Relationship between Nuclear Grade of Ductal Carcinoma in situ and Cell Origin Markers

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Abstract. Ductal carcinoma in situ (DCIS) is a group of heterogeneous lesions genetically, morphologically, and biologically. Recently, breast epithelium in the terminal ductal lobular unit has been sub-classified based on the expression of several cytokeratin markers as stem cells (CK5/6 +), luminal cells (CK8, CK18 +), and basal cells (CK14, CK17 +). In this study we describe the relationship between DCIS of different nuclear grades (non-high grade and high grade) and these cell origin markers. Fifty-three cases of non-high grade and 46 cases of high grade DCIS were selected, and representative sections from each case were stained with antibodies to these cytokeratin markers. High grade DCIS showed significantly higher rates of expression with stem and basal cell markers compared with non-high grade DCIS (p <0.05). The majority of DCIS, both high grade and non-high grade, expressed luminal cell markers (67% to 91%) and single type of cell origin marker (72% to 87%). High-grade DCIS more frequently co-expressed all three types of cell origin markers compared with non-high grade DCIS (p <0.05). In summary, a subset of high grade DCIS frequently rises from stem or-and basal cell populations; the subset is associated with poor prognosis in invasive breast carcinoma. Thus, these markers may be used to identify a potentially more aggressive subgroup of breast carcinoma at its pre-invasive stage (DCIS), and to manage it accordingly. Second, most DCIS express luminal cell markers, suggesting that malignant transformation occurs relatively late along the cell differentiation pathway, contrary to the traditional belief that most neoplasms arise from a more primitive stem cell population. Third, the majority of DCIS exclusively express one type of progenitor marker, indicating that in most incidences they may arise from a single progenitor population. Last, triple expression of all types of cell origin marker is frequently associated with high grade DCIS, suggesting that more complicated pathways are involved in these more aggressive lesions. Further studies are needed to delineate the relationships of cell origin markers in DCIS and invasive carcinoma to the clinical outcome.

Keywords: breast carcinoma, ductal carcinoma in situ, nuclear grade, intraductal carcinoma

Introduction

The incidence of DCIS has increased dramatically due to population-based mammographic screening, and it now comprises approximately 30% of the 200,000 newly diagnosed breast cancers annually in the United States. At present, the treatment for DCIS is not standardized, largely due to a limited understanding of these neoplasms [1-3]. The Van Nuys Prognostic Index (tumor size, margin width, pathological grade, nuclear grade, necrosis classification, patient age) is currently the most used and best validated prognostic index for DCIS [4,5].

Numerous studies have attempted to identify morphologic and molecular factors with prognostic value for DCIS. Among them, nuclear grade is one of the most important factors. In fact, it is the main criterion used for re-classifying DCIS as high, intermediate, or low grade [6-8]. We have previously

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reported ultrastructural differences at periductal regions between high grade and non-high grade DCIS, and we have shown that the escape of tumor cells of high grade DCIS from its original site can be detected by electron microscopy [9]. So far, molecular markers (ie, ER, PR, p53, Ki-67, Her-2/neu, bcl-2, and p21) demonstrate no independently significant prognostic value for DCIS [10]. Recently, a 21-gene expression signature has been shown to predict distant recurrence and response to chemotherapy in patients with small, ER positive, lymph node negative invasive breast carcinoma [11].

It is generally believed that breast carcinogenesis is a sequential multi-step progression from normal epithelium to invasive carcinoma via hyperplasia, atypical hyperplasia, low grade DCIS, and high grade DCIS, based on clinical, pathological, and molecular studies [12-14]. However, recent studies have shown that low grade DCIS frequently does not share any genetic alterations with high grade DCIS, suggesting that multiple, parallel, genetically distinct pathways may be present, and challenging the conventional multi-step linear pathway [15-17]. Sontag and Axelrod [18] proposed 4 possible pathways for progression of heterogeneous breast carcinomas: (i) linear (sequential multi-step), (ii) nonlinear, (iii) branched, and (iv) parallel, with the parallel pathway most closely simulating clinical observations.

