Histopathology

Histopathology 2016 DOI: 10.1111/his.12924

CK19 expression in breast tumours and lymph node metastasis after neoadjuvant therapy

Begoña Vieites, María Á López-García, Carolina Castilla, María J Hernández, Michele Biscuola, Lina Alfaro,[1] María R Atienza,[2] María Á Castilla[3] & José Palacios[4]

Department of Pathology, Hospital Universitario Virgen del Rocío, Sevilla, Spain, [1]Department of Gynaecology and Obstetrics, Hospital Universitario Virgen del Rocío, Sevilla, Spain, [2]Department of Oncology, Hospital Universitario Virgen del Rocío, Sevilla, Spain, [3]Laboratorio de Oncología Molecular y Nuevas Terapias, Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocío, Universidad de Sevilla, CSIC, Sevilla, Spain, and [4]Department of Pathology, Hospital Universitario Ramón y Cajal, Instituto de Investigación Sanitaria Ramón y Cajal (IRYCIS), Universidad de Alcalá, Madrid, Spain

Date of submission 17 September 2015
Accepted for publication 21 December 2015
Published online *Article Accepted* 28 December 2015

Vieites B, López-García MÁ, Castilla C, Hernández MJ, Biscuola M, Alfaro L, Atienza MR, Castilla MÁ & Palacios J

(2016) *Histopathology* DOI: 10.1111/his.12924

CK19 expression in breast tumours and lymph node metastasis after neoadjuvant therapy

Aims: Neoadjuvant therapy is used in many patients with breast cancer before surgery, with the aim of reducing the tumour size, allowing conservative resections. Sentinel node biopsy is a conservative procedure for handling the axilla in breast cancer; however, the use of this technique after neoadjuvant treatment is under discussion. For sentinel node assay, methods based on the detection of cytokeratin 19 (CK19) mRNA, such as one-step nucleic acid amplification (OSNA), are available. However, if systemic therapy could alter protein expression, then CK19 would not be a good target for analysing these nodes. The aim of this study was to evaluate the immunohistochemical expression of CK19 within different cancer types, and to compare its expression in breast tumours and axillary nodes before and after treatment.

Methods and results: CK19 immunostaining was studied in 162 tumour and node samples before and after treatment. Statistical studies using the McNemar test and chi-square test were performed. CK19 expression was found in 155 cases. We compared CK19 expression in tumour and node biopsies before and after treatment, and we found a lack of significant CK19 expression changes.

Conclusions: Our study has confirmed the preservation of CK19 protein expression in breast cancer cells after neoadjuvant therapy. On the basis of these results, quantification-based methods such as the OSNA CK19 assay, could be an accurate tool with which to analyse the sentinel nodes, regardless of whether they had been obtained before or after treatment.

Keywords: breast cancer, CK19, neoadjuvant therapy, OSNA

Introduction

Neoadjuvant systemic breast cancer therapy (NSBT) is a treatment modality used in eligible patients with breast cancer prior to surgery. Traditionally, it was used in an attempt to reduce the size of tumours with surgical difficulties regarding complete resection, such as inflammatory or locally advanced carcinomas, usually followed by radical mastectomy. However, its indications have increased, and the main objective of NSBT is now to downstage large tumours to allow for breast conservation.

Address for correspondence: B Vieites, Department of Pathology, Hospital Universitario Virgen del Rocío, 41013 Seville, Spain.
e-mail: mb.vieites.sspa@juntadeandalucia.es

Given that more conservative management of the breast has been adopted, the issue of radical treatment of the axilla has been brought up for discussion. In this context, the sentinel lymph node technique might be considered as a useful tool for the management of patients after neoadjuvant systemic therapy.[1–5]

If sentinel lymph node biopsy (SLNB) were to be selected, different histological procedures could be employed to analyse the sentinel nodes. In recent years, a novel standardized molecular method has been incorporated for clinical use. The one-step nucleic acid amplification (OSNA) assay (Sysmex, Kobe, Japan) allows us to analyse the whole lymph node and to obtain a semiquantitative result based on the detection and amplification of cytokeratin 19 (CK19) mRNA.[6–10]

CK19 is a luminal epithelial cell protein expressed by most (96.9–98.2%) breast cancer tumours, but its status after chemotherapy is unknown. According to previous studies, the immunohistochemical (IHC) expression pattern and intensity of different biomarkers may be altered after chemotherapy.[11–16] On the basis of these findings, breast cancer epithelial markers, such as CK19, might undergo similar changes, and consequently CK19 should not be used as a target to search for metastatic cells; however, whether these changes occur has not been evaluated until the present study.

The aims of this study were: (i) to systematically evaluate the IHC expression pattern and intensity of CK19 within a spectrum of different types of breast carcinoma; and (ii) to compare CK19 IHC expression in samples from breast tumours and axillary lymph nodes, both before and after neoadjuvant therapy. On the basis of the results, we will be able to determine whether CK19 expression in sentinel nodes still constitutes a reliable marker for the management of axillary lymphadenectomy in breast cancer patients after neoadjuvant systemic therapy. By determining whether CK19 expression is preserved after treatment, we will be able to evaluate the utility of OSNA assay as the elective method for analysing the SLNB in breast cancer after NSBT.

