A new clinical cut-off of cytokeratin 19 mRNA copy number in sentinel lymph node better identifies patients eligible for axillary lymph node dissection in breast cancer

UNIVERSITÀ DEGLI STUDI DI TORINO

A new clinical cut-off of cytokeratin 19 mRNA copy number in sentinel lymph node better identifies patients eligible for axillary lymph node dissection in breast cancer

Cristina Deambrogio[1], Isabella Castellano[2], Alessia Paganotti[3], Elisabetta Omodeo Zorini[4], Fabio Corsi[5], Riccardo Bussone[6], Roberto Franchini[7], Jlenia Antona[1], Umberto Miglio[1], Anna Sapino[2], Concetta Antonacci[4], Renzo Boldorini[1]

[1]Department of Health Science, School of Medicine, University of Eastern Piedmont "Amedeo Avogadro", Novara, Italy

[2]Department of Medical Sciences, University of Turin, Turin, Italy

[3]Unit of Pathology, Maggiore Hospital, Novara, Italy

[4]Unit of Pathology, Luigi Sacco Hospital, Milan, Italy

[5]Department of Surgery, Luigi Sacco Hospital, University of Milan, Milan, Italy

[6]Breast Unit, Azienda Ospedaliera Città della Salute e della Scienza di Torino, Turin, Italy

[7]Unit of Surgery, Maggiore Hospital, Novara, Italy

Corresponding to Professor Renzo Boldorini, Dipartimento di Scienze della Salute, via Solaroli 17, Novara 28100, Italy; renzo.boldorini@med.unipmn.it

Abstract

Aims

Cytokeratin 19 (CK19) mRNA copy number predicts the probability of tumour load in axillary lymph nodes (ALN) and can help in decision-making regarding the axillary dissection. The purpose of this study was to define a new cut-off of CK19 mRNA copy number using the one-step nucleic acid amplification (OSNA) assay on metastatic sentinel lymph nodes (SLN) in order to identify cases at risk of having one or more positive ALN.

Methods

1296 SLN from 1080 patients were analysed with the OSNA assay. 194 patients with positive SLN underwent ALN dissection and the mean value of CK19 copy number (320000) of their SLN was set as initial cut-off. Receiver operative characteristics curve identify a best cut-off of 7700 (sensitivity 78%, specificity 57%). A comparison between our and the traditional cut-off (5000) was performed.

Results

The cut-off of 7700 successfully identifies patients with positive ALN (p=0.001, false- negative cases: 17%). In the range between 5000 and 7700, one patient with positive ALN would not undergo axillary dissection, whereas eight patients with negative ALN would be correctly identified.

Conclusions

We suggest that the level of CK19 mRNA copy number could be the only parameter to consider in the intraoperative management of the axilla.

Introduction

Since the end of 1990s, sentinel lymph node (SLN) biopsy has replaced traditional axillary lymph node (ALN) dissection and has become the standard technique for nodal staging in patients with breast cancer (BC) and clinically-negative axilla.[1–4] Among patients with positive SLNs, ALN dissection (ALND) is the standard of care;[5–7] however, it has been recently discussed whether ALND can be avoided in patients at low risk of metastasis in ALN.[8] ,[9] Many nomograms have been proposed in order to predict the likelihood of ALN positivity,[10] ,[11] but all of these are based on specific features concerning the primary tumour, such as size, histotype, grading, lymphovascular invasion, multifocality, oestrogen and progesterone receptors, Her2 neu overexpression, and molecular subtypes.[11] Since in most of the Breast Cancer Units the preoperative management of BC is based on fine-needle aspiration biopsy,[12] the information needed for nomograms are available only after surgery of the primary tumour.

