Review Article

Sentinel lymph node as a target of molecular diagnosis of lymphatic micrometastasis and local immunoresponse to malignant cells

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(Received August 17, 2007/Revised October 31, 2007/Accepted November 4, 2007/Online publication December 9, 2007)

The sentinel lymph node (SLN) is defined as the lymph node(s) first receiving lymphatic drainage from the site of the primary tumor. The histopathological status of SLN is one of the most significant predictors of recurrence and overall survival for most clinical stage I/II solid tumors. Recent progress in molecular techniques has demonstrated the presence of micrometastatic tumor cells in SLN. There is now a growing body of data to support the clinical relevance of SLN micrometastasis in a variety of solid tumors. Increasing the sensitivity of occult tumor cell detection in the SLN, using molecular-based analysis, should enable a more accurate understanding of the clinical significance of various patterns of micrometastatic nodal disease. The establishment of metastasis to SLN might not be simply reflected by the flow dynamics of lymphatic fluid that drains from the primary site to the SLN, and the transportation of viable cancer cells. Recent studies have demonstrated that primary tumors can actively induce lymphangiogenesis and promote SLN metastasis. Moreover chemokine receptors in tumor cells may facilitate organ-specific tumor metastasis in many human cancers and some experimental models. In contrast, recent clinical and preclinical studies regard SLN as the first lymphoid organ to respond to tumor antigenic stimulation. SLN dramatically show morphological, phenotypical and functional changes that indicate immune suppression by tumor cells. The immune suppression in SLN results in failure of prevention or eradication of tumor metastasis. The mechanism of immunomodulation remains unclear; however, several regulatory molecules produced by tumor cells and tumor-associated macrophages or lymphocytes are likely to be responsible for inducing the immune suppression in SLN. Further studies may develop a novel immunotherapy that overcomes tumor-induced immune suppression and can prevent or eradicate SLN metastasis. (*Cancer Sci* 2008; 99: 441–450)

In the history of the surgical treatment of malignant tumors, the standard procedure has been to perform complete dissection of regional lymph nodes in addition to the primary tumor. This has been believed to improve patients' survival.[1] However, the clinical significance of prophylactic lymph node dissection for patients without lymph node metastasis has been the subject of controversy over the past 10 years.[2–4]

Given this background, the concept of the sentinel lymph node (SLN), intraoperative lymphatic mapping and sentinel lymphadenectomy appeared attractive.[5] The SLN is defined as the lymph node(s) first receiving lymphatic drainage from the site of a tumor (Fig. 1).[5] The pathological status of the SLN is thought to predict the status of all regional lymph nodes. If the SLN is recognizable and negative for cancer metastasis, unnecessary radical lymph node dissection could be avoided. The SLN hypothesis was advanced to specifically address those patients at high risk of having lymph node (LN) metastasis based on the characteristics of their primary tumors, but who had no evidence of clinically detectable regional metastatic disease.

The histopathological status of tumor-draining regional LN is one of the most significant predictors of recurrence and overall survival for most clinical stage I/II solid tumors, and is often used to justify stratification of patients for adjuvant therapy.[6,7] More efficient and accurate diagnosis of LN metastasis and prognostic information can be obtained from a small number of LN, by intraoperative lymphatic mapping and sentinel lymphadenectomy.[5,8]

SLN mapping and biopsy was first applied to melanoma, and was subsequently extended to breast cancer and, more recently, to many other solid tumors including colorectal, gastric, esophageal, gynecologic, head and neck, thyroid, urologic, and non-small cell lung cancers.[9–16] The SLN concept has revolutionized the approach to the surgical staging of both melanoma and breast cancer, and these techniques can yield patient benefit by avoiding various complications due to unnecessary prophylactic complete LN dissection in cases with negative SLN for cancer metastasis.

Hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining have been commonly used, in combination with thin serial sectioning of frozen and paraffin-embedded specimens, for the detection of micrometastatic disease in the SLN/LN.[17–19] The application of IHC has markedly improved the sensitivity of micrometastatic disease detection in the SLN/LN beyond the capability of routine H&E staining alone.[17–19] The antibodies against tumor markers of interest must be highly specific and sensitive for detection of tumor cells, and virtually non-reactive to the adjacent non-tumor cells in the SLN/LN. The most commonly used IHC target for epithelial carcinomas are the cytokeratins (CK), which are ubiquitously expressed as intermediate filaments in normal eukaryotic epithelial cells.[18–20] However, the risk of false positive results with the use of individual anti-CK antibodies and antibody cocktails has been described.[21]

In comparison, the use of IHC to detect micrometastatic deposits of melanoma has been less problematic due to the high specificity of antibodies to HMB-45 and S-100 proteins.[22] Because these antibodies also have their limitations, new antibodies such as melanoma antigens recognized by T-cells (MART-1) and microphthalmia-associated transcription factor (MITF) are being investigated.[23] IHC staining for detection of occult metastatic tumor cells in LN has been the gold standard.

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doi: 10.1111/j.1349-7006.2007.00672.x

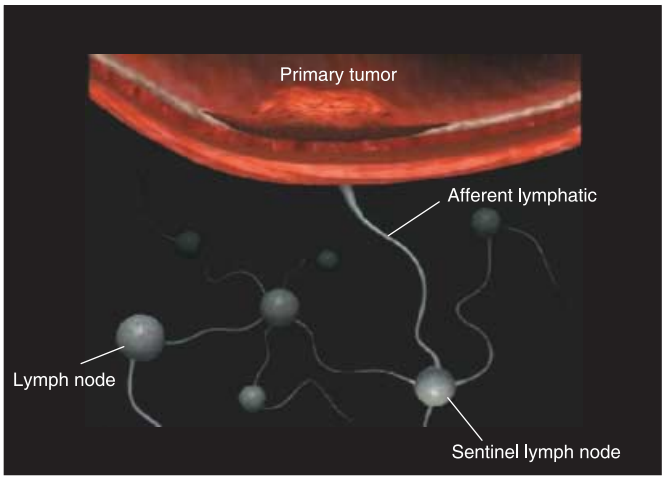

Fig. 1. Primary tumor and sentinel lymph node (SLN). The SLN is defined as one or more lymph nodes that first receive lymphatic drainage from the site of the primary tumor. For intraoperative lymphatic mapping and sentinel lymphadenectomy, blue dye and/or radioisotope-labeled colloid are injected intradermally (or submucosally) around the primary tumor site before surgery. Subsequently, the tracers pass through the afferent lymphatics, and blue-stained or radioactive nodes are regarded as the SLN.

