The THAI Journal of SURGERY 2014;35:42-49.
Official Publication of the Royal College of Surgeons of Thailand

Original Article

Efficacy of Intraoperative One-step Nucleic Acid Amplification Assay for Detection of Breast Cancer Metastases in Sentinel Lymph Node

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Abstract

Background: **Accurate intraoperative diagnosis of sentinel lymph node (SLN) metastases enables the selection of patients for axillary lymph node dissections during the same operation, reducing the need for a second operation. To find out whether the one-step nucleic acid amplification (OSNA) assay (Sysmex®) has potential for intraoperative alternative for SLN evaluation, we evaluated the efficacy of OSNA in comparison with frozen section and permanent histological findings. Turn-around time of intraoperative OSNA assay was also considered.**

Materials and Methods: **In total, 111 SLN samples from 62 patients with early stage breast cancers were analyzed. Each SLN was cut into 2-mm sections. Alternate sections were subjected to OSNA assay and histopathological evaluation. Binomial distribution analysis with 95% confidence interval (95%CI) was used for all diagnostic value analysis.**

Results: **The sensitivity and specificity of the OSNA compared with permanent section before discordant case analyses was 84.6% (95%CI, 57.8%-95.7%) and 91.8% (95%CI, 84.7%-95.8%) respectively. Sensitivity and specificity after discordant case analysis were 85.7% (95% CI, 60.0%-96.0%), 92.8% (95% CI, 85.8%-96.5%) and the accuracy was 91.9% (95% CI, 85.3%-95.7%). Turn-around time for OSNA assay was approximately 40 minutes.**

Conclusions: **The OSNA assay was proven to have high accuracy and reliability for evaluation of lymph node metastases in breast cancer patients with acceptable processing time. The assay may be used as an alternate method of frozen section especially in centers lacking of experienced pathologists.**

Keywords: **Breast cancer, nucleic acid amplification assay, sentinel node**

Introduction

Breast cancer causes high morbidity and mortality for women worldwide[1]. Clinical staging after cancer diagnosis is important for deciding further treatment in each patient. Axillary lymph node status is the important predictor for adjuvant treatment and also the prognostic factor of disease. Sentinel lymph node (SLN) biopsy is currently the standard procedure for non-palpable axillary lymph node evaluation. Intraoperative diagnosis of SLNs allows for completion

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of axillary lymph node dissection (ALND) at the time of primary breast surgery if found to be positive for metastatic tumor, eliminating the need for a second surgical procedure with its associated costs, morbidity and patient distress.

Intraoperative evaluation may include frozen section (FS) or touch imprint cytology (IC) or a combination. While the specificity of these techniques is high, the sensitivity varies widely, ranging from 50-75%[2-4]. There is also much variability in the protocols adopted by different laboratories for the final pathologic evaluation of the SLNs in breast cancer. In addition, pathologic assessments examine only a very small amount of the SLN and are subject to inter-observer variability in interpretation, prompting the development of standardized techniques.

To overcome these problems, various molecular techniques have been developed for SLN examination. Using real-time polymerase chain reaction (RT-PCR) technique, a number of specific genes such as *cytokeratin-19 (CK-19), mammaglobin (mam), prolactin inducible protein (PIP), epithelial mucin 1 (MUC1) and prostate-derived ets transcription factor (PDEF)* have been evaluated[5,6]. Molecular testing of SLNs can enable standardized, objective and rapid diagnosis. The one-step nucleic acid amplification (OSNA) (Sysmex®, Inc., Kobe, Japan) detects CK19 mRNA, an established epithelial cell marker for detection of metastatic carcinoma in the SLNs and uses the reverse transcription loop-mediated isothermal amplification (RT-LAMP) method as the amplification technology[7].

To find out whether OSNA has potential for intraoperative alternative for SLN evaluation, we performed the study to evaluate the sensitivity, specificity and accuracy between OSNA, frozen section and permanent histological findings. Turn-around time of intraoperative OSNA assay was also assessed.

Materials and Methods

Patients with newly diagnosed early invasive breast carcinoma at the Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, during July 2011 - January 2012 were prospectively enrolled in this study. The exclusion criteria were male patients, patients with palpable axillary lymph node or metastatic disease, previous axillary operation or contraindication for SLNB and sentinel lymph node not able to be identified intra-operatively. Ethical approval was obtained through the Siriraj Institutional Review Board before the study start date (Protocol No. 288/2554(EC4)). Informed written consents were obtained from all patients before entry into the study.

