特集　センチネルノードナビゲーション手術(SNNS)の進歩と展望

II. 各論

2. 消化器癌
b) 胃癌——SNNS導入による胃癌治療の変化*

二村浩史　矢永勝彦**

〔要旨〕胃癌センチネルノードナビゲーション手術(SNNS)は本邦から発信された臨床研究であり，その有用性が報告されている．リンパ流域切除は郭清範囲を術中に個別に同定することで，郭清と同等の効果が期待できる．内視鏡的粘膜下層剥離術後にリンパ節転移の可能性が否定できない場合を含め，今後の早期胃癌治療はSNNSと腹腔鏡下手術の融合によってSN転移陰性例に対する腹腔鏡下手術による手術の低侵襲化や縮小手術の個別化などを可能にすると見込まれる．

はじめに

1992年にMorton ら[1]が早期悪性黒色腫に対してセンチネルノードナビゲーション手術(SNNS)をはじめて臨床応用した．その後，乳癌での検証が行われており，今や乳癌手術においてはSNNSが標準治療となりつつある[2]．

本稿では，胃癌におけるSNNSの現状と導入後における胃癌治療の変化について述べる．

I. 胃癌SNNSの現状

胃癌SNNSは本邦から発信された臨床研究であり，良好な成績が報告されている[3~8]．2007年12月現在，PubMedで「sentinel node(SN)」で検索すると5,808件の論文がヒットする．このうち2006年以降は1,553件である．SNに検索用語「breast cancer」を加えると2,974件，うち2006年以降では687件で，SNに「gastric cancer」を加えると165件，2006年以降では43件がヒットする．このようにSNに関する英文論文ではその約半数が乳癌に関するものであり，胃癌に関するものはわずか3%にすぎない．これは欧米諸国に胃癌が少ない特徴からして当然のことかと考えられるが，2006年以降の胃癌SNNS 43件中29件が本邦からの報告であり，したがって胃癌SNNSに関してはわが国が主体となって研究がすすめられているといえる．今後胃癌SNNSは，SN生検と腹腔鏡下手術の融合により，SN転移陰性例に対して腹腔鏡下手術による低侵襲手術，個別化縮小手術の実践を目指していくものと考えられる．

現在わが国では，臨床応用に向けた胃癌SN生検手技の多施設共同臨床試験が二つ進行している．一つはトレーサーに色素indocyanine green(ICG)を用いて開腹下で胃の漿膜に注入する方法である，Japan Clinical Oncology Group

キーワード：胃癌，SNNS

* Changes in the surgical strategy for early gastric cancer by sentinel node navigation surgery (SNNS)

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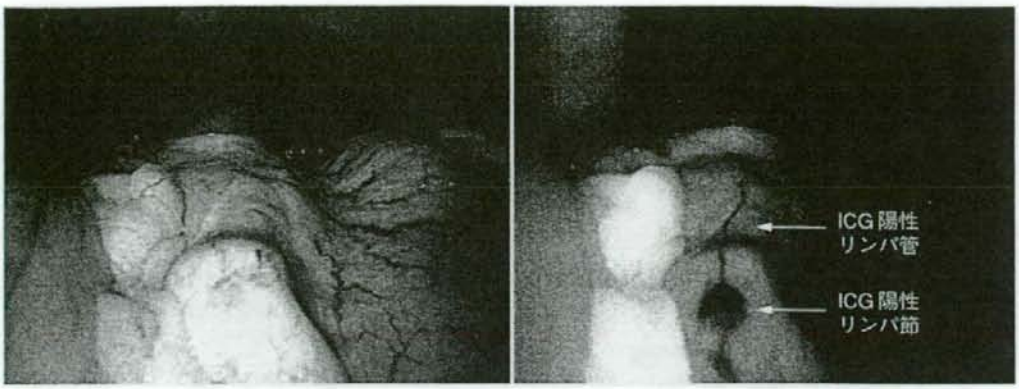


a．肉眼観察．ICG陽性リンパ管やリンパ節は視認できない．

b．赤外光観察．ICG陽性リンパ管，リンパ節が明瞭に観察できる．

図1．腹腔鏡下観察所見(肉眼 vs 赤外光)

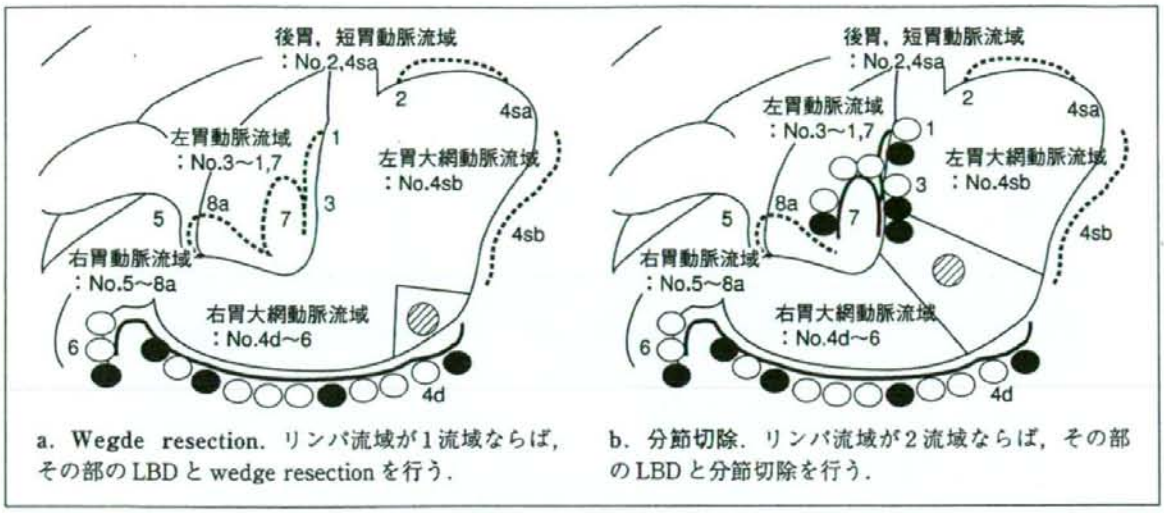
(JCOG)胃癌外科グループの「早期胃癌におけるセンチネルリンパ節生検の妥当性に関する研究(JCOG0302)」[9]であり，もう一つはトレーサーとして色素isosulfan blue(Lymphazurin)＋アイソトープ(RI)^{99m}Tcスズコロイドを用いて内視鏡にて癌周囲に局注する方法である，SNNS研究会・厚生労働省がん研究助成金研究班の多施設共同研究[10]である．それぞれトレーサーや投与経路，同定方法が違うので一概に単純比較はできないが，SNNSが胃癌で臨床応用できるか否かの重要な研究であることには間違いない．最終結果が待たれるところである．ただし，JCOGスタディで使用されるICGは，視認性に劣るため肉眼観察のみでは偽陰性率が高いと報告されている[8,11,12]．

われわれは偽陰性の発生を防ぐ目的で，ICGの吸収波長が赤外光の波長と一致する性質を利用して赤外線観察でのSNNSを行い，良好な成績を報告してきた(図1)[8,11,13~15]．乳癌では蛍光ICG赤外線観察の報告がある[16]．最近胃癌でもこの方法を応用したSNNSの報告もあり，各施設で偽陰性をなくすための新たなSN同定法が開発されてきている[17]．

しかし，これらの研究で有用性が証明されても胃癌におけるリンパ節の微小転移診断の有用性や意義が明確でないため，胃癌SNNSの術中転移診断法については議論の余地のあるところである[18~20]．

