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Original Article

Prevalence, Morphologic Features and Proliferation Indices of Breast Carcinoma Molecular Classes Using Immunohistochemical Surrogate Markers

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Abstract: There is dearth of studies that provide a practical working formulation of breast cancer gene expression analysis for the surgical pathologist. ER, PR, HER2 were used as surrogate markers to classify 205 breast carcinomas into molecular classes. Ki-67 labeling index was calculated using an image analysis system. The data was analyzed for molecular class prevalence, and inter-relationships amongst morphologic parameters, Ki-67 index, and molecular classes. Of the 205 tumors, 113 (55%) were classified as luminal A (strong ER+, HER2 negative), 34 (17%) as luminal B (weak to moderate ER+, HER2 negative), 32 (15%) as triple negative (negative for ER/PR and HER2), 8 (4%) as ERBB2 (negative for ER/PR but HER2+), 10 (5%) as luminal A-HER2 hybrid (strong ER+ and HER2+), and 8 (4%) as luminal B-HER2 hybrid (weak to moderate ER+ and HER2+). The average Ki-67 index was lowest in luminal A (15.8%), intermediate for ERBB2 (27.8%) and highest for triple negative tumors (>50%). Multivariate logistic regression analyses found the following associations: ERBB2 tumors with apocrine differentiation (p=0.0031); Triple negative tumors with high Ki-67 index (p<0.0001) and CK5 positivity (p<0.0001); HER2 negative-low receptor positive tumors (luminal B) with increased lymph node involvement (p=0.0141). The immunohistologic criteria were validated on a different set of 359 cases treated with neoadjuvant chemotherapy, which showed a pathologic complete response predominantly in ERBB2 and triple negative tumors. Immunohistochemistry is a reliable surrogate tool to classify breast carcinoma according to the gene expression profile classification.
Key Words: Molecular classes, breast carcinoma, immunohistochemical surrogate markers, Ki-67

Introduction

Gene expression analysis has demonstrated distinct classes of breast carcinomas based on the degree of expression of a select number of genes [1-3]. Some of the 'key' genes that have been studied in the context of expression analysis have been examined and routinely reported by diagnostic immunohistochemistry, yet there have been very few studies correlating gene expression analysis with protein expression immunohistochemistry and tumor morphology [4-6]. There is dearth of studies that attempt to generate a practical working formulation of breast cancer gene expression analysis for the surgical pathologist to use as a reporting apparatus. Gene expression analysis can be translated into diagnostically useful information for the surgical pathologist and oncologist using "genomic and theranostic immunohistochemistry" for protein expression analysis.

The aim of this study is to translate gene expression profiling results that have been published, into a reproducible, user-friendly, integrated method of protein expression analysis by immunohistochemistry (IHC) and tumor morphology. To meet our goals, first, we reviewed a relatively large number of breast carcinomas and classified them according to the molecular classes using immunohistochemical surrogate markers and identified morphologic parameters in each class. Secondly, we examined if there were

specific proliferation indices that identified specific molecular classes. We chose to study this parameter because difference in proliferation activity among the molecular classes was a key feature in the seminal gene expression profiling study [1]. Moreover, there are widely variable reports in the literature regarding the prognostic significance of Ki-67 index [7-18]. Last, but not the least, we tested the validity of our IHC based molecular classification criteria used in this study on a large data-set of patients treated with neo-adjuvant chemotherapy (NACT).

Materials and Methods

Tissue and Patient Data

The study was approved by the Institutional Review Board. Two hundred and five consecutive invasive breast carcinomas (study cases) diagnosed in a 6 month period (January to June 2005) with available resection specimens were retrieved from the files of our Hospital. Patients treated with neo-adjuvant chemotherapy (NACT) were excluded from this study group. All tumor slides from these cases were reviewed. Several morphologic characteristics were noted (detailed later). Estrogen receptor (ER), progesterone receptor (PR) and HER2 status was determined using IHC and/or fluorescence in situ hybridization (FISH) on initial diagnostic material. The results were available from the original pathology reports. At our institution, ER and PR results are reported using a semi-quantitative score (previously described as "H-score") which details the percentage of positive cells showing none, weak, moderate, or strong staining [19]. The score is given as the sum of the percent staining multiplied by an ordinal value corresponding to the intensity level (0 = none, 1 = weak, 2 = moderate, 3 = strong). With four intensity levels, the resulting score ranges from 0 (no staining in the tumor) to 300 (diffuse intense staining of the tumor). HER-2/neu protein was analyzed and scored using CB11 antibody and basic DAB detection on Benchmark® XT (Ventana, Tucson, AZ). HER-2/neu slides were reviewed and HER2 was considered positive with either 3+ immunoreactivity (diffuse strong reactivity in >30% of the tumor cells) or amplification by FISH (with a ratio of HER2 to chromosome 17 centromeric region > 2.2, using PathVysion Vysis dual color FISH by Vysis Inc., Downers Grove, IL). All 2+ cases by immunohistochemistry were followed by FISH. This is in accordance with the recently published College of American Pathologists/American Society for Clinical Oncology guidelines for HER2 testing [20]. Equivocal FISH result (ratio of 1.8-2.2) was considered as negative for HER2 in this study.

