Clinical Translational Research

Oncology

Oncology 2009;77:205–211
DOI: 10.1159/000236020

Received: November 5, 2008
Accepted after revision: April 15, 2009
Published online: September 4, 2009

The Utility of Rapid Diagnosis of Lymph Node Metastasis in Gastric Cancer Using a Multiplex Real-Time Reverse Transcription Polymerase Chain Reaction Assay

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Key Words
Gastric cancer · Lymph node metastasis · Micrometastasis · Isolated tumor cells · Sentinel node concept · Reverse transcription polymerase chain reaction · Multiple markers

Abstract
Background: Lymph node metastasis is the most important prognostic factor in gastric cancer. However, diagnosis by hematoxylin and eosin staining or immunohistochemistry is not always sufficient for the detection of cancer cells because only representative number of slices are examined. Cancer cells may, therefore, be missed by traditional histological methods. Recently, reverse transcription polymerase chain reaction (RT-PCR) methods have been introduced for improved detection of cancer cells. The purpose of this study was to evaluate the utility of a prototype RT-PCR assay run on the Cepheid SmartCycler® system compared to conventional RT-PCR using the LightCycler® system. ***Patients and Methods:*** Forty-seven overt metastatic lymph nodes from 8 patients with advanced gastric cancer and 22 benign lymph nodes from patients without malignant tumor who received surgery were obtained with informed consent. We examined the lymph nodes by RT-PCR, using markers for CEA and CK19 and the LightCycler and SmartCycler systems. ***Results:*** In the singlex assay, the sensitivity of CEA and CK19 was 91.5 and 70.2% in the LightCycler system, and 97.9 and 95.7% in the SmartCycler system, respectively. In the multiplex assay, the sensitivity was 91.5% in the LightCycler system and 100% in the SmartCycler system, respectively. ***Conclusion:*** In this study, rapid diagnosis using RT-PCR by the SmartCycler system had higher accuracy for detecting lymph node metastasis than the conventional LightCycler system. The SmartCycler system is more effective for the diagnosis of lymph node metastasis in gastric cancer when run with the prototype assay.

Introduction

Sentinel nodes (SN) are the first lymph nodes to receive lymphatic flow from the primary tumor and metastasis initially occurs at this site [1]. Thus, metastases are likely located in SNs as the first step of lymph node metastasis. For this reason, SN navigation surgery has been introduced for patients with breast cancer and malignant melanoma [2–5]. Recently, this concept has also been ap-

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0030–2414/09/0774–0205$26.00/0

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plied to gastrointestinal tract cancer [6–10]. We recently reported that the SN concept was acceptable for patients with early gastric cancer using immunohistochemical staining and real-time reverse transcription polymerase chain reaction (RT-PCR) using the LightCycler® System [11, 12].

Lymph node metastasis is one of the most important prognostic factors in a number of cancers [13–18]. However, the sensitivity of pre-operative diagnosis using multidetector-row helical computed tomography and abdominal ultrasonography of lymph node metastasis is insufficient [19, 20]. The diagnosis of lymph node metastasis during operations generally depends on hematoxylin and eosin (HE) staining at most institutions. At some institutions, it is possible to perform immunohistochemistry during the operation to detect micrometastasis in lymph nodes [21–23]. However, these methods can be highly laborious. Moreover, these methods are performed only on representative slices of the lymph nodes. If metastases exist in the remaining, uninspected mass of the lymph nodes, metastatic foci would not be detected. Isozaki et al. [24] examined lymph node metastasis in gastric cancer by histological examination using a serial sectioning method and concluded that serial sectioning enables more accurate evaluation of the extent of lymph node metastasis.

To avoid discrepant results, accurate and efficient diagnosis of lymph node metastasis during surgery is important. Additionally, the clinical significance of micrometastasis, including isolated tumor cells (ITC) is controversial. However, it has been shown that micrometastatic cells and ITC have proliferative activity [25, 26]. It is important to diagnose these tiny metastatic foci to clarify their clinical significance.

RT-PCR is a more sensitive method for the detection of micrometastasis than immunohistochemistry [27]. The sensitivity and specificity of RT-PCR depends on the markers selected. Using multiple markers in real-time RT-PCR may be a valid method for the detection of micrometastasis and ITC [28].

Recently, a prototype research assay using rapid real-time RT-PCR, (Veridex LLC, Raritan, N.J., USA) which runs on the Cepheid SmartCycler® system (Cepheid, Sunnyvale, Calif., USA) has been developed and the utility of this system has been reported [29–31]. This system is advantageous both due to its speed (approximately 40 min in total to obtain a result) and its ability to perform multiplex assays. The current study investigated the utility of the SmartCycler system compared to the LightCycler® system using carcinoembryonic antigen (CEA) and cytokeratin-19 (CK19) as markers in each system. Expression of these markers has previously been shown to have diagnostic importance in gastric cancer [12, 27, 28, 32, 33].

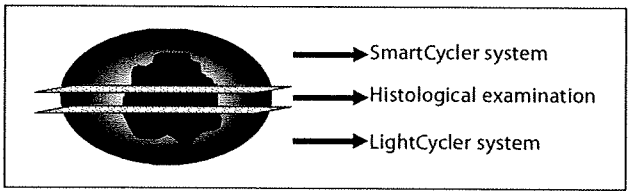

Fig. 1. All lymph nodes were cut into 3 uniform pieces. One third was used for HE staining and immunohistochemistry, one third for RT-PCR using the LightCycler system, and the remaining third for RT-PCR using the SmartCycler system.

Materials and Methods

Diseased and Benign Lymph Nodes

Forty-seven histologically diseased lymph nodes, as measured by HE staining and immunohistochemistry using an anti-CK antibody, were obtained with informed consent from 8 patients with advanced gastric cancer. Twenty-two benign lymph nodes were obtained with informed consent from patients with benign disease who underwent surgery.

Verification of Lymph Node Metastasis by HE Staining and Immunohistochemistry

All lymph nodes were cut into 3 uniform pieces. One third was used for HE staining and immunohistochemistry, one third for RT-PCR using the LightCycler system and the remaining third for RT-PCR using the SmartCycler system (fig. 1).

All lymph nodes were stained with HE and immunohistochemistry was performed using a monoclonal anti-CK antibody cocktail (AE1/AE3; Dako Corporation, Carpinteria, Calif., USA). The tissue 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 subsequently immersed in proteinase K (Dako Corporation) 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, the avidin-biotin complex and immunoperoxidase were applied (ABC method, Vectastain ABC Kit; Vector Laboratories Inc., Burlingame, Calif., 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. CK-positive normal gastric mucosa and primary tumor specimens were used as positive controls in all testing. Three independent observers (S.Y., Y.U. and H.A.) evaluated all immunohistochemically stained slides. Overt metastatic lymph nodes were verified as macrometastatic lymph nodes histologically.