Since 2007, one-step nucleic acid amplification (OSNA) has been approved as a diagnostic semiautomatic system for lymph node examination.[13] OSNA is an accurate tool for intraoperative assessment of the SLN status and quantitatively measures the presence of cytokeratin 19 (CK19) mRNA copy number, which, using predefined cut-offs,[13] distinguishes the absence of metastasis (less than 250 CK19 mRNA copies) from the presence of either micrometastasis (250–5000 CK19 mRNA copies or >0.2–2mm in diameter) or macrometastasis (more than 5000 CK19 mRNA copies or >2mm in diameter) (Union Internationale Contre le Cancer (UICC) Classification).[14]

Until now, about 30 studies have been published demonstrating the reliability of the molecular OSNA assay in BC routine clinical practice.[15–21] Among these, the study of Buglioni et al[22] demonstrated that a specific cut-off of 2000 CK19 mRNA copy numbers in the SLN could predict the likelihood of finding positive ALN, identifying patients who are eligible for ALND. This copy number was obtained from the molecular analysis of only a part of the SLN: indeed, this cut-off was based on a 4-slice model and only half of the SLN was used for OSNA. In addition, OSNA results were merged with histological and molecular variables of the BC subtypes, available only after surgery in the definitive surgical specimen.[22]

In this study, we tried to define a specific cut-off of CK19 mRNA copy number, useful in clinical practice. Indeed, below this cut-off, the risk of positive non-SLN should be low and thus ALND could be omitted, independently of the characteristics of the primary tumour.

Methods

Study population

This retrospective study included 1296 SLNs from 1080 patients (figure 1) with BC operated in three different hospitals between January 2009 and December 2012, in whom presurgical ultrasound and/or clinical examination as well as fine-needle aspiration biopsy of the axilla were negative. Patients (a) without any immunohistochemical expression of CK19 on the core biopsy of primary tumour, (b) with previous breast or axillary surgery or (c) receiving neoadjuvant chemotherapy were excluded from the study. In all the cases, breast surgery and SLN were performed. Tumours were graded according to Elston and Ellis grading system and staged according to the UICC tumour node metastasis system criteria.[14]

