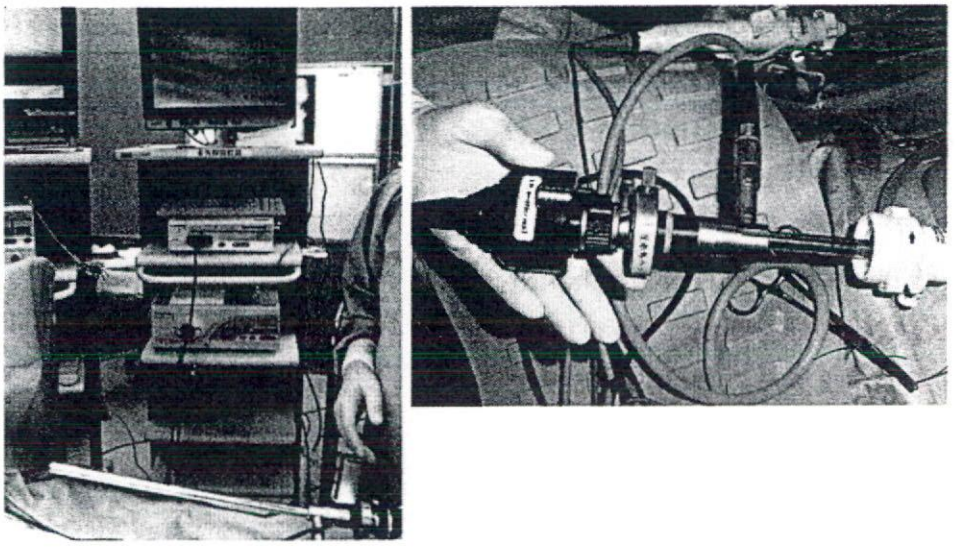

図2　赤外線腹腔鏡システム
〔二村浩史，他：消化器内視鏡　17；738-741，2005[7]より引用〕

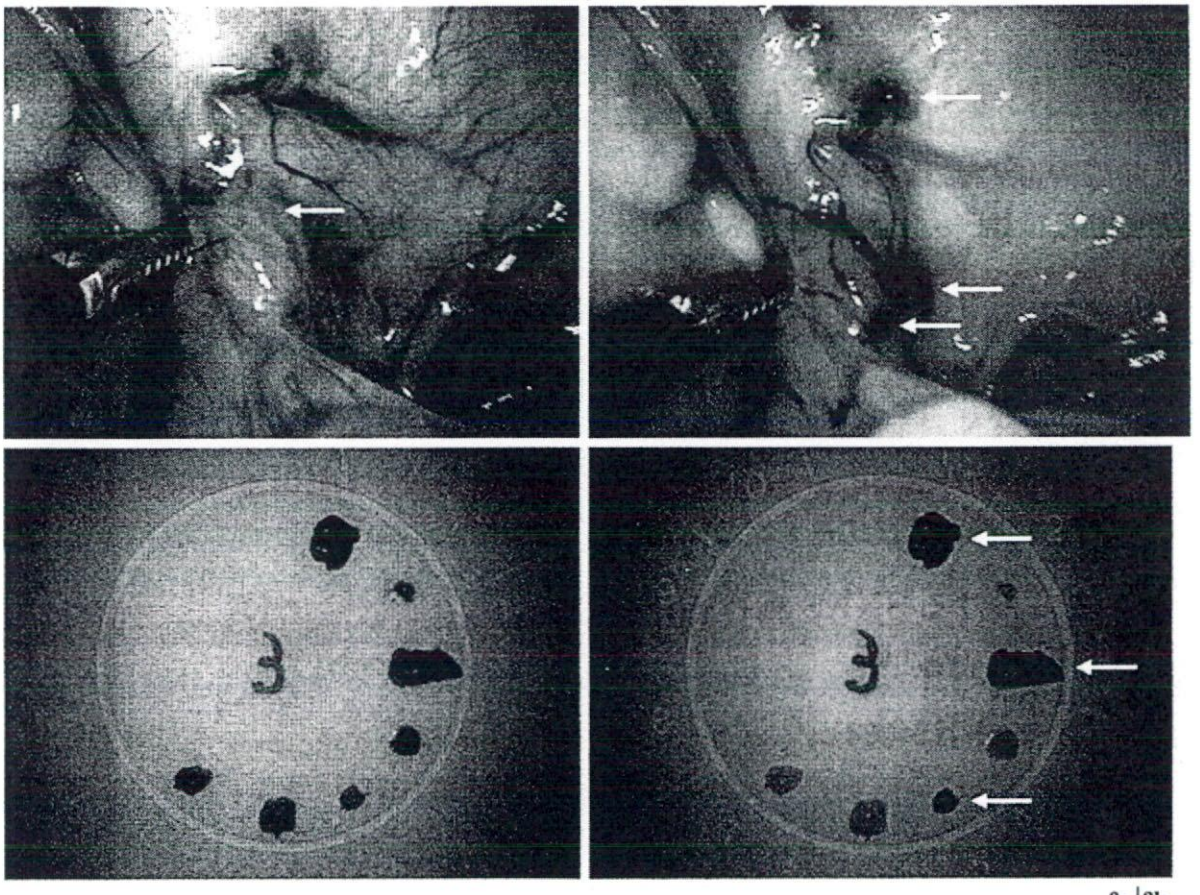

図3　肉眼観察と赤外観察
a：肉眼にて green のリンパ管が観察できる．
b：赤外では明らかに明瞭にリンパ管，リンパ節が観察できる．
c：肉眼で SN は視認できない．
d：赤外では明瞭に SN の同定ができる（矢印）．

節をSNとして同定する(図3c, d).

赤外法は現在まだ臨床応用前段階であるため，全例は術中凍結迅速病理診断に出してはいない．リンパ節病理診断法は最大割面のHE染色で行う．すべてホルマリン固定後サイトケラチン免疫染色(CAM 5.2, Becton Dickinson, San Jose, CA, USA)で微小転移の有無も確認する．

Ⅲ. 赤外法におけるSNNS施行対象と検討方法

これまで赤外法によるSNNSを施行した胃癌患者195人(うち腹腔鏡手術78人)を対象とした．

開腹および腹腔鏡下での赤外法におけるSN同定率，転移リンパ節検出感度を検索した．またEMRやESD後における赤外法SNNS 13人の状況を検討した．さらに2005年7月から2006年6月まで慈恵医大4附属病院合同で行った開腹下赤外法SNNS臨床試験24人の肉眼観察と赤外光観察におけるICG陽性リンパ流域数，SN個数，SN同定率，転移リンパ節検出感度を比較検討した．

Ⅳ. 赤外法におけるSNNS施行結果と肉眼観察との比較

1. 赤外法によるSNNS(表1)

この項のポイント
- 癌で完全に占居されて色素が入っていかないリンパ節以外に，赤外線観察では偽陰性は認めなかった．
- EMR, ESD後であっても腹腔鏡下で十分SNNSは可能であった．

胃癌全体ではSN同定率195人中194人(99%)，感度33人中32人(97%)であった．同定できなかった1人は胆石術後で癒着強固の患者であった．また転移リンパ節がICG陰性であった1人は，肉眼および触診で明らかに癌で置換されており，術中病理診断にても癌細胞による占居で転移診断された患者である．腹腔鏡下ではSN同定率78人中78人(100%)，感度10人中10人(100%)であった．とくに腹腔鏡下では薄いgreen nodeやICG陽性リンパ管の同定は困難であり，赤外法は有用であった(図3)．

表1 赤外観察SNNS

	赤外線観察SNNS		計
	開腹下	腹腔鏡下	
患者数	117	78	195
SN同定率	116(99%)	78(100%)	194(99%)
リンパ節転移例	23	10	33
転移検出感度	22(96%)	10(100%)	32(97%)

EMR, ESD後であってもSN同定率は13人中13人(100%)，感度は3人中3人(100%)であった．いずれも周囲臓器と癒着を認めたり，局注に際して粘膜下が切除後瘢痕による硬化で色素が入りづらいことがある．13人中7人に腹腔鏡下SNNSを施行したが，SNNSは十分可能であった．

2. 肉眼観察と赤外光観察の比較(表2)