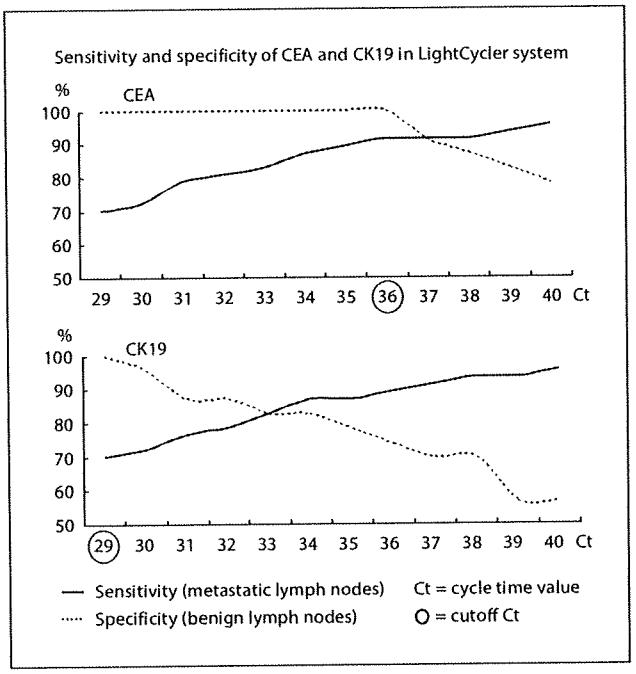

Fig. 2. Regarding the LightCycler system, we set up the provisional cutoff Ct value in the highest sensitivity with 100% specificity to avoid false positive results. The highest sensitivity with 100% specificity was 91.5% for CEA, using the cutoff value of 36 and the highest sensitivity with 100% specificity was 70.2% for CK19, using the cutoff value of 29.

Real-Time RT-PCR Assay by the LightCycler System

All samples were prepared for the LightCycler system according to the previously published manuscript [12]. Total RNA was extracted from homogenized lymph nodes using the guanidinium thiocyanate phenol-chloroform method from Isogen (Nippon Gene, Toyama, Japan). The concentration, purity and mass of total RNA were determined by measuring spectrophotometric absorption at 260 and 280 nm using GeneQuant pro UV/Vis Spectrophotometer (Amersham Pharmacia Biotech, Cambridge, UK). Total RNA was treated with DNase-I (Invitrogen, Life Technologies, Foster City, Calif., USA) to eliminate contamination with genomic DNA. Complementary DNA (cDNA) was synthesized using the Advantage RT-for-PCR kit (Clontech Laboratory Inc., Palo Alto, Calif., USA) according to the manufacturer's protocol and cDNA was then processed for PCR. This assay was performed based on the hybridization probe method. The CEA primers and probe were designed based on the method described by Gerhard et al. [34] and the CK19 primers and probe were designed at Nihon Gene Research Laboratories Inc. This assay is interpreted using threshold cycle (Ct) values.

Real-Time RT-PCR Assay by the SmartCycler System

The nodal tissue was homogenized and RNA was purified using the RNA Sample Preparation Kit (Veridex LLC). The RT-PCR assay, which included reverse transcription of the cDNA from target mRNA and amplification of the cDNA, was performed in one step using a prototype kit run on the SmartCycler system. This kit was designed to detect expression of CEA, CK19 and a housekeeping gene (internal control) and is based on the hydrolysis probe method and interpreted using Ct values. Each assay contained a positive and negative external control.

Table 1. Comparison between LightCycler and SmartCycler systems in singlex and duplex assay

		LightCycler	SmartCycler
Sensitivity	Single marker		
	CEA	91.5%	97.9%
	CK-19	70.2%	95.7%
	Double markers	91.5%	100%
Specificity		100%	100%

Statistical Analysis

For this study, the McNemar test was used to analyze data comparing the methods. $p < 0.05$ was considered significant.

Results

Expression of CEA and CK19 mRNA in Metastatic and Benign Lymph Nodes Using the LightCycler System

All lymph nodes were confirmed to express the housekeeping gene using the SmartCycler system. In benign lymph nodes, the specificity of CEA was 100% when using a Ct cutoff of 36, but when the Ct value was over 36, the specificity began to decrease. Specificity was 90.9% at a Ct of 37 (fig. 2). The highest sensitivity with 100% specificity was 91.5% for CEA, using a Ct cutoff value of 36 (table 1). We set up the provisional cutoff Ct value at 36 for CEA with the LightCycler system (fig. 2). In the same way, the specificity of CK19 was 100% up to a Ct cutoff of 29, but when the cycle time value was over 29, specificity began to decrease (fig. 2). The highest sensitivity obtained with 100% specificity was 70.2% with CK19 and a Ct cutoff set at 29 (table 1). We set up the provisional cutoff Ct value at 29 for CK19 with the LightCycler system (fig. 2).

Expression of CEA and CK19 mRNA in Metastatic and Benign Lymph Nodes Using the Prototype Assay System

In benign lymph nodes, the specificity of CEA was 100% at all times (fig. 3). The highest sensitivity with the lowest Ct cutoff of 35 was 97.9% (table 1). We set up the

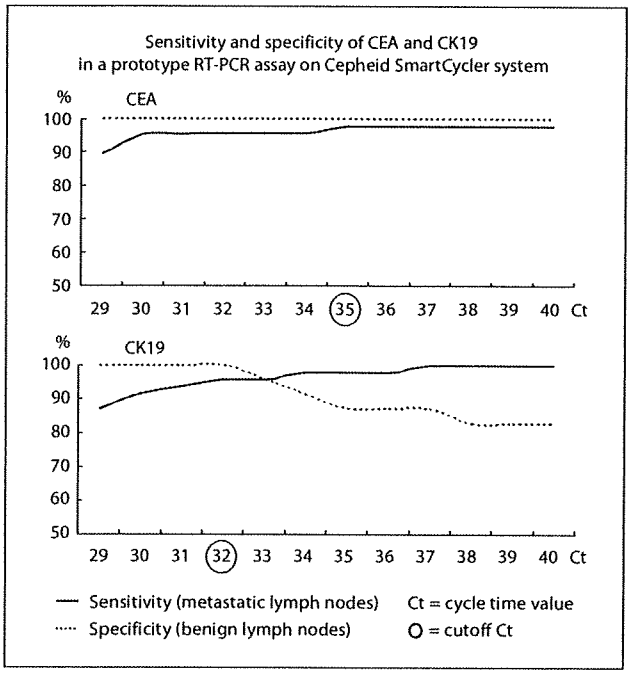

Fig. 3. Regarding the SmartCycler system, we set it up in the same way as the LightCycler system. The highest sensitivity with 100% specificity was 97.9% for CEA, using the cutoff value of 35 and the highest sensitivity with 100% specificity was 95.7% for CK19, using the cutoff value of 32.

