Table 2. Clinicopathologic characteristics of patients

	Number of patients
Stage	
0	5
I A/B	41
II A/B	49
III A/B/C	5
IV	1
Nodal status	
pN0	60
pN1	35
pN2	2
pN3	4
Histopathologic type	
Invasive ductal carcinoma	87
Neuroendocrine carcinoma	1
Matrix producing carcinoma	1
Mucinous carcinoma	2
Apocrine carcinoma	1
Invasive lobular carcinoma	4
Ductal carcinoma *in situ*	5

SDS (PAG Mini; Daiichi Pure Chemicals). After electrotransfer to Immobilon-FL polyvinylidene difluoride membranes (Millipore), the membrane was blocked with skim milk (BD Bioscience) for 1 h at room temperature. The primary antibody, anti-CK19 (A53-B/A2; Santa Cruz Biotechnology), was diluted 1:500 with TBS-Tween 20 (TBS-T) solution, and the membrane was incubated at 4°C overnight with anti-CK19 antibody. The membrane was then washed with TBS-T and incubated with a secondary antibody conjugated with horseradish peroxidase, which was diluted 1:2,000 with TBS-T. After washing the membrane twice with TBS-T, CK19-CK19 antibody complex was visualized using the ECL-Advance detection kit (GE Healthcare). The intensity of the signal in each band was evaluated by LumiAnalyst (Roche). CK19 protein concentration was determined based on a standard curve that was obtained by measuring known quantities of CK19 protein (Biodesign) of 0.15, 0.075, 0.038, and 0.018 ng/μL.

A cutoff value for CK19 protein expression between histopathologically positive and negative lymph nodes was determined by Western blot analysis of 37 histopathologically negative lymph nodes from 16 pN0 patients, 54 histopathologically negative lymph nodes from 17 pN1-3 patients, and 22 histopathologically positive lymph nodes from 12 patients (Figs. 3A and 5A). The cutoff value was determined by statistical analysis of the amount of CK19 measured by Western blot analysis of 37 histopathologically negative lymph nodes from 16 pN0 patients.

Results

Selection of the mRNA marker. We evaluated mRNAs for CK19, CEA, FOXA1, SPDEF, MUC1, and MGB1 using 11 histopathologically positive and 15 negative lymph nodes from 26 patients. The absolute mRNA expression levels of CEA and MGB1 in metastatic lymph nodes were not as high as expected, whereas the absolute expression levels of MUC1 mRNA in nonmetastatic lymph nodes was relatively high. For these reasons, CEA, MGB1, and MUC1 mRNAs were not selected for the OSNA assay.

The expression levels of CK19, FOXA1, and SPDEF mRNAs differed between histopathologically positive and negative lymph nodes. However, the lower limits of the expression levels of FOXA1 and SPDEF mRNAs in histopathologically positive lymph nodes were 4 to 30 times less than that of CK19 mRNA (Fig. 6). On the other hand, the detection limit of the OSNA assay was nearly equivalent to 32 threshold cycles of the RT-PCR system. An assay system should detect the upper limit of the expression levels of an mRNA marker in histopathologically negative lymph nodes. The upper limits of the threshold cycle of FOXA1 and SPDEF mRNAs were about 35 and 32,

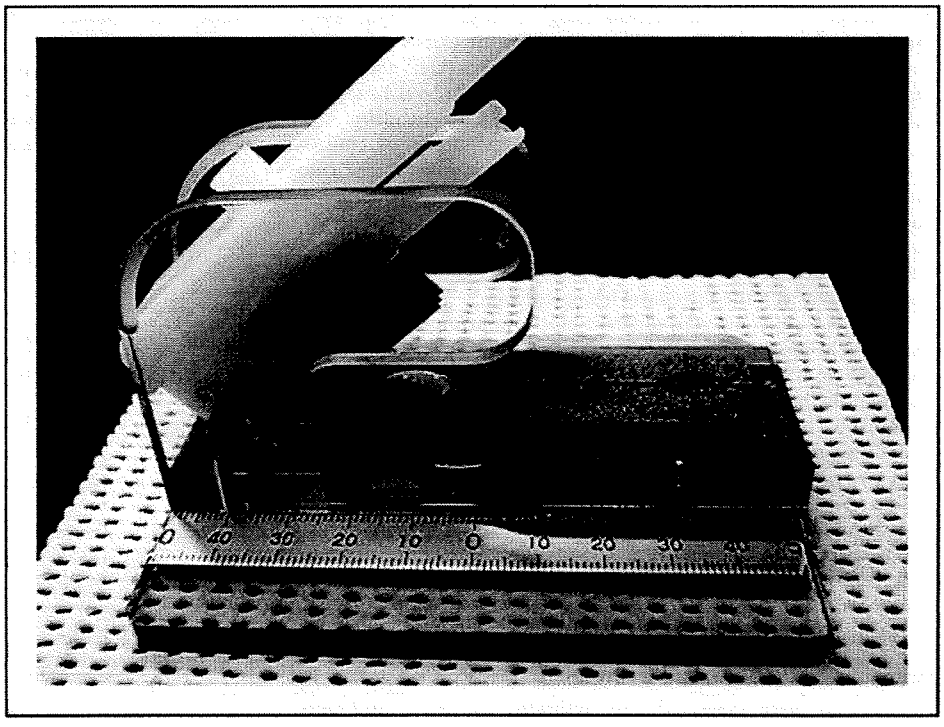

Fig. 4. Lymph node cutting device.

respectively. For these reasons, we determined CK19 mRNA to be the best marker for the OSNA assay.

OSNA assay. As shown in Fig. 1B, an inverse correlation between the threshold time in the RT-LAMP step and CK19 mRNA concentration was observed in a range of CK19 mRNA concentrations of 2.5×10^2 to 2.5×10^6 copies/μL, and both curves overlapped completely in the presence and absence of the lymph node lysate; the correlation coefficient value in both cases was 0.99. This result indicates that factors that may be present in lymph node lysates do not interfere with the OSNA assay.

Effect of lymph node size on the OSNA assay. The threshold time of the OSNA assay with 2.5×10^3 and 2.5×10^5 copies/μL of CK19 mRNA in a lysate obtained from 130 mg of lymph node was 10.9 and 9.6 min, respectively. The threshold time with 2.5×10^3 copies/μL of CK19 mRNA in a lysate obtained from a lymph node of 214, 354, and 428 mg was 10.7, 10.9, and 10.9 min, respectively, whereas the time with 2.5×10^5 copies/μL of CK19 mRNA in a lysate obtained from a lymph node of 214, 354, and 428 mg was 9.6, 9.7, and 9.7 min, respectively. The threshold times with 2.5×10^3 and 2.5×10^5 copies/μL of human CK19 mRNA in the lysates obtained from lymph nodes of 130, 214, 354, and 428 mg were within an acceptable error range. The results indicate that the OSNA assay is not influenced by lymph node size.

Amplification of genomic DNA by the OSNA assay. To exclude the possibility of genomic DNA amplification in the OSNA assay, we examined the OSNA assay using genomic DNA purified from lymph nodes. Genomic DNA was not amplified from either metastatic or nonmetastatic lymph nodes. The results indicate that the OSNA assay amplifies only CK19 mRNA.

Cutoff values. A cutoff value for the OSNA assay between histopathologically positive and negative lymph nodes was determined by the logarithmic normal distribution of CK19 mRNA copy numbers from 42 lymph nodes from pN0 patients. The average value of CK19 mRNA expression +3 SD was 2.5×10^2 copies/μL. Based on this analysis, we set the cutoff value at 2.5×10^2 copies/μL, which represents the upper limit of the copy numbers in the histopathologically negative lymph nodes from pN0 patients (Fig. 7A).

To validate the cutoff value, we examined CK19 mRNA expression in 42 histopathologically negative lymph nodes from 16 pN1-3 patients. Only one of these 42 cases showed $>2.5 \times 10^2$ copies/μL CK19 mRNA (Fig. 7B). This lymph node showed 3×10^3 copies/μL of CK19 mRNA. This suggested that micrometastatic foci in block a or c (Fig. 3A) of the lymph node were included in the sample. On the other hand, CK19 mRNA expression in all 24 pathologically positive lymph nodes from 10 patients exceeded the cutoff value (Fig. 7C).

To obtain a cutoff value for CK19 mRNA expression between macrometastasis with metastatic foci $>2^3$ mm^3 and micrometastasis, we compared CK19 mRNA expression in serial sections of a lymph node with an area of metastatic foci and roughly estimated macrometastasis to be $>5 \times 10^3$ copies/μL, which is the lowest value of CK19 mRNA expression found in metastatic foci of 2^3 mm^3 (Table 1).

Accordingly, for the OSNA assay, we defined macrometastasis (++) as $>5 \times 10^3$ copies/μL of CK19 mRNA, micrometastasis (+) as 2.5×10^2 to 5×10^3 copies/μL, and nonmetastasis (-) as $<2.5 \times 10^2$ copies/μL.

Clinical study. All OSNA assays were carried out during surgery and were completed within 30 min. H&E and CK19 immunohistochemistry were used in the histopathologic examination.

Isolated tumor cells (ITC) are widely used as one of indicators in a nomogram-aiding treatment decisions. In the American Society of Clinical Oncology guidelines (10), ITCs are described as having unknown clinical significance, and there are insufficient data to recommend appropriate treatment, including axillary lymph node dissection. For this reason, we viewed ITC as negative.

Table 3 shows the results of CK19 immunohistochemistry in all samples with the H&E results given in parenthesis. H&E-based histopathology failed to detect 1 of 40 cases of macrometastasis and 3 of 5 cases of micrometastasis. Overall, the sensitivity of H&E-based histopathology was 91.1% based on the results of CK19 immunohistochemistry–based histopathology. The sensitivities of the one- and two-level CK19

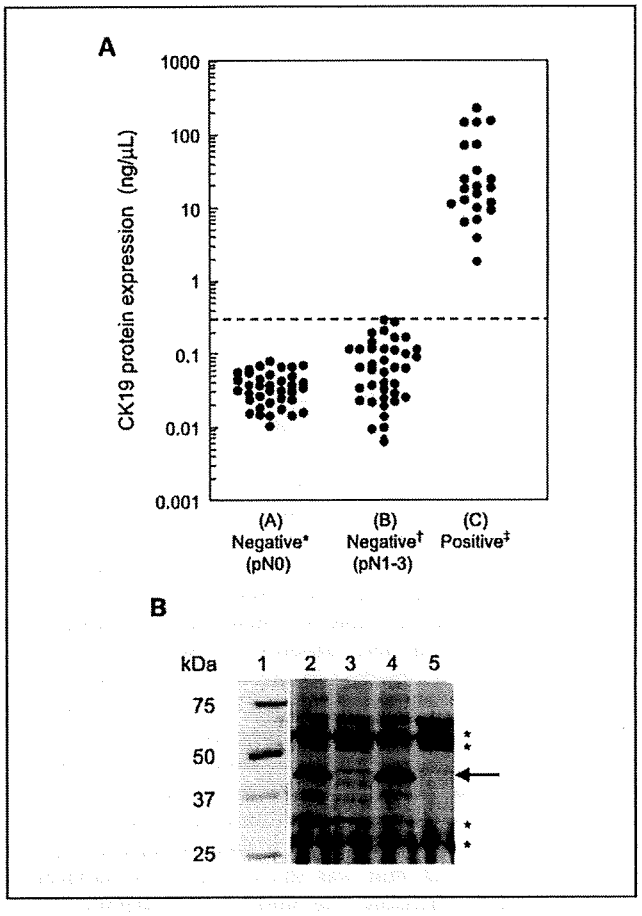

Fig. 5. CK19 protein expression in lymph node lysates. *A*, CK19 protein expression in histopathologically positive and negative lymph node lysates. *, histopathologically negative lymph nodes dissected from pN0 patients. †, histopathologically negative lymph nodes dissected from pN1-3 patients. ‡, histopathologically positive lymph nodes. The CK19 protein expression was determined by Western blot analysis (see Materials and Methods). Broken line, cutoff line between micrometastasis and nonmetastasis. The protein concentration of representative lymph node lysates used in this experiment was within the range of 8.7 to 11.6 μg/μL. *B*, a representative example of Western blot analysis of CK19 protein in lymph node lysates. Lane 1, molecular weight markers stained with Coomassie brilliant blue. Lanes 2 and 4, histopathologically positive lymph node lysate. Lanes 3 and 5, histopathologically negative lymph node lysate. Arrow, CK19 protein. *, nonspecific bands. The vertical scale shows molecular weights.

