INTRODUCTION

There is no gold standard treatment for lymphedema. Vascularized lymph node transfer (VLNT) is the most recent development, which becomes a new horizon in the physiologic treatment.1–6 Lymph nodes (LNs) from various sites have been selected: groin,7 submental,8 supraclavicular,9 thoracodorsal,10 lateral thoracic,11 internal mammary,12 deep inferior epigastric,13 lateral intercostal artery,14 gastroepiploic,15,16 jejunal mesentery,17,18 mesoappendix,19,20 and ileocecal area.21 The systematic reviews and meta-analysis have shown many satisfactory results.22–24 The mechanism of vascularized LN flap is still unsettled. The possible explanations fall either into pumping mechanism theory25–28 or lymphangiogenesis theory.25,29–31 Fortunately, both theories are grounded in the existence of LNs in the flap. The significance of quantity of transferred LNs has been demonstrated in animal32,33 and clinical studies.34 Apart from that, the success of the treatment is also attributed to donor site morbidity and anatomic reliability.34

In search of the optimal donor site, various methods have been applied to detect LNs: surgical exploration with the naked eye (NK) or surgical exploration under operative microscope8,35,36 and imaging studies.35,37,38 However, little is known about the sensitivity and specificity of each of these methods. The systematic reviews and meta-analysis have shown many satisfactory results.22–24 The mechanism of vascularized LN flap is still unsettled. The possible explanations fall either into pumping mechanism theory25–28 or lymphangiogenesis theory.25,29–31 Fortunately, both theories are grounded in the existence of LNs in the flap. The significance of quantity of transferred LNs has been demonstrated in animal32,33 and clinical studies.34 Apart from that, the success of the treatment is also attributed to donor site morbidity and anatomic reliability.34

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Technical Challenges in “Micro” Lymph Node Identification during Vascularized Submental Lymph Node Flap Harvesting

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doubts on the accuracy of previous data derived from the NK and surgical stereo microscopy (SM).

MATERIALS AND METHODS

This study aimed to assess the accuracy of 2 LN counting methods: the NK and SM. Both methods were compared with histological observation (HIS) set as gold standard. The counting was conducted in submental LN flap due to a large number of LN41 and anatomic complexities. The latter involves marginal mandibular nerves, digastric muscle, platysma muscle, and submandibular gland, which requires extra care during flap harvest.8,35,37

Forty vascularized submental LN flaps were obtained from 25 fresh cadavers self-donated to the Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University. Cadavers were of Thai nationality, with 10 being male and 13 being female specimens. The ages of these specimens ranged from 56 to 76 years. Exclusion criteria were (1) visible cranial and cervical deformity, (2) medical history of lymphatic diseases involving the head and neck, and (3) visible surgical wound to the cervical Ia/Ib sublevel. The procedure was approved by Siriraj Institutional Review Board (SiRB) with protocol number 366/2561 (Exempted).

Arterial injections were performed according to Piyaman et al.8 In brief, external carotid arteries of both sides were cannulated, irrigated with 0.9% saline solution and injected with red polycrylamide solution. The amount of injected solution was 80 ml per vessel, 160 ml per head. Flap design was elliptical skin paddle, where longitudinal axis ran from mental protuberance (gnathion) to angle of mandible (gonion). Medial curve was demarcated roughly by both bellies of digastric muscle, while lateral curve by inferior mandibular border. The dissection included (1) the anterior belly of digastric muscles, (2) the entire submental artery, (3) the segment of facial artery from its origin to mandibular border, and (4) the superficial part of the submandibular gland. Once the flaps were removed, they were fixed in 10% formalin and then sliced to 2-mm thickness. All slices were subjected to routine histological process: dehydration, paraffin embedding, and H&E staining. Each paraffin block representing a slice of a flap was cut into 5–200 microscopic slides for accurate LN count.

Counting the Lymph Nodes

LN counts were performed by 3 methods: NK, SM (equivalent to operative microscope), and HIS (Fig. 1). NK count was conducted during flap dissection by the qualified plastic surgeon (NY). Next, the anatomist (PP) fixed the harvested flap in 10% formalin and performed SM count at 10×–20× magnification. Then, the flaps were processes into series of H&E microscopic slides. The anatomist performed HIS count by tracing the slides. HIS was set as definite LN identification, a “gold standard” method against which accuracy of NK and SM count would be tested. Identification criteria for LNs were based on morphology; these were the presence of capsule, cortex, medulla capsular arteries, and hilar arteries8 (Fig. 2).

