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ARTICLE

Combining Contrast-Enhanced Ultrasound with Methylene Blue for Detection of Sentinel Lymph Nodes in Early Breast Cancer

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Abstract

Aims/Background Sentinel lymph nodes (SLNs) are an important prognostic factor for breast cancer patients, but traditional axillary lymph node dissection methods have many complications, while sentinel lymph node biopsy has been developed as a better method. This study aimed to evaluate the efficiency of combining contrast-enhanced ultrasound (CEUS) with methylene blue for identifying SLNs in early-stage breast cancer patients.

Methods This retrospective study included clinical data from 163 female patients with lymph node-negative and T1-2 early breast cancer admitted to China-Japan Friendship Hospital between August 2022 and November 2023. All patients received a periareolar injection of SonoVue followed by ultrasonography to identify SLNs. The methylene blue was used to detect SLNs during the surgery, and the patients underwent sentinel lymph node biopsy. We compared the methylene blue method with combined CEUS and methylene blue to identify the number of SLNs per patient. Furthermore, these two methods were compared to determine the number of SLNs and the number of SLNs positive in 34 SLNs positive patients.

Results This study included 163 patients with tumor (T)1-2 node (N)0-3 metastasis (M)0. The identification rate of SLNs was 100% for CEUS. We detected 376 SLNs using a combined CEUS and methylene blue method, with a median of 2 (1, 5). Furthermore, methylene blue identified 627 SLNs, with a median of 3 (1, 12). However, CEUS detected a significantly lower number of SLNs than those identified by methylene blue ($p < 0.001$). Additionally, metastasis frequency was substantially higher for the combined CEUS and methylene blue method (66.3%, 53/80) compared to methylene blue approach alone (39.5%, 58/147) ($p < 0.001$).

Conclusion Combining CEUS with methylene blue is expected to improve the accuracy of axillary staging in breast cancer patients while reducing surgical trauma and postoperative complications.

Key words: contrast-enhanced ultrasound; methylene blue; sentinel lymph nodes; breast cancer

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Introduction

Breast cancer is one of the most common malignant tumors, with the highest incidence rate among women both in China and worldwide (Siegel et al, 2022). The incidence of female breast cancer continues to increase, with an estimated 287,850 new cases and 43,250 deaths, making it the second leading cause of cancer-related death in the United States (Siegel et al, 2022). Axillary lymph node involvement is an important prognostic factor for breast cancer patients. In the past, axillary staging was based on histopathological findings following axillary lymph node dissection (ALND) (Barrio, 2024). This procedure often resulted in postoperative complications, including lymphedema, arm numbness, and limited shoulder mobility. The sentinel lymph node (SLN), which is the first lymph node or group of nodes involved in lymphatic drainage, works as an indicator of axillary lymph node status in clinically node-negative (cN0) breast cancer patients, as reported by numerous prospective randomized trials (Cserni et al, 2022). The implementation of the sentinel lymph node biopsy (SLNB) has revolutionized axillary surgery in breast cancer patients by avoiding the need for ALND in those without lymph node involvement. This development reduces the morbidity associated with ALND and improves the accuracy of staging (Ashikaga et al, 2010; Krag et al, 2010; Lyman et al, 2017; Tinterri et al, 2023). However, SLNB has been linked to postoperative complications, such as an 11% rate of sensory deficit and a 5% lymphedema, though these incidence rates were significantly lower than ALND (Jung et al, 2022). Therefore, performing SLNB with higher precision is vital to decrease surgical damage.

Several techniques have been devised to identify the SLN during the SLNB procedure. The initially recommended approach is a combination of methylene blue and a radioisotope. Although radioisotopes are highly effective, their high cost and potential risk of radiation exposure hinder their extensive use, particularly in developing countries. Methylene blue, a cost-effective chemical agent, has become the predominant alternative (Yang et al, 2024). Nevertheless, its accuracy in identifying SLNs ranges from 66% to 94%, with false-negative rates varying between 0 and 12% (Xu et al, 2022). Therefore, it is necessary to develop a novel method that, combined with methylene blue, can enhance SLN localization and improve the identification rates and accuracy.