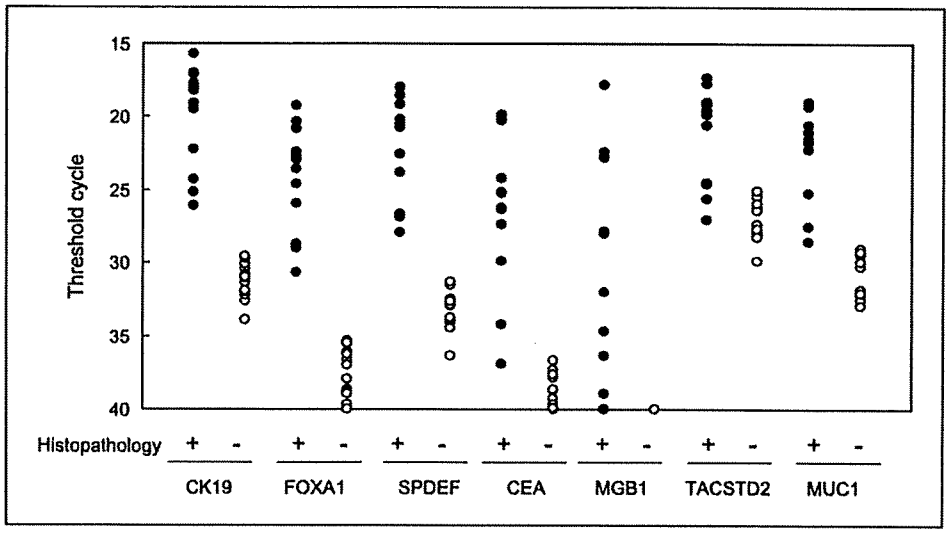

Fig. 6. Expression of mRNA markers in histopathologically positive and negative lymph nodes. The selected mRNA markers (CK19, FOXA1, SPDEF, CEA, MGB1, TACSTD2, and MUC1) were evaluated by QRT-PCR using 11 histopathologically positive (●) and 15 negative (○) lymph nodes from 26 patients.

immunohistochemistry-based histopathologies were 86.7% and 91.1%, respectively, based on the results of three-level CK19 immunohistochemistry-based histopathology (Supplementary Table S3).

The concordance rate between the OSNA assay and the CK19 immunohistochemistry-based three-level histopathology for 325 lymph nodes was 98.2%. The concordance rate for SLNs was 96.4%.

No false positive results were found with the OSNA assay of 144 histopathologically negative lymph nodes from 60 pN0 patients, in which neither micrometastasis nor macrometastasis was observed for serial sections from blocks b′ and d′ (Fig. 3C). Furthermore, the OSNA assay judged 13 ITC cases as negative. These results are summarized in Table 3.

Discordant cases. Six discordant cases were observed between the OSNA assay and CK19 immunohistochemistry-based histopathologic examination (Table 4). Four cases were micrometastasis according to the OSNA assay and were negative according to the CK19 immunohistochemistry-based histopathology. In any case, CK19 mRNA expression of $>10^3$ copies/μL was observed (Table 4). These four discordant cases came from pN1 and pN2 patients. In two of four cases, micrometastasis was observed in the multilevel examinations of blocks b′ and d′. On the other hand, two remaining cases (Table 4, *samples* 5 and 6) were negative according to the OSNA assay and micrometastasis according to the three-level histopathology. Samples 5 and 6 showed metastatic foci of 0.3 and 0.4 mm in the long axis, which were observed on surfaces i′ and ii′, respectively. When i′ and ii′ were histopathologically examined, about 0.2 mm was shaved from the surfaces of blocks b′ and d′. Therefore, the amount of metastatic foci in blocks a′ and c′ that were used for the OSNA assay (i.e., a′ and c′) could not be quantified.

We also measured the amount of CK19 protein by Western blot analysis of the lysate used in each discordance case. A cutoff value for CK19 protein expression between metastasis positive and negative lymph node was determined by the distribution of CK19 protein expression in 37 histopathologically negative lymph nodes from 16 pN0 patients. The distribution could be described as a logarithmic normal distribution. The statistical analysis indicated that an average value +3 SD was 0.13 ng/μL. Based on this analysis, the cutoff value was determined to be 0.3 ng/μL, which is the upper limit of the CK19 protein expression in 54 histopathologically negative lymph nodes from pN1-3 patients (Fig. 5A). Furthermore, CK19 protein expression in 22 histopathologically positive lymph nodes from 10 patients contained protein levels over the cutoff value.

Based on this cutoff value, we measured the amount of CK19 protein using quantitative Western blot analysis of the lysate for the OSNA assay of samples 1, 2, and 5. As described in Table 4, samples 1 and 2 showed an amount of CK19 protein expression equivalent to micrometastasis. Sample 5 exhibited no CK19 protein expression.

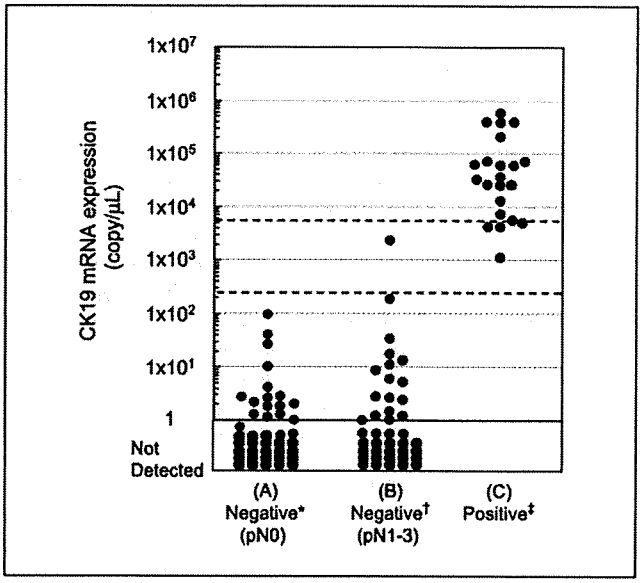

Fig. 7. CK19 mRNA expression in the OSNA assay carried out under the protocol A (Fig. 3 A). *, histopathologically negative lymph nodes dissected from pN0 patients. †, histopathologically negative lymph nodes dissected from pN1-3 patients. ‡, histopathologically positive lymph nodes. Top broken line, cutoff between macrometastasis and micrometastasis. Bottom broken line, cutoff between micrometastasis and nonmetastasis.

Table 3. Comparison of the OSNA assay with the histopathologic examination

Number of lymph nodes	OSNA*	Histopathologic examination †			
		Macrometastasis	Micrometastasis	ITC	Negative ‡
325 from 101 patients	++	34 (34)	0 (0)	0 (0)	0 (0)
	+	6 (5)	3 (1)	0 (0)	4 (0)
	-	0 (0)	2 (2)	13 (11)	263 (0)
81 SLNs from 49 patients	++	11 (11)	0 (0)	0 (0)	0 (0)
	+	1 (0)	2 (1)	0 (0)	1 (0)
	-	0 (0)	2 (2)	3 (2)	61 (0)
144 from 60 pN0 patients	++	0 (0)	0 (0)	0 (0)	0 (0)
	+	0 (0)	0 (0)	0 (0)	0 (0)
	-	0 (0)	0 (0)	3 (3)	141 (0)

*In the OSNA assay, (++), (+), and (-) show $>5 \times 10^3$, 2.5×10^2 to 5×10^3, and $<2.5 \times 10^2$ copies/μL of CK19 mRNA, respectively.
† Histopathologic examinations with H&E and CK19 immunohistochemistry were carried out in all samples. In cases where metastatic foci were observed in the histopathologic examination by either H&E or CK19 immunohistochemistry, the sample was categorized as macrometastasis, micrometastasis, or ITC. The results of the three-level CK19 immunohistochemistry–based histopathologic examination were determined by the consensus of three third-party pathologists. The number of lymph nodes judged to be positive based on the three-level H&E-based histopathologic examination is shown in parenthesis.
‡ No cancer cells were observed in either the immunohistochemistry- or H&E-based histopathologic examinations.

Discussion

The detection of lymph node metastasis by RT-PCR (37–40) and by QRT-PCR (12, 19–25) has been studied previously. CK19 mRNA has been described as having the highest sensitivity at nearly 90%. However, there are drawbacks using CK19 mRNA due to the concomitant amplification of pseudogenes in genomic DNA that lead to false positive results. For this reason, a combination of two or three markers has been used.

We evaluated 45 potential mRNAs and finally selected CK19 mRNA as the best marker for the OSNA assay. To use CK19 mRNA as a marker, we designed RT-LAMP primers that do not amplify the known CK19 pseudogenes (see Materials and Methods). In addition, the lymph node solubilization step in the OSNA assay was carried out at pH 3.5. At this pH, almost all genomic DNA precipitates out. Even when the sample still contained genomic DNA, DNA amplification is unlikely to occur in the OSNA assay because the RT-LAMP step is carried out at 65°C, a temperature at which genomic DNA typically does not denature. Indeed, purified genomic DNA from metastatic lymph nodes was not amplified in the OSNA assay.

In the present clinical study assessing 325 lymph nodes from 101 patients, an overall concordance rate between the OSNA assay and the CK19 immunohistochemistry–based three-level histopathology was 98.2%. A concordance rate of 96.4% was obtained with 81 SLNs from 49 patients. On the other hand, 1 of 40 macrometastatic cases and 2 of 5 micrometastatic lymph nodes, as defined by CK19 immunohistochemistry–based histopathology, were missed by H&E-based histopathology. Therefore, the sensitivity of three-level H&E-based histopathology was 93.3% based on the three-level CK19 immunohistochemistry–based histopathology. Furthermore, the sensitivity of one- and two-level CK19 immunohistochemistry–based histopathologies is 86.7% and 91.1%, respectively, based on the three-level CK19 immunohistochemistry–based histopathology (Supplementary Table S3). These results indicate that the performance of the OSNA assay is better than that of one- and two-level CK19 immunohistochemistry–based histopathologies and almost equivalent to three-level CK19 immunohistochemistry–based histopathology.

Chu and Wiess (41) reported that 98.2% of primary breast cancer tissues exhibit CK19 protein expression. Two of our authors (Tsujimoto and Tsuda) also examined the CK19 immunohistochemistry–based histopathologic examination of primary breast cancer tissues and found that there was no CK19 protein expression in 20 (2.2%) of 896 cases examined. However, low CK19 mRNA expression in lymph nodes has not been reported.

Table 4. Discordant cases between the OSNA assay and three-level histopathologic examination

Discordant case	CK19 mRNA (copy/μL)	CK19 protein (ng/μL)*	Histopathologic examination †	Nodal status
1	9.6×10^2	1.4	Negative	pN2
2	1.5×10^3	1.6	Negative	pN1
3	2.3×10^3	Not tested	Negative	pN1
4	3.6×10^3	Not tested	Negative	pN1
5	ND	0.04	Micrometastasis	pN1
6	ND	Not tested	Micrometastasis	pN1

Abbreviation: ND, not detected.
*Amount of CK19 protein was determined by Western blot analysis (see Materials and Methods).
† Results of CK19 immunohistochemistry–based histopathologic examination of the sections i′, ii′, and iii′ of protocol C (Fig. 3C).

In the present clinical study, CK19 immunohistochemistry-based histopathologic examination of two lymph nodes from one patient revealed metastatic foci smaller than macrometastasis despite the presence of macrometastasis defined by H&E-based histopathologic examination; the histologic type of this primary tumor was neuroendocrine carcinoma. These samples unequivocally had low CK19 expression. The OSNA assay of these samples was positive, indicating that CK19 mRNA was expressed despite the low protein expression found by CK19 immunohistochemistry.

In QRT-PCR studies in which several mRNA markers have been used (12, 19, 24, 25), the ability to quantitatively discriminate macrometastasis from micrometastasis has not been discussed. In the OSNA assay, the solubilization of a lymph node is followed by mRNA amplification. Regardless of the size of the lymph node, a constant portion of lysate is transferred to an RT-LAMP reaction. This indicates that the OSNA assay can, in principle, discriminate macrometastasis from micrometastasis and micrometastasis from nonmetastasis when the cutoff values of CK19 mRNA are properly set. To ensure the quantitative capacity of the OSNA assay, endogenous factors should not interfere with the RT-LAMP reaction. We showed that the presence of a lysate obtained from a lymph node (130-600 mg) did not interfere with the OSNA assay (Fig. 1B). A 600-mg sample of lymph node is equivalent to that having a diameter of about 1 cm. The presence of fat or the reagents that were used to identify SLNs, e.g., radioisotope colloid and blue dyes, did not also interfere with the reaction (data not shown).