provisional cutoff Ct value at 35 for CEA by the prototype assay system to avoid false positive results (fig. 3). With CK19, the specificity was 100% up to a Ct of 32, but when the Ct value was over 32, the specificity began to decrease (fig. 3). The highest sensitivity with 100% specificity was 95.7% with CK19 and a Ct cutoff of 32 (table 1). We set up the provisional CK19 cutoff Ct value at 32 for the prototype assay system (fig. 3).

Comparison of the Performance between the LightCycler and Prototype Assay Systems in Singlex and Duplex Assays

As a singlex assay, using both CEA and CK19, sensitivity of the prototype assay system was equal to or higher than that of the LightCycler system. With both systems, the sensitivity was improved for the multiplex assay (compared to the singlex assay). These results are shown in table 1. Both assays used the provisional cutoff Ct values for each marker in each system with a specificity of 100% in order to avoid false positive results (table 1). The concordance of the results in both systems was compared

Table 2. Evaluation of the results of the LightCycler and SmartCycler systems

	LC-CK19		p value
	negative	positive	
SC-CK19			
Negative	22	2	0.0027
Positive	14	31	
	LC-CEA		p value
	negative	positive	
SC-CEA			
Negative	23	0	0.0833
Positive	3	43	
	LC-CK19 or CEA		p value
	negative	positive	
SC-CK19 or CEA			
Negative	22	0	0.0455
Positive	4	43	

LC = LightCycler system; SC = SmartCycler system.

using the McNemar test (table 2). For CK19, the sensitivity of the prototype assay system was significantly higher than that of the LightCycler system ($p = 0.0027$). For CEA, there was no significant difference between the systems. In the multiplex assay, the sensitivity of the prototype assay system was significantly higher than that of LightCycler system ($p = 0.0455$).

Discussion

Accurate diagnosis for lymph node metastasis is essential for gastric cancer, but the sensitivity of preoperative diagnosis for lymph node metastasis is not always sufficient [19, 20]. An improvement in accuracy of the intraoperative diagnosis for lymph node metastasis may ultimately lead to a better prognosis for patients. We reported that the SN concept is applicable to patients with early gastric cancer even in cases where micrometastasis is detected by immunohistochemical staining and RT-PCR [11, 12]. While lymph nodes are a three-dimensional specimen, routine intraoperative histological examination is a two-dimensional diagnostic method. Hence, a comprehensive diagnosis for lymph node metastasis is needed.

RT-PCR is a sensitive method for the detection of micrometastasis in lymph nodes, but it has been very time consuming. In conventional RT-PCR assay, approximately 3 h were required to obtain our results in this study.

Genetic diagnostic methods such as RT-PCR have recently improved, and these methods have advantages for rapid diagnosis. The transcription-reverse transcription concerted reaction using CEA as a marker enables one to obtain the result within 1 h, and the performance of transcription-reverse transcription concerted assay is at least equivalent to the LightCycler system [35]. The one-step nucleic acid amplification (OSNA) assay is characterized by mRNA quantification and requires about 21 min to complete. OSNA using CK19 as a marker generated similar results to histological staining in 98.2% of lymph nodes in breast cancer patients [36]. The fully automated, multiplex quantitative RT-PCR assay and SmartCycler system requires less than 30 min to complete. The sensitivity and specificity of this system was 94 and 100%, respectively, compared to histological examination of lymph nodes from patients with breast cancer [37].

With these diagnostic methods, selection of markers is a very important issue. Gastric cancer is comprised of various histological types. Honda et al. [38] 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. Therefore, the diagnostic value of CEA for the detection of metastatic foci in lymph nodes becomes problematic. Okada et al. [28] suggested that multiple-marker RT-PCR assays are useful for detecting micrometastasis in regional lymph nodes with gastric cancer. Some markers have been reported for the diagnosis of lymph node metastasis with gastric cancer, such as CEA [12, 27, 28, 32, 39–41], CK18 [42], CK19 [33], CK20 [27, 28, 41], MUC1 [39], MUC2 [40], hTRT [39], TFF1 [43], mammaglobin B [44] and MAGE3 [28]. In the current study, assays were performed by using only CK19 and CEA.

In breast cancer and other types of solid tumors, CK19 is one of the most popular markers for the detection of circulating tumor cells, bone marrow and lymph nodes [45–52]. Though the utility of RT-PCR using CK19 was reported previously [33], the specificity of CK19 was lower compared with CEA in the LightCycler system. Rudd et al. [53] reported the existence of its pseudogene, and lower specificity compared with CEA may be caused by the pseudogene. However, we considered these 2 markers in combination (CK19 and CEA) to be useful for detecting epithelial cancer cells, and the issue of specificity is improved by using multiple markers.

In this study, the advantages of the prototype RT-PCR assay on the SmartCycler system compared with the conventional system are: the simplicity of the procedure, rapidity to get results and the improvement of the sensitivity as well as the specificity by using multiple markers in one assay.

In the near future, the improvement of genetic diagnostic methods offering both simplicity and reproducibility of the result, and newly discovered markers with high sensitivity as well as specificity may enable a more accurate diagnosis of lymph node metastasis, including micrometastasis.

In conclusion, the prototype assay system has potential benefits for diagnosis of lymph node metastasis compared with the conventional RT-PCR system because of its improved turnaround time, sensitivity and specificity.

Acknowledgements

We are grateful to Mr. Toshihito Furukawa, a statistician at Biostatistical Research Corporation (Tokyo, Japan), for the analysis of our data.

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Journal of Surgical Oncology 2009;99:148–153

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.

J. Surg. Oncol. 2009;99:148–153. © 2008 Wiley-Liss, Inc.

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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Received 20 August 2008; Accepted 26 November 2008

DOI 10.1002/jso.21228

Published online 30 December 2008 in Wiley InterScience (www.interscience.wiley.com).

