Evaluation of *RASSF1A* methylation in the lysate of sentinel lymph nodes for detecting breast cancer metastasis: A diagnostic accuracy study

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Abstract. The restriction enzyme-based digital methylation-specific polymerase chain reaction (RE-dMSP) assay is useful for diagnosing sentinel lymph node (SN) metastasis in patients with breast cancer, by detecting tumor-derived methylated Ras association domain-containing protein 1 (RASSF1A). In addition, this assay has high concordance (95.0%) with one-step nucleic acid amplification (OSNA). The present study aimed to perform RE-dMSP using OSNA lysate from more patients and to re-evaluate its clinical usage. Overall, 418 SNs from 347 patients were evaluated using both OSNA and RE-dMSP. The concordance rate was 83.3% (348/418). *RASSF1A* methylation of the primary tumors was negative in 36 patients. When these patients were excluded, the concordance rate improved to 88.2% (330/374). Of the 79 OSNA-negative cases, 19 were RE-dMSP-positive, although all were positive for cytokeratin 19 expression in the primary tumor, suggesting that RE-dMSP can detect tumor-derived DNA with a higher sensitivity. The percent of methylated reference of the breast tumors showed a wide variety in the 16 OSNA-positive/RE-dMSP-negative cases, and such variability of methylation could have affected the results in these patients. In conclusion, although RE-dMSP can diagnose SN metastasis with high sensitivity and accuracy, and can be a supplementary tool to OSNA in breast cancer, RE-dMSP showed certain discordance with OSNA and critically depended on the absence or heterogeneity of DNA methylation in breast tumors. Further research is expected to develop an assay targeting other DNA alterations, such as mutations.

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Abbreviations: SN, sentinel lymph node; OSNA, One-Step Nucleic acid Amplification; CK19, cytokeratin 19; LN, lymph node; TTL, total tumor load; RASSF1A, Ras association domain-containing protein 1; SNB, sentinel lymph node biopsy; dPCR, digital polymerase chain reaction; RE-dMSP, restriction enzyme-based digital methylation-specific polymerase chain reaction; FFPE, formalin-fixed paraffin-embedded; PMR, percent of methylated reference

Key words: SN, molecular diagnosis, RASSF1A promoter methylation, OSNA, breast cancer, dPCR

Introduction

Sentinel lymph node (SN) biopsy, commonly known for diagnosing lymph node metastasis among node-negative patients with breast cancer clinically (1-3). Rapid pathological examination and one-step nucleic acid amplification (OSNA) have been utilized for intraoperatively diagnosing SN metastasis in practice (4,5). Cytokeratin 19 (CK19) is expressed in breast cancer cells whereas normal lymph node (LN) cells do not express it. OSNA measures the amplification of CK19 mRNA in SN cells to evaluate the presence of SN metastasis with an accuracy similar to that of histopathological examination (4,5). OSNA can potentially quantify the total tumor load (TTL) in SNs as the summation of CK19 mRNA copies, reported to be significant for forecasting non-SN metastatic state (6-8) and patient prognosis (9). Though, TTL determination using OSNA does not sensitively project the total number of tumor cells in the SN because the copy number of CK19 mRNAs for every tumor cell differs substantially. Actually, a 30-times variance in CK19 mRNA copies amongst tumors of the same size has been reported (4).

The amount of DNA per tumor cell is considered less variable than mRNA; hence the identification of total SN tumor cells from tumor-derived DNA can more accurately ascertain the extent of LN metastasis. Promoter methylation of Ras association domain-containing protein 1 (RASSF1A) is an epigenomic change frequently observed in breast cancer (10,11). We have recently first developed an assay

