Human Pathology (2007) **38**, 197–204

ELSEVIER

Human PATHOLOGY

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Original contribution

Identification of a basal-like subtype of breast ductal carcinoma in situ☆

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Received 7 July 2006; revised 23 August 2006; accepted 24 August 2006

Keywords:
Basal-like subtype;
Ductal carcinoma in situ;
Estrogen receptor–negative;
Breast carcinoma

Summary Microarray profiling of invasive breast carcinomas has identified subtypes including luminal A, luminal B, HER2-overexpressing, and basal-like. The poor-prognosis, basal-like tumors have been immunohistochemically characterized as estrogen receptor (ER)–negative, HER2/neu–negative, and cytokeratin 5/6–positive and/or epidermal growth factor receptor (EGFR)–positive. The aim of this study was to determine the prevalence of basal-like ductal carcinoma in situ in a population-based series of cases using immunohistochemical surrogates. A total of 245 pure ductal carcinoma in situ cases from a population-based, case-control study were evaluated for histologic characteristics and immunostained for ER, HER2/neu, EGFR, cytokeratin 5/6, p53, and Ki-67. The subtypes were defined as: luminal A (ER+, HER2−), luminal B (ER+, HER2+), HER2, positive (ER−, HER2+), and basal-like (ER−, HER2−, EGFR+, and/or cytokeratin 5/6+). The prevalence of breast cancer subtypes was basal-like (n = 19 [8%]); luminal A, n = 149 (61%); luminal B, n = 23 (9%); and HER2+/ER−, n = 38 (16%). Sixteen tumors (6%) were unclassified (negative for all 4 defining markers). The basal-like subtype was associated with unfavorable prognostic variables including high-grade nuclei ($P < .0001$), p53 overexpression ($P < .0001$), and elevated Ki-67 index ($P < .0001$). These studies demonstrate the presence of a basal-like in situ carcinoma, a potential precursor lesion to invasive basal-like carcinoma.

1. Introduction

DNA microarray profiling studies on invasive breast tumors show distinct and reproducible subtypes of breast carcinoma associated with different clinical outcomes [1-10]. These subtypes include at least 2 types of estrogen receptor (ER)–negative tumors (basal-like and HER2+/ER−) and at least 2 types of ER+ tumors (luminal A and luminal B) [2,3]. The basal-like subtype is typically HER2− (ie, not

☆ This work was supported by an award to the University of North Carolina for a Breast Cancer Specialized Program of Research Excellence (SPORE) from the National Cancer Institute, Bethesda, MD, (CA58223), by the NCI (RO1-CA-101227-01) to C.M.P., and by the Breast Cancer Research Foundation, New York, NY.

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doi:10.1016/j.humpath.2006.08.017

amplified) and shows some characteristics of breast myoepithelial cells [11]. The basal-like subtype has been shown to have the highest proliferation rates and poor clinical outcomes [2,3] and has also been described as the prevalent subtype in *BRCA1*-related breast carcinomas [12].

Studies have used basal/myoepithelial cytokeratins and other markers to identify a subset of ER- and HER2− breast carcinomas that are associated with a poor prognosis, providing further evidence that basal-like breast cancer represents a distinct subtype of invasive carcinoma [13-18]. An association between epidermal growth factor receptor (EGFR) expression and the basal-like phenotype has been demonstrated [16]. Breast cancers showing immunoreactivity for cytokeratin 5/6 were also frequently found to coexpress EGFR. Nielsen et al [11] proposed a panel of four antibodies (ER, EGFR, HER2, and cytokeratin 5/6) to identify basal-like tumors in which the basal-like tumors were identified as those being ER−, HER2 not amplified, and positive for expression of cytokeratin 5/6 and/or EGFR.

Little is known with regard to the development of basal-like breast tumors. The aim of this study was to evaluate pure ductal carcinoma in situ (DCIS) cases from a large population-based series of incident cases to determine if a basal-like DCIS subtype exists and, if so, to determine the prevalence of this subtype and its correlations with histologic features and other immunohistochemical markers.