We observed no false positive result in the OSNA assay from 144 histopathologically negative lymph nodes (60 pN0 patients). In the statistical analysis of the copy numbers of CK19 mRNA in these 144 lymph nodes, the average value of CK19 mRNA expression +3 SD was $<2.5 \times 10^2$ copies/μL, indicating that the probability of negative lymph nodes showing $>2.5 \times 10^2$ copies/μL is low in the OSNA assay. In the OSNA assay, all 13 ITC cases were judged as nonmetastasis (Table 3).

Based on the serial sectioning experiment (Table 1), the average copy numbers equivalent to 0.2^3, 0.3^3, and 0.4^3 mm^3 can be calculated to be 3.9×10^1, 1.3×10^2, and 3.1×10^2 copies/μL, respectively. Therefore, the cutoff value of 2.5×10^2 copies/μL in the OSNA assay can theoretically detect metastatic foci of 0.3^3 to 0.4^3 mm^3.

The OSNA assay identified 34 cases of macrometastasis out of 40 macrometastatic lymph nodes defined by the permanent three-level CK19 immunohistochemistry-based histopathology. The concordance rate was 85.0%. The remaining six cases were identified as micrometastasis. This is the first example of a molecular biological method with the potential to quantify the size of metastatic foci in a lymph node.

Six discordant cases were observed between the OSNA assay and CK19 immunohistochemistry-based histopathologic examination (Table 4). The quantitative Western blot analysis of the discordant cases (samples 1 and 2) clearly showed the presence of an amount of CK19 protein equivalent to micrometastasis. Although the possible presence of benign epithelial cells such as glandular inclusions in the lymph nodes cannot be eliminated, the results may be better explained by the presence of metastatic foci in the lymph nodes in light of the results of the specificity study and the amount of CK19 protein expression. Two other cases (Table 4, *samples 5* and *6*) were negative according to the OSNA assay, but were judged positive for micrometastasis according to three-level histopathology. These two cases showed metastatic foci of 0.3 and 0.4 mm. Therefore, the amount of metastatic foci in blocks a′ and c′ used for the OSNA assay cannot be estimated exactly. Indeed, in sample 5, the quantitative Western blot analysis of CK19 protein showed no expression of CK19 protein (Table 4).

The results of the clinical study indicate that using one-half of a lymph node in the OSNA assay gave nearly the same results as the three-level histopathology. It became clear in the clinical study conducted at six facilities that the OSNA assay is rapid enough to be done during surgery. Furthermore, the assay would be convenient and objective compared with the intraoperative immunohistochemistry-based histopathologic examination, which is usually done by an experienced pathologist (42, 43).

Acknowledgments

We thank Dr. T. Notomi (Eiken Chemical, Japan) for providing the CK19 cDNA, Dr. Masashi Takeda (National Hospital Organization Osaka National Hospital), Dr. Kenichi Wakasa (Osaka City University Medical School), and Dr. Tsuyoshi Okino (Osaka Sailor Hospital) for conducting the histopathology as third-party pathologists, and Dr. Satoshi Teramukai (Kyoto University) for managing the clinical information. We also thank the staff of the clinical and pathologic laboratories at each facility for their support. Thanks also go to Yoshihito Yamamoto, Yasumasa Akai, Katsuhito Matsumoto, Masahiro Nishida, Dr. Junyi Ding, Dr. Hideki Takata, and Kayo Hiyama for supporting the construction of the OSNA assay system. Finally, we express special thanks to Dr. Tameo Iwasaki, Sysmex Corporation, for his helpful advice and encouragement.

References

1. Donegan WL. Tumor-related prognostic factors for breast cancer. CA Cancer J Clin 1997;47:28–51.
2. van Diest PJ, Peterse HL, Borgstein PJ, Hoekstra O, Meijer CJ. Pathological investigation of sentinel lymph nodes. Eur J Nucl Med 1999;26:S43–9.
3. Weaver DL, Krag DN, Ashikaga T, Harlow SP, O'Connell M. Pathologic analysis of sentinel and nonsentinel lymph nodes in breast carcinoma: a multicenter study. Cancer 2000;88:1099–107.
4. Sabel MS, Zhang P, Barnwell JM, Winston JS, Hurd TC, Edge SB. Accuracy of sentinel node biopsy in predicting nodal status in patients with breast carcinoma. J Surg Oncol 2001;77:243–6.
5. Stitzenberg KB, Calvo BF, Iacocca MV, et al. Cytokeratin immunohistochemical validation of the sentinel node hypothesis in patients with breast cancer. Am J Clin Pathol 2002;117:729–37.
6. Luini A, Gatti G, Ballardini B, et al. Development of axillary surgery in breast cancer. Ann Oncol 2005;16:259–62.
7. Cote RJ, Peterson HF, Chaiwun B, et al. Role of immunohistochemical detection of lymph-node metastases in management of breast cancer. International Breast Cancer Study Group. Lancet 1999;354:896–900.
8. Van Diest PJ, Torrenga H, Borgstein PJ, et al. Reliability of intraoperative frozen section and imprint cytological investigation of sentinel lymph nodes in breast cancer. Histopathology 1999;35:14–8.
9. Torrenga H, Rahusen FD, Meijer S, Borgstein PJ, van Diest PJ. Sentinel node investigation in breast cancer: detailed analysis of the yield from step sectioning and immunohistochemistry. J Clin Pathol 2001;54:550–2.
10. Lyman GH, Giuliano AE, Somerfield MR, et al. American Society of Clinical Oncology guideline recommendations for sentinel lymph node biopsy in early-stage breast cancer. J Clin Oncol 2005;23:7703–20.
11. Tanis PJ, Boom RP, Koops HS, et al. Frozen section investigation of the sentinel node in malignant melanoma and breast cancer. Ann Surg Oncol 2001;8:222–6.
12. Hughes SJ, Xi L, Raja S, et al. A rapid, fully automated, molecular-based assay accurately analyzes sentinel lymph nodes for the presence of metastatic breast cancer. Ann Surg 2006;243:389–98.
13. Leidenius MH, Krogerus LA, Toivonen TS, Von Smitten KJ. The feasibility of intraoperative diagnosis of sentinel lymph node metastases in breast cancer. J Surg Oncol 2003;84:68–73.
14. Fortunato L, Amini M, Frrina M, et al. Intraoperative

examination of sentinel nodes in breast cancer: is the lass half full or half empty? Ann Surg Oncol 2004;11: 1005–10.
15. Pugliese MS, Kohr JR, Allison KH, Wang NP, Tickman RJ, Beatty JD. Accuracy of intraoperative imprint cytology of sentinel lymph nodes in breast cancer. Am J Surg 2006;192:516–9.
16. Caemon M, Olsha O, Rivkin L, Spira RM, Golomb E. Intraoperative palpation for clinically suspicious axillary sentinel lymph nodes reduces the false-negative rate of sentinel lymph node biopsy in breast cancer. Breast J 2006;12:199–201.
17. Salem AA, Douglas-Jones AG, Sweetland HM, Mansel RE. Intraoperative evaluation of axillary sentinel lymph nodes using touch imprint cytology and immunohistochemistry. Part II. Results. Eur J Surg Oncol 2006;32:484–7.
18. Brogi E, Torres-Matundan E, Tan LK, Cody HS III. The results of frozen section, touch preparation, and cytological smear are comparable for intraoperative examination of sentinel lymph nodes: a study in 123 breast cancer patients. Ann Surg Oncol 2005;12:173–80.
19. Mitas M, Mikhitarian K, Walters C, et al. Quantitative real-time RT-PCR detection of breast cancer micrometastasis using a multigene marker panel. Int J Cancer 2001;93:162–71.
20. Inokuchi M, Ninomiya I, Tsugawa K, Terada I, Miwa K. Quantitative evaluation of metastases in axillary lymph nodes of breast cancer. Br J Cancer 2003;89: 1750–6.
21. Weigelt B, Bosma AJ, Hart AA, Rodenhuis S, van 't Veer LJ. Marker genes for circulating tumour cells predict survival in metastasized breast cancer patients. Br J Cancer 2003;88:1091–4.
22. Weigelt B, Verduijn P, Bosma AJ, Rutgers EJ, Peterse HL, van't Veer LJ. Detection of metastases in sentinel lymph nodes of breast cancer patients by multiple mRNA markers. Br J Cancer 2004;90:1531–7.
23. Sakaguchi M, Virmani A, Dudak MW, et al. Clinical relevance of reverse transcriptase-polymerase chain reaction for the detection of axillary lymph node metastases in breast cancer. Ann Surg Oncol 2003;10: 117–25.
24. Backus J, Laughlin T, Wang Y, et al. Identification and characterization of optimal gene expression markers for detection of breast cancer metastasis. J Mol Diagn 2005;7:327–36.
25. Nissan A, Jager D, Roystacher M, et al. Multimarker RT-PCR assay for the detection of minimal residual disease in sentinel lymph nodes of breast cancer patients. Br J Cancer 2006;94:681–5.
26. Notomi T, Okayama H, Masubuchi H, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 2000;28:E63.
27. Nagamine K, Hase T, Notomi T. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes 2002;16:223–9.
28. Parida M, Posadas G, Inoue S, Hasebe F, Morita K. Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile virus. J Clin Microbiol 2004;42:257–63.
29. Yoshikawa T, Ihira M, Akimoto S, et al. Detection of human herpesvirus 7 DNA by loop-mediated isothermal amplification. J Clin Microbiol 2004;42:1348–52.
30. Hong TC, Mai QL, Cuong DV, et al. Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. J Clin Microbiol 2004;42:1956–61.
31. Poon LL, Leung CS, Tashiro M, et al. Rapid detection of the severe acute respiratory syndrome (SARS) coronavirus by a loop-mediated isothermal amplification assay. Clin Chem 2004;50:1050–2.
32. Fukuda S, Takao S, Kuwayama M, Shimazu Y, Miyazaki K. Rapid detection of norovirus from fecal specimens by real-time reverse transcription-loop–mediated isothermal amplification assay. J Clin Microbiol 2006;44:1376–81.
33. Schmitt AO, Specht T, Beckmann G, et al. Exhaustive mining of EST libraries for genes differentially expressed in normal and tumour tissues. Nucleic Acids Res 1999;27:4251–60.
34. Mori Y, Nagamine K, Tomita N, et al. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem Biophys Res Commun 2001; 289:150–4.
35. Mori Y, Kitao M, Tomita N, et al. Real-time turbidimetry of LAMP reaction for quantifying template DNA. J Biochem Biophys Methods 2004; 59:145–57.
36. Gusterson BA. The new TNM classification and micrometastases. Breast 2003;12:387–90.
37. Min CJ, Tafra L, Verbanac KM. Identification of superior markers for polymerase chain reaction detection of breast cancer metastases in sentinel lymph nodes. Cancer Res 1998;58:4581–4.
38. Manzotti M, Dell'Orto P, Maisonneuve P, Zurrida S, Mazzarol G, Viale G. Reverse transcription-polymerase chain reaction assay for multiple mRNA markers in the detection of breast cancer metastases in sentinel lymph nodes. Int J Cancer 2001;95: 307–12.
39. Noguchi S, Aihara T, Nakamori S, et al. The detection of breast carcinoma micrometastases in axillary lymph nodes by means of reverse transcriptase-polymerase chain reaction. Cancer 1994;74: 1595–600.
40. Bostick PJ, Huynh KT, Sarantou T, et al. Detection of metastases in sentinel lymph nodes of breast cancer patients by multiple-marker RT-PCR. Int J Cancer 1998;79:645–51.
41. Chu PG, Wiess LM. Keratin expression in human tissues and neoplasms. Histopathology 2002;40: 403–39.
42. Lee IK, Lee HD, Jeong J, et al. Intraoperative examination of sentinel lymph nodes by immunohistochemical staining in patients with breast cancer. Eur J Surg Oncol 2006;32:405–9.
43. Nahrig JM, Richter T, Kuhn W, et al. Intraoperative examination of sentinel lymph nodes by ultrarapid immunohistochemistry. Breast J 2003;9: 277–81.

