

Inference of the Basal Epithelial Phenotype in Breast Carcinoma from Differential Marker Expression, Using Tissue Microarrays in Triple Negative Breast Cancer and Women Younger than 35

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■ **Abstract:** Basal-cell phenotype breast carcinoma has been associated with high-grade and metaplastic morphology, expression of basal-type cytokeratins, uniform negativity for ER and HER2, and decreased overall survival. Breast cancers occurring in young women are usually T2 disease at presentation, high-grade and of poor prognosis. We compared two groups of breast cancers, (a) ER-, PR-, HER2- (triple negative) [TNBrCa] and (b) non-triple negative breast cancers (non-TNBrCa) occurring in women under 35, using tissue microarray technology to characterize expression of the basal/myoepithelial cytokeratins (CK5/6, CK7, and CK14), luminal cytokeratins (CK8, CK18, and CK19), EGFR, p-cadherin, c-kit, p63, and p53. We also sought to identify characteristic histomorphologic features indicative of basal-like phenotype. The triple negative group showed preferential staining versus the age <35 group for CK5/6 (22% versus 4% $p = 0.05$), CK14 (44% versus 15%, $p = 0.013$), EGFR (83% versus 24%, $p < 0.0001$) and c-kit (19% versus 0% $p = 0.026$). Conversely, non-TNBrCa in women younger than 35 demonstrated increased expression of the luminal CK8 (92% versus 60%) compared with the triple negative patients ($p = 0.006$). The TNBrCa have characteristic histologic features including higher tumor grade, pushing tumor border, geographic necrosis, syncytial growth pattern, brisk mitotic activity, lack of/minimal in situ component, medullary-like and metaplastic differentiation. Invasive carcinomas in women younger than 35 usually have an associated in situ component, prominent nucleoli, central acellular fibrotic zone, and infiltrative tumor border. Triple negativity for ER/PR/HER2 coupled with EGFR, c-kit, and basal/myoepithelial cytokeratins (CK5/6, CK14) expression, and distinctive histomorphologic features predict morphology consistent with basal-cell phenotype. ■

Key Words: basal-like phenotype, breast cancer, basal cytokeratins, triple negative, younger women

BACKGROUND

Gene expression (cDNA) microarray data has facilitated the division of breast cancers into four main subtypes at the transcriptional level: luminal A, luminal B, normal breast-like, and basal-like (1–6). The basal-cell phenotype has been correlated with a poor prognosis (7), distinctive histopathologic features, lack of estrogen receptor (ER), progesterone receptor (PR), HER2 expression (8–13), and BRCA1 mutations

(14–19). Thus, any breast cancer occurring in a young patient is potentially of a basal-cell phenotype.

The basal-cell phenotype is associated with a distinct array of histopathologic features including high nuclear grade, geographic necrosis, pushing borders, brisk lymphocytic response, and metaplastic elements (8,12,13,20). Furthermore, immunohistochemical studies have associated expression of basal cytokeratins (CK5/6, CK7, and CK14) as well as other markers (p-cadherin, c-kit and p63) with the basal-cell phenotype (18,19,21–31). We attempted to identify markers characteristic of the basal-cell phenotype in cancers occurring in young patients (<35 years) and in those with triple-negative receptor status. In addition, we sought to identify characteristic histomorphologic features that identify triple-negative breast cancer (TNBrCa) from nontriple negative breast cancer (non-TNBrCa).

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MATERIALS AND METHODS

Tissue Specimens

Invasive breast carcinomas of women with TNBrCa [$n = 60$] of any age and non-TNBrCa [$n = 31$] in women younger than 35 were studied with approval from the Institutional Review Board of the Emory University School of Medicine. Clinicopathologic characteristics of the patients and tumors were obtained from the pathology reports and the patients' medical records.

Immunohistochemistry

Tissue microarray (TMA) were constructed for each population using two 1.0 mm cores from each cancer and from five normal breast tissues. Immunohistochemical stains used include: basal-cell associated cytokeratins (CK5/6, CK7, and CK14), luminal-associated cytokeratins (CK8, CK18, and CK19), and other immunomarkers (c-kit, epidermal growth factor receptor (EGFR), p-cadherin, p53, and p63).

