

Non-Invasive Evaluation of Axillary Lymph Node Status in Breast Cancer Patients Using Shear Wave Elastography

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Less invasive procedures are currently required to examine the axillary lymph node status. Shear wave elastography with acoustic radiation force impulse provides objective and reproducible quantification of the intrinsic property of the soft tissue. In this study, we measured shear wave velocity of the axillary lymph nodes of patients with breast cancer using Virtual Touch Tissue Quantification (VTTQ). The degree of lymph node metastasis was evaluated by measuring the expression level of cytokeratin 19 (CK19) mRNA, a specific marker for breast cancer cells. The one-step nucleic acid amplification (OSNA) was used to determine the copy number of CK19 mRNA in 149 lymph node specimens of 149 primary breast cancer patients. Axillary lymph node status according to OSNA (copy number/ μ l) were categorized as 0-249 copies (-), 250-5,000 copies (+), and copy number > 5,000 (++) . A category (-) represents no metastasis in the axillary lymph node. There were 121 patients with OSNA-, 9 with OSNA+ and 19 with OSNA++. The average velocities according to OSNA categories were 1.64 ± 0.42 m/second for OSNA-, 2.25 ± 0.78 m/second for OSNA+, and 2.79 ± 0.98 m/second for OSNA++. There were significant differences in the shear wave velocity between OSNA- and OSNA+ ($P = 0.040$) or OSNA++ ($P < 0.001$). The most optimal cutoff velocity to distinguish benign from metastasis is 1.44 m/second, as determined using the receiver operating characteristic method. The shear wave velocity measured with VTTQ could provide clinically useful information about axillary lymph node metastasis in patients with primary breast cancer.

Keywords: axillary lymph node; breast cancer; cytokeratin 19; one-step nucleic acid amplification; Virtual Touch Tissue Quantification

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Introduction

Breast cancer is the most common cancer in women worldwide (Tamaki et al. 2011, 2012; Youl et al. 2011). Breast cancer represents a major cause of morbidity and mortality but early detection and the use of optimal treatments, including surgical, radiation and chemoendocrine therapies, have successfully resulted in a decrease in the breast cancer mortality (Rosen et al. 1993; Mettlin 1999; Greenlee et al. 2001). The nodal status of breast cancer patients at the time of their initial clinical diagnosis is generally considered one of the most important factors for the prognosis of the patients (Fisher et al. 1993; Yenidunya et al. 2011). The sentinel lymph node (SN) is well known as the first lymph node to receive lymph drainage from the primary tumor and is also highly predictive for the status of the remaining axillary lymph node (Morton et al. 1992;

Wilson and Giuliano 2005). The SN biopsy has therefore reduced the morbidity associated with axillary lymph node dissection (ALND) and has readily evolved into the standardized staging procedure in clinically node negative breast cancer patients (Veronesi et al. 1999; Golshan et al. 2003; Lyman et al. 2005; Ferrari et al. 2006). For instance, previous studies demonstrated that morbidity of ALND including lymphedema, arm paresthesia, chronic pain and immobility occurs in 5% to 50% of the patients (Kuehn et al. 2000; Petrek et al. 2001; Silberman et al. 2004; Wilke et al. 2006). In addition, SN biopsy is an acceptable procedure alternative to ALND for staging the axillary lymph node status in patients with early stage breast cancer (Lyman et al. 2005). However, it is also true that post-SN-biopsy morbidity, including upper limb lymphedema, has been reported to occur from 4% to 15% of the patients (Temple et al. 2002; Mansel et al. 2006; Fleissig et al. 2006; Wilke et al.

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2006). Therefore, less invasive procedures are currently required to examine the axillary lymph node status.

Being the quantitative measurement of shear wave elastography based on acoustic radiation force impulse (ARFI), Virtual Touch Tissue Quantification (VTTQ) (Nightingale et al. 2002; Fahey et al. 2007; McAleavey et al. 2007; Tamaki et al. 2013) is a quantitative measurement of shear wave velocity generated by ARFI, allowing both the qualitative visual and quantitative evaluation of the elasticity of the tissue concerned. This method could evaluate the stiffness of tissues such as muscles, tendons, abdominal organ, breast and prostate, providing complementary mechanical characteristics of the tissue concerned, in addition to conventional physical and morphological information provided by sonogram. Unlike the conventional elastography that is performed in an operator-dependent manner, shear wave velocity measured with VTTQ is reproducible in an operator-independent manner. This potentially improves the characterization of tissues and focal lesions in an objective way. VTTQ thus represents a good tool for the quantification of the intrinsic property of the tissue, and could provide objective and reproducible data (Nightingale et al. 2002; Fahey et al. 2007; McAleavey et al. 2007; Tamaki et al. 2013).