この項のポイント
- 肉眼観察では偽陰性が生じるが，赤外光観察では癌で置き換わったリンパ節以外は偽陰性は認めなかった．
- 肉眼ではICG陽性リンパ流域を見逃す危険性がある．

慈恵医大4附属病院合同臨床研究での開腹下でのICG陽性リンパ流域数は，肉眼では24人中18人(75%)に観察でき，平均0.9流域(0～2流域)であった．赤外観察では24人中24人

表2　慈恵医大4附属病院合同開腹下赤外観察SNNS臨床試験

	肉眼観察	赤外観察
ICG陽性リンパ管同定率	18/24(75%)	24/24(100%)
ICG陽性リンパ流域数	0.9(0～2)	1.7(1～4)
SN同定率	16/24(67%)	24/24(100%)
SN個数	2.8(0～13)	8.5(3～28)
転移検出感度	3/8(38%)	7/8(88%)

(100%)に観察でき，平均1.7流域(1～4流域)であった．SN同定率は，肉眼24人中16人(67%)に対し，赤外では24人中24人(100%)であった．SN個数は，肉眼で平均2.8(0～13)個，赤外で平均8.5(3～28)個であった．転移リンパ節検出感度は，肉眼8人中3人(38%)に対して赤外では8人中7人(88%)であった．赤外で感度陰性であった1人は，先に述べた癌で置換されて色素が入っていけない転移リンパ節の患者である．

V．考察—赤外線腹腔鏡システムの有用性

胃癌SNNSにおいて色素法はもっとも簡便であり，とくにICGはわが国では安価で安全性も保たれており，使用しやすい色素である．しかし，表2のように肉眼のみでは明らかに視認できないICG陽性リンパ管やSNがあり，偽陰性となる危険性が高い．また，他の色素でも時間の経過とともにwash outされて視認できなくなってしまうという欠点がある．他の色素で偽陰性の報告もある[10]．その点，赤外観察は，ICGの濃度が薄くても黒く明瞭に視認できるため確実にSNの同定ができる[6)~8)]．とくに腹腔鏡下ではすべてのgreen nodeを視認することは困難であり，ICGを用いるならば赤外観察が必要と考える[8),11)]．そのうえ腹腔鏡下では部屋を暗くしなくてもよく，また一連の腹腔鏡観察の流れのなかでワンタッチで赤外観察に切り替えることができ，煩雑さがない．赤外観察でSN以外に転移を認めたとする報告がある[11]．また癌に占居されて色素が入らない転移リンパ節は偽陰性となるが，LBDを施行し掘り出したリンパ節を赤外観察したり，触診することで解決できると考える．アイソトープ法では視認できないため色素の併用が必要[3]であり，煩雑なうえアイソトープ室が必要である．さらに腹腔鏡下では側方線量のため同定が困難なことがある[12]．今後，一般病院においてもSNNSを応用するならば，赤外線腹腔鏡システムは有用と考えられる．

VI．EMR，ESDと腹腔鏡手術の接点

現在，ESDの進歩により内視鏡治療の適応が広がりつつある[13]．今後，ESDと腹腔鏡手術の接点が生まれるが，リンパ節郭清の必要性を判断するうえでSNNSが必要と考えられる．EMR後ICG肉眼観察のみの腹腔鏡下リンパ節郭清の報告もある[14]．われわれのデータからは偽陰性となる可能性が否定できない．アイソトープ法によるEMR後の機能温存縮小手術への応用も報告されている[15]．赤外観察は腹腔鏡下でリンパ流域が明瞭に観察でき，LBDの範囲が明確にできる点でも今後，ESDとのコラボレーションにおいてもっとも期待できる方法であろう．

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Summary

Detection of Sentinel Lymph Node—Observation with Infrared Ray

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Hideyuki Kashiwagi* and Katsuhiko Yanaga*

Compared to the conventional dye method, SNNS with infrared ray observation allows easy, much improved identification of SN. With this technique, the usefulness of combining ESD techniques and laparoscopic surgery is expected.

Key words: early gastric cancer, indocyanine green, infrared ray laparoscopy system, laparoscopic surgery, sentinel lymph node navigation surgery

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Morphological Distribution of Metastatic Foci in Sentinel Lymph Nodes with Gastric Cancer

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Background: The TNM classification defines micrometastasis (MM) and isolated tumor cells (ITC) in lymph nodes (LN). Sentinel node (SN) navigation surgery has been introduced in gastrointestinal cancer. Few reports have examined the morphological distribution of MM and ITC of SN in gastric cancer. The purpose of this study was to clarify the clinical significance of the morphological distribution of cancer cells in SNs according to metastasis (MA), MM, and ITC.

Methods: All dissected LNs obtained from 160 consecutive patients with mapped SNs arising from cT1–2 N0 tumors were examined. Metastasis in these LNs was examined by histology and cytokeratin staining. The distribution of MA, MM, and ITC was classified as marginal sinus (MS), intermediate sinus (IS), parenchymal (PA), and diffuse types (DF).

Results: Nodal metastases were detected in 65 SNs from 30 patients and MA, MM, and ITC accounted for 53.9%, 21.5%, and 24.6%, respectively. MS, IS, PA, and DF accounted for 57%, 6%, 17%, and 20.0%, respectively. Patients with metastasis of non-MS had more nodal metastasis in non-SNs ($P = .025$) and had nodal metastasis in second tier ($P = .009$), compared with the patients with metastasis of MS. The incidence of metastasis in non-MS was higher in tumors larger than 40 mm than those smaller than 40 mm ($P = .011$).

Conclusion: When performing SN navigation surgery in gastric cancer, we should keep in mind that the patients with tumor larger than 40 mm in size and nodal metastasis of non-MS may have non-SN metastasis and nodal metastasis in second tier.

Key Words: Micrometastasis—Isolated tumor cells—TNM classification—Sentinel node—Gastric cancer.

Lymph node metastasis is one of the most important prognostic factors in gastric cancer, even at the early stage.[1,2] Almost all lymph node metastasis occurs in the regional nodes. Lymph nodes play key roles as mechanical and biological barriers against migrating cancer cells in experimental rat model.[3,4] The presence or absence of lymph node metastasis is clinically important for selecting the treatment strategy. If no nodal metastasis is found before or during surgery, less invasive surgery such as endoscopic mucosal resection and reduction of lymphadenectomy are applied in early-stage gastric cancer. The concept of sentinel nodes (SNs) and isolated tumor cells (ITCs) was recently introduced in the Sixth TNM classification,[5] which separates lymph node metastases according to size as follows: metastasis (MA) (>2 mm), micrometastasis (MM) (0.2–2 mm),

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and ITC (< 0.2 mm). Although lymph node MM has been detected by immunohistochemistry or reverse transcription-polymerase chain reaction, the clinical significance remains controversial.

Several groups have applied SN navigation surgery to melanoma and breast cancer.[6-8] According to this concept, a sentinel node is the first lymph node to receive lymphatic flow from the primary tumor and is therefore the initial site of lymph node metastasis. Thus, MM and ITC are probably located in SNs at the first step of lymph node metastasis.[9,10] We previously described lymph node micrometastasis in SNs of gastric cancer.[11,12] Recently, the microanatomic distribution of metastasis within SNs predicts the non-SNs metastasis in melanoma.[13] We investigated the relationship between microanatomic distribution of SN metastasis and clinicopathologic factors in gastric cancer. The goal of the present study is to clarify the clinical significance of the morphological distribution of cancer cells in SNs according to MA, MM, and ITC based on the TNM classification.

PATIENTS AND METHODS

Patients

We enrolled 160 consecutive patients with gastric cancer, who were preoperatively diagnosed with clinical T1–T2 (cT1, 127 patients; cT2, 33 patients) and cN0. Written, informed consent was obtained from all of the patients based on a document approved by our institutional ethics committee. The patients were clinically diagnosed before surgery based on gastrointestinal fiberscopy, double contrast gastrography, endoscopic ultrasonography, and computed tomography. All underwent curative gastrectomy with lymphadenectomy at the Department of Surgical Oncology and Digestive Surgery, Kagoshima University Hospital, between 2001 and 2006. Patients with endoscopic mucosal resection were not enrolled in this study. None of the patients had undergone preoperative radiation therapy or chemotherapy.