Tissue Microarray

Invasive tumors were marked on one slide to be chosen for the construction of tissue microarray (TMA). On 7 cases, appropriate tissue block was not available for the construction of TMA. Therefore, 198 cases were represented on 4 TMAs. Each case was represented with 3 different 0.6 mm cores (3 fold redundancy).

Immunohistochemistry

IHC staining for the proliferation marker Ki-67 was performed on 5 micron TMA sections using an anti-Ki-67 antibody (clone K-2, Ventana Medical Systems Inc. Tucson, AZ). The protocol consisted of a pretreatment with CC1, pH 8.0 (Ventana) followed by incubation with anti-Ki-67 mouse monoclonal antibody. The antigen-antibody complexes were detected using an iVIEW™ DAB detection kit (Ventana). The Ki-67 labeling index (LI) was calculated using Ventana Image Analysis System (VIAS; Ventana Medical Systems, Tucson, AZ). Either the entire tumor on all 3 cores was scanned (for tumors with low cellularity and/or heterogeneous staining) or at least 6 high power fields (for cellular and homogeneously staining tumors) were scanned and the average Ki-67 proliferation index was calculated. The VIAS is semi-automatic, but also allows manual selection of neoplastic cells resulting in very accurate counts. The TMA sections were also subjected to CK5 (a superior marker of basal phenotype) [26] staining using anti-CK5 antibody (clone XM26; dilution 1:25; Novocastra-Vision Biosystems, Norwell, MA). Similar protocol was used as described above. Scoring was again performed using H-score like methodology described above. A score of 10 or less was considered as negative, and 11 or higher as positive. Whole tissue sections of cases belonging to ERBB2 tumor group were also stained with CK5/6 (clone D5/16B4; Ventana) and EGFR (clone 3C6; Ventana).

Tumor Classification

Table 1 Criteria used for tumor classification

Category	Criteria used in this study	Corresponding categories in Cheang *et al* [21]
Luminal A (LUMA)	ER score 200 or higher, HER2 negative	Luminal
Luminal B (LUMB)	ER score 11-199 or PR score >10, HER2 negative	Luminal
Triple Negative (TN)	ER and PR score 10 or less, HER2 negative	TNP; Core Basal if + for CK5/6 or EGFR; 5NP if negative for CK5/6 and EGFR
ERBB2	ER and PR score 10 or less, HER2 positive	HER2+/ER-/PR-
Luminal A-HER2 Hybrid (LAHH)	ER score 200 or higher, HER2 positive	Luminal/HER2+
Luminal B- HER2 Hybrid (LBHH)	ER score 11-199 or PR score >10, HER2 positive	Luminal/HER2+

TNP, triple negative phenotype; 5NP, five negative phenotype

Using IHC as a surrogate for expression profiling, the tumors were classified as follows: Luminal A (LUMA), Luminal B (LUMB), Triple Negative (TN), ERBB2, Luminal A-HER2 Hybrid (LAHH) and Luminal B-HER2 Hybrid (LBHH). A criterion for each category is summarized in **Table 1**. Our criteria correspond to the initial gene expression profiling studies [1-3], and also similar (but not identical) to the prior studies that used IHC as a surrogate for molecular classification [4, 6]. Our criteria primarily correspond to those used by Cheang *et al* [21], but we have sub-divided the luminal category into LUMA, LUMB, LAHH and LBHH based on ER expression level and HER2 positivity. In our classification, we have made an assumption that mRNA expression level as determined by expression profiling directly correlates to semi-quantitative protein expression level as determined by IHC. We support our hypothesis based on the results from prior studies that have correlated mRNA expression with protein expression by IHC [22-25]. Although it is difficult to define a cut-off, any ER+/HER2 negative tumor showing diffuse strong ER expression in 2/3rd of the tumor (an IHC score of 200 or higher) was considered as a LUMA tumor and the remainder of ER+/HER2 negative tumors were considered as LUMB. Although somewhat arbitrary, this simple cut-off keeps the category of LUMA tumors as pure as possible using IHC. The ER+/HER2+ tumors were similarly subdivided into luminal A-HER2 hybrid (LAHH) and luminal B-HER2 hybrid (LBHH) based on ER expression levels.

Criteria for Various Morphologic Parameters

The morphologic features included in this study are abbreviated from Fulford *et al*; however, the criteria have been slightly modified to be more objective [27]. All cases were reviewed by two observers using a double-headed microscope. All tumors were graded according to the Nottingham grading system [28]. The presence of geographic necrosis (large irregular areas of necrosis only) was noted as "yes" or "no". The tumor borders were classified as pushing if the tumor was well circumscribed, and infiltrative if there was any irregularity/infiltration into the surrounding parenchyma or fat. Lymphocytic infiltrate was categorized as none, mild (involving less than 25% of the tumor), moderate (25 to 50% of the tumor) and marked (>50% of the tumor). Nucleoli were classified as prominent if they were easily visible at low power. A tumor was classified as "pure apocrine carcinoma" if it had all the cytologic features of apocrine differentiation in ~100% of the tumor cells. If some, but not all the classic features were present, the tumor was considered to have "apocrine differentiation", but was not classified as apocrine carcinoma.