II. SNNS導入後における胃癌治療の変化

これまでわが国では，胃癌治療はD2郭清が標準術式であり，早期胃癌ではリンパ節郭清の一部を省略する縮小手術α，βがガイドラインで示されている[21]．先にも述べたが，SNNS導入後はSN転移陰性例に対して腹腔鏡下手術による低侵襲手術，個別化縮小手術へと治療が変化してくるものと考えられる．なお腹腔鏡下SNNSを色素＋RIで行う場合，色素染色リンパ節とRI集積リンパ節は必ずしも一致しない点[7]，開腹に比べていわゆるshine throughによる影響を強く受ける点[22]に注意を要する．これまで偽陰性とされた多くの症例は同一のリンパ流域(lymphatic basin)内にSNが同定されていること[23,24]，赤外線腹腔鏡システムを使用してもpick up法では偽陰性を生じること[25]，サイトケラチン染色やPCRでの検索でSN以外の微小転移リンパ節も同じbasin内にあるため，リンパ流域切除(lymphatic basin dissection；LBD)をすれば転移リンパ節の取り残しはないと報告されている[26]．このため，最近ではLBD法[27]によるSN同定が行われることが多くなってきた．胃の動脈リンパ領域から早期胃癌の至適郭清範囲を設定する報告[28]があるが，LBDは郭清範囲を術中に個別に同定し郭清することと



a. Wegde resection. リンパ流域が1流域ならば，その部のLBDとwedge resectionを行う．

b. 分節切除．リンパ流域が2流域ならば，その部のLBDと分節切除を行う．

図2．リンパ流域に基づく胃癌縮小手術
実線：リンパ流域，●：SN，○：流域内の非SN，▨：癌部

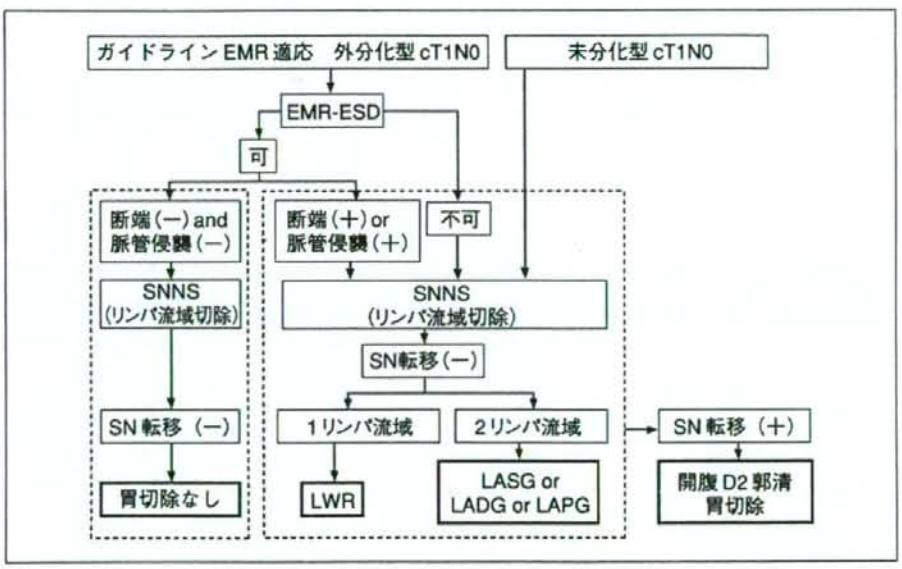


図3．今後のc0IIc胃癌の治療方針
LWR：laparoscopic wedge resection，LASG：laparoscopy-assisted segmental gastrectomy，LAPG：laparoscopy-assisted proximal gastrectomy

同等の効果が得られると考えられる．

すなわち，腹腔鏡下SNNSをLBDで行い，リンパ流域が1方向で術中リンパ節転移がなければwedge resection(図2a)，2方向で術中転移がなければ分節切除(図2b)，3方向なら幽門側胃切除，また術中に転移を認めた場合は定型手術に移行するという術式設定が成り立つ(図3)．現にこれに準じた手術を施行している施設もある[29,30]．

さらに，内視鏡的粘膜下層剝離術(ESD)の適応拡大に伴い，リンパ節転移の可能性があるカテゴリー，すなわちSM1・分化型・3 cm以内・脈管侵襲なしの条件を満たさない場合に腹腔鏡下SNNSを施行し，図3のような術式設定をすることができる[31]．このESD切除標本の病理検索，特にHE染色でリンパ管侵襲を見逃さないためにD2-40染色を施行したり，先進部が未分化型に変化していないかを確実に診断する必要がある[32,33]．最近術中にESDとリンパ節郭清を組み合わせて，胃を切除しないとする報告もある[34]．しかしわれわれは，連続切片ではじめてリンパ管侵襲陽性の診断がついて，SNNSによる手術の結果，切除胃にリンパ管侵襲陽性の遺残を認めた症例を経験している[35]．術中の脈管侵襲診断ができない以上，現時点ではまずESDを先行させて，断端陰性，脈管侵襲がないとされた先進部未分化型のもの以外は，basinに流れる胃壁内のリンパ管を切除するために図3のような胃切除は必要と考えられる．

おわりに

SNNS導入後は個別化により必要最小限の胃癌治療が可能となってくると考えられる．

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REVIEW

Detection of micrometastases in sentinel node navigation surgery for gastric cancer

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KEYWORDS
Gastric cancer;
Micrometastasis;
Isolated tumor cell;
Sentinel node;
Genetic diagnosis

Abstract
Although lymph node metastasis is one of the important prognostic factors for patients with gastric cancer, the clinical significance of micrometastasis remains controversial. In the 6th edition of the TMN classification, micrometastases were classified as micrometastasis (MM) and isolated tumor cells (ITC) according to its greatest dimension. The accurate diagnosis of micrometastases is required when considering less invasive surgery, especially in early stage of gastric cancer. Since generating useful information about micrometastases by conventional RT-PCR is time-consuming, this procedure is not useful for rapid diagnosis during surgery. Recently some new methods of genetic diagnosis have reduced the amount of time required to obtain information about micrometastases in lymph nodes to 30–40 min. Such methodology can be clinically applied during less invasive surgery. The sentinel node (SN) concept has recently been applied to gastric cancer and SN navigation surgery (SNNS) is ideal for reduction of lymphadenectomy in patients with early gastric cancer. However, we should think about some conditions to establish SN concept for gastric cancer: the particle size of radioisotope, relationship between metastatic area and RI uptake, and the diagnosis of micrometastases by various method such as histological examination, immunostaining and RT-PCR. Here, we described the current status of MM and ITC in the lymph nodes and the SN concept in gastric cancer.

Contents

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Clinical significance of micrometastasis in gastric cancer

The advocated concept of "so-called micrometastasis" based on morphological or methodological findings is also referred to as micrometastasis, microcarcinosis, occult metastasis, latent metastasis, microinvolvement, microenvironment and isolated tumor cells (ITC). The term "ITC" has recently been introduced into the TNM classification [1]. These are individual tumor cells or small cell clusters that do not exceed 0.2 mm in the greatest dimension, which are usually detected by immunohistochemistry (IHC) or molecular methods, but may be verified by hematoxylin and eosin (HE) staining. They do not typically show evidence of metastatic activity (e.g., proliferation or stromal reaction) or penetrate vascular or lymphatic sinus walls. When ITC are located in lymph nodes or at distant sites, they should be classified as N0 or M0, respectively. The same applies to cases with findings suggestive of tumor cells or associated components detected by non-morphological techniques such as flow cytometry or DNA analysis.

The clinical significance of lymph node micrometastases in gastric cancer remains controversial. Some authors have reported a significant difference between the presence of micrometastases and prognosis in gastric cancer [2–9] whereas others have not [10–12] (Table 1). Nakajo et al. defined micrometastases as "tumor micrometastasis – cluster formation of tumor cells with stromal reaction and tumor cell microinvolvement – the presence of individual tumor cells without a change in stroma". They suggested that the immunohistochemical incidence of micrometastases in the lymph nodes of patients with gastric cancer is quite high and that such metastasis is associated with the prognosis of patients with pN0 [7]. Doekhie et al. defined MM and ITC according to the 6th edition of the TNM classification and found that the number of examined lymph nodes and the ratios (%) of positive MM and ITC in the lymph nodes are independent risk factors for locoregional and distant recurrent disease [2].