Introducing microbubble contrast agents has made contrast-enhanced ultrasound (CEUS) a promising method for identifying SLNs. CEUS, an innovative noninvasive technique, was first utilized in 2004 to identify SLNs in a porcine melanoma model (Rubio and Sobrido, 2022). Omoto et al (2024), reported the application of this method in human breast cancer. Several studies have demonstrated the effectiveness of CEUS in enhancing SLNs identification and minimizing the surgical impact of SLNB procedure (Deng et al, 2023; Niu et al, 2022; Tang et al, 2024). However, some research findings indicate that using CEUS alone to guide SLNB could lead to false negative outcomes and limited diagnostic precision (Fan et al, 2023; Li et al, 2022). Therefore, our study aimed to assess the feasibility of using CEUS to identify SLNs and determine whether combining CEUS with methylene blue could enhance the accuracy of SLN detection in early-stage breast cancer.

Methods

Study Participants

This retrospective study included the clinical data from female patients with early-stage breast cancer who were admitted to China-Japan Friendship Hospital between August 2022 and November 2023. All surgeries were performed by the same surgeon (JL). Informed consent was obtained from each patient before the enrollment. This study was approved by the Ethics Committee of China-Japan Friendship Hospital (No. 2022-KY-095), and the study design adhered to the principles of the Helsinki Declaration. A flowchart of the study design is shown in Fig. 1.

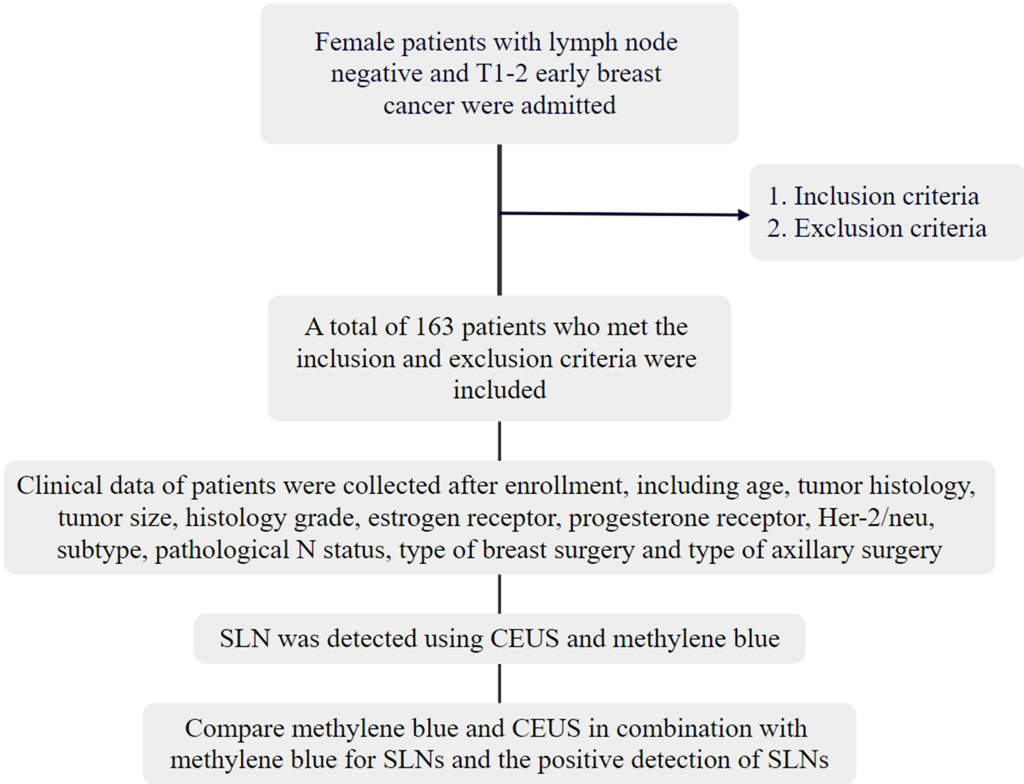

Fig. 1. A flowchart of the study design. Her-2, human epidermal growth factor receptor type 2; SLN, sentinel lymph node; CEUS, contrast-enhanced ultrasound.