Identification of Sentinel Lymph Node

One day before surgery, 3 mCi (2 mL) of ^{99m}Technetium (^{99m}Tc)-tin colloid was endoscopically injected into the submucosa of the gastric wall at four sites (0.5 mL each) around the tumor using a disposable 23-gauge needle (MAJ-75, Olympus, Japan). Curative surgery then proceeded according to the Japanese classification of gastric cancer.[14] Radioisotope (RI) uptake during surgery was measured in individual lymph nodes using Navigator GPS (TYCO HEALTHCARE, Ltd., Tokyo, Japan). SNs were defined as individual lymph nodes with 10-fold greater RI uptake than background. SNs were separately removed during surgery. After surgery, all dissected lymph nodes were mapped and RI uptake was measured again.

Evaluation of Lymph Node Metastasis by Hematoxylin-Eosin and Immunohistochemical Staining

We examined 3945 lymph nodes from 160 patients. The mean number of dissected lymph nodes per patient was 20 (range, 9–69). All lymph nodes were stained with hematoxylin-eosin (HE) and immunohistochemically using a monoclonal anticytokeratin (CK) antibody cocktail (AE1/AE3, DAKO Corporation, Carpinteria, CA, USA) that reacts with a broad spectrum of human CKs. The sections were deparaffinized in xylene, rehydrated with a graded series of ethanol, and then endogenous peroxidase activity was blocked by a 5-min incubation in 3% hydrogen peroxide in methanol. The sections were then immersed in proteinase K (DAKO Corporation, Carpinteria, CA, USA) to activate the antigen and incubated with CK monoclonal antibody diluted 1:200 for 30 min. After two 5-min washes with phosphate-buffered saline (PBS), the avidin-biotin complex and immunoperoxidase were applied (ABC method; VECTASTAIN ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA). Cells positive for CK were visualized using diaminobenzidine tetrahydrochloride, and the sections were lightly counterstained with hematoxylin. The negative controls consisted of sections processed in the same manner but without the primary antibody. Consistently CK-positive normal gastric mucosa and primary tumor specimens were used as positive controls. All immunohistochemical stained slides were evaluated by three independent observers (S.Y, S.N, and Y.U).

Based on the Sixth TNM classification, lymph node metastases were separated according to size: MA (> 2 mm), MM (0.2–2 mm), and ITC (< 0.2 mm). SN metastasis was classified into three types in the measurement of metastatic foci: cluster type in which grouping tumor cells were seen in single site (Fig. 1a), multiple cluster type in which grouping tumor cells were seen in multiple sites (Fig. 1b), and diffuse type in which scattered tumor cells were seen (Fig. 1c). Among these, we measured maximal size of metastatic foci in the cluster type, the sum of the maximal size of metastatic

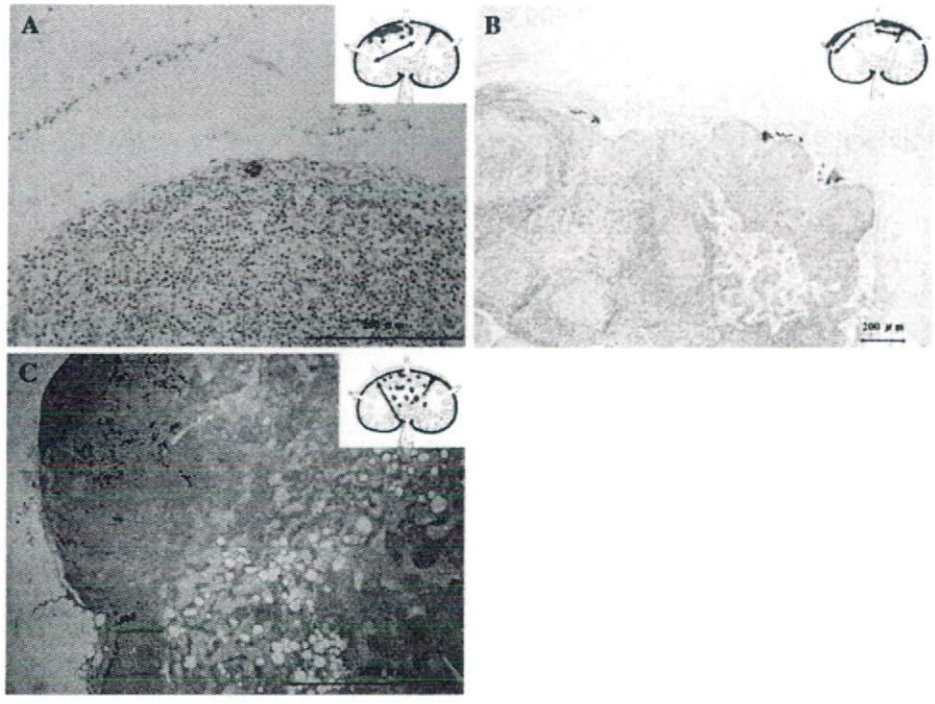

FIG. 1. Measurement criteria for metastatic foci in lymph nodes. Criteria: maximal size of metastatic foci in cluster type, sum of maximal sizes of metastatic foci in multiple cluster type and maximal size of area including cancer cells in noncluster types. (a) Cluster type (400×). (b) Multiple cluster type (100×). (c) Diffuse type (40×).

foci in the multiple cluster type, and the maximal size of the area including cancer cells in the noncluster type (Fig. 1). We classified the distribution of metastatic cancer cells in lymph nodes into four types: marginal sinus type in which tumor cells were present in marginal sinus alone (Fig. 2a), intermediate sinus type in which tumor cells were present in intermediate sinus alone (Fig. 2b), parenchymal type in which tumor cells were seen in parenchyma (Fig. 2c), and diffuse type in which tumor cells were scattered in lymph node (Fig. 2d).

Statistical Analysis

StatView statistical software version 5.5 (SAS institute, Cary, NC, USA) performed all statistical calculations. Data were statistically compared using the χ^2 test. A P value of $< .05$ was considered statistically significant.

RESULTS

Detection of Sentinel Lymph Nodes and Lymph Node Metastasis, Including Micrometastasis

The mean number of SN in 158 of 160 patients (99.7%) was 4.4 (range, 1–17). The rate of detection was 100% and 93.9% (31 of 33) in patients with 127 cT1 and cT2 tumors, respectively.

Lymph node metastasis in SNs was found in 18 of 160 patients (11.9%) by routine histological HE staining. A total number of nodal metastasis was 29. Furthermore, among 142 patients without metastasis by HE staining, CK staining detected 36 metastases in SNs from 12 patients. Accordingly, the total numbers of patients and metastases in SNs were 30 and 65, respectively. Since one patient had false negative result in our SN mapping, sensitivity was accordingly 96.7% (29 of 30) and accuracy rate was 99% (157 of 158).

According to the Japanese Classification of Gastric cancer,[14] 83.3% of patients (25 of 30) had nodal metastasis in first tier and 16.7% of patients (5 of 30) in second tier. Nine patients (30.0%) had nodal metastasis in both SN and non-SN.

Lymph Node Metastasis According to TNM Classification

In 65 nodal metastases, the incidences of MA, MM, and ITC diagnosed by HE staining and CK staining were 53.9% (35 of 65), 21.5% (14 of 65), and 24.6% (1665), respectively (Table 1).