Materials and methods

The reference material included 721 breast cancer tumours diagnosed in a 3-year time period at the Virgen del Rocío University Hospital-Seville. One hundred and eighty-four of the total population of patients received NSBT, and were therefore selected for the present study; because diagnostic biopsies or tumour resections from 22 patients were not available, the study finally included a total of 162 cases.

Most of the patients presented with a palpable breast mass or a mammographically suspected nodule detected within the breast cancer early detection programme. A core-needle biopsy of the breast lesion was performed in every patient prior to surgical excision. Clinical axillary status was evaluated by palpation and ultrasound imaging, and core biopsy was also performed in cases with suspicion of node metastases. Seventy-nine of 162 patients had metastatic axillary lymph nodes, but only 17 of them had a lymph node biopsy prior to NSBT. With the aim of classifying the type of breast cancer according to international recommendations,[17] IHC assays for oestrogen receptors (clone 6F11; NovoCastra, Newcastle, UK), progesterone receptors (clone 16; NovoCastra), HER2 (HercepTest; Dako, Glostrup, Denmark) and Ki67 (clone MIB-1; Dako) were performed on each breast biopsy specimen.

All tumours were resected after the completion of systemic treatment, including axillary lymph node resections in most of them (91.35%). Samples for the study included: core biopsies of the breast and some suspected nodes before NBST and breast tumour and metastatic nodes after treatment. Core biopsies were fixed in buffered formalin (10%) for 8 h and resection specimens for a median time of 24 h prior to histological processing.

The haematoxylin and eosin (HE)-stained sections from each case were reviewed by the pathologists involved in the study, and the tumours were reclassified according to both histological type and grade, with the aim of avoiding interobserver variability. The main clinical and pathological data were recorded, and are shown in Table 1: age, histological type, tumour size, axillary metastatic nodes, cancer stage (TNM), hormonal status, and pathological response to chemotherapy (according to the Miller and Payne classification[18]).

IHC ASSAY

Two 4-μm sections were obtained from each formalin-fixed and paraffin-embedded tissue block: one for HE staining, and one for CK19 assay. Prior to immunohistochemistry, the HE section was reviewed, and the presence of tumour tissue was confirmed in all samples obtained before and after NSBT. The IHC assay was performed according to standard procedures, by use of the Leica Bond-III automated

Table 1. Clinical and histological features of tumours after neoadjuvant therapy

	Patients, *n* (%)
Age (years)	
<50	78 (48.1)
≥50	84 (51.9)
Histological type	
IDC	137 (84.6)
ILC	14 (8.6)
Others	9 (5.6)
In situ	2 (1.2)
Tumour size (mm)	
≤20	74 (45.7)
20–50	61 (37.6)
>50	27 (16.7)
Lymph node metastases (pN)	
0	57 (35.2)
1–3	51 (31.5)
3–9	34 (21.0)
>9	20 (12.3)
TNM stage	
0	20 (12.3)
I	22 (13.6)
II	59 (36.4)
III	61 (37.7)
Hormonal status	
ER+	117 (72.2)
ER−	45 (27.8)
HER2 status	
Positive	44 (27.2)
Negative	118 (72.8)
Pathological response (according to Miller and Payne grading system[17])	
1	31 (19.1)
2	45 (27.8)
3	38 (23.5)
4	28 (17.3)
5 (CPR)	20 (12.3)

CPR, Complete pathological response; ER, Oestrogen receptor; IDC, Invasive ductal carcinoma; ILC, Invasive lobular carcinoma.

immunostainer system (Leica Biosystems, Wetzlar, Germany). Antigen retrieval was performed by heat-induced epitope retrieval, and the anti-CK19 monoclonal antibody (clone b170; NovoCastra) was applied at a dilution of 1:150 for 30 min at 25°C.

Positivity for CK19 expression was determined as any membrane, cytoplasmic or combined membrane–cytoplasmic staining present in at least 10% of the tumour cells, regardless of the intensity of the staining or whether this was complete or incomplete circumferential staining. Specific membrane or cytoplasmic immunostaining in <10% of tumour cells was defined as CK19-negative. The Allred scoring system for assessing the expression of steroid hormone receptors in breast cancer served as a pattern for our assessment of CK19 expression.[19] IHC interpretation was performed for each sample, and a proportion score (PS) and an intensity score (IS) were obtained. The PS was the percentage of CK19-stained tumour cells relative to the total number of cells, and was classified as: PS0 (0%), PS1 (>0–1%), PS2 (≥1–10%), PS3 (>10–33%), PS4 (>33–66%), and PS5 (>66–100%). The IS represented staining intensity determined by visual assessment, and was classified as: 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong) (Figure 1). The total score (TS) was calculated as the sum of the PS and IS, and ranged from 0 to 8.

For statistical studies, a TS of ≤4 was defined as low CK19 expression, and a TS of >4 was defined as high CK19 expression. The intensity of CK19 staining was considered to be low if the IS was ≤1 and high if the IS was >1, and proportional staining was considered to be high if the PS was ≥3. The same evaluation was performed in samples from lymph node metastases.

