Breast Cancer (2013) 20:181–186
DOI 10.1007/s12282-011-0324-z

ORIGINAL ARTICLE

Clinical application of the one-step nucleic acid amplification method to detect sentinel lymph node metastasis in breast cancer

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Received: 29 July 2011 / Accepted: 24 November 2011 / Published online: 28 December 2011

Abstract
Background The one-step nucleic acid amplification (OSNA) method can assess the expression level of cytokeratin 19 mRNA in sentinel lymph nodes in breast cancer. We compared the time required for diagnosis and concordance of results between the OSNA method and conventional intraoperative pathological examination. We then examined the relationship between the frequency of non-sentinel lymph node metastasis and (1) the expression level of CK19 mRNA in the sentinel lymph nodes and (2) clinico-pathological features of the primary tumor.
Methods In the comparison study, pairs of sentinel lymph node sections from 53 consecutive patients were examined: one section by hematoxylin-eosin staining and the other by OSNA assay. The latter involved reverse-transcription loop-mediated isothermal amplification of cytokeratin 19 mRNA, assessed quantitatively. In the second phase, 306 sentinel lymph nodes were removed from 248 consecutive patients, and whole sentinel lymph nodes were examined by OSNA assay alone.
Results OSNA assay was a little more time-consuming than conventional pathological diagnosis (34–45 vs. 22–25 min, $p < 0.0001$). Concordance between the two methods was 93%. The frequency of non-sentinel lymph node metastasis ($p < 0.0001$) and the total number of lymph node metastases ($p < 0.0001$) increased with the amount of cytokeratin 19 mRNA on OSNA assay. We found no significant relationship between the amount of cytokeratin 19 mRNA in sentinel lymph nodes and breast cancer immunohistochemical subtype.
Conclusions The OSNA method is suitable to detect sentinel lymph node metastasis and to predict the possibility of non-sentinel metastasis. This semi-automated quantitative analysis system reduces the burden on pathologists.

Keywords Breast cancer · Sentinel lymph node · CK19 · OSNA

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Introduction

Several large clinical trials have shown that sentinel lymph node biopsy (SNB) is associated with less comorbidity than axillary lymph node dissection. In trials with a long observation period, patients who underwent SNB alone had equivalent prognoses to those who had axillary lymph node dissection [1, 2]. As breast surgery has become less and less invasive in recent times, SNB without axillary lymph node dissection has also become standard treatment for clinically node-negative patients. The ACOSOG Z0011 trial showed that, even for patients who underwent breast-conserving surgery and in whom a metastatic sentinel lymph node was found, avoidance of axillary lymph node dissection did not diminish prognosis [2].

The key aim of SNB is to determine whether breast cancer metastasis exists in the sentinel lymph nodes. The

standard method has been pathological detection of sentinel lymph node metastasis stained by hematoxylin and eosin and categorized according to the American Joint Cancer Committee/Union Internationale Contre le Cancer (UICC/AJCC) TNM classification. The problem with this standard method is that pathologists can examine only a limited number of slides intraoperatively, so metastasis is sometimes missed.

The one-step nucleic acid amplification (OSNA) method for the detection of sentinel lymph node metastasis was first reported by Tsujimoto et al. [3]. It consists of solubilization of a lymph node followed by reverse-transcription loop-mediated isothermal amplification (RT-LAMP) of cytokeratin (CK) 19 mRNA. The RD-100i system (Sysmex, Kobe, Japan), which performs RT-LAMP of CK19 automatically, was approved for the detection of sentinel lymph node metastasis in Japan in 2008. The advantage of the OSNA method is that it can cover the whole lymph node, unlike step sectioning during pathological examination. Meanwhile, the anticipated drawbacks of the method are false-negative results due to low CK19 expression and false-positive results caused by inclusion of mammary luminal cells. This study aimed to investigate the clinical application of the OSNA method to detect sentinel lymph node metastasis.

This study had two phases. The aim of the first was to compare frozen pathological examination with the OSNA assay in terms of the time required for each procedure and the concordance rate for the two procedures (comparison study). The aim of the second was to investigate the relationship between the frequency of non-sentinel lymph node metastasis and (1) the expression level of CK19 mRNA in the sentinel lymph nodes and (2) clinico-pathological features of the primary tumor.