The counting was recorded as positive once LN was presented. Each node was counted only once; therefore, true positive counts would be matched with the number of confirmed nodes. On the other hand, a negative count was recorded once the node becomes absent in a finite area. The area covering 1 negative count was a 2-mm-thick slice of the flap. Each slice was subsequently processed into a paraffin block for serial histological sectioning and HIS count.

Grouping the Lymph Nodes (Micro-, Meso-, Macro-)

The LNs were classified by diameter into 3 groups. Firstly, micro-lymph node (micro-LN) had a diameter of <1.5 mm.40 To complete the classification, 2 new terms were introduced: meso-lymph node (meso-LN) and macro-lymph node (macro-LN). Meso-LNs had a diameter ranging from 1.5 mm to ≤5.0 mm. Lastly, macro-LNs had a diameter of 5.0 mm and larger. LNs were also classified by location into cervical sublevel Ia and Ib. This study assigned the lateral (posterior) border of the anterior belly of digastric muscles (ABDM) as the demarcation line between Ia and Ib sublevel. According to this demarcation, Ib nodes were categorically located lateral to anterior belly of digastric muscle, whereas Ia nodes were further subdivided into medial, superficial, and deep zones relative to the muscle.

Statistical Analysis

The number and the size of LNs in each submental LN flap were counted by 3 methods: (1) by using surgical exploration with the NK, (2) by using a 10× stereo microscope (SM), and (3) by HIS under a light microscope. The data were represented by arithmetic mean ± SD. For accuracy test, the results from the first 2 methods were compared with histological observation. The accuracy was represented by (1) sensitivity, (2) specificity, (3) false positive rate, and (4) false negative rate. The LNs were further classified by size (micro-, meso-, macro-) and neck sublevel (Ia, Ib). The subgroup analyses were performed by comparing the accuracy between subgroups.

RESULTS

From 40 flaps, 175 LNs were confirmed by HIS (+ + +, + +, +, +, and - - - in Fig. 3). The average number was 4.4 ± 1.8 nodes per flap. The average LN size was measured 4.4 ± 2.2 mm, ranging from 1.0 to 10.7 mm (Table 1). LNs were classified by size into 9 micro-LNs (5.1%), 113 meso-LNs (64.6%), and 50 macro-LNs (28.6%) (Table 2). LNs were classified by location into 52 nodes in Ia (29.7%) and 123 nodes in Ib (70.3%). All Ib nodes, by our definition, were located lateral to the ABDM and relatively close to the submandibular gland (SMG). On the other hand, Ia nodes were further subdivided by topographic relationship with the ABDM into 30 superficial nodes, 10 deep nodes, and 15 medial nodes.

Naked eye (NK) detected 1.9 ± 1.4 nodes per flap (Table 1), yielding 33.7% sensitivity (Table 3 and +++ in Fig. 3). Of all the nodes detected by NK, the size ranged from 1.7 mm to 10.9 mm, 5.3 ± 2.3 mm by average (Table 1).
Subgroup analysis showed that the sensitivities increased with size, from 0% for the micro-LN, 27.4% for the meso-LN to 54.0% for the micro-LN (Table 2). Subgroup analysis also shows different sensitivities between 2 sublevels; 21.2% in sub-level Ia and 39.0% in Ib sub-level (Table 3).

Stereo microscopy (SM) detected 3.4 ± 1.4 nodes per flap (Table 1), yielding 63.5% sensitivity (Table 3 and +++ in Fig. 3). Of all the nodes detected by SM, the size ranged from 1.6 to 10.9 mm, 5.0 ± 2.1 mm by average (Table 1). Sensitivities increased with size, from 0% for the micro-LN, 56.6% for the meso-LN to 92.0% for the macro-LN (Table 2). Sensitivities varied by location, from 50.0% in Ia sublevel to 69.1% in Ib (Table 3).