STATISTICS

Statistical analysis was performed with SPSS version 19.0 (IBM Corp., Armonk, New York, USA). Changes in CK19 IHC expression before and after NSBT were assessed with the McNemar test. Associations between CK19 IHC expression and clinical variables

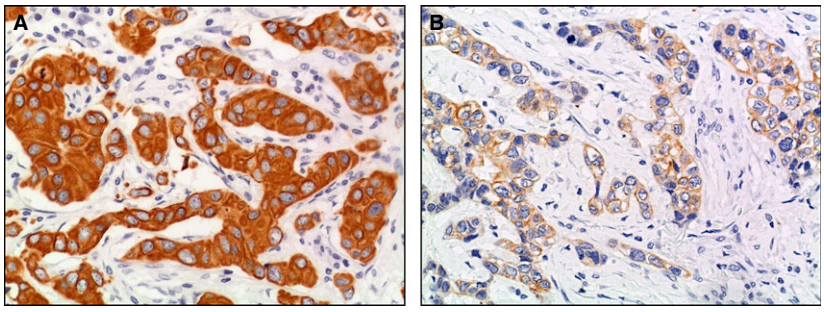

Figure 1. Intensity range of cytokeratin 19 immunohistochemical expression. **A**, Intensity score 3+ (strong). **B**, Intensity score 1+ (weak). Representative micrographs are shown.

were analysed with the chi-square test. Differences were considered to be significant at $P < 0.05$. Cases with a complete pathological response to neoadjuvant therapy were excluded from the statistical analysis, owing to the absence of tumour cells on which to evaluate CK19 expression.

Results

CLINICOPATHOLOGICAL FEATURES

The clinicopathological features of the 162 cases are summarized in Table 1. The patients ranged in age from 31 years to 84 years (mean: 50.48 years). The initial diagnosis was made on core-biopsy specimens obtained by ultrasound-guided core needle or vacuum-assisted breast biopsy. On the basis of the IHC study for hormonal receptors HER2 and Ki67, and according to the criteria suggested by Cheang *et al.*[20] and accepted by international consensus, tumours were classified as triple-negative (TN) phenotype in 28 of the 162 cases, HER2 phenotype in 17 of the cases, and luminal phenotype (43 luminal A and 74 Luminal B; 46 of the latter with luminal B-HER2 phenotype) in 177 of the cases.[17,20] Patients received different neoadjuvant treatments, depending on the type of tumour and its clinical and pathological features: most patients (75%) were treated with anthracycline and taxane-based regimens, either currently or sequentially; 82% of the HER2-positive breast cancer patients (82%) were treated with trastuzumab within the neoadjuvant schedule, and 7% were treated with hormonal therapy (aromatase inhibitors).

After histopathological revision, 139 of the 162 tumours were classified as invasive ductal carcinoma of no special type (IDC-NST), three were classified as mixed tumours (IDC-NST and mucinous differentiation), 14 were classified as invasive lobular carcinoma (ILC), two of these as pleomorphic ILC (P-ILC), three showed features of metaplastic carcinoma (MetapC), two showed features of apocrine carcinoma, and two were pure mucinous carcinomas (MucCs). Twenty cases previously diagnosed as invasive carcinoma showed a complete pathological response (CPR) after treatment, two of them including areas of residual *in-situ* carcinoma.

Tumour sizes after systemic therapy ranged from a lack of tumour cells (CPR) to 130 mm (mean: 28.4 mm). The pathological T (primary tumour) classification after neoadjuvant therapy according to the *AJCC Cancer Staging Manual* 7th edn[21] was ypT1 in 54 cases, ypT2 in 61 cases, and ypT3 in 27 cases. Residual invasive tumour was not detected in 20 breast resection specimens (ypT0), owing to a complete response to treatment. Fifty-five of 162 cases showed no axillary lymph nodes metastases, so axillary dissection might have been avoided in these cases. The pathological response to neoadjuvant therapy in the surgical resection specimens was measured according to the Miller and Payne classification system,[18] comparing cancer cellularity of the core biopsy (before treatment) with that of the resected tumour (after treatment), and grading between 1 (no reduction in the number of tumour cells) and 5 (lack of tumour cells). A CPR was found in 20 cases, a low response (grade 1 and 2) was found in 70 cases, and a medium to high response (grades 3 and 4) was found in 72 cases. Likewise, response to treatment was measured at the axillary lymph nodes: a lack of or minimal changes attributable to neoadjuvant therapy (types A and B) were found in 97 cases, and a moderate to high response (types C and D) was found in 65 cases.

IHC CK19 EXPRESSION

CK19 expression was found in 155 (95.67%) of the 162 breast cancer diagnostic biopsies. Seven cases (4.33%) did not express CK19: two corresponded to MetapC, and one to a P-ILC, all of them showing a TN phenotype, and the rest were IDC-NSTs showing HER2, luminal A and luminal B phenotypes. None of them showed a CPR. The IHC staining pattern in the tumour cells of biopsies prior to neoadjuvant

treatment was mixed (cytoplasmic with a membrane accentuation) in most of the positive cases (67.09%); 39 showed pure membranous staining (25.16%), and the remaining cases (7.75%) showed a diffuse cytoplasmic staining pattern. The intensity of the staining was low (IS: 1) in 26 (22.41%) cases, medium (IS: 2) in 75 (64.65%) cases, and strong (IS: 3) in 15 (12.93%) cases. The membranous–cytoplasmic staining pattern was maintained in most tumours. Changes in the pattern of expression, with more diffuse staining in the post-treatment samples as opposed to the previous pure membranous expression, were detected in 12 of 155 cases; those changes might have been attributable to differences in the preanalytical processing of the tissues (biopsy versus surgical specimen). The PS and IS in breast and metastatic lymph nodes before and after neoadjuvant treatment are summarized in Table 2.