Validation of Immunohistologic Criteria

In order to examine the validity of the IHC surrogate markers for molecular classification in a clinical setting, HER2 status and hormone receptor metrics were examined for 359 tumors (validation set-completely different from the study group of 205 cases described above) treated with standard NACT at our institution in the last 8 years. The list of these cases was obtained from the hospital tumor registry as part of a separate IRB approved project. Similar criteria as described in **Table 1** were used to categorize these tumors in different classes. ER/PR/HER2 data was retrieved from the pathology reports. The

pathology reports were also examined for pathologic complete response (pCR), i.e. absence of invasive carcinoma in the post-therapy breast resection specimen along with absence of metastasis in the regional lymph nodes. Average percentage tumor size reduction (based on pre and post therapy tumor size) was also calculated.

Statistical Analysis

Table 2 Morpho-immunohistologic findings in breast cancer molecular classes

	LUMA (n=113)	LUMB (n=31)§	TN (n=32)	ERBB2 (n=8)	LAHH (n=10)	LBHH (n=8)
Nottingham Grade:						
I	41 (37%)	10 (32%)	0 (0%)	0 (0%)	1 (10%)	1 (12%)
II	58 (52%)	17 (55%)	6 (19%)	3 (38%)	6 (60%)	4 (50%)
III	14 (11%)	4 (13%)	26 (81%)	5 (62%)	3 (30%)	3 (38%)
Necrosis:						
Absent	113(100%)	30 (97%)	24(75%)	5 (62%)	10(100%)	8(100%)
Present	0 (0%)	1 (3%)	8 (25%)	3 (38%)	0 (0%)	0 (0%)
Lymphoid						
Infiltrate:	66 (58%)	16 (52%)	4 (13%)	0 (0%)	4 (40%)	3 (38%)
None	39 (35%)	14 (45%)	10 (31%)	3 (38%)	6 (60%)	5 (62%)
Mild	6 (5%)	1 (3%)	15 (47%)	5 (62%)	0 (0%)	0 (0%)
Moderate	2 (2%)	0 (0%)	3 (9%)	0 (0%)	0 (0%)	0 (0%)
Marked						
Borders:						
Infiltrating	101 (89%)	27 (87%)	24 (75%)	8 (100%)	10(100%)	8 (100%)
Pushing	12 (11%)	4 (13%)	8 (25%)	0 (0%)	0 (0%)	0 (0%)
Apocrine						
Differentiation:	109 (96%)	29 (94%)	23 (72%)	1 (13%)	8 (80%)	6 (75%)
Absent	4 (4%)	2 (6%)	9 (28%)	7 (87%)	2 (20%)	2 (25%)
Present						
Nucleoli:						
Not prominent	109 (96%)	27 (87%)	23 (72%)	4 (50%)	8 (80%)	7 (87%)
Prominent	4 (4%)	4 (13%)	9 (28%)	4 (50%)	2 (20%)	1 (13%)
Nodal Status*:						
Negative	74 (70%)	12 (50%)	21 (78%)	5 (71%)	7 (70%)	2 (40%)
Positive	31 (30%)	12 (50%)	6 (22%)	2 (29%)	3 (30%)	3 (60%)
Tumor Size:						
Mean (cm)	1.56	1.62	2.14	1.6	1.5	2.3
Median (cm)	1.5	1.4	1.5	1.2	1.3	1.9
Range (cm)	0.6-4.5	0.5-3.5	0.8-7	0.6-2.5	0.8-3.2	1-6.5
Patient Age:						
Mean (yrs)	62	55	57	64	56	57
Median (yrs)	62	50	54	65	56	49
Range (yrs)	41-90	40-81	39-90	51-86	37-73	43-83
ER Score:			NA	NA		
Mean	261	172			242	156
Median	270	180			245	175
Range	200-300	90-195			200-290	60-190
PR Score:			NA	NA		
Mean	147	108			143	75
Median	150	101			150	53
Range	0-295	0-245			6-300	0-200
Ki-67 LI:						
Mean (%)	15.8	15.9	61.7	27.8	16.3	24.6
Median (%)	11	12	66	31	14	29
Range (%)	1-73	1-60	9-94	10-41	1-40	11-36
CK5*:						
Negative	107(100%)	25 (93%)	9 (29%)	3 (38%)	10(100%)	8 (100%)
Positive	0 (0%)	2 (7%)	22 (71%)	5 (62%)	0 (0%)	0 (0%)

NA, Not Applicable. *Data not available on all cases. §Three LUMB tumors showing sheet like growth pattern similar to "basal-like" carcinoma were excluded from the analysis.

Statistical analysis was performed using the SPSS software program (SPSS Inc. Chicago, IL). The comparison between mean values was performed using non-parametric 2-tailed t-test (Mann-Whitney test). The percentages were compared using Chi-square test. The differences between pCR among different molecular classes (validation set) were analyzed using ANOVA. Statistical significance was defined as a p-value less than 0.05. Multivariate analyses were performed for binary endpoints with logistic regression and for continuous endpoints with linear regression.