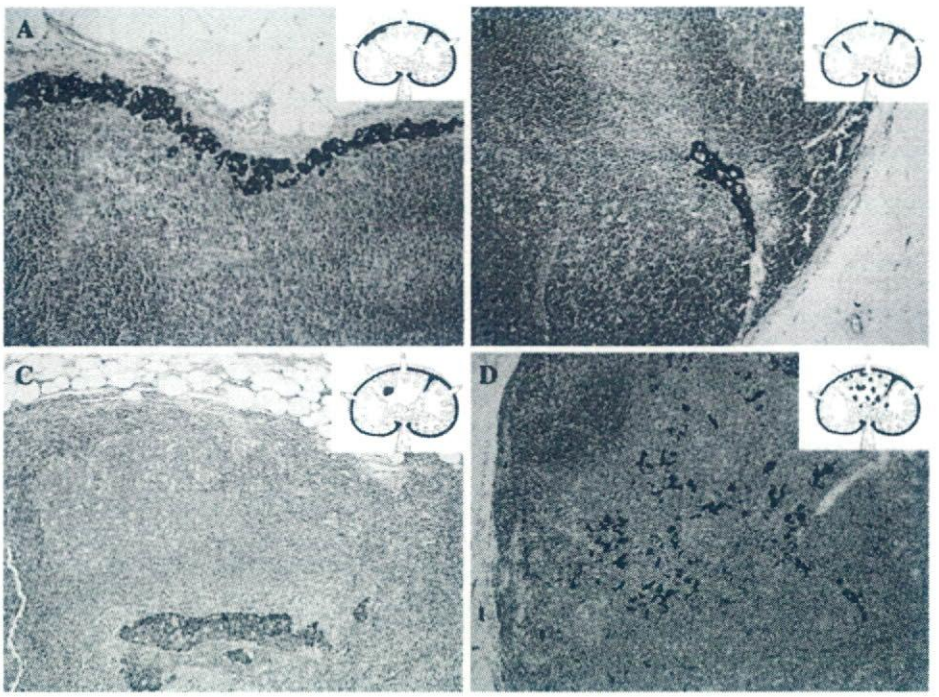

FIG. 2. Classification of distribution of metastatic foci. (a) Marginal sinus type (400×). (b) Intermediate sinus type (400×). (c) Parenchymal type (100×). (d) Diffuse type (100×).

TABLE 1. *Distribution of the metastatic foci and morphology*

	Marginal sinus type	Intermediate sinus type	Parenchymal type	Diffuse type
				
Type		Nonmarginal sinus type		
Incidence	37 (57%)	4 (6%)	11 (17%)	13 (20%)
MA (n = 35)	16 (46%)	1 (3%)	7 (20%)	11 (31%)
MM (n = 14)	10 (72%)	1 (7%)	1 (7%)	2 (14%)
ITC (n = 16)	11 (69%)	2 (12%)	3 (19%)	0

MA, macrometastasis; MM, micrometastasis; ITC, Isolated tumor cell.

Morphological Distribution of Metastatic Foci in Sentinel Nodes

The ratios of marginal sinus, intermediate sinus, parenchymal, and diffuse types of metastatic foci in 65 SNs were 56.9%, 6.2%, 16.9%, and 20.0%, respectively. In the marginal sinus type, the ratio of MA was 43.2%, MM was 27.0%, and ITC was 29.7%. Of metastatic foci, 57% were located in the marginal sinus of SNs (37 of 65 nodes) (Table 1). The remaining 28 nodes were classified as intermediate sinus (n = 4), parenchymal (n = 11), and diffuse (n = 13) types. In MA, 54% of SN metastasis was nonmarginal sinus type. On the other hand, the rate of nonmarginal sinus type in MM and ITC was 28% and 31%, respectively (Table 1).

Correlation between Clinicopathological Factors and Sentinel Node Metastasis

Clinicopathological factors were analyzed between patients with SN metastasis alone and those with SN and non-SN metastasis. The patients with tumor

larger than 40 mm had significantly more non-SN metastasis, compared with patients with tumor smaller than 40 mm (P = .006). Furthermore, the incidence of non-SN metastasis was significantly higher in patients with nonmarginal sinus type than in those with marginal sinus type (P = .025) (Table 2).

Correlation between Clinicopathological Factors and Distribution of Metastatic Foci in Sentinel Nodes

Nonmarginal sinus type was more frequently found in the patients with tumor larger than 40 mm (P = .011). According to the Japanese Classification of Gastric Carcinoma,[14] although the patients with marginal sinus type had no lymph node metastasis in second tier, 36% of patients with nonmarginal sinus type had lymph node metastasis in second tier (P = .009) (Table 3).

DISCUSSION

Lymphatic flows into the afferent lymphatics that connect to the marginal, intermediate, and medullary sinuses and then finally reaches other lymphatics via the efferent lymphatic vessels. Cancer cells detached from the primary tumor flow into intramural lymphatics and enter lymph nodes in the same manner as lymph itself. The SN concept is to detect the first lymph node metastasis using dye and RI colloid. The detection rate of SN in gastric cancer has ranged from 71% to 100%.[15–24] In contrast, we detected 100% and 93.9% of SNs from cT1 and cT2 tumors, respectively. Thus, the SN concept might be a useful diagnostic tool for detecting lymph node metastasis in early gastric cancer.

The concept of MM and ITC has been introduced in the Sixth Edition of the TNM classification. Since the method of measurement of cancer foci was not defined in this source, we propose considering cancer foci as cluster and noncluster types. Few investigators have examined morphological distribution according to TNM classification as MA, MM, and ITC. Understanding this morphological distribution of metastasis seems to be important for SN navigation surgery. Nagata et al. indicate that migrant cancer

TABLE 2. *Correlation between clinicopathological factors and SN metastasis*

Characteristic	SN metastasis	Non-SN metastasis	*P* value
Gender			.389
Male	15	5	
Female	6	4	
Age			.285
≥60	10	5	
< 60	11	4	
Tumor size			.006
< 40 mm	16	2	
≥40 mm	5	7	
Clinical T			.469
cT1	11	6	
cT2	10	3	
Pathological T			.091
pT1	14	3	
pT2–3	7	6	
Gross type			.523
Elevated	1	1	
Depressed	21	8	
Histology			.593
Differentiated type	3	2	
Undifferentiated type	18	7	
Lymphatic invasion			.139
Positive	13	8	
Negative	8	1	
Venous invasion			.690
Positive	10	5	
Negative	11	4	
SN status			.025
Marginal sinus type	14	2	
Nonmarginal sinus type	7	7	

TABLE 3. *Correlation between clinicopathological factors and distribution of SN metastatic foci*

Characteristic	Total No.	Marginal sinus type	Nonmarginal sinus type [a]	*P* value
Gender				.796
Male	119	11 (55%)	9 (45%)	
Female	41	5 (50%)	5 (50%)	
Age				.696
< 60	57	8 (57%)	6 (43%)	
≥60	103	8 (50%)	8 (50%)	
Tumor size				.011
< 40 mm	111	13 (72%)	5 (28%)	
≥40 mm	49	3 (25%)	9 (75%)	
Clinical T				.431
cT1	127	8 (47%)	9 (53%)	
cT2	33	8 (62%)	5 (38%)	
Pathological T				.153
pT1	134	11 (65%)	6 (35%)	
pT2–3	26	5 (38%)	8 (62%)	
Pathological N				.009
pN1	25	16 (64%)	9 (36%)	
pN2	5	0	5 (100%)	
Gross type				.118
Elevated	8	0	2 (100%)	
Depressed	152	16 (57%)	12 (43%)	
Histology				.743
Differentiated type	74	3 (60%)	2 (40%)	
Undifferentiated type	86	13 (52%)	12 (48%)	
Lymphatic invasion				.338
Positive	39	10 (48%)	11 (52%)	
Negative	121	6 (67%)	3 (33%)	
Venous invasion				.464
Positive	23	7 (47%)	8 (53%)	
Negative	137	9 (60%)	6 (40%)	

[a] Nonmarginal sinus type: intermediate, parenchymal, and diffuse type.

cells are initially arrested in the marginal sinus, where they evoke a biological response in a rat experimental model.[25] We found that 57% of metastases in SNs of gastric cancer were located in the marginal sinus with the remainder in the nonmarginal sinus. Our data based on the SN concept indicated that more than half of metastases were initially trapped in the marginal sinus, irrespective of metastatic modes (MA, MM, and ITC). However, some cancer cells were detected in the nonmarginal sinus. An examination of multiple sections of nodal metastasis might identify cancer cells even in the marginal sinus. Gaps and fragmentation of the superficial lymph node cortex are considered to provide intranodal shunt flow between afferent and efferent vessels in abdominal and pelvic nodes of elderly Japanese patients.[26]

When analyzing the relationship between microanatomical location of metastasis and clinicopathologic factors, significant correlation was found between tumor size and the distribution of metastatic foci in SNs. Although all patients with marginal sinus type had nodal metastasis in first tier alone, nonmarginal sinus type was found in all patients with pN2. Moreover, we evaluated the relationship between the distribution of metastatic foci in SNs and the incidence of non-SN metastasis. Interestingly, the incidence of non-SN metastasis was higher in the patients with SN metastasis of nonmarginal sinus type than in those with metastasis of marginal sinus type. Dewar et al.[13] suggested that the possibility of non-SNs involvement was extremely low in melanoma patients with the microanatomical location of metastasis with only subcapsular deposits in SNs. This result is in accord with the findings of our study.