To assess changes in CK19 expression before and after neoadjuvant therapy, a McNemar test was performed (Table 3). We compared CK19 expression in breast cancer and lymph node biopsies before and after NSBT (Figure 2). As shown in Table 3, CK19 expression did not change significantly after treatment in any of the studied conditions. Nine of 155 (5.80%) breast cancer samples showed changes in CK19 expression after NSBT; eight of them were IDC-NSTs, and the other was an MucC. Seven of these nine tumours showed a luminal phenotype, and the remaining two showed a TN phenotype.

Although the restrictive cut-off value used classified the cases in different expression groups (low versus high expression), these samples showed TS values that ranged from 4 to 7, and therefore did not represent large differences.

Next, a chi-square test was performed, in order to determine whether there were any statistical associations between CK19 expression in breast cancer biopsies post-NSBT and several clinical variables (age, histological type, grade, size, TNM stage, pathological response, lymph node response, and lymph node metastasis). As shown in Table 4, none of these variables showed a statistically significant association with CK19 expression.

Discussion

NSBT has become the standard treatment for some types of breast cancer, mainly inflammatory and locally advanced breast cancer prior to surgery. Its use has now been extended to tumours that are primarily eligible for mastectomy, with the aim of reducing their size and making conservative management possible. The St Gallen Consensus Conference[17,22] included the use of NSBT in the therapeutic options for breast cancer, and concluded that the reason for choosing this therapy should not only be the alteration of surgical indications, but also the systemic beneficial effects of this treatment.

The efficacy of NSBT was proved by different trials demonstrating disease-free survival and overall survival rates equivalent to those obtained with systemic treatments administered after surgical resection.[23,24] Moreover, an improvement in effectiveness as compared with adjuvant therapy in patients aged <50 years has been proposed.[24] The use of NSBT in early cancer has also been shown to have significant advantages, such as a hypothetical reduction in tumour dissemination during surgery, a decrease in local recurrence after surgical resection, and an early start of systemic treatment for micrometastatic disease. A crucial point regarding neoadjuvant treatment in relation to adjuvant therapy is the possibility of measuring the pathological response to certain drugs, allowing the identification of resistant or sensitive tumours.[25] Although breast-conserving surgery is the goal of the local treatment of breast cancer after NSBT, the management of the axilla is not clear. Given that the axillary status represents the most important independent prognostic factor in breast cancer, complete axillary lymphadenectomy has represented the choice of treatment, but in recent years conservative options with the sentinel node assay have been proposed.[1,2,26,27]

There is no consensus about the timing for performance of the SLNB. If sentinel nodes are evaluated prior to neoadjuvant treatment, accurate information about lymph node status is obtained, contributing to the design of precise radiotherapy and chemotherapy strategies. Otherwise, the evaluation of SLNB after NSBT allows determination of the effectiveness of systemic drugs on the lymph nodes; in these cases, if lymph node metastases are not detected, axillary lymphadenectomy may be avoided.[25,28,29] Actually, a high percentage of metastatic nodes become negative after neoadjuvant treatment; in these cases, an axillary lymph node dissection would not add benefits in terms of total survival.[3,30,31]

The possibility of a high rate of false-negative results in SLNB after neoadjuvant therapy has been postulated; probable reasons are limited sentinel node detection caused by an alteration in lymphatic drainage or a failure to detect tumour cells, owing to fibrosis or partial necrosis caused by systemic

Table 2. Immunohistochemical scores of cytokeratin 19 in breast cancer and lymph node biopsies before and after neoadjuvant systemic breast cancer therapy (NSBT)

	PS	*n* (%)	IS	*n* (%)	TS	*n* (%)
Breast pre-NSBT	0	7 (4.3)	0	7 (4.3)	0	7 (4.3)
	1	1 (0.6)	1	29 (18.0)	1	0 (0)
	2	3 (1.8)	2	78 (48.1)	2	0 (0)
	3	10 (6.2)	3	48 (29.6)	3	2 (1.2)
	4	55 (34.0)			4	8 (4.9)
	5	86 (53.1)			5	16 (9.9)
					6	40 (24.7)
					7	58 (35.8)
					8	31 (19.2)
Breast post-NSBT	0	7 (4.9)	0	7 (4.9)	0	7 (4.9)
	1	1 (0.7)	1	9 (6.4)	1	0 (0)
	2	2 (1.4)	2	48 (33.8)	2	0 (0)
	3	5 (3.6)	3	78 (54.9)	3	2 (1.4)
	4	35 (24.6)			4	1 (0.7)
	5	92 (64.8)			5	10 (7.0)
					6	15 (10.6)
					7	39 (27.5)
					8	68 (47.8)
Node pre-NSBT	0	0 (0)	0	0 (0)	0	0 (0)
	1	0 (0)	1	3 (17.6)	1	0 (0)
	2	0 (0)	2	12 (70.6)	2	0 (0)
	3	3 (17.6)	3	2 (11.8)	3	0 (0)
	4	10 (58.8)			4	1 (5.8)
	5	4 (23.6)			5	4 (23.5)
					6	8 (47.1)
					7	2 (11.8)
					8	2 (11.8)
Node post-NSBT	0	7 (7.1)	0	7 (7.1)	0	7 (7.1)
	1	0 (0)	1	4 (4.1)	1	0 (0)
	2	1 (1.0)	2	36 (36.7)	2	0 (0)
	3	0 (0)	3	51 (52.1)	3	1 (1.0)
	4	21 (21.5)			4	0 (0)
	5	69 (70.4)			5	2 (2.0)