2. Material and methods

2.1. Study participants

The Carolina Breast Cancer Study (CBCS) is a population-based, case-control study of invasive and in situ breast cancer conducted in 24 counties of central and eastern North Carolina [19]. Incident cases were identified using a Rapid Case Ascertainment System, in cooperation with the North Carolina Central Cancer Registry [20]. Controls were selected from Division of Motor Vehicles (women younger than 65 years) and United States Health Care Financing Administration lists (women 65 years or older). Patients of in situ breast cancer were enrolled between 1996 and 2001 and included women with DCIS, lobular carcinoma in situ, and DCIS with microinvasion to a depth of 2 mm. For this study, only cases of pure DCIS were included. There was no oversampling according to age and race, as was used for the invasive portion of the CBCS [21]. Race was classified according to self-report. Less than 2% of participants reported Native American or other race and were classified as non–African American. A total of 503 patients (106 African Americans, 397 non–African Americans) and 458 controls (70 African Americans, 388 non–African Americans) were enrolled. For cases, the contact rate was 99.3%, and the cooperation rate was 83.4%, yielding an overall response rate of 82.7%. For controls, the contact rate was 90.6%, the cooperation rate was 73.0%, for an overall response rate of 65.2%.

The current analysis is based upon pure DCIS cases from the in situ portion of the CBCS. Of the total of 503 in situ breast cancer cases, 446 had a diagnosis of pure DCIS, 28 were lobular carcinoma in situ, and 29 were DCIS with microinvasion. In-person interviews were conducted to obtain information on potential breast cancer risk factors, and permission to obtain medical records and tumor tissue. The study procedures for recruitment and enrollment were approved by the Institutional Review Board of the University of North Carolina School of Medicine.

Formalin-fixed, paraffin-embedded tumor blocks were requested for all cases. Of the 446 pure DCIS cases, 3 cases refused to allow tumor tissue to be obtained, referring pathologists declined to send blocks on 25 cases, and tumor tissue was insufficient for sectioning on 25 cases. Thus, for 393 pure DCIS cases (88% of the total), tumor blocks were cut in multiple 5-μm sections, and the first and last sections were used, stained with hematoxylin and eosin. Hematoxylin and eosin slides were used to conduct centralized review of pathology for each case. The diagnosis of each case was confirmed by either of 2 reviewing pathologists (C.A.L., D.M.) and further evaluated to determine DCIS histologic features. Nuclear pleomorphism was graded as mild (<2-fold variation), moderate (2-<3-fold variation), and marked (≥3-fold variation). Nuclear size was graded in relation to red blood cell (RBC) diameter: small (1×-1.5× RBC diameter), intermediate (1.5×-2× RBC diameter), large (2×-3× RBC diameter), and very large (>3× RBC diameter). Nucleoli were scored none, small/indistinct, prominent (readily visualized at 10×), and multiple. Other histologic features evaluated include presence/absence of necrosis, architecture pattern (solid, cribriform, micropapillary, papillary, or clinging), apocrine features, and presence/absence of calcifications.

A total of 245 pure DCIS cases (55% of the total) had sufficient tissue to conduct immunohistochemistry (IHC) assays for all 6 immunohistochemical markers used in the study (ER, HER2, p53, cytokeratin 5/6, EGFR, Ki-67). There were no significant differences in histologic features (eg, distribution of comedo versus noncomedo DCIS) between the cases with sufficient tumor tissue and the

Table 1 Panel of antibodies used in the study

Antibody	Clone	Dilution	Company
ER	Id5	1:20	Dako, Carpinteria, CA
HER2/neu	CB11	1:100	BioGenex, San Ramon, CA
EGFR	31G7	1:7	Zymed, South San Francisco, CA
Cytokeratin 5/6	D5/16B4	1:50	Zymed
p53	DO7	1:850	BioGenex
Ki-67	MIB-1	1:75	Dako

ER, estrogen receptor; EGFR, epidermal growth factor receptor.