Inclusion and Exclusion Criteria

The inclusion criteria for study participants were as follows: patients with (a) primary breast cancer; (b) no palpable axillary lymph nodes or no suspected metastatic axillary lymph nodes based on clinical, radiographic assessment such as mammography, breast ultrasound, or breast-enhanced MRI; and (c) a clinical T stage of 1–2 or Tis, as per the 7th edition of the American Joint Committee on Cancer (AJCC) manual (Rusch et al, 2010). Moreover, the exclusion criteria included the patients with (a) pregnancy or breastfeeding; (b) inflammatory breast cancer; (c) previous treatments such as chemotherapy, radiotherapy, or axillary surgery; (d) severe heart or lung disease; and (e) allergies to the contrast-enhanced agents.

Sample Size Calculation

Assuming bilateral $\alpha = 0.05$, a power of 80%, and a dropout rate of 10%, the minimum required sample size was calculated to be 75 cases. Including 163 patients in this study, the sample size requirements were met.

Experimental Approaches

Patient's ultrasound scans were conducted utilizing either Philips Elite Color Doppler (Philips, Amsterdam, the Netherlands) with L18-4 linear array probes or the ACUSON Sequoia Ultrasound System (Siemens Healthineers AG, Forchheim, Germany) with L10-4 linear array probes. Sentinel lymph nodes were independently examined by two imaging doctors based on the ultrasound results. In case of any discrepancies in the diagnosis, a senior imaging doctor intervened to reach a consensus.

Patients were positioned supine with their arms abducted to expose the axilla completely. Following regular disinfection, a suspension (0.5–0.8 mL) of the contrast agent SonoVue (F240401Z, Bracco, Milan, Italy) was administered percutaneously around the areola. The injection site was gently massaged to enhance the contrast agent's absorption into the lymphatic vessels. The lymphatic vessels from the injection site to the axilla were visualized using contrast-enhanced ultrasound, and the lymph nodes that absorbed the contrast agent were identified as contrast-enhanced SLNs. While the patient remained stable, the appearance, number, position, and enhancement pattern of the lymph nodes were dynamically observed and documented. Furthermore, surface positioning markers were utilized, and details such as the SLN's number, shape, dimensions, and location (including depth and distance from the lateral edge of the pectoralis major muscle) were carefully documented.

Following anesthesia administration, 2 mL of methylene blue solution was injected subcutaneously around the areolar area. SLNs were identified as lymph nodes that showed either partial or full methylene blue staining or were directly linked to a blue-stained afferent lymphatic vessel. After identifying lymphatic vessels and SLNs using methylene blue, they were compared to the SLNs identified by the preoperative CEUS localization method, and the results were documented. All SLNs identified by methylene blue or CEUS and any suspicious lymph nodes detected during the procedure were completely removed and sent for immediate frozen section examination. If the intraoperative frozen section analysis confirmed metastasis in SLNs, ALND was subsequently conducted. However, if no metastasis was found, only SLNB was performed.

In cases where the frozen section pathology of the SLN during surgery suggests no metastasis but postoperative paraffin pathology indicates metastasis, the SLN is classified as positive. However, when using a combination of CEUS and methylene blue, the SLN is classified as positive only when both methods give positive results.

Statistical Analysis

Statistical analysis was conducted using SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). Categorical variables were presented as numbers and percentages, continuous variables, which were tested for normality using the Kolmogorov-Smirnov (K-S) method, were then expressed as mean ± standard deviation ($\bar{x} \pm$ s) or median. The differences among categorical variables were assessed using chi-square tests, and Student's *t*-test was employed to analyze differences in mean values of continuous variables. A p-value of <0.05 was considered statistically significant.

STROBE Statement

The authors have reviewed the STROBE Statement checklist of items, and the manuscript was prepared and revised based on this checklist of items.