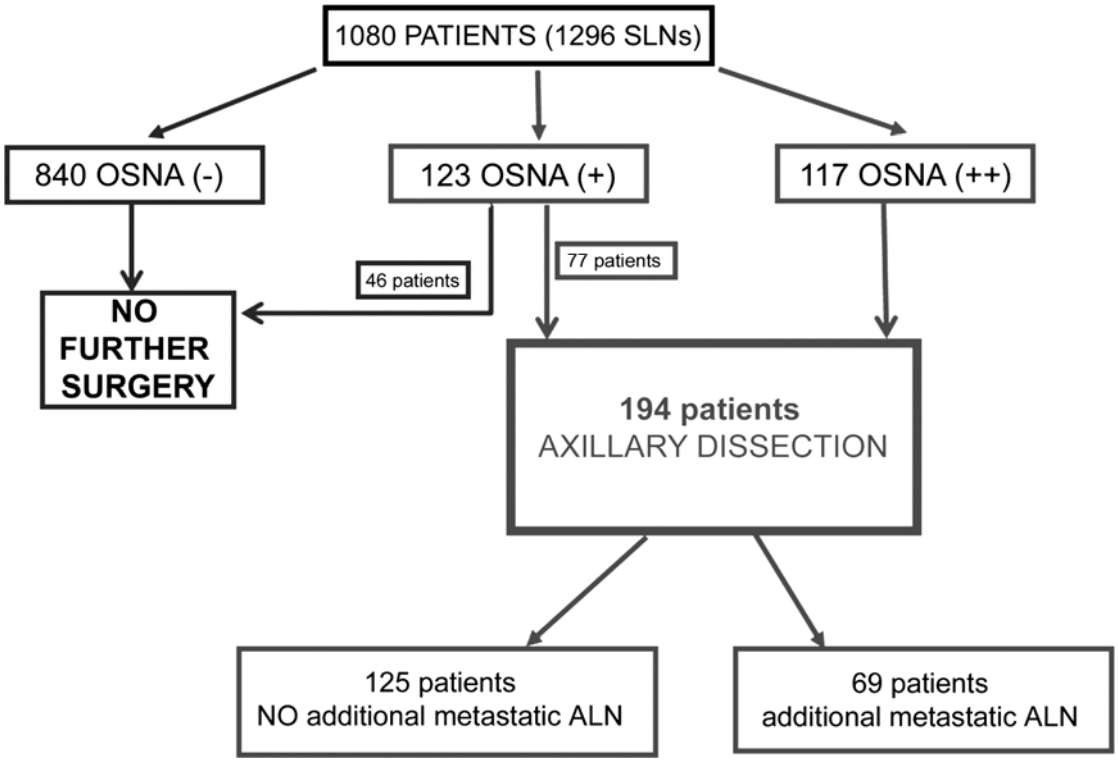

Figure 1
Diagram of patients' recruitment. SLN, sentinel lymph node; ALN, axillary lymph node; OSNA, one-step nucleic acid amplification.

Intraoperative SLN analysis with the OSNA assay

The identification of SLN was performed by injection of a radioactive (99mTc-labelled nanocolloid) tracer at the level of the tumour and Patent Blue retroareolar. SLNs were removed surgically using a handheld γ-ray detection probe. SLNs were excised before primary tumour surgery and immediately sent on ice to pathology units for examination. The mean number of SLNs was 1.2 per patient. The fresh SLNs were harvested from the fat tissue, weighted and cut along the short axis to obtain gross sections of 2.0 mm thickness. Following the standard procedure for OSNA analysis described by Tsujimoto et al,[13] SLN with a weight less than 50 mg were excluded from the study. The mean number of SLNs excluded from the study for the low weight was two per centre. SLNs weighting more than 600 mg were cut into two or more pieces and processed as separate samples. Imprint cytology was performed by touching the two sides of the cut surface of each gross section onto slides. Two of these slides were then fixed in methanol and stained with rapid H&E, and tested by rapid immunocytochemistry with anti-AE1/AE3 antibodies. Whole SLNs were examined by OSNA as previously described.[13] Results were expressed as CK19 mRNA copy number per microlitre, and metastatic load was assessed in accordance with the cut-off levels defined by Tsujimoto et al:[13] (+) symbol was defined as 'micrometastases' and the (++) symbol was defined as 'macrometastases'. A negative result was defined as (−).

Management of the ALNs after OSNA results

ALND was not performed in patients with negative SLN (<250 mRNA copy number). Complete ALND was performed during the same surgical session in all patients with macrometastasis in SLN. Patients with micrometastasis underwent immediately ALND only in two of the three institutions; in one, ALND was delayed after a multidisciplinary discussion concerning clinical and biomorphological features of primary BC.

Axillary non-SLNs were routinely examined by H&E staining and the number of positive lymph nodes was recorded.

Informed consent was obtained from all patients before inclusion in the current protocol for the whole SLN examination by OSNA assay. The main clinical and pathological characteristics of the population studied are summarised in table 1.

Table 1
Clinical and pathological findings of 194 patients with positive sentinel lymph node who underwent axillary dissection

Characteristics	**Patients (n#194)**	**Percentage**
Median age (range)	60.1 (31–89) years	/
Histotype		
Ductal	150	77.3
Lobular	30	15.5

Characteristics	Patients (n#194)	Percentage
Other	14	7.2
Grading		
1	27	13.9
2	114	58.8
3	47	24.2
Unknown	6	3.1
Tumour size		
T1	103	53.1
T2	77	39.7
T3	4	2
Unknown	10	5.2
Vascular invasion		
Absent	78	40.2
Present	116	59.8
Oestrogen receptor		
Negative	32	16.5
Positive	162	83.5
Progesterone receptor		
Negative	19	9.8
Positive	175	90.2
HER2 status		
Negative	173	89.2
Positive	21	10.8

Statistical analysis

Statistical analysis was performed using MedCalc statistical software, V.12.7.3 (MedCalc Software bvba, Ostend, Belgium). The association between categorical variables was determined using the $\chi 2$ or Fisher's exact test. Receiver operative characteristics (ROC) curves and Youden's index were performed in order to identify a new cut-off value. p Values of <0.05, with a 95% CI, were considered statistically significant.