Table 2. *(Continued)*

PS	*n* (%)	IS	*n* (%)	TS	*n* (%)
				6	13 (13.4)
				7	30 (30.6)
				8	45 (45.9)

IS, Intensity score; NSBT, Neoadjuvant systemic breast cancer therapy; PS, Proportion score.

Table 3. Changes in immunohistochemical cytokeratin 19 (CK19) expression after neoadjuvant therapy

	Breast post-NSBT				*P*	Total cases
Breast pre-NSBT	0 → 0	0 → 1	1 → 0	1 → 1	0.18	142
n	8	7	2	125		

	Node post-NSBT				*P*	Total cases
Breast pre-NSBT	0 → 0	0 → 1	1 → 0	1 → 1	0.063	96
n	8	5	0	83		
Breast post-NSBT	0 → 0	0 → 1	1 → 0	1 → 1	1.00	96
n	8	0	0	88		
Node pre-NSBT	0 → 0	0 → 1	1 → 0	1 → 1	1.00	13
n	0	1	0	12		

NSBT, Neoadjuvant systemic breast cancer therapy.
0, negative/low CK19 expression, and 1, high CK19 expression, on the line in which these numbers are connected by arrows. Four comparisons are shown: breast biopsies pre-NSBT and post-NSBT, breast biopsies pre-NSBT versus lymph node biopsies post-NSBT, breast and lymph node biopsies post-NSBT, and lymph node biopsies pre-NSBT and post-NSBT. *P*-values from the McNemar test.

therapy.[24] However, recent studies comparing two cohorts of patients, treated and not treated prior to surgery, have not demonstrated significant differences in the rate of successful sentinel node identification or in the results of the SLNB assay. These authors concluded that it is technically feasible to identify the sentinel nodes after NSBT, and that the accuracy of SLNB remains similar to that in early cancer not susceptible to neoadjuvant therapy.[2,3,26,27,32]

When an SLNB procedure is indicated, a new question may arise: which is the most appropriate technique with which to evaluate the nodes after systemic treatment? In recent years, different molecular methods for analysing sentinel nodes have been proposed. The OSNA assay is a standardized, automated and reproducible test with demonstrated accuracy based on quantitative real-time polymerase chain reaction amplification of CK19 mRNA.[6,7,9,10] It can be used to analyse whole lymph nodes, and yields semiquantitative results for the detection of clinically relevant nodal metastases. The OSNA assay can distinguish macrometastases and micrometastases from low-volume metastases corresponding to isolated tumour cells according to the *Cancer Staging Manual of the Union International Contre le Cancer* and the *American Joint Committee on Cancer Staging Manual* 7th edn.[21] This method has been validated, and it is widely accepted for clinical use; also, it allows accurate results to be obtained in an optimal time.[7,9,10,33,34] The total tumour load of sentinel nodes has been proposed as a good tool with which to predict the state of the non-sentinel axillary nodes; the use of the OSNA CK19 method to assay the sentinel nodes allows quantification of the remaining tumour load in the nodes after systemic treatment, and consequent selection of the appropriate surgical attitude for the axilla.[35]

However, some doubts may emerge about the use of this technique in patients who have previously received neoadjuvant treatment. Is CK19 still a good target for detecting metastatic cells on lymph nodes after systemic therapy? This question arises because of the demonstration of IHC alterations of different markers, such as SP1, lactoalbumin, and hormonal receptors, on breast cancer cells after treatment.[12,14–16] CK19 is a luminal epithelial cell marker expressed by most (96.9–98.2%) breast cancer cells, but not by haematopoietic cells.[11,36–39] It is employed for the detection of disseminated tumour cells in blood and bone marrow, and it is also the target of OSNA assay used to analyse sentinel lymph nodes. Some authors have postulated that this cytoskeleton protein modulates endoplasmic reticulum stress signalling, and that it might contribute to cell survival and dormancy in breast cancer cells.[40] CK19 mRNA was selected among 45 possible biomarkers, as the most sensitive marker for the detection of metastases in lymph nodes by OSNA. Although this keratin has been detected in all types of breast cancer, lack of

