Research

Phosphohistone 3 (PHH3) and lactate dehydrogenase 5 (LDH5) are expressed in ductal carcinoma in situ of the breast: possible clinical implications

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Abstract

Introduction/Background: Proliferation and pre-malignancy are typical features of DCIS. The expression of PHH3 as a marker for proliferation and LDH 5 as an indicator for oxygen independent energy metabolism was investigated in order to assess their potential for an improved characterisation of these lesions.

Methods: Archived tissue blocks and clinical records of 130 patients with DCIS. Immunohistochemistry for PHH3, LDH5, estrogen receptor (ER), progesterone receptor (PR), human EGF receptor 2 (HER2). Silverstein nuclear grading. Chi Square test for all factors.

Results: Percentage of positive patients was 69% for PHH3, 94% for LDH5, 76% for ER, 67% for PR, and 21% for HER2. Significant correlation was seen between PHH3 and LDH5 expression. No correlation could be found for any of the other comparisons.

Conclusion: This is the first description of PHH3 and LDH5 in DCIS. The biological significance of PHH3 remains to be determined in a larger set of patients. LDH5 may be a useful diagnostic marker in the future. Positive HER2 receptors were considerably less frequent than previously reported.

Keywords: DCIS, PHH3, LDH5, Immunohistochemistry, clinical implications.
Introduction

The term “ductal carcinoma in situ of the breast” (DCIS) encompasses a wide spectrum of histopathological and clinical pictures. Common denominator is the proliferation of neoplastic cells within mammary ducts. An estimated 14 – 53% of these lesions progress to invasive cancer [1-4], but there is an ongoing controversy over the factors that help in assessing the prognosis when counselling an individual patient for appropriate treatment. While local recurrence in 10-15% of cases, arguably due to treatment regimens of varied intensity, is a considerable concern [5-7], the _quoad vitam_ prognosis is excellent with a total mortality of about 2%.

All morphological and immunohistochemical factors that are used for characterisation of invasive breast cancer have also been examined in DCIS. A grading of low, intermediate and high nuclear malignancy has been proposed by Tavassoli and coworkers [8]. Addition of further characteristics (size, surgical margins) and their association with recurrence risk has led to the development of the Van Nuys Prognostic Index (VNPI) [9]. Since lower age seems to be an additional adverse factor, this was later added by Kelley and coworkers [10], to result in the USC/VNPI score. However, small size of the underlying cohort and retrospective nature of the analysis limit the clinical value of the score [5, 11].

Similar to invasive breast cancer, DCIS express estrogen and progesterone receptors in 60 - 75% of cases [12-14]. There is an inverse relation between grading/proliferation and receptor positivity [15-17]. These receptors seem to be relevant, as the NSABP B24 study showed tamoxifen to be beneficial in adjuvant therapy [18]. Furthermore NSABP P-1 and IBIS 1 demonstrated a preventive effect for the development of both DCIS and invasive breast cancer [19, 20]. Several studies, however, demonstrated a positive effect of tamoxifen only in ER-positive DCIS [15-17].

For HER2, considerably higher rates of positivity than in invasive cancer have been reported, i.e. 35-60% vs. 20%, with a positive correlation between grading and positivity [12, 16, 21]. An explanation for this difference is a putative role for EGF in the initiation of malignant transformation or progression from DCIS to full-scale malignancy [22, 23]. NSABP B-43 is a presently ongoing large trial examining the effect of trastuzumab as adjuvant treatment in high risk DCIS [24]. At present, the clinical significance of HER2 in DCIS is unclear. Surprisingly, a
large DCIS cohort with extensive follow-up demonstrated significantly improved long-term invasive disease-free survival for patients with HER2 positive disease in primary DCIS [25].

Phosphohistones are a group of proteins serving as sterical support of DNA during nucleosome formation and mitosis [26]. Phosphorylation of Phosphohistone 3 is crucial for chromosome condensation during mitosis [27]. Antibodies are highly specific for mitotic cells and do not bind to unphosphorylated histone H3 [28]. Immunohistochemical staining using Anti PHH3-antibodies facilitates identification and quantitation of mitotic cells, particularly in high grade tumours with a high cellular density. Since cells in prophase are equally detected by this method, the number of counted mitoses is higher but more reproducible than in established methods [29, 30]. In lymph node negative early invasive breast cancer, a high PHH3 index is associated with earlier and more frequent recurrence. Additionally, age, grading and hormone receptor status are correlated with PHH3 expression. Patients with low PHH3 index have a good prognosis [31, 32].