Results

Of the 205 tumors, 113 (55%) were classified as LUMA, 34 (17%) as LUMB, 32 (15%) as TN, 8 (4%) as ERBB2, 10 (5%) as LAHH, and 8 (4%) as LBHH. Mean Ki-67 LI was significantly different among LUMA, HER2 and TN molecular classes ($p<0.01$). Ki-67 LI correlated with the number of mitotic figures ($p<0.01$) irrespective of the molecular class of the tumor.

Multivariate linear regression analysis of Ki-67 LI found correlations with TN molecular class ($p<0.001$, mean 61.7±25.0% for TN versus 17.6±14.7% for others), necrosis ($p<0.0001$ mean 65.7±24.7% with versus 21.0±19.4% without) and extent of lymphoid infiltrate ($p<0.0001$, means: 14.8±13.8, 23.53±19.8, 49.9±28.2, and 61.8±24.6 for none, mild, moderate, and marked respectively).

Multivariate logistic regression analyses found the following associations: LUMB class with increased lymph node involvement (p=0.0141, 14/27 LUMB versus 45/154 others); ERBB2 class with apocrine differentiation (p=0.0031, 7/8 ERBB2 versus 19/197 others), lymphoid infiltrate (p=0.0118, 8/8 ERBB2 versus 104/197 others) and increased amount of ductal carcinoma in situ (p=0.0129, 5/8 tumors with >25% in situ carcinoma); TN class with both high Ki-67 index (p=0.011, mean 61.7±25.0% for TN versus 17.6±14.7% for others) and with CK5 positivity ($p<0.0001$).

Multivariate logistic regression for the presence versus absence of lymph node metastasis identified significant correlation only with increasing tumor diameter (p=0.0023, OR=1.99/cm) and LUMB class (p=0.0075, OR=4.69). To test the robustness of these findings, we repeated the analysis as a multivariate linear regression with extent of lymph node involvement scored 0, 1, 2, 3, or 4 for negative, pN1mi, pN1, pN2, or pN3, respectively as the end point. We found the same significant correlations for LUMB (p=0.009) and tumor diameter (p=0.001) with that analysis.

The Ki-67 LI, CK5 immunoreactivity, and comparative morphologic features for various molecular classes are shown in **Table 2**. Other pertinent morphologic details are provided below.

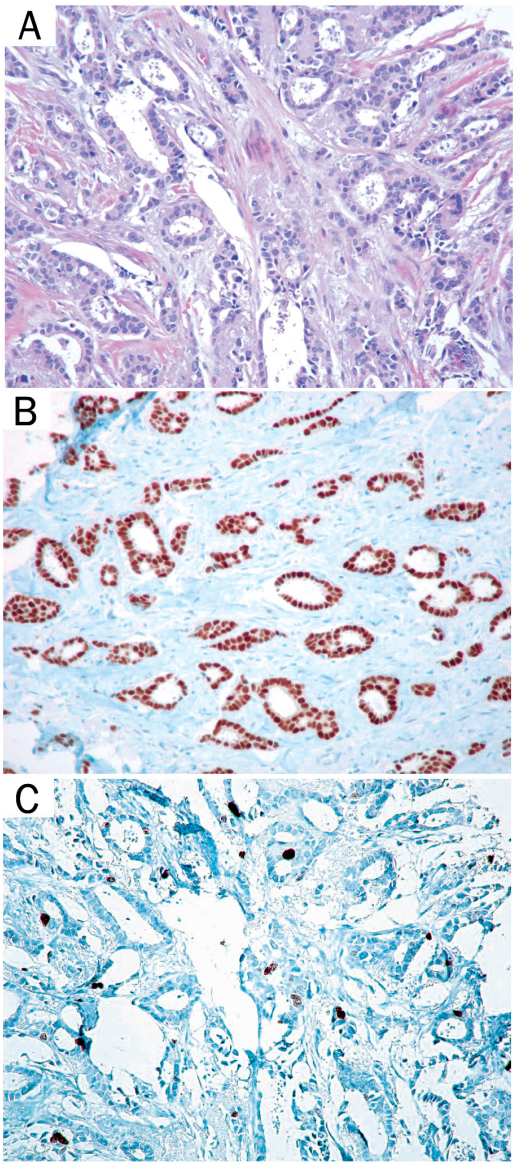

Figure 1 A prototype LUMA tumor (A-H&E; B-Anti-ER) with very low Ki-67 labeling index (C, Anti-Ki67).