Results

OSNA assay results and management of the axilla

On the whole, SLNs of 840 patients (77.8%) were negative with the OSNA assay and 240 (22.2%) were positive. Among these, 123 (51.2%) had micrometastasis and 117 (48.8%) macrometastasis; 194 patients underwent ALND, including all the patients with macrometastasis and 77 (77/123; 62.6%) patients with micrometastasis. In patients undergoing ALND, 125 (125/194; 64.4%) did not have any additional metastatic lymph nodes in ALN, whereas one or more positive ALNs were detected in 69 patients (69/194; 35.6%) (figure 1).

Correlation between CK19 mRNA copy number in positive SLN and the risk of axillary involvement

First, we calculated the mean value of CK19 mRNA copy number in all the positive SLNs of the patients who underwent ALND, independently to the distinction between micrometastasis and macrometastasis. The CK19 mRNA copy number ranged from 250 to 7400000; the mean value of 320000 CK19 mRNA copies was used as cut-off in order to distinguish patients with a high and low risk of metastasis in ALN. In the group of patients with a CK19 mRNA copy number higher than 320000 in SLN, 55.2% had one or more positive ALN (true-positive), whereas in patients with a CK19 copy number less than 320000 in SLN, 32.2% had one or more positive ALN (false-negative). Although the difference was statistically significant ($p=0.021$, table 2), the number of false-negative cases (53/165, 32%) was too high, probably related to the wide range of CK19 mRNA copy numbers and therefore not useful in clinical practice. In order to overcome the limitation of this result, we used the ROC curve analysis[23] to search for an optimal cut-off value with the highest sensitivity and specificity (figure 2). Across various cut-off points, Youden's index maximised the difference between sensitivity and specificity and between real-positive and false-positive subjects; thus, the optimal cut-off value was calculated. The total sum of sensitivity and 1-specificity of the single copy number cut-offs was represented by the ROC analysis, with a higher area under the curve (AUC) indicating the best cut-off for our objective. On the basis of ROC analysis, with an AUC of 0.69, the value for a cut-off was calculated to 7700 CK19 mRNA copies. Therefore, two groups were identified. The first group included 86 patients having ≤7700 of CK19 mRNA copies: 71 (82.6%) were ALN-negative and 15 (17.4%) ALN-positive, with one or more positive lymph nodes (false-negative cases, table 2). The main pathological findings of these 15 patients are reported in table 3. Except for patient 2, the CK19 mRNA copy number was always under 5000 copies, meaning that these cases would have been considered false-negative also using the classical cut-off of Tsujimoto et al.13 Interestingly, in four cases (n# 2, 7, 8 and 12), more than two additional ALNs were positive (pN2, table 3). The second group included 108 patients with more than 7700 CK19 mRNA copies; 54 (true-positive, 50%) were ALN-positive ($p=0.001$, table 2). The new cut-off showed 78% sensitivity and 57% specificity in differentiating patients with negative ALN or with one or more metastatic lymph nodes (table 4). Positive and negative predictive values of this new cut-off were 50% and 83%, respectively.

Table 2
Comparison between ALN s status of 194 patients and the cut-off obtained by: (A) mean value of CK19 mRNA copy number in all positive SLN, (B) ROC curve and (C) according to Tsujimoto et al[13]

	ALN+69 (%)	ALN—125 (%)	p Value	χ^2
A				
Cut-off (no distinction micrometastases/macrometastases)				
≤320 000 (165)	53 (32.2)	112 (67.8)	0.02	5.719
>320 000 (29)	16 (55.2)	13 (44.8)		
B				
New cut-off (ROC curve)				
≤7700 (86)	15 (17.4)	71 (82.6)	<0.001	22.145
>7700 (108)	54 (50)	54 (50)		
C				
Tsujimoto's cut-off				
≤5000 (77)	14 (18.2)	63 (81.8)	<0.001	16.839
>5000 (117)	55 (47)	62 (53)		

Table 3
Main pathological features of the 15 cases with a cytokeratin 19 (CK19) mRNA copy number ≤7700 and one or more metastatic axillary lymph nodes