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′; anti-sense, 5′-GCAAATGCTTTAAGGAAGAA GC-3′. The donor and acceptor probe sequences for CEA identification were 5′-CCTGAAATGAAGAAACTACACCAGGGC-fluorescein and 5′-LC-Red640-GCTATATCAGAGCAACCCCAACCAGC-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′-TCCACCACCCTGTTGCTGTA-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.

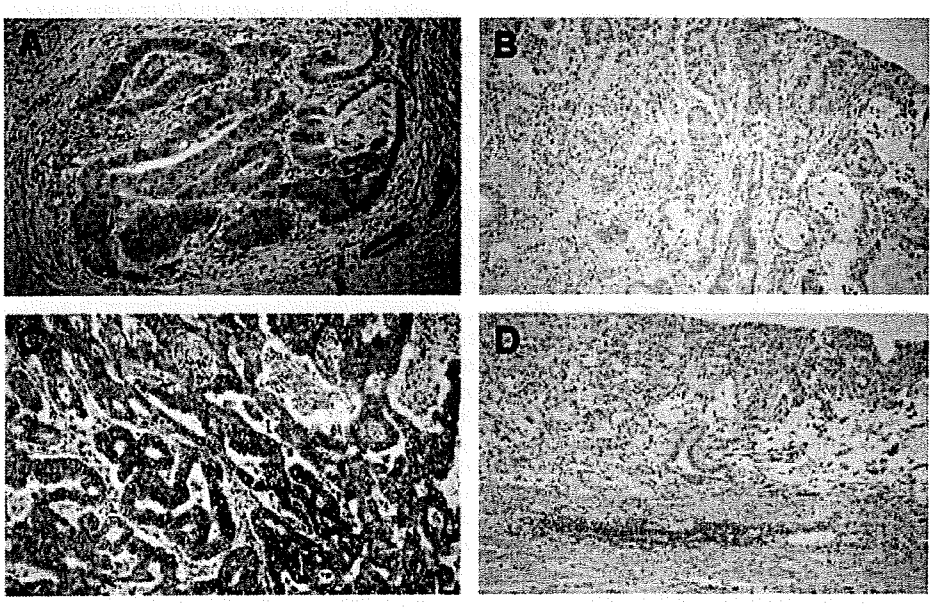

Fig. 1. Expression of VEGF-C and -D in cytoplasm of cells from early gastric cancer. **A,B**: High and low levels of VEGF-C expression, respectively. **C,D**: High and low levels of VEGF-D expression, respectively (magnification, 200×).

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

Variable	VEGF-C expression (%)			VEGF-D expression (%)		
	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-C expression	VEGF-D expression (%)		*P*-value
	Low (n = 63)	High (n = 17)	
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

Profile of VEGF-C and -D expression	Micrometastasis (%)		*P*-value
	Negative (n = 61)	Positive (n = 19)	
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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Selective lymphadenectomy of para-aortic lymph nodes for advanced gastric cancer

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Received February 3, 2009; Accepted March 13, 2009

DOI: 10.3892/or_00000464

Abstract. The Japanese randomized trial comparing standard D2 with D2 plus additional para-aortic lymph node (PAN) dissection for advanced gastric cancer (JCOG study 9501) did not demonstrate any difference in survival between the two groups. It is unknown whether there is any prognostic benefit in dissection for subgroups of PAN. Non-inferiority in survival of the patients with PAN metastasis to the patients having n2 metastasis was examined according to the subgroup of PANs and the tumor location. The survival curve of n2 patients (n=131) were retrospectively compared with that of patients with PAN metastasis (n=55) and also compared with that of patients with metastasis to subgroup of PANs by the location of primary tumor (regions U, M and L). Expectedly, the prognosis of the n2 patients is significantly better than that of the patients with PAN metastasis, but there was no difference in the survival times between the n2 (+) group and the a2-lat (+) or the b1-int (+) group, suggesting that the a2-lat or the b1-int dissection matched the D2 dissection. Furthermore, the importance in dissection of the a2-lat and the b1-int was investigated according to the primary tumor location. The patients with metastasis to a2-lat in the region U, a2-lat and b1-int in the region M and b1-int in the region L demonstrated prognostic non-inferiority to the patients having n2 metastasis. Selective lymphadenectomy of subgroups of PANs in which metastases are highly suspected according to the tumor location is one of treatment strategies to advanced gastric cancer.

Introduction

Though the incidence of gastric cancer has decreased in the world, it is still one of leading causes of cancer death in many countries of different areas, such as Eastern Asia, Eastern Europe and Latin America. Hematological and peritoneal metastases in gastric cancer are life-threatening diseases, which should be treated with chemotherapy. It is probable that surgical intervention is able to improve prognosis of the patients with lymph node metastasis. Various types of lymph node dissection including D1, D2 and D3 have been tried to control lymph node metastasis in gastric cancer. Extended lymphadenectomy disappointed the expectation of survival improvement by two European randomized control studies comparing the D1 procedure with the D2 procedure (1,2), whereas another randomized trial reported in 2006 showed a significant benefit in patient survival for a D2 or D3 procedure as compared with D1 dissection, without increased operative mortality (3). It was reported that 20-30% of patients with advanced gastric cancer had metastasis to para-aortic lymph nodes (PANs) (4) and the 5-year survival of such patients reached 13 to 40% (5-7). These data facilitated to launch a randomized study on the importance of dissection of PANs. The Japanese randomized trial comparing standard D2 with D2 plus additional para-aortic lymphadenectomy for advanced gastric cancer (JCOG study 9501) was carried out between July 1995 and April 2001. Unfortunately, the JCOG study 9501 did not demonstrate any difference in survival between the two groups (8).

It remains unknown whether there is any prognostic benefit in subsets into which patients are classified according to primary tumor location or subgroup of PAN. Non-inferiority in survival of the patients with PAN metastasis to the patients having metastasis in the second-tier lymph nodes (n2) was retrospectively examined according to the subgroup of PANs and the tumor location.