More recently, molecular techniques have provided new approaches and demonstrated undetected metastatic tumor cells.[24]

To date, SLN have been thought to be a preferential site of initial micrometastasis of solid tumors. In contrast, recent clinical and preclinical studies regard SLN as the first lymphoid organ to respond to tumor antigenic stimulation. However, immunologic roles of SLN in the development of tumor metastasis have not been elucidated yet. Tumor-induced immune modulation of SLN may facilitate LN metastasis by inhibiting immune cell activities. This review also focuses on the immunoresponse in the SLN against tumor metastasis.

Molecular Diagnosis for Micrometastasis of SLN

The molecular detection of tumor cells using RNA or DNA markers with various polymerase chain reaction (PCR) techniques has evolved exponentially in the last decade. The primary approach of molecular detection of tumor cells has been focused on the mRNA of tumor markers using reverse transcription (RT)-PCR assay. Detection of metastatic tumor cells has been clearly demonstrated in LN, organs, and body fluids. Using RT-PCR, it is now possible to reliably detect 1–10 tumor cells within a background of 10^6–10^7 normal cells.[20] The high sensitivity of the RT-PCR assay, compared with H&E and IHC, allows the detection of the occult tumor cells among the lymphoid cells in SLN/LN. However, molecular-based techniques require the stringent optimization of sample processing, reagents, molecular targets, RT and PCR reactions, and PCR cDNA product detection assays. Meticulous attention to techniques must be adhered to, throughout all stages of the assay, in order to ensure accurate results. One of the keys to the most efficient RT-PCR assay is the quality of the detection marker. Finding a good marker is of the utmost importance in molecular detection.

Quantitative RT-PCR assay is now being used more extensively to not only identify the presence of target mRNA but also to quantify the number of mRNA copies from tumor-associated genes. Quantitative RT-PCR analysis permits the rapid molecular analysis of multiple mRNA targets expressed in tumor cells, and these results can then be correlated to clinical outcomes in order to study the relationship between gene expression levels and outcome.[25] Real-time PCR assay, which enables rapid analysis, is currently being attempted for intraoperative molecular diagnosis. LN along the recurrent laryngeal nerves obtained from patients with esophageal cancer were assessed prospectively using intraoperative histopathologic examination and real-time RT-PCR assay using multiple markers (carcinoembryonic antigen [CEA], squamous cell carcinoma [SCC] antigen, and MAGE-A3).[26] The whole procedure takes only 2.5 h from the time of tissue sampling to completion of the real-time RT-PCR assay. Genetic diagnosis by intraoperative real-time PCR assay can predict cervical LN metastasis and may be used to indicate subsequent cervical lymphadenectomy. Further improvements of the assay may allow the PCR-based intraoperative diagnosis to be applicable to other cancer surgeries. Taniyama *et al*. reported that the newly developed one-step nucleic acid amplification (OSNA) assay may allow rapid assessment for intraoperative diagnosis of LN metastasis.[27] At the present time, however, the techniques still need further validation.

The current definition of SLN/LN micrometastasis is a deposit of tumor cells measuring ≤2 mm. However, this definition has become somewhat arbitrary due to the high degree of sensitivity of IHC and RT-PCR. With the advent of increasingly more sensitive detection assays for occult metastasis, the actual definition of micrometastasis may need to be reconsidered. It has been demonstrated that the metastatic potential of individual tumor cells varies and that not all embolic tumor cells are capable of progressing to functional metastatic tumors.[28] There is also evidence to suggest that the number of tumor cells in the LN/SLN, as well as the location of nodal micrometastasis (i.e. single or a few occult tumor cells *vs* clumps of cells, and cells located within the subcapsular sinus *vs* the nodal parenchyma), may be pathologically relevant factors.[23,29] Historically, the clinicopathological relevance of micrometastatic SLN/LN disease has been unclear and controversial. There is, however, growing evidence that LN/SLN micrometastasis may indeed mean a worse prognosis in many solid cancers, including breast, melanoma, colorectal, esophageal, gastric, lung, head and neck, gynecologic, and urologic cancers.

Breast cancer. A number of mRNA targets have been studied in breast cancer, including CEA, mammaglobin 1 and 2, MAGE-A, MUC-1, C-MET, β1→4-N-acetylgalactosaminyltransferase (β1→4-GalNAc-T), β-hCG, prostate specific antigen (PSA), c-myc, prolactin inducible protein (PIP), and various CK family markers.[30–37] However, the specificity of several of these markers for breast cancer, including CEA, MUC-1, and CK-19 appears to be poor, because they sometimes show positive results for RT-PCR performed on LN and blood in healthy volunteers without breast cancer.[38–40] MAGE-A3 may be a promising breast cancer molecular marker, although as it appears to be expressed by approximately 50% of breast cancers, but is not expressed in normal mammary epithelium, or in the LN or blood of healthy volunteer donors.[31]

Although not as extensive as the studies that were performed on melanoma, there is compelling evidence to suggest a clinically relevant impact of SLN/LN micrometastasis detected using molecular assays in breast cancer (Table 1). Bostick *et al*. reported significant correlation between the presence of positive RT-PCR markers β1→4-GalNAc-T, C-MET, and P97) in the SLN and primary tumor estrogen receptor status and Bloom–Richardson histopathological grade, both of which are known prognostic factors.[30]