Lactate dehydrogenase 5 (LDH 5), which is not expressed in normal epithelial cells, enables anaerobic glycolysis in malignant cells and thus renders the cells independent of oxygen supply [33-35]. LDH 5 expression in invasive breast cancer is associated with a poor prognosis [36].

Here, we describe for the first time the expression of PHH3 and LDH5 in DCIS and examine their possible role for an improved clinical understanding of these tumours.

Methods

Patients

Tissue blocks of 130 patients diagnosed and treated for DCIS at the certified Center for Breast Disease Suedbaden Freiburg and Emmendingen, Germany, between 2001 and 2008, were available for evaluation.

A mailed questionnaire was used to collect follow up data on adjuvant therapy, recurrence, last mammogram, and survival.

Institutional Review Board Approval was obtained.
Immunohistochemistry

First, tissue multi arrays (TMA) were constructed. Therefore, ductal carcinoma in situ (DCIS) was detected and marked within the H&E stained tissue samples used for routine diagnostics by an experienced pathologist (S.T.). Three different tissue punches with DCIS of each patient were used for the TMA. From each TMA acceptor block with previously formalin-fixed, 10% neutral-buffered and paraffin embedded patient’s tissue samples, section of 2 μm thickness were cut for immunohistochemical analyses.

For PHH3 and LDH5 immunohistochemistry, sections were mounted onto glass slides (SuperFrost® Plus slides, Langenbrinck, Emmendingen, Germany), dried overnight (58º C) followed by dewaxing in Xylene and rehydrating in decreasing alcohol concentrations. Incubation with TRS (Target retrieval solution, 1:10 dilution, Dako, Glostrup, Denmark) for 20 minutes (pH 9) and then cooled on ice for 10 minutes. Sections were washed in distilled water and in Dako wash buffer (1:10 dilution) 10 minutes each and encircled using a Dako-Pen.

PHH3: Rabbit polyclonal Anti-phospho-Histone H3 (Ser10) Mitosis Marker (Millipore #06-570; Billerica, MA, USA) was used at a dilution of 1:5000, diluted with Dako Real™ antibody solution and incubated with wash buffer (Dako) for 30 minutes. The immune complex was visualized with the Dako REAL™ Detection System, Alkaline/Phosphatase/RED, Rabbit/Mouse (K5005; Dako). Sections were incubated with Dako Link Biotinylated Secondary Antibodies (AB2, Dako) for 10 min followed by washing with wash buffer (10 minutes) and with Streptavidin Alkaline Phosphatase (AP, Dako, 15 minutes) and once again with wash buffer for 10 minutes. Finally, an incubation of the sections with chromogen (as described at the Dako REAL™ Detection System) was stopped by distilled water (10 minutes) and counterstained with hematoxylin. Dehydration in increasing alcohol and xylol followed before mounting and coverslipping.

LDH5: After treatment with Dako Real™ Proteinkinase K and its blocking by Dako Real™ Peroxydase-Blocking Solution sections were washed twice in Tris/Hcl buffer and incubated with 1% goat serum (30 min) followed by incubation with the polyclonal rabbit Laktat Dehydrogenase Isoenzyme 5 (LDH5) antibody (ab53010, Abcam plc, Cambrige, UK) for 30 min. After washing with Tris/Hcl buffer a goat-anti-rabbit secondary antibody was applied (incubation for 15 min). After washing with wash buffer (10 minutes) and with Streptavidin Alkaline Phosphatase (AP, Dako, 10 minutes) an incubation of the sections with chromogen
followed (as described at the Dako REAL™ Detection System) and it was stopped by distilled water (10 minutes). Finally, counterstaining with hematoxylin was performed followed by dehydration in increasing alcohol and xylol before mounting and coverslipping.

For ER, PgR and HER2/neu immunohistochemistry the following primary antibodies were used: monoclonal antibody to estrogen receptor (clone 1D5; Dako, Glostrup, Denmark; 1:200 dilution; pH 6); monoclonal antibody to progesterone receptor (PgR6636; Dako, Glostrup, Denmark at 1:200 dilution; pH 6), and the polyclonal antibody to the HER2/neu protein (Dako, Glostrup, Denmark; 1:350 dilution; pH 6). All stainings were performed on an autostainer by Dako (Dako Cytomation) according to the instructions of the company. A nuclear reactivity of ER and PgR in > 1% of the tumour cells was evaluated as a positive reaction. HER2neu overexpression (HER+) was defined as an intense and complete membrane staining in more than 30% of the tumour cells, using the definition that was valid at the time.