Table 2 Prevalence of breast cancer subtypes in DCIS

Subtype	Patients with DCIS (n = 245) (n [%])
Basal-like	19 (8)
Luminal A	149 (61)
Luminal B	23 (9)
HER2+/ER−	38 (16)
Unclassified	16 (6)

remaining DCIS cases. There were also no significant differences between the subset of DCIS cases with IHC marker data to the remaining cases with regard to age, race, family history, or other breast cancer risk factors.

2.2. Immunohistochemistry

Sections, measuring 5 μm, from formalin-fixed, paraffin-embedded tumors were cut and mounted onto Probe On Plus slides (Fisher Scientific, Hampton, NH). After deparaffinization in xylene, slides were rehydrated through a graded series of alcohol and placed in tris buffer. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase and methanol. Commercially available antibodies to ER, HER2/neu, cytokeratin 5/6, EGFR, p53, and Ki-67 were used in the study (Table 1). After tissue pretreatment (steam antigen retrieval for cytokeratin 5/6 and p53 and pepsin for EGFR) and protein block, slides were incubated with antibody, followed by incubation with streptavidin-conjugated HRP using Vectastain ABC Elite kit protocol (Vector Laboratories, Burlingame, CA). 3,3′ -diaminobenzidine tetrahydrochloride (DAB) or SG chromogen was used for the visualization of the antibody/enzyme complex. ER (DAB)/cytokeratin 5/6 (SG) and HER2 (SG)/Ki-67 (DAB) were performed as dual-stain assays. The dual-stain assay technique is a sequential immunohistochemical assay where the first primary antibody is directed against the membrane, cytoplasmic, or matrix protein followed by an antibody against the nuclear protein of interest. An acid dissociation step and