Wascher *et al*. assessed MAGE-A3 mRNA as a molecular marker for the detection of occult tumor cells in the SLN of breast cancer patients.[41] Serial frozen sections of SLN ($n = 121$) obtained from 77 American Joint Committee on Cancer (AJCC) stage I–IIIA breast cancer patients were assessed using RT-PCR and Southern blot analysis. Forty-one of 77 (53%) patients were positive for MAGE-A3. Interestingly, MAGE-A3 mRNA

doi: 10.1111/j.1349-7006.2007.00672.x

Table 1. Representative SLN/LN RT-PCR studies for breast cancer

Author	No. of patients	Lymph node	RT-PCR marker(s)	H&E/IHC positive (%)	RT-PCR positive (%)	Clinical correlation of micrometastasis detected using RT-PCR
Lockett (1998)[37]	35	RLN	CK-19, c-myc, PIP	0	40	Primary tumor size, decreased 5-year survival
Bostick (1998)[30]	41	SLN	β1→4-GalNAc-T, C-MET, p97	30†	95†	Estrogen receptor status, histopathological grade
Masuda (2001)[32]	129	RLN	CEA	0	31	Decreased 10-year survival
Wascher (2001)[41]	77	SLN	MAGE-A3	45	53	Infiltrating lobular carcinoma
Manzotti (2001)[33]	123	SLN	Maspin, CK-19, CEA, MUC-1, mammaglobin	33	53†	Progesterone receptor status, peritumoral vascular invasion
Sakaguchi (2003)[34]	108	SLN	CK-19, epithelial glycoprotein 2	26	30	No correlation with disease-free survival
Ouellette (2004)[35]	42	SLN	Mammaglobin B1, mammaglobin B2	40	52	ND
Gillanders (2004)[42]	489	RLN	CEA, mammaglobin, mammaglobin B, PIP, CK-19, MUC-1, PDEF	30	49	Histologic grade, St. Gallen risk category
Nissan (2006)[36]	28	SLN	CK-19, NY-BR-1, mammaglobin B	27†	50†	ND

†Percentage of total number of SLN found to be positive. β1→4-GalNAc-T, β1→4-N-acetylgalactosaminyltransferase; CEA, carcinoembryonic antigen; CK-19, cytokeratin-19; H&E, hematoxylin and eosin; IHC, immunohistochemistry; LN, lymph node; ND, not determined; PDEF, prostate-derived Ets transcription factor; PIP, prolactin inducible protein; RLN, regional lymph node; RT-PCR, reverse transcription-polymerase chain reaction; SLN, sentinel lymph node.

expression in the SLN occurred more frequently with infiltrating lobular carcinoma than with infiltrating ductal carcinoma.

Others have studied non-SLN axillary LN in breast cancer patients and have reported similar findings. Lockett *et al*. assessed 61 consecutive breast cancer patients with H&E/IHC and a multiple marker RT-PCR assay (CK-19, c-myc, and PIP).[37] A total of 15 of 37 (40%) patients with H&E/IHC-negative LN were positive by RT-PCR. An increasing number of positive RT-PCR markers significantly correlated with both increased primary tumor size and decreased predicted 5-year survival.

Masuda *et al*. evaluated 149 breast cancer patients with negative LN using both H&E and IHC evaluation.[32] RT-PCR was performed using CEA as a marker, and 40 of 129 (31%) patients were found to have RT-PCR positive LN. Patients with RT-PCR negative LN had a 10-year disease-free survival rate of 88% versus 66% for RT-PCR positive patients ($P = 0.0008$) and an overall 10-year survival rate of 94% versus 68%, respectively ($P = 0.0024$). On multivariate analysis, patients with RT-PCR-positive LN micrometastasis were found to have a hazard ratio of 3.99 for relapse and 4.29 for death due to cancer. In view of the definition of the SLN, these compelling findings in the study of axillary LN would be expected to be highly applicable with regard to the molecular status of the SLN as well.

In 2004, Gillanders *et al*. reported on the clinical relevance of molecular detection of micrometastasis in axillary LN in the results of a prospective multi-institutional cohort study for 489 patients with breast cancer.[42] Seven markers were used for real-time RT-PCR assay: CEA, mammaglobin, mammaglobin B, PIP, CK-19, muc1, and prostate-derived Ets transcription factor (PDEF). Patients who were histopathologically negative but PCR positive were significantly associated with traditional indicators of prognosis, including histologic grade and St Gallen risk category. They concluded that molecular markers could serve as valid surrogates for the detection of occult micrometastasis in axillary LN.

In general, the prognosis for breast cancer patients with early intervention is relatively more favorable than for other carcinomas. Therefore the prognostic significance of molecular detection of micrometastasis in SLN for breast cancer patients remains unclear. One major problem in evaluating the prognostic value of micrometastasis detection in SLN is that patients who had undergone sentinel lymphadenectomy are often treated with postoperative adjuvant therapy. At least 8 years are required for the follow up of a large number of patients to evaluate a significant number of events. Detection of occult tumor cells in the SLN has not shown clinical significance for patients with breast cancer to date.

Melanoma. The molecular detection of melanoma, using RT-PCR, is facilitated by the expression of melanogenesis-specific genes by melanoma tumors cells, including tyrosinase, MART-1, gp-100/pmel-17, MITF, and tyrosinase-related proteins 1 and 2 (TRP-1, TRP-2).[43–51] The expression of various mRNA transcripts of the human melanoma-associated antigen (MAGE-A) family have also been demonstrated in a variety of tumors, including melanoma and cancers of the breast and gastrointestinal tract.[31,46]

Several studies have reported the use of RT-PCR to detect micrometastatic melanoma in the SLN, and have shown that RT-PCR can significantly upstage patients with SLN that are negative by H&E and IHC (Table 2). In addition to the accurate detection of micrometastatic melanoma cells in the SLN using molecular assays, there is persuasive evidence that the detection of such micrometastases has prognostic significance. Regarding the SLN in particular, Shivers *et al*. followed 114 patients with melanoma for a mean duration of 28 months.[44] Patients with SLN that were histopathologically and RT-PCR negative had a recurrence rate of 2%, while patients with histopathologically negative but RT-PCR positive SLN had a 13% recurrence rate ($P = 0.02$).

