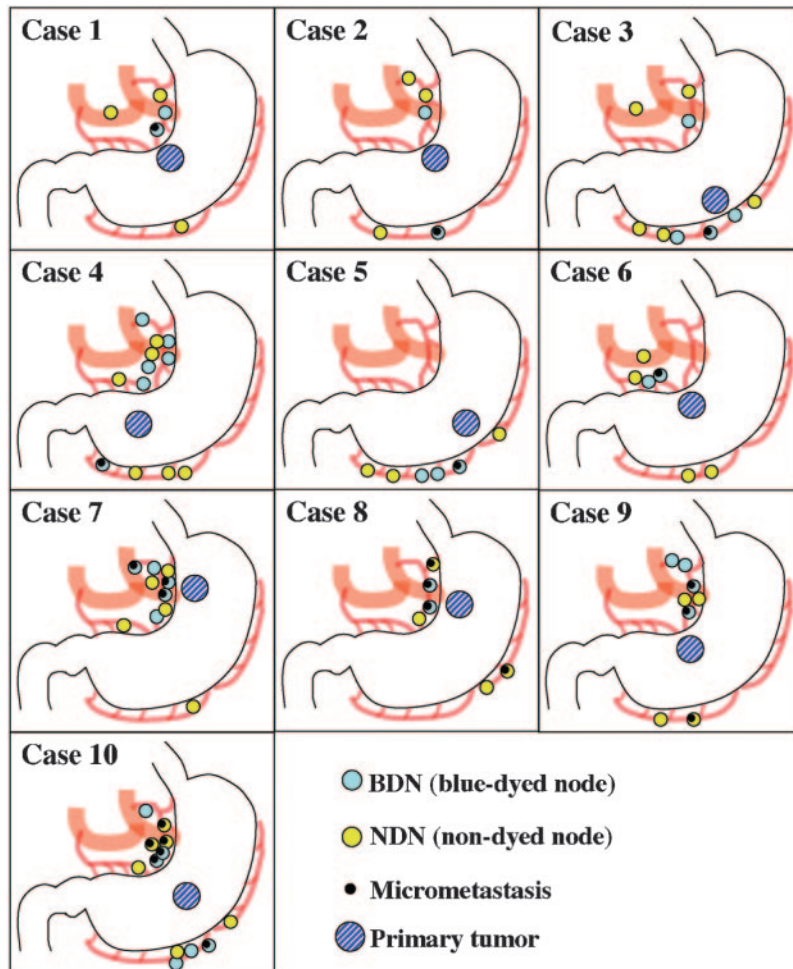


Fig. 5 Mapping of 10 cases with lymph node micrometastasis. Six (cases 1, 2, 3, 4, 5, and 6) of 10 cases were micrometastasis-positive in only one lymph node; all blue-dyed nodes. Three cases (8, 9, and 10) were micrometastasis-positive in both blue-dyed nodes and nondyed nodes.

mononuclear cell ratio (Fig. 3). When *CEA* or *CK20* mRNA level of the sample was over  $1:10^5$ , the mRNA expression was considered positive.

**Sensitivity of the RT-PCR.** *CEA* mRNA and *CK20* mRNA expression of all three cancer cell lines was positive. Positive rates of *CEA*, *CK20*, and either marker of primary gastric cancer were 93.1% (27 of 29), 86.2% (25 of 29), and 96.6% (28 of 29), respectively. Positive rates of *CEA*, *CK20*, and either markers of diseased lymph nodes were 100% (13 of 13), 76.9% (10 of 13), and 100% (13 of 13), respectively (Table 2). Lymph nodes positive for either *CK20* or *CEA* by RT-PCR were determined to be micrometastasis-positive.

**Micrometastasis of Blue-dyed Nodes and Nondyed Nodes.** Twenty-one (16 blue-dyed nodes and 5 nondyed nodes) of 345 lymph nodes from 57 patients, in which no metastasis was found using H&E staining, were over the cutoff value by RT-PCR (Fig. 4A). Eight of 21 micrometastasis-positive lymph nodes were confirmed to be positive for cytokeratin staining (Fig. 4B). These 21 lymph nodes were obtained from 10 patients; expression of *CEA* and *CK20* mRNA and immunohistochemical findings are shown in Table 3. Mapping of positive lymph nodes in the 10 cases is shown in Fig. 5. All 10 cases had micrometastasis in the blue-dyed nodes. Seven of 10 cases were micrometastasis-positive only in blue-dyed nodes (cases 1–7). Although micrometastasis of nondyed nodes was found in three cases (cases 8–10), these three cases included micrometastasis of blue-dyed nodes, such that there was no patient who only had micrometastasis in nondyed nodes (Table 4). Six of 10 cases were micrometastasis-positive in only one blue-dyed node (cases 1–6). Of the 21 micrometastasis-positive lymph nodes, 20 were perigastric, and one was located distantly (case 7). Two of 10 cases showed micrometastasis of nondyed nodes located in the different lymphatic basin from the blue-dyed node (case 8, case 9).

