Determination of sentinel lymph node status in primary breast cancer by prospective use of immunohistochemistry increases the rate of micrometastases and isolated tumour cells

Analysis of 174 Patients after SLN Biopsy

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Running title: Prospective use of immunohistochemistry ...

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Abstract

Aim: The objective of the present study was to evaluate the prospective use of immunohistochemistry (IHC) for histopathological diagnosis of sentinel lymph node(s) (SLN) in primary breast cancer using stage migration and non-SLN metastases as endpoints in relation to metastatic involvement.

Study design: Serial sectioning and prospective use of IHC were applied to SLN examination in addition to routine haematoxylin-eosin staining in 174 consecutive patients with unifocal T1-T2 breast cancer included in a National Sentinel Node Study. Axillary lymph node dissection (ALND) was performed in all cases with macrometastases, micrometastases and isolated tumour cells (ITC).

Results: The SLN was found in 173/174 patients and a metastatic foci was found in 50 patients including 28/50 with macrometastases, 16/50 with micrometastases and 6/50 with ITC. IHC detected 3/16 of the micrometastases and 4/6 of ITC. Stage migration from N0 to N1mi was encountered in 3/132 patients by use of IHC. Non-SLN metastases were noted in 15/28 of patients with macrometastases and in 3/16 of patients with micrometastases, whereas no patient with ITC had additional metastases ($p=0.007$).

Conclusion: The prospective use of IHC and serial sectioning for histopathological diagnosis of SLNs increased the detection rate of N1mi and ITC, but only 3/132 of patients were stage-migrated by use of IHC. Patients with ITC did not have any risk of non-SLN metastases, supporting that ALND can safely be omitted in this group of patients.

Introduction

Axillary lymph node status is still the most important prognostic factor in primary breast cancer despite the development of advanced analysis of prognostic tumour markers (1, 2). Histopathological examination after surgical removal of at least ten axillary lymph nodes (ALN) is the standard staging procedure for determination of the axillary lymph node status (3). Today the sentinel lymph node (SLN) biopsy technique is introduced as an accurate staging procedure for small unifocal breast tumours with clinically negative axilla (4). For patients without metastases in the SLN, axillary lymph node dissection (ALND) can be omitted and the technique seems to offer minimal arm morbidity without compromising clinical outcome in early-stage breast cancer (4).

Histopathological examination of the SLN(s) is more detailed than traditional examination of axillary lymph nodes, because the SLN is the only node involved in the majority of patients and the decision on further axillary surgery is based on the status of the SLN (1). Serial sectioning of the SLN and use of immunohistochemistry (IHC) with cytokeratin have led to increased detection of minimal lymph node involvement classified as micrometastases (>0.2 mm $\leq$ 2 mm; pN1mi) and isolated tumour cells (ITC) ($\leq$ 0.2 mm; pN0(i+)) (5). Initially, non-standardised protocols for pathological examination of the SLNs were used, but uniform protocols for pathological evaluation of the SLN are currently being developed assuring that the above definitions are being used (1, 6). Patients with micrometastases are recommended ALND and systemic treatment is advocated to follow the guidelines for node-positive patients, although the exact prognostic role of micrometastatic involvement of the SLN is unknown (2). Micrometastatic involvement of the ALN is correlated to a worse prognosis compared to node-negative patients in some reports (7, 8), although this is still an unresolved issue. Regarding SLN micrometastases and relation to clinical outcome, there is limited data based on retrospective examination by IHC of the SLN (9). Patients with ITC are recommended adjuvant treatment as node-negative patients (2), whereas the role of ALND in this group of patients is still under debate since the risk of additional axillary nodal involvement is yet not clarified (10, 11, 12, 13).

The aim of the present study was to evaluate the prospective use of IHC in conjunction with serial sectioning and haematoxylin and eosin (HE) staining for SLN examination in relation to the rate of false negative intraoperative cases, stage migration and non-SLN metastases

using a modification of a published protocol (14). All patients with metastatic involvement of the SLN, as well as patients with ITC, had complementary ALND and thesize of the metastatic foci were measured retrospectively.

