Sentinel Node and Mechanism of Lymphatic Metastasis

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The validity and clinical usefulness of the sentinel node (SN) concept for breast cancer has been confirmed, and individualized limited surgery based on diagnosis of SN metastasis is presently performed. In the future, SN navigation surgery (SNNS) will be actively applied to the treatment of early gastric cancer, and an intraoperative real-time reverse transcription-polymerase chain reaction (RT-PCR) assay to detect SN micrometastasis of gastric cancer is under development. Not only anatomical factors, but also many other factors such as local immunosuppression in the SN and lymphoangiogenesis may be involved in development of SN micrometastasis, and clarification of the mechanisms of metastasis and development of treatment methods are awaited. (*English Translation of J Jpn Col Angiol 2008; 48: 137-142.)

Keywords: sentinel node, RT-PCR, micrometastasis, lymphoangiogenesis, chemokine

INTRODUCTION

In general/gastroenterological surgery, sentinel node navigation surgery (SNNS) aiming at limiting/omitting lymph node dissection has attracted attention as minimally invasive function-preserving surgery. The sentinel node (SN) is a lymph node directly receiving lymph flow from primary tumor lesion and where lymph node micrometastasis first develops (Fig. 1). Therefore, if lymph node metastasis is absent in the SN, it is highly likely that cancer has not yet metastasized to any other lymph nodes. Pathological or molecular biological diagnosis of metastasis to the SN allows economical and time efficient diagnosis of lymph node micrometastasis. SNNS aims to limit or omit lymph node dissection in individual patients using SN mapping and the presence/absence of metastasis confirmed by SN biopsy as a parameter, and also to minimize the resection range.

For malignant melanoma and breast cancer, the validity and clinical usefulness of the SN concept have already been confirmed,1–4) and individualized limited surgery based on diagnosis of SN metastasis is presently performed. In the gastroenterological cancer field, there are still technical problems for SN identification until diagnosis of metastasis. However, for early gastric cancer among gastroenterological cancers, research of the SN concept is the most advanced, and its application to gastric function-preserving individualized limited surgery based on diagnosis of SN metastasis is expected.

1. Present Status and Prospects of the Clinical Application of SNNS in General/Gastroenterological Fields

1) Breast cancer

Many feasibility studies on breast cancer have confirmed the validity of the SN concept in breast cancer, and at present, SNNS is a standard surgical technique for cN0 breast cancer in the world.3,4) SNNS for breast cancer led to the development of an epoch-making treatment method that omits conventional axillary lymph node dissection based on results of SN biopsy, providing great benefits to patients. In cN0 breast cancer, the SN is generally present in the axilla. When SN metastasis is absent, dissection of axillary lymph nodes other than the SN can be omitted,
and this omission significantly reduced the incidences of postoperative lymphedema, sensory disturbance, and impaired elevation of the affected upper limb that had previously frequently developed.

For breast cancer compared with other solid cancers, combinations of various treatment methods such as chemotherapy, hormone therapy, radiotherapy, and molecular target therapy are possible, and new multidisciplinary treatment methods may be developed in the future. For breast cancer, pathological or molecular biological diagnosis of metastasis, concentrating on the SN, allows accurate and efficient diagnosis of lymph node micrometastasis, and multidisciplinary treatment methods based on diagnosis of lymph node metastasis and localization diagnosis are expected to be developed.

Concerning the significance of lymph node micrometastasis according to the AJCC Classification, although it was reported that lymph node micrometastasis (≤ 2 mm) does not always have clinical importance as axillary lymph node metastasis in breast cancer, further accurate evaluation is necessary. Hansen et al. performed SN biopsy in 790 patients with breast cancer and observed SN micrometastasis in 54 (6.8%) and isolated tumor cells (ITC) in 84 (10.6%), but no difference in outcomes was seen between these patients and those without SN metastasis.5)

In addition, it has been clarified that many patients with breast cancer show metastasis only to the SN. Identification of such patients and whether axillary lymph node dissection is necessary is a problem. Giuliano et al. performed a randomized controlled trial (RCT) in which patients with breast cancer with 1 to 2 SNs containing metastases underwent SN biopsy with or without further axillary lymph node dissection, and reported no differences in outcomes between the two treatment groups.6) Since the number of registered patients did not reach the planned number in their study, similar studies are necessary to evaluate the importance of axillary lymph node dissection in patients positive for SN metastasis.