	CK19 mRNA (copy/µL)	Histotype	Grading	Tumour size	SLN pos	non-SLN pos	LVI	Oestrogen receptor %	PgR%	HER2 status	Ki67%
1	3.60E+02	Ductal	G2	pT1	1	1	Absent	100	100	Neg	10
2	4.70E+02	Lobular	G2	pT1	1	3	Present	95	75	Neg	4
3	4.90E+02	Ductal	G2	pT1	1	1	Present	99	70	Neg	14
4	9.30E+02	Other	G2	pT2	1	1	Absent	0	0	Pos	20
5	1.30E+03	Ductal	G2	pT1	1	1	Absent	95	82	Neg	14

	CK19 mRNA (copy/µL)	Histotype	Grading	Tumour size	SLN pos	non-SLN pos	LVI	Oestrogen receptor %	PgR%	HER2 status	Ki67%
6	1.70E+03	Ductal	G3	pT1	1	1	Present	80	30	Neg	70
7	2.10E+03	Ductal	G2	pT1	1	4	Absent	100	100	Neg	10
8	2.10E+03	Lobular	G3	pT1	1	3	Absent	95	0	Neg	30
9	2.50E+03	Ductal	G2	pT1	1	1	Absent	0	0	Pos	40
10	2.50E+03	Ductal	G2	pT2	1	1	Absent	100	70	Neg	10
11	2.70E+03	Lobular	G1	pT1	1	1	Absent	100	80	Neg	14
12	2.80E+03	Lobular	G3	pT3	1	4	Present	90	35	Neg	42
13	3.10E+03	Other	G2	Unknown	1	1	Absent	20	10	Neg	5
14	4.10E+03	Ductal	G2	pT2	1	1	Absent	100	100	Neg	12
15	7.40E+03	Ductal	G2	pT2	1	1	Absent	95	38	Neg	32

Table 4
Comparison between the cut-off of cytokeratin 19 mRNA copy number in sentinel lymph node proposed by Tsujimoto et al[13] and our cut-off

	Tsujimoto's cut-off (5000) (%)	Our cut-off (7700) (%)
Positive predictive value	47	50
Negative predictive value	82	83
Sensibility	80	78
Specificity	50	57
False-negative cases	18	17
False-positive cases	53	50

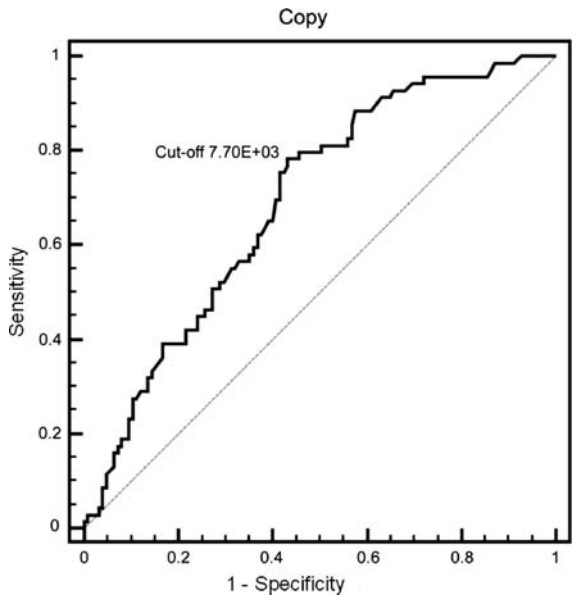

Figure 2
The new best cut-off of cytokeratin 19 mRNA copy number obtained by receiver operative characteristics curve analysis.

Evaluation of the comparison between the traditional cut-off and the new cut-off value

Finally, our case series was further analysed by using the classical cut-off of 5000 CK19 mRNA copy number proposed by Tsujimoto et al.[13] As shown in table 4, only one patient with CK19 mRNA copy number between 5000 and 7700 had one or more positive ALN, whereas eight patients had negative ALN.