Bostick *et al*. reported on their study of the SLN of 72 patients with early stage melanoma, using a multiple marker RT-PCR assay (tyrosinase, MART-1, and MAGE-3).[46] Bisection and serial sectioning of SLN, prior to H&E/IHC and molecular analysis, was performed to reduce the false negative rate associated with random or limited sampling of the SLN. Twenty of 55 patients (36%) with SLN negative by H&E and IHC stains were positive for at least two of the three markers in the panel (44% of these 55 patients expressed MAGE-3, 36% expressed MART-1, and 29% expressed tyrosinase). By multivariate analysis, the presence of two or more RT-PCR markers in the SLN correlated with a significantly increased risk of recurrence.

Blaheta *et al*. evaluated 214 SLN from 116 patients with melanoma using IHC and single marker (tyrosinase) RT-PCR.[47] Using H&E and IHC alone, 15 of 116 (13%) patients were confirmed to have SLN micrometastasis. Of the remaining 101 patients with histopathologically negative SLN, 36 (36%) SLN were positive by RT-PCR for tyrosinase. Of the 15 patients with histopathologically detected SLN micrometastases, 10 (67%)

Table 2. Representative SLN/LN RT-PCR studies for melanoma

Author	No. of patients	Lymph node	RT-PCR marker(s)	H&E/IHC positive (%)	RT-PCR positive (%)	Clinical correlation of micrometastasis detected by RT-PCR
Goydos (1998)[43]	45	SLN	Tyrosinase, MART-1	22	29	ND
Shivers (1998)[44]	114	SLN	Tyrosinase	20	61	Decreased disease-free and overall survival
Blaheta (1999)[45]	73	SLN	Tyrosinase	18	49	Primary tumor thickness
Bostick (1999)[46]	72	SLN	Tyrosinase, MART-1, MAGE-A3	24	50†	Increased risk of recurrence
Blaheta (2000)[47]	101	SLN	Tyrosinase	0	36	Increased risk of recurrence
Ribuffo (2003)[48]	134	SLN	Tyrosinase, MART-1	11	63	Decreased disease-free survival
Goydos (2003)[49]	175	SLN	Tyrosinase	19	58	Increased risk of recurrence
Kuo (2003)[50]	77	SLN	Tyrosinase, MART-1, TRP-1, TRP-2	48	55†	Decreased disease-free and overall survival
Morton (2003)[51]	215	SLN	Tyrosinase, MART-1, TRP-2, MITF	25	47	Decreased disease-free and overall survival

†Percentage of two or more markers positive. H&E, hematoxylin and eosin; IHC, immunohistochemistry; LN, lymph node; MART, melanoma antigens recognized by T-cells; MITF, microphthalmia-associated transcription factor; ND, not determined; RT-PCR, reverse transcription-polymerase chain reaction; SLN, sentinel lymph nodes; TRP, tyrosinase-related proteins.

Table 3. Representative SLN/LN RT-PCR studies for gastrointestinal cancers

Author	Tumor	No. of patients	Lymph node	RT-PCR marker(s)	H&E/IHC positive (%)	RT-PCR positive (%)	Clinical correlation of micrometastasis detected by RT-PCR
Noguchi (1996)[63]	Gastric	12	RLN	CK-19	7	21	ND
Ichikawa (1998)[55]	Colon	15	RLN	MMP-7	19	26	ND
Kijima (2000)[64]	Esophagus	21	RLN	CEA	52	86	ND
Bernini (2000)[57]	Colorectal	43	RLN	MUC-2	0	28	Advanced T-factor
Bilchik (2001)[11]	Colorectal	40	SLN	β-hCG, C-MET, MAGE-A	35	60	Advanced T-factor
Yoshioka (2002)[26]	Esophagus	50	RLN	CEA, SCC, MAGE-A3	20 (intraoperative diagnosis)	48 (intraoperative diagnosis)	Predict cervical LN metastasis
Noura (2002)[58]	Colorectal	64	RLN	CEA	55	30	Decreased disease-free and overall survival
Matsuda (2004)[66]	GI cancers	51	SLN	CK-19	25	45	ND
Arigami (2006)[65]	Gastric	53	SLN	CEA	0	25	ND

CEA, carcinoembryonic antigen; CK-19, cytokeratin-19; H&E, hematoxylin and eosin; IHC, immunohistochemistry; LN, lymph node; MMP, matrix metalloproteinase; ND, not determined; RLN, regional lymph node; RT-PCR, reverse transcription-polymerase chain reaction; SCC, squamous cell carcinoma antigen; SLN, sentinel lymph node.

had recurrence, compared with an overall recurrence rate of 20% among all 116 patients. During a 19-month median follow-up period, the recurrence rate for patients with RT-PCR-positive SLN was 25%, and the recurrence rate in patients with negative SLN using H&E, IHC and RT-PCR was only 6% ($P = 0.01$). By multivariate analysis, histopathological and RT-PCR SLN tumor status were the only significant predictors of disease-free survival.

Goydos *et al*. studied 175 patients with stage I or II melanoma using single marker (tyrosinase) RT-PCR.[49] At a median follow up of 34 months, 17 of 34 (50%) patients with histologically positive SLN had a recurrence. Of the 141 patients with histologically negative SLN, 73 patients were negative for tyrosinase using RT-PCR, and none of these patients had a recurrence. Of the 68 patients with histologically negative but RT-PCR-positive SLN, 14 patients (21%) had a recurrence.