The presence or absence of carcinoma metastasis should be evaluated by histological evaluation of lymph nodes but several new methods of evaluating the carcinoma metastasis have been introduced into the clinical practice. Among these newly developed methods, the one-step nucleic acid amplification (OSNA) involves the homogenization of entire lymph node specimens, followed by real-time PCR amplification and quantification of cytokeratin 19 (CK19) mRNA (Tsujimoto et al. 2007). CK19 mRNA is a suitable marker for identifying breast cancer deposits in lymph nodes because virtually all breast cancers express this cytoskeleton protein (Tsujimoto et al. 2007). The RD-100i system (Sysmex, Kobe, Japan), which could automatically perform the reverse-transcription loop-mediated isothermal amplification of CK19, was approved for the detection of sentinel lymph node metastasis (Tsujimoto et al. 2007). The advantage of the OSNA method included that it could examine the whole lymph node unlike step section during the pathological examination (Tsujimoto et al. 2007), although histological information, such as viability of carcinoma cells, is lost. To the best of our knowledge, this is the first study to examine the correlation between the shear wave velocity of axillary lymph node measured with VTTQ and the degree of lymph node metastasis judged by OSNA. We examined whether VTTQ is a potential procedure to reduce an unnecessary axillary lymph node biopsy.

Materials and Methods

Patients

Table 1 summarizes the relevant clinicopathological information of the 149 patients enrolled in the present study. Between October 2011 and September 2012, we examined 149 axillary lymph nodes of

Table 1. Clinicopathological information of the 149 patients.

Total patients number	149
Median age (range)	57 (29-83)
Median SN number (range)	1 (1-4)
Patients number according to OSNA status	
OSNA -	121
OSNA +	9
OSNA ++	19
Average copy number (range)	
OSNA -	250 >
OSNA +	1,835.6 (360-4,600)
OSNA ++	13,963.2 (7,400-480,000)

primary breast cancer with VTTQ using the ARFI technology and OSNA. We excluded 27 patients with neoadjuvant chemotherapy. All specimens sampling was performed at Nahanishi Clinic Okinawa. The median age of the 149 enrolled patients was 57 years (age range, 29-83). The study protocol was approved by the Ethics Committee at Nahanishi Clinic Okinawa (NNCEC2012017). All participants gave informed consent.

Measurement of shear wave velocity with VTTQ

Conventional ultrasound (US) was performed using the ACUSON S2000 US System (Mochida Siemens Medical System, Tokyo, Japan) equipped with a large-format 50-mm linear array transducer with a bandwidth of 6-18 MHz (Yoneda et al. 2010; Tozaki et al. 2011). ARFI imaging (Virtual Touch Tissue Quantification, Mochida Siemens) was performed using the linear array transducer (9L4, Mochida Siemens) with a bandwidth of 4-9 MHz (Yoneda et al. 2010; Tozaki et al. 2011). An acoustic push pulse and detection pulse were used to calculate the shear wave velocity (measured in m/second), which increases with the tissue stiffness (Yoneda et al. 2010; Tozaki et al. 2011). VTTQ involves targeting the lesions to be examined for its elastic properties with a region-of-interest (ROI) cursor having a fixed dimension of 5 × 5 mm (Yoneda et al. 2010; Tozaki et al. 2011). In all patients, these US examinations were performed by one breast surgeon (K.T., 10 years of experience in breast US) at Nahanishi Clinic Okinawa. This investigator was blinded to the OSNA data of the patients. We examined the stiffness of two areas including central and cortical areas of the lymph nodes. We pointed out the fastest velocities as the hotspots of these lesions (Spiratos et al. 2002). In addition, when we could evaluate more than two sentinel nodes, we defined the fastest velocities as the hotspot and examined the correlation between the results of these hotspots and the corresponding lymph nodes.