Immunostains were performed using the antibodies and positive controls listed in Table 1. Negative controls were run simultaneously and had primary antibody replaced with buffer. HER2 and EGFR immunostains were performed using FDA-approved kits (HER2: HercepTest; EGFR: PharmDX) in accordance with manufacturer instructions regarding antigen retrieval and method of immunostaining. Antigen retrieval for all other antibodies, except CK8, was conducted in citrate buffer at pH 6.0 under a pressure of 15 pounds per square inch for 3 minutes. For

CK8, EDTA of pH 8.0 was used instead of citrate buffer. The EnVision + Dual Link Kit (Dako Corp., Carpinteria, CA) was used for all immunostaining, with diaminobenzidine as the chromogen, hematoxylin as the counterstain, and the Dako Autostainer.

Antibody staining was nuclear for ER, PR, p53, and p63, membranous for HER2, EGFR and P-cadherin, and cytoplasmic, with or without membranous staining, for CK5/6, CK7, CK14, CK8, CK18, CK19, and c-Kit.

Histomorphologic Analysis

The hematoxylin and eosin stained sections of all available tissue blocks on all cancer patients (non-TNBrCa and TNBrCa) were reviewed by two pathologists (AN and CC) for the following histologic features: tumor histologic grade (32), mitotic activity per 10 high power fields, in situ component, nuclear features, geographic necrosis, tumor border, tumor stromal lymphocytic infiltrate, apocrine differentiation, metaplastic features, atypical medullary features, central acellular fibrotic zone, syncytial growth pattern and lymphovascular invasion.

Statistical Analysis

Fisher's exact test was used to compare antigen frequencies between TNBrCa and women younger than 35. A Student's t-test was used to compare means between the two groups. For both tests, results were considered statistically significant when the p-value was <0.05 .

Table 1. Antibodies Used for the Immunohistochemical Analysis

Antibody	Clone	Dilution	Source	Positive control	Cutoff values
ER	1D5	1:50	DakoCytomation*	Breast carcinoma	$\geq 10\%$
PR	PgR 636	1:400	DakoCytomation*	Endometrial carcinoma	$\geq 10\%$
HER2	FDA-approved kit	prediluted	DakoCytomation*	Breast carcinoma	$\geq 30\%$
CK5/6	D5/16 B4	1:80	DakoCytomation*	Mesothelioma	$\geq 5\%$
CK7	0B-TL 12/30	1:40	DakoCytomation*	Nonsmall cell carcinoma of lung	$\geq 5\%$
CK14	LL002	1:40	Novocastra [†]	Placenta	$\geq 5\%$
CK8	35BH11	1:40	DakoCytomation*	Pancreatic carcinoma	$\geq 5\%$
CK18	DC10	1:10	DakoCytomation*	Carcinoid tumor	$\geq 5\%$
CK19	BA17	1:50	DakoCytomation*	Colon carcinoma	$\geq 5\%$
c-Kit (CD117)	Polyclonal	1:800	DakoCytomation*	Gastrointestinal stromal tumor	$\geq 1\%$
EGFR	FDA-approved kit	prediluted	DakoCytomation*	Colon carcinoma	$\geq 1\%$
P-cadherin	56C1	1:40	Novocastra [†]	Placenta	$\geq 10\%$
p53	D07	1:80	DakoCytomation*	Colon carcinoma	$\geq 10\%$
p63	4A4	1:160	Neomarkers [‡]	Squamous cell carcinoma of skin	$\geq 1\%$

ER, Estrogen receptor; PR, Progesterone receptor; EGFR, Epidermal growth factor receptor.

*DakoCytomation, Carpinteria, CA.

[†]Novocastra, Newcastle-Upon-Tyne, United Kingdom.

[‡]Neomarkers, Fremont, CA.