Several recent studies support the concept of multiple distinct genetic pathways for breast carcinogenesis. Double labeling immunohistochemical studies have shown that at least 3 distinct cell types exist in the normal terminal duct lobular unit (TDLU): stem cells (CK5/6 positive); luminal glandular cells (CK7/8/18/19 positive); and basal myoepithelial cells (CK14, 17, SMA positive), along with 2 intermediate cell populations with dual expression of either stem and luminal cell markers (CK5/6 + and CK8 and 18 +) or stem and basal cell markers (CK5/6+ and CK14 and 17+) [19,20]. Furthermore, studies have shown that a subgroup of invasive breast carcinomas expressing CK5 and/or CK17 correlates with poor clinical outcome [21,22]. Sorlie et al [23,24] have shown that invasive breast carcinoma has 5 distinct gene expression patterns: 1 basal-like type, 2 luminal types (A and B), 1 ERBB2 type, and 1 normal breast tissue type, which roughly correspond to the different cell types identified in TDLU, and also correlate with clinical outcome.

Thus, one may theorize that different types of progenitor cells in the TDLU give rise to DCIS of different nuclear grades, which in turn give rise to invasive carcinoma of different histologic grades, which have different clinical outcomes. If this hypothesis is true, the direction of a pre-invasive lesion (DCIS) progression may be predetermined from the beginning of cell proliferation based on the type of progenitor cell from which it has arisen. If true, there would be a great advantage to use cell origin markers to identify and direct the treatment of these subgroups of DCIS lesions appropriately before they are invasive and life threatening.

Nuclear grade of DCIS is a key histological factor with prognostic significance. So far, no data are available regarding the relationship between the different types of progenitor cells and DCIS of different nuclear grades. The objective of the current study was to determine the precise relationships between DCIS of different nuclear grade and the 5 cell origin cytokeratin markers. The answer to this question will not only provide insight into critical issues in breast carcinogenesis, but will also identify molecular markers of potential prognostic value.

Materials and Methods

Ninety-nine cases of DCIS with no co-existing invasive carcinoma were retrieved from the files of the Pathology Department at Strong Memorial Hospital, Rochester, NY. Fifty-three cases of non-high grade and 46 cases of high grade DCIS were selected by consensus among three pathologists using criteria proposed by Holland [7] and Silverstein [8]. Immunohistochemical stains were performed on formalin fixed paraffin embedded tissue obtained from a single representative section selected from each case, using antibodies to the 5 cell origin cytokeratin markers (stem cells: CK5/6; luminal cells: CK8 and 18; basal cells: CK14 and 17). Positive staining was defined as strongly cytoplasmic staining in ≥10% of the tumor cells.

The source, dilution, and pretreatment for each antibody are listed in Table 1. Pretreatments consisted of enzyme digestion, or other retrieval methods such as steam or pressure cooker. Sections were stained on a Dako Autostainer using either a labeled monoclonal polymer, HRP (Envision Plus System, DakoCytomation, Carpinteria, CA) or Horse Anti-Mouse IgG-Biotin (Vector Laboratories, Burlingame, CA), Streptavidin-HRP (Jackson Laboratory, Bar Harbor, ME), and AEC (DakoCytomation), and counterstained with
Table 1. Information about antibodies used in this research.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Vendor</th>
<th>Pre-treatment</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK5/6</td>
<td>D5/16B4</td>
<td>Dako</td>
<td>PK/TRS</td>
<td>1:50</td>
</tr>
<tr>
<td>CK8</td>
<td>35BH11</td>
<td>Dako</td>
<td>PK</td>
<td>1:100</td>
</tr>
<tr>
<td>CK18</td>
<td>DC10</td>
<td>Dako</td>
<td>PK</td>
<td>1:100</td>
</tr>
<tr>
<td>CK14</td>
<td>LL002</td>
<td>Noracstra</td>
<td>PC(6)</td>
<td>1:60</td>
</tr>
<tr>
<td>CK17</td>
<td>E3</td>
<td>Dako</td>
<td>PK/TRS</td>
<td>1:60</td>
</tr>
</tbody>
</table>

hematoxylin. Fisher’s exact test was used to assess statistically significant differences of percentages between high grade and non-high DCIS. Statistical calculations were performed with SAS software (Statistical Analysis System Inc., Cary, NC).