Measurement of CK19 mRNA copy number by OSNA

OSNA was performed according to the manufacture's instructions (Sysmex, Kobe, Japan) (Tsujimoto et al. 2007). The SN slices were homogenized in 4-ml homogenizing buffer Lynorhag (Sysmex) (Tsujimoto et al. 2007). Twenty microliters of this homogenate were further used for automated amplification of CK19 mRNA via reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Tsujimoto et al. 2007). Real-time PCR amplification was accomplished with the Lyoamp Kit (Sysmex) on RD-100i (Sysmex) (Tsujimoto et al. 2007). The degree of amplification was detected via

by-product of the reaction, pyrophosphate (Tsujimoto et al. 2007). The resulting change in turbidity, upon precipitation of magnesium pyrophosphate, was in turn correlated to CK19 mRNA copy number/ μ l of the original lysate via a standard curve which was established beforehand with three calibrators containing different CK19 mRNA copy numbers (Tsujimoto et al. 2007). A standard positive control sample containing 5,000 copies / μ l of CK19 mRNA and a negative control sample not containing any CK19 mRNA were used for quality assurance in every assay run in this system. Lymph nodes that exceeded the specified maximum weight of 600mg were cut into two or more pieces and processed as separate nodes. Up to four lymph nodes could be analyzed in one run in this system (Tsujimoto et al. 2007). All the results were presented on the RD-100i in qualitative categories (-, +, ++) and further specified by CK19 mRNA copy number/ μ l: 0-249 copies (-), 250-5,000 copies (+), and copy number > 5,000 (++) , respectively (Tsujimoto et al. 2007). A category (-) indicates no metastasis in the axillary lymph node, whereas categories (+) and (++) are comparable to the presence of micrometastasis and macrometastasis, respectively (Tsujimoto et al. 2007).

Statistical analysis

Statistical analyses were performed using StatMate IV for Windows (ATMS, Tokyo, Japan). We compared VTTQ according to the qualitative categories of OSNA with Student's *t* test. The optimal cutoff value for VTTQ to distinguish benign from metastasis was selected using the receiver operating characteristics (ROC) method, by minimizing the sum of the observed false-positive and false-negative errors with bootstrapping methodology. In addition, we also

examined the correlation between VTTQ velocity and the corresponding copy number of OSNA. A *p* value of less than .05 was considered to indicate a statistically significant difference.

Results

The correlation between the results of VTTQ and OSNA

The median quantification velocity was 1.49 (0.93-3.94) m/second. The average of quantification velocities according to OSNA qualitative categories were 1.64 ± 0.42 m/second of OSNA-, 2.67 ± 0.95 m/second of lymph node metastasis including OSNA+ and ++, 2.25 ± 0.78 m/second of OSNA+, and 2.79 ± 0.98 m/second of OSNA++. There were statistically significant differences between OSNA- and lymph node metastasis ($P < 0.001$, 95%CI [3.43-66.42]), OSNA+ ($P = 0.040$, 95%CI [1.13-76.54]), or OSNA++ ($P < 0.001$, 95%CI [2.70-161.51]) (Fig. 1). There were 121 patients with OSNA-, 9 with OSNA+ and 19 with OSNA++ (Table 1). Thus, the lymph node metastasis was present in 28 patients.

The VTTQ cutoff points to distinguish benign from metastasis

Quantification velocity was continuous variable, but both biologically and clinically relevant cutoff point could be determined by the ROC method described above. Among 121 patients without metastasis and 28 patients with lymph node metastasis, the best cutoff value for the