Table 2. Clinicopathologic Parameters and Immunohistochemical Differential Expression between Triple Negative Breast Cancer and Cancer in Women Younger than 35

	Triple negative	Age under 35	Fisher p-value
Mean age	53.2 (25–88)	29.4 (23–35)	
>1 + axillary LN	16/41 (39%)	12/17 (71%)	0.043
ER	0/56 (0%)	16/29 (55%)	<0.0001
PR	0/56 (0%)	14/29 (48%)	<0.0001
HER2	0/56 (0%)	18/29 (62%)	<0.0001
CK5/6	12/54 (22.2%)	1/26 (3.8%)	0.051
CK7	49/53 (92.5%)	26/27 (96.3%)	0.658
CK14	24/54 (44%)	4/26 (15%)	0.013
CK8	29/48 (60%)	24/26 (92%)	0.006
CK18	49/49 (100%)	26/27 (96.3%)	1
CK19	42/52 (80.8%)	23/25 (92%)	0.317
c-kit	10/51 (19%)	0/26 (0%)	0.026
EGFR	46/54 (83%)	6/25 (24%)	<0.0001
p-cadherin	52/52 (100%)	26/26 (100%)	1
p53	39/52 (75%)	21/27 (77.8%)	1
p63	10/54 (18.5%)	1/26 (3.8%)	0.093

ER, estrogen receptor; PR, progesterone receptor; CK, cytokeratin, EGFR, epidermal growth factor receptor.

RESULTS

Immunohistochemical Analysis (Table 2)

The triple negative group showed preferential staining versus the age <35 group for CK5/6 (22% versus 4%, $p = 0.051$; Fig. 1a), CK14 (44% versus 15%, $p = 0.013$; Fig. 1b), c-kit (19% versus 0%, $p = 0.026$) and EGFR (83% versus 24%, $p < 0.0001$; Fig. 1c).

Nontriple negative carcinomas in women younger than 35 demonstrated increased expression of the luminal CK8, (92% versus 60%, $p = 0.006$; Fig. 1d) than the triple negative patients. Results were not statistically significant ($p > 0.05$) for differences in staining for other basal/myoepithelial markers (CK7, p63, and p-cadherin; Fig. 1e–g), the luminal cytokeratins (CK18, CK19), and p53 (Fig. 1h,i). Compared with nontriple negative cancers in women under 35, the triple negative cancers showed a lower rate of regional lymph node metastases ($p = 0.043$). Furthermore,