Results

Analysis of Baseline Characteristics

We included 163 patients diagnosed with T1-2N0-3M0 breast cancer, according to the 7th edition of the AJCC manual. The patients' ages ranged from 47 to 68 years, with a median age of 53. The average BMI of the patients was 23.59 ± 3.67. Tumor histology indicated invasive ductal carcinoma (n = 119, 73%), invasive lobular carcinoma (n = 11, 6.7%), ductal carcinoma *in situ* (n = 28, 17.2%), papillary carcinoma (n = 2, 1.2%), and mucinous adenocarcinoma (n = 3, 1.8%). Furthermore, 106 (65%) patients underwent a mastectomy, and 57 (35%) patients have received breast-conserving surgery. For the type of axillary surgery, the number of patients who have undergone SLNB was 132 (81%), and 31 (19%) have done SLNB with axillary dissection, Demographic and clinical characteristics of the study participants are given in Table 1.

Number of SLNs Detected

All the study participants received periareolar injections of SonoVue and methylene blue. Intraoperatively, the SLNs identified by CEUS were also detected through methylene blue. The identification rate of SLNs was 100% when combining CEUS with methylene blue.

The methylene blue method identified 627 SLNs across all the study participants, while CEUS combined with the methylene blue method detected 376 SLNs. The median number of SLNs detected per patient using the combined CEUS and methylene blue method was 2 (mean, 2.31; standard deviation, 0.88; range, 1–5), compared to 3 SLNs per patient detected using the methylene blue method (mean, 3.85; standard deviation, 1.96; range, 1–12). The combined method identified a significantly lower number of SLNs than the methylene blue alone ($p < 0.001$). Table 2 shows the number of SLNs per patient identified by combined CEUS and methylene blue and by methylene blue alone. Representative results are shown in Fig. 2, illustrating that SLNs receiving draining from SonoVue or methylene blue can be visualized.

Assessing Metastasis in SLNs

Among 163 patients included in this study, 34 patients exhibited positive SLNs. The frequency of metastasis in SLNs detected using the combined CEUS and methylene blue method was 66.3% (53/80), significantly higher than the rate for SLNs detected by methylene blue alone (39.5%, 58/147), suggesting a statistically significant difference ($p < 0.001$, Table 3).

The study participants with positive SLNs (n = 34) exhibited at least one SLN detected positive using SonoVue and methylene blue. Furthermore, methylene blue successfully detected all positive SLNs among these 34 patients. In 5 patients, the

Table 1. Baseline characteristics of the study participants.

Variables	No. of patients
Age, years	
<50	40 (24.5%)
≥50	123 (75.5%)
Tumor histology	
Invasive ductal carcinoma	119 (73%)
Invasive lobular carcinoma	11 (6.7%)
Ductal carcinoma *in situ*	28 (17.2%)
Papillary carcinoma	2 (1.2%)
Mucinous adenocarcinoma	3 (1.8%)
T stage	
Tis	8 (4.9%)
T1	112 (68.7%)
T2	41 (25.2%)
Unknown	2 (1.2%)
Histology grade	
1	27 (16.6%)
2	64 (39.3%)
3	45 (27.6%)
Unknown	27 (16.6%)
Estrogen receptor	
Positive	130 (79.8%)
Negative	31 (19.0%)
Unknown	2 (1.2%)
Progesterone receptor	
Positive	98 (60.1%)
Negative	59 (36.2%)
Unknown	6 (3.7%)
Her-2/neu	
Positive	35 (21.5%)
Negative	121 (74.2%)
Unknown	7 (4.3%)
Subtype	
Luminal A/B	101 (62.0%)
Her-2-enriched	35 (21.5%)
Triple-negative	17 (10.4%)
Unknown	10 (6.1%)
Pathological N status	
Negative	127 (78.0%)
1 positive node	18 (11.0%)
≥2 positive nodes	18 (11.0%)
Type of breast surgery	
Mastectomy	106 (65%)
Breast-conserving surgery	57 (35%)
Type of axillary surgery	
SLNB	132 (81%)
SLNB plus axillary dissection	31 (19%)

SLNB, sentinel lymph node biopsy.

Table 2. The number of SLNs per patient identified using CEUS or methylene blue.