Morton *et al*. assessed the paraffin-embedded SLN of 215 patients with AJCC stage I/II melanoma using a multiple marker quantitative real-time PCR assay.[51] Tyrosinase, MART-1, TRP-2 and MITF were used as specific mRNA markers. Among 162 patients with histopathologically negative SLN, 49 (30%) patients showed PCR-positive and were upstaged. These patients had a significantly increased risk of disease recurrence and death compared with both histopathology and PCR-marker-negative patients using multivariate analysis. These studies demonstrated the clinicopathological utility of detecting micrometastatic melanoma in SLN.

The 5-year survival rate approaches 90% for patients with AJCC stage I malignant melanoma, and 70% for stage II melanoma, but decreases significantly to 25–50% for stage III melanoma. Therefore, accurate staging is highly important for optimal management of early stage disease. The clinicopathological relevance of micrometastatic melanoma in SLN detected using RT-PCR assay is still controversial, because melanoma mRNA markers for RT-PCR assay are often expressed in melanocytes or nevus cells. A recently reported large-scale multicenter study failed to prove the clinical relevance of molecular upstaging using RT-PCR in patients with melanoma.[52] A series of previous studies that reported prognostic significance of micrometastatic melanoma in SLN detected using RT-PCR, however, may show the clinical significance of molecular detection of micrometastasis in SLN.[53] Future investigations will validate the clinicopathological importance of micrometastatic melanoma in SLN.

Colorectal cancer. The application of molecular analysis to the SLN in colorectal cancer (CRC) is currently at an early stage (Table 3). However, with the recent and successful application of the SLN concept to this disease, preliminary data from molecular-based assays is now being generated. Molecular markers for SLN/LN analysis in CRC studied so far include CK, CEA, MAGE-A, C-MET, β-hCG, MUC-2, and matrix metalloproteinases.[11,31,54–58]

CRC is being studied to evaluate the prognostic impact of micrometastatic SLN/LN disease. Liefers *et al*. evaluated 192

doi: 10.1111/j.1349-7006.2007.00672.x

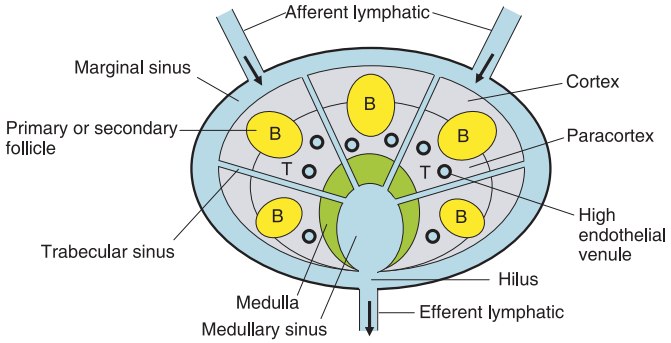

Fig. 2. Schema of lymph node. B, B-cell area; T, T-cell area.

LN from 26 stage II CRC patients using nested RT-PCR and CEA as a molecular marker.[56] In this study, 14 of 26 (54%) patients had LN that were RT-PCR positive. The 5-year survival rate for these 14 patients was 50%, while the survival rate among the remaining 12 patients was 91% ($P = 0.03$).

Bernini *et al*. recently studied the LN of 43 CRC patients, using MUC-2 as a molecular target for RT-PCR.[57] They found a correlation between RT-PCR LN positivity and the size of the primary tumor. None of the 10 Tis/T1 tumors and one of six (17%) T2 tumors were shown to be LN positive using RT-PCR, while 10 of 25 (40%) T3 tumors and one of two (50%) T4 tumors were positive using RT-PCR. These results are of clinical significance, because primary tumor T-stage is a known prognostic factor for CRC.

Interim results from the first multicenter phase II trial evaluating the molecular staging of the SLN in early colon cancer (clinical stage I/II) were recently reported by Bilchik *et al*.[11] Forty patients with histopathologically negative (by H&E) SLN were assessed using IHC for CK, and using a multiple-marker RT-PCR panel (β-hCG, C-MET, and MAGE-A3). In 10 (25%) cases, the SLN was positive by H&E. In four (10%) cases, the SLN were positive by IHC and negative by H&E. Of the remaining 26 patients with negative SLN by H&E and IHC, 12 (46%) were positive for at least two RT-PCR markers. This study also demonstrated a correlation between the number of markers detected and the tumor T-stage, which is, by itself, a significant prognostic factor for colon cancer. These results suggest that molecular staging can be successfully and meaningfully applied to the SLN in CRC, in addition to melanoma and breast cancer.

Noura *et al*. recently reported a comparative study of detection of micrometastasis using IHC and RT-PCR assay in H&E-negative LN of 64 AJCC stage II CRC patients.[58] CEA was used for RT-PCR assay and compared to an IHC study with anti-CK antibody. Micrometastases were detected in 19 of 64 (30%) patients using RT-PCR and in 35 of 64 (55%) patients using IHC. Patients who were PCR-positive in LN showed significantly worse disease-free and overall survival than PCR-negative patients. However, micrometastasis in LN using IHC did not correlate with prognosis. Although a larger prospective study may be required for the validation of the assay, the results suggested the prognostic utility of molecular detection of micrometastasis in LN/SLN of CRC patients.

Other carcinomas. Gastric, esophageal, prostate, biliary, head and neck, lung and gynecologic cancers have also been upstaged following RT-PCR analysis of LN/SLN and, in many cases, these results have correlated with known prognostic factors (Table 3).[14,59–66] Arigami *et al*. reported that 13 of 53 (25%) gastric cancer patients with histopathology-negative SLN were upstaged using RT-PCR assay.[65] They concluded that the SLN concept is applicable to patients with cT1 and cN0 gastric cancer, even when including the molecular diagnosis of micrometastasis. Other groups also suggested that nodal micrometastasis detected using RT-PCR assay has some clinical significance in gastrointestinal cancers.[66] Molecular assessment of the SLN may be a variable tool to complement histological examination for gastrointestinal cancers.

