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Evaluation of a novel diagnostic kit for detecting metastatic sentinel lymph nodes in breast cancer patients using the semi-dry dot blot method combined with an automatic reader: A single-center prospective study

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Research Article

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Abstract

Background: A semi-dry dot blot (SDB) detects lymph node (LN) metastases by measuring cytokeratin (CK) in LN lavage fluid using an anti-CK antibody. We evaluated a SDB kit that combines an anti-CK19 antibody with an automatic reader for the intraoperative diagnosis of LN metastasis in patients with breast cancer. This exploratory study evaluated the diagnostic performance and identified the optimal cut-off values for detection.

Methods: We analyzed 226 LNs dissected from 108 breast cancer patients who underwent sentinel LN (SLN) biopsy at Nagasaki University Hospital between January and December 2020. The SDB kit assayed proteins extracted from SLNs and an automatic reader measured CK19 absorbance. The results were compared with those of hematoxylin and eosin staining and CK19 immunohistochemistry, interpreted independently and blindly by pathologists. Cut-off values for CK19 absorbance were determined post-hoc using receiver operating characteristic (ROC) curve analysis.

Results: Among the 226 SLNs, histopathology identified 14 macrometastases (6.2%), 7 micrometastases (3.1%), and 205 metastasis-negative nodes, including one with isolated tumor cells (90.7%). Using a cut-off CK19 absorbance of 9.9 milli-absorbance units (mABS), sensitivity, specificity, and overall agreement of the SDB kit were 85.7%, 80.5%, and 81.0%, respectively. With a cut-off of 60.0 mABS to distinguish macrometastases from non-macrometastases, these values were 92.9%, 100%, and 99.6%, respectively. The assay required approximately 20 min, costing <$30 USD per test.

Conclusions: This SDB kit combined with an automatic reader provided results quickly and accurately, with potentially lower implementation costs than pathologist-only diagnoses of metastases without LN tissue loss, permitting concurrent histopathology.

Introduction

Sentinel lymph node (SLN) biopsy is a standard surgical procedure for clinically node-negative breast cancer [1–3]. Historically, patients with lymph node (LN) metastases were treated with axillary LN dissection (ALND), and macrometastases with a maximum diameter exceeding 2.0 mm were targeted for ALND based on data from the International Breast Cancer Study Group (IBCSG) 23 – 01 trial [4]. ALND was traditionally performed for macrometastatic SLNs; however, its use markedly declined after the American College of Surgeons Oncology Group (ACOSOG) Z0011 trial found no survival benefit in patients with clinical T1–T2 breast cancer, clinically node-negative axillae, and up to two SLN macrometastases, who underwent breast-conserving surgery without neoadjuvant therapy [5]. Accordingly, in clinically node-negative patients, an intraoperative diagnosis of SLN macrometastases remains crucial to ensure appropriate intraoperative axillary surgery. Intraoperative pathological evaluation using frozen sections stained hematoxylin and eosin (H&E) is the most prevalent method of identifying SLN macrometastases; however, it reportedly yields false-negative results at a substantial rate of approximately 7–10% [6–8]. Furthermore, the workload associated with performing intraoperative pathological diagnoses places a significant burden on pathologists and technical staff in Japan [9–11].

Automated molecular diagnostic tests, such as the One-Step Nucleic Acid Amplification (OSNA) assay (Sysmex, Kobe, Japan), are required to reduce this burden. However, the loss of LN tissue due to homogenization to obtain cytokeratin (CK) 19 mRNA and the substantial cost of the associated diagnosis, encompassing both equipment and operational expenses, remains a significant consideration [12–14]. The semi-dry dot blot (SDB) method is another diagnostic technique used to detect LN metastases, in which metastatic involvement is identified by the presence of CK proteins is detected using an anti-CK antibody in the lavage fluid of sectioned LNs. This test is based on the principle that epithelial components, such as CKs, are absent in normal LNs [15]. We have previously reported that the original SDB method using a modified dot blot technique achieved 93.3% sensitivity, 96.9% specificity, and 96.6% overall agreement [16]. Subsequently, we developed a kit-based version using a newly developed anti-CK19 antibody, simplifying the procedure and demonstrating at 94.5% sensitivity, 95.2% specificity, and 95.0% overall agreement for identifying macrometastases [17].