Materials and methods

Identification of sentinel lymph nodes

First 0.5 ml of the radio-pharmaceutical compound technetium (99mTc)-albumin colloid (Nanocoll; GE Healthcare, Little Chalfont, England) mixed with 0.5 ml of 1% lidocaine hydrochloride was injected into the dermis of the areola 4–7 h before the surgery. All patients underwent preoperative static scintigraphic imaging in anterior and oblique projections using a dual-head gamma camera (Millennium VG, General Electronic Co., Fairfield, CT, USA) with a low-energy, high-resolution collimator (4-min acquisition in a 256 × 256 matrix) approximately 30 min to 1 h after injection of the radiocolloid tracer. The locations of axillary sentinel lymph nodes were then marked on the patient's skin. The patient was administered general anesthesia in the operating room, and 2 ml of Patent Blue V dye (Laboratoire Guerbet, Aulnay-sous-Bois, France) diluted to a total volume of 5 ml with saline was injected into the dermis of the areola immediately before the first incision. Intraoperative identification of sentinel lymph nodes was based on blue dye mapping and a handheld gamma probe (Navigator GPS, Radiation Monitoring Device Instruments Corp., Watertown, MA, USA) detection. All nodes that stained blue or those with radioactive counts more than 50 times the background count were defined as sentinel lymph nodes. During this study, we omitted axillary dissection in the patients with no metastases in the sentinel lymph nodes (SNs) and performed axillary dissection on the patients with micro- or macrometastases in SNs.

Clinical application of the OSNA method to detect sentinel lymph node metastasis in breast cancer

In the comparison study, a sentinel lymph node was sliced to give a pair of sections, each with a thickness of 2 mm. One of the paired sections was then stained with the standard hematoxylin-eosin method, and the other was examined by OSNA assay. The former section was fixed in 4% buffered formaldehyde and embedded in a single paraffin block. One pathologist histologically examined all prepared slides from this section without information about the results of the OSNA assay. The definition of sentinel lymph node metastasis was based on the 7th UICC/AJCC TNM classification.

Detailed procedures of the OSNA assay are described by Tsujimoto et al. [3]. Briefly, sentinel lymph nodes were homogenized with 4 ml of a lysis buffer solution and centrifuged at 10,000*g* at room temperature. The RD-100i system was used to analyze 2 μl of the supernatant with an RT-LAMP method. Lymph nodes were assessed as negative when there were fewer than 2.5×10^2 copies/μl of CK19 mRNA; positive (1+) when there were between 2.5×10^2 copies/μl and 5.0×10^3 copies/μl; and positive (2+) when there were more than 5.0×10^3 copies/μl. Because some substances interfere with the RT-LAMP reaction, we always prepared the dilute solution of homogenized lymph node. The positive (i+) was determined positive under the inhibition of the RT-LAMP reaction, and CK19 mRNA was >250 copies/μl in the 10-time diluted solution. Positive (1+) was considered as micrometastasis and positive (2+), positive (i+) as macrometastasis.

Immunohistochemistry method

The following immunohistochemistry (IHC) antibodies were used in this study: estrogen receptor (ER) α (clone

1D5, monoclonal, 1:50 dilution; Dako, Glostrup, Denmark), progesterone receptor (PgR) (clone PgR636, monoclonal, 1:800 dilution; Dako), human epidermal growth factor receptor (HER) 2 (Hercep test, Dako), and Ki67 (clone MIB-1, 1:100, Dako). Positivity for ER or PgR was defined as immunoreactivity in >1% of tumor cell nuclei, as recommended in the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) guidelines [4]. Positivity for HER2 IHC was defined as 3+, uniform, intense membrane staining of >30% of invasive tumor cells [5]. We also performed FISH for HER2 in cases in which membrane staining was either non-uniform or weak in intensity but with obvious circumferential distribution in at least 10% of cells. Breast cancer subtypes were classified in terms of ER, PgR, and HER2 status and Ki67 labeling index. ER- and/or PgR-positive and HER2-negative breast cancer was classified into Luminal A and Luminal B subtypes using the Ki67 label index. The cutoff point of the Ki67 labeling index was set at 20%; cases with Ki67 of less than 20% were classified as Luminal A, and cases with Ki67 greater than or equal to 20% were classified as Luminal B.

Ethical considerations

This study was conducted following the principles of the Helsinki Declaration and was approved by the ethics committee of our hospital. We obtained informed consent from all patients who participated in this clinical study.