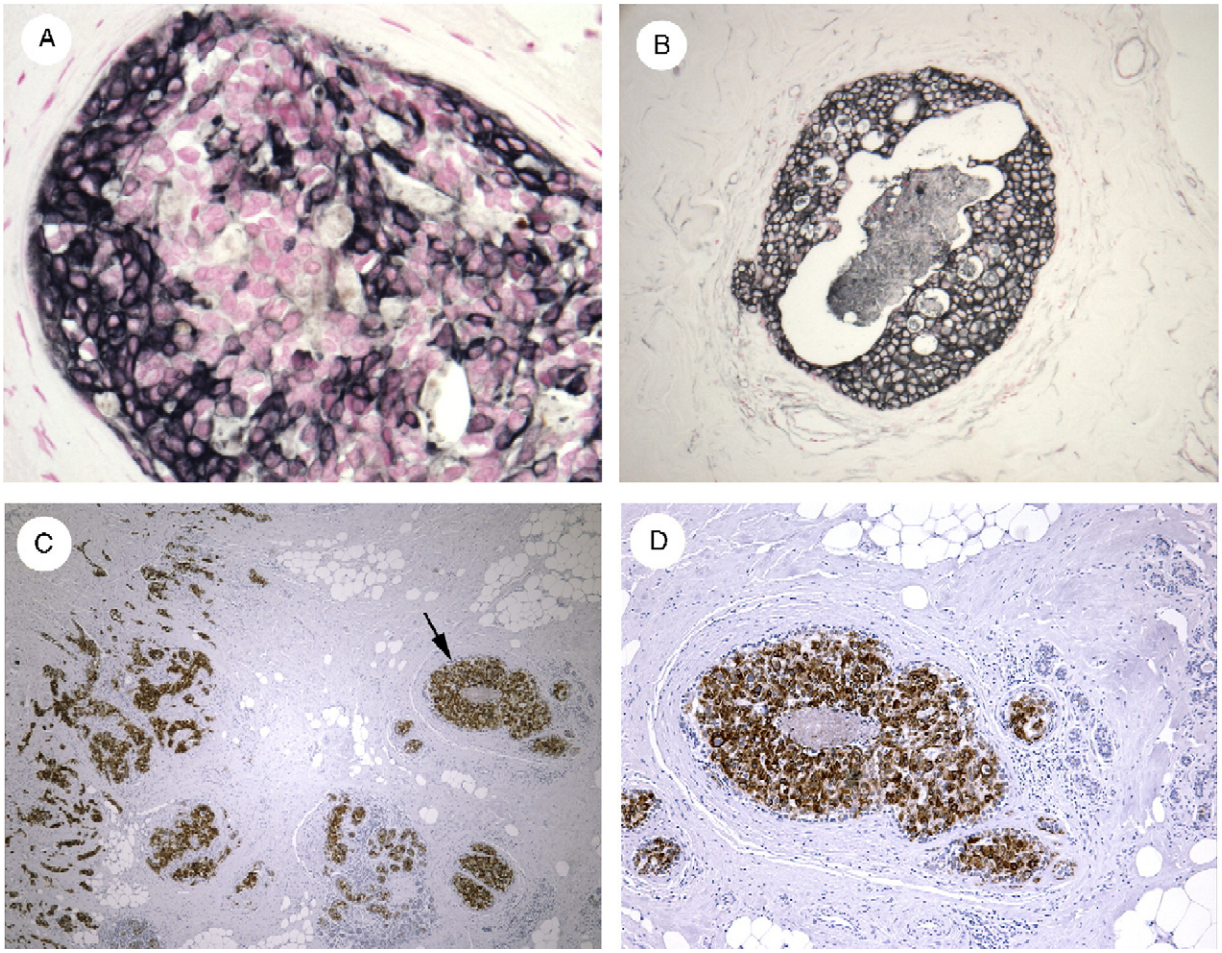

Fig. 1 Cytokeratin 5/6 and EGFR expression in basal-like ductal carcinoma in situ. A, This DCIS showed strong cytokeratin 5/6 expression in most tumor cells and a complete lack of ER expression (cytokeratin 5/6 and ER dual stain). B, EGFR membranous expression was typically strong and present in nearly all tumor cells. C, This invasive basal-like carcinoma showed strong cytokeratin 5/6 expression in both the invasive (left side of field) and in situ (arrow) components. D, Higher magnification of DCIS admixed with the invasive basal-like carcinoma confirmed identical strong cytokeratin 5/6 expression.

postfixation step using zinc formalin is inserted between primary applications. Interpretation is enhanced by using contrasting chromogens and their complementary counterstain.

2.3. Definitions

High nuclear grade histology was defined according to the 1997 Consensus Conference of the Classification of DCIS [22], based on the presence of marked nuclear pleomorphism, large or very large nuclei, and prominent nucleoli. Tumors with Allred score above 2 ER nuclear staining were classified as ER+ [23]. Tumor HER2 membranous staining equivalent to 3+ intensity with DAB chromogen and 2+ or 3+ intensity with the SG chromogen in more than 10% of cells was scored as overexpression. Any degree of cytoplasmic staining for cytokeratin 5/6 and any degree of distinct membranous staining for EGFR were counted as positive for expression. High Ki-67 index was defined as nuclear staining in more than 10% of tumor cells. p53-positive was defined as intensity of weak or higher, percent positive of 10 or higher, and localization of stain to nucleus or nuclear and cytoplasm.

In a previous study, we developed IHC-based surrogates for some of the breast tumor gene expression subtypes by performing both microarray analysis and IHC for ER, HER2/neu, EGFR, and cytokeratin 5/6 on 115 breast cancers and then validated these IHC surrogates using a 930-case tissue microarray from the University of British Columbia [11]. For this study, the immunohistochemical subtype definitions used were the refined definitions used in the analysis of the invasive CBCS cases that were: luminal A (ER+, HER2−), luminal B (ER+, HER2+), HER2 positive (ER−, HER2+), and basal-like (ER−, HER2−, EGFR and/or cytokeratin 5/6+) [24].