Nevertheless, unresolved issues in the evaluation remain; specifically, the visual assessment of reaction positivity is subjective and lacks sufficient precision, particularly for detecting subtle reactions, which limits the widespread and commercial application of the original SDB kit. To overcome this limitation and to enable a more objective assessment, we developed an automatic reader for the SDB kit. Our aim was to evaluate the performance of SDB kits combined with an automatic reader for diagnosing SLN metastasis and distinguishing macrometastases from non-macrometastatic lesions in SLN biopsy specimens from patients with breast cancer.

Patients and Methods

Patients and study design

This study enrolled patients who underwent SLN biopsy at Nagasaki University Hospital between January 2020 and December 2020. This study was approved by the Institutional Ethics Committee for Medical Research of Nagasaki University Hospital (18101513) and was conducted in accordance with the Declaration of Helsinki. All procedures adhered to the official guidelines of the ethical committee for informed consent and disclosure. All patients were provided the necessary information regarding the trials, and only LNs from patients who provided written consent to participate were included.

The eligibility criteria were patients with breast cancer who underwent SLN biopsy at our institution during the study period. Patients were excluded if their SLNs could not be identified intraoperatively or if they received neoadjuvant chemotherapy or endocrine therapy. SLNs were identified intraoperatively using blue dye and radioisotopes or blue and fluorescent dye in combination. In total, 226 SLN were obtained from 115 axillae of 108 patients with breast cancer treated at Nagasaki University Hospital, including seven patients with bilateral breast cancer, as illustrated in the schematic diagram of the study (Fig. 1).

During surgery, the SLNs were sectioned at 2-mm intervals and rinsed with phosphate-buffered saline. Cells suspended in the lavage fluid of sectioned LNs were collected by centrifugation and lysed to extract proteins. LNs were evaluated by pathologists in a blinded manner using H&E staining of formalin-fixed, paraffin-embedded (FFPE) sections, and the results were compared with those of the SDB test. As this was an exploratory study designed to evaluate the performance of the kit and automated reader and to identify candidate cut-off values, no formal *a priori* sample size calculation was performed. Instead, the sample size was pragmatically determined based on the number of consecutive eligible patients accrued during the 12-month study period.

Pathological examination

The pathological diagnosis based on H&E and immunohistochemical (IHC) analysis was deemed negative when the specimen contained no tumor cells or isolated tumor cells (ITCs) no more than 0.2 mm in maximum diameter. Micrometastases were diagnosed when tumor cells were larger than 0.2 mm, reaching up to 2.0 mm, while macrometastases were diagnosed when tumor nests were larger than 2.0 mm maximum in diameter, in accordance with the criteria of the 9th edition of the tumor–node–metastasis classification of the Union for International Cancer Control [18]. In the present study, non-macrometastases were defined as micrometastases, ITCs, or the absence of tumor cells. Lymphatic and venous invasions were assessed using H&E-stained sections and D2-40 IHC staining for lymphatic invasion and Elastica van Gieson staining for venous invasion. Nuclear grade was determined according to the nuclear pleomorphism criteria of the Nottingham grading system [19]. Estrogen receptor and human epidermal growth factor receptor 2 (HER2) statuses were evaluated by IHC using the antibody clones and positivity criteria defined in the American Society for Clinical Oncology/College of American Pathologist guidelines [20, 21].

SDB kit and automatic reader development

As previously described, we developed two versions of the immunochromatography test using newly developed anti-CK19 antibodies with low and high ability to detect LN metastases [14]. To differentiate between macrometastases and non-macrometastases, a low-power detection method with a minimal concentration of CK19 protein (5 ng/mL) was appropriate for objective evaluation by an automatic reader using quantitative immunochromatography.