Immunoresponses in the SLN against Tumor Metastasis

The SLN are also known to be the first lymphoid organ to respond to tumor antigenic stimulation. The SLN is the site where immunoreactive lymphocytes initially encounter tumor-specific antigens and develop antitumor immunity. The SLN may have critical roles in the development of local immunity that could reject and eradicate metastatic cancer cells. Immune dysfunction in the SLN does not directly reflect generalized immune suppression against cancer. However, there are many possible mechanisms that may explain the reasons why the SLN has limited capability to prevent cancer metastasis.

Nagata *et al*. have made a metastasis model in the rat mesenteric LN, and visualized the migration of cancer cells *in vivo*.[67] Migrant cancer cells were initially arrested in the marginal sinus in the tumor-draining LN; therefore the marginal sinus was supposed to constitute a mechanical barrier against cancer cell passage (Fig. 2). The cancer cells filled the marginal sinus, and no cancer nests were found in the cortex, paracortex, or medulla before the marginal sinus was filled. Cancer cells subsequently invaded to the cortex and paracortex over the inner linings of the marginal sinus. Cytokines such as tumor necrosis factor-α, interleukin (IL)-1β and IL-2 secreted by macrophages markedly increased at the early stages of metastasis, but gradually decreased according to the tumor proliferation in the LN. They suggested that parasinus macrophages may play a crucial role in the transient antimetastatic capability of the nodes, and deterioration of cytokine induction may be responsible for subsequent cancer proliferation.

Individual LN show the heterogeneous reactivity, frequency, and density of T-cells, dendritic cells (DC), and other lymphocytes in the paracortical area. Cytokine generation and cytotoxicity against tumor cells might vary among individual nodes. Cochran *et al*. have extended a series of studies to compare the cellular phenotype and physiology of metastasis-susceptible SLN with non-SLN from the same patient in melanoma and breast cancer.[68] They demonstrated that the immunoreactivity of SLN is entirely or segmentally down-regulated compared with non-SLN, that is to say that SLN are likely to be immune-modulated by tumor cells. Tumor-induced down-regulation of SLN immunity is certainly related to the survival of tumor cells and the development of clinically significant metastasis in SLN.

The cancer cells in the primary site produce immunomodulators that can lead to immunosuppression of the SLN affected via the direct lymphatic drainage pathway from the primary tumor (Table 4). In contrast, non-SLN in the regional basin are regarded to be less affected by the immunomodulators from the primary tumor site. The down-regulation of immunoreactivity in SLN is particularly obvious in the density and maturity of paracortical DC (PDC) and T-cells.[51] Huang *et al*. reported a significant reduction in the aggregate area of the paracortex occupied by PDC, and less frequency, density and maturity of PDC in the SLN compared with non-SLN in studies of various solid tumors, in particular breast cancer and melanoma.[69] DC with long dendrites are considered to be more effective at antigen presentation; however, SLN show dendritic cells with short or no dendrites that are likely to reflect the down-regulation of antigen presentation. Cochran *et al*. also found that the formation of dendritic meshworks that are related to the interaction between mature DC and T-cells was markedly reduced in SLN compared with non-SLN.[68]

Table 4. Representative factors that relate to sentinel lymph node metastasis produced by tumor cells

Factors	Molecules
Cytokines	IL-6, IL-8, IL-10, TGF-α, TGF-β
Growth factors	VEGF-A, VEGF-C, VEGF-D, basic fibroblast growth factor, platelet-derived growth factor, placental growth factor, hepatocyte-growth factor
Chemokine receptors	CCR7, CCR10, CXCR4

IL, interleukin; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

Huang *et al*. showed that T-cell density and activation markers for T-cells are markedly reduced within the paracortical area in the SLN compared with the non-SLN.[70] Naïve T-cells, which arrive to the paracortical area through the endothelium of high endothelial venules (HEV), are known to encounter antigen-presenting DC in the paracortex. Recent studies have demonstrated that HEV in SLN are fewer than in non-SLN, and also transendothelial migration of naïve T-cells is markedly reduced in SLN.[71]

Other studies also supported the concept that primary tumors enable suppression of the immune functions in the SLN and facilitate the development of LN metastasis. In SLN an increased level of IL-10, which is secreted from the primary tumor, markedly inhibits DC maturation, migration and translocation of major histocompatibility complex (MHC)-class II peptide complex into the plasma membrane of DC.[72] This result is consistent with other reports that IL-10 concentrations in SLN are significantly higher that those in non-SLN.[73–76]

Torisu-Itakura *et al*. investigated the nodal profile of immunoregulatory cytokines using cDNA microarray to confirm the identity of the SLN and non-SLN.[76] As a result, 57 genes were expressed at significantly different levels in SLN and non-SLN. The expression levels of IL-13, leptin, lymphotoxin β receptor, and macrophage inflammatory protein 1b were significantly higher for tumor-positive SLN and compared with tumor-negative SLN, and the expression of IL-11Ra was significantly lower for tumor-positive SLN. They concluded that SLN show a different immunoregulatory cytokine profile from non-SLN. Further investigations will be needed to clarify immunomodulation by the tumor cells for the development of nodal metastasis.

New Insights into the Mechanism of SLN Metastasis

Recent advances in molecular oncology have shown that various factors such as oncogenes, tumor suppressor genes, growth factors, apoptotic factors, adhesion molecules, angiogenic factors, and cytokines are related to the development of tumor metastasis. The SLN is directly situated in the lymphatic drainage pathway from the primary tumor site. Therefore the establishment of metastasis to SLN may be simply reflected by the flow dynamics of lymphatic fluid that drains from the primary site to the SLN, and the transportation of viable cancer cells.