Non-LN structures, confirmed by HIS, detected 647 true negative counts in total (+ + -, - + - and + - - and - - - in Fig. 3). Among these, NK detected 634 true negative counts, yielding 98.0% specificity. SM detected 622 true negative counts, yielding 96.1% specificity (Table 3).

There were totally 30 false positive counts by any methods (+ + -, - + -, and + - - in Fig. 3). Most of them, 26 counts,
were located in Ib sublevel. Accordingly, Ib sublevel had a higher false positive rate than Ia. The causes of false positivity included fat, SMG, muscle (Fig. 4), and double counting. The latter occurred when single extensive LNs were mistaken as 2 separate nodes. False positivity was categorized into 3 scenarios (Fig. 5): (1) double falsified, (2) corrected-by-SM, and (3) corrupted-by-SM. Double falsified

Table 1. Size and Number of LNs Classified by Detection (Counting) Methods

| Detection methods | NK       | SM      | HIS      |
|-------------------|----------|---------|----------|
| No. LN per flap   | 1.9 ± 1.4| 3.4 ± 1.4 | 4.4 ± 1.8 |
| Size of LN (mm)   | 5.3 ± 2.3| 5.0 ± 2.1 | 4.4 ± 2.2 |
|                   | [range, 1.7–10.9] | [range, 1.6–10.9] | [range, 1.0–10.7] |

Data are represented by mean ± SD unless otherwise specified.

Table 2. Sensitivity of NK and SM in LN Detection Classified by Size (Subgroup Analysis)

| Detection Methods | Overall | Micro-LN (<1.5 mm) | Meso-LN (≥1.5, <5 mm) | Macro-LN (≥5 mm) |
|-------------------|---------|--------------------|-----------------------|------------------|
| No. LN (%)        | 175 (100) | 9 (5.1)            | 113 (64.6)           | 50 (28.6)        |
| NK sensitivity    | 33.7     | 37.0               | 27.4                  | 54.0             |
| SM sensitivity    | 63.4     | 68.5               | 56.6                  | 92.0             |

Table 3. Accuracy of NK and SM in LN Detection Classified by Cervical Sublevel (Subgroup Analysis)

| Detection Methods | Cervical Sublevels |
|-------------------|---------------------|
|                   | Ia + Ia | Ia | Ib      |
| Sensitivity (%)   | NK       | 33.7 | 21.2 | 39.0 |
|                   | SM       | 63.5 | 50.0 | 69.1 |
| Specificity (%)   | NK       | 98.0 | 100.0 | 96.3 |
|                   | SM       | 96.1 | 98.7 | 93.9 |
| False positive rate (%) | NK | 7.4 | 0.0 | 10.6 |
|                   | SM       | 14.3 | 7.7 | 17.1 |
| False negative rate (%) | NK | 17.9 | 13.7 | 21.6 |
|                   | SM       | 9.9 | 8.7 | 11.0 |

Table 3. Accuracy of NK and SM in LN Detection Classified by Cervical Sublevel (Subgroup Analysis)
scenario, 8 counts, occurred when both NK and SM mistook fat or SMG for the LN. The corrected-by-SM scenario, 5 counts, occurred when NK mistook fat or SMG for the LN at first, but the structures were identified correctly later by SM. Lastly, the corrupted-by-SM scenario, 17 counts, occurred when non-LN structures were correctly identified by NK at first, but they were counted as the LN by SM later.

**DISCUSSION**

LN count under the NK (the conventional method) shows a very low sensitivity. The method missed two-thirds of LNs of all size and failed to detect any micro-LN smaller than 1.5 mm. The sensitivity was still inadequately low to count the node larger than 5 mm by missing half of them. Therefore, the naked eye is not suitable for LN detection. The result is supported by Okamura’s study, which showed only 13.8% accuracy of macroscopic (NK) detection of LNs under 5 mm. However, an average detectable size of metastatic LN in stomach cancer was reduced to 4.06 ± 0.95 mm. The reduction may be due to harder consistency of metastatic LNs, making LN detection easier.

SM showed average detectable size at 5.0 mm, similar to NK at 5.3 mm. SM also failed to detect any micro-LN as well as NK. Nevertheless, the overall SM sensitivity is twice as much as NK and is exceptionally high for LNs > 5.0 mm. SM could detected 3.4 ± 1.4 nodes per flap. The number was consistent with the surgical exploration in the previous studies (Table 4).