An automatic reader from Hamamatsu Photonics (Hamamatsu, Japan) was adapted to function with the SDB kit. Calibration was performed at the beginning of the evaluation using standard materials. Following a 10-min interval after the application of a protein extract droplet from the cell pellet to the SDB kit, the completed reaction kit was placed on an automatic reader. The absorbance of the kit, expressed in milli-absorbance (mABS), was calculated within approximately 15 s. During this evaluation, both the reaction intensity and distance from the baseline of the kit were documented. The records

registered a control reaction for all samples and a test reaction in instances where the kit identified the CK19 protein. The cost of an automatic reader is estimated to be approximately $3,000 USD.

CK19 IHC staining

In cases of discordance between the SDB kit and the pathological evaluation, additional IHC analysis was conducted. In false-positive cases (SDB-positive/pathology-negative), IHC staining was performed using the same anti-CK19 antibody used in the SDB kit to identify occult metastases that were not detectable by H&E staining. In false-negative cases (SDB-negative/pathology-positive), IHC staining with anti-CK19 antibodies was performed to evaluate CK19 protein expression the metastatic lesions. FFPE sections (4-μm thick) were mounted on silane-coated slides, deparaffinized, and treated with 0.3% hydrogen peroxide in methanol for 15 min at room temperature to block endogenous peroxidase activity. Sections were incubated overnight at 4°C, with an anti-CK19 primary antibody identical to that used in the SDB kit, followed by detection using the Histofine Simple Stain MAX-PO(M) Kit (Nichirei, Tokyo, Japan), following the manufacturer's instructions. The slides were counterstained with hematoxylin.

Endpoints and statistical analysis

Diagnoses based on the SDB kit and automatic reader findings were compared with those made by histological examination of H&E-stained, FFPE LN sections. Sensitivity, specificity, and overall agreement were evaluated to distinguish macrometastases from non-macrometastases and LN metastases from LN metastasis-negative cases, including those with ITCs.

The statistical program JMP software (version 16; SAS Institute Inc., Cary, NC, USA) was used to analyze the data. Sensitivity, specificity, and overall agreement were determined by comparing the SDB kit results with permanent pathological examinations using 95% confidence intervals (CIs). Continuous data are expressed as medians and were compared using the Wilcoxon rank-sum test. Categorical data were compared using Fisher's exact test.

Receiver operating characteristic (ROC) curve analysis was conducted to evaluate the diagnostic performance of the SDB kit in distinguishing macrometastases from non-macrometastases and LN metastases from LN metastasis-negative cases, including ITCs. The area under the ROC curve (AUC) was calculated as a measure of accuracy. Sensitivity, specificity, and overall agreement were determined using various cut-off values. Statistical significance was set at $P < 0.05$.

Results

Patient background

Table 1 summarizes the clinicopathological characteristics of enrolled patients. The median ages were 62.0 and 63.5 years in the non-macrometastasis and macrometastasis groups, respectively, with no

significant difference between the two groups (P = 0.43). Patients with macrometastases had a significantly higher proportion of pathological T-stage ≥ pT2 (P = 0.02) and lymphatic invasion (P < 0.01) than those without macrometastases. In contrast, there were no significant between-group differences in venous invasion (P = 0.17), nuclear grade (P = 0.28), estrogen receptor status (P = 0.21), or HER2 status (P = 0.35).

Among the 226 SLNs eligible for analysis, permanent histopathological examination with H&E staining identified 14 macrometastases (6.2%), seven micrometastases (3.1%), and 205 SLNs negative for cancer cells, including one case with ITCs (90.7%).