Sentinel lymph nodes from breast cancer patients were sent to the Department of Pathology. The nodes were cut and then delivered to the Department of Clinical Pathology for OSNA processing. The first 67 nodes were kept at -70 °C and processed in batch (4 nodes were processed at a time). The next 44 nodes were immediately processed with OSNA assay after receiving the samples and the start-finish time was recorded. The sentinel nodes were processed as follows: each node was weighted and labeled, then sliced along the minor axis into 4 pieces (Figure 1) with 2 mm-width triple-blade type tissue cutter TC-10 (Sysmex®). Slices a and c weighted between 0.05-0.6 g were sent for

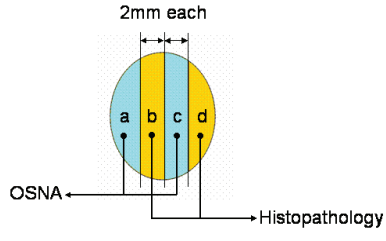

Figure 1 Each sentinel lymph node was cut into four pieces with a special tissue cutter. Two pieces from the node were used for routine intraoperative and histopathological examination (pieces b and d). The rest of the node (pieces a and c) were sent to molecular lab for OSNA processing.

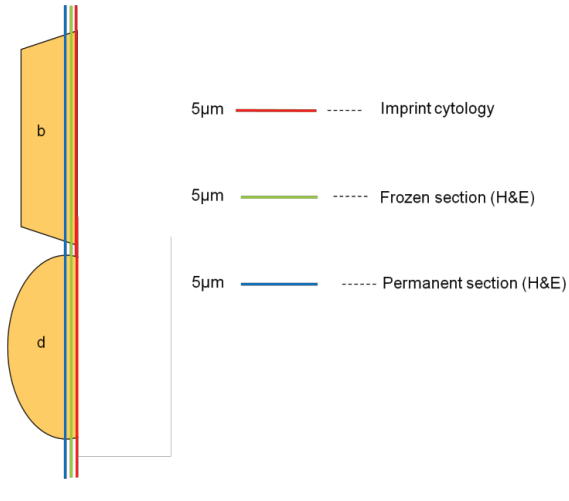

Figure 2 Histopathological examination of slices b and d. Both slices were cut for imprint cytology, frozen section and permanent section examination.

Table 1 Judgment Criteria of Histopathological Examination

1. Positive : "Macrometastasis" or "Micrometastasis" was confirmed by both or either of intraoperative histopathological examination with imprint cytology and frozen section and postoperative histopathological examination with permanent tissue specimen.	
Macrometastasis	Metastasis > 2mm
Micrometastasis	Metastasis > 0.2mm and ≤ 2mm
2. Negative: Both intraoperative histopathological examination with imprint cytology and frozen section and postoperative histopathological examination with formalin-fixed paraffin-embedded sections confirmed "no cancer cell" or "ITC", or either of examination confirmed "no cancer cell" and the other "ITC".	
ITC	Metastasis ≤ 0.2mm
No cancer cell	No cancer cell was observed.

OSNA processing. The remaining two slices (b and d) were subjected to routine intra-operative and post-operative histopathological examination. Imprint cytology was performed on slices b and d. Then both slices were fixed with OTC compound and one frozen section from each slice b and d were prepared. The remaining portions were thawed and fixed again in formalin, embedded in paraffin. Each of slices b and d were sectioned and stained with H & E (Figure 2). The diagnostic criteria of histopathological examination were shown in Table 1.

One step nucleic acid amplification (OSNA) assay was processed as follows: cytokeratin 19 mRNA was used as a target marker for detection of metastasis cancer in sentinel lymph nodes. Before starting to process the lymph nodes, a standard curve, together with a positive and a negative control, must be run using 3 concentrations of CK19 mRNA and reagents from Lynoam BC for CK19 mRNA amplification (Sysmex®). Pieces a and c were homogenized with disposable Lynoprep blade set (Sysmex®) and Polytron® PT1,300D (Kinematica AG, Switzerland) in 4 ml of glycine buffer (Lynorhag, Sysmex) at 10,000 rpm for 60 sec. One ml of the suspension was transferred to a 1.5 ml Eppendorf tube. The tube was then centrifuged at 10,000 xg for 1 min and 200 µl of supernatant was transferred to a new Eppendorf tube. Twenty µl of the supernatant was diluted to 1:100 and 1:10,000 with lysis buffer to achieve the final volume of 200 µl and analyzed with the RD100i system (Sysmex®). For each run, four nodes were analyzed together with a positive and a negative control. Amount of CK19 mRNA copies in sentinel lymph node were calculated from the previously construct standard curve and reported as

