The Usefulness of a Fluorescent Technique Using Indocyanine Green for Evaluating Patterns of Lymph Flow and Facilitating Sentinel Node Biopsy in Patients with Skin Cancer

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Running title: SLNB with ICG method for skin cancer

Abstract

Background: Sentinel lymph node biopsy (SLNB) is an established minimally invasive procedure for detecting micro-metastasis in regional nodes in patients with cutaneous malignancies, including malignant melanoma. Because in some cases they cannot be detected and in others regional metastases are found despite negative sentinel lymph nodes (SLNs), a more accurate means of detecting them is needed.

Methods: We evaluated the usefulness of a fluorescent technique using indocyanine green (ICG) in combination with conventional tracers, namely blue dye and radioisotopes, for SLNB in 26 patients with skin cancers. Furthermore, we evaluated the correlation of primary site and the anatomic pattern of lymph flow draining into basin using ICG technique.

Results: In 19 cases (73%), more SLNs were detected with ICG than with the conventional tracers. The average number of SLN using conventional tracers with or without ICG was 3.9 versus 2.9 per case, respectively ($p < 0.01$) and 3.3 versus 2.4 per basin, respectively ($p < 0.01$). The average number of basins detected per case was 1.24

and 1.08, respectively (p = 0.043). We found a single flow in subjects with distal limb primary lesions, whereas subjects with proximal limb and trunk lesions tended to have more than two lymph flows draining into basins (p = 0.002).

Conclusion: The ICG technique may minimize overlooking of SLNs in patients with lesions on sites with multiple lymph flows such as the proximal limb and trunk, as well as head and neck.

Key words: Skin cancer, Sentinel node biopsy, Indocyanine green, Lymph flow

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Introduction

Sentinel lymph node biopsy (SLNB) is a minimally invasive procedure aimed at detecting micro-metastases in regional nodes of patients with skin cancers and thus determining whether to perform lymph node dissection. SLNB is widely performed in patients with cutaneous malignant melanoma (MM) [1]. It is also reportedly useful in patients with other skin cancers such as cutaneous squamous cell carcinoma (cSCC), Merkel cell carcinoma (MCC), and extramammary Paget's disease (EMPD) [2-5]. Conventional SLNB has mainly been performed using a combination of blue dye (BD) and radioisotope (RI) as tracers, the detection rate being about 95% [6]. But, in remaining 5% cases, no SLN were detected and some cases acquired lymph node metastasis after the negative SLNB, which was considered to be false negative [7-10]. A more reliable method of SLNB is needed to minimize such patients.

Indocyanine green (ICG), a reagent that generates near-infrared fluorescence, is widely used in various procedures such as retinal angiography. Intradermal ICG injection

enables visualization of lymph flows and nodes through the skin by using a near-infrared camera.

Recently, a few researchers have reported that using ICG in patients with skin cancer is safe and effective in the detection of SLNBs [11-17], however, this procedure has not yet been widely accepted. We retrospectively evaluated the efficacy of a combination of ICG and conventional tracers in detecting SLNBs in patients with skin cancers and also assessed patterns of lymph flow by region of primary lesion, which may be one of the factors of the false negative results.

Materials and Methods

Patients

Patients with pathological diagnoses of skin cancer (MM, cSCC, MCC, EMPD) and evidence of dermal or subcutaneous invasion on incisional or excisional biopsies at the Fukuoka University Hospital between August 2012 and January 2015 were enrolled in this study. Cases of MM with clinically obvious invasion were eligible without biopsy, whereas cases with clinically apparent lymph node metastases or known distant

metastases before SLNB were excluded. Informed consent was obtained from all patients prior to SLNB. This study was approved by the Institutional Review Board of Fukuoka University Hospital (approval number 13-8-01).

Twenty-six patients (mean age, 64 years; range, 36–85 years) underwent SLNB with ICG in combination with BD and RI. Fifteen of them had MM, eight cSCC, two EMPD and one MCC. The primary tumor was located on the head and neck in two patients, trunk in three, extremities in 18, and genitals in three (Table 1).