Patients and methods

All patients enrolled in this study were histologically proven gastric adenocarcinoma. A total of 937 patients who were assessed equal to or higher than clinical stage II received D2 (n=715) or D3 (n=222) surgery with the grade of residual tumor, R0 and R1 (9), in Kanazawa University Hospital between April, 1973 and December, 2002. D3 operation was defined as D2 plus PAN lymphadenectomy in the present study. PAN was subgrouped into a1, a2-lat, a2-int, b1-lat, b1-int and b2 (Fig. 1). The clinical and pathological findings except for factors H and P were based on the guidelines of the

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Key words: para-aortic lymph node, extended lymphadenectomy, gastric cancer

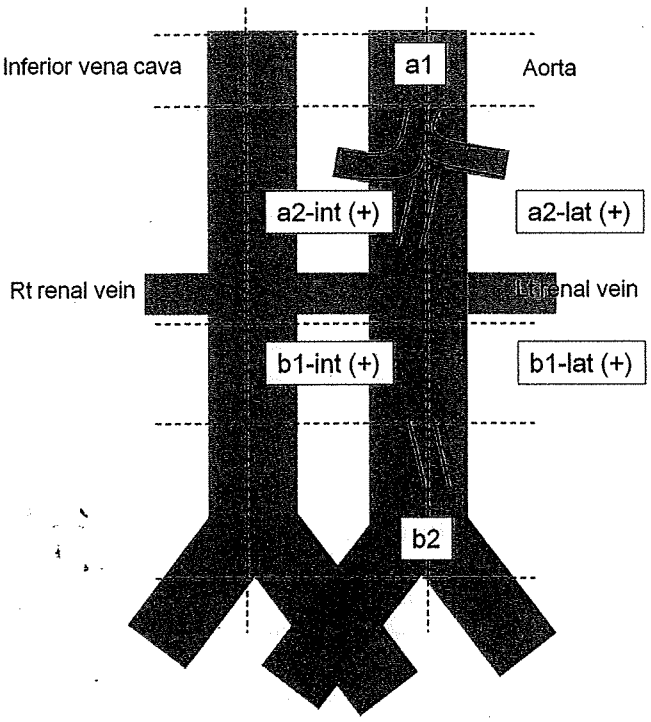

Figure 1. Subgroup of the para-aortic lymph nodes. The 'a1' lymph node is defined as node located between aortic foramen and upper margin of celiac axis in the craniocaudal direction. The 'a2' lymph node is defined as node located between upper margin of celiac axis and lower margin of left renal vein (LRV). The 'b1' lymph node is defined as node located between lower margin of LRV and upper margin of inferior mesenteric artery (IMA). The 'b2' lymph node is defined as node located between upper margin of IMA and bifurcation of abdominal aorta. The '-int' and '-lat' lymph nodes in the lateral direction are defined as nodes located between center of aorta and center of inferior vena cava and nodes located on the left side to center of aorta, respectively.

second edition of General Rules of Gastric Cancer, edited by the Japanese Research Society for Gastric Cancer (10). The factors H and P were described according to the guidelines of the first edition (11).

To examine metastatic rates in subgroups of the PANs, 222 patients receiving D3 operation were analyzed. Survival curve of patients with PAN metastasis [PAN (+) patients] was compared with that of patients with metastasis to n2 [n2 (+) patients]. A total of 131 n2 (+) patients consisted of 71 and 60 patients undergoing D2 and D3 operations, respectively, while all 55 PAN (+) patients received D3 operation. Furthermore, survival curves of n2 (+) patients and patients with metastasis to subgroup of PANs were compared by the location of primary tumor (regions U, M and L).

The significant difference in proportions between groups was determined with the Chi-square test. Patient survival was calculated with the Kaplan-Meier method and survival curves were compared with the log-rank method. Statistical significance was defined as a p-value <0.05.

Results

Patient demographics of n2 (+) patients and PAN (+) patients is summarized in Table I. The patients in the n3 group had tumor with deep invasion, peritoneal dissemination, extended level of lymph node metastasis intraoperatively estimated (sN)

Table I. Patient characteristics.

	n2 (+) (n=131)	PAN[a] (+) (n=55)	P-value
Gender			
Man/Woman	79/52	34 /21	0.98
Average age (range)	59 (19-70)	59 (18-71)	0.42
Gross type			
0	11	3	0.091
1	5	1	
2	48	12	
3	42	28	
4	14	9	
5	11	2	
Tumor location			
U/M/L	37/44/50	19/16/20	0.67
Tumor depth			
1/2/3/4	33/19/58/21	4/6/30/15	0.018
H			
0/1	130/1	53/2	0.43
P			
0/1	123/8	45/10	0.023
sN			
0/1/2/PAN[a]	14/37/68/12	0/13/15/27	<0.001
Histology			
D/Ud/Sq[b]	65/65/1	16/39/0	0.026
Surgery			
Total gastrectomy	61	41	<0.001
Distal gastrectomy	60	8	
Proximal gastrectomy	6	1	
Pancreatoduodenectomy	3	3	
Others	1	2	

PAN[a], Para-aortic lymph node; D/Ud/Sq[b], differentiated/undifferentiated/squamous.

and undifferentiated-type cancer, comparing with those in the n2 group. Distal gastrectomy in the n2 (+) group was more frequently performed than in the PAN (+) group.

Metastatic rates in subgroups of the PANs were as follows; 9.4% (20/212) in a2-lat, 6.5% (13/199) in a2-int, 7.4% (14/188) in b1-lat, 6.5% (13/200) in b1-int, 7.7% (1/13) in a1 and 57% (4/7) in b2. In the D2 group, 472 patients showed n0; 114, n1; 107, n2 and 20, n3, while 51 patients showed n0; 56, n1; 60, n2 and 55, n3, in the D3 group. The metastasis to PANs in the D2 group was diagnosed by sampling of PANs.

The survival was compared between the PAN (+) patients and the n2 (+) patients (Fig. 2). The 1- and 5-year survival rates of the n2 (+) patients were 78 and 43%, while the 1- and 5-year survival rates of the PAN (+) patients were 63 and 22%. The prognosis of the n2 (+) patients is significantly better than that of the PAN (+) patients. Then, the survival curve of the n2 (+) patients was compared with that of the patients with metastasis to each subgroup of PANs (Fig. 3). The 1- and 5-year survival rates of the patients with metastasis

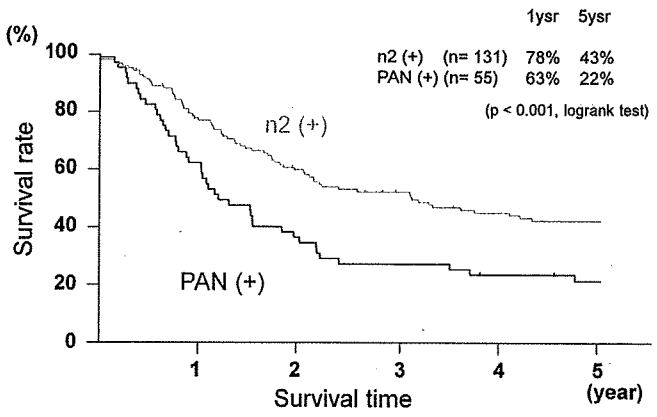

Figure 2. Survival curves of the n2 (+) group and the PAN (+) group.

in a2-lat or b1-int were 60 and 35% or 92 and 54%, respectively. There was no difference in the median survival times between the n2 (+) group and the a2-lat (+) or the b1-int (+) group, suggesting that the a2-lat or the b1-int dissection matched the D2 dissection in the prognosis. On the other hand, the 1- and 5-year survival rates of the patients with metastasis in a2-int or b1-lat were 62 and 15% or 50 and 14%, respectively, thus significantly lower than those of the patients with the n2 (+) group. These data indicated that the a2-lat and the b1-int were candidates for selective lymphadenectomy in PAN dissection.