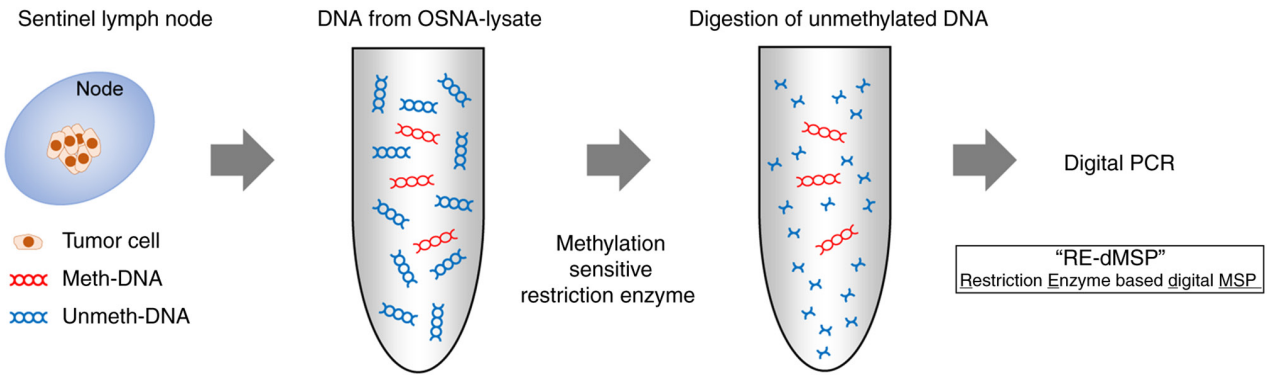

Figure 1. Schematic representation of 'RE-dMSP' for the measurement of methylated RASSF1A DNA in OSNA lysate. Incubation of DNA from OSNA lysate with methylation-sensitive restriction enzyme to digest the unmethylated RASSF1A DNA (non-tumor DNA; blue) and subsequent subjection to digital PCR to measure the methylated RASSF1A DNA (tumor-derived DNA; red). RE-dMSP, restriction enzyme-based digital methylation-specific polymerase chain reaction; RASSF1A, Ras association domain-containing protein 1; OSNA, one-step nucleic acid amplification.

for detecting *RASSF1A* promoter methylation following restriction enzyme-based digital methylation-specific polymerase chain reaction (RE-dMSP). RE-dMSP is adequately sensitive to detect ≥3 copies of methylated *RASSF1A* per assay. In our previous study, 161 SN lysates from 71 patients were analyzed using RE-dMSP and showed high concordance of 95% with the results of OSNA (12). The study also demonstrated that the variation in methylated *RASSF1A* copy number determined using RE-dMSP was remarkably lesser than in CK19 mRNA (2.8 folds vs. 10.5 folds) in 11 breast cancer cell lines (12). Thus, RE-dMSP was indicated to estimate tumor burden of LN metastasis more precisely than OSNA.

This previous study suggested that RE-dMSP is a supplementary method to OSNA in identifying and quantifying axillary metastatic lymph nodes by quantifying methylated *RASSF1A* copy number. However, a further study with more patients is required for validation. This study was conducted under the hypothesis that RE-dMSP is a comparable in sensitivity and specificity to OSNA and is useful in the diagnosis of SN metastases and designed to perform OSNA and RE-dMSP using SN lysate from several patients with breast cancer and to re-assess the clinical usage of RE-dMSP.

Materials and methods

Patients and samples. This study was a diagnostic accuracy study and included 347 consecutive breast cancer patients who underwent surgery with sentinel lymph node biopsy (SNB) and whose sentinel nodes were all assessed using OSNA at Hakuaikai Sagara Hospital between November 2014 and October 2019. Approval for this research was issued by the Ethical Review Board of Osaka University Hospital (approval date/number: January 29, 2019/#18396). All patients provided opt-out consent for the use of their samples in the current study. SNB was performed using both dye (patent blue or indocyanine green) and radiocolloid (technetium-99m tin colloid). The whole SN tissue was used for OSNA and homogenized to 4-ml Lynorhag solution (Sysmex Corporation, Kobe, Japan), of which 2-μl lysate was used for assay. The remaining lysate was kept at -80˚C until RE-dMSP was carried out. The classification of CK19 mRNA copy number per assay, which is listed below in the present study: >5,000, (++); >250 and ≤5,000, (+); >0 and ≤250, (-); and 0, (N.D.). As recommended by the manufacturer of OSNA (4), (++) and (+) were considered positive, and >0 and ≤250 were regarded negative for SN metastasis though the amplification of CK19mRNA was found. In the analysis, 418 SNs from 347 patients were included, and 520 lysates were analyzed (the SN was separated into two lysates in 62 SNs, three lysates in 14 SNs, and four lysates in five SNs because of its large size).