Sentinel lymph node biopsy

SLNB was performed using ICG as an additional tracer in combination with BD and RI. First, a total of 0.4–0.6 mL of an RI tracer, Tc^{99m}-labeled phytic acid, was injected intradermally into four to six sites around the primary tumor or scar 1 day before planned surgery. The location of the RI tracer was scanned as dynamic image immediately after injection, and subsequently as static image (3 h after injection) by lymphoscintigraphy. Intraoperatively, a total dose of 0.6–0.8 mL of 2% patent blue solution and ICG were injected intradermally into six to eight sites around the primary

tumor or scar. Immediately thereafter, the flow and accumulation of ICG were visualized with a near-infrared camera (PED neo; Hamamatsu Photonics, Hamamatsu, Japan) on a display monitor and marked with a pen on the skin. The skin incision was designed directly on the basis of the radioactive hot spots by gamma probe. If the RI tracer had failed to detect any lymph nodes because the basin was too close to the primary site (shine-thorough phenomenon), or lymph nodes detected only by ICG, the incision was designed according to the point of accumulation of ICG. Nodes with accumulation of BD or RI or both with at least one-tenth of the count of the node with maximum accumulation according to hand-held gamma probe readings were regarded as SLNs and removed. Nodes with accumulation of ICG only were also defined as SLNs. Lymph vessels were ligated as much as possible when removing SLNs because leakage of ICG otherwise contaminated the operative field, making it difficult to identify other SLNs visually through the near-infrared camera.

Pathological examination

The removed SLNs were formalin fixed and paraffin embedded and stained with hematoxylin and eosin. If the specimens were negative for metastases, immunohistochemical examination was performed with antibodies to S-100 protein, HMB-45, and MART-1 for melanoma, cytokeratin (CK) for cSCC, CK-20 for MCC, and CK-7 for EMPD.

Statistical analysis

Statistical analyses were performed with IBM SPSS statistics version 20 (IBM, Chicago, IL, USA). The Student's paired *t*-test, the Kruskal–Wallis test, and the Fisher's exact test were used to determine statistical significance. Multiple comparisons were performed when comparing more than two groups. $P < 0.05$ was considered statistically significant.

Results

Evaluation of the number of SLNs and basins detected by ICG

A summary of all SLNBs is shown in Table 2. There were no serious adverse events related to SLNB. At least one SLN was detected (detection rate 100%) in each of the 26 patients. In 19 of 26 (73%) patients, there were SLNs identified only by ICG but not with BD nor RI. BD or RI or both detected 75 SLNs in all and ICG detected an additional 25 nodes, making the total 100. The mean number of SLNs per case was 2.9 by conventional methods and 3.9 with the addition of ICG ($p < 0.01$). Twenty-eight basins were detected using BD + RI and 32 with the addition of ICG. The mean number of basins per case was 1.08 and 1.24, respectively ($p = 0.043$).The mean number of SLNs per basin was 2.4 by BD + RI and 3.3 with the addition of ICG ($p < 0.01$), the additional basins being mostly in the groin ($p < 0.01$) and axilla ($p = 0.047$). In four of the 19 patients (21%) with additional SLNs detected by ICG, those SLNs were located in the additional basins detected only by ICG. One of these four had a MM on the cheek: lymphoscintigraphy showed hot spots in the submandibular and parotid glands, whereas ICG detected not only these nodes but also additional lymph flow to the submental area, resulting in removal of an SLN from this region. Another of these cases

had a MM in the lateral thoracic region; a SLN was detected in one side of the axilla by RI. However, ICG identified two lymph routes, not only to the axilla but also the ipsilateral groin; SLNs were removed from both of these basins. The remaining two cases had penile lesions (cSCC and EMPD); SLNs were detected in one side of the groin by RI in both. However, ICG revealed that both patients had independent lymph routes to both groins, resulting in removal of SLNs from both groins. In an additional two cases in whom SLNs could not be detected by lymphoscintigraphy or gamma probe because the relevant basin was too close to the primary site (shine-thorough phenomenon), SLNs were identified by ICG. Overall, lymph node metastases were found in six cases (23%) and eight (8%) nodes. All SLNs with metastases were identified by BD + RI.