2) Gastric cancer

At present in the gastroenterological field, cT1N0 gastric cancer is the most suitable target for function-preserving individualized limited surgery based on the SN status. After introduction of endoscopy-guided tracer injections and the radioisotope (RI) method, the SN identification procedure for gastric cancer has markedly improved. Many
single-center studies have been performed, and high SN identification rates (90%–100%) and metastasis detection sensitivities (85%–100%) have been reported.7–9)

To evaluate results of SN biopsy of gastric cancer, a multicenter trial was performed using a dual tracer method in which a dye and RI were submucosally injected under endoscopy (Table 1). As a result, about 400 patients were registered and the SN identification rate was 98%, sensitivity to detect lymph node metastasis was 93%, the false-negative rate was 7%, and the rate of correct diagnosis of lymph node metastasis based on the SN was 99%. These favorable results were comparable to those reported by previous single-center trials.10)

A recent meta-analysis on SN biopsies of gastric cancer (38 papers, 2128 patients) showed an SN identification rate of 94%, a correct diagnosis rate of 92%, and particularly favorable results in patients with T1. This analysis also showed that the dual tracer method (RI + dye) facilitates SN identification and has high sensitivity, and endoscopy-guided tracer injections into the submucosal layer are useful.9)

Assuming that the SN concept can be applied to early gastric cancer, early gastric cancer without SN metastasis as well as breast cancer without SN metastasis can be theoretically treated by local resection of the stomach (primary lesion) alone with complete omission of lymph node dissection. However, at present, since there are cases negative for SN metastasis, but positive for metastasis to other lymph nodes (false-negative cases), a surgical technique that has a larger safety zone and does not impair curability is necessary. Therefore, selective dissection of the lymphatic basin containing the SN (SN basin: Fig. 1) is recommended.11,12) Even when false-negative cases occur due to technical problems of SN identification and overlooked findings of intraoperative rapid cytodiagnosis, actual lymph node metastasis is present in the SN basin and can be frequently identified.13) In patients with clear SN metastasis, since the possibility that metastasis is also present outside the SN basin cannot be excluded, standard resection and dissection are necessary at present.

For future clinical application of SNNS to the treatment of early gastric cancer, improvements in the intraoperative SN identification technique and micro-metastasis diagnosis technique are indispensable. At present, we consider that SN basin dissection is necessary and development of an intraoperative real-time reverse transcription-polymerase chain reaction (RT-PCR) assay is important.

Improvements in laparoscopic surgery for gastric cancer have technically allowed the combination of endoscope-guided minimally invasive surgery and function-preserving limited surgery using SNNS (Fig. 2).11,12,14) If the validity of the SN concept for gastric cancer is confirmed, and a standard SN biopsy technique is established, limited surgery using SNNS will be actively incorporated into laparoscopic surgery for gastric cancer in the future.

2. INTRAOPERATIVE RAPID DIAGNOSIS OF SN METASTASIS OF GASTRIC CANCER

For carcinomas on the body surface such as malignant melanoma and breast cancer, regional lymph node dissection can be added later based on results of pathological

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Table 1 Standard protocol utilized in the feasibility study for sentinel node mapping in gastric cancer (Japanese Society for Sentinel Node Navigation Surgery)