Analysis of Sentinel Node Involvement in Gastric Cancer

DAISAKU MORITA,* HITOSHI TSUDA,*,# TAKASHI ICHIKURA,‡ MIKIHIKO KIMURA,* SHINSUKE AIDA,§ SHIGERU KOSUDA,¶ JOHJI INAZAWA,‖,# HIDETAKA MOCHIZUKI,‡ and OSAMU MATSUBARA*

*Department of Basic Pathology, ‡Department of Surgery I, §Department of Laboratory Medicine, and ¶Department of Radiology, National Defense Medical College, Tokorozawa, Saitama; ‖Department of Molecular Cytogenetics, Medical Research Institute and School of Biomedical Science, Graduate School, Tokyo Medical and Dental University, Bunkyo, Tokyo; and #Core Research for Evolutional Science and Technology of Japan Science and Technology Corporation, Kawaguchi, Saitama, Japan

Background & Aims: **Sentinel node navigation surgery (SNNS) is performed for patients with early gastric cancer. Because sentinel nodes (SNs) to gastric cancer exist but they have not been well-described, we attempted to validate the SN concept at the micrometastasis level.** ***Methods:*** **For 53 patients who underwent curative gastrectomy for T1/T2 (<4 cm) N0 gastric cancer, SNNS was performed with radioactive tin colloid and/or indocyanine green, and subsequent modified D1 lymphadenectomies were added. Whole formalin-fixed paraffin-embedded tissues of all resected lymph nodes from these patients were cut into 5-μm thick serial step sections at 85-μm intervals, and occult metastases were examined immunohistochemically.** ***Results:*** **Metastases were detected in 3 (1.5%) of 204 SNs and 3 (0.33%) of 901 non-SNs in pN0 cases and in 18 (46%) of 39 SNs and 3 (1.9%) of 158 non-SNs in pN1 cases. On a patient basis, metastases were detected in 4 (9%) of 46 pN0 patients, 2 (4%) each in SNs and non-SNs, and in 7 pN1 patients, of whom 7 and 4 had SN and non-SN metastases, respectively. The sensitivity, false-negative rate, and accuracy of SN identification by SNNS were 82%, 18%, and 96%, respectively, at the occult metastasis level. However, on the basis of the concept of the sentinel lymphatic station (SLS), which represents all lymphatic stations to which SNs belong, metastases were always limited to the lymph nodes in SLS in the 11 cases with metastases. Non-SN metastases occurred in 3 (60%) of 5 patients with SN metastases >2.0 mm in diameter but not in 4 patients with SN metastases ≤2.0 mm in diameter.** ***Conclusions:*** **The sentinel node concept held true at the occult metastasis level in 96% of patients with gastric cancer, and the accuracy of SNNS was elevated to 100% by introducing the concept of the sentinel lymphatic station. The size of SN metastasis was a predictive factor for metastasis beyond the sentinel node.**

Advances in diagnostic technology have made it possible to detect gastric cancer at an early stage. Currently, stage I gastric cancer accounts for approximately 61% of all surgically resected cases in Japan.[1] Gastrectomy with lymph node dissection, usually D2 lymphadenectomy, is accepted in Japan as the standard surgical procedure for gastric cancer and contributes to improvement in patient outcome. Because lymph node metastasis occurs in only 10%-16% of patients with early gastric cancer, reduction or omission of regional lymph node dissection would be reasonable if it were possible to predict the extent of lymph node metastasis in each patient.[2-4]

The sentinel node navigation surgery (SNNS) is now widely applied as reduction surgery for various types of cancer, such as cancers of the breast, colorectal region, prostate, lung, female genital tract, and stomach.[5-10] A sentinel node (SN) is defined as the lymph node that is first to receive the flow of lymphatic fluid from the area containing the primary tumor in an organ. According to the SN hypothesis, lymph node dissection can be omitted when no metastases are detected in SNs. In breast cancers and skin melanomas, the SN hypothesis has been shown to be mostly applicable clinically, and the procedure of SNNS with radioisotope-guided and/or dyeing methods is now established. The accuracy of SN detection by these methods is reported to be 83%-100% in SNNS for breast cancer.[6,11-21] Some authors have also reported successful SNNS for gastric cancer, but it is still unclear whether the concept of the SN is valid, or whether the SN hypothesis holds true in surgery for gastric cancer.[22-25] To answer these questions, we performed a study to examine occult metastasis in serial step sections of whole formalin-fixed paraffin-embedded tissue blocks of all dissected SNs and non-SNs and compared the status of "true" metastasis between the SN and non-SN in 53 patients with early, T1/T2 (<4 cm) N0, gastric cancer.

Patients and Methods

Sentinel Lymph Node Navigation Surgery and Lymph Node Dissection

This study was reviewed and approved by the internal review board of the National Defense Medical College, Japan, and informed consent was obtained from all patients. The patients were diagnosed as having gastric carcinoma by endoscopic examination with histopathologic confirmation. By preoperative examinations, eg, barium radiography, endoscopy, ultrasonography, and computed tomography, we confirmed that the largest diameter of the tumor was less than 4 cm, and that lymph node metastasis or distant metastasis was not detectable. A total of 53 patients who underwent curative gastrectomy for primary gastric cancer received SNNS at the Department of Surgery I, National Defense Medical College Hospital, between October 2000-October 2003. The identification of SNs was carried out with radioactive tin colloid (Nihon Mediphysics, Tokyo, Japan) and/or indocyanine green (Daiichi Pharma-

Abbreviations used in this paper: SLS, sentinel lymphatic station; SN, sentinel node; SNNS, sentinel node navigation surgery.

1542-3565/07/$32.00
doi:10.1016/j.cgh.2007.05.001

ceutical, Tokyo, Japan). Endoscopically, 2.0 mL of technetium-99m tin colloid (74 MBq/mL) was injected into the submucosal layer of the stomach at 4 sites around the tumor 21 hours before surgery. Just after laparotomy, 4 mL of 1.25% indocyanine green was delivered endoscopically into the same areas as the radiocolloid injection. A hand-held gamma-detector probe (Navigation; Tyco Healthcare Japan, Tokyo, Japan) was used to identify hot nodes and guide the surgery intraoperatively. An SN was defined as any hot node whose ex vivo radioactivity count was at least 10 times higher than the background count and/or any node for which green dye uptake could be identified visually.[26] All hot nodes and/or green nodes were subjected to intraoperative pathologic examination.

As shown in a previous publication, the double staining method with dye and radioisotope is the most reliable.[24] We performed the double staining method in principle. However, the method with dye alone or with technetium alone was performed when the radioactive reagent or an intraoperative endoscopist was not available.

For the patients in whom no metastasis to SNs was evident by frozen section examination, modified D1 lymphadenectomy was performed after SNNS. If an SN was found to include metastasis by frozen section examination, D2 lymphadenectomy was added. Because this is a retrospective study, we followed up the patients even if occult metastases were newly detected in permanent sections of SNs or non-SNs in the present study. For all SNs, intraoperative histopathologic diagnosis was performed routinely. Each SN was mounted in OCT compound (Sakura Finetek USA, Torrance, CA) and frozen immediately without being cut; 1 section was subjected to histologic diagnosis. After intraoperative diagnosis, the residual lymph node tissues were fixed in formalin and processed for routine permanent histologic diagnosis. After gastrectomy, all lymph nodes dissected were also fixed with formalin, embedded with paraffin, and processed for permanent histologic diagnosis. For each dissected lymph node, 1 section was prepared from a representative cut surface. By permanent histologic diagnosis, lymph node metastasis was absent (pN0) in 46 patients but was detected in 1–6 lymph nodes (pN1) in 7 patients. The locations of all SNs and non-SNs were classified to any of the lymphatic stations defined in the Japanese Classification of Gastric Carcinoma, 2nd English edition, by the Japanese Gastric Cancer Association (Figure 1).

The numbers of patients who underwent the combination method with dye and radioisotope together, dye method only, and radioisotope method only were 35, 2, and 16, respectively. Total gastrectomy, proximal gastrectomy, distal gastrectomy, and pylorus-preserving gastrectomy were performed on 4, 6, 29, and 14 patients, respectively. Patient characteristics are shown in Table 1. A total of 1302 lymph nodes, 24 per patient on average, were obtained. There were 243 SNs and 1059 non-SNs or 4.6 and 20.0 per patient on average, respectively.

Serial Step Sections

All tissue blocks of dissected lymph nodes were serially enumerated for identification and classified into those ≥2.0 mm and those <2.0 mm in diameter. Nine tissue microarray blocks were constructed by using 360 lymph nodes that were less than 2.0 mm in diameter. In addition, 942 lymph nodes with a diameter of 2 mm or larger were rearranged in 40 tissue blocks and re-embedded in paraffin. From all these tissue

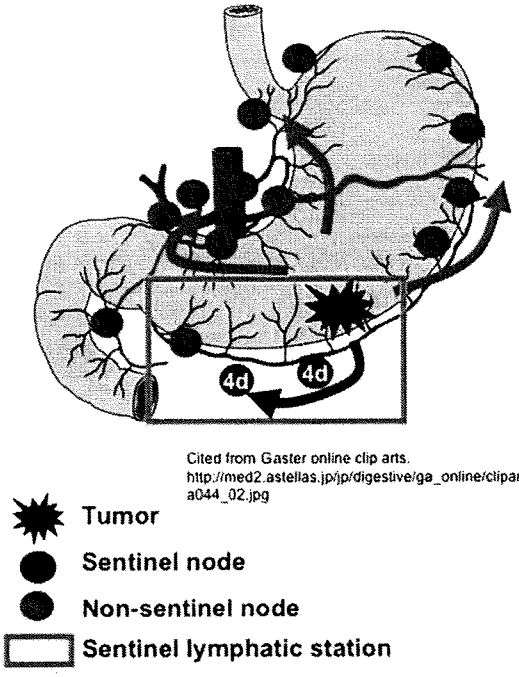

Figure 1. Concepts of the SN and SLS in the stomach. *Numbers* stand for lymphatic stations, which are defined in the Japanese Classification of Gastric Carcinoma, 2nd English edition. *Each number* corresponds to 1 lymphatic station. Perigastric lymphatic stations are classified as nos. 1–6. If 2 SNs are identified in lymphatic station no. 4d (*blue*), and no. 4d contains not only these SNs but also another non-SN (*red*), these 3 nodes are regarded as belonging to the SLS. *No. 1*, right paracardiac lymph nodes (*LNs*); *No. 2*, left paracardiac LNs; *No. 3*, LNs along the lesser curvature; *No. 4sa*, LNs along the short gastric vessels; *No. 4sb*, LNs along the left gastroepiploic vessels; *No. 4d*, LNs along the right gastroepiploic vessels; *No. 5*, suprapyloric LNs; *No. 6*, infrapyloric LNs; *No. 7*, LNs along the left gastric artery; *No. 8a*, LNs along the common hepatic artery.

blocks, triplet 5-μm thick serial sections were obtained every 85 μm until all the tissue in the block had been used. These sections were mounted on silane-coated glass slides (Muto Pure Chemicals, Tokyo, Japan). The total number of sections obtained was the triplets of 980 (mean, 19.6 slides per block). One of the triplet sections was stained with hematoxylin-eosin and subjected to histopathologic examination and the second to immunohistochemical study. The third sections were stocked as spare sections.

Immunohistochemistry. These sections were deparaffinized in xylene, rehydrated with ethanol, and reacted with 5% hydrogen peroxide for 10 minutes. Antigen retrieval of the sections was performed by exposure to microwave radiation in 10 mmol/L citrate buffer (pH 6.0) at 95°C for 5 minutes 3 times. The slides were then incubated with 10% normal goat serum (Nichirei, Tokyo, Japan) for 30 minutes, followed by anti-human cytokeratin antibodies (clone: AE1+AE3, dilution: 1/200; Dako, Glostrup, Denmark) at room temperature for 2 hours. The slides were then reacted with EnVision polymer reagent (Dako) at room temperature for 30 minutes. The slides were washed in phosphate-buffered saline with 0.1% Tween 20 (Sigma-Aldrich, Steinheim, Germany) 3 times and subsequently reacted with 3, 3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide as a chromogen. Two observers (D.M., H.T.)

Table1. Clinicopathologic Characteristics of Study Population

Measure	Number
Patients	53
Dissected lymph nodes	1302
(Average ± SD)/patient	24.5 ± 5.3
SNs	243
(Average ± SD)/patient	4.6 ± 1.7
SLSs	88
(Average ± SD)/patient	1.7 ± 0.7
Age (*y*)	61.6 ± 8.4
Sex ratio (male:female)	38:15
Depth of tumor invasion	
pT1 (mucosa)	23
pT1 (submucosa)	26
pT2 (muscularis propria)	3
pT2 (subserosa)	1
Type of surgery	
Total gastrectomy	4
Proximal gastrectomy	6
Distal gastrectomy	29
Pylorus-preserving gastrectomy	14
Method for detection of SNs	
Dyeing only	16
Radioactive only	2
Dyeing and radioactive	35

SD, standard deviation.

independently judged the presence of tumor cells in hematoxylin-eosin-stained sections and immunohistochemistry sections. If 1 or more cancer cells were revealed by hematoxylin-eosin or if 1 or more cells were positive by immunohistochemistry, we judged the lymph node to be positive for occult metastasis. If there was a discrepancy in judgment between 2 observers, consensus was acquired by using a discussion microscope.