Furthermore, CK staining during operation is recommended[27] and improves the diagnosis of SNs.

In conclusion, we demonstrated the microanatomical distribution of cancer foci in SNs. More than half of metastatic foci were located in the marginal sinus, but if the patients had SNs metastasis with nonmarginal sinus type, we should pay attention to the possibility of not only SN metastasis but also non-SN metastasis and pN2. SN navigation surgery in gastric cancer should be carefully performed in such patients.

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Sentinel Node Micrometastases Have High Proliferative Potential in Gastric Cancer

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Background. **The 6th edition of the TNM classification has recently defined "sentinel nodes (SN)," "micrometastasis," and "isolated tumor cells (ITC)." The present study examines the frequency and proliferative activity of such metastases with focus on the SNs of gastric cancer.**

Methods. **We enrolled 133 patients with cT1-2 tumors (cT1: 104, cT2: 29) and mapped SNs. Lymph node metastases were examined by routine histology and by immunohistochemistry with anti-cytokeratin. We used the Ki-67 antibody to detect the primary tumor and lymph node metastases to evaluate proliferative activity.**

Results. **The number of patients with SNs metastases and metastatic SNs was 19 and 52, respectively. The frequencies of macrometastasis, micrometastasis, and ITC were 48%, 25%, and 27%, respectively. Ki-67 expression in the tumor closely correlated with lymphatic invasion ($P = 0.0001$), venous invasion ($P < 0.0001$), and lymph node metastasis ($P < 0.0001$). Cells in 96% of macrometastases, 92% of micrometastases, and 29% of ITCs were Ki-67 positive.**

Conclusions. **We showed that micrometastasis and some ITCs in SNs had proliferative activity. We suggest that micrometastasis and ITCs should be removed, especially during SN navigation surgery, until their clinical significance is clarified.**

Key Words: **micrometastasis; isolated tumor cells; TNM classification; gastric cancer; sentinel node.**

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INTRODUCTION

The incidence of early gastric cancer has recently increased and surgeons have taken several approaches to treat this condition, including endoscopic mucosal and submucosal resection, laparoscopic surgery, and reduction of lymphadenectomy. Sentinel node navigation surgery (SNNS) is among the less invasive surgical options for cancer, and it has recently been introduced for the treatment of gastric cancer [1, 2]. For SNNS to be effective, the presence or absence of lymph node metastases, including micrometastases, must be determined. However, the clinical significance of lymph node micrometastasis remains controversial [3–11]. Various types of micrometastases, such as sentinel node (SN), pNX (sn), pN0 (sn), and pN1 (sn) have recently been added to the 6th edition of the TNM classification system. Furthermore, lymph node metastasis has now been subclassified into three types according to the size of the metastatic foci: metastasis (>0.2 cm), micrometastasis (between 0.2 cm and 0.2 mm), and isolated tumor cells (ITCs; <0.2 mm) [12].

The SN is the first node to receive lymphatic drainage from a primary tumor, leading to the notion that micrometastasis or ITC develops first in the SN [13]. However, whether such metastatic cancer cells can implant and proliferate remains obscure. Ki-67 expression detected by the use an anti-Ki-67 antibody is frequently used to assess proliferative activity in tumor cells because the protein is usually detectable throughout the cell cycle except during the G0 phase [14]. Moreover, Ki-67 expression closely correlates with tumor progression in gastric cancer [15, 16]. The present study uses immunohistochemistry to identify microme-

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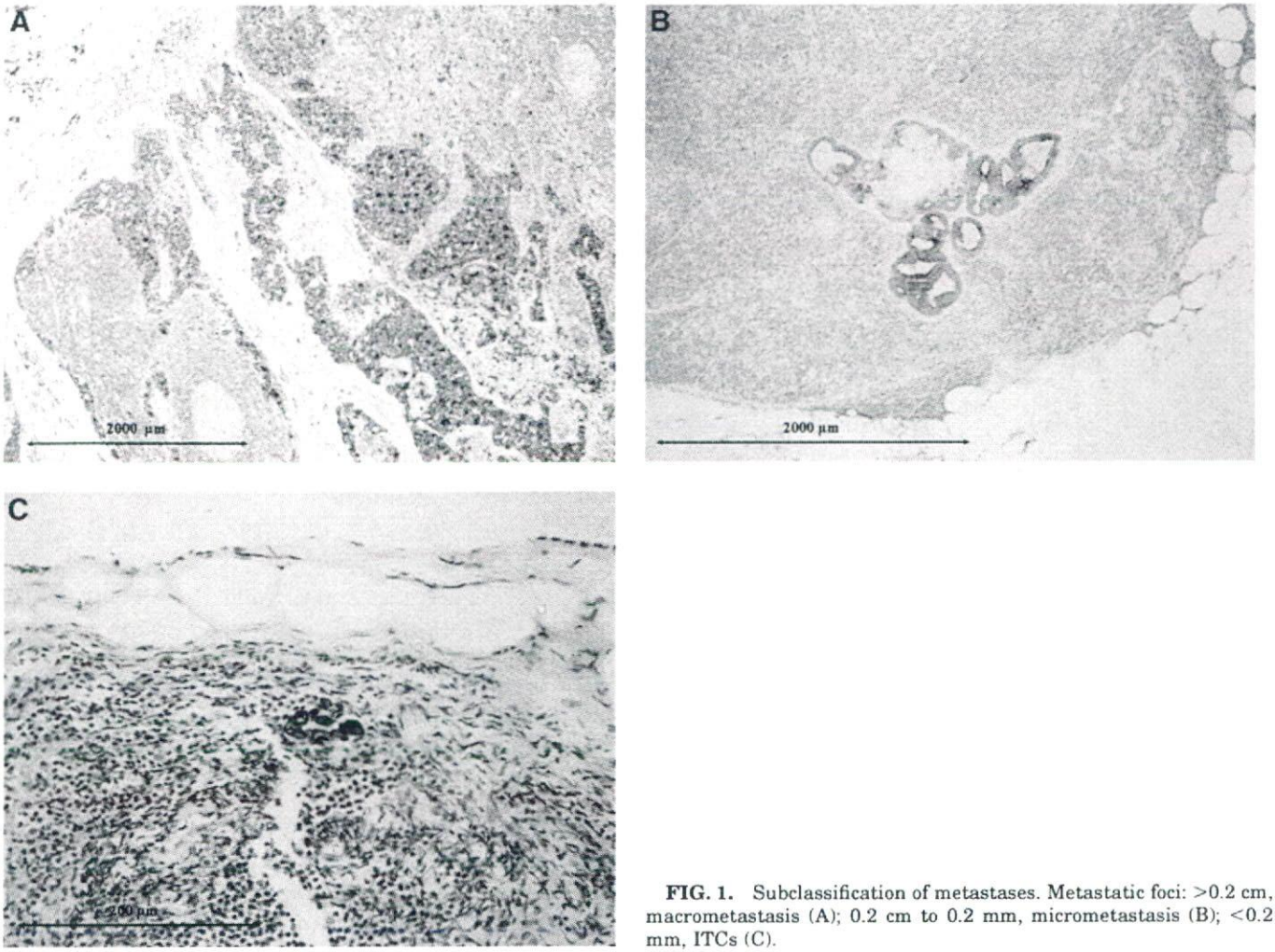

FIG. 1. Subclassification of metastases. Metastatic foci: >0.2 cm, macrometastasis (A); 0.2 cm to 0.2 mm, micrometastasis (B); <0.2 mm, ITCs (C).

tastasis and ITC and then elucidates the proliferative activities of metastatic foci in SNs of gastric cancer.

MATERIALS AND METHODS

Patients

We enrolled 133 consecutive patients with gastric cancer who had been preoperatively diagnosed with clinical grade T1 (n = 104) or T2 (n = 29) between 2001 and 2005. All of them underwent curative gastrectomy with lymphadenectomy and provided written, informed consent to participate based on a document approved by our institutional ethics committee.