Identification of lymph flows by ICG

ICG readily enables visualization of lymph flows intraoperatively in real time. We used ICG to evaluate the anatomical patterns of lymph flows running from primary sites towards the relevant basins and identified the following three distinct patterns of lymph

flow (Fig. 1. a−c): (a) several small peripheral lymph flows around the primary site running towards proximity and joining to form a single large flow that drained into a single basin; (b) two or more independent flows draining into a single basin; and (c) two or more independent flows draining into different basins. Pattern (a) was observed in 14 of the 26 cases, in 13 (93%) of whom the primary lesions were on distal limb. Pattern (b) was observed in six cases, two of whom had lesions on proximal limb, two on trunk (including one genital), and two on the head and neck; none of these cases had primary lesions on their distal limb. Pattern (c) was observed in six cases, three of whom had lesions on the trunk and lymph flow to two different basins. The other three had lesions on the distal limb and SLNs were detected in both the typical basins (axilla or groin) and interval nodes (cubital or popliteal fossae) (Table 3). Furthermore, the number of final lymph flows draining into each basin was assessed. Patients with pattern (a) had only one lymph route, whereas those with pattern (b) or (c) had two or more. The relationship between primary sites and number of lymph flows was also evaluated (Fig. 2. a). Analysis of variance showed statistically significant differences between primary

sites and patterns of lymph flow (Kruskal–Wallis test, $p = 0.01$). Multiple comparison showed that patients with primary lesions on the trunk had multiple lymph flows more frequently than those with primary lesions on distal limb ($p = 0.048$). Furthermore, patients with primary lesions on the proximal limb and trunk (b + c) had multiple lymph flows significantly more frequently than those with distal limb lesions (a) (Fisher's exact test, $p = 0.002$) (Fig. 2. b).

Discussion

SLNB is reportedly useful in the management of skin cancers, including MM [2-5]. Although the rate of detection of SLNs is more than 95% with conventional BD + RI methods, SLNs are not detected in some cases.

Morton *et al.* reported an overall rate of detection of SLNs in patients with MM of 95.3%, with a lower detection rate in patients with MM in the head and neck region (84.6%) [6]. The lower detection rate in the head and neck was possibly attributable to the complexity of lymph routes, the tendency to small lymph nodes in this area, and the shine-through phenomenon [18]. Furthermore, in some cases, regional recurrence

develops after a negative SLNB, suggesting that some SLNs were not detected. The false-negative rate is reportedly 5.7−32% for MM and 13−20% for MCC [3,4, 7-10], being particularly high for MM in the head and neck (32%). Very importantly, patients with false-negative SLNBs have a poorer prognosis than those with positive SLNs because the negative results justify not performing lymph node dissection [9, 10]. Thus, inadequate identification of SLNs can worsen rather than improve the prognoses of these patients. Clearly, a more reliable method of identifying SLNs for SLNB is needed to minimize these patients.

We have been using ICG in combination with dye and RI since 2012.

The fluorescent reagent ICG is widely used in various procedures such as retinal angiography [11]. ICG injected into the body binds albumin and the resultant complex generates fluorescence with a peak wavelength of 850 nm (near-infrared) when irradiated by near-infrared rays. The flow and accumulation of ICG in lymph vessels and nodes can be visualized through the skin with a near-infrared camera. Because ICG is smaller (molecular weight of 774.96 Da), it flows faster with the lymph flow than the

RI tracer and dye [11]. Furthermore, small amounts of ICG produce very high fluorescence intensity and can therefore be visualized with a near-infrared camera. A few studies of SLNB using the fluorescent method in breast cancer and skin cancer were recently reported; most of them evaluated the number of SLNs detected and the safety of the procedure [11-15]. Fujisawa *et al*. reported that the ICG method is useful for finding both additional (occult) basins and additional (occult) SLNs [16]. They detected occult SLNs in occult basins in five of 34 cases with MM with ICG. Nakamura *et al*. reported using ICG + BD + RI to facilitate SLNB in 12 patients with skin cancers on the head and neck. They detected SLNs with ICG in both sides of the neck in four cases, whereas they detected SLNs on both sides by BD + RI in only two of 18 cases [17]. Thus, ICG enables detection of occult SLNs in basins that are not detected by the conventional BD + RI technique.