Detection of RASSF1A methylation using RE-dMSP. *RASSF1A* gene methylation was detected using RE-dMSP assay as reported in our previous study (Fig. 1) (12). Briefly, DNA was extracted from 100-150-μl OSNA lysate using the QIAamp Circulating Nucleic Acid Kit (Qiagen GmbH, Hilden, Germany) and eluted in 50-μl desalted water. Then, 6.6-μl DNA solution was mixed to 20 μl volume with following solutions: 1X ddPCR Supermix for probes (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 900 nM each primer, 250 nM probe, and to completely digest unmethylated DNA, three methylation-sensitive restriction enzymes, 10 U *Hha*I, *Hpa*II (New England BioLabs, Inc., Ipswich, MA, USA), and *BstU*I (Thermo-Fisher Scientific, Inc., Waltham, MA, USA). The final 20-μl mixture was incubated for 16 h at 37˚C. Methylation analytical process was undertaken using three wells per assay. 2.0-μl DNA solution was also incubated without restriction enzymes as a control to ensure the presence of DNA (Fig. S1). The sequence of primers and probes was as follows: forward 3'-AGCTGGCACCCGCTGG-5', reverse 3'-GTGTGGGGTTGCACGCG-5', and probe 3'-CTCCAGCC-5' (Universal Probe Library #19; Roche #04686926001). Following incubation, droplet generation oil was added, and subsequently the mixture was transferred onto a QX100 droplet generator (Bio-Rad Laboratories, Inc.). Then, 40-μl emulsified mixture was subjected to polymerase chain reaction (PCR) using a T100 thermal cycler (Bio-Rad Laboratories, Inc.) at 95˚C for 10 min, followed by 40 cycles at 94˚C for 30 sec and 60˚C for 1 min and 98˚C for 10 min. The data analysis was performed with the QX100 droplet reader and QuantaSoft software, version 1.7.4 (both Bio-Rad Laboratories, Inc.). The presence of two or more

dots per well was considered positive result, and the copy numbers of three positive wells were summated. For cases divided into multiple lysates, the results were summed.

For analysis of methylation status in primary breast tumors, DNA was extracted from five 10-μm formalin-fixed paraffin-embedded (FFPE) tumor sections using the QIAamp DNA FFPE kit (Qiagen GmbH), and RE-dMSP was carried out. The cutoff for methylation in primary tumors was set at 4% based on previous reports to distinguish cancer tissues from noncancer tissues (13-15).

Analysis of CK19 with Immunohistochemistry. The protein expression of CK19 in primary tumors was examined using immunohistochemistry with 4-μm FFPE tissue sections. As reported in our former study (16), each section was immunohistochemically stained with mouse monoclonal anti-CK19 primary antibody (clone, RCK 108; 1:100; Dako; Agilent Technologies, Inc.) and a peroxidase-conjugated secondary antibody (catalog number: 414131F; Histofine Simple Stain MAX PO (M); Nichirei, Tokyo, Japan). The sections were visualized subsequently with 3,3-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and counterstained with hematoxylin.

Statistical analysis. R, version 4.1.1, was used for statistical processing. Spearman's rank correlation coefficient was used to assess the correlation between methylated DNA copy number and CK19 mRNA copy number. $P<0.05$ was considered significant. Regarding the sample size in this study, at least 214 cases were needed to estimate a 95% confidence interval with a specificity of 85% and an accuracy of 80 to 90% (interval width 10%). In this study, 420 SNs were provided from Hakuaikai Sagara Hospital as much as possible.

Results

RE-dMSP using SN lysates for OSNA. The clinicopathological characteristics of the 347 patients in this study are presented in Table I. Overall, 418 SNs were analyzed: 284 patients had one SN, 56 had two SNs, and seven had three or more SNs. The amount of total DNA in the SN lysates ranged from 4,800 to 5,920,000 copies per 100-μl lysate, confirming successful DNA extraction from all samples. SN metastases were detected intraoperatively using OSNA in 284 of the 418 SNs (67.9%), and 266 (63.6%) SNs were metastatic according to RE-dMSP results. The concordance rate between the OSNA and RE-dMSP results was 83.3% (Table SIA). In 418 SNs, the amounts of CK19 mRNA and methylated *RASSF1A* were significantly related ($r=0.744$; $P<0.01$) (Fig. 2A). Of 134 OSNA-negative [(-) and N.D.] SNs, 26 (19.4%) were RE-dMSP-positive. Of 284 OSNA-positive [(++) and (+)] SNs, 44 (15.5%) were RE-dMSP-negative (Fig. 2B). Of 91 patients having OSNA-negative [(-) and N.D.] SNs, 19 (20.9%) had RE-dMSP-positive SNs. Of 256 patients having OSNA-positive [(++) and (+)] SNs, 40 (15.6%) had RE-dMSP-negative SNs (Fig. 2C).