In contrast, Fukagawa et al. defined micrometastases as the presence of tumor cells detected by cytokeratin-specific IHC and not by ordinary HE staining. They concluded that the immunohistochemical presence of micrometastases in the regional lymph nodes does not affect the survival of gastric cancer patients [10]. Hong-Jo et al. defined micrometastases as individual epithelial cells or epithelial cell clusters detected immunohistochemically within the capsule of the lymph node either in the sinus or in the lymphatic vessels and they concluded that IHC with an anticytokeratin antibody seems to be of little prognostic value in patients with submucosal gastric carcinoma [11].

Yokoyama et al. suggested that ITC in the regional lymph nodes regressed by removal of the primary tumor mainly via natural killer cell-mediated anti-tumor activity and that micrometastases in the lymph nodes could be effectively eliminated by the postoperative chemotherapy by animal model [13]. Yonemura et al. immunohistochemically evaluated ITC and found that they are highly proliferative and have the potential to induce established lymph node metastasis; the prognosis was significantly worse among patients with, than without ITC. They also suggested that highly proliferative ITC are destined to become clinical metastases [14]. We evaluated the proliferative activity of MM and ITC in the sentinel nodes with gastric cancer immunohistochemically using anti-Ki-67 antibody. Anti-Ki-67 antibody is frequently used to assess proliferative activity in tumor cells because the protein is usually detectable throughout the cell cycle expect during the G0 phase [15]. It was found that 92% of MM, and 29% of ITC were Ki-67 positive and we considered that there was great importance in diagnosis of MM and ITC [16]. There were some problems regarding the difference of used antibody, identification between micrometastases and immune cells and estimation of micrometastases, especially in single cells. The clinical significance of micrometastases should be further studied in a large population of patients with the same disease stage.

Sentinel node concept in gastric cancer

According to the sentinel node (SN) concept, SN is the first lymph node to receive lymphatic flow from the primary tumor and metastasis initially occurs at this site [17]. Thus, micrometastases are probably located in SNs as the first step of lymph node metastasis. SN navigation surgery (SNNS) has been clinically introduced for patients with breast cancer and malignant melanoma and it can also assess lymph node dissection areas [18–21]. The SN concept has recently been applied to cancers of the gastrointestinal tract [22–26]. Several authors have reported that the SN concept is applicable to patients with clinical T1 and N0 gastric cancers [27–30]. The incidence of patients with early gastric cancer is increasing in Japan thanks to early endoscopic discovery. Minimally invasive surgery based on SNNS might become an option if the SN concept could be applied to patients with early clinical T1 and N0 gastric

Table 1 Clinical immunohistochemical investigation of micrometastases with gastric cancer

Author	Patient	Definition	Significance
Doekhie et al. [2]	40 With disease recurrence 41 Without disease recurrence	6th Edition of TNM classification	Yes Disease recurrence
Morgagni et al. [3]	300 With pN0 early gastric cancer	Single or small clusters of cancer cells identified only by IHC staining	No
Yasuda et al. [4]	64 With histologically node-negative gastric cancer (T2, T3)	Single or small clusters of cancer cells identified only by IHC staining	Yes Survival
Lee et al. [5]	46 With early gastric cancer 107 With advanced gastric cancer	Single or small clusters of cancer cells identified only by IHC staining	Yes Survival
Cai et al. [6]	162 With early gastric cancer	Tumor cells detected only by cytokeratin immunostaining	Yes Survival
Nakajo et al. [7]	67 Diagnosed as pN0 by routine histological examination	Tumor micrometastasis: cluster formation of tumor cells with stromal reaction Tumor cell microinvolvement: presence of individual tumor cells without a change in the stroma	Yes Survival
Harrison et al. [8]	25 Patients withT1-4N0M0 gastric and gastroesophageal junction adenocarcinoma	Immunohistochemical detected lymph node micrometastasis with histologic node negative	Yes Survival
Maehara et al. [9]	34 Patients with node-negative early gastric cancer who died of a recurrence	Cytokeratin-positive cells in lymph nodes of patients with node-negative early gastric cancer	Yes Survival
Fukagawa et al. [10]	107 Patients with pT2N0M0	The presence of tumor cells detected only by cytokeratin immunostaining (1) Single cell type and (2) Cluster type	No
Choi et al. [11]	88 With submucosal gastric cancer with D2 lymph node dissection	Any tumor cells, single or in groups, detected by cytokeratin IHC and missed in HE staining	No
Morgagni et al. [12]	139 With early gastric cancer diagnosed as pN0	Defined as single or small clusters of neoplastic cells identifiable only by IHC staining	No

cancer. When patients preoperatively staged as T1 and N0, do not show any evidence of micrometastases in SNs during surgery, reduction of gastrectomy such as preserving gastrectomy, segmental resection, wedge resection and limited proximal gastrectomy is possible. Similarly, SN biopsy and basin dissection of lymph nodes might also reduce the extent of lymphadenectomy. A key factor in SNNS is the accurate diagnosis of micrometastases during surgery.

Selection of tracer for sentinel node detection

Several authors have described SN mapping in gastric cancer. However, the SNs were detected by radioisotope (RI)-labeled colloid guided, dye-guided and both RI and dye guided methods. The most popular RI tracer is ^{99m}Technetium (^{99m}Tc) in forms such as ^{99m}Tc-tin colloid, ^{99m}Tc-sulfur colloid, ^{99m}Tc-serum albumin and ^{99m}Tc-phytate [31–34]. The accuracy rate of dye method in SN mapping using patent blue is 98% [29]. Indocyanine green (ICG) can also detect SN [35,36] and infrared electronic endoscopy with ICG staining can easily detect SN [37]. Furthermore, the accuracy rate of SN detection is improved by using both RI and dye tracers [30,38]. However, in the dye method, the visceral fat and the time elapsed after injection of dye may affect the detection of SN. Therefore, we regard the RI method as important.

Several questions are associated with the RI method of SN detection. Which RI tracers are suitable for SN detection? Which particle sizes of colloid easily move to lymph nodes and remain in the SNs? How many SNs are proper in SNNS? Colloid particle size might be an important factor in determining how certain tracers move from injection sites to lymph nodes via lymphatics [39]. We investigated the relationship between colloid particle size and the detection rate of SN in early gastric cancer. In this study, ^{99m}Tc-tin colloid was used because the particle size can be changed easily by varying the ratio between the isotonic sodium chloride and the tin solution [40]. We preoperatively injected three size of ^{99m}Tc-tin colloid (50, 100 and 500 nm) into the submucosa of the stomach under endoscopic control. The uptake of RI and total RI-uptake in SNs was highest when using particles of 100 nm and at least one lymph node metastasis (including micrometastases) was included in such SNs. Our results indicated that colloid particles of 100 nm were suitable for detection of SNs in early gastric cancer. Although small (50 nm) and large (500 nm) particle sizes were not proper for detection of SNs in gastric cancer, the optimal colloid size might vary among organs [26].