Material and Methods

Patients

Patients with unifocal primary breast cancer < 3 cm and a clinically negative axilla were included prospectively within the National Swedish Sentinel Node Study at the Department of Surgery in Helsingborg from March 2001 to March 2003. The eligibility of patients as well as the procedure for the sentinel node biopsy technique followed the criteria in the national protocol described previously (15). The study was approved by the Ethics Committee at the University of Lund, Sweden, and written informed consent was obtained from all patients. The study included 184 patients and stopped when the national guidelines for SLN diagnosis recommended IHC only in doubtful cases. Patients with non-palpable lesions were preoperatively indicated using ultrasound or mammographic guidance. Patients with predominantly ductal carcinoma in situ with small invasive foci (n=5), medullary carcinomas (n=2) or very small primary tumours not allowing further analyses (n=3) were excluded from the protocol. No patient received preoperative treatment. Postoperative radiotherapy to the breast was delivered to all patients having breast-conservative surgery 131/173. Patients were followed by annual mammography and biannual clinical investigation according to the study protocol.

The clinicopathological characteristics of the patients are given in Table 1.

Sentinel lymph node mapping

The sentinel node was identified using radiolabelled isotope (50MBq $^{99m\,Tc}$-labelled colloid, Solco Nanocoll®; Nycomed, Amersham, UK) and 1 ml vital blue dye (Patent Blue V®; Guerbet, Paris, France) injected intradermally above the tumour. Preoperative lymphoscintigraphy was performed in all patients and the sentinel node was defined as a hot and/or blue node by using a hand-held gamma-probe (Neoprobe 2000; Neoprobe Corporation, Dublin, OH) as previously reported (15) .

Histopathological analysis of lymph nodes

The SLN(s) were analysed by frozen sections (FS) intraoperatively. Lymph nodes 4 -10 mm diameter were bisected and if > 10mm diameter divided into < 4 mm slices, all examined by two consecutive sections. The remaining tissue of the SLNs was formalin-fixed and paraffin-embedded. Parallel 4 µ sections were performed at three different levels 60-100µ apart in each paraffin block. The sections from each level were stained with haematoxylin-eosin (HE) and immunohistochemically for cytokeratins. The monoclonal antibody MNF 116 (Dakocytomation, Copenhagen) was used in 1:200 dilution, with protease pretreatment and staining performed on a Ventana NexES staining machine and only permenant sections were subjected to IHC. The protocol is a modification of a publication using IHC on a limited number of sections (14).

Macrometastases (N1) were defined as a tumour infiltrate larger than 2 mm and micrometastases (N1mi) defined as tumour involvement >0.2 mm but $\leq$ 2 mm according to the UICC TNM Classification of Malignant Tumours. Tumour involvement of 0.2 mm or less (N0(i+)) was classified as isolated tumour cell involvement (ITC). Tumour involvement detected by IHC was verified by the morphological diagnosis of epithelial tumour cells and cytokeratin-positive deposits which could not be identified on routine stains were not encountered. Sentinel nodes with metastatic involvement were retrospectively reviewed by a pathologist (LS), giving exact measurement for the SLN metastases according to a protocol described (13).

The axillary lymph nodes were analysed according to the standard protocol at the institution; bisecting nodes larger than 5 mm and stained by HE after fixation and embedding.

Statistical Analyses

The relation between nodal involvement and clinicopathological characteristics was calculated by Chi-square test for categorised variables and by Kruskal-Wallis test for continuous variables.

Recurrence free survival was defined as local, distant or breast cancer specific death as primary event. The Cox Proportional Hazards Model was used for estimation of univariate hazard ratios (HRs).

All calculations were performed in SPSS version 11.0. (SPSS inc., Ill., USA).

Results

Diagnosis of SLN metastases and isolated tumour cells

One or more SLNs were detected in all but one patient who had metastatic growth in fourteen axillary lymph nodes, giving a detection rate of (173/174) in this cohort. The median number of SLNs examined was 2 (range 0-6) and 26/173 patients had metastatic involvement diagnosed by frozen section (FS) intraoperatively. After definitive histopathological examination with HE and IHC with cytokeratin staining, 50/173 patients had metastatically involved SLNs, including 28/50 patients with macrometastases, 16/50 patients with micrometastases and 6/50 patients with ITC. The median size of the micrometastatic lesion was 0.90 mm (0.25-1.90) and for ITC 0.10 mm (0.10-0.15). No patient had more than two metastatically involved SLNs and for N1mi and N0(i+) only one involved SLN was recorded (Table 2).