Discussion

The OSNA assay allows the intraoperative determination of the SLN tumour load as a basis for decision-making in order to perform surgical axillary dissection.[15–19] Moreover, CK19 mRNA copy number, associated with histological parameters (such as vascular invasion, receptor expression) and molecular subtypes (luminal A, luminal B, HER2-positive, triple-negative), correlates with the risk of tumour metastasis in other ALN, as recently demonstrated by Buglioni et al.[22]

Our study took its origin essentially from two considerations: (a) OSNA assay is able to differentiate micrometastasis and macrometastasis using criteria derived from histology[13] ,[14] and (b) in the majority of Breast Cancer Units, presurgical investigations of BC are currently obtained by means of cytological examination[12] and therefore neither histological parameters nor molecular subtypes of BC are available before surgery. Starting from the idea that the bidimensional histological distinction of micrometastasis and macrometastasis in SLN might not fully reflect a molecular tridimensional concept and an SLN intraoperative assay can be really useful in decisions concerning axillary dissection,[15–18] we constructed an analysis to assess the risk of positive ALN in correlation with CK19 mRNA copy number in SLN as detected by OSNA, without further distinction in micrometastasis or macrometastasis.

Our case series consisted of 1296 SLNs from 1080 BC patients; among these, 194 ALNDs were performed following positive OSNA results (micrometastasis or macrometastasis). This group of patients was the object of the study. The 194 cases with positive SNL showed a PR positivity rate slightly higher than ER positivity rate (90.2% vs 83.5%). This finding, quite unusual for breast carcinoma, could be explained with the lack of uniformity in the preanalytical phase, staining procedure or in the use of different antibodies among the three centres.

Using the cut-off obtained from the mean value of CK19 mRNA copy number in all the SLN-positive patients, independently by the distinction in micrometastasis or macrometastasis, our results had statistical significance for the cut-off value of 320000 CK19 mRNA copy number. Unfortunately, this result was clinically unsatisfactory because of the high percentage of false-negative cases (32%), meaning that, using this cut-off, the surgical treatment would not have been applied to more than a third of the patients with positive ALN. Moreover, the mean value was modified by the presence of high levels of CK19 mRNA copy number in some lymph nodes; indeed in our range, very high values (millions copy) of CK19 copy number were included, suggesting that there was a wrong presurgical selection during the axillary ultrasound or clinical examination in a few cases.

To overcome the limitation of this model, we constructed a ROC curve[23] to establish the best cut-off value in term of sensitivity and specificity allowing us to identify patients with one or more positive non-SLN. We found that the value of 7700 CK19 mRNA copies may be useful to this aim; however, 18% of patients were classified as false-negative. These cases were separately analysed in order to search for any other factors associated with this metastatic profile. Interestingly, considering the CK19 mRNA copy number, 14 out of 15 cases were diagnosed as micrometastasis by OSNA, meaning that in the majority of the Breast Units, these patients would not have done any additional ALND. In addition, three patients had more than three positive ALNs (pN2) and, among these, two were lobular carcinoma, further confirming the need of a more careful follow-up for this histotype.

The current cut-off of Tsujimoto and collegues[13] that identifies macrometastasis is settled as 5000 copies/uL; for this cut-off value generally, there is still a strong uniformity in surgery, as patients with macrometastatic SLN are still now always operated in Italy, differently from micrometastasis.

We have implemented a comparison between our cut-off derived from the calculation with ROC curves23 and the cut-off of Tsujimoto and coworkers;[13] our data were analysed according to both the cut-offs. Below 5000 CK19 mRNA copies in SLN, 14 patients had a positive ALN and 63 a negative ALN. Below 7700 copies (our cut-off) in SLN, 15 patients had a positive ALN and 71 a negative ALN. Thus, the further step to our cut-off demonstrates that only one patient with positive ALN would not have been profited from a complete surgical dissection, but eight patients with negative ALN would not have been submitted to a useless operation on the axilla. Compared with the current cut-off established by Tsujimoto et al,[13] our cut-off had higher specificity to identify patients who do not need axillary dissection. Our cut-off identifies better false-positive and false-negative cases and true-positive and true-negative cases, addressing more precisely to the complete surgery patients who really need the ALND from patients who can avoid it. Heilmann et al found that a cut-off CK19 mRNA copy number of 7900/μL obtained using ROC analysis indicates a positive non-SLN result with the highest sensitivity and specificity.[24] This result was obtained by processing separately 1 mm middle slice for histology and the rest for OSNA, and therefore it might not represent the real copy number of CK19 mRNA of the entire lymph node.