<table>
<thead>
<tr>
<th>A. Indication</th>
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<tbody>
<tr>
<td>T1/T2 N0M0 gastric cancer (single lesion, no previous treatments)</td>
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<tr>
<td>Diameter of primary lesion &lt;4.0 cm</td>
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<tr>
<th>B-1. Radio-guided method</th>
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<tr>
<td>Tracer: 99m Technetium tin colloid (0.3 mCi at the time of surgery)</td>
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<tr>
<td>Administration: Endoscopic submucosal injection (0.5 mL × 4 points)</td>
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<tr>
<td>Timing of administration: the day before surgery</td>
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<tr>
<td>SN detection: Gamma probing (GPS Navigator; RMD Instruments LLC, Watertown, MA)</td>
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<th>B-2. Dye-guided method</th>
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<tr>
<td>Tracer: 1% Isosulfan blue (Lymphazurin, TycoHealth Care, Japan)</td>
</tr>
<tr>
<td>Administration: Endoscopic submucosal injection (0.5 mL × 4 points)</td>
</tr>
<tr>
<td>Timing of administration: the day before surgery</td>
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<tr>
<td>SN detection: Identification of blue stained nodes within 15 min</td>
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examination of permanent specimens of biopsied SN. However, to perform limited surgery based on diagnosis of SN metastasis for carcinomas in intraabdominal organs such as gastric cancer, intraoperative diagnosis of SN metastasis is indispensable. In conventional intraoperative rapid pathological diagnosis to detect micrometastasis of gastric cancer, slides of a few sections including the maximum cut surface were examined under a microscope. The detection sensitivity of hematoxylin and eosin (H & E) staining alone was limited. Addition of immunostaining with an anti-cytokeratin antibody was reported to increase the sensitivity in detecting SN micrometastasis. However, only a limited number of slides are examined using this immunostaining as well as H & E staining, and pathological diagnosis is difficult using frozen sections compared with paraffin-embedded sections. Therefore, the possibility that SN micrometastasis is overlooked cannot be completely excluded.

In recent years, detection of cancer cells that cannot be morphologically identified have been detected using molecular biological methods such as RT-PCR and one-step nucleic acid amplification (OSNA). These methods are generally more sensitive than conventional pathological diagnosis methods, and allow examination of not only a few slides, but also entire lymph nodes. However, in the process from mRNA extraction to RT and PCT in the RT-PCR method, contamination (false-positive results), false-negative results due to inaccurate manipulation, or poor PCR reproducibility occurs, and therefore, examination for micrometastasis using molecular biological methods requires extremely high level skills.

There have been some studies on examination for SN micrometastasis of gastric cancer using RT-PCR. Osaka et al. evaluated a total of 345 lymph nodes (150 blue-dyed nodes and 195 non-blue-dyed nodes) resected from 57 patients with early gastric cancer. Resected lymph nodes were cut into halves, with one half being used for histopathological examination, and the other being subjected to real-time RT-PCT using CEA and CK20 as markers. All lymph nodes were negative for metastasis using routine H & E staining, but 8 were positive using anti-cytokeratin staining. A total of 21 lymph nodes (10 patients) were metastasis-positive using RT-PCR. Since in the 10 patients, at least one of the blue nodes was metastasis-positive using RT-PCR, they concluded that there were no false-negative patients even at the molecular level, and blue-dyed nodes could be regarded as SNs.

Arigami et al. compared findings of histopathological examination and those of real time RT-PCR in a total of 1410 lymph nodes obtained by SN biopsy from 61 patients with ct1 or ct2N0 gastric cancer. Metastasis was positive in 5 patients (8.2%) using H & E staining of SN and in 8 (13.1%) using anti-cytokeratin staining. Of the 53 metastasis-negative patients using anti-cytokeratin staining, 13 (25%) were positive using RT-PCR of SN. Of 40 patients negative for metastasis using RT-PCR of SN, only 1 with ct2 cancer was positive using RT-PCR of non-SN nodes, i.e., false negative at the molecular level. These results show an absence of false-negative results even at the molecular level in patients with ct1N0 gastric cancer, confirming the
validity of the SN concept at the molecular level.