Furthermore, as well as detecting SLNs, ICG enables us to observe the anatomy of lymph flows in real time. Lymphoscintigraphy can also depict lymph flows, however, it

has limitation to detect small or peripheral ones in detail and nodes with the primary site close to the basin.

To our knowledge, there are no published reports evaluating patterns of lymph flows from the primary sites to SLNs and their basins using ICG. As shown in Figure 1, we have identified the following three patterns of lymph flows: (a) a single lymph flow draining into a single basin; (b) two or more lymph flows draining into a single basin; (c) two or more lymph flow draining into different basins. These patterns of lymph flow may explain the cause of false-negative results of SLNB using conventional methods. SLNs are rarely overlooked in pattern (a) because the injected tracer identifies a single central lymphatic vessel that flows directly to SLNs in one basin. However, with pattern (b) and (c), SLNs can be overlooked because the site(s) of injection determine whether the tracer enables identification of all the lymphatic vessels. Morton *et al*. reported 59 cases of false-negative SLNBs in patients with MM. Recurrences developed in the biopsied basins in 48 of these 59 cases (81%) and 11 had recurrences in basins other than the biopsied one [6]. Our model may explain this: the former cases possibly had

pattern (b) and the latter, pattern (c). Although it is well known that the rate of false-negative SLNBs is high for primary MM on the head and neck, the frequency of false negatives with primary lesions in other sites such as the trunk or limbs has not yet been clarified. In our study, 13/16 cases (81%) with primary lesions on their distal limbs had pattern (a). In patients with primary lesions in sites closer to their basins, such as proximal limb and trunk, the tracer can run into more than two central lymphatic vessels immediately after injection and drain into a single basin through two or more flows: pattern (b). Depending on the position of their lesions on the trunk, tracers may drain into bilateral and/or both axillae and groins in patients with primary lesions on the trunk: pattern (c). Our findings may explain that the risk of false-negative results can be higher in patients with primary lesions on the proximal limb and trunk than in those with distal limb lesions. We postulate that use of ICG for SLNB has advantages for primary skin cancers located on the proximal limb and trunk as well as head and neck.

Last, this study had several drawbacks. First, because the particles are very small, ICG can accumulate in secondary nodes as time passes. Because intradermally injected ICG

reaches secondary nodes within 20−30 min [16], the SLNs should be removed within this time period. In this study, we removed lymph nodes which were stained with ICG only as SLN in the field after the first SLNs had been removed, however, these lymph nodes might include secondary nodes in part. Second, because ICG has very high fluorescence intensity, even a small leakage of ICG can contaminate the operative field and disturbed surgeon to visually find other stained lymph nodes. Ligation of lymphatic vessels prevents this problem; however, it remains a technical limitation. Third, ICG is suitable for detecting lymph flows in shallow part (<1.5cm in depth from the surface of skin) but not ones in deep part. Compressing surface of skin when observing lymph flows with a near-infrared camera can enable us to observe lymph flows in middle or deep part of subcutaneous tissue depending on the degree of obesity of individual, however it remains limitation.

In summary, injection of ICG with conventional BD + RI is useful for identification of SLNs in patients with skin cancers, especially in the presence of shine-through phenomenon and in subjects with primary lesions on the trunk, proximal limb, and head

and neck. As this was a relatively small study, our results should be verified with a larger sample.

Acknowledgment: We would like to thank Associate Professor Yasushi Yamauchi (Department of Gastroenterological Surgery, Faculty of Medicine, Fukuoka University) for allowing us to use the near-infrared camera.