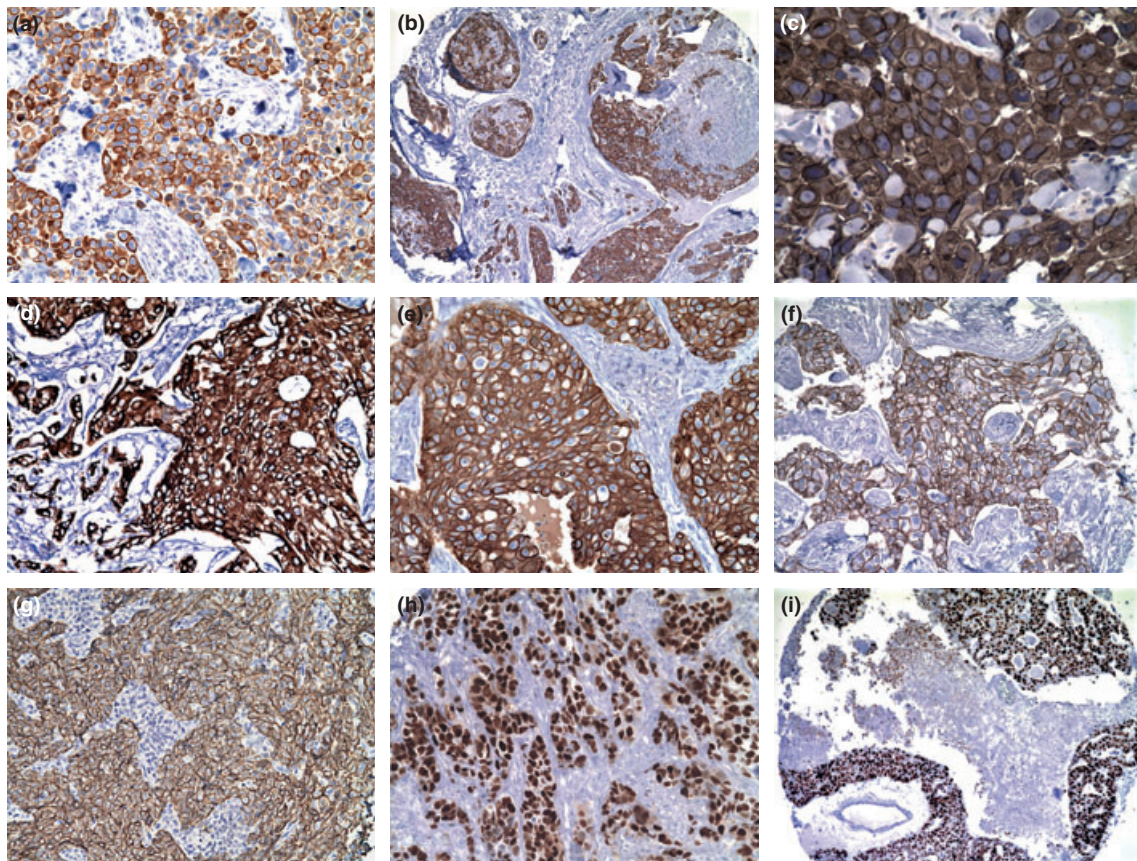


Figure 1. (a) CK5/6 immunostain in a TNBrCa (20 $\times$). (b) CK 14 immunostain in a TNBrCa (10 $\times$). (c) EGFR immunostain in a TNBrCa (40 $\times$). (d) CK8 immunostain in a non-TNBrCa (40 $\times$). (e) CK 7 immunostain in a TNBrCa (10 $\times$). (f) p-cadherin immunostain in a TNBrCa (10 $\times$). (g) p-cadherin immunostain in a non-TNBrCa (40 $\times$). (h) p53 immunostain in a TNBrCa (20 $\times$). (i) p-53 immunostain in a non-TNBrCa (20 $\times$).

approximately half of non-TNBrCa in young women expressed ER, PR, and HER2.

Histomorphologic Features (Table 3)

Most tumors in the triple negative group showed high nuclear grade. Compared to non-TNBrCa in younger women, TNBrCa are usually of higher grade (grade 3), with brisk mitotic activity (>25/10 HPF; Fig. 2a), usually not associated with an in situ

component, have inconspicuous nucleoli, with associated geographic tumor necrosis (Fig. 2b), and demonstrate tumor pushing borders, and syncytial growth pattern. In addition, they sometimes exhibit characteristic histomorphologic features such as metaplastic (Fig. 2c) and atypical medullary-like features (Fig. 2d). On the other hand, non-TNBrCa in young women is usually associated with an in situ component, prominent nucleoli, central acellular fibrotic zone (Fig. 2e), and infiltrative tumor borders.

Table 3. Histologic Features between Triple Negative Breast Cancer and Women Younger than 35