We also applied the new TNM classification, 6th edition[27] for the lymph node status of the 53 patients. Routinely defined pN0 and pN1 were reclassified into pN0, pN0 i+, pN1mi, and pN1 (>2 mm). The pN1 was classified into 2 subgroups according to the TNM classification of breast cancer: pN1 (>2 mm) and pN1mi were defined as statuses with metastatic foci >2 mm in diameter and with those >0.2 mm but ≤2 mm in diameter, respectively.[27] Tumor cell nests or single cells <0.2 mm in diameter were defined as isolated tumor cells or pN0 i+. By the present method, pN1 (>2 mm), pN1mi, and nests of pN0 i+ 90 mm or larger were always detectable. If a single tumor cell was speculated to approximately 10 mm in diameter, at least 9 or more tumor cells were estimated to be always identified in the lymph node by the present method.

Concept of the Sentinel Lymphatic Station

We validated the sentinel lymph node concept in gastric cancer from the viewpoint of both individual SNs and the sentinel lymphatic station (SLS). A lymphatic station denotes a group of lymph nodes that are localized in an identical area that is numbered in the Japanese Classification of Gastric Carcinoma, 2nd English edition (Figure 1). The SLS is defined here as the area of 1 or more lymphatic stations at which lymphatic flow arrives first from the primary lesion of gastric cancer. When SNs were identified, all lymphatic stations that contained the SNs were regarded as SLSs. In the present study, a total of 88 SLSs were detected (mean, 1.7 per patient).

Statistical Analysis

A "true metastasis" was defined as a metastasis that was detected by routine examination or occult metastasis detected by the present serial step section method. We compared the rate of metastases detected by routine examination with that of true metastases at the patient level and at the lymph node level. We also calculated sensitivity, specificity, positive and negative predictive values, and overall accuracy of SNNS with regard to true metastasis at the SN level and at the SLS level.

Mean values between groups were compared by *t* test. Fisher exact test or the Mann-Whitney *U* test was used to examine differences between groups. Differences at $P < .05$ were considered to be statistically significant. All data were analyzed with the Statistical Package for JMP version 5.1.1J software package (SAS Institute, Cary, NC).

Results

Detection of Occult Metastasis by Serial Section Examinations

The results of both hematoxylin-eosin and immunohistochemistry were always concordant, and the level of interobserver agreement for these results was 100%. Occult metastases were detected by serial section examination in 4 of 46 patients with pN0 (Table 2), 2 in SNs only, and 2 in non-SNs only. In these 4 pN0 patients, occult metastases were detected in 6 lymph nodes comprising 3 SNs and 3 non-SNs. In 7 patients with pN1, metastases were detected in 21 lymph nodes; 18 were detected by routine examination and 3 occult. The average size of nodes with metastases was 5.3 ± 3.8 mm (standard deviation) and 5.0 ± 3.3 mm in these 4 pN0 cases and 7 pN1 cases, respectively. However, the average sizes of the metastases in these 2 groups were 0.45 ± 0.33 mm and 3.3 ± 3.2 mm, respectively, and the difference was significant ($P = .0005$).

Table 2. Correlation Between Routine Diagnosis and Serial Step Section Diagnosis of SN and non-SN Metastasis

		No. of patients with lymph node metastases		
	Total	Serial step section diagnosis[a] (occult metastasis)[a]		Routine diagnosis[a]
pN0 (n = 46)				
SN	46	2 (4.4%)	(2)	0
Non-SN	46	2 (4.4%)	(2)	0
pN1 (n = 7)				
SN	7	7 (100%)	(1)	7
Non-SN	7	3 (43%)	(2)	1
Total	53	11		7

[a]Two pN0 cases with metastasis in SN deleted by serial step section diagnosis and 2 pN0 cases with metastasis in non-SN detected by serial step section diagnosis were always occult metastases and were exclusive together. Three pN1 cases with non-SN metastasis, 2 of which were detected by serial step section diagnosis and 1 of which was diagnosed routinely, also had SN metastasis.

Table 3. Demographics of Metastatic Lymph Nodes

Case no.	No. of SNs detected	SLS	Detection in routine pathologic diagnosis	Lymph node status	Location	Size of a lymph node (*mm*)	Size of metastasis (*mm*)
pN0							
1	3	No. 4d	−	SN	No. 4d	12	0.2
2	5	No. 3, 4d, 5	−	Non-SN	No. 4d	5	0.8
3	7	No. 6	−	SN	No. 6	2	0.9
			−	SN	No. 6	2	0.1
4	2	No. 1, 3	−	Non-SN	No. 3	7	0.2
			−	Non-SN	No. 3	4	0.5
pN1							
5	7	No. 3, 4d, 6, 8a	+	SN	No. 3	1.5	0.7
6	5	No. 4d	+	SN	No. 4d	6	0.1
			+	SN	No. 4d	5	3
			+	SN	No. 4d	4	2
7	9	No. 3, 4d	+	SN	No. 3	12	12
			+	SN	No. 3	10	10
			+	SN	No. 3	5	5
			+	SN	No. 3	2.5	2.5
			+	SN	No. 3	2	2
			+	SN	No. 3	1.5	1.5
			−	SN	No. 3	1.5	1.5
			+	Non-SN	No. 4d	3	3
8	3	No. 6	+	SN	No. 6	4	4
			+	SN	No. 6	2	0.5
9	2	No. 3, 8a	+	SN	No. 3	8	0.05
10	3	No. 3, 4sb	+	SN	No. 3	2.5	2.5
			−	Non-SN	No. 3	10	0.9
11	10	No. 3, 4d	+	SN	No. 4d	8	8
			+	SN	No. 4d	4	4
			+	SN	No. 4d	3	2
			−	Non-SN	No. 3	10	5

At the lymph node level, true metastases were detected in 21 (8.6%) of 243 SNs and 6 (0.57%) of 1059 non-SNs. The rate to metastasis to SNs was approximately 15 times as high as that to non-SNs. The rate of true metastasis to SN was high (46%, 18 of 39 SNs) in pN1 patients, but it was only 1.5% (3 of 204 SNs) in pN0 patients. The rate of true metastasis to non-SNs was also low, 1.9% (3 of 158 non-SNs) and 0.33% (3 of 901 non-SNs), in pN1 and pN0 patients, respectively.

Accuracy of Routine Pathologic Diagnosis

True metastases to SNs were detected in 9 patients, by routine pathologic diagnosis in 7 and occult metastases in 2. With regard to SNs, the false-negative rate, sensitivity, specificity, and overall accuracy of routine pathologic diagnosis against the status of true metastasis were 22% (2 of 9), 78% (7 of 9), 100% (44 of 44), and 96% (51 of 53). Likewise, true metastases to non-SNs were detected in 5 patients, 3 (43%) of 7 pN1 patients, and 2 (4%) of 46 pN0 patients. Only 1 of these 5 non-SN metastases was detected by routine diagnosis. With regard to all lymph nodes dissected, true metastases to SNs or to non-SNs were detected in 11 patients, by routine pathologic diagnosis in 7, and occult metastases in 4. The false-negative rate, sensitivity, specificity, and overall accuracy of routine pathologic diagnosis against the status of true metastasis were 36% (4 of 11), 64% (7 of 11), 100% (42 of 42), and 92% (49 of 53) of lymph nodes as a whole.

Validation of the Sentinel Node Concept on a Serial Step Section Basis

"Skip metastases," defined as metastases to non-SNs without presence of metastasis in any SNs, were detected in 2 (4%) of 53 patients. Therefore, the false-negative rate, sensitivity, specificity, and overall accuracy of SNNS at the occult metastasis level were 18% (2 of 11), 82% (9 of 11), 100% (42 of 42), and 96% (51 of 53). Thus, the sentinel node theory was applicable to 96% of the patients in this study.

Validation of the Sentinel Lymph Node Concept From the Viewpoint of Sentinel Lymphatic Station

The distribution of SLS was investigated in 11 patients with true metastases to SNs and/or non-SNs (Table 3). Two (cases 1 and 3) of 4 pN0 patients had occult metastases to SNs, including a metastasis to no. 4d in case 1 and 2 metastases to no. 6 in case 3. The other 2 pN0 patients (cases 2 and 4) had occult skip metastases to non-SNs: In case 2, a metastasis occurred in a no. 4d non-SN, and 5 SNs without metastases were located in no. 3 (2 nodes), no. 4d (2 nodes), or no. 5 (1 node). In case 4, metastases occurred in no. 3 non-SNs, and 2 SNs without metastases were located in no. 1 or no. 3. Therefore, in both cases 2 and 4, occult skip metastases always belonged to SLSs. In 7 pN1 patients, 21 nodes had metastases, 18 of which were detected routinely in SNs, whereas the other 3 were occult metastases to SNs or non-SNs (Table 3). All

Table 4. Patient and Tumor Characteristics According to SN and/or non-SN Status

		SN/non-SN status			
	No. of cases	With Metastasis(%)	[With occult Metastasis]	Without Metastasis	*P* value
Age (*y*)					
<60	23	5 (22)	[2]	18	.9623
≥60	30	6 (20)	[2]	24	
Histologic type					
Well-differentiated tubular	16	0 (0)	[0]	16	.0077[a]
Moderately differentiated tubular	17	7 (41)	[3]	10	
Poorly differentiated adenocarcinoma	18	3 (17)	[1]	15	
Undifferentiated carcinoma	2	1 (50)	[0]	1	
Depth of invasion					
T1 (mucosa)	23	1 (4)	[0]	22	.0253[b]
T2 (submucosa)	26	8 (31)	[4]	18	
T2 (muscularis propria or subserosa)	4	2 (50)	[0]	2	
Location of tumor					
Upper	8	1 (13)	[1]	7	.7561
Middle	25	5 (20)	[1]	20	
Lower	20	5 (25)	[2]	15	
Lymphatic vessel invasion					
Negative	32	1 (3)	[0]	31	<.0001
Positive	21	10 (48)	[4]	11	
Blood vessel invasion					
Negative	45	7 (16)	[3]	38	.0942
Positive	8	4 (50)	[1]	4	
Method for SN identification					
Dyeing only	16	4 (25)	[2]	12	.4740
Radioactive only	2	1 (50)	[1]	1	
Dyeing and radioactive	35	6 (17)	[1]	29	
Type of surgery					
Total gastrectomy	4	0 (0)	[0]	4	.0440
Proximal gastrectomy	6	1 (17)	[1]	5	
Distal gastrectomy	29	10 (35)	[3]	19	
Pylorus-preserving gastrectomy	14	0 (0)	[0]	14	

[a]Well-differentiated tubular adenocarcinoma vs moderately differentiated tubular adenocarcinoma, poorly differentiated adenocarcinoma, and undifferentiated carcinoma.
[b]T1 (mucosa) vs T1 (submucosa) and T2 (muscularis propria or subserosa).

metastases were included in SNs in 4 pN1 patients (cases 5, 6, 8, and 9), whereas metastases were detected in both SNs and non-SNs in the other 3 (cases 7, 10, and 11). In case 10, both a routinely detected SN metastasis and an occult non-SN metastasis were located in no. 3. In case 7, all 9 SNs were located in either no. 3 or 4d, and 7 routinely detected SN or non-SN metastases and another occult SN metastasis were also located in no. 3 or no. 4d. In case 11, all 10 SNs were located in either no. 3 or no. 4d, and 3 routinely detected SN metastases and 1 occult non-SN metastasis were also located in no. 4d, or in no. 3. Therefore, in 7 pT1 cases, metastatic lymph nodes were always included in SLSs, irrespective of whether the metastatic nodes were SN or non-SN, and non-SLSs examined did not have metastasis. Thus, false-negative rate, sensitivity, specificity, and accuracy of SNNS based on the SLS concept were 0%, 100%, 100%, and 100%, respectively.