Ratios of metastatic areas and radioisotope uptake in sentinel nodes

SNs are identified by RI or dye uptake during surgery. However, even if macroscopically overt nodal metastases are found during operation, RI or dye is not always contained in such nodes. Miwa et al. reported that false negative results were found in 4 of 211 patients with early-stage gastric cancer using the dye method. All four patients with a false negative finding had a large clinical node [29]. Martin et al. reported that the highest RI count (in SNs) was not predictive of nodal metastasis in breast cancer [41]. The problem arises due to the relationship between RI count and lymphatic flow or nodal metastasis. We investigated whether the RI count reflects the ratio of metastatic areas of sentinel nodes in gastric cancer, with the aim of decreasing false negative rates in the radioisotopic identification of SNs. The mean ratio of the metastatic area was significantly higher in RI (−) (68.3 ± 20.5%) than in RI (+) (15.1 ± 20.8%; $p < 0.0001$). Therefore, the presence of lymph node metastases without RI uptake should be considered, particularly for lymph nodes with a metastatic area of >60% [42]. A diagnosis of lymph node metastasis should be confirmed using both preoperative imaging and SN detection.

SN mapping in patients with clinical T1 and N0 gastric cancer

SN mapping by the RI method has been performed in patients with clinical T1 and N0 gastric cancer since 2000. Lymph node metastases are examined by conventional HE and IHC using cytokeratin antibody as described [26,28,43]. At the present time we have enrolled 139 patients with clinical T1 and N0 gastric cancer in our institute. On the day prior to surgery, about 3 mCi of ^{99m}Tc-tin colloid was injected endoscopically into the submucosa at four sites around the tumor using a 23-G needle. Lymph node mapping was performed after surgery. RI uptake in all dissected lymph nodes was measured after surgery using Navigator GPS™ (Tyco Healthcare, Tokyo, Japan) [26–28,42,43]. 'Hot' nodes were defined as individual lymph nodes with a signal ten times greater than the background level, and these were considered to be SNs. All dissected lymph nodes were examined by HE staining and IHC. The average number of SN was 4.3 per patient and the detection rate of SN was 97.8% (136/139). Lymph node metastases were detected in 8% of patients (11/136) and the sensitivity of detecting lymph node metastases in SN was 100%. In patients without nodal metastases by HE staining, lymph nodes metastases were detected by IHC in 10.4% (13/125) of 125 patients. In the patients with nodal metastases detected by both HE staining and IHC, 23 patients had lymph node metastasis in SNs and one patient was false negative case because the patient had lymph node metastasis in non-SNs only. Thus, the sensitivity and accuracy rate of detecting lymph node metastases, including micrometastases, in SN mapping was 95.8% (23/24) and 99.3% (135/136), respectively.

The 10.4% incidence of lymph node metastases that we detected by IHC alone was high, even in patients with pathological N0 in this series. According to the results of SN mapping, both conventional HE and IHC staining should be performed when considering reducing the extent of lymphadenectomy based on the SN concept.

Diagnosis of lymph node metastases including micrometastases

In SNNS, there is a problem regarding the diagnosis of lymph node metastases including micrometastases. As mentioned above, not all metastases are detected by routine histological examination. As lymph node metastasis is one of the most important prognostic factors in gastric cancer [9,44], accurate diagnosis of lymph node metastasis before and during surgery is indispensable for SNNS. Although lymph nodes are three-dimensional structures, histological and IHC investigations are usually performed on planar slices. Metastatic foci located in the center of lymph nodes result in a correct positive identification, but when they are not located in the hilus of the lymph node, the results are deemed negative (Fig. 1). Serial sectioning of lymph nodes for HE staining results in a more accurate evaluation of the extent of lymph nodes metastases [45], but this procedure is clinically laborious and expensive. A comprehensive diagnostic method is required for the accurate diagnosis of lymph node metastases, especially for detecting micrometastases.

Owing to progress in genetic detection, micrometastases in lymph nodes have been identified in patients in whom histological examinations indicated absent nodal involvement [46,47]. We investigated lymph node micrometastases determined by real-time reverse transcription-polymerase chain reaction (RT-PCR). The relationship between micrometastases and lymphatic invasion, evaluated by IHC using a specific marker for the lymphatic vessels (D2-40) was analyzed [48]. We examined 1862 lymph nodes obtained from 80 patients with node-negative gastric cancer. All dissected lymph nodes were examined by RT-PCR in addition to HE staining and IHC. The resected primary specimens were immunostained using the D2-40 monoclonal antibody, and the presence or absence of lymphatic invasion was compared

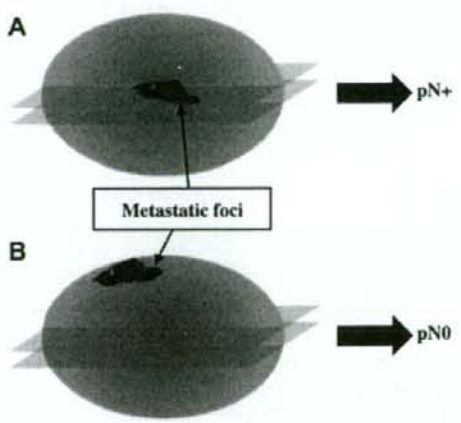

Figure 1 The metastatic foci in (A) are diagnosed accurately, but in (B) diagnosed inaccurately. Histological examination of lymph node metastasis is not always accurate because of the distribution of metastatic foci in the lymph node.

with that determined by HE staining. The incidence of lymph node micrometastases determined by IHC and by RT-PCR was 11.3% (9/80) and 31.3% (25/80), respectively. Although routine HE staining revealed lymphatic invasion in 11.3% (9/80) of patients, D2-40 staining uncovered new invasion in 23.8% (19/80) of patients. Lymph node micrometastases were more frequent among patients with pT2, than with pT1 tumors ($P = 0.0042$). In the diagnosis of HE and D2-40 staining, the incidence of lymph node micrometastases was significantly higher in patients with lymphatic invasion than in those without lymphatic invasion ($P = 0.0150$ and $P < 0.0001$, respectively). Lymph node micrometastases correlated more closely with D2-40, than with HE staining. We concluded that the incidence of both micrometastases and lymphatic invasion was high and that they correlated even in pN0 gastric cancer. The presence of micrometastases should be considered when planning less invasive treatment strategies. From the results of this study we conclude that although RT-PCR is non-morphological diagnostic method, an RT-PCR positive lymph node indeed may indicate the presence of nodal metastases.

RT-PCR can detect micrometastases in the lymph node more sensitively than HE and IHC. A few investigators have examined micrometastases of SN in gastric cancer using RT-PCR. In our institute, we examined the applicability of the SN concept to 61 patients with gastric cancer who were preoperatively diagnosed with clinical T1–T2 and N0 (cN0) based on lymph node micrometastases determined by IHC and RT-PCR. During surgery, RI uptake in the lymph node was measured using Navigator GPS™. All dissected lymph nodes were examined by RT-PCR in addition to HE and IHC. Sentinel nodes were identified in all patients (100%). The incidences of metastasis determined by HE and IHC were 8.2% (5/61) and 13.1% (8/61), respectively. Micrometastases undetectable by IHC were identified in 14 patients (23.0%) by RT-PCR. Only one patient had micrometastases detectable by RT-PCR in lymph nodes other than SN, but this patient had a cT2 tumor. In patients with cT1 and cN0 tumors, the false negative and accuracy rates were 0% and 100%, respectively. We concluded that although the incidence of micrometastases detected by RT-PCR was quite high, SN navigation identified such metastasis in all patients except one. Thus, the SN concept was applicable to patients with cT1 and cN0 gastric cancer, even when micrometastases were detectable by RT-PCR [28].

Selection of markers in genetic diagnosis using RT-PCR

Several RT-PCR markers can detect micrometastases (Table 2), and carcinoembryonic antigen (CEA) is very popular. However, Honda et al. reported that 21.7% of differentiated tumors, 34.7% of undifferentiated tumors with a tubular component and 69.7% of pure undifferentiated tumors all express high levels of CEA [49]. Since gastric cancer comprises various histological types, markers other than CEA might be required to detect micrometastases by RT-PCR. Cytokeratin-19 (CK19) is a candidate for detecting micrometastases in lymph nodes [50], but a CK19 pseudogene can be associated with false positive findings [51]. Hoon et al. [52] suggested that a PCR assay with multiple markers is more reliable and sensitive than a single-marker assay for detecting melanoma cells in the blood of patients. Similarly, Okada et al. [53] suggested that the multiple-marker RT-PCR assays are useful for detecting micrometastases in regional LNs among patients with gastric carcinoma. As with immunohistochemical methods, these markers are not cancer cell-specific. The problems associated with RT-PCR methods are multiple: marker selection, contamination of normal epithelial cells, presence of pseudogenes, and amplification of suitable settings.