Evaluation of PHH3 and LDH5

The number of PHH3-detected mitoses in cancer cells of the pre-treatment biopsies was counted by S.E and S.T. using a light microscope. Although inter observer agreement occurred frequently, all results of both independent observers were controlled and re-evaluated. Only PHH3-positive nuclei in the prophase, metaphase, anaphase and the telophase of mitosis were noted. Interphase figures with an irregular granular PHH3-staining were excluded. Signals in malignant epithelial tumour cells were remarked per 10 high power fields HPF (four-hundredfold magnification); those in tumour stromal cells or blood cells were ignored. This evaluation method was described before in detail [31].

Absence of proliferative activity was noted as 0, one to 5 mitoses were recorded as weak and > 6 mitoses were counted as strong activity. Since the TMA contained three DCIS punches, the average value of PHH3 detected mitoses per each patient was taken for evaluation.

For the evaluation of LDH5 expression, immunohistochemical stains were graded qualitatively to 0 (no cytoplasmatic staining), 1 (light), 2 (moderate), and 3 (intense). Since, again, 3 punches were available per patient and staining sometimes varied among these, the most intense staining was used for analysis.
For statistical analysis, Pearson’s chi square test was used, p values < 0.05 were regarded as significant. Calculations were performed using BMDP Statistical Software, Release 7.1.

Results

Clinical Data

Our cohort consisted of 130 patients treated at the certified Center for Breast Disease Suedbaden Freiburg and Emmendingen, Germany between 2001 and 2008. Compliance with the national guideline in effect at the time [37] was ascertained by a tumour board recommendation in all cases. Mean age at diagnosis was 62 (range 40 – 89 years). There was no correlation between patient’s age with neither PHH3 expression nor LDH5 expression.

The duration of follow up was 3.9 years (mean, range 2 – 9). Of 130 patients, 124 were available for analysis. One patient had deceased, the reason could not be elucidated.

58 patients had postoperative radiotherapy and 62 had been treated with tamoxifen, of which 51 had ER-positive DCIS. Nine women had stopped tamoxifen prematurely or were switched to a different medication. 89 women had undergone regular follow-up mammograms.

Four patients developed a recurrence (DCIS or invasive cancer) during follow-up, one had primarily been graded G2 and three were G3. Of note, all of these were both PHH3 and LDH5 positive. However, there was no significant correlation between grading (p = 0.31) and recurrence nor between either PHH3 (p= 0.19) or LDH5 (p=0.62) positivity and recurrence.

Immunohistochemistry

Due to the limited size of the specimens, it was not possible to perform all stainings on all specimens. Therefore, figures do not add up to 130, except for grading. <Table 1> summarises the findings.
Nuclear grading according to Silverstein was classified as low (G1) in 21, intermediate (G2) in 57 and high (G3) in 52 cases, respectively. ER was positive in 78, PR in 71, and Her2neu in 27 cases resp.

69% (n=78) of 113 specimens available for evaluation were PHH3-positive (any positive score). PHH3 positivity correlated neither with grading nor with ER, PR, nor HER2, respectively. <Figure 1> represents PHH3 positive DCIS cells in different phases of mitosis.

93.8% (n=106) of 113 specimens available for evaluation were LDH5 positive (any positive score). This correlated significantly with PHH3 positivity ($p = 0.0225$) <Table 2>. No correlations were found with any other parameter. <Figure 2> displays sample slides of LDH positive specimens.

<table>
<thead>
<tr>
<th>Grading</th>
<th>n</th>
<th>PHH3 pos</th>
<th>PHH3 neg</th>
<th>p (chi²)</th>
<th>LDH5 pos</th>
<th>LDH neg</th>
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Table 1: Correlation between established and new immunohistochemical markers.
Figure 1: A: PHH3-stained mitotic figures in different mitotic phases like prophase/early metaphase (B); late metaphase (C) and ana-/telophase (D); PHH3 immunohistochemistry, 40x magnification. Stained interphase nuclei and uncertain mitotic figures (arrows) were not respected.

<table>
<thead>
<tr>
<th></th>
<th>PHH3 negative</th>
<th>PHH3 positive</th>
<th>Total</th>
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<tr>
<td>LDH5 negative</td>
<td>3 (2,9%)</td>
<td>1 (1%)</td>
<td>4 (3,8%)</td>
</tr>
<tr>
<td>LDH5 positive</td>
<td>24 (23,1%)</td>
<td>76 (73,1%)</td>
<td>100 (96,2%)</td>
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<tr>
<td>Total</td>
<td>27 (26%)</td>
<td>77 (74%)</td>
<td>104 (100%)</td>
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**Table 2: Correlation between PHH3 and LDH5.**
Figure 2: A: LDH5 immunohistochemistry, 10x magnification DCIS with no cytoplasmic staining B: weak staining, C: moderate expression, and D: strong staining.
Discussion

The incidence of DCIS has risen considerably in the last three decades, mainly due to the implementation of mammography screening programs. Nowadays, in the US, every year over 60,000 women are diagnosed with DCIS, equalling more than 20% of all new breast cancer diagnoses [38]. The relation between invasive and ductal in situ carcinomas seems to be similar in Germany [39]. More than 80% of these lesions are detectable only by mammogram [4, 40].