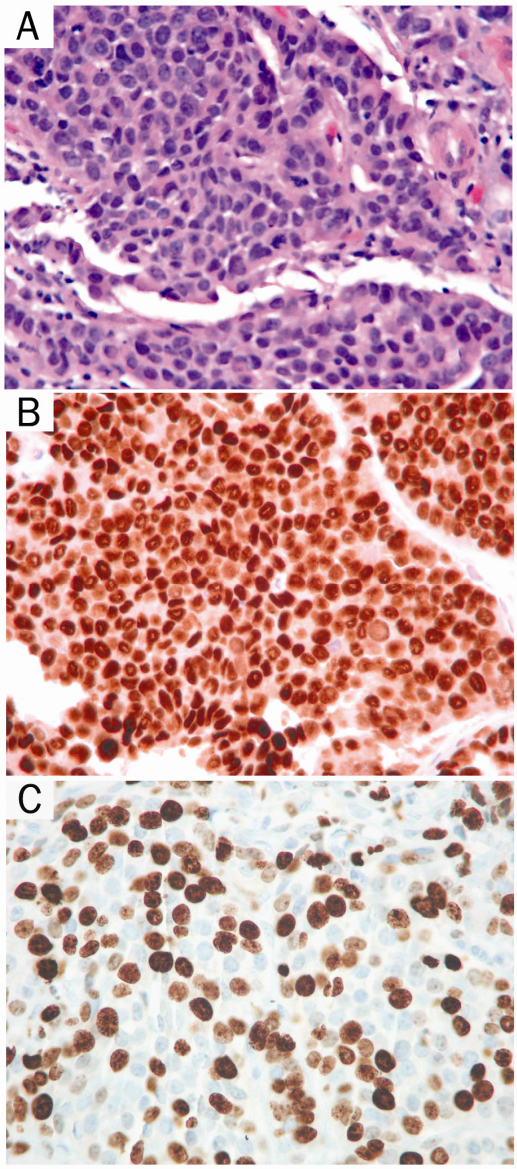

Figure 2 A higher grade LUMA type of invasive ductal carcinoma (A-H&E; B-Anti-ER) showing an increased Ki-67 labeling index (C, Anti-Ki67) that correlates to the mitotic activity of the tumor.

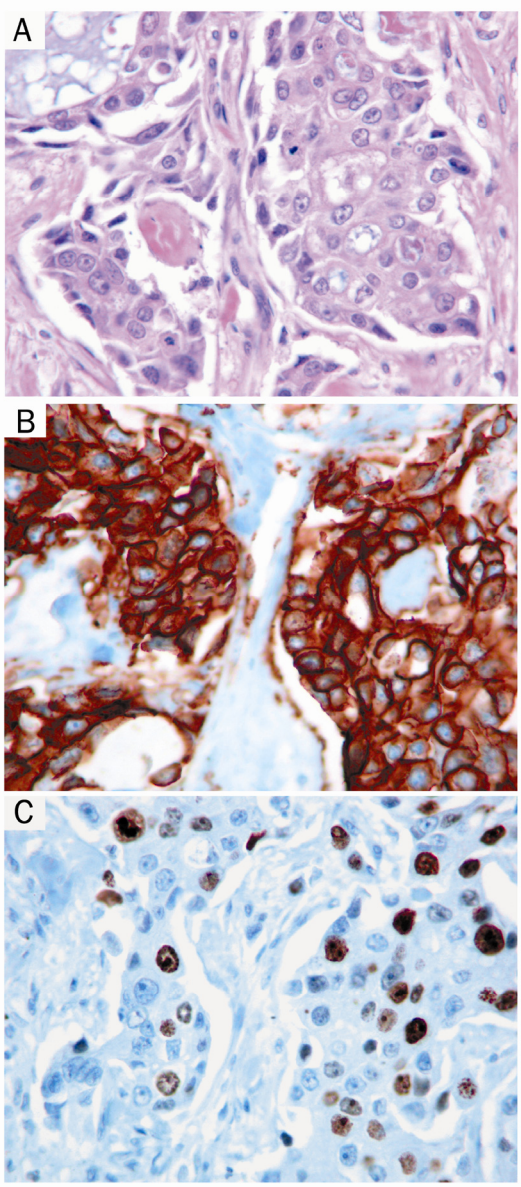

Figure 3 An ERBB2 tumor showing apocrine differentiation (A-H&E; B-Anti-HER2) and moderate increase in Ki-67 labeling index (C, Anti-Ki67).

LUMA Tumors: Of the 113 LUMA tumors, 101 (89%) were ductal, 10 (9%) lobular and 2 (2%) were mixed ductal and lobular types. The ductal tumors were composed of 85 no special type (NST), 3 pure mucinous, 3 pure tubular, 3 solid papillary, 3 mixed NST and mucinous, 1 pure cribriform, 1 NST and cribriform, 1 NST and papillary, 1 tubular and mucinous carcinoma. Among lobular cancers, both classic (6 cases) and pleomorphic (4 cases) tumors were identified in the LUMA category. Although, the average Ki-67 LI was the lowest among all the molecular classes (**Figure 1**), few cases showed a high labeling index which correlated with high mitotic activity in these cases (**Figure 2**).