Identification of SNs

We mapped SNs as described [17–19]. In brief, 3 mCi (2 mL) of ^{99m}technetium-tin colloid was endoscopically injected into the submucosa of the gastric wall at four sites around the tumor 1 d before surgery. During surgery, radioisotope uptake in each lymph node was measured using Navigator GPS (TYCO Healthcare, Ltd., Tokyo, Japan). All dissected lymph nodes were mapped after surgery and radioisotope uptake was measured once again. Lymph nodes with signals that were 10-fold above background were considered to be SNs.

Diagnosis of Lymph Node Metastasis

Lymph nodes were cut at the plane of the largest dimension, fixed in 10% formaldehyde and embedded in paraffin for sectioning. Some of the sections (3 μm) were stained with hematoxylin and eosin (H and E) and others were immunohistochemically examined. Lymph nodes were stained using AE1/AE3 (20:1 mixture of AE1 to AE3; Boehringer, Mannheim, Germany), a monoclonal antibody cocktail that reacts against a broad spectrum of human cytokeratins (CK). All sections were incubated at 60°C overnight, deparaffinized in xylene and rehydrated through a series of graded ethanols. The sections were incubated in citrate buffer (pH 6.0) for 6 min under pressure, immersed in CK monoclonal antibody diluted 1:100, and then CK reactivity was detected using alkaline phosphatase. We classified lymph node metastases into three categories according to the TNM classification, (Fig. 1) as macrometastasis (>0.2 cm), micrometastasis (between 0.2 cm and 0.2 mm), and ITCs (<0.2 mm). Three independent observers evaluated all of the immunostained slides (SY, SN, and YU).

Detection of Cytokeratin and the Ki-67 Antigen

After lymph node metastasis was determined by CK staining, sections on glass slides were immersed in xylene for a few days to remove the coverslips. The sections were then rehydrated with a graded series of ethanols, autoclaved in 10 mM sodium citrate (pH

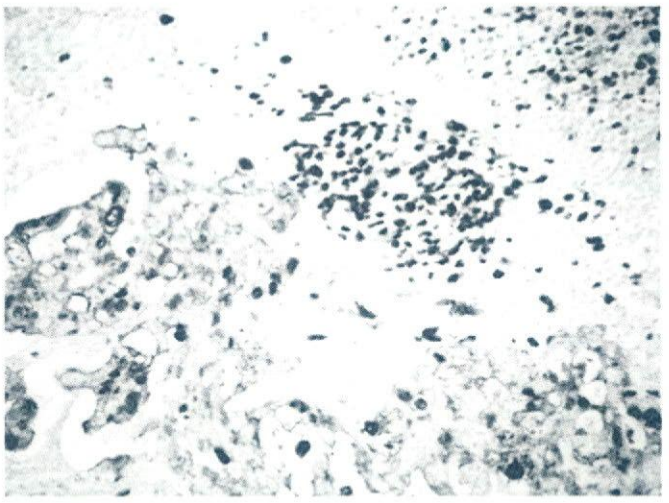

FIG. 2. Proliferative activity in metastatic foci of lymph nodes detected with Ki-67 antigen. Germinal center of the lymph node expresses Ki-67 antigen in nuclei (red) and thus serves as positive control. Cancer cells are framed in cytokeratin (brown) and express nuclear Ki-67 antigen (red).

6.0) for 15 min, and cooled at room temperature. The sections were sequentially incubated at room temperature with 1.5% bovine serum albumin for 30 min, mouse monoclonal Ki-67 antibody (DakoCytomation, Copenhagen, Denmark) diluted 1:50 for 60 min, three washes with PBS for 3 min each and biotin-labeled secondary antibody (VECTASTAIN ABC kit, Vector Laboratories, Inc., Burlingame, CA) diluted 1:200 for 30 min. The sABC steps (DakoCytomation) proceeded at room temperature for 30 min. Alkaline phosphatase (AP) was visualized using fuchsin substrate with the endogenous AP inhibitor, levamisole. Nuclei were not stained with hematoxylin to optimally visualize those that were Ki-67 positive.

The positive control was the germinal center of lymph nodes expressing red nuclear Ki-67 antigen. Cancer cells were framed in brown cytokeratin and nuclear Ki-67 antigen was stained red (Fig. 2).

The Ki-67 labeling index (LI; Ki-67 positive cancer cells/total cancer cells × 100) was determined by observing 1000 tumor nuclei and all nuclei of lymph node metastases in areas of sections with the most intense labeling. Three independent observers evaluated all immunostained slides (SY, SN, and YU).

RESULTS

Detection of Sentinel Node and Lymph Node Metastases

We identified SNs in 131 of 133 patients (detection rate: 98.5%), in all cT1 patients (n = 104; 100% detection rate) and in 27 cT2 patients (n = 29; 93% detection rate). The number of SNs per patient ranged from 1 to 17 (mean, 4.3); we obtained 3264 lymph nodes from 133 patients (range, 13 to 69 per patient; median, 27). Staining with H and E identified lymph node metastasis in 22 SNs from 14 patients. Thirty additional metastatic SNs from five patients were H and E negative but CK positive. Thus, 19 patients had lymph node metastases, and 52 lymph nodes were positive for metastases, including both micrometastases and ITCs in SNs. The overall detection rates of SNs in cT1 and cT2 were 100% and 90%, respectively. All patients in this series with nodal metastasis had lymph node metastasis in SNs. Thus, the detection rate (i.e., sensitivity) for metastasis in SNs in both cT1 and cT2 was 100% and the false-negative rate was 0%.

The incidence of macrometastasis, micrometastasis, and ITCs according to the TNM classification was 48% (25/52), 25% (13/52), and 27% (14/52), respectively. Of these, 16 of 25 macrometastasis (64%), 3 of 13 micrometastasis (23%), and 3 of 14 ITCs (21%) were HE positive. The remainder was CK positive.

Detection of Ki-67 Antigen in Primary Tumors and Metastatic Foci of Lymph Nodes

A comparison of Ki-67 antigen expression and clinicopathologic findings for primary tumors revealed significant differences in tumor depth (P = 0.0119), lymphatic invasion (P = 0.0001), venous invasion (P < 0.0001), and lymph node metastasis (P < 0.0001; Table 1). Nineteen patients had lymph node metastasis, including micrometastasis and ITCs. Cells were Ki-67 positive in 96% of macrometastases (Fig. 3A), 92% of micrometastases (Fig. 3B), and 29% of ITCs (Fig. 3C). In addition, Ki-67 positivity was significantly more frequent in macrometastasis and micrometastasis than in ITCs (P < 0.001; Table 2).

DISCUSSION

Recently, SNNS has been introduced in gastrointestinal intestinal tract cancer, and "sentinel nodes", "micrometastasis," and "isolated tumor cells" have been included in the 6th edition of the TNM classification [20]. For SNNS to be genuinely helpful, lymph node metastases, including micrometastases, must be cor-

TABLE 1

Correlation Between Expression of Ki-67 and Clinicopathological Factors

Variable	Expression of Ki-67 (mean ± S.D.)	P-value
Tumor depth		
pT1	26.3 ± 16.6	0.0119
pT2	36.9 ± 17.2	
Histological type		
differentiated	27.1 ± 15.9	0.7948
undifferentiated	27.9 ± 18.0	
Lymphatic invasion		
negative	24.9 ± 15.3	0.0001
positive	38.2 ± 19.1	
Venous invasion		
negative	25.2 ± 15.4	<0.0001
positive	46.6 ± 16.8	
Lymph node metastasis		
negative	25.1 ± 15.4	<0.0001
positive	49.3 ± 15.2	