Establishing marker cut-off values

Metastatic and benign nodes without cancer cells should be compared to establish cut-off values of markers. Kubota et al. compared cut-off values assessed as CEA and CK20 expression between histologically proven nodal metastasis and benign nodes obtained from patients without cancer. Their results indicated that duplex quantitative real-time RT-PCR using CEA and CK20 primers is the most sensitive method of detecting micrometastases and useful for evaluating the prognostic significance of lymph node micrometastasis in patients with gastric cancer [46]. Osaka et al. established cut-off values by evaluating CEA and CK20 expression in primary gastric cancer tumors, metastatic lymph nodes, histologically benign lymph nodes, normal blood from healthy volunteers, and in the cancer cell lines, MKN-45, OCUM-2M, and OCUM-2M-D3. The sensitivity of detecting primary tumors was 93.1% for CEA, 86.2% for CK20 and 96.6% for either CEA or CK20. These values for detecting nodal metastasis were 100%, 76.9% and 100%, respectively, and all cell lines were positive for both CEA and CK20 [47]. These results indicated that the sensitivity was improved by measuring the expression of both markers rather than that of either alone.

After cut-off values for multiple markers are established by evaluating the expression of each marker individually in benign lymph nodes, multiplex assays might improve both the sensitivity and specificity of detecting nodal metastasis.

The discovery of a cancer-specific primer will eventually lead to only a single assay being sufficient to detect micrometastases in lymph nodes.

Clinical application of the diagnosis of micrometastases

The presence of micrometastases in lymph nodes of gastric cancer has been confirmed by various methods. The concept of micrometastases should be applied not only to the prediction of prognosis and adjustment of staging migration but also to actual clinical practice. Intraoperative detection of micrometastases in lymph nodes is useful for determining the extent of lymphadenectomy. Conventional RT-PCR requires several steps and thus can generate information after 2–3 h. Thus, a more rapid procedure is required to obtain valuable results during surgery.

The new RT-PCR-based system, the GeneSearch BLN Assay (Veridex, LLC, Warren, NJ) has been developed for clinical applications. This system also generates results within about 30 min. Compared with histological staining, the GeneSearch BLN Assay detected 98% of metastases

Table 2 Diagnosis of lymph node metastasis with gastric cancer by genetic methods

Author	Source of lymph nodes	Method	Marker
Noguchi et al. [50]	100 From 12 gastric cancer patients	RT-PCR	CK-19
Aihara et al. [65]	37 From 2 gastric cancer patients	RT-PCR	Mammaglobin B
Okada et al. [53]	435 Nodes from 28 gastric cancer patients	RT-PCR	CEA, CK-20, MAGE-2
Matsumoto et al. [64]	312 From 50 gastric cancer patients with node-negative gastric cancer	RT-PCR	CEA
Kubota et al. [46]	392 From 21 gastric cancer patients	RT-PCR	CEA, CK-20
Ajisaka and Miwa [59]	213 From 35 gastric cancer patients with SN mapping	RT-PCR	CEA, CK-18, hTRT, MUC-1
Sonoda et al. [60]	305 From 28 gastric cancer patients	RT-PCR	MUC-2, CEA
Osaka et al. [47]	345 From 57 gastric cancer patients with SN mapping	RT-PCR	CEA, CK-20
Xu et al. [61]	298 From 35 gastric cancer patients	RT-PCR	CK-18
Sonoda et al. [62]	310 Lymph nodes from 33 patients with pN0 early gastric cancer	RT-PCR	MUC-2, TFF1
Arigami et al. [28]	1410 Lymph nodes from 61 gastric cancer patients with SN mapping	RT-PCR	CEA
Horibe et al. [63]	92 Lymph nodes from 9 gastric cancer patients	RT-LAMP	CK-19

RT-PCR, reverse transcriptase-polymerase chain reaction; RT-LAMP, reverse transcription loop-mediated isothermal amplification.

>2 mm and 88% of metastases >0.2 mm during surgery using CK19 and mammaglobin as markers [54].

Improvements to the RT-PCR procedure such as the transcription-reverse transcription concerted reaction (TRC) [55] have resulted in results being generated within 1 h. The quantitation, sensitivity, and reproducibility of TRC and conventional RT-PCR were similar when CEA expression was evaluated in a gastric cancer cell line and in metastatic lymph nodes of gastric cancer [56].

The one-step nucleic acid amplification (OSNA) assay is characterized by mRNA quantitation, high specificity, the absence of genomic DNA amplification and requires only about 21 min to complete. This method using Cytokeratin-19 (CK19) generated similar results to histological staining in 98.2% of lymph nodes in breast cancer patients [57]. The fully automated, multiplex quantitative RT-PCR system, GeneXpert (Cepheid, Sunnyvale, CA) is useful and requires <30 min to complete. The sensitivity and specificity of this system using two markers was 94% and 100%, respectively, compared with histological staining of lymph nodes from patients with breast cancer [58].

These novel technologies seem productive for diagnosing micrometastases, since such metastases can be detected during surgery and the accuracy rates seem at least as high as those of HE or IHC staining of frozen sections.

Conclusion and outlook

The presence of micrometastases in lymph nodes might be related to recurrence and prognosis in patients with gastric cancer. The clinical significance of micrometastases should be surveyed in a large patient population with the same disease stage who undergo the same types of surgery. Recent progress in RT-PCR technology means that about 30–40 min is required to determine micrometastases status. Since this is a relatively short time in which to reach an intraoperative diagnosis of micrometastases in lymph nodes, the extent of lymphadenectomy could be individualized based on such findings.

SNNS is an ideal method of reducing the extent of lymphadenectomy. The SN concept is applicable to patients at the early stages of cancer, including gastric cancer. The following conditions should be satisfied to establish the SN concept in gastric cancer. The absence of overt metastasis should be confirmed by preoperative imaging, because RI uptake is undetectable in massive metastases in SN. Suitable RI particles should be selected because the RI count and the numbers of SN differ according to particle size. The accurate diagnosis of micrometastases is essential for SN navigation surgery in gastric cancer, because of the presence of micrometastases among the patients without pathological nodal metastasis. SN navigation surgery, based on the rapid diagnosis of micrometastases, using new RT-PCR technology might soon change the concept of lymph node dissection in early gastric cancer.

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Vascular Endothelial Growth Factor-C and -D Expression Correlates With Lymph Node Micrometastasis in pN0 Early Gastric Cancer

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Background and Objectives: Vascular endothelial growth factors (VEGF)-C and -D play an important role in lymphangiogenesis, and the expressions of these factors are related to lymphatic invasion and lymph node metastasis in various malignant neoplasms. The present study investigates the expression of VEGF-C and -D in early gastric cancer and analyzes its relationship to lymph node micrometastasis determined by reverse transcription-polymerase chain reaction (RT-PCR).

Methods: We examined 1,828 lymph nodes obtained from 80 patients with node-negative early gastric cancer. All dissected lymph nodes were examined by RT-PCR for CEA mRNA in addition to hematoxylin–eosin staining. The resected primary specimens were immunostained using anti-VEGF-C and -D polyclonal antibodies.

Results: The incidence of lymph node micrometastasis determined by RT-PCR was 23.8% (19/80). The high expression of VEGF-C and -D was found in 27.5% (22/80) and in 21.3% (17/80), respectively. The expression of VEGF-C and -D was closely related to lymph node micrometastasis ($P = 0.0390$ and 0.0213, respectively).