We also developed and previously reported a real-time RT-PCR assay using CK19, CK20, and CEA as markers for diagnosis of SN micrometastasis of gastric cancer.13) This method requires only 60–80 minutes from collection of specimens to acquisition of results, and is also useful for intraoperative rapid diagnosis. We have performed diagnosis of SN and non-SN metastasis in 103 patients who underwent SN biopsy, and observed 28 patients (27.2%) negative for pathological diagnosis of the SN and positive for RT-PCT, and 7 false-negative patients (6.8%) at the molecular level. However, all non-SNs that caused false-negative results (positive for RT-PCR) were present in the same lymphatic basin as that of the SN. Therefore, in individualized limited surgery based on diagnosis of SN metastasis, SN basin dissection may be indispensable to assure safety as the most important point.

The clinical importance of micrometastasis of cancer and ITC in early gastric cancer is still unclear, and requires further studies including those on long-term prognosis.

3. MECHANISM OF SN METASTASIS OF CANCER

It is a question whether the establishment of cancer metastasis to the SN directly receiving lymph flow from primary tumor lesions can be explained only by the anatomical reason that cancer cells invade intratumoral and peritumoral lymphatic microvessels, and are transported to the SN by lymphatic flow. The SN is lymph tissue that first receives information on tumor-specific antigens from primary tumors, and plays an extremely important role as the place of local immune responses. The reason that the SN cannot block cancer metastasis is of great interest. Although local immunity in the SN does not always reflect systemic immunosuppression by cancer, recent studies have gradually clarified the mechanism of suppression of local immunity in the SN by tumor cells for lymphogenous metastasis.15)

Nagata et al.19) observed changes in morphology and cytokines in lymph nodes at the time of the establishment of lymph node metastasis using a rat mesenteric SN model. Cancer cells migrating to lymph nodes were initially present in the marginal sinus, suggesting that the marginal sinus constitutes a mechanical barrier against cancer cell passage through the SN. Cancer cells filled the marginal sinus, and then invaded to the cortex and paracortex as if a dam had broken. Tumor necrosis factor (TNF)-α, interleukin (IL)-16, and IL-2 produced by macrophages in the SN markedly increased in the early stages of metastasis, but were inhibited with an increase in tumor size in the lymph nodes with time. They speculated that these macrophages play an important role in inhibiting establishment of SN metastasis.

In the paracortical area, there are dendritic cells and various types of lymphocytes. Their number and activity differ among individual lymph nodes.15) Recent studies comparing the SN and non-SN have shown significant decreases in the number and maturity of paracortical dendritic cells in the SN or a significant decrease in dendritic cell meshwork in the SN.20,21) Other studies have shown decreases in the number and activity of T cells in the SN compared with non-SN22) and a significant decrease in the number of high endothelial venules (HEV) through which naïve T cells pass to recognize dendrite cells expressing antigens in the SN compared with non-SN.23) These results suggest that the SN undergoes immune modulation from primary tumor lesions, resulting in an environment facilitating cancer cell survival and establishment of metastasis in the SN.

Other studies have also supported the idea that immune function in the SN is inhibited by certain signals from primary lesions and metastasis to SN is promoted. IL-10 released from primary lesions has been known to be increased in the SN, inhibiting dendritic cell maturation and migration.23) This result is also consistent with significantly higher IL-10 levels in the SN than in non-SN shown by recent studies.24–27) A recent study also indicated that SN tumor burden markedly correlated with immunosuppressive dendritic cells and Foxp3-expressing regulatory T-cells within the SN of melanoma patients.28) The data are consistent with the theory that melanoma induces expressions of specific cytokines, which in turn, stimulate immune suppressors within the SN. In particular, the study demonstrated that IL-10 and IFNγ co-regulated immunosuppressive dendritic cells within the SN.28)

In addition, expression levels of IL-13, leptin, lymphotoxin β receptor, and macrophage inflammatory protein 1b were significantly higher for tumor-positive SN compared with tumor-negative SN, and expression of IL-11Ra was significantly lower for tumor-positive SN.27) They concluded that SN indicates a different immunoregulatory cytokine profile from non-SN. Further investigations will be needed to clarify immunomodulation by tumor cells for developing nodal metastasis.