The OSNA assay is an optimal molecular approach to define a rapid diagnosis of SLN status.[15–18] It allows an intraoperative decision about the surgical management of the patients without the need of presurgical histological parameters such as the tumour subtypes.[15] On the other hand, the histological concept of micrometastasis and macrometastasis should be abandoned in favour of an objective evaluation of CK19 mRNA copy number.

The proposed cut-off value of 7700 CK19 mRNA copies may represent a useful tool in the selection of patients in which ALND could be recommended due to the risk of having additional metastatic ALN. Prospective studies are needed to determine the clinical impact of this variable in the management of BC patients.

Take home messages

The decision of performing axillary lymph node dissection in breast cancer relies on the status of the sentinel lymph node treated by histological or molecular approach. The distinction between micrometastasis and macrometastasis in sentinel lymph node, based on established cut-offs (2 mm or 5000 copy number of cytokeratin 19 (CK19) mRNA), helps surgeons to identify patients eligible for axillary lymph node dissection, but some of the patients with micrometastasis could be undertreated.

A recently proposed cut-off of 2000 copy number of CK19 mRNA, along with histological and molecular features of the breast cancer, could better identify patients with high risk for axillary lymph node metastasis. Since these findings are available only after surgery in breast cancer units using fine-needle aspiration biopsy as preoperative management, these data have only a limited clinical application.

Using a receiver operative characteristics curve analysis, we calculated the best cut-off of 7700 copy number of CK19 mRNA obtained by one-step nucleic acid amplification examination of metastatic sentinel lymph node independently to the distinction between micrometastasis and macrometastasis and the findings of primary tumour. This cut-off identifies successfully patients with positive axillary lymph nodes with a low negative rate, suggesting that the level of CK19 mRNA copy number could be the only parameter to consider in the intraoperative management of the axilla.

Acknowledgments

We thank Lega Italiana Lotta contro i Tumori (LILT) section of Novara for partial financial support.

Footnotes

Contributors CD: acquisition of data for the work, drafting the work. IC: revising the work critically. AP: acquisition and analysis of data for the work, drafting the work. EOZ: acquisition and analysis of data for the work. FC, RiB, RF: performed the surgery. JA: acquisition of data for the work. UM: statistical analysis. AS, CA: revising the work critically. ReB: design of the work, drafting the work and final approval of the version to be published.

Funding Lega Italiana Lotta contro i Tumori (LILT) section of Novara.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

References

1. Veronesi U, Paganelli G, Viale G, et al. *Sentinel lymph node biopsy as a staging procedure in breast cancer: update of a randomized controlled study*. Lancet Oncol 2006;7:983–90.
2. Benson JR, Della Rovere GQ.*Management of the axilla in women with breast cancer*. Lancet Oncol 2007;8:331–48.
3. Golshan M, Martin WJ, Dowlatshahi K. *Sentinel lymph node biopsy lowers the rate of lymphedema when compared with standard axillary lymph node dissection*. Am Surg 2003;69:209–11.
4. Reitsamer R, Peintinger F, Prokop E, et al. *Sentinel lymph node biopsy alone without axillary lymph node dissection—follow up of sentinel lymph node negative breast cancer patients*. Eur J Surg Oncol 2003;29:221–3.
5. Chua B, Ung O, Taylor R, et al. *Treatment implications of a positive sentinel lymph node biopsy for patients with early-stage breast carcinoma*. Cancer 2001;92:1769–74.
6. Morrow M. *Is axillary dissection necessary after positive sentinel node biopsy? Yes!*. Ann Surg Oncol 2001;8(Suppl 9):74S–6S.
7. Kamath VJ, Giuliano R, Dauway EL, et al. *Characteristics of the sentinel lymph node in breast cancer predict further involvement of higher-echelon nodes in the axilla: a study to evaluate the need for complete axillary lymph node dissection*. Arch Surg 2001;136:688–92.
8. Damle S, Teal CB. *Can axillary lymph node dissection be safely omitted for early-stage breast cancer patients with sentinel lymph node micrometastasis?*. Indian J Surg Oncol 2010;1:216–17.
9. Galimberti V, Botteri E, Chifu C, et al. *Can we avoid axillary dissection in the micrometastatic sentinel node in breast cancer?* Breast Cancer Res Treat 2012;131:819–25.
10. Meretoja TJ, Leidenius MH, Heikkilä PS, et al. *International multicenter tool to predict the risk of four or more tumour-positive axillary lymph nodes in breast cancer patients with sentinel node macrometastases*. Breast Cancer Res Treat 2013;138:817–27.
11. Meretoja TJ, Leidenius MH, Heikkilä PS, et al. *International multicenter tool to predict the risk of nonsentinel node metastases in breast cancer*. J Natl Cancer Inst 2012;104:1888–96.
12. Popli MB, Sahoo M, Mehrotra N, et al. *Preoperative ultrasound-guided fine-needle aspiration cytology for axillary staging in breast carcinoma*. Australas Radiol 2006;50:122–26.