Conclusions: We demonstrated a close relationship between micrometastasis and VEGF-C and -D expression of the primary tumor. Thus, levels of VEGF-C and -D expression might be useful for predicting micrometastasis in patients with early gastric cancer.

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KEY WORDS: **vascular endothelial growth factor (VEGF)-C; VEGF-D; lymph node micrometastasis; reverse transcription-polymerase chain reaction; gastric cancer**

INTRODUCTION

Lymph node metastasis represents one of the most important prognostic factors for patients with gastric cancer [1–5]. Therefore, D2 lymph node dissection for gastric cancer has become established as a standard procedure [6]. The 5-year survival rates of postoperative patients with early mucosal and submucosal gastric cancer are 95–100% and 85–95%, respectively [7–9]. Early gastric cancer is frequently treated by endoscopic mucosal resection (EMR) without lymphadenectomy [10], yet routine hematoxylin–eosin (HE) staining has revealed lymph node metastasis in 2–20% of such patients [8,9,11–13].

Immunohistochemical and biological techniques are now sufficiently sensitive to detect very low numbers of cancer cells, and real-time reverse transcription-polymerase chain reaction (RT-PCR) can identify lymph node micrometastasis in patients with gastrointestinal tract cancers [14,15]. Some authors have reported the clinical significance of lymph node micrometastasis determined by immunohistochemistry [8,16–18]. Accordingly, the control of lymph node metastasis, including micrometastasis, is the principal indicator for surgical treatment. However, to precisely diagnose pre-operative lymph node metastasis, especially micrometastasis, by imaging is difficult.

How lymph node metastasis arises from the primary tumor is obscure. Recent studies have demonstrated a principal role of molecules such as lymphatic endothelial markers in the mechanism of lymphogeneous metastasis [19–21]. Vascular endothelial growth factor receptor (VEGFR)-3 is one of such molecular markers and its first lymphatic-specific endothelial ligand to be discovered was vascular endothelial growth factor (VEGF)-C [22,23]. Similarly, VEGF-D is also a ligand for VEGFR-3 [24]. The expression of VEGF-C and -D in tumor cells is closely connected with lymphangiogenesis and closely correlates with lymphatic invasion and lymph node metastasis in gastrointestinal tract cancers, including gastric cancer [25–30]. However, little is understood about the relationship between VEGF-C and -D expression and lymph node micrometastasis (considered the initial stage of lymph node metastasis) in early gastric cancer.

The purpose of the present study was to investigate the presence of lymph node micrometastasis using RT-PCR in patients with node-negative (pN0) early gastric cancer. In addition, we analyzed relationships between clinicopathological findings including lymph node micrometastasis and VEGF-C and -D expression.

MATERIALS AND METHODS

Patients

We enrolled 80 patients (58 men and 22 women; age range 41–84 years; average 64 years) who were diagnosed with pN0 early gastric

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cancer (mucosal and submucosal tumors) by routine HE staining. All patients underwent curative gastrectomy with lymphadenectomy at the Department of Surgical Oncology and Digestive Surgery, Kagoshima University Hospital, between February 2003 and March 2005. None of the patients had received radiation therapy or chemotherapy before undergoing distal (n = 50), proximal (n = 6), total (n = 11), or partial (n = 13) gastrectomy. Based on the Japanese classification of gastric carcinoma [31], 21, 7, and 52 patients underwent D1, D2, and modified D2 (removal of all perigastric and other nodes along the left gastric, common hepatic and celiac arteries) lymphadenectomy, respectively. Fourteen, 44, and 22 tumors were located in the upper, middle, and lower thirds of the stomach, respectively. Fifty-two and 28 patients had mucosal and submucosal tumors, respectively, that were histopathologically classified as differentiated (n = 39; papillary, well and moderately differentiated tubular adenocarcinomas) and undifferentiated (n = 41; poorly differentiated adenocarcinoma, mucinous adenocarcinoma, and signet-ring cell carcinoma) types. All patients provided written informed consent to participate in all procedures associated with the study in accordance with our institutional guidelines.

Lymph Nodes

We examined 1,828 lymph nodes from 80 patients with pN0 early gastric cancer. The mean number of dissected lymph nodes was 23 (range 2–57). Positive controls comprised 21 lymph nodes from 10 patients with advanced gastric cancer and histologically evident metastasis. Negative controls comprised 30 lymph nodes from 14 patients without cancer (gall bladder stone, n = 6; gastric adenoma, n = 4; gastric ulcer, n = 3; Crohn's disease, n = 1). The lymph nodes were cut into two blocks at the plane of the largest dimension. Half of each lymph node was suspended in 1 ml of Isogen (Nippon Gene, Toyama, Japan) and immediately stored at −80°C. The other half was fixed in 10% formaldehyde, embedded in paraffin, cut into 3 μm sections and stained with HE.

Immunohistochemical Staining and Evaluation

The resected primary tumors were immunostained using polyclonal antibodies against VEGF-C and -D. Specimens of primary tumors were also fixed in 10% formalin, embedded in paraffin, sectioned, and then deparaffinized on slides with xylene and rehydrated with a graded series of ethanol. Endogenous peroxidase was blocked by immersing the slides in methanol containing 3% hydrogen peroxide for 30 min. After three 5-min washes with phosphate-buffered saline (PBS), non-specific binding was blocked at room temperature for 30 min with 1% bovine serum albumin in PBS. The sections were autoclaved in citrate buffer (0.01 mol/L, pH 6.0) at 120°C for 15 min to activate the antigen and incubated at 4°C overnight with anti-VEGF-C and -D polyclonal antibodies (Santa Cruz Laboratory, Santa Cruz, CA) diluted 1:100 in PBS. After three 5-min washes in PBS, the reactions for VEGF-C and -D were developed using avidin–biotin complex immunoperoxidase (ABC method; VECTASTAIN ABC kit, Vector Laboratories, Inc., Burlingame, CA) [32] and visualized using diaminobenzidine tetrahydrochloride. Negative controls were performed in all cases by omitting the first antibody.

Two independent investigators (T.A. and S.N.), who were blinded to the clinicopathological data of the patients, evaluated the immunoreaction of VEGF-C and -D. The presence of VEGF-C and -D immunoreactivity in over 10% of the cancer cells was defined as the high expression [26,29]. The expression of VEGF-C and -D was evaluated in 10 fields each containing 100 cells using high-power (200×) microscopy.

Cell Lines

MKN-45, an adenocarcinoma cell line that produces carcinoembryonic antigen (CEA) derived from a gastric cancer, was cultured in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum (Mitsubishi Kasei, Tokyo, Japan) and 100 U/ml each of penicillin and streptomycin.