The function of intratumoral and peritumoral lymphatic vessels for cancer metastasis has been discussed
in recent years. Intratumoral lymphatic vessels may not be functional in terms of lymphatic fluid transport and cancer metastasis.\(^{15,29-31}\) However, several studies have indicated that peritumoral lymphatic vessels are more functional and important for promoting lymphatic metastasis.\(^{32,33}\) LYVE-1, podoplanin, and PROX-1 are useful markers for identifying lymphatic epithelial cells.\(^{34-36}\) Expression of these specific lymphatic markers in intratumoral and peritumoral lymphatic vessels has been known to vary heterogeneously, according to the maturity of lymphatic vessels and tumor progression.

Many recent studies have revealed the close involvement of expression of vascular endothelial growth factor (VEGF)-C/D as a lymphatic vessel growth factor in tumors and VEGF-A as a blood vessel growth factor in lymphogenous metastasis of cancer.\(^{15}\) VEGF-C and VEGF-D are known as the first specific lymphangiogenic factors among the VEGF family.\(^{37,38}\) Several studies have shown that VEGF-C or VEGF-D produced by tumor cells enable not only the induction of lymphangiogenesis, but also enhance lymphatic metastasis to SN.\(^{39-42}\) VEGF receptor (VEGFR)-3 is a lymphatic growth factor receptor among four VEGF receptors, and specifically binds to VEGF-C and VEGF-D, but not to VEGF-A.\(^{43}\) VEGFR-3 is usually expressed in the lymphatic epithelium in normal tissues.\(^{36,44}\) Activation of VEGFR-3 is known to promote lymphatic endothelial cell proliferation, migration, and cell survival through several signal pathways such as the phosphatidylinositol 3-kinase/AKT pathway.\(^{45}\) Recent studies have reported that VEGFR-3 is expressed in some types of cancer cells, and that generation of a paracrine loop involving VEGF-C and VEGF-3 may promote cancer cell survival, lymphangiogenesis, and nodal metastasis.\(^{46}\) Several studies have revealed that lymphangiogenesis and lymphatic metastasis promoted by VEGF-C or VEGF-D are significantly suppressed by blocking the VEGF-R3 signaling pathway.\(^{40,47,48}\) Skobe et al. reported that the human breast carcinoma cell line transfected with VEGF-C significantly promoted peritumoral and intratumoral lymphangiogenesis and lymphatic metastasis.\(^{40}\) Other groups have also demonstrated that another human breast carcinoma cell line, MCF-7, transfected with VEGF-C cDNA was significantly correlated with lymphangiogenesis and lymphatic metastasis in SCID mice models.\(^{42}\) Moreover, tumor-associated lymphangiogenesis promoted by VEGF-C significantly inhibited VEGFR-3 fusion protein,\(^{41}\) suggesting that the VEGFC (or VEGF-D) and VEGFR-3 pathway may be the therapeutic target of inhibiting tumor lymphangiogenesis.

Recent studies suggest that binding of VEGF-C and VEGF-D to VEGFR-2 also may stimulate lymphangiogenesis, and VEGF-A, which binds to VEGFR-2, markedly promotes tumor lymphangiogenesis.\(^{32,47}\) These results suggest that VEGF-A and/or VEGFR-2 may be therapeutic targets of inhibiting tumor lymphangiogenesis. Other molecular markers including hepatocyte growth factor, platelet-derived growth factor (PDGF), fibroblast growth factor-2, angiopoietin-1, and insulin-like growth factors 1/2 were recently identified as potent lymphangiogenic factors.\(^{43}\) However, it is still unknown whether these newly identified lymphangiogenic factors markedly induce cancer metastasis to SN.