Results

In the areas of morphologically normal breast tissue on the same slides as the tumors, the cell origin markers stain the corresponding normal cells in the terminal ductal lobular unit; thus, the luminal cell markers (CK8 and CK18) stain the inner epithelium layer, while the stem cell marker (CK5/6) and the basal cell markers (CK14 and CK17) stain the outer basal/myoepithelial layer. These staining patterns are more clearly demonstrated in the ductal structure than in the acinar structure of the lobules (data not shown). The differential cytokeratin patterns in high grade and non-high grade DCIS are illustrated in Fig. 1.

A significant subgroup of high grade DCIS arises from stem or/and basal progenitor cells. The staining patterns for non-high grade and high grade DCIS are significantly different, with more staining for stem cells marker (26% vs 7%; p = 0.015) and basal cell markers (24-26% vs 7%; p = 0.028 for CK14 and 0.015 for CK17) in high-grade DCIS (Table 2). These results suggest that a significant subset of high grade DCIS frequently arises from stem cell or basal cell populations.

Most DCIS express luminal cell markers. The majority of both high grade (67%-91%) and non-high grade DCIS (85%-87%) stain with luminal cell markers CK8 and CK18 (Table 2), suggesting that most intraductal carcinomas evolved from a later stage along the cell differentiation pathway.

Most DCIS express exclusively one type (stem or luminal or basal) of cell origin markers. Thus, 72% of high grade DCIS and 87% of non-high grade DCIS express exclusively one type of cell origin marker (Table 3). These results indicate that

Table 2. Expression of the cell origin markers in all 99 cases of DCIS (including 46 high grade lesions and 53 non-high grade lesions).

<table>
<thead>
<tr>
<th>Markers of cell origin</th>
<th>DCIS grade</th>
<th>No. of positive cases</th>
<th>% of positive cases</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK5/6</td>
<td>non-high</td>
<td>4/53</td>
<td>7</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>12/46</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>CK8</td>
<td>non-high</td>
<td>45/53</td>
<td>85</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>31/46</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>CK18</td>
<td>non-high</td>
<td>46/53</td>
<td>87</td>
<td>0.537</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>42/46</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>CK14</td>
<td>non-high</td>
<td>4/53</td>
<td>7</td>
<td>0.028</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>11/46</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>CK17</td>
<td>non-high</td>
<td>4/53</td>
<td>7</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>12/46</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Expression of cell origin markers (S = stem cell, L = luminal cell, B = basal cell) singly or in combinations in 99 cases of DCIS (including 46 high grade lesions and 53 non-high grade lesions).

<table>
<thead>
<tr>
<th>Markers of cell differentiation</th>
<th>DCIS grade</th>
<th>No. of positive cases</th>
<th>% of positive cases</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S or L or B (single marker)</td>
<td>non-high</td>
<td>46/53</td>
<td>87</td>
<td>0.081</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>33/46</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>CK5/6 (stem cell marker)</td>
<td>non-high</td>
<td>0/53</td>
<td>0</td>
<td>0.465</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>1/46</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>CK8 + 18 (luminal cell)</td>
<td>non-high</td>
<td>46/53</td>
<td>87</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>31/46</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>CK14 + 17 (basal cell)</td>
<td>non-high</td>
<td>0/53</td>
<td>0</td>
<td>0.465</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>1/46</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>L+B, S+L, S+B (dual markers)</td>
<td>non-high</td>
<td>2/53</td>
<td>4</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>2/46</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>S+L+B (triple cell markers)</td>
<td>non-high</td>
<td>1/53</td>
<td>2</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>10/46</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Null (no cell marker expression)</td>
<td>non-high</td>
<td>4/53</td>
<td>7</td>
<td>0.369</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>2/46</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

* p value = 0.003 for overall differences in cell differentiation expression between the 2 groups (non-high grade vs high grade).
Fig. 1. Differential cytokeratin expression patterns in high grade and non-high grade DCIS (original magnification x20).
most intraductal breast carcinomas may originate from a single progenitor cell population.

A significant subgroup of high grade DCIS arises from cells with all 3 types of cell origin markers. High grade DCIS shows significantly higher frequency of triple expression (22% vs 2% for non-high grade DCIS; p = 0.002). High-grade DCIS comprises the majority of the stem/basal cell marker-positive population in this group, indicating a more complex pathway with extensive interactions among the 3 types of cell origin markers. The incidences of dual expression and null expression were not significantly different between high grade and non-high grade groups of DCIS (Table 3).