LUMB Tumors: These tumors were of both ductal and lobular types, generally moderately differentiated, with some degree of PR expression and relatively low Ki-67 LI (**Table 2**). However, 3 of these 34 LUMB tumors showed morphology consistent with the "basal-like" tumors described above and previously in the literature. Two of these tumors were also positive for CK5. These tumors were classified

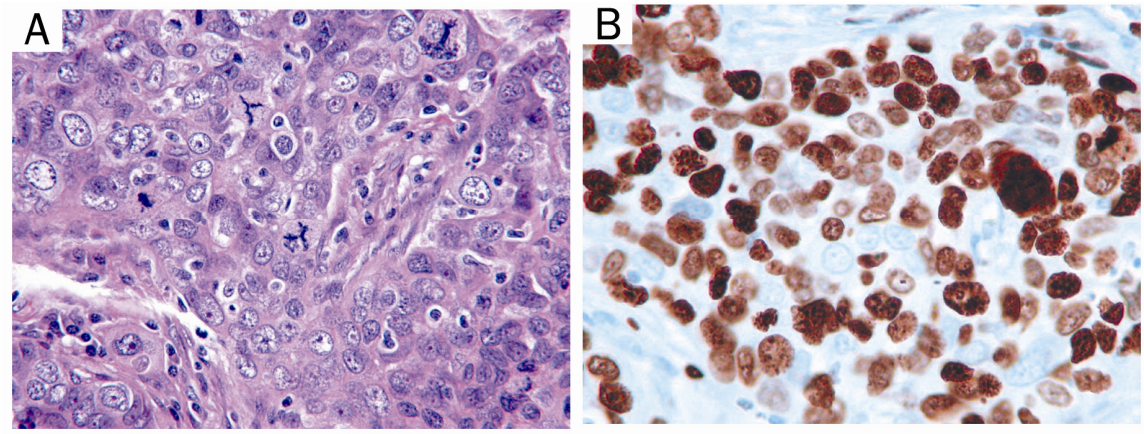

Figure 4 A TN tumor showing sheet like growth pattern (A, H&E) and a high Ki-67 labeling index (B, Anti-Ki67).

as LUMB because the ER IHC scores for these 3 tumors were 20, 45 and 60, respectively.

ERBB2 Tumors: These tumors in our series were mainly high-grade with the majority showing at least some degree of apocrine differentiation (**Figure 3**). CK5 immunoreactivity was seen in 5 of 8 cases (63%). All of these 5 positive cases showed apocrine differentiation and reactivity with EGFR antibody. Four of these 5 cases were also positive with CK5/6. Some feature classically ascribed to TN-basal like carcinomas such as necrosis and moderate intra-tumoral lymphoid infiltrate [5] were seen in 37% and 63% cases respectively. The average Ki-67 LI was 27.8%, which was intermediate between labeling index for LUMA and TN tumors.

TN Tumors: Using morphologic criteria, TN tumors could be classified into 3 groups-tumor with sheet-like growth pattern (n=15; 47%, **Figure 4**); ductal NST (n=13; 40%, **Figure 5**); and apocrine carcinomas (n=4; 3%). These included 2 tumors that showed spindle cell metaplastic features. The classic morphologic features of "basal-like" breast carcinoma described in the literature [5] were predominantly seen in tumors with sheet-like growth pattern (SLGP). However, the CK5 reactivity was seen in 73% (11/15) cases of tumors with SLGP, in 62% (8/13) cases of ductal NST and in 75% (3/4) of apocrine carcinomas. The average Ki-67 LI was highest (71.3%) in tumor with SLGP, slightly lower (58.5%) in ductal NST, and lowest (27%) in apocrine carcinomas.

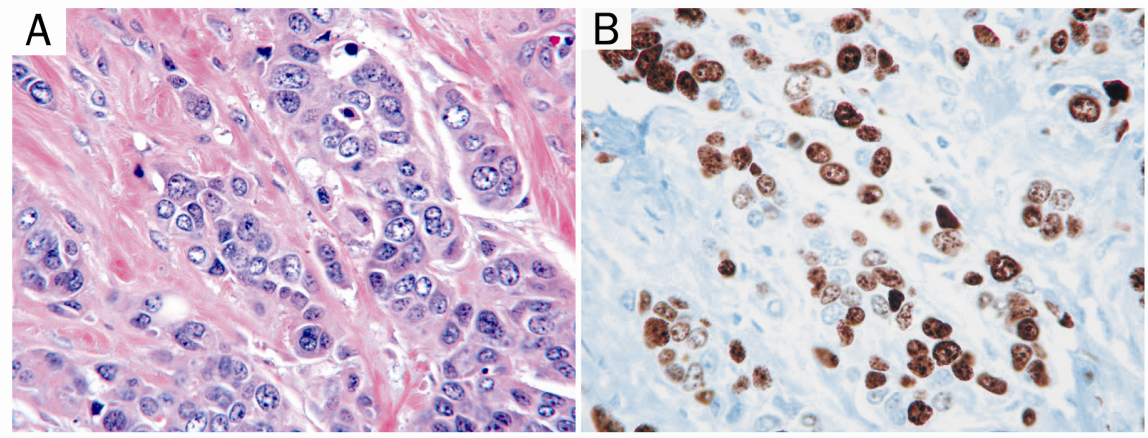

Figure 5 A TN tumor without sheet like growth pattern (A, H&E) and a high Ki-67 labeling index (B, Anti-Ki67).