Chemokines, grouped into CXC and CC subfamilies based on arrangement of the two NH2-terminal cysteine residues, are small secreted proteins that regulate the chemotactic response for a variety of cells.\(^{49}\) These ligands and receptors have been predominantly investigated in lymphoid cells. Of particular interest is CCL21/SLC, also referred to as 6Ckine or exodus, which is involved in recruiting CCR7 (+) naïve T-cells, natural killer cells, memory T-cells, and dendritic cells.\(^{15}\) CCL21/SLC is constitutively expressed in the HEV of lymph nodes and lymphatic endothelial cells, Peyer’s patches, thymus, spleen, and mucosal tissue.\(^{50}\) It has a high affinity for CCR7, a member of the seven transmembrane-spanning G protein coupled receptor family.\(^{51-53}\) CCR7 is prevalent in various subsets of T-cells and DC. The release of CCL21/SLC by HEV cells recruits CCR7 (+) cells to draining lymph nodes.\(^{15,50,54}\)

The concept that chemokine receptors promote organ-specific tumor metastasis was first experimentally addressed by Muller et al.\(^{55}\) They demonstrated that the chemokine receptor CXCR4 was highly expressed in human breast cancer, and its specific ligand CXCL12/SDF-1 was expressed in a variety of tissues such as bone marrow, lung, and lymph nodes where breast cancer cells preferentially metastasize. Moreover, breast cancer cell lines showed chemotactic migration to CXCL12 in vitro, and a SCID mouse model showed that experimental metastasis of a breast cancer cell line to lymph nodes is significantly inhibited by neutralizing antibodies against CXCR4. Human melanoma cells have been shown to express the chemokine receptors CCR7 and CXCR4.\(^{54,56}\) Both chemokine receptors were functional to their specific ligands, CCL21 and CXCL12/SDF-1, respectively. On the other hand, lymph nodes are known to produce the chemokines CXCL12 and CCL21. Activation of these chemokines attracts antigen-presenting cells, such as dendritic cells
and T-cells, to help an immune response in the nodes.\textsuperscript{56} We hypothesized that metastatic tumor cells may take advantage of chemokines activated in lymph nodes. To determine this, we examined SN in melanoma patients with micrometastasis and those without it.\textsuperscript{54} Our studies demonstrated that CXCL12 and CCL21 production by SN correlated with metastasis involvement. Interestingly, as the tumor burden increased in the SN, chemokines were more suppressed.\textsuperscript{54} The results suggested that metastatic tumor cells or factors may suppress chemokine production through direct or indirect mechanisms.\textsuperscript{15} These mechanisms may be similar to inflammatory responses in lymph nodes in that, after initial activation, nodes do not continually expand by recruiting immune cells. There appears to be a physiological mechanism of cells populating lymph nodes that regulate chemokine production.

Many subsequent additional studies have shown that cancer cells express chemokines and chemokine receptors, and acquired the ability to invade and metastasize by interacting with immunocompetent cells and fibroblasts in the microenvironment around them.\textsuperscript{57,58} At present, therapeutic strategies targeting the chemokine and chemokine receptor network are being evaluated.\textsuperscript{59}

CONCLUSIONS

SNNS represented by surgery without axillary dissection for breast cancer as function-preserving limited surgery provides great benefits to patients, and will be actively applied to the treatment of gastroenterological cancers, particularly early gastric cancer. For this, the development of a clinically applicable intraoperative real-time RT-PCR assay to detect SN micrometastasis of gastric cancer is urgently required.

The SN is the first place where cancer cells, as an assassin, and the host fight each other. In the establishment of SN micrometastasis, not only anatomical factors, but also many other factors, such as inhibition of local immunity and lymphoangiogenesis in the SN, are considered to be involved. Clarification of this mechanism is expected to lead to development of novel treatment methods aimed at inhibiting lymphogenous metastasis or targeting metastatic lesions in the SN.

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