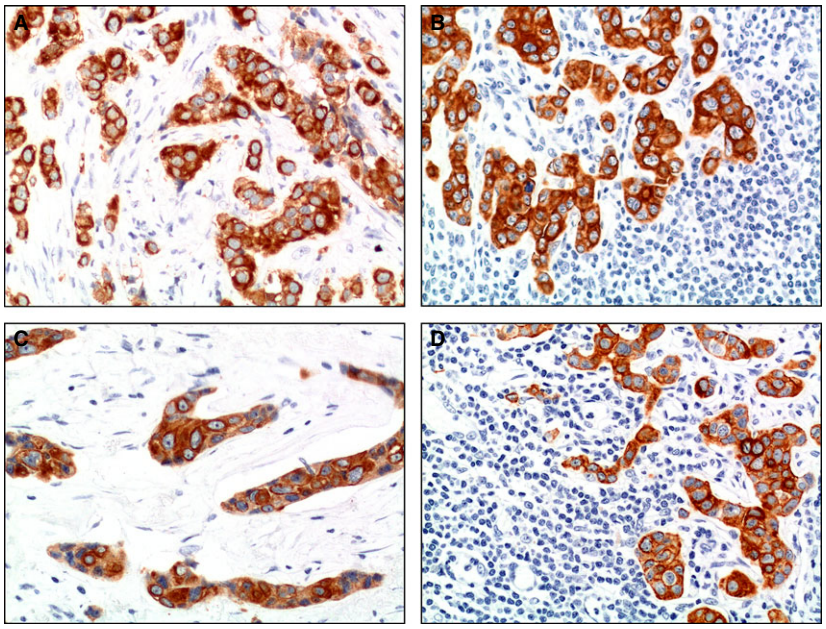

Figure 2. Immunohistochemical expression of cytokeratin 19 (CK19) in an invasive carcinoma of no special type: maintenance of CK19 expression after neoadjuvant therapy. **A**, Primary tumour biopsy [pre-neoadjuvant systemic breast cancer therapy (NSBT)]. **B**, Lymph node biopsy (pre-NSBT). **C**, Primary tumour resection (post-NSBT). **D**, Lymph node resection (post-NSBT). Representative micrographs are shown.

staining has been found in 3.4–20.5% of breast tumours; Parikh *et al.* have found a statistically significant association between lack of CK19 and the TN phenotype (30% TN), but other authors have demonstrated similar proportions of CK19 negativity in luminal cancers.[36,38,39]

In our institution, 18 of 721 (2.49%) breast cancers within the last 3 years did not express CK19; these tumours showed luminal A (44.44%), luminal B (11.11%), TN (38.88%) or HER2 (5.55%) phenotypes. In the present study, seven of the 162 tumours (4.33%) did not express CK19, and also showed different immunophenotypes and histological subtypes. These results are in agreement with results previously published.[36,38,39] Owing to the possibility of a CK19-negative tumour, and with the aim of avoiding false-negative results in sentinel lymph node assay by OSNA, the study of the expression of CK19 prior to surgery is mandatory. However, to the best of our knowledge, nothing has been reported about CK19 expression in tumours with previous systemic therapy.

After comparing CK19 protein expression on samples from primary tumours and lymph node metastases, before and after NSBT, we have found that this expression does not significantly change; CK19-negative tumours did not become positive after neoadjuvant treatment, and CK19-positive tumours did not become negative. Under the conditions of this study, and because of the restrictive cut-off employed for the statistical analysis, only nine breast cancer biopsies showed changes in the CK19 expression level pre-NSBT and post-NSBT. These subtle differences have been detected in the percentage of stained cells or intensity of CK19 staining, which may be attributable to differences in the preanalytical phase (time of fixation, type of fixative, ischaemic time, etc.) between biopsy and resection samples, or tumour heterogeneity. No statistically significant associations were found between the CK19 expression level in breast cancer biopsies post-NSBT and the clinical variables analysed.

In summary, our study has confirmed the preservation of CK19 protein expression in breast cancer cells after systemic treatment, with no significant differences being found in the pattern or intensity of staining in samples before and after chemotherapy. On the basis of these results, CK19 could be used as a target to evaluate the presence of tumour cells in breast and lymph nodes in those patients with breast cancer subjected to neoadjuvant therapy prior to surgery.

According to some authors,[1,2,26,27,32] the selective biopsy of sentinal lymph nodes is a feasible method for examining axillary status in those breast cancers under neoadjuvant treatment. On the basis of the results of our study, and provided that protein

Table 4. Association between cytokeratin 19 (CK19) immunohistochemical expression in breast cancer biopsies after neoadjuvant systemic breast cancer therapy and clinicopathological variables

		CK19 expression, *n* (%)		
	No. of patients	Low	High	*P*
Age (years)				
<50	58	0 (0)	58 (43.0)	0.125
≥50	77	3 (2.2)	74 (54.8)	
Histological type				
IDC	115	3 (2.2)	112 (83.0)	0.467
Others	20	0 (0)	20 (14.8)	
Grade				
1	13	0 (0)	13 (9.6)	0.569
2–3	122	3 (2.2)	119 (88.2)	
Size (mm)				
<50	108	2 (1.5)	106 (78.5)	0.554
≥50	27	1 (0.7)	26 (19.3)	
TNM stage				
0, IA–B	26	0 (0)	26 (19.3)	0.394
IIA–B, IIIA–C, IV	109	3 (2.2)	106 (78.5)	
Pathological response				
1–3	102	3 (2.2)	99 (73.4)	0.312
4–5	33	0 (0)	33 (24.4)	
Lymph node response				
A	35	1 (0.7)	34 (25.2)	0.785
B, C, D	100	2 (1.5)	98 (72.6)	
Lymph node metastasis				
Yes	40	2 (1.5)	38 (28.2)	0.152
No	95	1 (0.7)	94 (69.6)	

IDC, invasive ductal carcinoma.
P-values from the chi-square test.

expression is maintained after NSBT, mRNA quantification-based methods such the OSNA CK19 assay could be accurate tools with which to analyse axillary sentinel nodes, regardless of whether SLNB was performed before or after treatment. Further studies would be necessary to confirm this.