13. Tsujimoto M, Nakabayashi K, Yoshidome K, et al. *One-step nucleic acid amplification for intraoperative detection of lymph node metastasis in breast cancer patients*. Clin Cancer Res 2007;13:4807–16.

14. Sobin L, Gospodarowicz M, Wittekind C. *UICC Classification of malignant tumours. New Yersey: Wiley Blackwell*, 2009.

15. Castellano I, Macrì L, Deambrogio C, et al. *Reliability of whole sentinel lymph node analysis by one-step nucleic acid amplification for intraoperative diagnosis of breast cancer metastases*. Ann Surg 2012;255:334–42.

16. Cserni G. *Intraoperative analysis of sentinel lymph nodes in breast cancer by one-step nucleic acid amplification*. J Clin Pathol 2012;65:193–9.

17. Le Frère-Belda MA, Bats AS, Gillaizeau F, et al. *Diagnostic performance of one-step nucleic acid amplification for intraoperative sentinel node metastasis detection in breast cancer patients*. Int J Cancer 2012;130:2377–86.

18. Tamaki Y. *One-step nucleic acid amplification assay (OSNA) for sentinel lymph node biopsy*. Breast Cancer Published Online First: 9 August 2012. doi:10.1007/s12282-012-0390-x.

19. Klingler S, Marchal F, Rauch P, et al. *Using one-step nucleic acid amplification (OSNA) for intraoperative detection of lymph node metastasis in breast cancer patients avoids second surgery and accelerates initiation of adjuvant therapy*. Ann Oncol 2013;24:2305–9.

20. Ohi Y, Umekita Y, Sagara Y, et al. *Whole sentinel lymph node analysis by a molecular assay predicts axillary node status in breast cancer*. Br J Cancer 2012;107:1239–43.

21. Osako T, Iwase T, Kimura K, et al. *Sentinel node tumour burden quantified based on cytokeratin 19 mRNA copy number predicts non-sentinel node metastases in breast cancer: Molecular whole-node analysis of all removed nodes*. Eur J Cancer 2013;49:1187–95.

22. Buglioni S, Di Filippo F, Terrenato I, et al. *Quantitative molecular analysis of sentinel lymph node may be predictive of axillary node status in breast cancer classified by molecular subtypes*. PLoS One Published Online First: 22 March 2013. doi:10.1371/journal.pone.0058823.

23. Hajian-Tilaki K. *Receiver Operating Characteristic (ROC) curve analysis for medical diagnostic test evaluation*. Caspian J Intern Med 2013;4:627–35.

24. Heilmann T, Mathiak M, Hofmann J, et al. *Intra-operative use of one-step nucleic acid amplification (OSNA) for detection of the tumour load of sentinel lymph nodes in breast cancer patients*. J Cancer Res Clin Oncol 2013;139: 1649–55.