Menopausal status was defined based upon in-person interview data. Women younger than 50 years who had undergone natural menopause, bilateral oophorectomy, or irradiation to the ovaries were classified as postmenopausal. In women older than 50 years, menopausal status was assigned based upon cessation of menstruation.

2.4. Statistical analysis

χ^2 and Fisher exact tests were used to compare frequencies in contingency tables. Statistical analyses were performed with SAS statistical software, Version 8.2 (SAS Institute Inc, Cary, NC).

Table 3 Characteristics of DCIS subtypes

Total (n = 245)	Basal-like (n = 19)	Luminal A (n = 149)	Luminal B (n = 23)	HER2+/ER− (n = 38)	Unclassified (n = 16)
Age at diagnosis					
Mean (SD)	57.6 (12.3)	55.7 (11.1)	50.7 (10.8)	56.1 (9.9)	55.6 (10.1)
$P^a = .26$					
Race					
African American	4 (21%)	38 (26%)	4 (17%)	7 (18%)	4 (25%)
Non–African American	15 (79%)	111 (75%)	19 (83%)	31 (82%)	12 (75%)
$P^b = .84$					
Menopausal status					
Premenopausal	6 (32%)	45 (30%)	8 (35%)	8 (21%)	4 (25%)
Postmenopausal	13 (68%)	104 (70%)	15 (65%)	30 (79%)	12 (75%)
$P^b = .77$					
Histology nuclear grade					
High-grade	16 (84%)	42 (28%)	14 (61%)	35 (92%)	13 (81%)
Intermediate/low grade	3 (16%)	107 (72%)	9 (39%)	3 (11%)	3 (19%)
$P^b < .0001$					
p53 status					
Positive	12 (63%)	20 (15%)	11 (52%)	21 (60%)	7 (44%)
Negative	7 (37%)	118 (85%)	10 (48%)	14 (40%)	9 (56%)
$P^b< .0001$					
Ki-67					
Low	6 (33%)	120 (83%)	8 (35%)	9 (24%)	10 (63%)
High	12 (67%)	25 (17%)	15 (65%)	28 (76%)	6 (37%)
$P^b < .0001$					
Apocrine features					
Present	1 (5%)	5 (3%)	2 (9%)	2 (5%)	0
Absent	18 (95%)	144 (97%)	21 (91%)	36 (95%)	16 (100%)
$P^c = .53$					

[a] Analysis of variance test for difference in means.
[b] χ^2 Test.
[c] Fisher exact test.

3. Results

Characteristics of the 245 pure DCIS cases with sufficient tumor tissue were as follows: African American (n = 57, 23%), non–African American (n = 188, 77%); premenopausal (n = 71, 29%), and postmenopausal (n = 174, 71%). Approximately 30% of DCIS cases were negative for ER, and approximately 25% of DCIS cases were positive for HER2/neu overexpression.

The prevalence of the breast cancer subtypes in the 245 DCIS patients is presented in Table 2. A basal-like immunophenotype (ER−, HER2−, and cytokeratin 5/6–positive and/or EGFR+) was identified in 19 cases (8%). All of the basal-like carcinomas had an Allred score of 0. Cytokeratin 5/6 expression in the basal-like carcinomas was highly variable, ranging from less than 5% to 75% positive tumor cells (Fig. 1A). EGFR membranous expression in the basal-like cases was typically strong in intensity and present in most cells (Fig. 1B). There were 149 (61%) ER+/HER2− (luminal A) cases, 23 (9%) ER+/HER2+ (luminal B) cases, 38 (16%) ER−/HER2+ (HER2) cases, and 16 (6%) unclassified (negative for all 4 markers) cases (Table 2).

The prevalence of the basal-like DCIS subtype was 7% in African American women and 8% in non–African American women ($P = .84$). The prevalence of the basal-like subtype was 9% in premenopausal and 8% in postmenopausal women ($P = .77$). Considering both race and menopausal status, the prevalence of the basal-like subtype was 9% in premenopausal African-American women, 6% in postmenopausal African-American women, 8% in premenopausal non–African-American women, and 8% in postmenopausal African-American women ($P = .94$).