Despite the differences between DCIS and invasive breast cancer, treatment, except for axillary staging, is quite similar in DCIS and, at least luminal A, invasive cancer. Many women will be advised to go on tamoxifen and will undergo a strict radiological follow up, with a considerable personal and socioeconomic burden. Women with DCIS experience the same level of anxiety as those with invasive breast cancer, despite the excellent prognosis of the former condition [38].

Therefore, investigating for prognostic markers is highly relevant when trying to “choose wisely” among different levels of treatment intensity. For example, in low grade DCIS, even active surveillance might be an option [41]. However, the issue of discriminating precisely between under- and overtreatment is still unsolved [42]. This may, in part, be due to the fact that the excellent prognosis and low incidence of adverse events would necessitate impractically high numbers of patients treated, assessed, and followed by a strict standard.

As opposed to invasive breast cancer [32, 36], prognostic factors in DCIS are not well established [37]. About 60 - 75% of lesions express estrogen and progesterone receptors [12]. The results in our cohort are in line with these findings. A recent study on 1667 patients with DCIS and a similar distribution of hormone receptor positivity showed a somewhat higher rate of Her2neu positivity (33.5%) compared to our cohort (21.3%) - despite a lower proportion of low grade lesions in their population. This study showed Her2 positivity to be associated with a higher risk of local in situ recurrence but not invasive disease [44].

Here, we report, for the first time, on the presence of PHH3 and LDH5 in DCIS. The expressions of both parameters have been positively correlated with a poor prognosis in invasive breast cancer [31, 32, 36].
The vast majority of lesions stained positive for LDH5. This enzyme is a marker for intratumoural hypoxia [34] and has been associated with a poorer prognosis in breast cancer and a range of solid tumours. [35, 45-47]. The consistent presence of LDH5 in DCIS underscores the malignant nature of this disease.

The expression of LDH5 correlated significantly with PHH3 positivity (p = 0.02). All 4 patients with recurrent disease in our series expressed both PHH3 and LDH5. Numerically, more ER negative lesions were associated with PHH3 expression than vice versa. One may speculate that these two observations point towards PHH3 being an indicator for a more aggressive lesion. The lack of statistical significance might be due to the relatively small sample size. However, as discussed above, other immunohistochemical markers have equally failed to serve as reliable tools for clinical counselling.

Beyond the description of PHH3 and LDH5 in DCIS, the implications of our study for the clinical management of this condition remain to be determined; however, the same applies to other immunohistochemical features in DCIS. Three possible applications of our findings are conceivable and may be the focus of future investigations:

1. easier identification of DCIS in histological slides through LDH 5 staining;
2. assistance in the differential diagnosis between atypical ductal hyperplasia and DCIS;
3. emergence of a clinical significance in a considerably larger series with longer follow-up.

Recently, other innovative approaches to differentiate biological properties of different breast cancers have been described. Immunohistochemistry for PTEN identified a 100% correlation between the wild type of this gene and intact mismatch repair mechanisms [48]. Computer assisted morphometry on the grounds of cytoplasmic changes in breast lesions was able to discriminate between malignant and benign but not malignant and atypical lesions [49]. Here, LDH5 with its high sensitivity might be a way of overcoming this problem. Lastly, complex algorithms capable of integrating a multitude of morphological features in breast cancer may identify patterns that will help in the prognostic differentiation of the broad range of (pre-) malignant breast diseases. Our results might add another piece to this jigsaw puzzle.
Authors' contribution to the manuscript

M. Sillem: Project development, manuscript writing
S. Baum: Manuscript writing/editing
S. Engist: Data collection/management, Data analysis
G. Kayser: Manuscript writing/editing
M. Werner: Manuscript writing/editing
S. Timme-Bronsert: Project development, manuscript writing

Compliance with ethical standards

Conflict of Interest: All authors declare that they have no conflict of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Institutional Review Board Approval was obtained.

Informed consent: Since the study was performed on archived pathological material and no interventions were performed on patients, it was deemed unnecessary to obtain informed consent.

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