However, recent studies have demonstrated that primary tumors can actively induce the formation of lymphatic vessels from host vessels, and that lymphangiogenesis is correlated with enhanced (sentinel) LN metastasis in various human carcinomas and some experimental models.[77–81] Moreover, tumor cells can induce peritumoral lymphangiogenesis and lymphatic vessel growth within SLN before and after they metastasize to the SLN, likely promoting further development of cancer metastasis.[82,83]

The functionality of intratumoral and peritumoral lymphatic vessels for cancer metastasis has been discussed in recent years. Intratumoral lymphatic vessels might not be functional with regard to lymphatic fluid transport and cancer metastasis.[81,84,85] However, several studies have shown that peritumoral lymphatic vessels are more functional and important for promoting lymphatic metastasis.[83,84,86] LYVE-1, podoplanin, and PROX-1 are useful markers for identifying lymphatic epithelial cells.[87–89] The 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.

Vascular endothelial growth factor (VEGF)-C and VEGF-D are, among the VEGF family, known as the first specific lymphangiogenetic factors.[90,91] Many studies have shown that VEGF-C or VEGF-D produced by tumor cells enable not only to induce lymphangiogenesis but also to enhance lymphatic metastasis to SLN.[77,78,84,92,93] VEGF receptor (VEGFR)-3 is a lymphatic growth factor receptor of four VEGF receptors, and specifically binds to VEGF-C and VEGF-D, but not to VEGF-A.[94] VEGFR-3 expression is restricted to the lymphatic epithelium in normal tissues; however, some tumor blood vessels also express VEGFR-3.[89,95] 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.[96] 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 VEGFR-3 may promote cancer cell survival, lymphangiogenesis and LN metastasis.[97]

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.[78,79,98] Skobe *et al*. reported that the human breast carcinoma cell line transfected with VEGF-C significantly promoted peritumoral and intratumoral lymphangiogenesis (but had no effect on angiogenesis) and lymphatic metastasis.[78] Other groups have also showed 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.[93] Moreover the tumor-associated lymphangiogenesis promoted by VEGF-C significantly inhibited VEGFR-3 fusion protein,[92] suggesting that the VEGF-C (or VEGF-D) and VEGFR-3 pathway may be the therapeutic target of inhibiting tumor lymphangiogenesis.

Recent studies also suggest that binding of VEGF-C and VEGF-D to VEGFR-2 may stimulate lymphangiogenesis, and VEGF-A, which binds to VEGFR-2, markedly promotes tumor lymphangiogenesis.[83,98] These results suggest that VEGF-A and/or VEGFR-2 may be another therapeutic target of inhibiting tumor lymphangiogenesis. Other molecular markers including hepatocyte growth factor, fibroblast growth factor-2, platelet-derived growth factor (PDGF), angiopoietin-1, and insulin-like growth factors 1/2, were recently identified as potent lymphangiogenetic factors.[94] However, it is still unknown whether these newly identified lymphangiogenetic factors markedly induce cancer metastasis to the SLN.

There is significant evidence that tumors of specific histology preferentially metastasize to LN.[99,100] This preferential metastasis cannot be explained simply by the lymphatic drainage pattern from the tumor. The observation of an orderly, systematic targeting of organs by metastatic breast cancer led Paget to hypothesize the 'seed and soil' theory of cancer metastasis.[101] In this model, organs that provide suitable environmental conditions for cancer growth are the preferential sites of cancer metastasis. Since Paget's original report more than one century ago, others have attempted to test, challenge, or supplement this theory. Recently, a novel mechanism for cancer metastasis has emerged that highlights the role of chemokines. There is evidence that antigen-presenting cells such as DC, T-cells, Langerhans cells, and

doi: 10.1111/j.1349-7006.2007.00672.x

natural killer (NK) cells bearing chemokine receptors migrate from skin to the draining LN in response to specific chemotactic factors referred to as chemokines.[102–107] In this signaling 'homing' mechanism, SLN produce and release specific chemokines that attract cancer cells bearing specific corresponding receptors in primary sites. The recent demonstration of specific chemokine receptors on tumor cells and respective chemokines has provided some insight into how tumor cells may home to SLN. Chemokine receptors have been suggested to play a pivotal role in regulating the recruitment of solid tumor cells to SLN.[108]

Chemokines, grouped into CXC and CC subfamilies based on the arrangement of the two NH_2-terminal cysteine residues, are small secreted proteins that regulate the chemotactic response for a variety of cells.[106] These ligands and receptors have been predominantly investigated on lymphoid cells. Of particular interest is CCL21/SLC, also referred to as 6Ckine or exodus, which is involved in recruiting CCR7(+) naïve T-cells, NK, memory T-cells, and DC.[102–107] CCL21/SLC is constitutively expressed in the HEV of LN and lymphatic endothelial cells, Peyer's patches, thymus, spleen and mucosal tissue.[105,109] It has a high affinity for CCR7, a member of the seven transmembrane-spanning G protein coupled receptor family.[110–112] CCR7 is prevalent in various subsets of T-cells and DC.[103,110–112] The release of CCL21/SLC by HEV cells recruits CCR7(+) cells to draining LN.[103,107,109]

The concept that chemokine receptors promote organ-specific tumor metastasis was first experimentally addressed by Muller *et al*.[113] 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 LN where breast cancer cells preferentially metastasize. Moreover, breast cancer cell lines enabled to show chemotactic migration to CXCL12 *in vitro*, and a SCID mouse model showed that experimental metastasis of a breast cancer cell line to LN is significantly inhibited by neutralizing antibodies against CXCR4.