Real-Time RT-PCR

Thawed lymph nodes were homogenized using FastPrep (Qbiogene, Inc., Carlsbad, CA) and then total RNA extracted according to the manufacturer's instructions was dissolved in 20 μl of water treated with diethylpyrocarbonate. The concentration, purity, and amount of total RNA were determined by measuring absorption at 260 and 280 nm using a GeneQuant pro UV/Vis Spectrophotometer (Amersham Pharmacia Biotech, Cambridge, England). To avoid contamination with genomic DNA, 0.5 μg of total RNA was digested at 37°C for 15 min with 1 U of DNase-I (Invitrogen, Life Technologies, Foster City, CA), which was then inactivated by heating with 1 μl of 25 mM ethylenediamine tetra-acetic acid (EDTA) at 65°C for 15 min. Complementary DNA (cDNA) was synthesized using the Advantage™ RT-for PCR Kit (Clontech Lab., Inc., Palo Alto, CA) according to the manufacturer's protocol and then stored at −20°C. A CEA-specific oligonucleotide primer was designed based on that described by Gerhard et al. [33]: sense, 5′-TGTCGGCATCATGATTGG-3′; antisense, 5′-GCAAATGCTTTAAGGAAGAA GC-3′. The donor and acceptor probe sequences for CEA identification were 5′-CCTGAAAT-GAAGAAACTACACCAGGGC-fluorescein and 5′-LC-Red640-GCT-ATATCAGAGCAACCCCAACCAGC-phosphorylation, respectively. Amplification of CEA by PCR using a quantitative fluorescence LightCycler™ (Roche Diagnostics, Mannheim, Germany) proceeded in a 20 μl reaction mixture containing 2 μl of LightCycler™ FastStart DNA Master Hybridization Probes (Roche Diagnostics), 3.0 mM $MgCl_2$, 0.5 μM sense and anti-sense primers, 0.4 μM fluorescent probe, 0.2 μM LC-Red probe, and 5 μl of undiluted template cDNA in LightCycler™ capillaries (Roche Diagnostics). Before amplification, 0.32 μl of anti-Taq DNA polymerase antibody (TaqStart™ antibody, Clontech Lab., Inc.) was added to the reaction mixture, which was then incubated at room temperature for 5 min to avoid primer prolongation. The amplification profile consisted of denaturation for one cycle at 95°C for 10 min followed by 35 cycles of 95°C for 10 sec, 60°C for 15 sec, and 72°C for 5 sec. Real-time PCR was monitored by measuring fluorescent signals at the end of the annealing phase for each cycle. We quantified and confirmed the integrity of the RNA by comparison with the results of real-time RT-PCR of the amplified glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) housekeeping gene. The sense and anti-sense primers for GAPDH were 5′-TGAACGGGAAGCTCACTGG-3′ and 5′-TCCACCACCCTGTTGC-TGTA-3′, respectively. The donor and acceptor probes for GAPDH were 5′-TCAACAGCGACACCCACTCCT-3′-fluorescein and 5′-LC-Red640-CACCTTTGACGCTGGGGCT-3′-phosphorylation, respectively. The GAPDH gene was amplified in 20 μl of the reaction mixture described above in a LightCycler™ capillary (Roche Diagnostics). The amplification profile consisted of one denaturation cycle at 95°C for 10 min followed by 45 cycles of 95°C for 15 sec, 60°C for 15 sec, and 72°C for 12 sec. All primers and probes were synthesized and purified by reverse-phase high-performance liquid chromatography and the Nihon Gene Research Laboratories (Sendai, Japan) established the optimal reagent concentrations and PCR cycling conditions. Each RT-PCR reaction included positive controls synthesized from MKN-45 cells and negative controls from RNA-negative samples. Quantification data were analyzed using the LightCycler™ software (Roche Diagnostics).

We tested the sensitivity of the RT-PCR assay by spiking a series of 10-fold dilutions of MKN-45 cells (10^6–10^0) into 1×10^7 peripheral blood mononuclear cells (PBMCs) from a healthy volunteer who did not express CEA mRNA. Total RNA extracted as described above was assayed by real-time RT-PCR. These results were used as external standards for analyses of quantification data. Levels of CEA mRNA were assessed using the LightCycler™ from the crossing point, marking the cycle when fluorescence of a sample increased above the background to give the maximal slope by log-linear amplification. Relative CEA mRNA levels of samples were calculated with reference to standard curves generated from plots of crossing points versus the initial number of MKN-45 cells.

Statistical Analysis

StatView statistical software version 5.5 (SAS Institute, Inc., Cary, NC) performed all statistical calculations. Data were statistically compared using chi-square and Fisher's exact tests. A P-value of <0.05 was considered statistically significant.

RESULTS

Sensitivity of RT-PCR in Cell Dilution Study and CEA mRNA Level in Control Samples

We detected CEA mRNA in as little as 10^1 tumor cells/10^7 PBMCs in MKN-45 cancer cell lines. The mean CEA mRNA levels corrected for GAPDH mRNA levels (CEA mRNA/GAPDH mRNA $\times 10^5$) in histologically evident metastatic (positive controls) and benign (negative controls) lymph nodes were 5.9×10^4 (range 4.9×10^0–9.1×10^5) and 0.0, respectively. Based on these results, samples with corrected CEA mRNA levels of >0.0 were classified as positive.

Lymph Node Micrometastasis and Clinicopathological Factors

Lymph node micrometastasis was identified in 19 of 80 patients (23.8%), in 43 of 1,828 nodes (2.4%), in 15 of 74 patients without (20.3%), and in 4 of 6 patients with (66.7%) lymphatic invasion by RT-PCR. The incidence of lymph node micrometastasis was significantly higher in patients with, than without lymphatic invasion ($P = 0.0261$; Table I). However, gender, tumor location, histological type, depth of tumor invasion, and venous invasion did not significantly differ.

TABLE I. Relationship Between Lymph Node Micrometastasis and Clinicopathological Factors in 80 Patients With pN0 Early Gastric Cancer

Variable	Micrometastasis (%) Negative (n = 61)	Positive (n = 19)	P-value
Gender			
Male	47 (81.0)	11 (19.0)	0.1412
Female	14 (63.6)	8 (36.4)	
Tumor location			
Upper	12 (85.7)	2 (14.3)	0.6045
Middle	32 (72.7)	12 (27.3)	
Lower	17 (77.3)	5 (22.7)	
Histological type			
Differentiated	32 (82.1)	7 (17.9)	0.2970
Undifferentiated	29 (70.7)	12 (29.3)	
Depth of tumor invasion			
Mucosal invasion	41 (78.8)	11 (21.2)	0.5828
Submucosal invasion	20 (71.4)	8 (28.6)	
Lymphatic invasion			
Negative	59 (79.7)	15 (20.3)	0.0261
Positive	2 (33.3)	4 (66.7)	
Venous invasion			
Negative	59 (76.6)	18 (23.4)	0.5620
Positive	2 (66.7)	1 (33.3)	

Expression of VEGF-C and -D

The expression of VEGF-C and -D was found in the cytoplasm of cancer cells. The high expression of VEGF-C and -D was present in 22 (27.5%; Fig. 1A,B) and in 17 (21.3%; Fig. 1C,D), respectively, of the 80 patients.

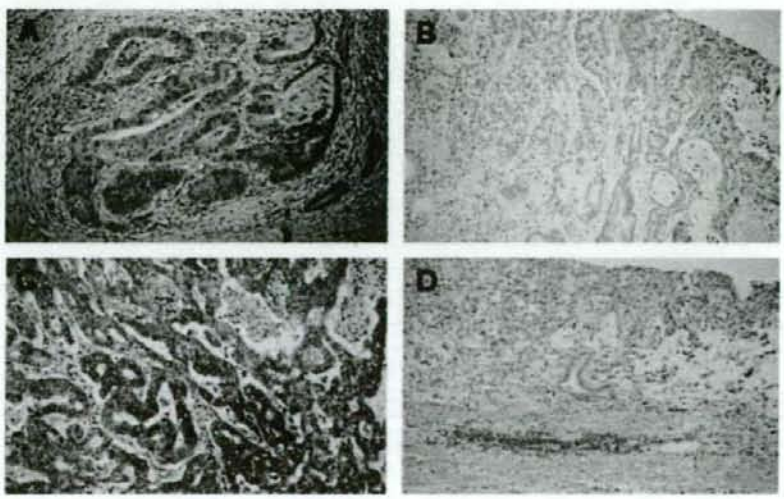

Fig. 1. Expression of VEGF-C and -D in cytoplasm of cells from early gastric cancer. **A,B**: High and low levels of VEGF-C expression, respectively. **C,D**: High and low levels of VEGF-D expression, respectively (magnification, 200×).