Table 2 Judgment Criteria of OSNA Assay

1. Positive: "++," "+" or "reaction inhibited (+i)"	
++	CK19 mRNA (copy number) in the sample (1) is ≥ 5,000 copies/µL
+	CK19 mRNA (copy number) in the sample (1) is < 5,000 and ≥ 250 copies/µL
Reaction inhibited (+i)	CK19 mRNA (copy number) in the sample (1) is < 250 copies/µL , and CK19 mRNA (copy number) in the sample (2) (10-fold diluted solution of the sample (1)) is ≥ 250 copies/µL
2. Negative: "–" and "–L"	
–	CK19 mRNA (copy number) in the sample (1) and in the sample (2) are < 250 copies/µL, and CK19 mRNA copy number in the sample (1) is determined within designated time
–L	Small amount of CK19 mRNA copy number in the sample (1) is detected, but falls in the range of 100 - 250 copies

Note: Total volume of lymph node slices applied to homogenization for 1 test in OSNA assay (4mL lymph node-homogenization buffer) must be less than 600mg. In case of total volume of lymph node slices exceed 600mg, these slices must be divided into 2 or more slices, and these divided slices are individually applied to homogenization and gene amplification reaction. Judgment of the lymph node is negative in case all the measurement results of those divided lymph node slices are "-" or "-L", and judgment is positive in case any of those are "++", "+" or "+i". In case any of slices are judged as "++", the lymph node is judged as "++".

(++), (+i), (+), (-) or (-L) for metastasis (Table 2). The molecular results were compared with results from frozen section and permanent section to evaluate concordant between these results. Turnaround time was recorded from arrival of the nodes in the molecular lab to getting complete results. The analyzer was calibrated to designate samples containing ≥ 250 copies/μl of CK19 mRNA as positive for metastatic tumor. A positive result was further classified into 2 categories: "+" and "++". The "+" signal was generated when the CK19 mRNA number was ≥ 250 copies/μl and ≤ 5,000 copies/μl. A "++" result was generated when the CK19 mRNA number was > 5,000 copies/μl.

Discordant analysis

In case of discordant judgments between OSNA assay and histopathological examination (discordant case) on lymph node basis, the following items were analyzed, and the validity of judgment by OSNA assay is discussed. For negative OSNA but positive histology, expression of CK19 protein in the remaining tissue submitted for histopathological is examined immunohistochemically with anti-CK19 antibody. In case of positive OSNA but negative histology, the remaining portion of the lymph node slices b and d that had been submitted for the histopathological examination are sectioned further to examine the absence/presence of metastatic foci.

Outcome evaluation

The accuracy, sensitivity and specificity of OSNA assay were evaluated compared with histopathological examination as the primary outcome. The turnover time of intraoperative OSNA was analyzed as the secondary outcome.

Statistical analysis

Diagnostic values were determined by comparing the results of the OSNA assay and histopathological examination using statistical program, Confidence Interval Analysis version 2.0, 2000. Binomial distribution analysis with 95% confidence interval (95%CI) was used for all diagnostic values analysis. Mean processing time of intraoperative OSNA was compared using One-way ANOVA test. A *P* value of <0.05 was considered statistically significant.

Results

Patient Characteristics and Sentinel Lymph Nodes

Sixty-four female patients were enrolled in the study and 119 sentinel lymph nodes were investigated by both OSNA assay and histopathology. Two patients

Table 3 Patient Characteristics

Patient Characteristic	Number	Percentage (%)
Enrolled	64	
Excluded	2	
Analyzed	62	100
Age (y)		
Median (range)	48 (28-86)	
< 45	16	26
≥ 45	46	74
Breast surgery		
Conservative	23	37
Mastectomy	39	63
Axillary node assessment		
SLNB	46	74
ALND	16	26
SN identification		
Dye alone	62	100
T classification		
T0	3	5
Tis	9	15
T1mic	3	5
T1a	2	3
T1b	5	8
T1c	14	23
T2	22	35
T3	3	5
Unknown	1	2
Histological type		
Invasive ductal carcinoma	48	77
Invasive lobular carcinoma	4	6
Ductal carcinoma in situ	9	15
Unknown	1	2
Nuclear grade		
1	4	6
2	36	58
3	21	34
Unknown	1	2
Angiolymphatic invasion		
+	10	16
–	27	44
Unknown	25	40
Estrogen receptor		
+	43	69
–	17	27
Unknown	2	3
Progesterone receptor		
+	43	69
–	16	26
Unknown	3	5
HER-2 receptor		
+	12	19
–	38	61
Unknown	12	19

with eight lymph nodes were excluded from data analysis due to previous neoadjuvant chemotherapy. Operation and data analysis were performed on a total of 62 patients. The demographic characteristics of the patients enrolled in the study are presented in Table 3.