Other HER2-positive Tumors: Morphologically, both LAHH and LBHH tumors were generally moderately differentiated and predominantly of the ductal, no special type. Average PR expression was higher in LAHH tumors compared to LBHH tumors. LAHH tumors showed an average Ki-67 LI of 16.3% compared to 24.6% LI for LBHH tumors.

Validation of Immunohistologic Criteria: Of the 359 tumors (validation set) treated with NACT, 110 (30.7%) were LUMA, 74 (20.4%) were LUMB, 79 (22%) were TN, 57 (16%) were ERBB2, 15 (4.2%) were LAHH and 24 (6.7%) were LBHH. Complete pathologic response was identified in 33% of ERBB2, 30.3% in TN, 8.3% of LBHH, 1.8% of LUMA, 1.4% of LUMB, and 0% of LAHH tumors ($p<0.0001$). Average percentage tumor size reduction was also highest in the TN (75%) and ERBB2 (68%) tumors and was statistically significant compared to other classes ($p<0.05$). The average percentage tumor size reduction in other tumors was as follows: 47% in LBHH, 33% in LAHH, 30% in LUMB, and 23% in LUMA. Further details regarding specific chemotherapy and outcome data is a subject of separate publication (manuscript in preparation).

Discussion

Unlike some previous studies that consider all ER+ tumors to be equal [4, 6, 21], we have used semi-quantitative hormone receptor IHC results, along with HER2 immunohistochemistry and FISH to classify tumors into 6 categories that correspond to molecular classes. This approach is necessary not only to be concordant with the molecular classification, but also to distinguish between weak ER+ tumors from strong ER+ tumors since these tumors respond differently to hormonal therapy [25, 29, 30]. According to the "intrinsic" gene set-based molecular classification [2], the tumors are classified as luminal A, ERBB2, Basal-like, Normal-breast like, and luminal B (which now also includes initially described luminal C). The initial gene expression study demonstrated all luminal B tumors to be HER2 negative [2]. However, subsequent study by the same investigative group eliminated the luminal C and included this category into luminal B [3]. Defined as such, some of the luminal B tumors became HER2 positive, but the vast majority of them were still negative, and even the initially described luminal C tumors were not all positive for HER2 [2]. In spite of these facts, ER+/HER2+ tumors were classified as luminal B in the Carolina Breast Cancer Study [4]. We have classified these tumors slightly differently based on semiquantitative immunohistochemistry (see **Table 1**). We believe this distinction is necessary before studies utilizing surrogate IHC markers are undertaken as HER2+ tumors need to be separated from pure luminal tumors which should be further categorized as luminal A and luminal B tumors. The tumors classified as LBHH tumors in our classification scheme are HER2 positive that show weak to moderate ER expression and at least some of these tumors correspond to the initially described luminal C molecular class. The tumors classified as LAHH represent HER2+ tumors that are also strongly positive for ER. These tumors are not uncommonly identified in routine practice, but do not seem to have a distinct molecular correlate. Currently, there are no known immunohistologic surrogate markers to definitively identify normal breast-like tumors. According to the expression profiling studies, normal breast-like tumors not only express genes characteristic of adipose tissue and other non-epithelial breast elements, but also strongly express basal epithelium genes [3]. Recently, it has been questioned whether these tumors represent poorly sampled tumor tissue or a distinct, clinically important group [31]. In the event these tumors really exist, we suspect that normal breast-like tumors are CK5+, but lack the characteristic morphology of "basal-like" tumors. In our series of cases, these tumors will predominantly fall in the TN group of tumors that lacks the sheet-like growth pattern. Considering these tumors as normal breast-like, morphologically they are moderate to poorly differentiated NST ductal carcinomas, with a relatively high Ki-67 LI. This correlation explains the relatively poor prognosis of normal breast-like tumors in gene expression profiling studies [2].

This classification enabled us to study several morphologic parameters and Ki-67 LI within each molecular class (**Table 2**). Numerous studies have been published regarding proliferation activity of breast carcinomas, many of which date back to the pre-expression profiling era. Investigators have used either flow cytometry to determine S-phase fraction or immunohistochemistry to study expression of proliferating cell nuclear antigen (PCNA) or

Ki-67 [10, 12]. There has been good correlation between different methodologies. Many studies analyzing Ki-67 LI have shown high LI to be a poor prognostic factor in breast cancer [14, 15]. However, different cut-off points have been used to define high proliferation index. In addition, different techniques have been used to determine the labeling index. Due to these factors, it is somewhat difficult to compare these studies and likely explain the reluctance to universally accept Ki-67 LI as a prognostic marker in breast cancer. The very first gene expression profiling study not only revealed "molecular portraits" but also identified genes responsible for the biologic differences between the tumor types [1, 32]. One of the largest distinct gene clusters identified by expression profiling was of the proliferation genes and included both PCNA and Ki-67. Since then, no study has primarily focused on the issue of Ki-67 LI and its correlation to all the molecular classes.