Furthermore the importance in dissection of the a2-lat and the b1-int was investigated according to the primary tumor location. The 1- and 5-year survival rates of the n2 (+) patients were 77 and 36%, respectively, while these rates of the patients with metastasis in a2-lat were 86 and 57%, respectively, in region U (Fig. 4). There was no difference in the median survival times between the n2 (+) group and the a2-lat (+) group, suggesting that the a2-lat dissection matched the D2 dissection in the prognosis in region U. Though the survival time of the b1-int (+) group was marginally shorter than that of the n2 (+) group, no conclusion could be made because of very few patients with the b1-int (+) group. Similar analyses were carried out for the cancers of regions M and L (Figs. 5 and 6). These analyses indicated that the a2-lat and b1-int dissection in the region M and the b1-int dissection in region L matched the D2 dissection in the prognosis.

Discussion

The Japanese randomized trial for D2 plus para-aortic lymphadenectomy in advanced gastric cancer (JCOG study 9501) failed in showing prognostic benefit. However, only 8.5% of patients had pathological metastasis in PANs and the 5-year survival rate reached 18.2% (8). The positive rate of PAN-metastasis in the JCOG study was much lower than expected, while the survival rate of their study is consistent with ones of previous studies. The 5-year survival rate of patients with liver metastases or peritoneal dissemination is as low as <5%, much worse than that of patients with PAN metastasis. These results supported that dissection of PANs is likely to improve the survival of the patients with PAN metastasis other than liver metastases or peritoneal dissemination. Taken together, the JCOG study 9501 could not prove superiority in the prognosis of patients with PAN-metastasis receiving PAN dissection to D2 dissection, but only

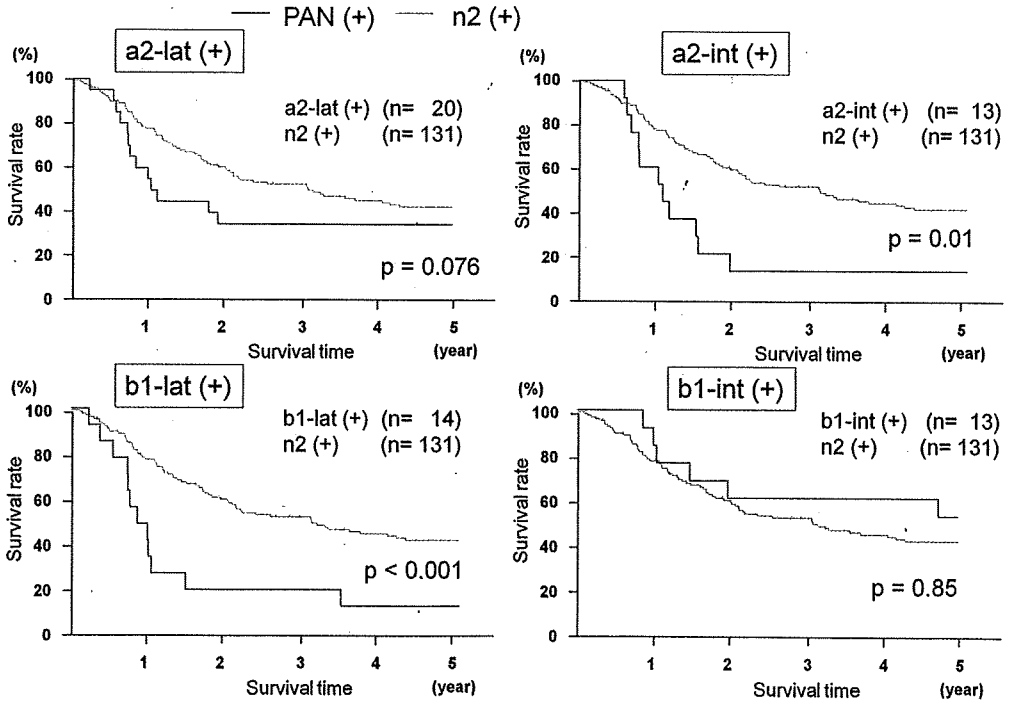

Figure 3. Survival curves of the n2 (+) group and the PAN (+) group according to the subgroup of PANs. There was no difference in the median survival times between the n2 (+) group and the a2-lat (+) or the b1-int (+) group, while the survival time of the n2 (+) group was significantly longer than that of the a2-int (+) or b1-lat (+) group.