Statistical analysis

JMP® 9.0 (SAS Institute Inc., Cary, NC, USA) software was used for the statistical analysis in this study. We compared the time required of each method using Student's *t* test, and analyzed the frequency of non-sentinel lymph node metastasis according to the OSNA method using Pearson's chi-square test with a two-sided significance level of 2.5%. The relationship between the results of OSNA and the number of axillary lymph node metastases was compared using a multi-way ANOVA for continuous variables. Logistic regression analysis was performed to investigate the relationship between the rate of non-sentinel lymph node metastasis and following the clinico-pathological features of the patient's age, invasive tumor size, peritumoral lymph vessel invasion, ER, PgR, HER2, grade, and Ki67.

Results

1. Comparison study: between June and July 2009, 53 patients without clinical lymph node metastasis underwent surgical resection of sentinel lymph nodes (61 nodes in total). The detection rate of sentinel lymph nodes was 100% (53/53). To investigate the suitability of the OSNA assay as an alternative to routine pathological examination, we determined the required time (as a measure of clinical usability in an intra-operative setting) from detection of the sentinel node in the pathology laboratory to reporting the result, and we also confirmed the concordance rate of results for the two tests.

It took a little more time to obtain sentinel lymph node results using OSNA (average 36 min; 95% CI 34–37) as compared to pathological examination (average 20 min; 95% CI 19–21; $p < 0.0001$). The concordance rate between the results of the two tests was 93.4% (Table 1). There were four cases in which the results were inconsistent between the two tests; one case was positive by OSNA assay and negative by pathological examination, whereas three cases were negative by OSNA assay and positive by pathological examination. Among the latter three cases, one showed low expression of CK19, and the other two showed normal expression of CK19 at the primary breast cancer site.

2. Relationship between the rate of non-sentinel lymph node metastasis and (a) the expression of CK19 mRNA in sentinel lymph nodes and (b) the clinico-pathological features of the primary site.

After the comparison study, we assumed the consistency rate was high enough to apply the OSNA method in the clinical setting. Thus, whole sentinel lymph nodes were examined by OSNA assay alone from August 2009 to February 2010. A total of 306 sentinel lymph nodes were removed from 248 patients without clinical lymph node metastasis; the average number of sentinel lymph nodes was 1.23 per patient. The detection rate of sentinel lymph nodes was 100% (248/248). Mean age of patients was 57 years old, and most patients had stage I or II disease (Table 2).

Application of the OSNA method to the 248 patients revealed that 198 (79.8%) had negative sentinel lymph nodes and 50 (20.2%) had positive nodes (Table 3). Among the positive cases, 17 (6.9%) were 1+, 26 (10.5%)

Table 1 Comparison of results between OSNA and pathological examination

53 patients	Pathological examination	
61 sentinel lymph nodes	Positive	Negative
OSNA		
Positive	9	1
Negative	3	48

OSNA one-step nucleic acid amplification

Rate of accordance: 93.4%

Table 2 Patient characteristics

	Number of patients	Proportion (%)
Total	248	100
Median age	57	
cStage		
0	23	9.2
I	110	44
II	111	44.8
III	4	1.6
ER		
Positive	213	85.9
Negative	35	14.1
HER2		
3+	24	9.6
2+	18	7.3
1+ or 0	173	69.8
Not determined	33	13.3
Ki 67		
≥20%	109	44
<20%	94	38
Not determined	45	18.1

ER estrogen receptor, *HER2* human epidermal growth factor receptor 2

Table 3 OSNA results in the second phase of the study

OSNA results	CK19 mRNA copies/ml	Number of patients (%)
Negative		
−	$<2.5 \times 10^2$	198 (79.8)
Positive		
2+	$>5.0 \times 10^3$	26 (10.5)
+	2.5×10^2 to 5.0×10^3	17 (6.9)
+i	$>2.5 \times 10^2$ in 10-time diluted solution	7 (2.8)

OSNA one-step nucleic acid amplification, +i positive with reaction inhibited

were 2+, and 7 (2.8%) were +i. Figure 1 shows the relationship between the OSNA assay results and the number of axillary lymph nodes removed at surgery. The mean number of lymph nodes removed was 3 in sentinel lymph node-negative patients, 7 in +i patients, 11 in 1+ patients, and 12 in 2+ patients.

The relationship between OSNA assay results and the frequency of non-sentinel lymph node metastasis is shown in Table 4. Three sentinel lymph node-negative patients (1.5%) demonstrated non-sentinel lymph node metastasis, and the frequency of non-sentinel lymph node metastasis increased with an increasing degree of positivity amount of CK19 mRNA on the OSNA assay. The amount of CK19 mRNA in sentinel lymph nodes was also correlated with the total number of lymph node metastases ($p < 0.0001$) (Fig. 2).