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Legends for Figures

Fig. 1 Patterns of lymph flows running from a primary lesion to sentinel lymph nodes observed with indocyanine green. (a) Several small peripheral lymph flows around the primary site run toward proximity and join to form a single large flow that drains into a single basin. (b) Two or more independent flows drain into a single basin. (c) Two or more independent flows drain into different basins.

Fig. 2 (a) Relationship between primary location and number of lymph flows. Analysis of variance shows statistically significant differences between them (Kruskal–Wallis test; $p = 0.01$). Multiple comparison among primary locations showed the trunk had more than two lymph flows significantly more often than distal limbs ($p = 0.048$).

(b) Proximal limb and trunk lesions had more than two lymph flows significantly more often than distal limb lesions (Fisher's exact test; $p = 0.002$).

Table 1 Patients characteristics

		N
Total		26
Age(range)		64(36-85)
Sex	Men	14
	Female	12
Primary tumor	MM	15
	cSCC	8
	EMPD	2
	MCC	1
Primary location	Head and neck	2
	Trunk	3
	Extremity	18
	Genital	3

MM, malignant melanoma cSCC, cutaneous squamous cell carcinoma

MCC, merkel cell carcinoma EMPD, extramammary paget's disease

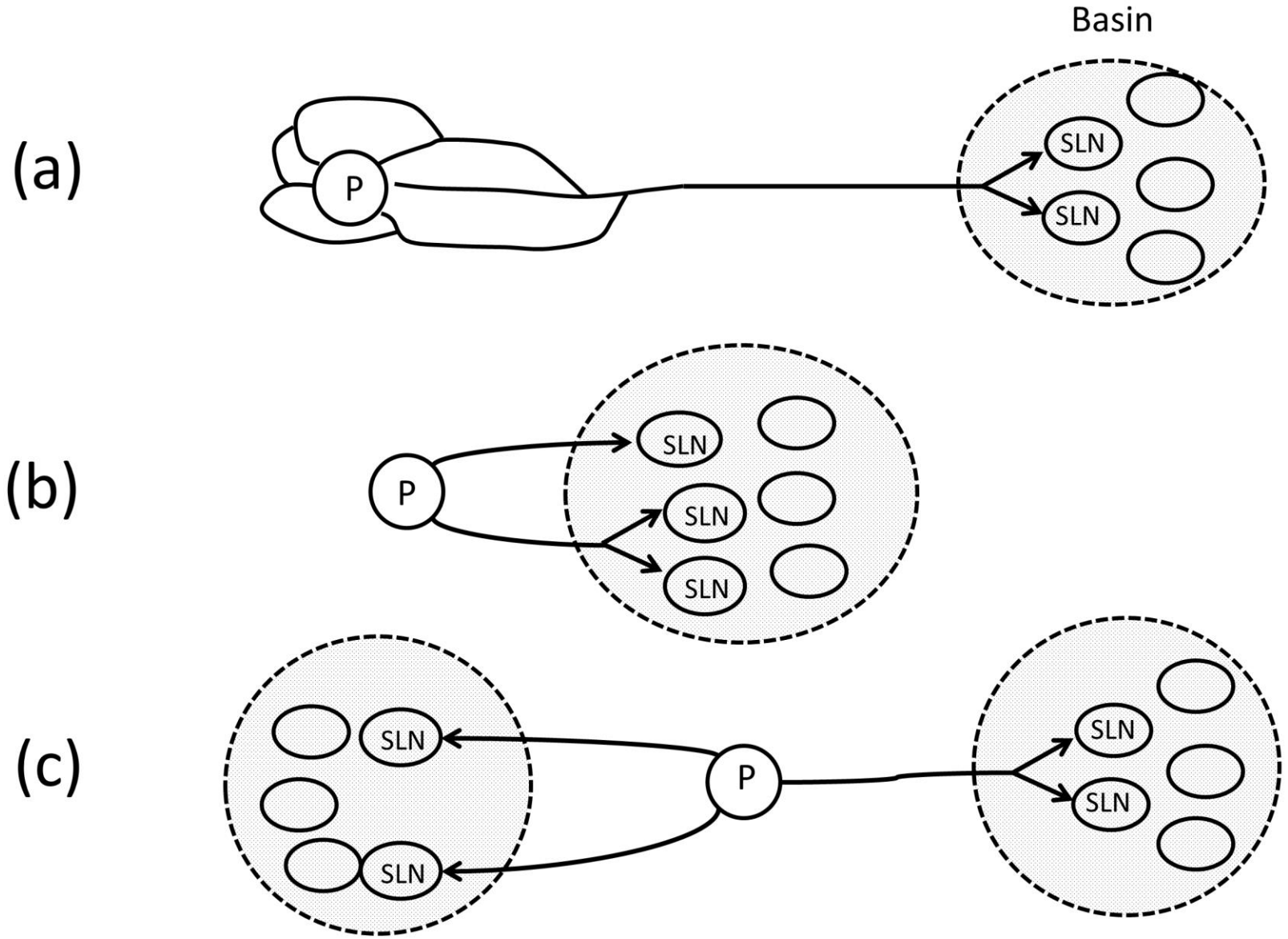
Table 2　Summary of SLNBs

	Dye+RI	Dye+RI+ICG	*P*value ※
Cases with SLN identified (detection rate)	26(100)	26(100)	
Total no. of SLN	75(75)	100	
Mean no. of SLN per case	2.9	3.9	<0.01
Total no. of basins	28(88)	32	
Groin	17(85)	20	NS
Axilla	6(86)	7	NS
Neck	2(100)	2	NS
Popliteal	2(100)	2	NS
Cubital fossa	1(100)	1	NS
Mean no. of basin per case	1.08	1.24	0.043
Mean no. of SLN per basin	2.4	3.3	<0.01
Groin	2.5	3.4	<0.01
Axilla	2.0	3.14	0.047
Neck	4.00	5.5	NS
Other (popliteal+cubital fossa)	1.6	1.6	NS
Number of positive SLN	8	8	NS
Number of negative SLN	67	92	NS
False negative	0	0	NS