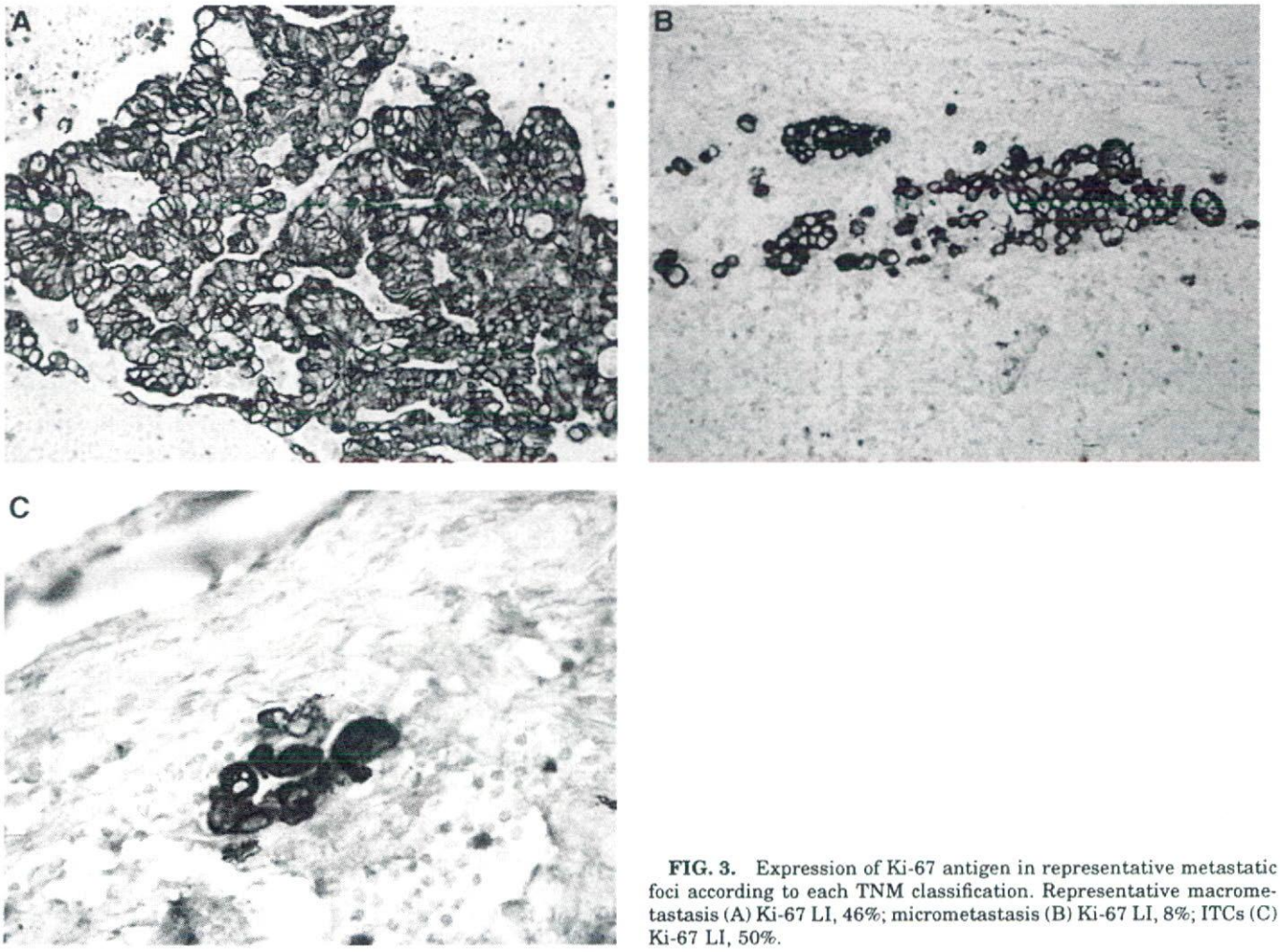

FIG. 3. Expression of Ki-67 antigen in representative metastatic foci according to each TNM classification. Representative macrometastasis (A) Ki-67 LI, 46%; micrometastasis (B) Ki-67 LI, 8%; ITCs (C) Ki-67 LI, 50%.

rectly diagnosed. We selected patients with cT1 or cT2 tumors for the present study because the SN concept seems to be appropriate for patients without nodal metastases [19, 21]. We mapped SNs and used CK staining to detect sparse cancer cells. All lymph node metastases, including micrometastases and ITCs, were contained in SNs.

The clinical significance of lymph node micrometastasis is still debatable. Some authors have described a close relationship between micrometastasis and prognosis, whereas others do not support this notion [3–11].

TABLE 2

Lymph Nodes with Ki-67-Positive Cells

	Ki-67 expression cancer cells	*P*-value
Macrometastasis (n = 25)	96%	<0.001
Micrometastasis (n = 13)	92%	
ITCs (n = 14)	29%	

Natsugoe *et al.* and Harrison *et al.* found that lymph node micrometastasis is clinically significant [3, 4], whereas others have found otherwise [6, 7]. However, the definition of micrometastasis and the stages of patients differed in each of these reports. Since micrometastasis and ITC have been defined in the TNM classification. Lee *et al.* reported that the size and pattern of lymph node metastases could yield prognostic information [11]. Further studies are needed to determine the prognostic significance of micrometastasis and ITC.

The presence or absence of micrometastases is currently an important problem for SNNS, particularly in early gastric cancers for which less invasive surgery is planned [21]. Unresolved issues concerning SNs include the frequency and size of micrometastasis, as well as the proliferative potential of small cancer foci.

Here, we found that HE staining detected metastases in 10% of 133 patients. We clustered metastases by the size of the metastatic foci according to the 6th edition of the TNM classification. Approximately 80%

of micrometastases and ITCs were detected by CK staining alone. Thus, at least an immunohistochemical study seems to be essential for SNNS. However, whether such cancer cells in lymph nodes have proliferative potential remained to be determined. We therefore measured proliferative ability as Ki-67 antigen expression, which reflects the proliferating cell cycle. Ki-67 expression in primary tumors correlates with various tumor progression factors such as tumor depth, lymphatic invasion, venous invasion, and lymph node metastasis, and our results agreed with those of others [15, 16]. When we next examined proliferative activity in various lymph node metastases, Ki-67 expression was positive in 96% of metastasis, 92% of micrometastasis, and in 29% of ITCs. Although proliferative activity was evident even in micrometastasis and ITCs, whether such metastases will become embedded and grow in lymph nodes remains unclear [22]. Yokoyama *et al.* reported that ITCs in regional lymph nodes of an animal model regressed after removal of the primary tumor mainly via natural killer cells [23]. We suggest that micrometastasis and ITCs be removed during less invasive types of surgery including SNNS, from the viewpoint of risk for recurrence, until the clinical significance of such metastases is clarified.

Previous studies and the data presented here suggest that SNNS would be an appropriate strategy with which to treat early-stage gastric cancer [19, 24]. Patients with gastric cancer should benefit from SNNS as the extent of both lymphadenectomy and gastrectomy can be reduced. The goal of SNNS should be to perform curative resection and prevent recurrence. Thus, the detection and subsequent diagnosis of micrometastases with proliferative activity is important when considering SNNS. To help bring this about, we recently introduced a method for rapid immunohistochemical staining during surgery [25]. We predict that rapid RT-PCR methods will soon be routinely applied to diagnose micrometastases during SNNS.

CONCLUSIONS

We identified micrometastases and ITCs and provided evidence of their proliferative activity in SNs. Since the clinical significance of micrometastasis and ITCs in lymph nodes remains obscure, we suggest that, for the time being, such metastases should be removed during the reduction of lymphadenectomy, including SNNS.

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特集 センチネルリンパ節理論の消化器癌への応用

5 センチネルリンパ節生検応用の拡大
(1) EMR/ESD への応用

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要 旨

早期消化管癌に対するendoscopic mucosal resection(EMR)やendoscopic submucosal dissection(ESD)は臓器温存，機能温存の観点から優れた治療法の一つである．しかし，これらの治療を選択するうえでリンパ節転移の有無はもっとも重要な因子である．画像診断で診断される明らかなリンパ節転移以外に分子生物学的，遺伝子学的解析により発見される微小転移がある．近年，センチネルリンパ節の概念が消化管癌でも導入されている．センチネルリンパ節を同定し，微小転移を含めたリンパ節転移を診断することは縮小治療には有用である．今後，早期消化管癌に対するEMRやESDの適応拡大に際してはセンチネルリンパ節生検を併用することにより，積極的にかつ安全に施行できると考えられる．

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Ⅰ．センチネルリンパ節の上部消化管癌への臨床応用

この項のポイント
- センチネルリンパ節の同定は治療の個別化の一つである．

センチネルリンパ節理論は乳癌やメラノーマではすでに臨床応用として導入され，リンパ節郭清の標準術式の一つとして確立されている．上部消化管癌にも次第に臨床導入が試みられつつあるが，どのような症例に応用可能であろうか？

センチネルリンパ節は最初に転移が起こると想定されるリンパ節である．血管と同様にリンパ管にも変異があり，さらにリンパ節に流入するリンパ管の数や走行，あるいは腫瘍によるリンパ流の変化により修飾されるため，個々の症例でセンチネルリンパ節は異なると考えられる．また臓器によっても差異があると考えられ，食道癌では早い時期から頸部・胸部・腹部のさまざまな領域に転移がみられる．一方，早期胃癌の場合にはある程度，占居部位に応じて

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lymphatic basin と呼ばれる転移領域が推測できる場合が多い[1].