Intraoperative false negative rate

The false negative rates for intraoperative diagnosis of metastases in the SLN for macrometastases, micrometastases and ITC using IHC are given in Table 2. The micrometastases diagnosed by FS exceeded 1.00 mm in diameter, whereas no ITC was diagnosed intraoperatively. There was no statistically significant difference in the rate of IHC-detected tumour involvement in relation to histopathological subgroups (i.e. ductal 5/123, lobular 2/38, tubular 0/9 or mixed 0/3, p=0.7).

Stage migration and IHC

Stage migration from N0 to N1mi after definitive histopathological diagnosis including IHC was noted in 3/132 patients diagnosed as N0 by use of HE (Table 2). In addition, the fraction of patients classified as N0(i+) increased from 2 by HE to 6 by HE and IHC.

Non SLN metastases

All patients with a metastatic foci in the SLN, including patients with ITC, had an ALND including level I-II harvesting in median 13 nodes with no difference in the number of analysed ALNs between the three groups (Table 2). Non-SLN metastases were found in 15/28 of patients with macrometastases, whereas 3/16 of patients with micrometastases had additional metastatic nodal involvement and 0/6 of the patients with ITC had a non-SLN metastasis (p=0.007). All patients diagnosed with more than one positive SLN had non-SLN metastases. One of the N1mi diagnosed by IHC measuring 1.00 mm had a non-SLN metastasis.

Relation between clinicopathological characteristics and lymph node status

Tumour size and PR negativity were related to metastatic SLN involvement, whereas histological grade, histopathological type and age were not (Table 1).

Clinical outcome

After a median of follow-up36 months (0-56 months, mean 37 months), ten relapses and nine deaths were recorded. Six breast cancer related deaths were diagnosed, two due to other malignancies (lung and haematological) and one due to liver disease. The recurrence free survival (RFS) for node-positive patients (n=28) was 86%, with four distant recurrences. Three patients died of breast cancer after diagnosis of a distant recurrence. For the 16 patients with micrometastases in the SLN, one distant recurrence was recorded (94% RFS), but no breast-cancer-related death occurred. For node-negative patients not classified as N0(i+) (n=123), four distant recurrences were diagnosed (97% RFS) and three patients died of breast cancer. There rate of distant recurrences were significantly related to the SLN status defined as N1, N1mi and N0 (Table 1). Within the ITC group (n=6), one local recurrence within a mastectomy scar was diagnosed but no distant recurrence or breast-cancer-related death. No axillary recurrence was recorded in any group including the N0 patients.

Using the Cox Proportional Hazards Univariate analyses tumour size (T2 *vs* T1), Nottingham histological grade (III *vs* I-II), number of involved nodes, non-SLN metastases and nodal metastatic size were predictors of breast cancer recurrence and death (Table 3).

Discussion

Protocols for histpopathological diagnosis of SLNs

The introduction of the SLN biopsy technique for T1-T2 breast cancer as a staging procedure is justified by the low false negative rate of detecting SLN and SLN is accepted as a reliable method for evaluation of axillary node status in this group of patients (4). Axillary staging still qualifies as the single most important prognostic factor in primary breast cancer, making the histopathological diagnosis of the SLN of great importance. Guidelines for histopathological diagnosis including serial sectioning and IHC have been developed for the optimal assessment of metastatic involvement of the SLN (1). Use of serial sectioning of the SLN is established, but the routine use of IHC is not generally recommended due to the increased detection of false positive “tumour” deposits and detection of metastases of no biological relevance (1). Cytokeratin-positive deposits of non-malignant origin have been attributed to debris from previous diagnostic procedures and a confirmation of malignant diagnosis based on morphological criteria is therefore mandatory in all IHC-positive cases (1). Several protocols have been developed for the diagnostic procedure of the SLN, making comparison between studies difficult regarding stage migration and non-SLN metastases (16). In this report with prospective examination of all SLNs by IHC, the diagnosis of N1mi and N0(i+) was confirmed by morphological characteristics of malignant cells and the patients with IHC-based diagnosis were 7/50, including 4 cases with ITC. IHC-detected tumour cells classified as N1mi or N0(i+) in the SLN are reported to occur in 4% - 62% of patients in protocols not using IHC consecutively (8, 11, 17, 18). The exploratory nature of the retrospective examination of the slides and the ambitious protocols presented in some of the studies may be a possible explanation for the difference in the detection rate. The applied protocol described here enabled us to make a relatively superficial examination of the blocks and the effect of IHC was to enhance detection of tumour deposits.