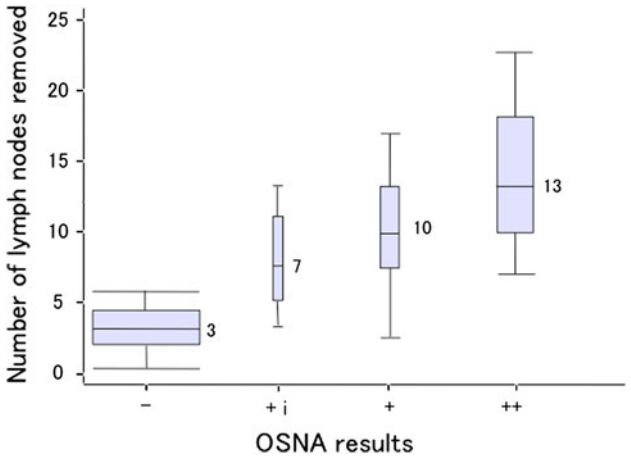

Fig. 1 Number of lymph nodes removed according to OSNA results. The *numbers* on the table represent median number of lymph nodes removed

Table 4 Frequency of non-SLN metastasis according to OSNA result ($p < 0.0001$)

OSNA results in SLN	Non-SLN metastasis		
	Negative	Positive	Frequency (%)
–	195	3	1.5
+i	6	1	14.3
+	11	6	35.3
2+	11	14	56

OSNA one-step nucleic acid amplification, *SLN* sentinel lymph node, *non-SLN* non-sentinel lymph node

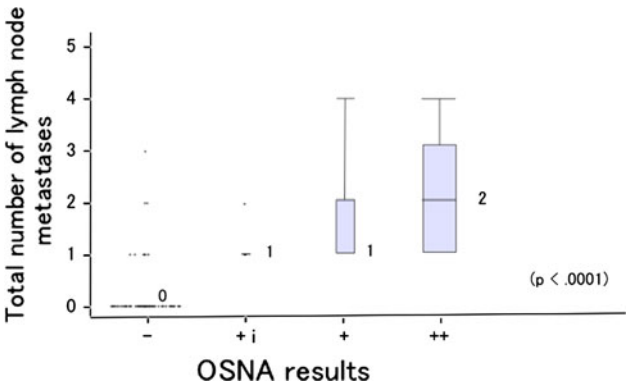

Fig. 2 Relationship between OSNA results and total number of lymph node metastasis. *Dots* represent each case of OSNA result and the number of total lymph node metastasis. The *bar* and *numbers* on the table represent median number of total lymph node metastasis

The relationship between other clinico-pathological features of primary breast cancer and the rate of non-sentinel lymph node metastasis is as follows. On univariate analysis, tumor size ≥2.0 cm (odds ratio 11.0; $p < 0.0001$), positive peritumoral lymph vessel invasion (odds ratio 34.2; $p < 0.0001$), and Ki67 labeling index ≥20% (odds ratio 3.10; $p < 0.01$) were significantly correlated with the frequency of non-sentinel lymph node metastasis. On multivariate analysis, peritumoral lymph node invasion was the sole significant predictor of non-sentinel lymph node metastasis ($p < 0.0001$). We also investigated the relationship between breast cancer immunohistochemical subtype and frequency of sentinel lymph node metastasis detected by the OSNA method. Two hundred eleven cases' subtype was identified except the following cases: DCIS (23 cases), micro-invasive breast cancer (4 cases), and immunohistochemistry results (ER, HER2, Ki67) were not known fully (10 cases). We found no difference between breast cancer subtypes in terms of sentinel lymph node results by the OSNA method ($p = 0.19$) (Table 5). Although there is a significant difference in CK19 mRNA amount between Luminal A and Luminal B ($p = 0.01$), there isn't a significant difference between luminal subtypes and triple negative subtype ($p = 0.50$).