Treatment group	No. of SLNs per patient												*p*-value
	1	2	3	4	5	6	7	8	9	10	11	12	
Methylene blue	10	31	41	34	18	16	5	3	2	1	1	1	0.000*
C+B	25	80	44	11	3	0	0	0	0	0	0	0	

SLN, sentinel lymph node; CEUS, contrast-enhanced ultrasound; C+B, CEUS in combination with methylene blue. *Student's *t*-test.

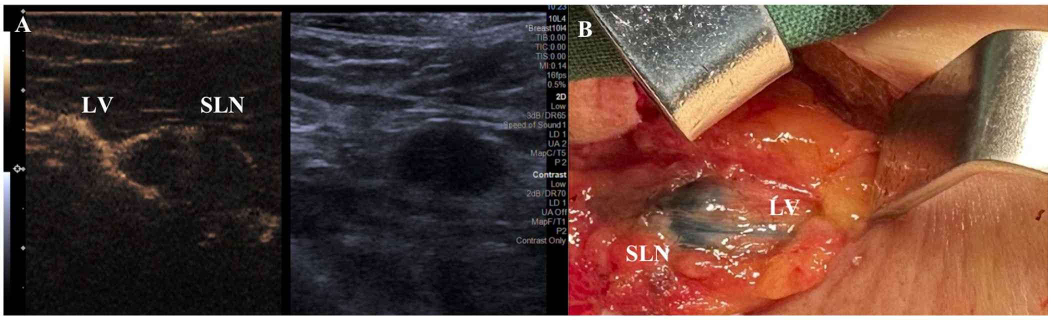

Fig. 2. SLNs identified through CEUS or methylene blue. (A) LV and SLN identified using CEUS. (B) Blue-stained SLN with afferent LV. CEUS, contrast-enhanced ultrasound; LV, lymphatic vessel; SLN, sentinel lymph node.

number of SLNs identified by CEUS was fewer than the number of positive SLNs (2/3, 2/3, 2/3, 3/4, and 2/3). The study showed that 34 patients had positive SLNs, and 129 had negative SLNs using CEUS. These patients were observed to have identical positive and negative SLN status when detected using methylene blue, resulting in a 100% agreement between the two methods.

Additionally, there was one case where micrometastasis and macrometastasis were confirmed in two distinct positive SLNs. Furthermore, one case was categorized as pN0, which was confirmed to have isolated tumor clusters.

Safety

We did not see any adverse events following the periareolar administration of SonoVue or methylene blue. The safety evaluation included the absence of skin responses near the injection site or allergic responses, both immediately after the surgery or at the 1-month follow-up.

Discussion

SLNB was initially introduced in the 1990s for breast cancer patients with clinically node-negative status. For early-stage breast cancer without axillary lymph node metastasis, the St Gallen International Expert Consensus recommended SLNB in 2009 (Gentilini et al, 2023). Results from the National Surgical Adjuvant Breast and Bowel Project (NSABP) trial B-32 suggested that SLNB and ALND yielded equivalent outcomes regarding overall survival, disease-free survival, and regional control. For clinically negative lymph node breast-cancer patients, no further axil-

Table 3. Metastasis in SLNs across 34 patients with positive SLNs detected using the CEUS or methylene blue method.

Detection method	No. of SLNs	No. of positive SLNs	Positivity (%)
methylene blue	147	58	39.5%
C+B	80	53	66.3%
chi-square value		14.885	
p-value		<0.001	

SLN, sentinel lymph node; CEUS, contrast-enhanced ultrasound. C+B, CEUS in combination with methylene blue.

lary dissection is considered appropriate, safe, and effective if the sentinel node is negative (Krag et al, 2010). Furthermore, the American Society of Clinical Oncology (ASCO) clinical guideline recommends that clinicians avoid using ALND for women with early-stage breast cancer who do not have nodal metastases (Lyman et al, 2017). Nowadays, SLNB is considered a viable alternative to ALND for axilla staging, offering a comparable prognosis outcome with lower morbidity (Ashikaga et al, 2010; Krag et al, 2010; Lyman et al, 2017; Tinterri et al, 2023).