Lower sensitivity in Ia sublevel compared with Ib sublevel was probably due to interposition of Ia LN with the ABDM. In the attempt to harvest Ia nodes, the surgeon has to choose how to approach the ABDM. Whether the approach is proceeded on the superficial or deep aspect
of the ABDM, the harvest would inevitably miss the node located on the opposite side of the muscle. The superficial approach seems preferable due to the larger number of the node located on superficial side. However, one should be aware that these superficial nodes usually receive arterial supply from the deep aspect of the muscle.42

Lower specificity in Ib sublevel compared with Ia was probably caused by the confusion among LNs, fat lobules, SMG lobules, and the muscle (Fig. 4). Their morphologies seem straightforwardly different; however, the pattern of the vasculatures complicated the identification. Submental artery supplies Ia/Ib sublevel as multiple minute branches. Hilar branches supplying the LN may come directly from the submental artery; the direct route, or indirectly from submental perforator; sharing route pattern.42 In case of direct route, hilar branch may be confused with the glandular branch piercing capsule of salivary gland or even muscular branch piercing the muscular fascia. On the other case, hilar artery may run in the sharing route pattern, mimicking lobular branches from the perforator to fatty tissue. Most of the confusions occurred during SM counting despite higher visual resolution compared with the NK. Recently, a study has developed a new harvest technique that claimed to obtain more LNs near SMG.46 Another study has shown that LNs located near SMG are supplied by glandular

Fig. 5. Sankey diagram of false positivity. Sankey diagram represents how all false positive structures flowing through the counting process from left to right. The first 3 columns represent 3 counting methods, whereas the last column represents the locations of each structure that are classified by cervical sublevels. The horizontal bands reflect how each structure is counted, re-counted, and finally identified as non-LN structures (eg, fat, SMG, muscle). Most of the false positive counts were fat and submandibular glands, which are mostly located in the Ib sublevel. The SM causes more false positivity than NK. Three false positive scenarios were double falsified (+++), corrupted by SM (+-), and corrected by SM (++) scenarios.

Table 4. Previous Clinical and Anatomical Studies on Submental LN Flap

| Study          | Study Type | Number of Flaps | No. LN by Method of Study |
|----------------|------------|-----------------|---------------------------|
|                |            |                 | Imaging | NK or SM | HIS         |
| Cheng 201246   | Anatomy    | 12              | —       | 2.3 ± 0.8 | 3.3 ± 1.5   |
|                | Clinical   | 6               | —       | —        | —           |
| Patel 201545   | Clinical   | 12              | 3.2 ± 1.0 (US) | 3.0 ± 0.6 | —           |
| Tzou 201742    | Anatomy    | 18              | 3.2 ± 1.1 (US) | 3.1 ± 0.6 | —           |
| Asuncion 201847| Clinical   | 19              | 5.2 ± 1.9 (CT) | 7.2 ± 2.4 (MRI) | —           |
| Gustafsson 201844| Clinical | 35             | 3.9 ± 1.9 (US) | —        | —           |
| Nonomura 201849 | Anatomy   | 4               | —       | 2–3      | —           |
| Paulus 202048  | Clinical   | 104             | —       | 2–3      | —           |

Data are reported as mean ± SD, unless otherwise specified.
CT, computed tomography; CTA, computed tomography angiography; MRI, magnetic resonance imaging; US, ultrasonography.
branch from the facial artery. Unfortunately, above mentioned studies did not include histological observation. According to our study, identification of LNs near SMG should be conducted with great caution and not to be confused with glandular branches supplying the lobe of salivary gland.

**Accuracy of Imaging Studies**

Compared with previous imaging studies (Table 4), higher LN counts were reported from MRI (7.2 ± 2.4), CT studies (5.2 ± 1.9), and CT-angiography (CTA) (5.3 ± 2.0). On the other hand, ultrasound (US) count showed a significantly lower number (3.2 ± 1.0 and 3.2 ± 1.1). The larger number from the MRI study seems to be the benefit from more extensive count, including jugulodigastric node in Ia sublevel and the additional submandibular node nestled deep to the submandibular gland. Both LN groups are supplied by facial arteries and still relevant to the flap harvest.