According to the TNM classification 6th edition, 1 and 3 of 4 pN0 patients were classified as pN0 i+ and pN1mi, and 1, 1, and 5 of 7 pN1 patients were classified as pN0 i+, pN1mi, and pN1 (>2 mm), respectively. We investigated the risk factors of non-SN metastasis among 9 patients with SN metastases. Six patients without non-SN metastases had SN metastases 1.48 mm in diameter on average. In contrast, 3 patients with non-SN metastases had SN metastases 7.5 mm in diameter on average ($P = .0695$). Three (60%) of 5 patients with pN1 (>2 mm) to SNs also had metastases in non-SNs, whereas none of 4 patients with pN1mi or pN0 i+ to SNs had metastasis to non-SNs. Between the group of pN1 (> 2 mm) and the group of pN1mi or pN0 i+, the F value was 0.010, and the mean diameter of SN metastases differed significantly by *t* test ($P = .033$).

Clinicopathologic Correlation

In the 11 patients with true lymph node metastases, tumor invasion was deeper and the ratio of lymphatic vessel invasion was higher than in patients without metastases (Table 4). In the 4 pN0 patients with occult metastases, tumor invasion was limited to the mucosa or submucosal layer. In 7 pN1 patients, tumor invasion was limited to the mucosa or submucosal layer in 5 but reached the muscularis propria or deeper in 2.

In all of these 11 pN0 patients, the primary tumors were moderately or poorly differentiated adenocarcinoma, and no case was well-differentiated adenocarcinoma. There were no significant correlations of true lymph node metastasis with patient age, tumor size, tumor location, blood vessel invasion, or SN detection method. At present, none of the 53 patients has

shown relapse or metachronous metastasis (mean follow-up period, 36 ± 13 months).

Discussion

Intraoperative use of dye for lymph node mapping was described >50 years ago.[28,29] In the present study with currently used radioactive labeling and/or dyeing methods, it was possible to identify lymph nodes in which initial metastasis had developed in 96% (51 of 53) of the patients examined. The sensitivity and accuracy of SNNS for gastric cancer at the routine diagnostic level are reported to be 92% and 98% with radioactive labeling and 83%-89% and 96%-98% with dyeing, respectively.[22-25] At the occult metastasis level, the sensitivity and accuracy were also 82% and 96%, respectively, and in addition, the rate of metastasis to SNs was approximately 15 times higher than that to non-SNs. From these results, we were able to show that SNs did exist in most of the patients, thus providing histopathologic proof of the validity of SNNS for gastric cancer. Even with the present serial sectioning method, there were 2 patients with skip metastases, indicating that initial metastasis to lymph nodes cannot always be detected perfectly by the current SNNS procedure. To overcome this situation, we suggest that the SLS concept be applied to gastric cancer surgery. In the 11 patients with any type of lymph node metastasis, all the affected lymph nodes belonged to the SLS. Therefore, metastatic cancer cells from the primary site always reached lymph nodes in the SLS first. We believe that the SN theory based on the SLS concept is applicable to patients with gastric cancer. In 9 patients with SN metastasis, we investigated factors correlated with non-SN metastases. Non-SN metastasis was detected in 60% of the patients with pN1 (>2 mm), but it was not detected in the patients with pN1mi or pN0 i+. These results suggested that the size of SN metastasis based on the new TNM classification was useful for predicting non-SN metastasis. Therefore, it seems useful to prepare sections every 2 mm from SNs or lymph nodes in SLSs for intraoperative pathologic diagnosis when the diameter of these lymph nodes is >2 mm.

The present results validate the rationale of SNNS for early gastric cancer on the basis of the SLS concept. If there are no metastases in lymph nodes in SLSs, no further dissection would be necessary. Furthermore, the possibility of metastasis beyond the SLSs would appear low if there are no lymph nodes with metastases >2.0 mm in diameter in SLSs. It would be worth studying the validity of omitting further dissection of non-SLSs in this situation.

To detect all clinically significant metastases in SLSs intraoperatively, it would be necessary to extend pathologic examination to all lymph nodes belonging to SLSs. This would naturally increase the work burden for histopathology staff. Thus, whether it is possible to diagnose all lymph nodes in SLSs by using a sectioning interval of 2 mm in routine pathologic practice is problematic. To minimize the burden of intraoperative pathologic diagnosis, the application of novel techniques for detecting lymph node metastasis would be helpful. Reverse transcription polymerase chain reaction and other molecular diagnostic modalities have been developed and are emerging as quantitative and highly sensitive methods for rapid detection of metastasis.[30,31] The application of these molecular diagnostic tools will facilitate the spread of SNNS as a standard procedure for gastric cancer worldwide as well as improving the accuracy of intraoperative diagnosis.

In the present study, all 11 patients with 1 or more lymph node metastases underwent curative resection, and none of them has shown relapse yet. Skip metastasis foci to the 3 non-SNs in these 2 patients were 0.2, 0.5, and 0.8 mm in diameter and were not pN0 i+ but pN1mi. It is unclear whether these occult metastases would have caused cancer recurrence if they had not been dissected. Lee et al[32] reported that 5-year overall survival rates differed significantly between patient groups with gastric cancer with and without micrometastasis to regional lymph nodes (49% and 76%, respectively). Their results suggest that surgical resection of micrometastases is mandatory to improve cure rate of patients with early gastric cancer.

SNNS is usually performed for the patients with T1 or T2 gastric cancer without clinically detectable lymph node metastasis. It is shown and is the consensus that adjuvant chemotherapy is not necessary for the patients with pT1 or pT2 pN0 gastric cancer.[1] The utility of adjuvant chemotherapy to patients undergoing gastrectomy with D1 or D2 lymphadenectomy to gastric cancer has been shown by randomized clinical trials, but pT1 or pT2 pN0 cases were excluded from these studies.[1] Therefore, at present, there is no evidence for adjuvant chemotherapy to most of gastric cancers' SNNS procedures.

In conclusion, the accuracy of the current SNNS procedure for detecting SNs in patients with early gastric cancer was 96% at the occult metastasis level. By application of the SLS concept, the accuracy of SN detection would be improved further. The size of SN metastasis was a predictive factor of non-SN metastasis. SNNS is a valid and practically useful method for reduction surgery in patients with early gastric cancer.

References

1. Japanese Gastric Cancer Association. JGCA gastric treatment guidelines. Available at: http://www.jgca.jp/PDFfiles/GL2004.PDF; Accessed August 28, 2006.
2. Inoue K, Tobe T, Kan N, et al. Problems in the definition and treatment of early gastric cancer. Br J Surg 1991;78:818-821.
3. Sano T, Sasako M, Kinoshita T, et al. Recurrence of early gastric cancer: follow-up of 1475 patients and review of the Japanese literature. Cancer 1993;72:3174-3178.
4. Lee HJ, Kim YH, Kim WH, et al. Clinicopathological analysis for recurrence of early gastric cancer. Jpn J Clin Oncol 2003;33: 209-214.
5. Morton DL, Wen DR, Wong JH, et al. Technical details of intraoperative lymphatic mapping for early stage melanoma. Arch Surg 1992;127:392-399.
6. Giuliano AE, Kirgan DM, Guenther JM, et al. Lymphatic mapping and sentinel lymphadenectomy for breast cancer. Ann Surg 1994;220:391-398.
7. Lechner P. Probe-guided surgery of colorectal carcinoma. Acta Med Austriaca 1997;24:68-72.
8. Wawroschek F, Vogt H, Weckermann D, et al. The sentinel lymph node concept in prostate cancer: first results of gamma probe-guided sentinel lymph node identification. Eur Urol 1999;36: 595-600.
9. Little AG, DeHoyos A, Kirgan DM, et al. Intraoperative lymphatic mapping for non-small cell lung cancer: the sentinel node technique. J Thorac Cardiovasc Surg 1999;117:220-224.
10. Burke TW, Levenback C, Tornos C, et al. Intraabdominal lymphatic mapping to direct selective pelvic and paraaortic lymphadenectomy in women with high-risk endometrial cancer: results of a pilot study. Gynecol Oncol 1996;62:169-173.
11. Krag DN, Weaver DL, Alex JC, et al. Surgical resection and

radiolocalization of the sentinel lymph node in breast cancer using a gamma probe. Surg Oncol 1993;2:335–339.
12. Albertini JJ, Lyman GH, Cox C, et al. Lymphatic mapping and sentinel node biopsy in the patient with breast cancer. JAMA 1996;276:1818–1822.
13. Giuliano AE, Jones RC, Brennan M, et al. Sentinel lymphadenectomy in breast cancer. J Clin Oncol 1997;15:2345–2350.
14. Veronesi U, Paganelli G, Galimberti V, et al. Sentinel-node biopsy to avoid axillary dissection in breast cancer with clinically negative lymph-nodes. Lancet 1997;349:1864–1867.
15. Cox CE, Pendas S, Cox JM, et al. Guidelines for sentinel node biopsy and lymphatic mapping of patients with breast cancer. Ann Surg 1998;227:645–651.
16. Krag D, Weaver D, Ashikaga T, et al. The sentinel node in breast cancer: a multicenter validation study. N Engl J Med 1998;339: 941–946.
17. Canavese G, Gipponi M, Catturich A, et al. Sentinel lymph node mapping opens a new perspective in the surgical management of early-stage breast cancer: a combined approach with vital blue dye lymphatic mapping and radioguided surgery. Semin Surg Oncol 1998;15:272–277.
18. Veronesi U, Paganelli G, Viale G, et al. Sentinel lymph node biopsy and axillary dissection in breast cancer: results in a large series. J Natl Cancer Inst 1999;91:368–373.
19. Sobin LH, Wittekind CH, eds. TNM classification of malignant tumours: International Union Against Cancer. 5th ed. New York: John Wiley & Sons, 1997.
20. Noguchi M, Tsugawa K, Miwa K, et al. Sentinel lymph node biopsy in breast cancer using blue dye with or without isotope localization. Breast Cancer 2000;7:287–296.
21. Motomura K, Inaji H, Komoike Y, et al. Combination technique is superior to dye alone in identification of the sentinel node in breast cancer patients. J Surg Oncol 2001;76:95–99.
22. Miwa K. Sentinel node concept and its application for cancer surgery (in Japanese). Nippon Geka Gakkai Zasshi 2000;101:307–310.
23. Hiratsuka M, Miyashiro I, Ishikawa O, et al. Application of sentinel node biopsy to gastric cancer surgery. Surgery 2001;129: 335–340.
24. Kitagawa Y, Fujii H, Mukai M, et al. Radio-guided sentinel node detection for gastric cancer. Br J Surg 2002;89:604–608.
25. Ichikura T, Morita D, Uchida T, et al. Sentinel node concept in gastric carcinoma. World J Surg 2002;26:318–322.
26. Sato K, Tamaki K, Shigekawa T, et al. Clinically useful detection criteria for sentinel nodes in patients with breast cancer using a radioisotope technique. Jpn J Clin Oncol 2002;32:403–406.
27. UICC 1997 Wittekind. TNM classification of malignant tumors. 6th ed. New York: Wiley-Liss, 2002.
28. Weinberg J, Greaney EM, Rawlings B, et al. The use and toxicity of pontamine sky blue. Science 1951;114;41–42.
29. Levine EA, Cohn I. Early work in lymphatic mapping. Surgery 2000;127:596.
30. Yamamoto N, Kato Y, Yanagisawa A, et al. Predictive value of genetic diagnosis for cancer micrometastasis: histologic and experimental appraisal. Cancer 1997;80:1393–1398.
31. Lockett MA, Metcalf JS, Baron PL, et al. Efficacy of reverse transcriptase-polymerase chain reaction screening for micrometastic disease in axillary lymph nodes of breast cancer patients. Am Surg 1998;64:539–543.
32. Lee E, Chae Y, Kim I, et al. Prognostic relevance of immunohistochemically detected lymph node micrometastasis in patients with gastric carcinoma. Cancer 2002;94:2867–2873.

Address requests for reprints to: Hitoshi Tsuda, MD, Department of Basic Pathology, National Defense Medical College, Namiki 3-2, Tokorozawa 359-8513, Japan. e-mail: htsuda@ndmc.ac.jp; fax: +81-4-2996-5193.