CEUS, a cutting-edge method for mapping lymph nodes, has been employed in the SLNB procedure for breast cancer across numerous studies (Jin et al, 2022; Omoto et al, 2024; Pang et al, 2024; Shi et al, 2022; Mori et al, 2024). However, the detection rate and the average number of SLNs identified through CEUS differed among different studies. Liu et al (2023) reported that the identification rate of SLNs using CEUS was 71.17%, with percutaneous CEUS localization accuracy of 94.67% for axillary SLNs. Furthermore, they reported that the average number of SLNs detected by CEUS was 1.5, while combined CEUS and methylene blue detected an average of 2.42 SLNs (Li et al, 2022). Cui et al (2023), reported a CEUS detection rate of 96.3%, with an average of 1.3 SLNs detected by CEUS, 2.5 by methylene blue.

Our study indicated a 100% identification rate for SLNs using CEUS, which aligns with previous results, such as the 98% reported by Fan et al (2023) and 100% by Matsuzawa et al (2015). The periareolar administration of SonoVue for SLN detection is a straightforward procedure, with enhanced lymphatic vessels easily visible following periareolar injection. SonoVue, a microbubble contrast agent encapsulated in a lipid membrane with sulfur fluoride (SF_6) gas, has been used for the diagnosing lesion in abdominal and superficial organs lesions and has shown promising clinical safety (Wang et al, 2022; Zhang et al, 2020).

Our study also showed that the average number of SLNs (2.31) identified by CEUS combined with methylene blue was significantly lower than the average number (3.85) identified by methylene blue alone. This outcome aligns with previous studies. For instance, Sun et al (2021) and Fan et al (2023) found average SLNs identified rates of 1.56 and 1.52, respectively, using CEUS. Furthermore, Wu et al (2020) found that the combination of methylene blue and CEUS identified an average rate of 3.13 SLNs.

Several reasons may contribute to the difference in the number of SLNs identified using CEUS compared to methylene blue. First, the average diameter of

SonoVue microbubble particles is 2.5 µm, ranging from 1.0 to 10.0 µm, which is more significant than methylene blue. These microbubbles are easily trapped by reticuloendothelial cells within the lymph node, preventing them from traversing through subsequent nodes (Fan et al, 2023; Xu et al, 2022). Moreover, the transition time of SonoVue from injection to its appearance in the SLN is typically 15 to 45 seconds, where it remains for 1 to 3 minutes before being cleared (Zhang et al, 2023). This rapid clearance may result in a quick exit, potentially leading radiologists to miss the true SLN. Conversely, methylene blue allows for detecting blue-stained lymphatic vessels and SLNs (Ou et al, 2023), which enhances the visibility of nodes within the adjacent adipose tissue. However, methylene blue can result in false positive results if the dye spreads to the tissue that are not true SLN or infiltrates secondary lymphatic vessels, leading to unnecessary exploration of the axilla and overexcision of lymph nodes. Lastly, we also removed the suspected lymph nodes during the methylene blue method, even if they were not the real SLNs. As a result of these features, the number of SLNs identified using CEUS was substantially lower than those detected by the methylene blue method.

In our study, all SLNs preoperatively marked by CEUS were identified by methylene blue tracing during the procedure, consistent with the findings reported by Cui et al (2023), where all contrast-enhanced SLNs were stained. Additionally, we considered SLNs detected by methylene blue methods but not by CEUS, along with suspected lymph nodes. Among the study participants (n = 163), 34 patients were found to have positive SLNs. The frequency of metastasis for SLNs identified by combining CEUS and methylene blue method was 66.3% (53/80), significantly higher than that for SLNs detected by methylene blue alone (39.5%, 58/147). The result is consistent with the previous study showing a higher metastasis frequency for SLNs identified by CEUS (73.2%) compared to those identified by methylene blue (56.9%) (Fan et al, 2023). Since the number of SLNs detected by CEUS is smaller than those identified by methylene blue, it can be speculated that CEUS more accurately detects true SLNs. This speculation is supported by our findings, where all SLNs detected by CEUS were also identified by methylene blue.