The basal-like subtype was associated with high-grade nuclei (84%, $P < .0001$), positive p53 expression (63%, $P < .0001$), and high Ki-67 index (67%, $P < .0001$) (Table 3). The ER− subtypes consisting of basal-like, HER2+/ER−, and unclassified showed higher rates of high-grade nuclei, compared with the ER+ subtypes (luminal A and B).

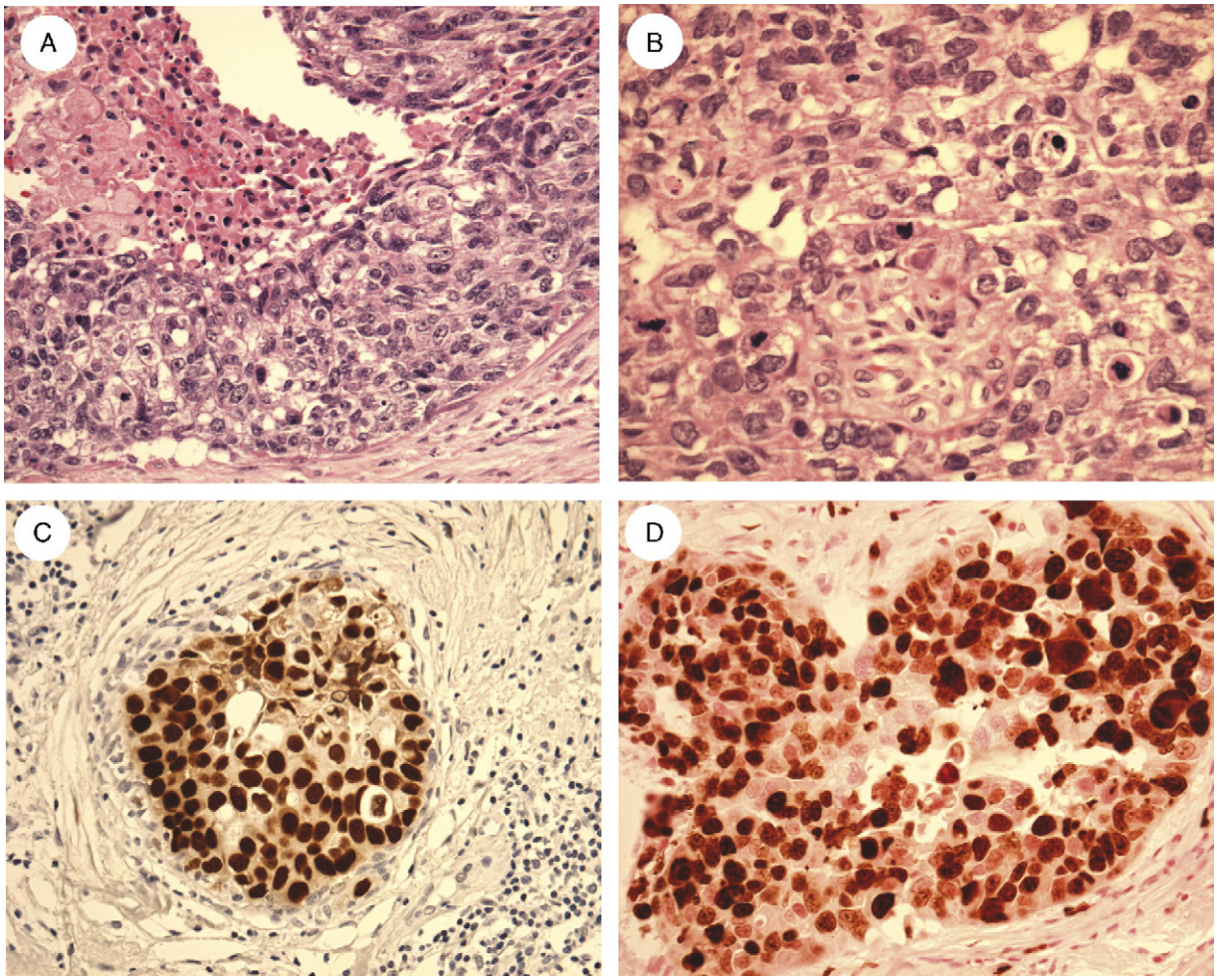

Fig. 2 Histology, p53 expression, and Ki-67 proliferative index associated with basal-like ductal carcinoma in situ. A, Hematoxylin and eosin–stained slide shows high-grade nuclei and central necrosis. B, Higher magnification of another case shows readily identifiable mitotic figures and apoptotic bodies. C, Many cases showed strong nuclear p53 expression. D, Most cases showed positive nuclear Ki-67 staining in more than 10% of tumor cells indicative of a high proliferative index for these tumors.

However, the basal-like and HER2+/ER− subtypes showed higher rates of positive p53 expression and high Ki-67 index, compared with the unclassified group. Sixteen of the 19 basal-like DCIS cases showed high-grade nuclei and evidence of a high proliferation rate including mitotic figures and apoptotic bodies. Three basal-like DCIS cases showed intermediate-grade nuclei. None of the low-grade DCIS cases showed a basal-like subtype. There were no specific histologic clues in the high-grade DCIS lesions to predict the basal-like DCIS subtype. Apocrine features were identified in only 1 basal-like tumor (Table 2). Examples of representative basal-like lesions are presented as follows: basal-like cases showing high-grade nuclei and a high proliferative rate (Fig. 2A and B) and basal-like cases showing positive expression of p53 (Fig. 2C) and a high Ki-67 index (Fig. 2D).