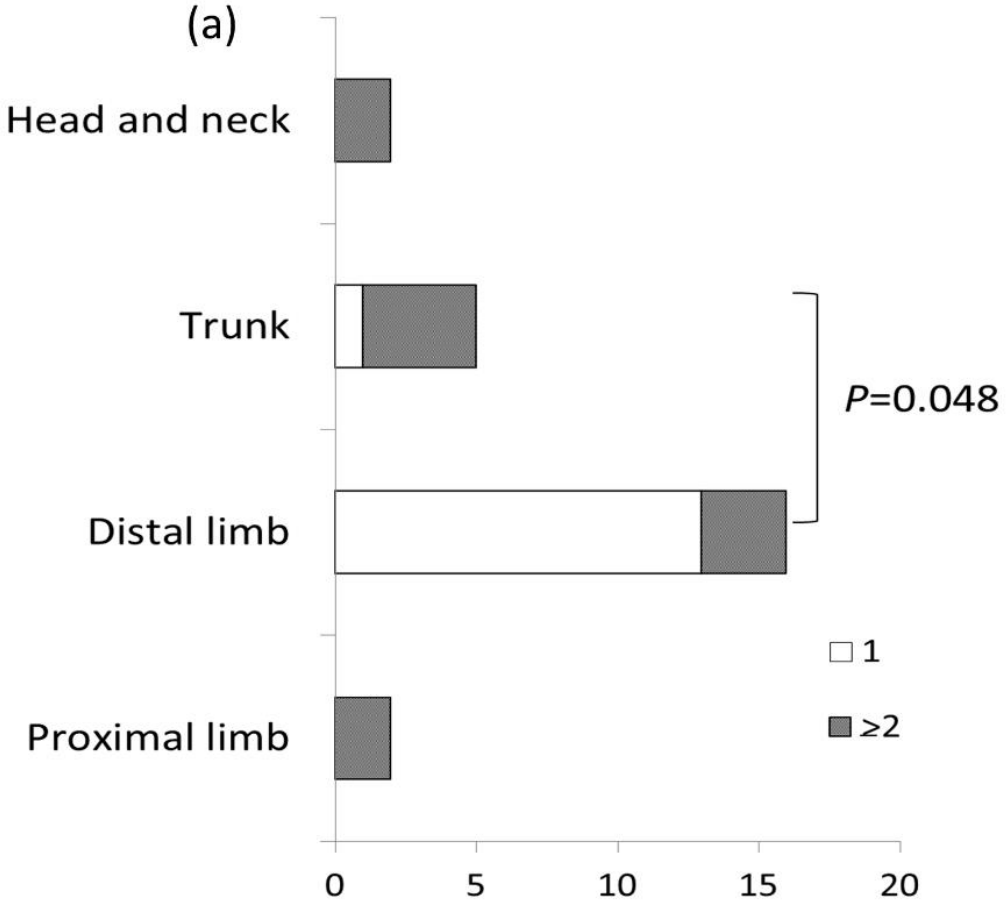
※ Paired t-test

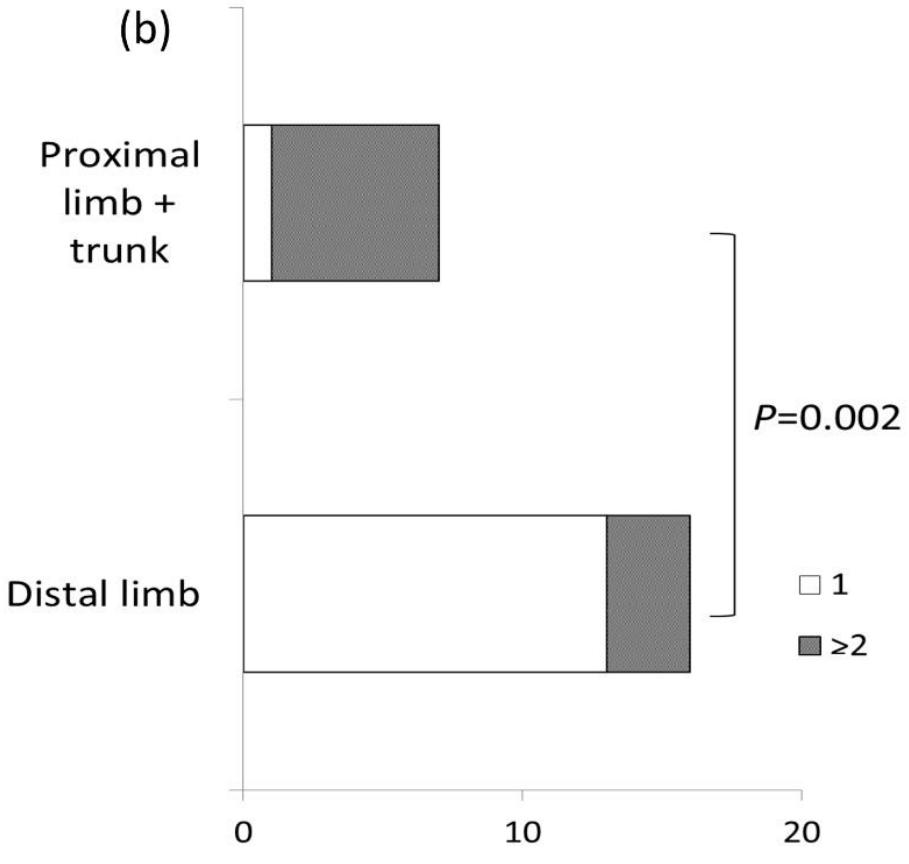
Table 3　Primary sites and anatomical patterns of lymph flows

	Patterns of lymph flows			
	a	b	c	Total
Primary site				
Head and neck	0	2	0	2
Proximal limb	0	2	0	2
Distal limb	13	0	3	16
Trunk	1	2	3	6
Total	14	6	6	26



SLN, sentinel lymph node p, primary lesion


(a)
Head and neck
Trunk
Distal limb
Proximal limb
P=0.048
1
≥2
0
5
10
15
20


(b)
Proximal limb + trunk
Distal limb
P=0.002
1
≥2
0
10
20