Efficacy of automated SDB kit evaluation

The measured absorbance of CK19 protein for each SLN corresponding to its permanent histopathological diagnosis is presented in Figure 2. We conducted a ROC analysis to distinguish macrometastases from non-macrometastases, and LN metastases, including micrometastases, from LN metastasis-negative cases, including ITCs. To distinguish macrometastases from non-macrometastases, the cut-off absorbance value was 60.0 mABS and the AUC was 0.950 (Supplementary Figure S1). To distinguish LN metastases from LN metastases-negative cases including ITCs, the cut-off was 9.9 mABS and the AUC was 0.858 (Supplementary Figure S2). With a cut-off CK19 absorbance value of 9.9 mABS for detecting LN metastases, excluding ITCs, the SDB kit achieved a sensitivity, specificity, and overall agreement of 85.7%, 80.5%, and 81.0%, respectively (Table 2). With a cut-off CK19 absorbance value of 60.0 mABS for distinguishing macrometastases from non-macrometastases, the corresponding values were 92.9%, 100%, and 99.6%, respectively (Table 3).

Assessment of discrepancies between the SDB kit and histopathological findings

In one case, the SDB kit yielded an absorbance of 3.9 mABS, which was within the non-macrometastatic range. However, histopathological evaluation by H&E staining and CK19 IHC identified a macrometastasis measuring 2.5 mm maximum in diameter. Strong CK19 expression was also observed in the corresponding primary breast tumors. Additionally, two other SLNs demonstrated CK19 absorbance values below 9.9 mABS, yet micrometastases measuring 0.6 mm and 0.5 mm in diameter were detected.

Furthermore, detailed permanent pathological assessment identified one case of epithelial contamination without true metastasis within the SLN tissue. This was likely caused by epithelial cell exfoliation from the subcutaneous fat or mammary gland tissue during mastectomy performed before SLN biopsy. Despite the absence of metastasis, the SDB kit yielded an absorbance of 57.2 mABS in this case, which was still classified as non-macrometastatic disease (Figure 3).

Procedure time and cost of the kit and automatic reader

The total turnaround time for the SDB assay was approximately 20 min. The preparation of the specimens required 7–10 min, depending on the sample size, followed by a 10-min reaction time. The

absorbance measurements were performed within approximately 15 s using the automatic reader.

The cost of the SDB kit was estimated to cost approximately $20 USD per test, whereas the automatic reader cost approximately $3,000 USD. Overall, this method provides a rapid turnaround with potentially lower implementation costs than the conventional molecular diagnostic techniques.

Discussion

In this single-center, prospective study, we evaluated a novel diagnostic kit that employs the SDB method combined with an automatic reader to detect SLN metastasis in patients with breast cancer. Overall, our findings demonstrate that the kit achieved high sensitivity, specificity, and overall agreement for distinguishing macrometastases from non-macrometastases with a rapid turnaround time of approximately 20 min at minimal cost. Importantly, the SDB method does not require homogenization of LN tissue, thus preserving the specimen for concurrent or subsequent histopathological assessments.

Our method offers several potential advantages over the commercially available OSNA assays in Japan and Europe. OSNA provides high diagnostic accuracy but requires complete homogenization of the LN, resulting in the loss of tissue for histological examination and incurring relatively high equipment and running costs. In contrast, the SDB kit preserves LN tissue, enables integration with conventional histopathology, and can be implemented at a substantially lower cost. Furthermore, our automated reader minimizes the subjectivity in interpreting chromogenic reactions, potentially improving reproducibility and facilitating broader adoption.

The relevance of intraoperative SLN assessment has been discussed in the recent SOUND [22] and INSEMA trials [23], both of which suggested that SLN biopsy may be omitted in selected low-risk, clinically node-negative patients undergoing breast-conserving surgery and postoperative irradiation. Nevertheless, intraoperative diagnosis remains important in patients who do not meet these criteria, particularly in settings where immediate axillary management is indicated or in regions where guidelines and surgical practice differ. Our results indicated that the SDB kit with automatic reading could provide a practical, accurate, and resource-efficient option for such cases.