Furthermore, we removed secondary lymph nodes, which may have been over-identified by methylene blue but not identified by CEUS. While methylene blue can reduce false negative rates, it also elevates the risk of postoperative complications, contradicting the main aim of SLNB. Our results showed a 100% concordance between CEUS and methylene blue in detecting positive SLNs, with 34 patients exhibiting positive SLNs and 129 indicating negative SLNs as determined by both methods. Although in 5 patients, the number of SLNs identified by CEUS was less than the number of positive SLNs, this difference did not affect the treatment decision, as completion of ALND was performed accordingly.

Regarding axillary surgery, previous clinical trials have reported that isolated tumour cells (ITCs) or micrometastases do not substantially affect patients' survival (Weaver et al, 2011). This study followed the ASCO clinical guideline recommendation (Lyman et al, 2017) and patients' willingness. For women with early-stage breast cancer who have one or two SLN metastases and are receiving breast-conserving surgery with conventionally fractionated whole-breast radiotherapy, ALND was not recommended.

Additionally, we administered a dose of 0.5–0.8 mL of contrast agent, with no skin reactions or allergic responses observed, indicating the safety of percutaneous SonoVue injection. The contrast agent injection dose varied among different studies, and it is commonly accepted that higher doses caused more obvious enhancement effects (Hwang et al, 2021). A meta-analysis revealed that diagnostic metrics improved up to doses below 1 mL, suggesting that a dose of less than 1 mL may be optimal (Liu et al, 2023).

Our study has several limitations. First, while we marked the locations of SLNs identified by CEUS and compared them to the SLNs dissected using the methylene blue method, there was no direct, one-to-one correspondence established to confirm that the SLNs identified solely by CEUS were indeed the exact same SLNs that were subsequently traced and identified by the combined CEUS and methylene blue approach during surgery. This lack of direct evidence limits the ability to fully assess the accuracy and consistency of the two methods when used in combination. Using clips (Chen et al, 2023; Siso et al, 2023) or hook wires (Kim et al, 2023) may enhance the precision of intraoperative localization of preoperatively marked SLNs. However, it is crucial to consider the potential risks, such as slippage of the metal wire or clip and the invasive nature of wire placement. Furthermore, it was a single-center study with limited sample size. A larger, multi-center study is needed to validate our findings. Additionally, a learning curve is crucial for improving the accuracy of CEUS, methylene blue methods, and the SLNB technique. Lastly, this study focused on the feasibility and efficiency of CEUS without assessing the relationship between different CEUS enhancement patterns and SLN metastase frequency.

Conclusion

In conclusion, this study revealed the potential of percutaneous CEUS as an effective method for accurate SLN localization, serving as a valuable adjunct to SLNB surgery. The combination of CEUS with methylene blue significantly improves the effectiveness of SLNB compared to methylene blue alone. This combined approach of CEUS and methylene blue enhances identification accuracy and offers reliable diagnostic performance, indicating its potential as a non-radioactive alternative for dual SLN tracking. However, further research and refinement are necessary to fully optimize this method for wider clinical adoption and application.

Key Points

- CEUS achieved a 100% detection rate for SLNs in patients with early breast cancer.
- The number of SLNs detected by CEUS was significantly lower than that identified by methylene blue.
- Combining CEUS with methylene blue significantly improved the positive detection rate of SLNs.
- Combining CEUS with methylene blue is expected to improve the accuracy of axillary staging of breast cancer while reducing surgical trauma and postoperative complications.

Availability of Data and Materials

The raw data are publicly available upon request on the corresponding author.

Author Contributions

Conception and design: JL, JZ; Administrative support: BZ, MY; Provision of study materials or patients: JL, BZ; Collection and assembly of data: TZ, JZ; Data analysis and interpretation: JZ, TZ, BZ, MY; Manuscript writing: All authors; Editorial changes of important content: All authors; Final approval of manuscript: All authors; Being accountable for all aspects of the work: All authors.

Ethics Approval and Consent to Participate

All patients provided written informed consent for their information to be stored in the hospital database and used for research. The study was approved by the Ethics Committee of China-Japan Friendship Hospital (2022-KY-095).

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Conflict of Interest

The authors declare no conflict of interest.

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