It was noted during the microscopic review of the invasive basal-like carcinomas described in Carey et al [23] that some tumors contained an in situ component that showed strong cytokeratin 5/6 expression (Fig. 1C and D) similar to the invasive carcinoma. This finding prompted review of 207 invasive basal-like carcinomas immunostained for cytokeratin 5/6, revealing 9 cases in which both the invasive and in situ carcinoma components showed expression of cytokeratin 5/6.

4. Discussion

The designation of a molecular subtype of invasive breast carcinomas as basal-like was based on the finding that the gene expression patterns between these tumors and normal breast basal (myoepithelial) cells showed some similarities that included the expression of keratins 5, 6, and 17 [1-3]. Numerous studies have since confirmed the presence of a group of ER− invasive breast tumors that express basal cytokeratins and are associated with poor prognosis [13-18]. It has been shown previously that most invasive basal-like carcinomas are ductal carcinomas not otherwise specified [25]. It is therefore presumed that an in situ precursor lesion exists for at least some of these tumors.

The aim of this study was to determine the prevalence of DCIS with basal-like features in a population-based setting. To date, only 1 study has reported on the identification of DCIS with a basal-like phenotype [26]. Bryan et al evaluated 66 cases of DCIS using immunohistochemical markers ER, PR, HER2, 3 basal cytokeratins, EGFR, and c-KIT. The DCIS cases chosen for their study were all high-nuclear-grade lesions retrieved from their pathology files and do not comprise a population-based series of incident cases. They identified 4 cases (6%) that demonstrated a "triple negative" phenotype, each of which was positive for either a basal cytokeratin or EGFR. Given that invasive breast carcinomas typically share immunophenotypic features with the DCIS from which they arise, they proposed that the hormone receptor–negative, basal cytokeratin and/or EGFR+ DCIS lesions identified may represent the precursor lesion to invasive basal-like carcinomas.