In general, Ki-67 LI correlated well with the number of mitotic figures irrespective of the molecular class of the tumor. TN tumors have the highest number of mitotic figures, and expectedly also showed the highest Ki-67 LI. This observation is in concordance with some recent studies that also identified a high Ki-67 LI in triple negative tumors [33, 34]. The ERBB2 tumors were a distant second followed by hormone receptor positive tumors (LBHH>LAHH>LUMB>LUMA). Within the hormone receptor positive tumors, not all tumors had low Ki-67 LI and showed a wide range. This difference in proliferation activity coupled with quantitative difference in ER expression has been exploited in the development of a commercial assay (OncotypeDx™) for predicting breast cancer prognosis and treatment [35]. It has been recently reported that a comparative morpho-immunohistologic analysis could be predictive of the recurrence score [23].

One surprising aspect of our study was the finding of only a moderate increase in Ki-67 LI in the ERBB2 group of tumors. HER2 positive tumors generally have lack of tubule formation, and show high-grade pleomorphic nuclei. On our review, we realized that a mitosis score of 3 is seen only in less than one-quarter of all HER2 positive tumors. Therefore, the Nottingham score is generally 7 or 8. This explains only moderate increase in Ki-67 LI. The interesting finding in the ERBB2 group, however, was the overwhelming presence of apocrine features in these tumors. It is also of interest to note that review of photomicrographs of the tumors used for expression profiling by Perou *et al* [1] also demonstrate apocrine features of the ERBB2 tumors (http://genome-www.stanford.edu/breast_cancer/molecularportraits/histology.shtml). At least 2 of the 5 ERBB2 tumors (Norway 53 and Norway 101) appears to be apocrine carcinoma, while the other 3 (Norway 57, Stanford 2, and Norway 47) shows some degree of apocrine differentiation.

As far as expression of basal markers is concerned, CK5 expression was predominantly seen in the TN group of tumors. Other tumors that showed CK5 expression belonged either to ERBB2 (5 cases) or the LUMB (4 cases) group. All five CK5+ ERBB2 tumors and one of four CK5+ LUMB tumors showed apocrine differentiation. Expression of CK5 in apocrine tumors is not a completely unexpected finding as cytokeratin polypeptide fragments 4-6 were shown to be expressed in "apocrine and sweat glands of the skin and mammary gland" by Moll *et al* in their seminal study [36]. Additionally, 2 of the 4 CK5+ LUMB tumors showed a "basal-like" morphology and very low ER IHC scores. CK5 expression was not seen in any of the LUMA, LAHH or LBHH tumors. It is of interest to note that pure apocrine tumors belong either to TN or ERBB2 group of tumors. The frequent expression of CK5 in apocrine tumors suggests a close kinship between this subset of TN and ERBB2 tumors that need to be further explored.

Comparing the current molecular classification with morphologic classification of the past, it appears that TN tumors with "sheet-like growth pattern" ("basal-like" morphology) comprise tumors of the medullary and atypical medullary types, based on patient demographics, association with BRCA1 mutants, and morphologic and IHC features [3, 4, 37-42]. However, one area of concern is the poor prognosis of "basal-like" carcinomas as recently reported by gene-expression studies compared to relatively good prognosis of pure medullary carcinomas described in the past [43, 44]. On critical review of our study cases, we found that only one of the 205 cases would fulfill the strict criteria set out by Ridolfi and colleagues [44] for diagnosing medullary carcinoma. Given the rarity of classic

medullary type carcinoma, it is of no surprise that clinical follow up data on medullary carcinoma differs from the "basal-like" carcinoma as a group.

We tested the usefulness of our IHC classification by demonstrating significant differences in response rates to NACT among different molecular classes. These differences were marked when TN and ERBB2 tumors were compared with the other groups. Significant difference in pCR was also seen between the two HER2+/ER+ groups with higher pCR in LBHH tumors compared to the LAHH group (8.2% versus none). Although the difference in pCR between the two ER+/HER2 negative groups was not significant, there was a subtle difference with respect to percentage tumor volume reduction between the two groups (30% in LUMB and 23% in LUMA). Our data is very comparable to the response rates obtained by gene expression profiling [45]. However, we agree with Rouzier *et al* that the mechanism of response is different between TN and ERBB2 tumors [45] given that significant difference exists in the proliferation rate between the two groups.

In conclusion, breast cancer is a heterogeneous disease at morphologic, immunohistologic and molecular level. LUMA tumors are generally well-differentiated with lowest Ki-67 LI. ERBB2 tumors show moderate Ki-67 LI and are generally of apocrine type. Conversely, 'pure' apocrine carcinomas either belong to ERBB2 or TN tumors, but have only moderate elevation of Ki-67 LI. TN tumors with "sheet-like growth pattern" have the highest Ki-67 LI among all breast tumors and are easily identified on morphologic examination. Basal cytokeratin (CK5) expression is limited to either TN/basal-like tumors or tumors that demonstrate apocrine differentiation. Breast cancer molecular classification using IHC surrogate markers is feasible and that these classes encompasses different pathologic features which indicates differences in biologic behavior. Furthermore, it provides useful clinical information and can be applied in routine practice.

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