The possible explanations for the few discordant results observed in this study warrant further investigation. Although IHC staining could detect 2.5 mm macrometastasis with obvious CK19 IHC staining in the false-negative cases in this study, one potential factor is the variation in CK19 expression. It has been well established that approximately 2–3% of breast carcinomas, particularly certain histological subtypes such as metaplastic and lobular carcinomas, lack CK19 expression [24, 25]. Lower CK19 protein expression has also been reported in younger patients. Although the CK19 protein expression rate in breast cancer is high (~ 98%) [26], rare subtypes such as metaplastic and encapsulated papillary carcinomas demonstrate lower positivity (80% and 85%, respectively). In most cases, performing CK19 IHC staining on a diagnostic core needle biopsy specimen can preemptively identify CK19-negative tumors, allowing for alternative intraoperative diagnostic approaches. Notably, no rare histological subtypes were observed in the discordant cases.

One possible explanation for the false-negative results when distinguishing macrometastases is the difference between the tissue surfaces used for cell collection in the SDB method and those used for pathological examination. In the SDB method, cell collection was performed immediately following SLN sectioning at 2-mm intervals by rinsing the cut surface with phosphate-buffered saline. In contrast, pathological examination required the preparation of 4-μm thick sections from FFPE SLN tissue, exposing a different tissue surface. In this case, only a small number of tumor cells may have been present on the cut surface used in the SDB method, whereas a greater number of tumor cells may have been exposed on the thin histological sections, leading to discrepant results between the SDB kit and permanent histopathological examination.

We observed one atypical result due to contamination by epithelial components outside the tissue, which resulted in a false-positive finding. In this case, we confirmed exfoliation of the skin and mammary glands before SLN biopsy. Because the SDB method can detect any epithelial component, whether from the mammary gland or cut surface of the LN tissue, we recommend that SLN biopsy be performed before breast surgery.

Our study had several limitations. First, the study was conducted at a single institution and had a small sample size. Second, although our kit demonstrated excellent performance in detecting macrometastases, further validation in a multicenter setting is required to confirm its generalizability. Third, we did not evaluate long-term clinical outcomes, such as recurrence or survival, in patients whose axillary management was guided by this method.

Overall, our novel SDB kit with an automated reader demonstrated high accuracy and rapid turnaround, with potentially lower implementation costs than pathologist-based evaluation for detecting SLN metastases in patients with breast cancer without the loss of LN tissue, thereby allowing concurrent histopathological examination. This method is particularly reliable for the identification of macrometastases. To obtain government approval for insurance coverage in Japan and validate its diagnostic performance across diverse practice settings, we plan to conduct a multicenter prospective clinical trial in the near future.

Declarations

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Conflict of interest: Nippon Gene Co., Ltd. provided the SDB kits used in this study but had no role in the study design, data collection, statistical analysis, interpretation of the results, manuscript preparation, or decision to submit the manuscript. Professor Rin Yamaguchi is a Deputy Editor-in-Chief of *Breast Cancer*; however, he was not involved in editorial handling or peer review of this manuscript. The other authors declare no conflict of interest.

Compliance with Ethical Standards: All procedures involving human participants were performed in accordance with the ethical standards of the institutional and/or national research committee and the 1964 Helsinki Declaration and its later amendments. Informed consent was obtained from all individual participants included in the study. This article does not contain any studies with animals performed by any of the authors.

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Tables

Table 1: Clinicopathological characteristics of the enrolled patients

	Macrometastases (N=10)	**Non-Macrometastasis (N=105)**	**P value**
Age (Median)	63.5	62.0	0.43
Pathological T factor (is 1 or 2)	4/6	81/24	0.02
Lymphatic invasion (/)	8/2	18/86	<0.01
Venous invasion (/)	3/7	14/91	0.17
Nuclear Grade (1 or 2/3)	9/1	72/30	0.28
Estrogen Receptor (/)	10/0	81/22	0.21
HER2 (/)	0/10	12/70	0.35

Comparison of the clinicopathological factors between patients with and without macrometastases. Continuous variables are expressed as median values, while categorical variables are presented as counts and percentages. Statistical comparisons were performed using the Wilcoxon rank-sum or Fisher's exact test, as appropriate.