臨牀消化器内科 2007 Vol.22 No.8
©日本メディカルセンター

特集 センチネルリンパ節理論の消化器癌への応用

3 リンパ節転移の術中診断の精度とその向上策

上田 重人* 津田 均**

Key words : 乳癌リンパ節

要旨

わが国における乳癌，消化器癌のセンチネルリンパ節ナビゲーション手術(SNNS)においては，センチネルリンパ節(SLN)転移の診断はおもに術中病理診断ないし細胞診で行われるが，約10～40%の頻度で転移見落とし，とくに微小転移巣(長径>0.2～2 mm)の見落としが起きる．微小転移見落としを防ぐためSLNを半割，3分割，あるいは2 mmごとの切片作製などこまかい検索を行う施設も増えてきた．しかし病理部門のマンパワーの面で限界があるのも事実である．微小転移検出の補助としてサイトケラチン(CK)免疫組織化学(IHC)法は有用であるが，IHC法のみで見つかりルーチン病理診断では検出困難な遊離腫瘍細胞(長径≦0.2 mm)の意義は，現時点では不明とされる．最近では分子生物学的手法によるSLNの術中転移診断法が開発されている．

はじめに

センチネルリンパ節(SLN)は癌原発巣周囲のリンパ流が最初に流れつくリンパ節と定義される．癌細胞が増殖・浸潤して原発巣から離れ，その周囲組織のリンパ管に侵入すると，これらの癌細胞はまずSLNに到達する．悪性黒色腫，乳癌などにおいて色素や放射性薬剤が腫瘍周囲から最初に流れ込むSLNは，確かに存在することが示されてきた[1),2)]．

乳癌のsentinel node navigation surgery (SNNS)では，SLNを同定して転移の有無を調べ，センチネル理論に基づいて転移がなければほかの腋窩リンパ節(非SLN)の郭清を省略し，転移があれば腋窩郭清を追加している．

わが国では，SLN生検と乳癌原発巣の手術を1回の手術枠内で行うことがほとんどであり，SLNの転移診断は通常，術中迅速病理診断または細胞診によって行われる．術中病理診断は臨床的にきわめて有用性の高い診断法であるが，ルーチンで行われる永久病理診断(ホルマリン固定パラフィン包埋組織ブロックから作製された切片で診断する．標本作製から結果が出るまで数日かかる)と比べると診断の質，精度が低くなる．

胃，食道，大腸など消化器癌においてもSNNSを行う施設が増えつつあり，センチネル理論が確立されつつある．

本稿ではSLN転移の術中病理診断の利点と問題点並びに，その解決法について考えてみたい．

*防衛医科大学校外科学(乳腺内分泌外科)
**同 病態病理学
(〒359-8513 埼玉県所沢市並木3-2)

Ⅰ．術中病理診断の利点と問題点

この項のポイント
- 術中病理診断は，一期的にSLN生検と原発巣の手術を行うためには不可欠である．
- 術中病理診断は，転移診断の偽陰性がしばしば起こりうる問題点がある．

術中病理診断の際には，通常，SLNを長軸方向に平行に半割～4分割して，各々の組織をコンパウンドに埋め込み急速に凍結して，クライオスタットで薄切し凍結切片を作製する．凍結切片を短時間で固定しヘマトキシリン-エオジン(HE)染色を施してリンパ節当り2～4割面の切片を作製し，術中組織診断を行う．施設やリンパ節の大きさによっては割を入れずそのまま1割面のみの切片を作る場合もある．胃癌SLNでは，数も多く2mm以下のSLNも多いので，術中病理診断の際に割を入れないことが多い．術中病理診断後のSLN組織は解凍し，一晩ホルマリン固定したのちルーチンどおりパラフィン包埋し，薄切，HE染色を行って永久病理診断に供する．

術中病理診断は30分以内に結果報告がなされ，必要に応じて腋窩郭清を追加でき，一期的にSLN生検と原発巣の手術を行うためには不可欠な方法である．事実，転移の術中病理診断は多くの場合さほど困難ではない．

しかし，術中病理診断は，病理医不在の施設では行うことができず，また件数が増えると病理スタッフの負担を増やす結果ともなる．さらに，作製された標本の質が十分ではなく，正しい評価が困難な場合もありうる．通常は，必要最小限の検索であることが多いため，転移陰性と報告したが，永久標本で癌細胞が見つかる，いわゆる転移診断の偽陰性(false negative)がしばしば起こりうる．

乳癌SLNの術中病理診断における偽陰性の頻度に関する諸家の報告を表に示す[3]．永久標本での転移陽性を真の陽性，転移陰性を真の陰性とすると，真の陽性に対する術中病理診断の陽性率は57～89%，したがって転移見落とし率は11～43%と報告されている．

表　センチネルリンパ節生検の術中迅速病理診断の偽陰性率(乳癌)

Hingston/Flett(1999年)	11%
Turner(1999年)	26.3%
Veronesi(1999年)	5.5%
Zurrida(2000年)	32%
Canavese(1998年)	27%
Van Diest(1999年)	13%
Rahusen(2000年)	43%
Weiser(2000年)	42%
Chao(2002年)	32%

註：Veronesi論文での偽陰性率が著しく低いのは，本文に述べたようにextensiveな術中評価を行ったことによる．
〔Chao, C., et al.：Am. J. Surg. 182；609-615, 2002[3]より〕

この頻度は胃癌，大腸癌でも同程度である．われわれの胃癌の検討でもSLN術中病理診断の転移見落とし率は22%(2/9)であった．

Ⅱ．術中病理診断の偽陰性

この項のポイント
- 微小転移の見落としを防ぐためには，SLNをなるべくこまかくスライスし，丁寧に検索するのがもっとも有効である．

偽陰性の大部分は，転移巣が検索した割面に含まれていないことで生じる(図1)．次いで，生検時の熱変性や圧挫によって診断がしにくい組織である場合，癌細胞が検索割面に出ていても異型や細胞数が少ないため見落とす場合，標本の質の問題などがある(図2)．

SNNSが試みられるようになった初期のころは，他臓器癌のリンパ節転移の病理診断と同様，一割面だけの検索を行う施設が多かった．しかし，SLNはほかのリンパ節と比べて転移

の頻度が高く，とくに微小転移巣(長径＞0.2 mm，≦2 mm)が高率で検出される．SNNSにおける術中病理診断の偽陰性率を減少させるためには，SLNをなるべくこまかくスライスし，各々についてきれいな標本を作製して丁寧に検索するのがもっとも有効である．極端な例では，Veronesiらのように乳癌のSLNを最初の15枚の切片を50 μmの厚さごとに，その後は100 μmの厚さごとに切片を作製している施設もある[4]．以下に，術中病理診断のためのSLN標本の切り出し法について述べる．

Ⅲ．術中病理診断におけるセンチネルリンパ節の切り出し法

この項のポイント
- SLNの切り出し法は，実際にはsingle sectioningかslice sectioningが用いられる．

術中病理診断のためのSLNの切り出し法にはsingle sectioning，slice sectioning，serial step sectioningなどがある(図1)．実際にはsingle sectioningかslice sectioningが用いられる．

1. single sectioning

single sectioningとは，SLNに割を入れずに包埋して，代表一割面のみの切片を作製し検索することをいう．リンパ節全体の1%以下(厚さ5 mmのリンパ節のうち5～10 μmで0.1～0.2%)を検索しているにすぎず，微小転移のみならず2 mm径より大きなマクロ転移も正確に診断できない可能性がある．

1999～2001年の間に当施設で経験したT1-T2，N0-N1a乳癌患者131名を対象に，RI法によりSLNを同定・生検し，single sectioning法により術中病理診断を行った．1名当りSLNは1.7個(1～6個)摘出された．106名が術中病理診断にて転移陰性とされ，そのうち11名(10.4%)に術後検索により新たな転移が判明した．真の転移陽性例は36例で，術中病理診断による偽陰性率は31%(11/36)となった．見落とされた転移巣はすべて微小転移巣であった．

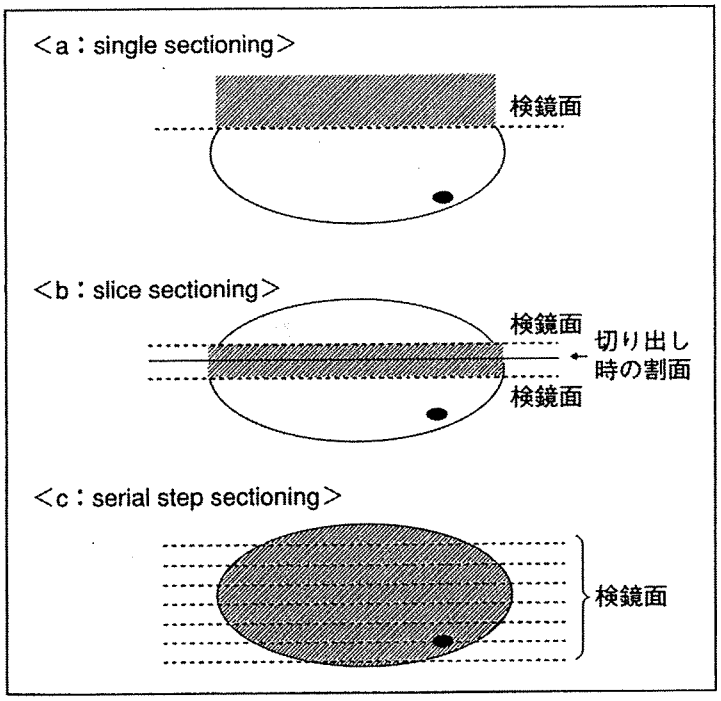

図1　センチネルリンパ節(SLN)の切り出し法
a：single sectioning．SLNをそのまま包埋し，急速凍結してクライオスタットにて面をある程度削り取り(斜線部)，面が出たら切片を作製し(破線部)，術中病理診断を行う．微小転移巣(黒玉)を見落とす可能性が高い．
b：slice sectioning(ここでは半割)．SLNで1カ所割を入れ(実線部)，二つに分かれた組織を包埋し，急速凍結してクライオスタットにてある程度面を削り取り(斜線部)，適切なところで2カ所の切片を作製(破線部)，術中病理診断を行う．この場合も微小転移を見落とす可能性はある．
c：serial step sectioning(ここでは7カ所で切片作製)．SLNをそのまま包埋し，急速凍結してクライオスタットにて，たとえば500 μmごとに切片を作製していく(破線部)．二つの異なる面の切片の間の組織はクライオスタットで削り取っていき，組織がなくまるまで等間隔で切片を作っていく．微小転移はほぼ確実に見つけられるが，実際的な方法ではない．

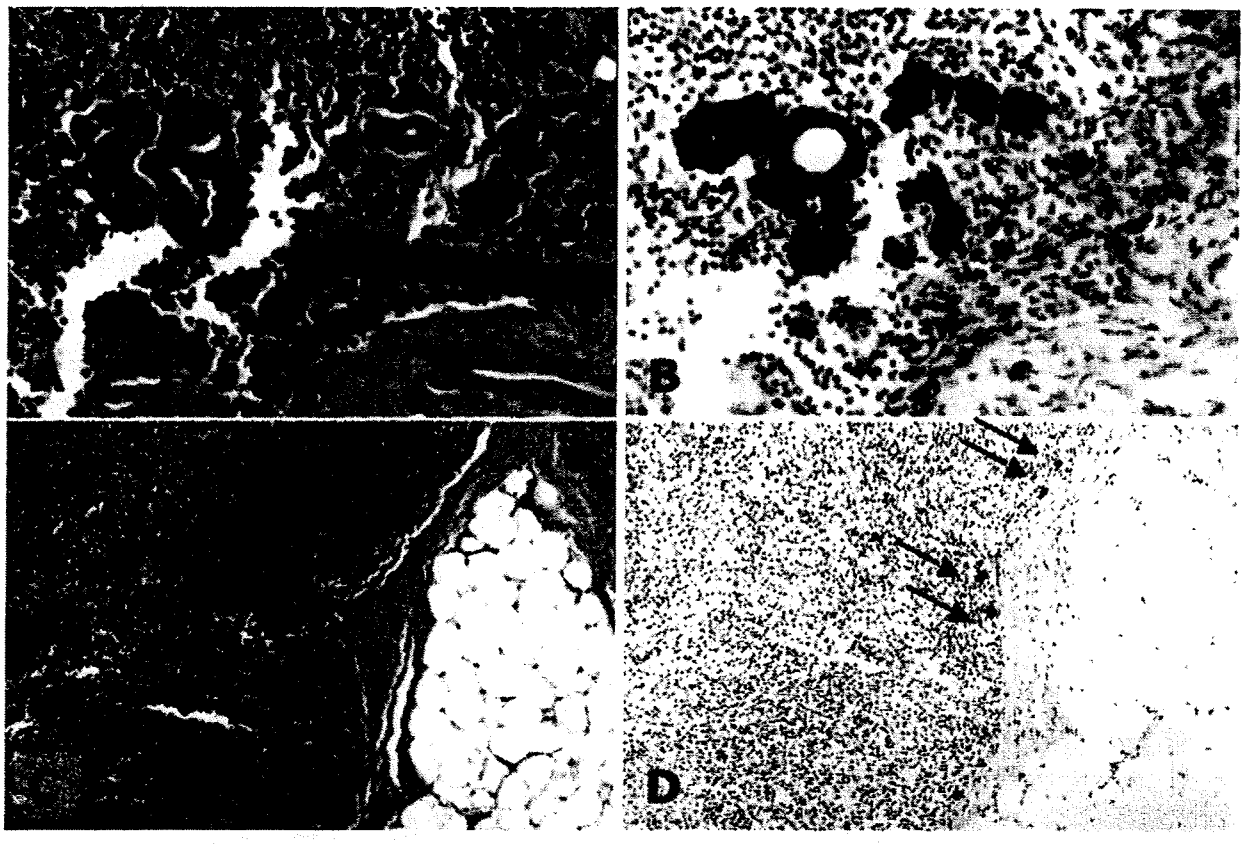

図2　センチネルリンパ節の乳癌微小転移と遊離腫瘍細胞(ITC)
A：HEで判定しにくい微小転移.
B：Aと連続的な切片で，サイトケラチン(CK)の免疫組織化学(IHC)法にて癌細胞を検出したもの．微小転移巣が黒褐色に染色され判定が容易となる．(原倍率 ×200)
C：HEで判定不可能なITC．浸潤性小葉癌の1例.
D：Cと連続的な切片にIHC法を施し，CKを検出したもの．個々バラバラに分布する癌細胞が同定された(矢印)．(原倍率 ×100)

当施設において，胃癌のSLN single sectioningによる転移の見落とし率は22%(2/9)であった.