Acknowledgements

This work was supported by grants from the Instituto de Salud Carlos III (ISCIII, grant number PI13/02477, RD12/0036/0064 and RD12/0036/0017), co-financed by the European Development Regional Fund ('A way to achieve Europe': grant number RD06/0020/0013) and by the Junta de Andalucía (Consejería de Salud, PI0581/2009) and ISCIII-Red de Biobancos PT13/0010/0056.

Author contributions

B. Vieites and J. Palacios designed the research study. B. Vieites and M. Á. López-García reviewed the histopathological data and interpreted all of the immunohistochemical results. L. Alfaro and M. Atienza reviewed the clinical and surgical data of the series. M. J. Hernández and M. Biscuola performed the immunohistochemical techniques. C. Castilla and M Á Castilla performed statistical analyses. B. Vieites, M. Á. López-García and C. Castilla wrote the manuscript.

Ethics

The study was performed according to the Declaration of Helsinki. The procedure was approved by the local ethics committee (approval date 10 April 2015, Code 0292-N-15, by Comité de Ética de la Investigación-HHUU Virgen Macarena-Virgen del Rocío).

Conflicts of interest

The authors declare that they have no conflicts of interest.

References

1. Shimazu K, Noguchi S. Sentinel lymph node biopsy before versus after neoadjuvant chemotherapy for breast cancer. *Surg. Today* 2011; **41**; 311–316.
2. Tan V, Goh B, Fook-Chong S, Khin LW, Wong WK, Yong WS. The feasibility and accuracy of sentinel lymph node biopsy in clinically node-negative patients after neoadjuvant chemotherapy for breast cancer—a systematic review and meta-analysis. *J. Surg. Oncol.* 2011; **104**; 97–103.
3. Alvarado R, Yi M, Le-Petross H *et al.* The role for sentinel lymph node dissection after neoadjuvant chemotherapy in patients who present with node-positive breast cancer. *Ann. Surg. Oncol.* 2012; **19**; 3177–3184.
4. Morrow M, Dang CT. Sentinel node biopsy after neoadjuvant chemotherapy: a new standard for patients with axillary metastases? *JAMA* 2013; **310**; 1449–1450.