Even though the US-based method is operator-dependent, which leads to lower sensitivity than other imaging modalities, the ultrasonography can provide specificity equal to or higher than that of CT/48 and MRI. Additionally, the method is more suitable for intraoperative usage. Recent developments in the resolution and application of color, power doppler, and 3D modalities have increased advantage of this method for LN detection.

**Roles of ICG Lymphography**

ICG (indocyanine green dye) is suitable for real time and intraoperative evaluation of lymphatic drainage. ICG lymphography has been applied for LN detection in sentinel LN biopsy for 2 decades. Later, the method has been incorporated into lymphedema management as a tool for diagnosis and treatment monitoring. The method has visualized the basic mechanism of VLNT and improved LN selection called reverse lymphatic mapping. The selection has been developed to prevent iatrogenic lymphedema of donor site in axillary and inguinal VLNT. Focusing on submental VLNT, the clinical application of ICG is still limited. The LN detections have been conducted preoperatively by magnetic resonance imaging, computed tomography, computed tomography angiography, or ultrasonography. The reverse lymphatic mapping has not been applied to the area yet probably due to zero incidence of iatrogenic lymphedema. The lymphatic vasculature in the area has been established by a similar method of lymphangiography. Hence, the focus of recent anatomic studies was on blood vasculature of the LNs. ICG lymphography, nevertheless, should be incorporated in future anatomic studies for 2 purposes. Firstly, accuracy of the ICG method for LN detection could be tested. Secondly, the complete vasculature of the area (artery, vein, and lymphatic) could be elaborated.

**Micro-lymph Node: Detect the Undetectable**

A peri-gastric LN study has introduced the term “micro-lymph node” describing the node < 1.5 mm. Undetectability of this LN group had raised a concern in the field of gastric cancer study, contributing to 36% of all LNs in Japanese station No. 4 (greater curvature nodes). In 2018, gastroepiploic vascularized LN flap has been introduced as lymphedema treatment and the micro-LN has been re-investigated. According to our data, Ia/Ib sublevel contains a much lesser proportion of micro-LNs compared with gastroepiploic LNs (4% versus 36%). Ia/Ib LNs are more likely to be activated by immune reaction and subsequently become hyperplasia. The wider range of activation could come from upper gastrointestinal tract, upper respiratory tract, and the others in the head and neck areas.

Recently, lymph vessel-only vascularized transfer, as known as lymphadiposal flaps, has been demonstrated with favorable outcome. The possible mechanism is relied on pumping of healthy lymphatic vessels of donor site to drain fluid into the vein in recipient site. In case of superficial circumflex iliac artery perforator flap (SCIP) as a donor site, the real mechanism is in doubt. The existence of micro-LN in SCIP has never been proved; thus, undetectable node may play hidden function, as described in “lymph node” flap. A further investigation into micro-LN in SCIP area is highly recommended.

**Limitations of This Study**

Firstly, this study was conducted in fresh cadavers, some of which were proceeded after another surgical workshop. In this case, decomposition certainly complicated differentiation of LNs from the surroundings. The latter was likely to lower the actual accuracy of the NK and SM, to some degree. Secondly, despite a prominent advantage of histology, implementing this method for LN count requires a lot of time and manpower. In the future, incorporation of artificial intelligence with the HIS or SM observation may shorten the study process.

Secondly, this study did not include lymphatic vasculature of the flap. The ICG lymphography or lymphangiography should be incorporated to the future anatomic study to complete the vasculature of the area. Additionally, the accuracy of the ICG method for LN detection could be tested.

**CONCLUSIONS**

Due to false positivity of NK and SM, the identification of the LN during the flap harvest should be performed with care. The tracing of hilar artery to the LNs may be misled to either glandular branch (artery) supplying lobe of salivary glands or lobular artery supplying fat lobe. Due to the low sensitivity of NK and SM, the pre-operative imaging modalities (ultrasonography, CT, CTA, or MRI) are advisable. We do not recommend harvesting the flap as large as possible to get the undetectable nodes. The extension of the flap into Ia sublevel, if necessary, should include submental artery and hilar branches, which are usually located deep (superior) to the digastric anterior belly. On research perspective, the future study on LN distribution should incorporate histology or imagings to increase the accuracy of the data.
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