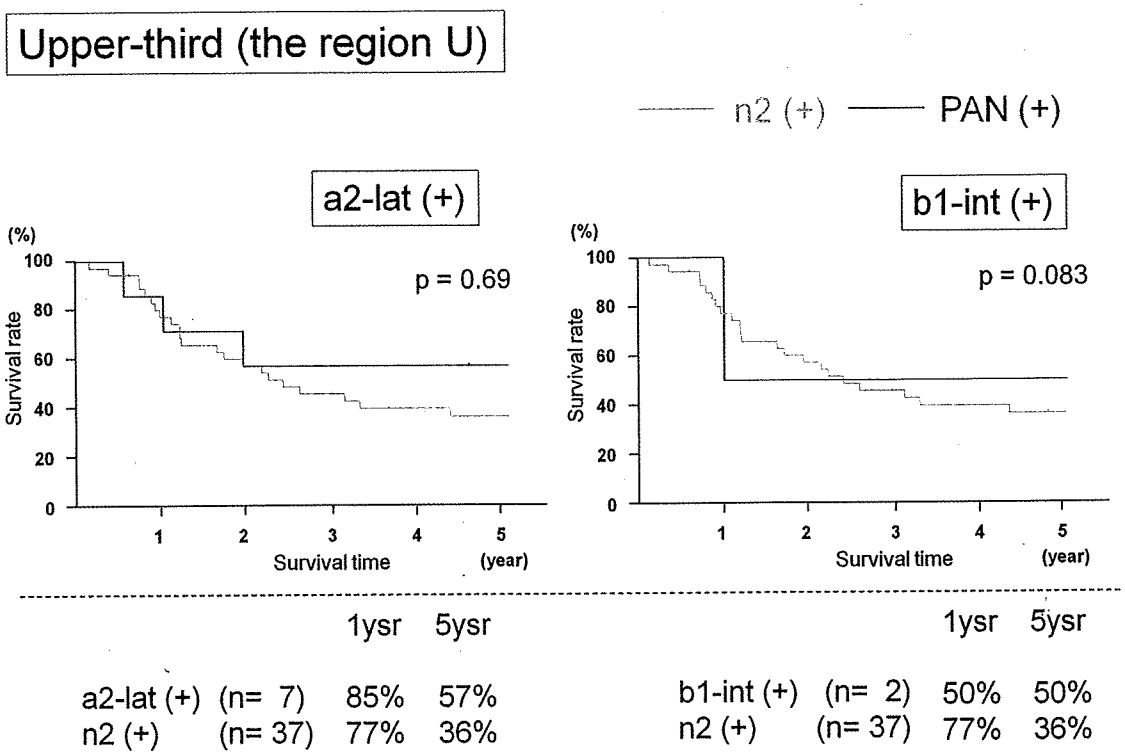

Figure 4. Survival curves of the n2 (+) group and the a2-lat (+) or the b1-int (+) group of the upper-third gastric cancer. There was no difference in the median survival times between the n2 (+) group and the a2-lat (+) group. The survival time of the b1-int (+) group was marginally shorter than that of the n2 (+) group.

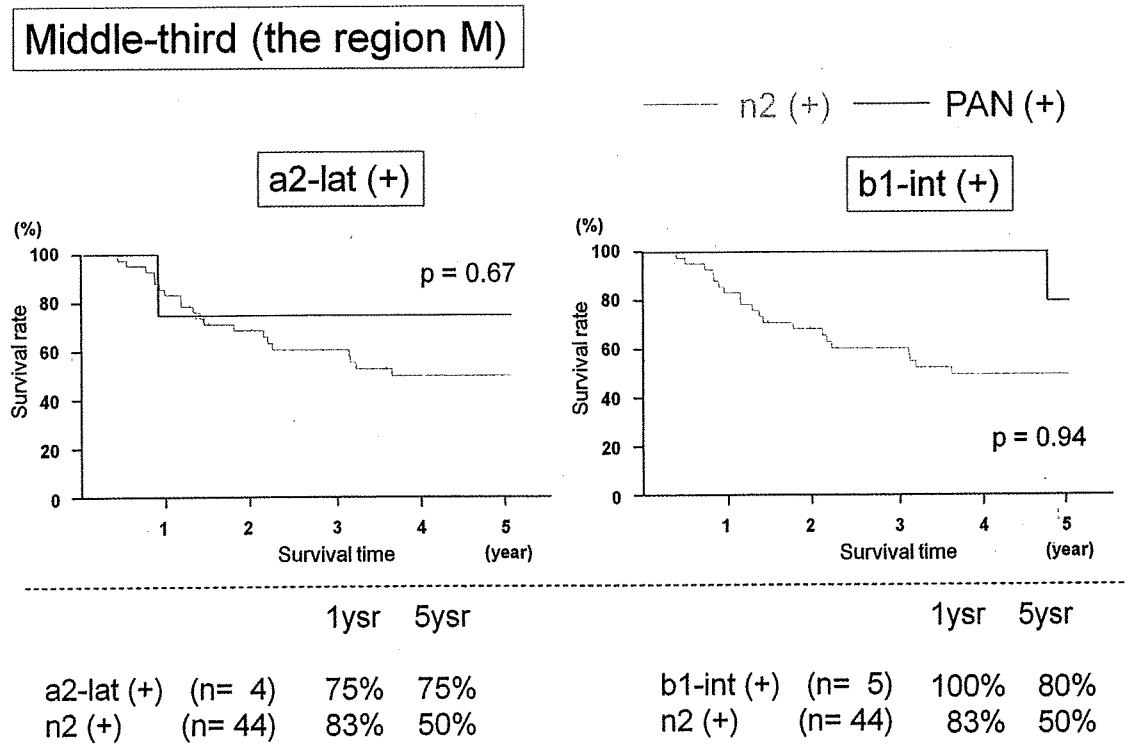

Figure 5. Survival curves of the n2 (+) group and the a2-lat (+) or the b1-int (+) group of the middle-third gastric cancer. There was no difference in the median survival times between the n2 (+) group and the a2-lat (+) or the b1-int (+) group in the middle-third stomach.

disclose difficulty in selection of real candidates for PAN dissection, that is, patients with PAN metastasis.

There are many studies describing risk factors pre-operatively predicting PAN metastases. Macroscopic N stage (N2 to N4) and tumor size (≥5 cm in diameter) were associated with PAN metastasis in the JCOG study 9501 (12). But only 20% of patients were N2 to N4 and 13% having tumor with >5 cm had actual PAN metastasis in this study. Thus, these