TABLE II. Relationship Between VEGF-C and -D Expression and Clinicopathological Factors in 80 Patients With pN0 Early Gastric Cancer

	VEGF-C expression (%)			VEGF-D expression (%)		
Variable	Low (n = 58)	High (n = 22)	*P*-value	Low (n = 63)	High (n = 17)	*P*-value
Gender						
Male	43 (74.1)	15 (25.9)	0.5877	48 (82.8)	10 (17.2)	0.2199
Female	15 (68.2)	7 (31.8)		15 (68.2)	7 (31.8)	
Tumor location						
Upper	12 (85.7)	2 (14.3)	0.4667	14 (100.0)	0 (0.0)	0.0705
Middle	31 (70.5)	13 (29.5)		34 (77.3)	10 (22.7)	
Lower	15 (68.2)	7 (31.8)		15 (68.2)	7 (31.8)	
Histological type						
Differentiated	27 (69.2)	12 (30.8)	0.6191	31 (79.5)	8 (20.5)	>0.9999
Undifferentiated	31 (75.6)	10 (24.3)		32 (78.0)	9 (22.0)	
Depth of tumor invasion						
Mucosal invasion	41 (78.8)	11 (21.2)	0.1155	42 (80.8)	10 (19.2)	0.5759
Submucosal invasion	17 (60.7)	11 (39.3)		21 (75.0)	7 (25.0)	
Lymphatic invasion						
Negative	58 (78.4)	16 (21.6)	0.0002	61 (82.4)	13 (17.6)	0.0168
Positive	0 (0.0)	6 (100.0)		2 (33.3)	4 (66.7)	
Venous invasion						
Negative	57 (74.0)	20 (26.0)	0.1818	61 (79.2)	16 (20.8)	0.5167
Positive	1 (33.3)	2 (66.7)		2 (66.7)	1 (33.3)	
Lymph node micrometastasis						
Negative	48 (78.7)	13 (21.3)	0.0390	52 (85.2)	9 (14.8)	0.0213
Positive	10 (52.6)	9 (47.4)		11 (57.9)	8 (42.1)	

VEGF-C and -D Expression and Clinicopathological Factors

The patients with high levels of VEGF-C and -D had a significantly higher incidence of lymphatic invasion ($P = 0.0002$ and 0.0168, respectively) and lymph node micrometastasis ($P = 0.0390$ and 0.0213, respectively), compared with those with low levels of VEGF-C and -D (Table II). However, the expression levels of VEGF-C or -D did not correlate with any other clinicopathological factors (Table II).

Lymph Node Micrometastasis and Combined Analysis of EGF-C and -D Expression

The correlation between VEGF-C and -D expression was significant ($P = 0.0001$; Table III), although these expressions were discrepant in 11 patients. Based on the expression profiles of VEGF-C and -D, all patients were divided into groups (Table IV) expressing high levels of both VEGF-C and -D (high group), low levels of both VEGF-C and -D (low group), and of either VEGF-C or -D (intermediate group). According to this combined analysis, the high group had a significantly higher incidence of lymph node micrometastasis compared with the low group ($P = 0.0135$).

DISCUSSION

Immunohistochemical and biological techniques have become sufficiently sensitive to detect a few occult cancer cells such as lymph node micrometastasis. Among these methods, RT-PCR is the most sensitive [14,15]. Mori et al. [14] detected CEA mRNA expression in 47 of 87 lymph nodes from patients diagnosed with node-negative malignant neoplasms and showed by RT-PCR that the rate of node-positivity was 66% among patients with gastrointestinal or breast carcinomas, compared with 26% among the same group of patients determined by a histological analysis. Here, we examined all dissected lymph nodes by RT-PCR in addition to HE staining and identified lymph node micrometastasis in 23.8% of patients with pN0 early gastric cancer. This result indicates that lymph node micrometastasis is extant at a high incidence during the early stage of gastric cancer.

Although the clinical significance of lymph node micrometastasis in gastric cancer remains controversial [18], some authors have reported that lymph node micrometastasis is a prognostic factor [8,16–18]. The immunohistochemical study of Cai et al. [8] showed that patients with submucosal gastric cancer and micrometastasis had a significantly worse prognosis than those without micrometastasis (82% vs. 100%), and that lymph node micrometastasis was closely associated with tumor size, macroscopic type, lymphatic invasion, and depth of submucosal invasion. Their study differed from the present investigation in terms of patients and methodology. We examined lymph node micrometastasis in both mucosal and submucosal types of cancer using

TABLE III. Relationship Between Expression Level of VEGF-C and -D

	VEGF-D expression (%)		
VEGF-C expression	Low (n = 63)	High (n = 17)	*P*-value
Low (n = 58)	55 (68.8)	3 (3.8)	<0.0001
High (n = 22)	8 (10.0)	14 (17.5)	

TABLE IV. Relationship Between Lymph Node Micrometastasis and VEGF-C and -D Expression Profile

	Micrometastasis (%)		
Profile of VEGF-C and -D expression	Negative (n = 61)	Positive (n = 19)	*P*-value
Low (n = 55)	46 (83.6)	9 (16.4)	0.0135
Intermediate (n = 11)	8 (72.7)	3 (27.3)	
High (n = 14)	7 (50.0)	7 (50.0)	

Low, low level of both VEGF-C and -D expression; intermediate, high level of either VEGF-C or -D expression; high, high level of both VEGF-C and -D expression.

RT-PCR. We found that lymph node micrometastasis was related only to lymphatic invasion and not to other clinicopathological factors including tumor size, macroscopic type, and depth of tumor invasion. However, 15 of 74 patients (20.3%) without lymphatic invasion had lymph node micrometastasis. Thus, we examined VEGF-C and -D expression to clarify the mechanism of initial nodal metastasis.

The VEGF family members VEGF-C and -D are associated with the lymphatic spread of cancer cells [25–30]. VEGFR-3, which is the receptor for VEGF-C and -D, is specifically expressed in cells of the lymphatic endothelium [22–24]. Since these signals promote the multiplication, migration, and luminal formation of lymphatic vessels, VEGF-C and -D expression induces lymphangiogenesis. Yonemura et al. [25] reported that VEGF-C expression is closely related to lymph node metastasis, lymphatic invasion, venous invasion, and tumor infiltration in gastric cancer. They also reported that the prognosis of patients was significantly poorer in the presence of high, than low levels of VEGF-C expression and that VEGF-C tissue status is an independent prognostic factor according to multivariate analysis [25]. On the other hand, little is known about VEGF-D expression in patients with gastric cancer. Ishikawa et al. [28] reported that the expression of VEGF-D is significantly correlated with histological type, tumor depth, lymphatic invasion, and lymph node metastasis in early gastric cancer. We found that high levels of VEGF-C and -D expression were significantly correlated with lymphatic invasion and lymph node micrometastasis. These results suggested that VEGF-C and -D expression could predict lymph node micrometastasis. We also found that lymph node micrometastasis more closely correlated with the expression of both VEGF-C and -D compared with that of either alone.

EMR has recently been applied to treat early gastric cancer [10]. When planning such therapy, the presence or absence of lymph node micrometastasis is an important issue. From the results of this study, to examine VEGF-C and -D expression in resected specimens of EMR could offer useful information regarding lymph node micrometastasis. Meticulous follow-up or sentinel node sampling under laparoscopy should be considered for patients with high levels of VEGF-C and -D expression.

In conclusion, we demonstrated that lymph node micrometastasis detected by RT-PCR, which was the initial stage of lymph node metastasis, was closely associated with lymphatic invasion and with VEGF-C and -D expression by the primary tumor. The presence of micrometastasis is an important factor when less invasive strategies such as EMR or reduction of lymphadenectomy are under consideration for patients with early gastric cancer, and could be indicated by the levels of VEGF-C and -D expressed by the primary tumor.

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Ⅰ．総　論

2. SNNSの歴史と進歩

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