Stage migration by use of IHC

In line with the finding in this study that prospective use of IHC detected additional metastatic SLN infiltration at a low rate, stage migration from N0 to N1mi by cytokeratin staining was noted in 3/132 of N0 patients. Stage migration is previously reported to be a

significant finding associated with IHC protocols, extended to a larger fraction of patients (9% or more) initially diagnosed as node-negative (13, 21). Using the protocol described, the prospective use of IHC had a minimal impact on stage migration and treatment decisions, supporting data in a recent publication (19). All macrometastases were diagnosed by HE, whereas 4/6 of patients diagnosed with ITC were detected by IHC and thus upstaged to N0(i+).

Risk of non SLN metastases

Non-SLN metastatic involvement is correlated to the size of the metastatic lesion in the SLN and ALND is therefore recommended for all patients with N1 disease (1, 2). Other markers with possible relation to non-SLN metastases include tumour size, number of SLNs examined and number of metastatically involved SLNs (21). SLN metastases diagnosed by routine HE have a higher risk of non-SLN metastases than IHC-detected metastases, reflecting the larger metastatic size seen in the former group (11). In this report evaluating prospective use of serial sectioning and IHC for SLNs, all patients with metastatic infiltration in the SLN had a backup ALND, making a complete evaluation of the risk of non-SLN metastases possible even with minimal nodal involvement. The rate of non-SLN metastases in patients with macrometastatic SLN was 15/28 and for patients with N1mi 3/16. In the patients with N1mi and non-SLN metastases, the metastatic lesion of the SLN was 1.00 mm or larger, emphasizing that the size of the SLN metastases seems to be of importance for predicting the risk of non-SLN metastases. The risk of non-SLN metastases in patients with IHC-detected tumour infiltration was 1/7 defined by the largest metastases (1.00mm) among the IHC-detected N1mi in the SLN.

The risk of non-SLN metastases for macro- and micrometastases was in line with previous reports (13), but we observed no risk of non-SLN metastases in the ITC group. Non-SLN involvement of axillary nodes is reported to be 14.8% in patients with ITC in a recent large retrospective report from the European Institute of Oncology (13). In the present report including only 173 patients with prospective use of IHC, no patient diagnosed with ITC had additional axillary nodal involvement diagnosed after ALND of level I-II. The conflicting results reported for ITC and the risk of further axillary nodal involvement could be explained by the sparse use of IHC in the published study as proposed by Cserni (23), as well as the level of axillary surgery routinely used in Sweden harvesting only level I-II of ALNs and the

small size of our study population. ALND is omitted at many institutions for patients with N0(i+) supported by reports of a low recurrence rate in this group of breast cancer patients (2). Based on the limited series of patients here including SLN biopsy technique and ALND procedures in all patients with SLN involvement, ALND can be omitted in patients with ITC and micrometastases smaller than 1.00 mm.

Metastatic size in the SLNs and clinical outcome

The UICC have revised the TNM classification, introducing micrometastases N1(mi) with metastases $\leq$ 2.00 mm and > 0.2 mm with an increased risk of non-SLN metastases justifying ALND and adjuvant treatment in line with N1 patients. Isolated tumour cells (ITC) and tumour deposits $\leq$ 0.2 mm have a lower risk of non-SLN metastases and are recommended adjuvant treatment as for node-negative patients. The exact prognostic role of N1mi and N0(i+) in SLN is unknown and the proposed increased risk of breast cancer related events due to nodal micrometastases is based on data from ALNs often examined extensively (7, 8), which has not been confirmed by retrospectively detected micrometastases in SLNs (9, 21, 22). The role of minimal disease in the SLN and clinical outcome is to be defined by future studies and uptil now, no increased risk of breast cancer events is correlated to micrometastases in the SLN compared to node negative patients (9, 21, 22). In the present study, the size of the metastasis in the SLNs was significantly related to outcome even after 36 months of follow-up and further follow-up is necessesary to evaluate the clinical significance of the finding. The distinction between N1mi and ITC did not provide any biologically important information in this study and the cutpoint for the risk of non-SLN metastases as well as distant recurrences was a metastatic foci $\geq$ 1.00mm in the SLN, which is in line with data by Viale et al (13). The biologically relevant cutpoint of minimal disease in the SLN and clinical outcome has to be defined by future studies.

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Conflict of interest

There are no declared conflict of interests.