Discussion

The presence of true stem cells in breast epithelium has long been a controversial issue. Physiologically, it is clear that the mammary gland needs at least stem cell-like activity (self renewal and multipotent differentiation capacity) for routine tissue renewal and for massive expansion during pregnancy and lactation. Traditional studies (eg, clear fat-pad transplantation [25], retroviral tagging [26], and x-linked chromosome inactivation [27]) demonstrated the presence of stem cell-like activity in normal mammary gland. Recently, successful isolation and in vitro growth of stem cells from mice and humans have given this issue new insights [28-33]. These cells are capable of renewing themselves, differentiating into luminal epithelium and basal myoepithelium, and forming elaborate terminal duct lobular unit-like structures within a reconstituted basement membrane.

Based upon immunostaining, cells in TDLU exhibit distinctive keratin phenotypes for luminal cells (CK7, 8, 18, 19) and for basal cells (CK5, 14, 17) [34-37]. A small number of CK5/6 positive cells have been proposed to have stem cell properties because of the identification of 2 intermediate cell populations: luminal precursor cells expressing CK5/6 and CK8, 18, and 19, and basal precursor cells expressing CK5/6 and CK14 and 17 [19,20,39]. Availability of these antibodies makes it possible to study human breast carcinoma in intact tissue sections.

Several studies have explored the relationships of cell origin markers and other biological markers to the clinical outcome in patients with invasive breast carcinoma [39-41]. They have shown that in general tumors with luminal cell phenotypes are associated with a low proliferation rate, high ER and PR positivity, and better prognosis, while tumors with stem or basal cell phenotypes are associated with high proliferation rates, low ER and PR positivity, and worse prognosis. Expression microarray studies show that there are 5 subtypes of invasive breast carcinoma (2 luminal types, 1 ERBB-2 over-expression type, 1 basal type, and 1 normal-like type), and that significantly worse prognosis is associated with basal and Her-2/neu over-expressing types [23,24]. It is unknown at present what is the precise relationship between these cell origin markers and DCIS, especially to DCIS of different nuclear grades, which is an important factor in the Van Nuys Prognostic Index [1,5].

DCIS is a group of heterogeneous lesions. Even with the current classification based on nuclear grade, subpopulations from each group (high grade, intermediate grade, and low grade) still behave differently from the rest within the same group. In the present study, we found that a significant subgroup of high grade DCIS expresses stem cell and/or basal cell markers compared to low grade DCIS, which may lead to different clinical outcome for this subset of DCIS. If the theory of multiple distinct pathways is true, it implies that high grade DCIS gives rise to high grade invasive ductal carcinoma, while low grade DCIS gives rise to low grade invasive ductal carcinoma. This may mean that for any given lesion, its progression pathway may be largely predetermined by the type of progenitor cells from which it has arisen. If so, we may be able to use these markers to determine progenitor information, as well as other morphological and molecular information (such as ER, PR, Her-2/neu), and to treat each subgroup of these heterogeneous lesions effectively before it becomes invasive and life threatening.

We have found that most DCIS (both high grade and non-high grade) express luminal cell markers, suggesting that malignant transformation occurs relatively late along the cell differentiation
pathway. This is contrary to the current belief that most carcinomas evolve from a more primitive stem cell stage. Moreover, it suggests that besides the type of progenitor cell, there are other factors that are important in determining the progression of DCIS. Although most DCIS express only one type of cell origin marker, indicating they may evolve from one progenitor population, triple expression of all 3 markers is frequently associated with high grade DCIS. Evidently, a more complicated carcinogenesis pathway (trans-differentiation) may be involved in this subgroup of high grade DCIS.

In many cases, staining patterns of the cytokeratin markers are focal, with foci of densely positive cells scattered through the entire lesion (data not shown). Thus, tissue microarray analysis may not be the most suitable strategy for studying these markers. Further studies are needed to determine the precise relationships between cell origin markers, DCIS, and invasive carcinoma. We envision that the different expression patterns of cell origin markers in non-invasive DCIS and invasive carcinomas may engender a better understanding of the molecular pathways of carcinogenesis, and promote the development of appropriate therapeutic strategies to prevent the transformation from in situ carcinoma to invasive carcinoma.

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References