2. slice sectioning

slice sectioningではSLNを長軸方向に平行に半割～数分割して約1～2 mm間隔で各組織片から切片を作製して検索を行う．マクロ転移の見落としはなくなり微小転移もかなり検出できるが，微小転移を見逃す可能性は残る.

2001～2004年の間に当施設でSNNSを施行した乳癌患者185名，334個のSLNに対して半割～3分割してslice sectioningによる術中病理診断を行った．139名が術中に転移陰性と診断され，そのうち転移の見落としはすべて微小転移巣であり，7名(5.0%)にみられた．真の転移陽性は46名，偽陰性率は13%(7/53)で，single sectioningによる術中病理診断成績と比べて改善している.

ASCO(American Society of Clinical Oncology)ガイドラインなどでは，SLNを最大2 mm間隔slice sectioningで病理標本を作製することを推奨している[5),6)]．この方法は理論的に2 mm径以上のマクロ転移を見逃さず，微小転移もかなりの頻度で検出できることと，実際にルーチンで行うことが可能である点から，現実的であると考えられる．今後，2 mm間隔での切り出しが普及するものとみられる.

3. serial step sectioning

serial step sectioningは，SLN全体を等間隔(間隔には50～500 μmまでさまざまな報告

がある)で，マイクロトームを用いて組織がなくなるまで連続的に組織切片を作製していく方法である[7]．国際対がん連合(UICC)のTNM分類で定義される微小転移が直径＞0.2 mm～≦2 mmであるから，微小転移を見逃さないためには200 μm間隔以下でserial step sectioningを行う必要が生じる．2 mmの厚さのリンパ節であれば10枚，5 mmの厚さのリンパ節組織であれば25枚の連続切片が作製されることになる．正確な転移診断が可能になる一方で，マンパワーの限界から通常は臨床実地で行うことは難しい．

上述のように，Veronesiらは乳癌SLNを最初の15枚の切片を50 μmごとに，その後は100 μmごとに切片を作製し，術中に検索している．彼らはこのexhaustiveな方法によってSLN当り計60枚以上の切片を作製し，術中に40～50分かけて病理学的評価を行い，転移診断の偽陰性率を5.5%に低下させたと報告している(表)[4),7)]．

4. complete serial sectioning

serial sectioningはリンパ節組織全部を連続的にマイクロトームでスライスし，全部の組織切片を作製する方法である．微小転移のみならず長径0.2 mm以下の遊離腫瘍細胞(isolated tumor cells；ITC)をも検出可能となるが，実地臨床の場で行ったという報告はない．

Ⅳ．術中迅速細胞診

この項のポイント
- 細胞診における微小転移の見落としの頻度は，組織診とほぼ同程度と考えられる．

術中迅速細胞診の場合は，摘出したリンパ節を2 mm間隔にスライスし，各割面をスライドグラスに捺印してパパニコロウ染色またはギムザ染色を行い，癌細胞の有無をスクリーニングする．残りの組織はホルマリン固定し，組織切片を作製してHE染色を行い，永久病理診断に供する[8)]．

捺印細胞診は迅速病理診断と比較して，標本作製が簡単で，検査時間が短い，薄切による組織の損失がない，などの利点がある．一方で診断に熟練を要するため，経験豊富な細胞診スクリーナが常勤していることが必須である．

細胞診における微小転移の見落としの頻度は，組織診とほぼ同程度と考えられる．細胞診のほうが転移診断の偽陰性率が低いという施設もある．尾浦らは，乳癌SLN生検の際に多数切片の術中病理診断にて転移が検出不能であったが捺印細胞診で癌細胞を同定しえた症例を4%に認めたと報告している[9)]．

Ⅴ．センチネルリンパ節の微小転移，ITCの定義と意義

この項のポイント
- 微小転移は，非SLN転移を伴う確率が比較的高い．

TNM分類第6版における乳癌所属リンパ節の病理学的分類(pN)では，微小転移(長径＞0.2 mm，≦2.0 mm)はpN 1 miと表記され，転移陽性として扱われる[10)]．一方，長径0.2 mm以下の単一の腫瘍細胞群や小さな細胞集塊はITCと定義され，転移活性の確証が得られないとして，pN 0に分類されている[10)]．ITCはおもにサイトケラチン(CK)などを検出する免疫組織化学(immunohistochemistry，IHC)法にて見つかるが，HEで検出されることもある．

現在も微小転移，ITCの臨床的意義について議論がなされている．微小転移については非SLN転移を伴う確率が比較的高いことがわ

かっている[11),12)]．非SLN転移予測因子としてのSLN微小転移巣の意義についてもいくつかの検討がなされた．Violeらは，上述のexhaustiveな術中病理検索によって，SLNに微小転移が見つかった患者の22%に非SLN転移がみられたと報告している[13)]．さらに，非SLN転移の頻度はSLN微小転移巣の長径>1 mmの例で36%，≦1 mmの例で16%であった．

私どもは臨床的にN0-N1aでSLN生検と腋窩郭清が行われた91例について，レトロスペクティブにSLN転移巣の非SLN転移に対する意義を検討した．ルーチン病理診断にて転移が見つからなかったリンパ節にはすべて100 μmごとのserial step sectioningを行い，HEとIHCを併用して転移を検索した[14)]．SLNへのマクロ転移，微小転移，ITCは各々26，10，5例にみられたが，各群における非SLN転移例数はITCを含むと各々17(65%)，4(40%)，1(20%)であり，ITCを除くと15(58%)，4(40%)，0(0%)であった．例数が少ないこと，追跡時間が短いことなどから参考にとどまるが，これらの結果はSLN微小転移の臨床的重要性を表すものと考えられる．

胃癌におけるレトロスペクティブな検討でも，SLNマクロ転移の群と，微小転移あるいはITCの群において，非SLN転移の頻度は各々60%(3/5)と0%(0/4)であった．胃癌におけるSLN転移巣の長径は非SLN転移の予測因子となりうると考えられる[15)]．

Ⅵ．免疫組織化学(IHC)の意義

この項のポイント
- HEで転移診断が困難なときは，IHCを追加することがある．

術中，永久にかかわらずSLNの病理診断にはHE染色が用いられるが，HEで転移診断が困難なときは，IHCを追加することがある．乳癌や消化器癌の細胞はサイトケラチン(CK)を発現しているため，抗CK抗体を用いたIHCにてSLNの転移診断が可能である．

IHC法により，HEで転移陰性とされた症例の7～31%でリンパ節にCK陽性の癌細胞が見つかるという報告がある[16)]．HE染色では細胞数が少なかったり異型が乏しいため観察できないような癌細胞は，IHCで簡単に発見される．乳腺の浸潤性小葉癌や胃の低分化腺癌の転移巣で細胞数が少なく個々バラバラに分布している場合は，HEで転移を見つけられないことが多い(図2)．

IHCのみで検出されるリンパ節転移巣は通常ITCであるが，ITCが予後に影響するか否かについては判定材料がない[17)]．TNM分類第6版やASCOガイドラインでは，IHCや分子レベルでのみ検出されるものは微小転移に含めるべきでないとし，それらの意義は臨床試験NSABP B-32の結果をみて決定することが望ましいとしている[5),18)]．

Ⅶ．分子生物学的手法によるセンチネルリンパ節転移の検出

この項のポイント
- 分子生物学的方法は感度がきわめて高く，将来有用な検査法である．

CK 18，CK 19は上皮細胞に陽性であり，ほかのリンパ節固有の細胞には発現されないことから，リンパ節への癌転移の優れたマーカーとなりうる．近年，分子生物学的手法により，癌細胞を含むリンパ節における癌細胞特異的分子のmRNAを検出して転移診断を行う方法が開発されている．

Schoenfeldらは，RT-PCR法により乳癌患者のSLNからCK 19 mRNAを検出し転移診

断を行ったところ，ルーチンのHE染色にて転移陰性と診断された例の15%がRT-PCRで転移陽性となったと報告している[19]．

PCRを行わず一定温度で目的の遺伝子断片を増幅できる直接遺伝子増幅法(one step nucleic acid amplification；OSNA法)が開発され，注目を集めている．OSNA法は，リンパ節を可溶化し試薬と反応させるだけで目的分子のmRNAを増幅・検出でき，約30分で転移診断を行える．現行の病理診断法と比較する多施設臨床試験においても，OSNA法は感度，特異度ともに良好であった[20]．微小転移もほぼ確実に検出できるため，従来の病理診断に代わる術中リンパ節転移検査として実用化が期待される．さらに胃癌，大腸癌でも検討が計画されている．

分子生物学的方法は感度がきわめて高く，ITCも十分検出されうる．したがって，微小転移以上が選択的に検出されるようカットオフ値の設定を厳密に行っている．ITCの意義が未だ不明なことから，ITCの解釈が変わってくればカットオフ値を変更していく必要があろう．

OSNA法では現在CK 19をマーカーに用いているが，CK 19発現陰性の乳癌がごくまれに存在し，その場合，転移診断の結果が偽陰性となる可能性がある．このような例に対しては，別のマーカーとの組み合わせなどを考慮する必要がある．また，全部のSLN組織を分子生物学的手法で用いてしまうと転移巣の長径などの情報が失われてしまうので，現時点では，組織を4等分し2片を分子生物学的手法に用い，残り2片をルーチンのHEに供して形態学的評価と合わせて判断するなどの併用法が良いと考えられる．

おわりに

SLNにおける微小転移の臨床的意義が示されたことから，現時点では病理部門の最重要課題は微小転移の見落としを減少させることであろう．そのためには，SLNの2 mm間隔切り出しなど，術中病理検索をより詳しく丁寧に行うのが最善と考える．また近い将来には分子生物学的手法の導入により，術中転移診断を行える施設の増加も期待できる．病理部門のマンパワーの問題など課題は多いが，各施設で可能な策を講じることで，術中病理診断の精度向上は十分可能であると考えられる．外科，放射線科と病理部門とが協力関係を維持して診療に当たることが肝要と考えられる．

文　献

1) Krag, D. N., Weaver, D. L., Alex, J. C., et al.：Surgical resection and radiolocalization of the sentinel lymph node in breast cancer using a gamma probe. Surg. Oncol. 2；335-339, 1993
2) Giuliano, A. E., Kirgan, D. M., Guenther, J. M., et al.：Lymphatic mapping and sentinel lymphadenetomy for breast cancer. Ann. Surg. 220；391-401, 1994
3) Chao, C., Wong, S. L., Ackermann, D., et al.：Utility of intraoperative frozen section analysis of sentinel lymph nodes in breast cancer. Am. J. Surg. 182；609-615, 2002
4) Veronesi, U., Zurrida, S., Mazzarol, G., et al.：Extensive frozen section examination of axillary sentinel nodes to determine selective axillary dissection. World J. Surg. 25；806-808, 2001
5) Lyman, G. H., Giuliano, A. E., Somerfield, M. R., et al.：American Society of Clinical Oncology guideline recommendations for sentinel lymph node biopsy in early-stage breast cancer. J. Clin. Oncol. 23；7703-7720, 2005
6) Schwartz, G. F.：Clinical practice guideline for the use of axillary sentinel lymph node biopsy in carcinoma of the breast：current