Using IHC surrogates, we observed the presence of basal-like in situ carcinoma, both as a pure form and in association with invasive basal-like carcinoma in multiple cases from the CBCS. The basal-like subtype in this study was associated with high-nuclear-grade histology, p53 overexpression, and a high Ki-67 proliferative index. Apocrine differentiation, a feature associated with ER− status, was not prominent in the basal-like carcinomas. These histologic and immunohistochemical findings are similar to those observed for invasive basal-like carcinomas [25].

The prevalence of basal-like DCIS in this study was 8%. One strength of this study is that the prevalence is based on a population-based study rather than selection of cases from our pathology archives. The selection process of using only DCIS cases with sufficient tumor for multiple IHC studies may have biased our evaluation toward larger DCIS cases, and it is possible that we could have overestimated the true prevalence of basal-like DCIS. Regardless, the prevalence of the basal-like phenotype observed in DCIS in this study (8%) is significantly less than the prevalence of basal-like phenotype for invasive carcinoma from this same CBCS (20%) [23]. The cause of this discrepancy is unknown; however, it should be noted that a reverse relationship was seen for the HER2+ group (HER2+ and ER−) where the DCIS prevalence was 16% and the invasive carcinoma prevalence was 7%. One possible explanation for this discrepancy between DCIS and invasive tumors is that the natural history of the basal-like DCIS lesions is to rapidly progress, and therefore, more invasive carcinomas may develop per DCIS for the basal-like subtype relative to the HER2+. If this were the case, the pure DCIS stage would be shorter and underrepresented in a population-based study. Another possibility is that the basal-like phenotype is an acquired phenotype during tumor progression. If some invasive basal-like carcinomas arise from dedifferentiation of ER+ precursor lesions, the incidence of the basal-like phenotype may be higher for invasive lesions than for pure in situ lesions. This hypothesis would suggest that some ER− tumors arise from ER+ precursors. In this study, the ER− rate for invasive carcinomas was 12% higher than the ER− rate observed for in situ carcinomas. This finding suggests the possibility that some ER− invasive carcinomas may arise from ER+ DCIS as a result of tumor progression; however, we favor the hypothesis that most ER− invasive carcinomas arise from ER− precursors. This is based in part on the finding of basal-like DCIS admixed with invasive basal-like carcinomas.

The identification of basal-like DCIS intimately admixed with invasive basal-like carcinoma strongly suggests that the pure basal-like DCIS cases we have identified could serve as a precursor lesion for invasive basal-like carcinoma. Earlier precursor lesions (like atypical ductal hyperplasia [ADH]) for basal-like in situ carcinoma have yet to

be identified. It is thought that the evolution of most invasive breast cancers follows a stepwise sequence of ductal hyperplasia (DH) to ADH to DCIS to invasive carcinoma. The known "early" breast lesions, DH and ADH, are ER+. If breast tumor subtypes represent distinct biologic entities, the subtypes may have alternative mechanisms of carcinogenesis, and it is unknown if basal-like DCIS lesions arise from an ER+ DH or ADH. It is unknown whether alternative progression schemes exist for invasive basal-like breast carcinoma, such as de novo progression from ER− stem cells. Future studies examining the cellular hierarchy of normal breast epithelium and its relationship to in situ and invasive carcinomas are needed to explore this possibility.

The identification of a basal-like subtype of DCIS may also have therapeutic implications. Given that DCIS now accounts for approximately 20% of breast cancers diagnosed by mammography, detailed classification of DCIS lesions to guide therapy is increasingly important [27,28]. Pathologic features, such as large-size and high-grade histology, have been shown to provide prognostic information [29]. Molecular characterization of DCIS lesions also provides treatment information for patients. Patients with ER− DCIS are not candidates for tamoxifen treatment. However, the frequent strong expression of EGFR in the basal-like DCIS lesions may provide other treatment options. Preclinical studies have suggested that EGFR is central in driving proliferation in ER−/EGFR+ carcinomas [30,31]. Clinical trials will eventually determine whether EGFR-inhibitors have a role in the prevention and treatment of ER− breast carcinomas.

Acknowledgments

The authors thank Elaine McSherry for her critical review.

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