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TABLE 1 Clinical and tumour characteristics in the cohort

CHARACTERISTIC	ALL (N=174) N0 (%)	N1 (N=28)	N1(mi) (N=16)	N0(i+) (N=6)	N0 (N=123)	P *
Age (years)						
median	60 (36-86)					
≥50	19 (11)	4 (14)	4 (25)	0	11 (9)	0.2
> 50	155 (89)	24 (86)	12 (75)	6 (100)	112 (81)	
Tumour size (mm)						
median	15 (6-40)					
T1b	29 (17)	2 (7)	1 (6)	0	26 (21)	
T1c	114 (65)	17 (61)	15 (94)	4 (67)	78 (63)	
T2	31 (18)	9 (32)	0	2 (33)	10 (16)	0.02
NHG						
NHG 1	35 (20)	6 (21)	4 (25)	0	25 (20)	
NHG 2	102 (58)	15 (54)	11 (69)	3 (50)	72 (58)	
NHG 3	37 (21)	7 (25)	1 (6)	3 (50)	26 (22)	0.4
Histopathological type						
Ductal CA	124 (71)	19 (69)	12 (75)	5 (83)	87 (71)	
Lobular CA	38 (22)	8 (28)	4 (25)	1 (17)	25 (20)	
Tubular	9 (5)	1 (3)	0	0	8 (6)	0.9
Mixed	3 (2)	0	0	0	3 (3)	
ER status						
ER positive	157 (90)	25 (89)	16 (100)	6 (100)	109 (89)	
ER negative	17 (10)	3 (9)	0	0	14 (11)	0.4
PR status						
PR positive	119 (68)	15 (54)	1 (6)	6 (100)	82 (67)	
PR negative	55 (32)	13 (46)	15 (94)	0	41 (33)	0.02
Distant recurrence						
No distant recurrence		24 (86)	15 (94)	6 (100)	119 (97)	
Distant recurrence		4 (14)	1 (6)		4 (3)	0.02

Abbreviations:
NHG=Nottingham Histological Grade, ER= oestrogen receptor, PR=progesterone receptor
N1=macrometastases, N1(mi)=micrometastases, N0(i+)= isolated tumour cells
* Comparisons between groups by Chi-square test

TABLE 2 Histopathological diagnosis of sentinel lymph nodes and axillary lymph nodes

	N1 (N=28)	**N1(MI) (N=16)**	**N0(i+) (N=6)**	**P-VALUE***
Number of SLNs Median (range)	2.0 (1-4)	2.0 (1-6)	2.5 (1-3)	0.8
Metastasis detected by FS	23	3	0	<0.001
Metastasis detected by HE	5	10	2	0.04
Metastasis detected by IHC	0	3	4	0.002
Intraoperative false negative rate	18%	81%	100%	<0.001
Number of metastatic SLNs Median (range)	1 (0-2)	1 (1-1)	0	0.5
Size of SLN metastasis (mm)	7.00 (2.10-18.00)	0.90 (0.25-1.90)	0.10 (0.10-0.15)	<0.001
Number of SLNs and ALNs Median (range)	13 (4-25)	11 (5-24)	13 (6-27)	0.9
Number of metastatic nodes (no) Median (range)	2 (1-20)	1 (0-3)	1 (1)	0.003
Non SLN metastases				
Median (range)	1 (0-19)	0 (0-2)	0	
0	13	13	6	
1-3	11	3		
4-	4			0.007

Abbreviations:
SLN = sentinel lymph node
FS = frozen section
HE = hematoxylin and eosin
IHC = immunohistochemistry
N1=macrometastases, N1(mi)=micrometastases, N0(i+)= isolated tumour cells
* Comparisons between groups by Chi-square test for categorised variables and by Kruskal-Wallis test for continuous variables

TABLE 3 Univariate Recurrence free Survival by Cox Proportional Hazard Analyses

COVARIATE	**RFS**		
	HR	**95% CI**	**p-value**
Tumour size	5.11	1.48-17.67	0.01
T2 vs T1			
NHG	6.95	1.94-24.01	<0.01
3 vs 1-2			
SLN status			
Metastatic size (mm)	1.11	1.01-1.23	0.03
ALN status			
Metastatic nodes (no)	1.41	1.19-1.67	<0.001
Non SLN metastatic nodes (no)	1.48	1.18-1.86	0.001

Abbreviations:
T=Tumour, NHG=Nottingham histological grade, SLN= sentinel lymph node, RFS=recurrence free survival, HR= hazard ratio, CI=confidence interval
* Cox univariate analyses