CK19 expression in primary tumors with OSNA-negative/RE-dMSP-positive SNs. In 19 patients whose SNs were OSNA-negative/RE-dMSP-positive, immunohistochemical staining for CK19 expression in primary tumors revealed strong homogeneous expression of CK19 in all tumors (Fig. S2).

RASSF1A methylation in primary tumors with OSNA-positive/RE-dMSP-negative SNs. In 40 patients having

Table I. Clinicopathological characteristics of 347 patients with breast cancer.

Characteristic	No. of patients
Age, years	
<50	251
≥50	96
Type of surgery	
Bt	160
Bp	187
No. of SLN	
1	284
2	56
≥3	7
ALND	
No	148
Yes	199
Tumor histology	
IDC	287
ILC	34
Others[a]	26
Tumor size	
T1	218
T2, 3	129
Histological grade	
1, 2	307
3	40
LVI	
Positive	267
Negative	80
Subtype	
HR+[b]/HER2-	280
HER2+	55
TNBC	5
Unknown	7
Recurrence	
No	343
Yes	4

[a]Including 11 mucinous, 7 microinvasive, 4 invasive micropapillary, 3 apocrine and 1 unknown carcinoma. [b]Represented ER+ or PgR+. Bt, total mastectomy; Bp, partial mastectomy; SLN, sentinel lymph node; ALND, axially lymph node dissection; LVI, lymphovascular invasion; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; HR, hormone receptor; TNBC, triple-negative breast cancer.

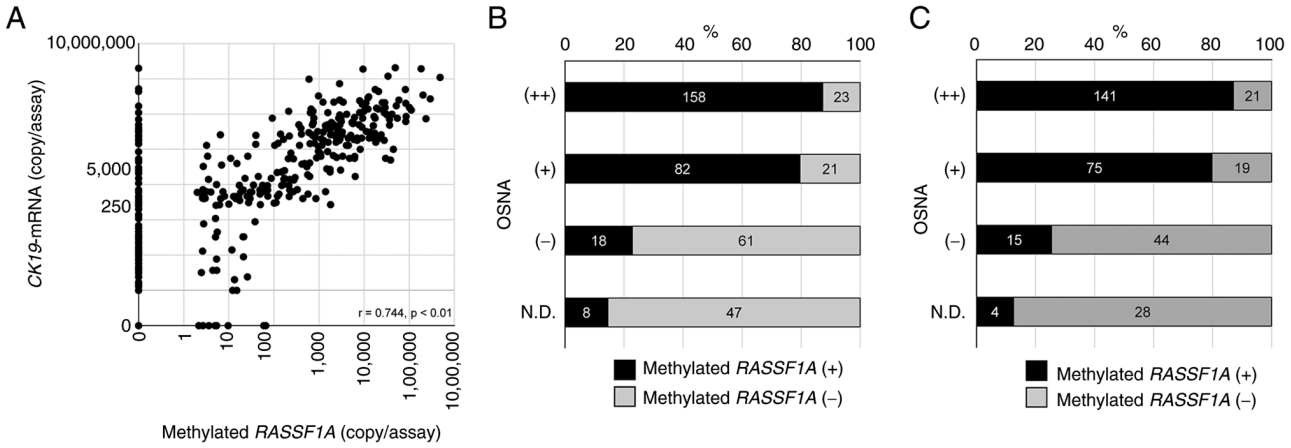

Figure 2. Association between CK19 mRNA expression and methylated RASSF1A copy number of 418 SNs in 347 patients. (A) Correlation between CK19 mRNA expression as determined by OSNA and the copy number (copies/assay) of methylated RASSF1A as determined by RE-dMSP in SNs. (B) Number of methylated RASSF1A-positive and -negative SNs stratified by OSNA diagnosis. (C) Number of methylated RASSF1A-positive and -negative patients stratified by OSNA diagnosis. CK19, cytokeratin 19; RASSF1A, Ras association domain-containing protein 1; SN, sentinel lymph node; OSNA, one-step nucleic acid amplification; RE-dMSP, restriction enzyme-based digital methylation-specific polymerase chain reaction.