Histologic feature	Triple negative breast cancer (%)	Breast cancer in women (<35 years; %)	Fisher's p-value
Tumor grade 3	60/60 (100)	16/31 (51.6)	<0.0001
Tumor grade 2	0/60	15/31 (48.4)	<0.0001
Mitosis (>25/10 hpf)			
Yes	30/60 (50)	7/31 (22.6)	0.014
No	30/60 (50)	24/31 (77.4)	
In situ component			
Yes	17/60 (28.3)	21/31 (67.7)	<0.0001
No	43/60 (71.7)	10/31 (32.3)	
Prominent nucleoli			
Yes	5/60 (8.3)	25/31 (80.6)	<0.0001
No	55/60 (91.7)	6/31 (19.4)	
Geographic necrosis			
Yes	23/60 (38.3)	2/31 (6.5)	<0.0001
No	37/60 (61.7)	29/31 (93.5)	
Vesicular Chromatin			
Yes	49/60 (81.7)	22/31 (71)	0.289
No	11/60 (18.3)	9/31 (29)	
Coarse chromatin			
Yes	11/60 (18.3)	9/31 (29)	0.289
No	49/60 (81.7)	22/31 (71)	
Pushing tumor border			
Yes	25/60 (41.7)	5/31 (16.1)	0.018
No	35/60 (58.3)	26/31 (83.9)	
Infiltrative tumor border			
Yes	36/60 (60)	25/31 (80.6)	0.061
No	24/60 (40)	6/31 (19.4)	
Tumor stromal lymphocytic infiltrate			
Yes	25/60 (41.7)	9/31 (29)	0.262
No	35/60 (58.3)	22/31 (71)	
Apocrine differentiation			
Yes	3/60 (5)	1/31 (3.2)	1.000
No	57/60 (95)	30/31 (96.8)	
Metaplastic features			
Yes	14/60 (23.3)	0/31 (0)	0.009
No	46/60 (76.7)	31/31 (100)	
Atypical medullary features			
Yes	15/60 (25)	3/31 (9.7)	0.082
No	45/60 (75)	28/31 (90.3)	
Central acellular fibrotic zone			
Yes	2/31 (6.5)	14/60 (23.3)	0.045
No	29/31 (93.5)	46/60 (76.7)	
Syncytial growth pattern			
Yes	36/60 (60)	9/31 (29)	0.005
No	24/60 (40)	22/31 (71)	
Lymphovascular invasion			
Yes	26/60 (43.3)	9/31 (29)	0.183
No	34/60 (56.7)	22/31 (71)	

DISCUSSION

As cDNA TMA technology is not practical to perform as a routine clinical test, surrogate markers to detect the basal-cell phenotype could serve as a practical means to provide useful clinical information. The basal-like phenotype defined as basal cytokeratin expression, absence of ER and lack of HER2 expression is observed by Nielson et al. (33) in about 15% of tumors. Breast cancers with a basal epithelial phenotype are more likely than nonbasal breast cancers to present between regular annual mammograms (interval breast cancers) (34). TNBrCa are increased in African American women regardless of age or body mass index (35). They show lower rates of metastasis to the bone, liver and axillary lymph nodes, but a higher rate of brain metastasis (6). Relative to other grade III tumors, basal-like tumors appear to have a relatively good long-term survival, but survival following metastases is poor (7).

Our data suggest that within the appropriate clinical and histopathologic context, the basal-cell phenotype of breast carcinoma can be predicted using a constellation of triple negative receptor status, positivity for the basal cytokeratins (CK14, CK5/6), c-Kit and EGFR expression. Our findings are similar to those of other investigators (18,20,22,26,27,31,36).

Basal marker expression is significantly related to poor prognosis, ER negativity, and younger patient age (31). Multivariate analysis has shown that CK5/6 expression is an independent indicator of reduced relapse free interval (25,31). All basal markers' expression show significant positive correlation with histologic grade, tumor size, Nottingham prognostic index (NPI), local and regional recurrence, distant metastasis, and patient death (31). On the other hand, expression of luminal markers is associated with good prognostic tumor characteristics and outcome (31). P-cadherin, a marker for basal-like breast cancers is