5. Untch M, Konecny GE, Paepke S, von Minckwitz G. Current and future role of neoadjuvant therapy for breast cancer. *Breast* 2014; **23**; 526–537.
6. 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**; 4807–4816.
7. Visser M, Jiwa M, Horstman A *et al.* Intra-operative rapid diagnostic method based on CK19 mRNA expression for the detection of lymph node metastases in breast cancer. *Int. J. Cancer* 2008; **122**; 2562–2567.
8. Schem C, Maass N, Bauerschlag DO *et al.* One-step nucleic acid amplification—a molecular method for the detection of lymph node metastases in breast cancer patients; results of the German study group. *Virchows Arch.* 2009; **454**; 203–210.
9. Bernet L, Cano R, Martínez M *et al.* Diagnosis of the sentinel lymph node in breast cancer: a reproducible molecular method: a multicentric Spanish study. *Histopathology* 2011; **58**; 863–869.
10. Feldman S, Krishnamurthy S, Gillanders WG *et al.* A novel automated assay for the rapid identification of metastatic breast carcinoma in sentinel lymph nodes. *Cancer* 2011; **117**; 2599–2607.
11. Chu PG, Weiss LM. Keratin expression in human tissues and neoplasms. *Histopathology* 2002; **40**; 403–439.
12. Kennedy S, Merino MJ, Swain SM, Lippman ME. The effects of hormonal and chemotherapy on tumoral and non neoplastic breast tissue. *Hum. Pathol.* 1990; **21**; 192–198.
13. Sharma S, Hiran KR, Pavithran K, Vijaykumar DK. A pilot study to assess the feasibility of evaluation of markers of response to chemotherapy at one day 21 days after first cycle of chemotherapy in carcinoma of breast: a prospective non-randomized observational study. *World J. Surg. Oncol.* 2009; **7**; 35.
14. Zhang N, Moran MS, Huo Q, Haffty BG, Yang Q. The hormonal receptor status in breast cancer can be altered by neoadjuvant chemotherapy: a meta-analysis. *Cancer Invest.* 2011; **289**; 594–598.
15. Lee SH, Chung MA, Quddus MR, Steinhoff MM, Cady B. The effect of neoadjuvant chemotherapy on estrogen and progesterone receptor expression and hormone receptor status in breast cancer. *Am. J. Surg.* 2003; **186**; 348–350.
16. Lee HC, Ko H, Seol H *et al.* Expression of immunohistochemical markers before and after neoadjuvant chemotherapy in breast carcinoma, and their use as predictors of response. *J. Breast Cancer* 2013; **16**; 395–403.
17. Goldhirsch A, Winer EP, Coates AS *et al.* Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. *Ann. Oncol.* 2013; **24**; 2206–2223.
18. Ogston KN, Miller ID, Payne S *et al.* A new histological grading system to assess response of breast cancers to primary chemotherapy: prognostic significance and survival. *Breast* 2003; **12**; 320–327.
19. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J. Clin. Oncol.* 1999; **17**; 1474–1481.
20. Cheang MC, Chia SK, Voduc D *et al.* Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. *J. Natl Cancer Inst.* 2009; **101**; 736–750.
21. Edge SB, Byrd DR, Compton CC *et al. The AJCC cancer staging manual*. 7th ed. New York: Springer, 2010.
22. Gnant M, Harbeck N, Thomssen C. St Gallen 2011: summary of the consensus discussion. *Breast Care (Basel)* 2011; **6**; 136–141.
23. Mauri D, Pavlidis N, Ioannidis JP. Neoadjuvant versus adjuvant systemic treatment in breast cancer: a meta-analysis. *J. Natl Cancer Inst.* 2005; **97**; 188–194.
24. Rastogi P, Anderson SJ, Bear HD *et al.* Preoperative chemotherapy: updates of National Surgical Adjuvant Breast and Bowel Project Protocols B-18 and B-27. *J. Clin. Oncol.* 2008; **26**; 778–785.
25. Makhoul I, Kiwan E. Neoadjuvant systemic treatment of breast cancer. *J. Surg. Oncol.* 2011; **103**; 348–357.
26. Stell VH, Flippo-Morton TS, Norton HJ, White RL Jr. Sentinel lymph node biopsy after neo-adjuvant chemotherapy in breast cancer. *Breast J.* 2010; **17**; 71–74.
27. Reitsamer R, Menzel C, Glueck S, Rettenbacher L, Weismann C, Hutarew G. Sentinel lymph node biopsy is precise after primary systemic therapy in stage II–III breast cancer patients. *Ann. Surg. Oncol.* 2010; **17**(Suppl. 3); 286–290.
28. Liu SV, Melstrom L, Yao K, Russell CA, Sener SF. Neoadjuvant therapy for breast cancer. *J. Surg. Oncol.* 2010; **101**; 283–291.
29. Fontein DB, van de Water W, Mieog JS, Liefers GJ, van de Velde CJ. Timing of the sentinel lymph node biopsy in breast cancer patients receiving neoadjuvant therapy—recommendations for clinical guidance. *Eur. J. Surg. Oncol.* 2013; **39**; 417–424.
30. Klauber-DeMore N, Ollila DW, Moore DT *et al.* Size of residual lymph node metastasis after neoadjuvant chemotherapy in locally advanced breast cancer patients is prognostic. *Ann. Surg. Oncol.* 2006; **13**; 685–691.
31. Pugliese MS, Stempel MM, Cody HS, Morrow M, Gemignani ML. Surgical management of the axilla: do intramammary nodes matter? *Am. J. Surg.* 2009; **198**; 532–537.
32. Navarro-Cecilia J, Dueñas-Rodríguez B, Luque-López C *et al.* Intraoperative sentinel node biopsy by one-step nucleic acid amplification (OSNA) avoids axillary lymphadenectomy in women with breast cancer treated with neoadjuvant chemotherapy. *Eur. J. Surg. Oncol.* 2013; **39**; 873–879.
33. Tamaki Y, Sato N, Homma K *et al.* Routine clinical use of the one-step nucleic acid amplification assay for detection of sentinel lymph node metastases in breast cancer patients: results of a multicenter study in Japan. *Cancer* 2012; **118**; 3477–3483.
34. Snook KL, Layer GT, Jackson PA *et al.* Multicentre evaluation of intraoperative molecular analysis of sentinel lymph nodes in breast carcinoma. *Br. J. Surg.* 2011; **98**; 527–535.
35. Peg V, Espinosa-Bravo M, Vieites B *et al.* Intraoperative molecular analysis of total tumour load in sentinel lymph node: a new predictor of axillary status in early breast cancer patients. *Breast Cancer Res. Treat.* 2013; **139**; 87–93.
36. Vilardell F, Novell A, Martin J *et al.* Importance of assessing CK19 immunostaining in core biopsies in patients subjected to sentinel node study by OSNA. *Virchows Arch.* 2012; **460**; 569–575.
37. Alvarenga CA, Paravidino PI, Alvarenga M *et al.* Expression of CK19 in invasive breast carcinomas of special histological types: implications for the use of one-step nucleic acid amplification. *J. Clin. Pathol.* 2011; **64**; 493–497.
38. Abd El-Rehim DM, Pinder SE, Paish CE *et al.* Expression of luminal and basal cytokeratins in human breast carcinoma. *J. Pathol.* 2004; **203**; 661–671.

39. Parikh RR, Yang Q, Higgings SA, Haffty BG. Outcomes in young women with breast cancer of triple-negative phenotype: the prognostic significance of Ck19 expression. *Int. J. Radiat. Oncol. Biol. Phys.* 2008; **70**; 35–42.

40. Bambang IF, Lu D, Li H *et al.* Cytokeratin 19 regulates endoplasmic reticulum stress and inhibits ERp29 expression via p38 MAPK/XBP-1 signaling in breast cancer cells. *Exp. Cell Res.* 2009; **315**; 1964–1974.