SNs of OSNA-positive/RE-dMSP-negative SNs, *RASSF1A* methylation in primary tumors were analyzed using RE-dMSP, and of them, 24 (60%) showed RE-dMSP-negative.

RE-dMSP using SN lysates for OSNA limited to patients with RASSF1A methylation-positive primary tumors. The status of the 112 patients whose SNs were RE-dMSP-negative regardless of OSNA results was determined, and *RASSF1A* methylation (the percent of methylated reference (PMR) >4%) was positive in 76 (67.9%) of 112 tumors (Fig. 3). The other 235 RE-dMSP-positive patients could be considered methylation-positive for primary breast cancer, and therefore, of the 347 patients, 311 (89.6%) were positive for *RASSF1A* methylation in primary tumors (Fig. 3). SN metastases were detected intraoperatively using OSNA in 258 of the 374 SNs (68.9%). Then, 266 (71.1%) SNs were positive according to RE-dMSP results (Table SIB). Only in cases where the primary tumors were positive for methylation (n=311), the copy number of CK19 mRNA by OSNA and *RASSF1A* methylation by RE-dMSP assay showed a better correlation than that in all cases (r=0.834; P<0.01) (Fig. 4A). The concordance rate between the OSNA and RE-dMSP results was 88.2% (Table SIB). Of 116 OSNA-negative [(-) and N.D.] SNs, 26 (22.4%) were RE-dMSP-positive. Of 258 OSNA-positive [(++) and (+)] SNs, 18 (6.9%) were RE-dMSP-negative (Fig. 4B). Of 79 patients having OSNA-negative [(N.D.) or (-)] SNs, 19 (24.1%) had RE-dMSP-positive SNs. Of 232 patients having OSNA-positive [(++) or (+)] SNs, 16 (6.9%) had RE-dMSP-negative SNs (Fig. 4C).

Correlation PMR of the primary tumors with tumor size in OSNA-positive/RE-dMSP-negative SNs. In 16 patients whose SNs were OSNA-positive/RE-dMSP-negative, the PMR of the primary tumors of them ranged from 4.25 to 75.1% (median=22.4%) and showed a positive correlation with tumor size (r=0.405) (Fig. S3).

Discussion

We have previously reported the use of RE-dMSP in detecting tumor-derived DNA in SNs from 71 patients. This study was conducted to validate this finding with more SNs. In this study, 418 SNs from 347 patients with breast cancer were analyzed, and we found a high correlation between the results of OSNA and RE-dMSP. RE-dMSP could be a supplementary tool to OSNA in diagnosing SN metastasis of breast cancer.

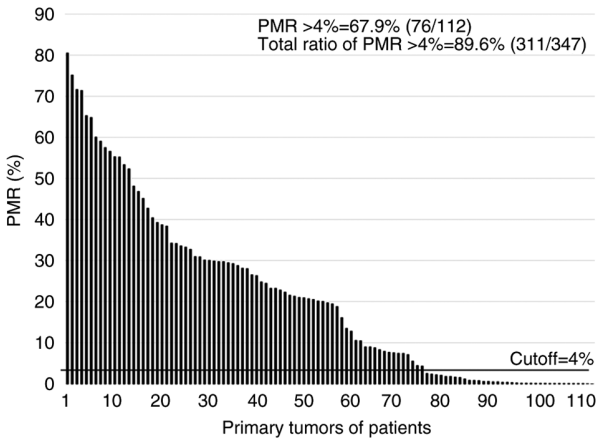

Figure 3. Methylation status of RASSF1A gene in primary tumors of 112 patients with RE-dMSP-negative SNs. RASSF1A, Ras association domain-containing protein 1; SN, sentinel lymph node; RE-dMSP, restriction enzyme-based digital MSP; PMR, percent of methylated reference.