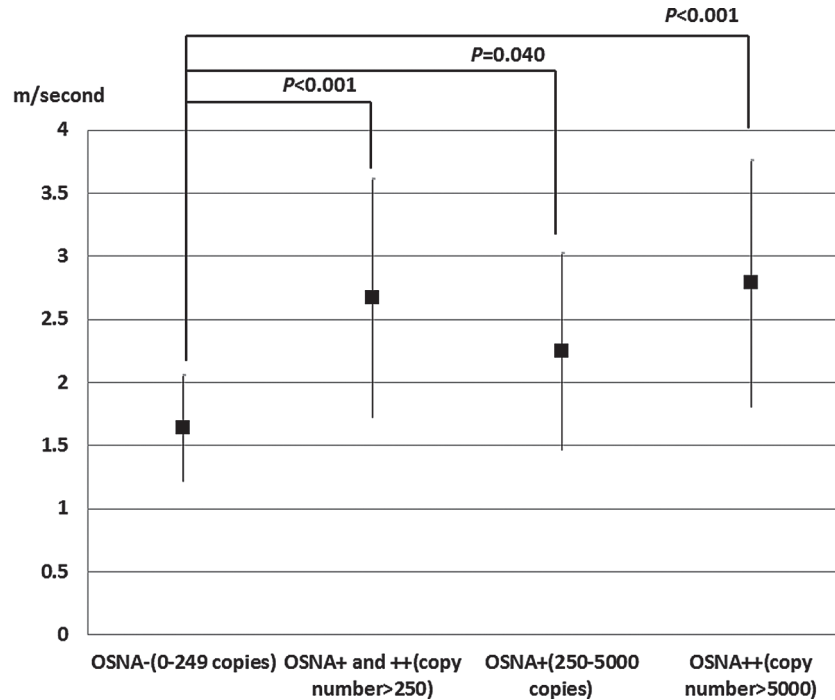


Fig.1. The correlation between the shear wave velocity and the copy number of CK19 mRNA.

The shear wave velocities were measured with VTTQ in 149 patients with primary breast cancer and are shown according to the axillary lymph node status: OSNA-, OSNA+ and ++, OSNA+, and OSNA++. The values were obtained from 149 patients with primary breast cancer. There were statistically significant differences between OSNA- and lymph node metastasis ($P < 0.001$, 95%CI [3.43-66.42]), OSNA+ ($P = 0.040$, 95%CI [1.13-76.54]), or OSNA++ ($P < 0.001$, 95%CI [2.70-161.51]).

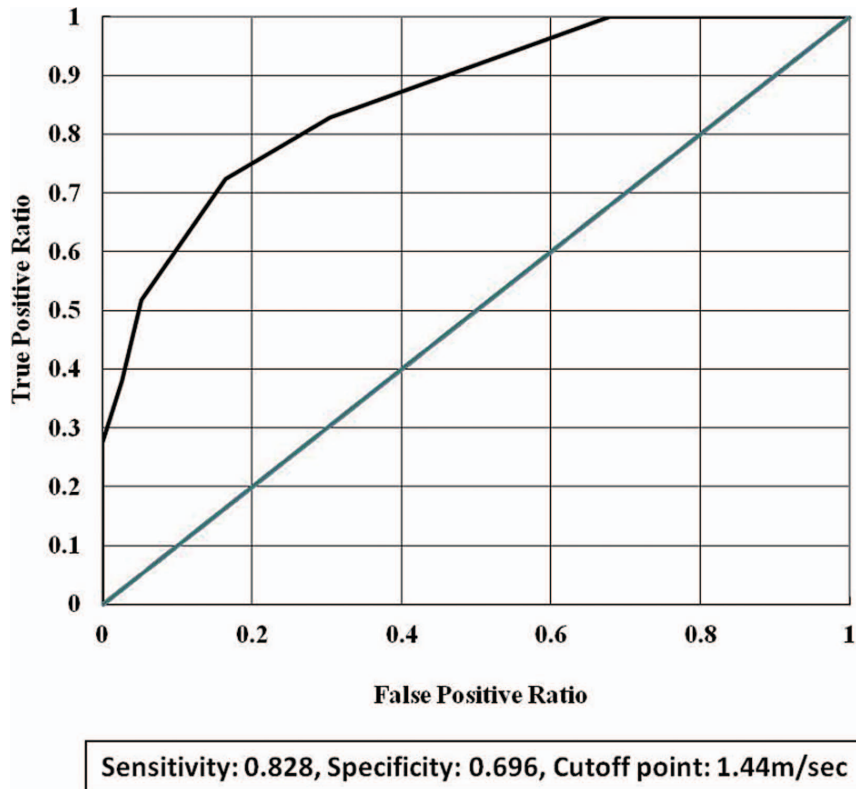


Fig. 2. The optimal VTTQ cutoff points to distinguish benign from metastasis in the axillary lymph nodes of the patients. The x and y axes of ROC curve represent the true and false positive rates, respectively. The true positive rate corresponded to sensitivity and false one to specificity. Establishment of the VTTQ velocity cut points was also included. The true positive rate represents sensitivity and the false one, specificity. ROC analysis was performed to identify benign and metastasis lymph nodes defined by VTTQ velocity using 121 breast cancer patients without lymph node metastasis and 28 patients with lymph node metastasis. The most optimal cut point for the VTTQ velocity according to the results of our present study was 1.44 m/sec.

velocity to distinguish benign from metastasis turned out to be 1.44 m/second (Fig. 2). The sensitivity of the quantification velocity with this cutoff value was 82.8% and the specificity was 69.6%.