Discussion

In the normal mammary gland, luminal cells mainly express CK7, CK8, CK18, and CK19, whereas basal/myoepithelial cells express CK 5/6, CK14, and CK17. Meanwhile, in breast adenocarcinoma, the CK19 expression rate is reported to be as high as 98.4% [6]. CK19 is a low-molecular-weight keratin of 40 kDa, which is also expressed in simple glandular epithelia. Recently, CK19 mRNA has been widely used to detect breast tumor cells in blood and bone marrow, and it has been shown useful as a prognostic marker in several clinical trials [7–10]. Bostick et al. [7] reported that CK19 could not be a marker for the detection of metastases in sentinel lymph nodes, because it was also expressed in normal lymph nodes and blood. Thereafter, Tsujimoto et al. [3] reported the one-step nucleic acid amplification (OSNA) method. In that study, they defined a CK19 cutoff value of 2.5×10^2 copies/μl, which represented the upper limit of the copy number in pathologically negative lymph nodes. With this cutoff value, no false-positive results were found when the OSNA assay was conducted for 144 histopathologically negative lymph nodes from 60 patients. Tamaki et al. [8] performed a comparison study between the OSNA method and the conventional pathological method in detecting metastasis in sentinel lymph nodes. They reported the OSNA assay could detect lymph node metastasis as accurately as the pathological method and could be an alternative method of intraoperative sentinel node detection. They divided sentinel lymph nodes into four pieces, with two being examined by OSNA and two by the pathological method. Veys et al. [9] also evaluated the usefulness of the RT-PCR assay to estimate the risk of metastasis in non-sentinel lymph nodes with the same method. Furthermore, Osako et al. [10] reported OSNA could detect more micrometastasis than the conventional pathological method. Accordingly, the present study is the first to examine whole sentinel lymph nodes by the OSNA method and to validate the relationship between the quantity of CK19 mRNA in a sentinel lymph node and the frequency of non-sentinel lymph node metastasis.

Application of the OSNA method in clinical practice can ease the burden on the pathologist, as most processes are automatic and the results are quantifiable. Hence, a skilled technician can undertake all steps of the process from system setting to determination of sentinel node metastasis. This was also the first study to compare the time taken for OSNA versus the conventional pathological method; the OSNA method took a little longer, at 30–45 min, but nonetheless was rapid enough to be of intraoperative use. The present concordance rate of 93.4% is similar to that noted in a previous study [8]. There are three possible reasons for discordance between the two methods. The main reason is that the methods examine sentinel lymph nodes in different locations. Another potential reason, in the situation where a case is OSNA positive and pathologically negative, is the limited number of slices that can be examined pathologically. The last reason, in the situation where a case is OSNA negative and pathologically positive, is loss of CK19 expression. Of CK5/6-positive basal breast cancers, 5% are negative for CK19 expression [11]. The present study found no significant relationship between the amount of CK19 mRNA in sentinel lymph nodes and breast cancer subtype as classified by the IHC. The number of triple negative breast cancer was only 17 cases, so we must accumulate more cases to examine the relation between subtype and the amount of CK19 mRNA.

Table 5 Relationship between OSNA results and subtypes of breast cancer ($p = 0.19$)

OSNA results in SLN	Subtypes of breast cancer			
	Luminal A	Luminal B	HER2	Triple negative
−	81	58	11	14
+i	3	4	0	0
+	3	10	1	2
2+	7	15	1	1

OSNA one-step nucleic acid amplification, *SLN* sentinel lymph node, *non-SLN* non-sentinel lymph node

When the amount of CK19 mRNA is less than 2.5×10^2 copies/μl, the frequency of non-sentinel lymph node metastasis was as low as 1.5%, but when it exceeded 5.0×10^3 copies/μl, the rate of non-sentinel lymph node metastasis was as high as 56% ($p < 0.0001$). The amount of CK19 mRNA in sentinel lymph nodes was significantly correlated with the frequency of non-sentinel lymph node metastasis. The present study suggests that the OSNA method is suitable to detect sentinel lymph node metastasis and also to predict non-sentinel metastasis. Moreover, the workload of pathologists can be reduced when OSNA is used to examine sentinel lymph node metastasis. However, we should investigate not only the relationship between the outcome and amount of CK19 mRNA in sentinel lymph nodes, but also the role of axillary lymph node dissection in the case of moderate-to-high expression of CK19 mRNA in sentinel lymph node metastasis. We are planning a randomized controlled trial to compare axillary dissection and omission of dissection in cases of moderate expression of CK19 mRNA in sentinel lymph nodes.

Acknowledgments We are grateful to the entire staff of our pathological laboratory and data management division, especially Ms. Manami Kaikura, Mrs. Taeko Kukita, and Ms. Sinobu Haraguchi, for their excellent technical assistance.

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