## DISCUSSION

Most carcinoma cells express both *CEA* (14) and *CK20* mRNA (15), but lymphocytes do not. Therefore, we used RT-PCR of *CEA* and *CK20* for detection of very small numbers of cancer cells in lymph nodes as reported previously (16, 17). In this study, blue-dyed nodes were detectable in 98% (57 of 58) of gastric cancer patients by the dye-guided method using Lymphazurin, which indicated that Lymphazurin was highly sensitive for detection of blue-dyed nodes in gastric cancer. Micrometastases were detected in 21 of 345 lymph nodes in which no metastasis was found by H&E staining. These 21 lymph nodes were obtained from 10 of 57 patients. All 10 cases had micrometastasis in the blue-dyed nodes. There was no patient who was detected micrometastasis at nondyed nodes without a detection of micrometastasis at blue-dyed nodes. Six of 10 cases were micrometastasis-positive in only one blue-dyed node. These findings suggest that the blue-dyed node detected by the dye-guided method is the true sentinel node in early gastric cancer. It has been reported the sentinel node biopsy using the dye-guided methods is useful for diagnosis of lymph node metastasis in gastric cancer (8, 9). However, no study has shown that dye flows into lymph in the same manner as cancer cells

Table 4 Correlation between micrometastasis of blue-dyed nodes and nondyed nodes

	Micrometastasis	Nondyed lymph nodes micrometastasis	
		Negative	Positive
Blue-dyed nodes	Negative	47	0
	Positive	7	3

NOTE. There were ten cases with micrometastasis of lymph nodes. Seven of ten cases were micrometastasis-positive only in blue-dyed nodes. Three of ten cases were micrometastasis-positive in both blue-dyed nodes and nondyed lymph nodes. There was no patient who only had micrometastasis in nondyed nodes.

from the viewpoint of micrometastasis. This is the first report to confirm that a lymph node detected by the dye-guided method should be the true sentinel node by the analysis of micrometastasis. Appropriate minimal surgery with sentinel node navigation using this dye-guided method would be made available for patients with early gastric cancer.

Ajisaka *et al.* (18) reported that all additional metastases were detected in non-sentinel nodes located in the same lymphatic basin as the previously detected sentinel nodes, and they also suggested that lymph node dissection of early-stage gastric cancer in the lymphatic basin may be mandatory even for patients without histologically detectable metastases in sentinel nodes. Carlini *et al.* (19) reported that gastric adenocarcinoma has its own lymphatic basin in which metastasis can occur. In contrast, two of 10 cases showed micrometastasis of nondyed lymph nodes located in the different lymphatic basin from the sentinel nodes detected previously in this study (case 8, case 9). It has been reported that the incidence of nodal basin recurrence after sentinel node biopsy was found to be 0.6% in cutaneous melanoma (20). Additional study may be needed for the clinical indication of lymph node dissection in the lymphatic basin.

In the follow-up within 3 years, there was no patient who died or recurrent in 57 patients. Therefore, no significant difference in survival and recurrence was found between micrometastasis-positive cases and micrometastasis-negative cases. The reasons for no difference might be that the follow-up period was short, and that these micrometastasis-positive lymph nodes were dissected in operation. However, in case 9, serum *CEA* was increased from 6.3 to 9.7 ng/ml at 2 years after operation. This case showed micrometastasis in both blue-dyed node and nondyed node, whereas no histological metastasis was found by routine H&E staining. A careful follow-up may be needed for this case. It has been reported that a 5- to 10-year survival rate of early gastric cancer patients without lymph node metastasis by routine H&E staining was 91 to 98% (21, 22, 23). It is important for 57 patients to follow up for a long period.

In this study, *CEA* mRNA and/or *CK20* mRNA was expressed in 97% (28 of 29) of primary tumors and 100% (13 of 13) of diseased lymph nodes (H&E staining). These findings suggested that RT-PCR using *CEA* and *CK20* is a sensitive method for detection of lymph node involvement in gastric cancer. The cutoff value was determined as the mean plus 2 SD of *CEA* and *CK20* mRNA in benign lymph nodes ( $n = 345$ ). According to the cutoff value, 1 (3.4%) of 29 primary tumors

was negative for *CEA* or *CK20* by RT-PCR. About 3 to 4% of carcinoma did not express *CEA* and *CK20* mRNA, which suggests that a false negative rate of micrometastasis might be about 3 to 4% of lymph nodes by RT-PCR of *CEA* or *CK20*. Lymph node detected by the dye-guided method is proved to be the true sentinel node; however, sentinel node navigation surgery could have the risk for a false negative of sentinel node micrometastasis in early gastric cancer.

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