OSNA Versus Permanent Section Before Discordance Analysis

Of 111 lymph nodes, 19 nodes were positive (11 were + and 8 were ++) and 92 nodes were negative for OSNA. Comparing to the results of permanent section as gold standard for lymph node metastatic evaluation, 10 samples were discordant. Eight of them were positive for OSNA assay but negative for histopathological examination. Other two were negative for OSNA but positive for histopathological examination. Further discordance analysis was performed in these samples. The results of sensitivity, specificity and accuracy were 84.6% (95%CI, 57.8%-95.7%), 91.8% (95%CI, 84.7%-95.8%) and 91.0% (95%CI, 84.2%-95.0%) respectively. Table 4 showed the results of OSNA versus permanent section before discordance analysis.

Frozen section Versus Permanent Section Before Discordance Analysis

Thirteen lymph nodes were positive for frozen section and permanent section and 98 were negative for both of them. All sample results were concordant between frozen and permanent section. The sensitivity, specificity and accuracy were 100% (95%CI, 77.2%-100%), 100% (95%CI, 96.2%-100%) and 100% (95%CI, 96.7%-100%), respectively. Table 5 showed the results.

Table 4 OSNA Versus Permanent Section Results Before Discordant Analysis

		Permanent Section	
		Positive	**Negative**
OSNA	Positive	11	8
	Negative	2	90

Table 5 Frozen Section Versus Permanent Section Results Before Discordant Analysis

		Permanent Section	
		Positive	**Negative**
Frozen	Positive	13	0
	Negative	0	98

Discordant Case Analysis

The 10 lymph node samples, which were discordant, were further investigated by the pathologists as described in Table 6. The two, negative for OSNA but positive for pathological examination, were positive for CK19 expression in immunohistopathological results. Both of them were also positive when the pathologists reviewed the section. One was diagnosed as micrometastasis. Another one presented as distinct tumor clusters in frozen permanent section, and the pathologists diagnosed positive result. The sample No.

Table 6 Discordant Case Analysis

Node No.	OSNA	Frozen Section	Permanent Section	Frozen Node		CK19 immunohisto-chemistry	Permanent Section		Summary
				Review Section	Smear		Review Section	Re-cut Section	
1	-	+	+	+	-	+	+	+	Micrometastasis
2	+	-	-	-	-	+	-	+	Micrometastasis
3	-	+	+	ITC	Distinct tumor clusters	+	Distinct tumor clusters	-	Distinct tumor clusters
4	+	-	-	red fade	-	ITC	ITC	ITC	Negative
5	+	-	-	-	-	ITC	-	-	Negative
6	+	-	-	-	-	-	-	-	Negative
7	+	-	-	-	-	-	-	-	Negative
8	+	-	-	-	-	-	-	-	Negative
9	+	-	-	-	-	-	-	-	Negative
10	+	-	-	-	-	-	-	-	Negative

2 was positive for OSNA but negative for frozen and permanent section. When this sample was re-cut, it was positive for CK19 expression and permanent section as micrometastasis. Other five samples that were positive for OSNA but negative for histopathological results had the same results after re-cut section. The sample No.4 and 5 were positive for CK19 expression.

OSNA Versus Permanent Section After Discordance Analysis

After discordant case analysis, one discordant data (OSNA: positive/Permanent: negative) was changed into concordant result. The sensitivity, specificity and accuracy were 85.7% (95% CI, 60.0%-96.0%), 92.8% (95% CI, 85.8%-96.5%) and 91.9% (95% CI, 85.3%-95.7%), respectively (as shown in Table 7).

Frozen Section Versus Permanent Section After Discordance Analysis

The sensitivity, specificity and accuracy after discordance analysis were 92.9% (95%CI, 68.5%-98.7%), 100% (95%CI, 96.2%-100%) and 99.1% (95%CI, 95.1%-99.8%), respectively. Table 8 showed the results.

OSNA in Neoadjuvant Cases

The eight lymph nodes from neoadjuvant patients were analyzed using OSNA, frozen section and permanent section. All the results were positive for OSNA, frozen and permanent section concordantly.

Table 7 OSNA Versus Permanent Section Results After Discordant Analysis

		Permanent Section	
		Positive	**Negative**
OSNA	Positive	12	7
	Negative	2	90

Table 8 Frozen section Versus Permanent Section Results After Discordant Analysis

		Permanent Section	
		Positive	**Negative**
Frozen	Positive	13	0
	Negative	1	97

Turnover Time of Intraoperative OSNA Assay

The overall time (from receiving the node until complete data analysis) ranged from 20 to 90 min (mean of 47.3 min). We divided data into three phases of learning experience. After gaining the experience, the mean processing time was reduced (as Figure 3). The first, second and third stage mean time were 53.4, 46.2 and 42.2 min, respectively ($p = 0.001$).