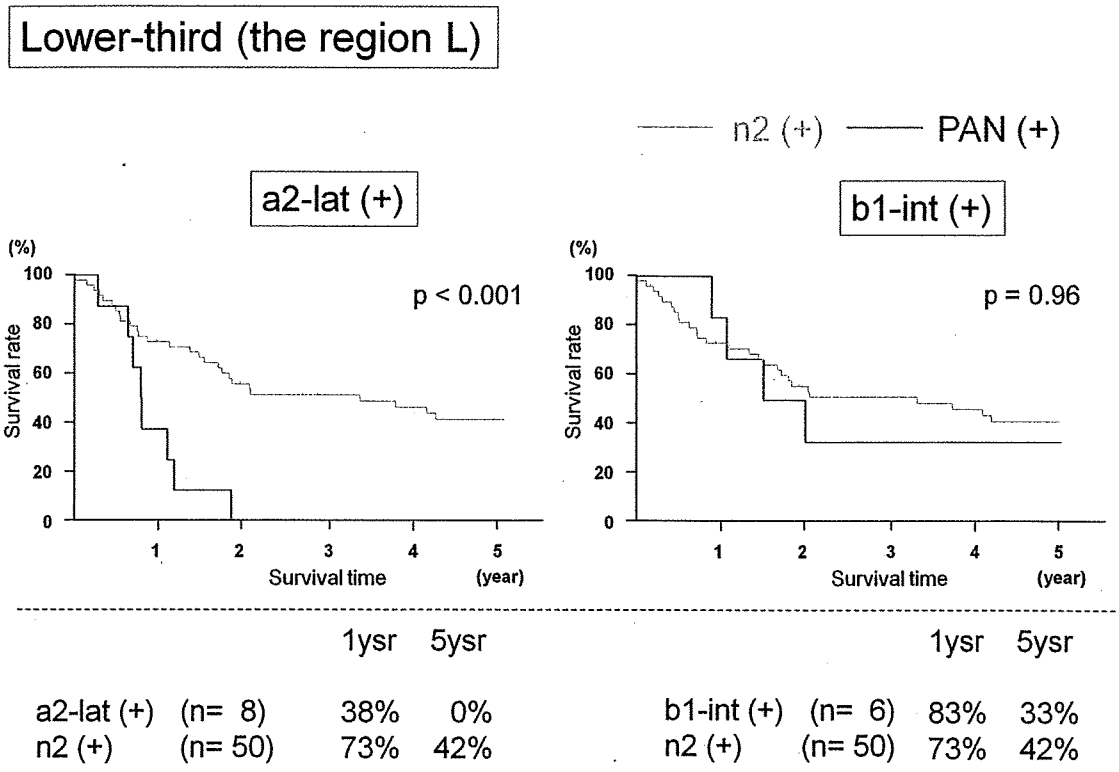

Figure 6. Survival curves of the n2 (+) group and the a2-lat (+) or the b1-int (+) group of the lower-third gastric cancer. There was no difference in the median survival times between the n2 (+) group and the b1-int (+) group, but the survival time of the n2 (+) group was significantly longer than that of the a2-lat (+) group.

predictive factors may be necessary conditions, but not sufficient ones. Other risk factors included depth of tumor invasion (13), total number of metastatic lymph nodes (14) and lymphatic metastases to the stations #7 and #8 (15). It is difficult, however, intraoperatively to prove whether patients fit these risk factors. On the other hand, several factors indicating good prognosis of the patients with PAN metastasis, including number of metastatic PANs (<3 or 4) (6,16) and total number of metastatic lymph nodes (<11) (16). But, again, it is hard to obtain such information before or during operation. These facts explain the difficulty in the patient selection for PAN dissection. We have performed PAN dissection to patients of gastric cancer without severe serosal invasion, but with lymph node metastasis to No. 3, 7, or 9 and showing H0, P0, M0 and Cy0 (17).

Lymphatic flow from the stomach drains into the perigastric nodes, next the node around the celiac axis or its main branches and finally into the PANs before joining the cisterna chyli as a systemic circulation. The main lymphatic route from the stomach to the PAN differs between the primary tumor locations. Research on lymphatic routes from perigastric nodes to PANs have been carried out using various tracers such as dyes, charcoals and radioisotopes. Yonemura (5) classified these lymphatic flows into 4 routes: i) Left subdiaphragmatic pedicle; ii) Celiac pedicle; iii) Superior mesenteric pedicle; and iv) Retropancreatic pedicle. The left subdiaphragmatic pedicle is characteristic of upper-third gastric cancer, especially, cardia cancer. This lymphatic flow reaches PANs next to the left side of aorta, that is, a2-lat. The lymphatic flow of the upper-third of the stomach drains into a2-lat not only along this left subphrenic artery, but also along the left gastric artery and the celiac axis. In this sense, a2-lat is likely to be important nodes for the upper-third gastric cancer. On the other hand, both the superior mesenteric and retropancreatic pedicles are characteristic of the lower-third gastric cancer. These lymphatic channels finally connect to the PANs next to the right side of aorta including a2-int and b1-int. The celiac pedicle is supposed to be a route common to whole area of the stomach. Metastasis to the PANs is strongly related with these lymphatic routes to the PANs. It is reported that PAN metastasis in the upper-third gastric cancer frequently occurs on the left side of aorta, while PAN metastasis in the lower-third gastric cancer tends to occur on the right side of aorta (14,18).

Nishi reported that the prognosis of a2-lat metastasis in the upper-third gastric cancer is very close to the one of n2 metastasis (18). Sasako *et al* (19) summarized a nationwide questionnaire asking location tendency in subgroups of PANs in the patients who received para-aortic lymphadenectomy and survived longer than 5 years after surgery. This study indicated that the most and the second most of the survivors in the 61 patients had metastases in a2-lat (n=31) and b1-int (n=11), respectively. Most of the survivors according to the sub-groups by the tumor location were registered at the a2-lat in the upper third and the middle third cancer (n=16 and n=7, respectively) and b1-int in the lower third cancer (n=7). These data strongly support our conception of selective lymph-adenectomy of subgroups of PANs according to the tumor location.

Sano *et al* (4) reported that there was no significant difference in postoperative morbidity or mortality between D2 and D3, but in the D3 group volume of blood loss was high

and the hospitalization was longer compared to the D2 group in the JCOG study 9501. Another randomized study conducted by Yonemura *et al* (20) demonstrated that both morbidity and mortality in the D3 group were significantly higher than those in the D2 group. If an advantage in elongation of survival would be achieved by the D3 operation, a little higher post-operative morbidity may be allowable, but two major randomized studies have failed in demonstrating survival benefits by PAN dissection (8,21). In this situation, whole lymphadenectomy of the PANs should be avoided for all the patients with advanced gastric cancer. It is important to balance survival advantage with postoperative morbidities in the treatment of these patients.

Acknowledgements

The authors are grateful to Koichi Miwa, ex-professor of Kanazawa University and Yutaka Yonemura for their performance of surgery.

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早期胃癌に対する胃横断手術（胃分節切除術）

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■手術を行うにあたってのポイント

われわれはESD適応外の早期胃癌に対してlymphatic mappingを行い，sentinel nodeに転移がない場合に，リンパ節郭清範囲を縮小するとともに，各種の機能温存手術（胃局所切除術，胃横断切除術，噴門側胃切除術，小範囲幽門側胃切除術）（表1）を施行している[1]。

本稿では，このうち胃横断切除術（胃分節切除術）を概説するが，対象はM（ML）領域

表1　lymphatic basin dissectionに基づいた縮小手術（機能温存根治手術）

占拠部位	流域（basin）		術式
	数	部位	
M, L	1	右胃大網動脈	胃局所切除術
U, M	2	左胃動脈 後胃動脈	噴門側胃切除術
M, L	2	左胃動脈 右胃大網動脈	胃横断切除術
L	2	右胃動脈 右胃大網動脈	小範囲幽門側胃切除術

（文献1）より転載）

図1　胃横断切除術