The concordance rate between the OSNA and RE-dMSP results for SNs in this study was lesser compared to our former study, although there was a predominant correlation. OSNA is an assay that targets CK19 mRNA, whereas RE-dMSP targets methylated *RASSF1A*. Considering the differences between the OSNA and RE-dMSP results in SNs, the expression of CK19 and *RASSF1A* methylation in the primary tumor are important.

It has been reported that 3.0-20.5% of patients with breast cancer show low expression of CK19 (17-22). In this study, OSNA-negative/RE-dMSP-positives SNs were observed in 19 patients, and CK19 expression was strongly positive in all these primary tumors, indicating that this concordance

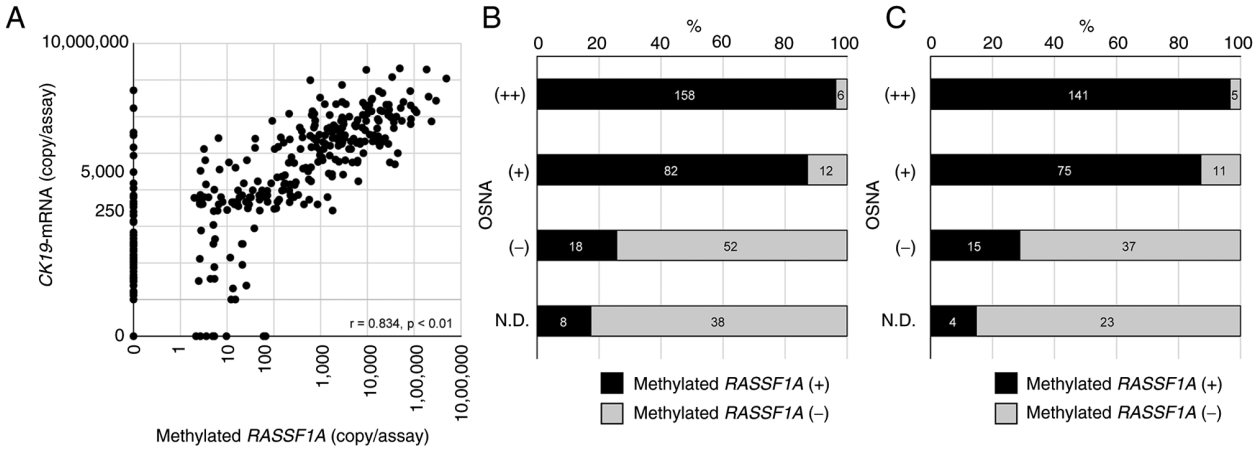

Figure 4. Association between CK19 mRNA expression and the methylated RASSF1A copy number of 374 SNs in 311 patients with primary tumors harboring RASSF1A methylation. (A) Correlation between CK19 mRNA expression as determined by OSNA and the copy number (copies/assay) of methylated RASSF1A as determined by RE-dMSP in SNs. (B) Number of methylated RASSF1A-positive and -negative SNs stratified by OSNA diagnosis. (C) Number of methylated RASSF1A-positive and -negative patients stratified by OSNA diagnosis. CK19, cytokeratin 19; RASSF1A, Ras association domain-containing protein 1; SN, sentinel lymph node; OSNA, one-step nucleic acid amplification; RE-dMSP, restriction enzyme-based digital methylation-specific polymerase chain reaction.

was unlikely to be attributable to the lack of CK19 expression. Four of these 19 patients had N.D. SNs according to the OSNA results. These OSNA-negative/RE-dMSP-positive SNs may be false negatives when assessed using OSNA. Furthermore, RE-dMSP may have identified true metastases that could not be identified using OSNA. RE-dMSP can identify as few as three copies of methylated *RASSF1A* per assay, which corresponds to 150 tumor cells per node, which is much smaller than micro-metastasis (>200 μm in diameter) (12). Therefore, OSNA-negative/RE-dMSP-positive is probably because of the high sensitivity of RE-dMSP. We examined 374 SNs from 311 patients with *RASSF1A* methylation in the primary tumor and found an even higher correlation than all 347 patients.