Table 2: Comparison between the SDB kit and the pathology in the detection of lymph node metastasis in patients with breast cancer (mABS=9.9)

	SDB metastases (+)	SDB metastases (-)	Total
Pathologically metastases (+)	**18**	**3**[b]	21
Pathologically metastases (-)	**40**[a]	**165**	205
	58	168	226

[a] Includes epithelial contamination (Figure 3).

[b] Includes two micrometastases (0.6mm, 0.5mm)

Sensitivity: 85.7% (95%CI: 0.668–0.949)

Specificity: 80.5% (95%CI: 0.786–0.814)

Overall agreement: 81.0% (95%CI: 0.775–0.827)

Comparison of SDB kit results and permanent histopathological diagnosis for detecting lymph node metastasis using a cutoff cytokeratin 19 absorbance of 9.9 mABS. Sensitivity, specificity, and overall agreement are presented with 95% confidence intervals (CIs).

Abbreviations: mABS, milli-absorbance; SDB, semi-dry dot blot.

Table 3: Comparison between the SDB kit and pathology in the detection of macrometastases in patients with breast cancer (mABS=60)

	SDB Macrometastases	SDB Non-Macrometastases	Total
Pathologically macrometastases	**13**	**1**[a]	14
Pathologically on-macrometastases	**0**	**212**[b]	212
	13	213	226

[a] Macrometastasis measuring 2.5 mm in maximum diameter

[b] Includes seven micrometastases

Sensitivity: 92.9% (95%CI: 0.782–0.929)

Specificity: 100% (95%CI: 0.842–1.000)

Overall agreement: 99.6% (95%CI: 0.977–0.996)

Comparison of SDB kit results and permanent histopathological diagnosis to distinguish macrometastases from non-macrometastases using a cut-off cytokeratin 19 absorbance of 60 mABS. Sensitivity, specificity, and overall agreement are presented with 95% confidence intervals (CIs).

Abbreviations: mABS, milli-absorbance; SDB, semi-dry dot blot.