Discussion

With over 30,000 SLNs tested to date globally, the OSNA Breast Cancer System performance is comparable to a very detailed histopathological examination of the SLN. This molecular assay is useful for the intraoperative assessment of SLNs by providing rapid, standardized and objective testing of the lymph nodes. Previous studies have reported the high diagnostic values of OSNA assay. The sensitivity and specificity were range from 91%-100% and 95%-99%, respectively. The concordance between OSNA and pathological results was also high[8-12]. According to our study, the sensitivity was 85.7% (95% CI, 60.0%-96.0%). This may be due to the small sample size.

Only 10 cases from our study showed discordant results. After discordant case analysis, 3 nodes that were previously negative in pathological findings were positive in CK19 expression in immunohistochemical examination. One was micrometastasis in re-cut slices. This case shows that OSNA is more sensitive than permanent histology. Other two presented the isolated tumor cells. The five of eight cases from OSNA positive/Permanent section were negative. These five

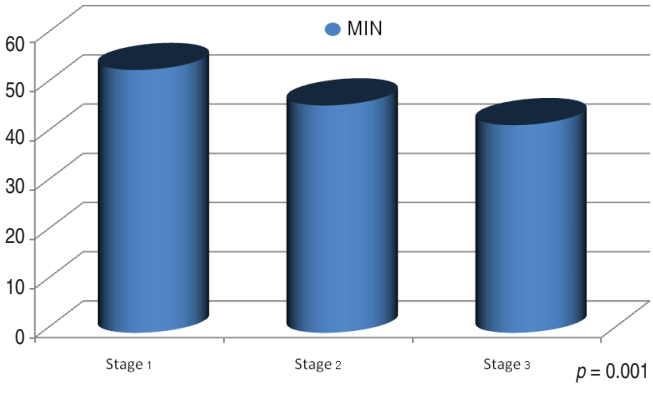

Figure 3 Mean time in the three stages of the learning period

cases seems to be false positive by OSNA. Alternatively, they could be viewed as false negative by even permanent histology. In the evaluation in Singapore and Australia, patient happens to have another sentinel node which was positive by both OSNA and permanent histology, suggesting that OSNA may be more sensitive than permanent histology. False positive results may occur from presence of pseudogenes or benign epithelial cells contamination[9]. In this regard, it might also be observed that this assay adopted the RT-LAMP method developed by Notomi et al[7]. The amplification process is isothermal by means of six primers and can detect CK19 mRNA quantitatively without pseudogenes interference. The assay can differentiate contamination of a few benign epithelial cells and the presence of ITCs from clinically significance by using a verified cutoff value[13].

Two cases of OSNA-/Permanent section+ were positive of CK19 expression in immunohistochemistry. This could be the technical error of OSNA processing. One of the cases has micrometastasis of 0.8 mm by permanent histology on H & E, which is a very small metastasis, while the other case is distinct isolated tumor cells by permanent histology. The latter case had "-L" for OSNA (Table 2), and did not reach the cut-off criteria for positive OSNA. These two cases may be sampling bias where the metastasis presented in the tissue submitted for histology but absent in the tissue submitted for OSNA.

We also evaluated the SNLN from previous neoadjuvant chemotherapy patients. Total 8 nodes were evaluated using OSNA, frozen and permanent sections. The results were concordantly positive. This is a pilot study applying OSNA to evaluate SLNB in patients with breast cancer treated with neoadjuvant chemotherapy. Previous study showed that OSNA had high accuracy in lymph node metastatic evaluation.14 Thus, OSNA could be used in SLNB for breast cancer patients who receive neoadjuvant chemotherapy. Further prospective study should be performed on the role of OSNA in SLN evaluation in breast cancer patients treated with neoadjuvant chemotherapy.

The turnaround time of OSNA was around 40 minutes, which was similar to previous studies[11,15]. OSNA has its particular time of processing. After gaining the experience, the time spending is less[15]. Furthermore, OSNA is feasible and applicable in a hospital where pathologist are not available for frozen section evaluation. One study showed that OSNA method reduces the admission days, duration of surgery, and is cost saving[16].

In conclusion, the OSNA assay proved to have high accuracy and reliability for evaluation of lymph node metastases in breast cancer patients. This tool can be used intra-operatively because the processing time is within acceptable limits. We believe that OSNA will be useful in the hospital where pathologists are not available for frozen section evaluation.

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