Discussion

Numerous clinicopathological factors and novel molecular markers have been investigated to improve the prediction of clinical outcome of the patients with breast cancer but one of the most important prognostic factors is still considered axillary lymph node status (Fisher et al. 1993). Therefore, the identification of the axillary lymph node status is considered important to predict the patients' prognosis and to guide the selection of the patients for adjuvant and/or neoadjuvant therapies (Fisher et al. 1993). ALND has been reported to be associated with complications such as pain, lymphedema and shoulder stiffness (Ernst et al. 2002). Therefore, SN biopsy is a less invasive technique than ALND. Therefore, SN biopsy is currently considered an accepted alternative to ALND for staging the axilla in patients with early stage breast cancer (Lyman et al. 2005). However, some morbidity after SN biopsy has been also reported in previous studies (Temple et al. 2002; Mansel et al. 2006; Fleissig et al. 2006). Less invasive pro-

cedures are thus needed to examine the axillary lymph node status at this juncture.

The shear wave velocity, measured by ARFI imaging with VTTQ, can provide new insights into the evaluation of tissue characteristics in a non-invasive fashion (Nightingale et al. 2002; Fahey et al. 2007; McAleavey et al. 2007). Positive correlation of the stiffness of tissue with VTTQ velocity has also been demonstrated (Nightingale et al. 2002; Fahey et al. 2007; McAleavey et al. 2007). VTTQ also could classify the biological features of the tissues according to the quantity of shear wave velocity (Nightingale et al. 2002; Fahey et al. 2007; McAleavey et al. 2007). The wave propagation speed is generally known to represent an intrinsic and reproducible property of a given tissue (Nightingale et al. 2002; Fahey et al. 2007; McAleavey et al. 2007). Unlike conventional elastography that is based on mechanically induced deformation (strain), acoustic radiation force imaging is operator independent, reproducible, and quantitative. The VTTQ velocity generated by ARFI is therefore an objective and reproducible data of the intrinsic tissue characteristics (Nightingale et al. 2002; Fahey et al. 2007; McAleavey et al. 2007). To the best of our knowledge, the correlation between tumor stiffness according to VTTQ velocity and the axillary lymph

node status has not been reported at all in the previous studies. This is therefore the first study to examine the correlation between VTTQ velocity and OSNA of axillary lymph node.

Results of our present study demonstrated that there were higher VTTQ velocities in axillary lymph node metastasis cases than in non-metastasis cases with statistically significant differences. In addition, there was a statistically significant difference in VTTQ velocities between OSNA- and OSNA+. Therefore, VTTQ is considered to have the potential to diagnose a breast cancer micrometastasis of axillary lymph node. We also examined the optimal cutoff value for VTTQ to distinguish non metastasis from metastasis using the ROC method, by minimizing the sum of the observed false-positive and false-negative errors with bootstrapping methodology. Results of our study demonstrated that the best cutoff value for the velocity to distinguish non metastatic from metastatic lymph nodes was 1.44 m/second. There is a high possibility of axillary lymph node metastasis if the value is more than 1.44 m/second of VTTQ velocity. VTTQ is therefore a potential procedure to reduce the unnecessary axillary lymph node biopsy in the patients with breast cancer.

In our present study, the number of patients is relatively small but the results still could suggest a potential value of VTTQ examination in the clinical management of breast cancer axillary lymph node status. VTTQ has huge potential for examining the axillary lymph node status instead of SN biopsy. Further investigations employing larger numbers of patients with longer periods of clinical follow-up may be required to refine the benefits of VTTQ for breast cancer patients in many situations.

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Conflict of Interest

The authors have no conflict of interest.

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