Figures

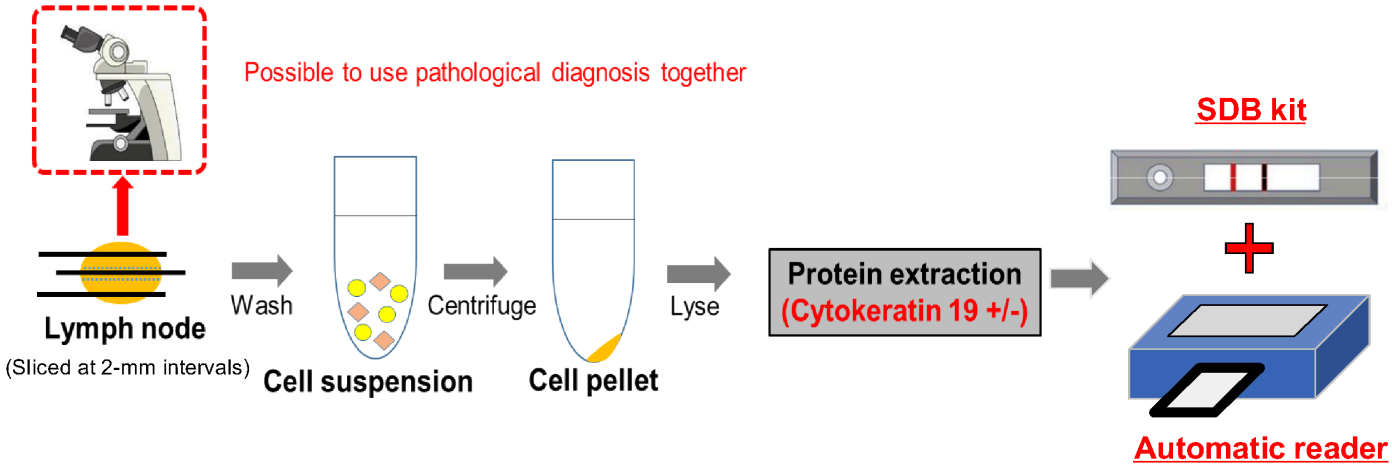


Figure 1

Schematic diagram of the study workflow

Sentinel lymph nodes (SLNs) were sectioned at 2-mm intervals and rinsed with phosphate-buffered saline to collect cellular components. Proteins extracted from the lavage fluid were analyzed using an SDB kit and absorbance was measured using an automatic reader. The results were compared with permanent histopathological diagnoses based on hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) for cytokeratin (CK) 19

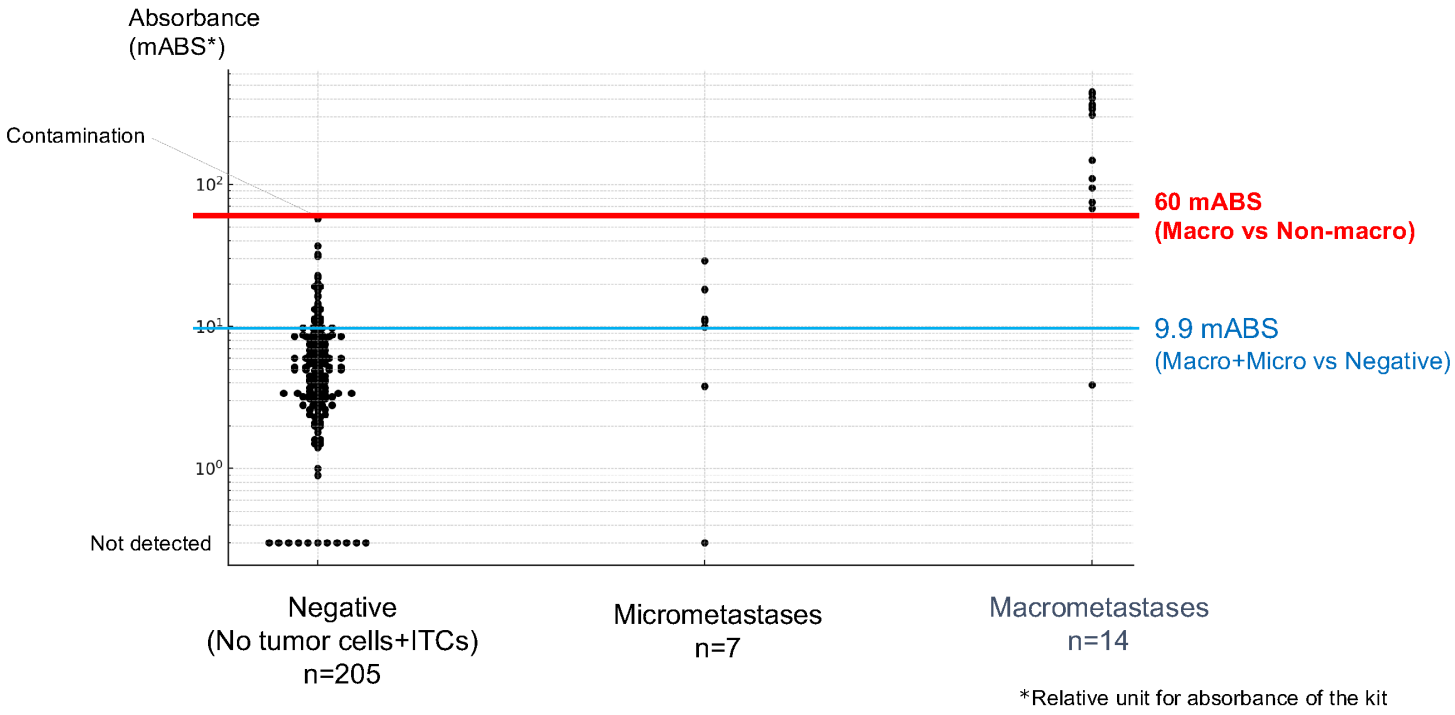


Figure 2

Relationship between cytokeratin (CK) 19 absorbance and histopathological diagnosis