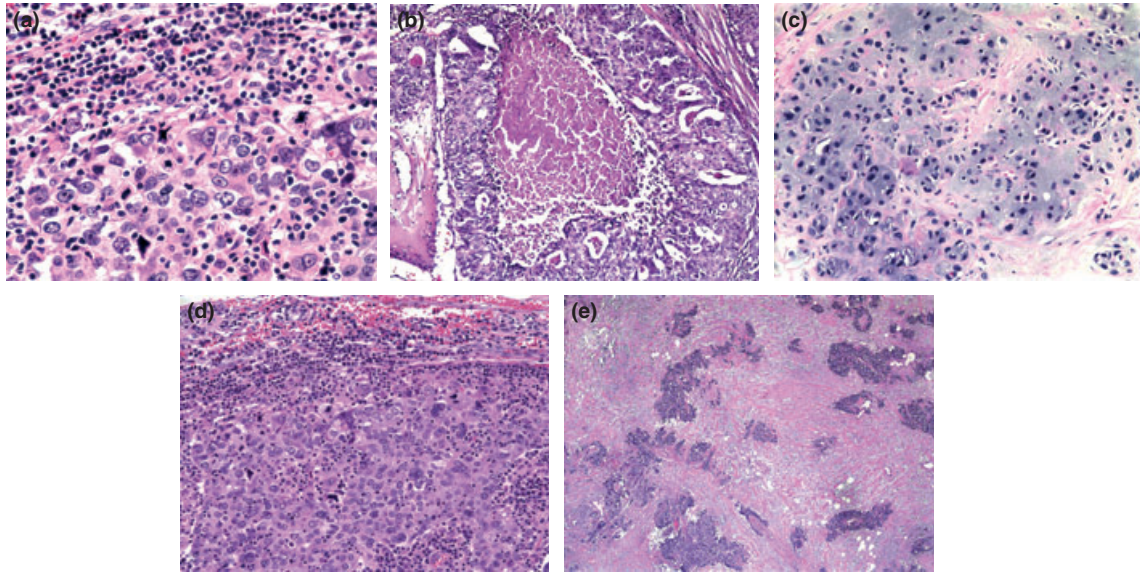


Figure 2. (a) IDC in a TNBrCa with high grade nuclei and brisk mitosis (H&E – 60×). (b) Invasive ductal carcinoma (IDC) of a TNBrCa with geographic tumor necrosis (H&E – 40×). (c) IDC with metaplastic features in a TNBrCa (H&E – 20×). (d) IDC in a TNBrCa with pushing border, and brisk lymphoplasmacytic infiltrate (medullary-like features) (H&E – 40×). (e) IDC with acellular fibrotic zone in a non-triple negative women <35 (20×).

strongly associated with the presence of a BRCA1 mutation, and is an adverse prognostic factor particularly in small, node-negative breast cancers (29). Furthermore, p-cadherin expression is associated with more aggressive tumors and with shorter patient survival (28).

Although in the current study, TNBrCa are associated with a lesser degree of in situ carcinoma (DCIS), other groups are proposing that DCIS with triple-negative status, basal-keratin and/or EGFR expression might represent a true precursor to triple-negative invasive breast cancer (37,38). Other studies have shown that some (~60–67%) of the tumors with BRCA-1 mutation represent basal-phenotype breast cancer (2,6,10,14–19,30,39,40).

All of the non-TNBrCa studied in women under 35 years show expression of HER2 and half express ER and/or PR. As a group, these cancers show greater expression of the luminal cytokeratin CK8 and less frequent EGFR expression versus the triple negative group. Basal cytokeratins and vimentin are known to be expressed in breast cancers of young patients, and both are associated with more aggressive tumor features and poor disease prognosis (27).

Women younger than 35 with breast cancer, usually have invasive disease, with T2 disease at presentation, and of poor histologic grade (grade III), and a greater proportion have node positive disease (61%)

(41). Their breast cancers are usually associated with DCIS, and, as in this study, about half of the cases express ER and PR, with a significant number exhibiting HER2 amplification (29.6%) (41). Progesterone receptor negativity and high Ki-67 labeling index are associated with distant metastasis in younger patients (42). Furthermore, PR, Ki-67, tumor stage, and nodal status are prognostic factors for distant failure in early-stage breast cancer in young patients (42).

Similar to other investigators (8,12,13,20), we have shown that TNBrCa have characteristic histomorphologic features such as higher histologic grade (grade 3), brisk mitotic activity (>25/10 HPF), absent to minimal in situ component, inconspicuous nucleoli, geographic tumor necrosis, pushing tumor borders, syncytial growth pattern, metaplastic and atypical medullary like features. There is compelling evidence to suggest that basal-like breast carcinomas are heterogeneous and encompass tumors with distinct histologic features, namely invasive ductal carcinomas, medullary carcinomas, metaplastic carcinomas, and tumors with overt myoepithelial differentiation (3,9–11,20,43,44).

In conclusion, the basal-cell phenotype breast cancer can be inferred from a constellation of immunohistochemical markers (basal cytokeratins, EGFR, c-kit) and histomorphologic features (high nuclear grade, syncytial growth pattern, pushing tumor borders, geographic tumor necrosis, metaplastic differentiation).

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