Human melanoma cells have been shown to express the chemokine receptors CCR7 and CXCR4.[114,115] The expression of these receptors is variable among melanomas, as shown by molecular analysis, both in cell lines and in microdissected tumor tissues.[114,115] Both chemokine receptors were shown to be functional to their specific ligands, CCL21 and CXCL12/SDF-1, respectively. To further examine the role of these chemokine receptor-ligands in metastasis, SLN were assessed because metastasis often occurs initially at these proximal tumor-draining LN.[114] LN are known to produce the chemokines CXCL12 and CCL21. Activation of these chemokines attracts antigen-presenting cells, such as DC and T-cells, to help orchestrate an immune response in the nodes.[115,116] We hypothesized that metastatic tumor cells may take advantage of chemokines activated in LN. To determine this, we examined SLN in melanoma patients with micrometastasis and those without it. Our studies demonstrated that CXCL12 and CCL21 production by SLN correlated with metastasis involvement. Interestingly, as the tumor burden increased in the SLN, chemokines were more suppressed.[114] The results suggested that metastatic tumor cells or factors may suppress chemokine production through direct or indirect mechanisms. These mechanisms may be similar to inflammatory responses in LN in that, after initial activation, the nodes do not continually expand by recruiting immune cells. There appears to be a physiological mechanism of cells populating LN that regulate chemokine production.

Wiley *et al*. showed that the functional expression of CCR7 enhances the metastasis of B16 murine melanoma to SLN compared with control, and that the metastasis is inhibited by neutralizing antibodies against CCL21.[117] Other groups have demonstrated that CCR7-positive cancer cells significantly correlated with a high incidence of LN metastasis in gastric[118] and esophageal carcinomas.[119]

Therapeutic Implications for SLN Metastasis

Malignant melanoma is one of the candidates for the investigation of immunotherapy because it is clinically resistant to chemotherapy and radiotherapy, and expresses many kinds of immunogenic molecules. To date, *Mycobacterium bovis* Bacillus Calmette-Guérin, IL-2, type II interferon (IFN), and granulocyte–macrophage colony-stimulating factor (GM-CSF) have been reported to have antitumor effects for melanoma after intratumoral injection.[120–123] GM-CSF has been supposed to provide an antitumor effect by acting on DC, T-cells, and macrophages. GM-CSF is known to cause mature DC to migrate to regional LN and increase their resistance to apoptosis,[124] and also to induce T-cell mediated antitumor immunity by activated DC. Vuylsteke *et al*. reported that preoperative peritumoral injections of GM-CSF resulted in enlargement of DC and T-cell areas in the SLN.[125] These results suggest that GM-CSF may have the potential to prevent or eradicate tumor metastasis in the SLN. Many other molecules, including IL-13 and IFN-α, have been reported to be candidates for immunomodulation in the SLN.[126,127] Further studies will prove the clinical significance of these immunomodulators for the treatment of SLN metastasis.

Several studies have shown that the VEGFR-3 and/or VEGFR-2 pathway might be the therapeutic target of inhibiting tumor lymphangiogenesis and cancer metastasis to SLN. To date, several antibodies and molecules including anti-VEGFR-3 antibody, anti-VEGF-C antibody, anti-VEGF-D antibody, soluble VEGFR-3 fusion protein, small interfering RNA to reduce VEGF-C mRNA expression, and a number of small molecule kinase inhibitors of VEGFR-2 have been investigated in animal models.[79,92,94,98,128] Most of the studies using these antibodies or molecules have demonstrated that specific inhibition of the VEGFR-3 and/or the VEGFR-2 pathway markedly reduces lymphangiogenesis and lymphatic cancer metastasis, and likely also reduces the incidence of distant organ metastasis.

Chemokine receptors will be also as a target of therapeutic intervention using antibodies or small molecule inhibitors. Anti-CXCR4 monoclonal antibody significantly inhibits the metastasis of human breast carcinoma cells to the LN of SCID mice.[92] Systemic administration of the CXCR4 antagonist AMD3100, a potent blocker of HIV cell entry, inhibited the growth of intracranial glioblastoma and medulloblastoma xenografts by inducing tumor cell apoptosis.[129] However, systemic inhibition of CXCL12-CXCR4 signaling may have adverse effects on the hematopoietic stem cells, primitive germ cells and neural precursors.[130]

Conclusion

The development of the SLN concept has radically altered the field of diagnosis and treatment of many solid tumors. As this paradigm shift receives validation from melanoma studies, greater attention on the histopathological microstaging of the SLN formalized the concept of LN/SLN micrometastasis. The use of serial sectioning and IHC analysis, and more recently, the use of RT-PCR, has enabled investigators to further study the potential clinical significance of micrometastatic LN/SLN disease. There is now a growing body of data to support the clinical relevance of LN/SLN micrometastasis in a variety of solid tumors. Increasing the sensitivity of occult tumor cell detection in the SLN, using molecular-based analysis, should enable a more accurate understanding of the clinical significance of various patterns of micrometastatic nodal disease. In the future, molecular staging of SLN should benefit and improve patient management.

Lymphangiogenesis and the 'chemokine-chemokine receptor network' are responsible for promoting lymphatic cancer metastasis. Metastasis to SLN might not be simply reflected only by the flow dynamics of lymphatic fluid that drains from the primary site to the SLN, but also by the diplomatic and active behavior of cancer cells. SLN dramatically show morphological, phenotypical and functional changes that indicate immune suppression by tumor cells. The immune suppression in SLN results in failure of prevention or eradication of tumor metastasis. The mechanism of immunomodulation remains unclear; however, several regulatory molecules produced by tumor cells and tumor-associated macrophages or lymphocytes are likely to be responsible for inducing immune suppression in the SLN. Further preclinical and clinical studies may achieve the reversal of tumor-induced immune suppression that can prevent or eradicate LN metastasis.

Acknowledgments

The authors are indebted to Professor J. Patrick Barron of the International Medical Communications Center of Tokyo Medical University for his review of this manuscript.

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doi: 10.1111/j.1349-7006.2007.00672.x