Scatter plot showing CK19 absorbance values (mABS) measured using the SDB kit for each sentinel lymph node according to the permanent histopathological diagnosis. Cut-off values of 60 mABS (for distinguishing macrometastases from non-macrometastases) and 9.9 mABS (for distinguishing any metastases from metastasis-negative cases, including isolated tumor cells [ITCs]) are indicated. Non-macrometastases were defined as micrometastases, ITCs, or the absence of tumor cells

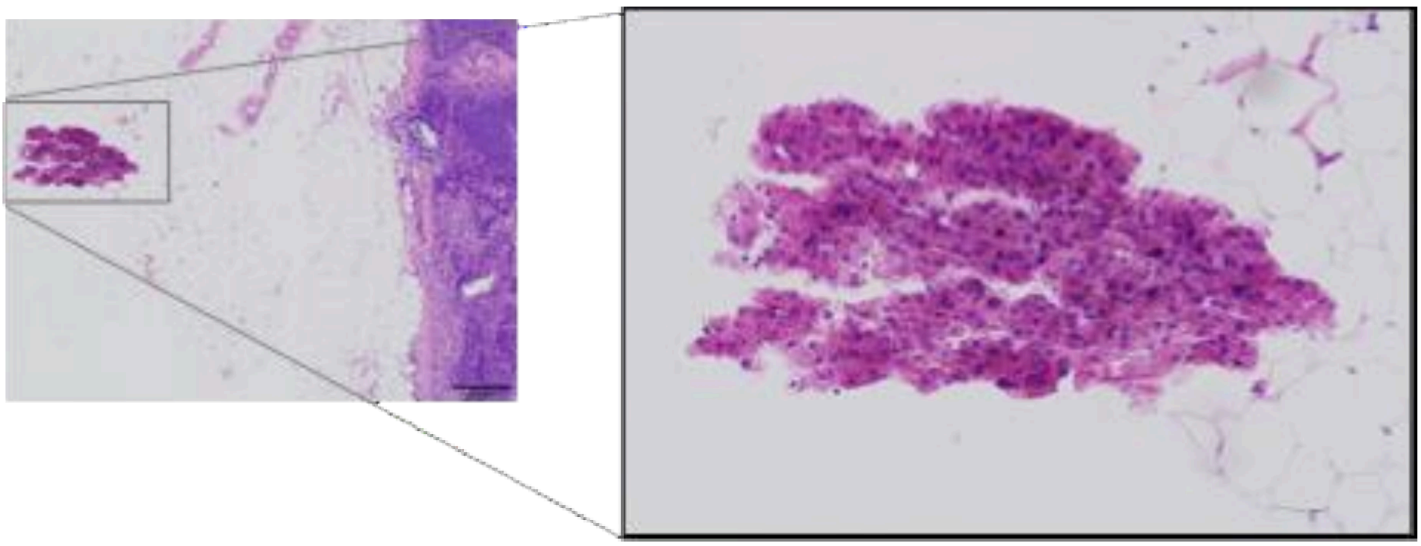


Figure 3

Epithelial contamination outside the lymph nodes

A representative histopathological image showing epithelial contamination outside the sentinel lymph node tissue, likely caused by epithelial exfoliation from the subcutaneous fat or mammary gland tissue during the biopsy procedure. The contaminated epithelial components have a diameter of approximately 0.6 mm. No metastases were identified in the sentinel lymph node tissue

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- fig4.png
- fig5.png