しかしながらセンチネルリンパ節理論の基本は，各個人固有のもっとも転移をきたしやすいリンパ節を同定することである．したがってセンチネルリンパ節の同定は個別化治療，とくにリンパ節郭清の個別化に有用である．早期癌では，センチネルリンパ節の転移の有無により郭清の省略化が可能になると思われる．

II．EMR，ESD の適応と適応拡大

この項のポイント
- 現在の適応拡大は手術例の結果に基づく retrospective な解析結果である．

早期消化管癌では endoscopic mucosal resection(EMR)や endoscopic submucosal dissection(ESD)の進歩により，多くの粘膜癌症例では局所制御が可能となってきている．『食道癌診断・治療ガイドライン』による EMR の適応に関して，絶対的適応は食道癌のうち深達度が M 1 あるいは M 2 で周在性 2/3 以下と診断された症例とされている，深達度が M 1(EP)や M 2(LPM)に留まるものには，リンパ節転移や脈管侵襲はまれで，EMR によって多くの症例で根治が可能である．また，相対的適応として，壁深達度が粘膜筋板に達したもの，粘膜下層に浸潤するもの(200 μm まで)ではリンパ節転移の可能性を認めるが，臨床的にリンパ節転移がない症例では粘膜切除が可能であり，相対的適応となる．また，粘膜切除が全周性になる病変でも相対的適応となる．と記載されている[2]．したがって，適応拡大となるのは M 3(MM)，SM 1 の一部の症例と考えられる．

『胃癌治療ガイドライン』による EMR の適応の原則はリンパ節転移の可能性がほとんどなく腫瘍が一括切除できる大きさと部位にあること，と記載されている．具体的な適応条件は 2 cm 以下の肉眼的粘膜癌(cM)と診断される病変で，組織型が分化型(pap，tub 1，tub 2)．肉眼型は問わないが，陥凹型では UL(−)に限る，となっている[3]．臨床研究として行うべき適応がいわゆる適応拡大の範疇に入ってくると考えられる．すなわち，① 分化型腺癌，M 癌，UL(−)，大きさを問わず，② 分化型腺癌，M 癌，UL(+)，3 cm 以下，③ 分化型腺癌，SM 1 癌，3 cm 以下，④ 未分化型腺癌，M 癌，UL(−)，2 cm 以下である[4]．

これらの食道癌，胃癌の適応拡大の基準は，これまでの多数の手術例をもとにリンパ節転移の有無に関して，原発巣を解析することによって得られた結果に基づくものである．これらの検討から適応を拡大する場合には，いくつかの問題点がある．多くのこれらの検討は原発巣およびリンパ節転移巣も代表一切片で行われており，ある意味きわめて曖昧な検討である．実際，食道表在癌切除例で深切り追加切片を作製して深達度を再評価すると 15.5%の症例で深達度の変更がみられる[5]．さらにリンパ節に関しても追加切片を作製し検討すると通常の HE 染色標本においても 10.5%に微小転移が発見される[6]．TNM 分類の第 6 版ではすでに微小転移の項目が取り上げられており，次項で述べるこれらの微小転移の検討も是非必要である[7]．また，リンパ節郭清が行われた手術例を基にした retrospective な解析であるために今後，リンパ節郭清を行わない場合には follow-up のありかたや再発への対処が重要な課題となる．

Ⅲ. センチネルリンパ節の微小転移

この項のポイント

- 早期癌に対する縮小治療には微小転移の診断が必須である.

前述したようにリンパ節の検索切片数を増やすとリンパ節微小転移がHE染色によってもかなりの頻度で認められる. 近年, 免疫組織染色による分子生物学的方法およびreverse transcriptase-polymerase chain reaction (RT-PCR法)による遺伝子学的方法の進歩により, 通常の組織標本では発見できないリンパ節微小転移巣の存在が明らかになってきた[8),9)]. EMRやESDの対象となる病変は早期癌であるが, そのうち画像診断でリンパ節転移陽性例は除外される. したがってEMRやESD症例のリンパ節転移診断は, 究極的には微小転移の有無をいかに正確に診断できるかということにある.

リンパ管侵襲はリンパ節転移を予測する重要な因子である. 病理診断で転移陰性(pN 0)と診断された80例の胃癌で, 微小転移とリンパ管侵襲について検討した[10)]. リンパ節微小転移はサイトケラチン(CK)染色とCEA-mRNAを用いたRT-PCR法により調べられた. リンパ管侵襲は通常の組織標本に加え, D 2-40を用いた免疫染色を行い解析した. CK染色とRT-PCRで陽性になった症例は各々9例(11.3%)と25例(31.3%)であった. また, リンパ管侵襲は組織診断で80例中9例に陽性であったが, 残る陰性71例中, D 2-40染色で11例が陽性と判定された(**図1**). 微小転移との関連をみると, 微小転移陽性25例中6例のみが組織学的リンパ管侵襲陽性であった. 一方, D 2-40染色では25例中16例にリンパ管侵襲陽性と診断された(**表**). この結果より, 微小転移を含めたリンパ節転移を予測するとき,

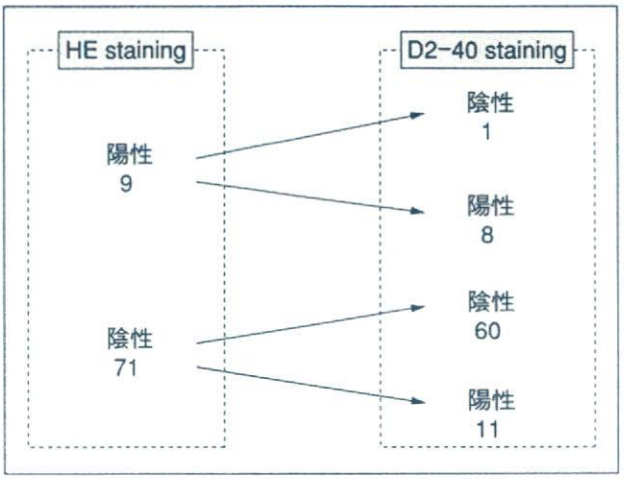

図1 HE(hematoxylin and eosin)染色とD 2-40染色の比較

表 リンパ節微小転移とリンパ管侵襲

リンパ管侵襲	微小転移(%)		p-value
	陰性(n=55)	陽性(n=25)	
HE染色			
陰性	52(73.2)	19(26.8)	0.0150
陽性	3(33.3)	6(66.7)	
D 2-40染色			
陰性	52(85.2)	9(14.8)	<0.0001
陽性	3(15.8)	16(84.2)	

HE : Hematoxylin and eosin staining

EMRやESD症例の原発巣のリンパ管侵襲の評価には通常の組織診断のみならずリンパ管の特異的染色も是非必要と考えられる.

術前に深達度T 1, T 2(cT 1, cT 2)と診断された胃癌104例について, センチネルリンパ節の同定とCK抗体を用いた免疫染色による微小転移を含めたリンパ節転移について検討した[11)]. センチネルリンパ節の同定は初期の注入不適の3例を除き, cT 1とcT 2で各々99%(78/79例)と95%(21/22例)であった. リンパ節転移は通常の組織検査ではcT 1で7例に認められたが, すべてセンチネルリンパ節に含まれていた. また, cT 1ではCK染色による微小転移が8例に認められたが, これらはすべて