The prevalence of low *RASSF1A* methylation in the primary tumors has been reported to be 14.8-24% (13,20). In this study, low *RASSF1A* methylation of primary tumors was found in 10.4% (36/347), which was almost consistent with those reported in previous studies. Of the 232 patients with OSNA-positive SNs, 16 (6.9%) were RE-dMSP-negative (Fig. 4C). The total copy number of CK19 mRNA in the SNs of the 16 patients ranged from 340 to 521,700 copies, including 11 OSNA (+) and 5 OSNA (++). The PMR of the primary tumors of these 16 patients showed a positive correlation with tumor size (r=0.405). This indicated that the discordance of OSNA-positive/RE-dMSP-negative tended to be observed in small tumors with low methylation or highly methylated but large tumors, as previously reported (23). The existence of regional and spatial heterogeneity of methylation within the same tumor has been reported (24). A study revealed heterogeneity within a tumor by showing differences in the rate of methylation between blocks of the same tumor and between regions of a block within the same tumor. In this study, the PMR in each case was assessed in the representative portion of the tumor. Therefore, based on the correlation between tumor diameter and the PMR, it is likely that larger tumors contain unmethylated tumor cells in other areas not used for PMR evaluation.

Therefore, OSNA-positive/RE-dMSP-negative SNs can be attributable to metastasis of unmethylated tumor cells from the partially or heterogeneously methylated primary tumors. Considering that four of them had non-SN metastases after axillary dissection, OSNA-positive/RE-dMSP-negative is probably a false-negative result of RE-dMSP. Additionally, as discussed in a previous section, 16 patients were ineligible because the methylation in the primary tumor was partial or heterogeneous. This means that almost 15% (51/347) of the patients cannot undergo RE-dMSP assay because of unfavorable methylation status. In contrast, the loss of CK19 mRNA expression was reported to be much less frequent than that of *RASSF1A* methylation. Even though RE-dMSP can provide more accurate TTL, it tends to yield false-negative results compared with OSNA. Using RE-dMSP alone to diagnose SN metastases is difficult. Additionally, RE-dMSP is not suggested for intraoperative diagnosis. The use of this assay should be investigated for its potential contribution to prognosis prediction and treatment strategy development.

This study has some limitations. We cannot examine the OSNA and RE-dMSP false-positive results. Furthermore, the other limitation of this assay may be due to its ability to target *RASSF1A* methylation only. A previous report by Abe *et al* (12) has analyzed *PIK3CA* mutation and *RASSF1A* methylation in the SN lysates of patients with *PIK3CA* mutation-positive tumors and reported the completed agreement between mutation and methylation status. Moreover, whole-genome/exon sequencing can identify at least one mutation in breast tumors (25), suggesting that mutation can be a more appropriate target for DNA-based SN diagnosis than *RASSF1A* methylation, where it can cover all patients.

In conclusion, RE-dMSP can diagnose SN metastasis with high sensitivity and accuracy and can be a supplementary tool to OSNA. However, it was revealed that false-negative results because of heterogeneous methylation and RE-dMSP's inability to target all patients with breast cancer had a nonnegligible effect on the results. Therefore, it is not a perfect complement to OSNA. Targeting genomic mutations will be

a solution to these problems, and studies on this solution are required.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to information that could compromise the privacy of the research participants but are available from the corresponding author on reasonable request.

Authors' contributions

SAP participated in data analysis and interpretation, and wrote the manuscript. NK was involved in designing the experiments and drafted the manuscript. NM, YO, NG, KA, TY, YoS, TM, TT, MS, and YaS were responsible for providing the resources, analyzing and interpretating the data, and revising the discussion. KS conceptualized and supervised the study. SAP and NM confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study involving human samples was approved by the Ethics Review Board of the Osaka University Hospital and was conducted according to The Declaration of Helsinki. All patients provided opt-out consent for participation and the use of their samples in the current study. This form indicated that their personal data could be utilized for academic or paper presentations, with the assurance of maintaining absolute anonymity.

Patient consent for publication

Not applicable.

Competing interests

NK received a research grant from AstraZeneca. KS received a research grant from AstraZeneca and ROHTO Pharmaceutical Co., Ltd. KS has received honoraria from Sysmex and AstraZeneca. KA, TY, and TM have received honoraria from AstraZeneca. The other authors declare that they have no competing interests.

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