Published in final edited form as:

Am J Surg Pathol. 2009 February ; 33(2): 163–175. doi:10.1097/PAS.0b013e31817f9790.

Most Basal-like Breast Carcinomas Demonstrate the Same Rb-/p16+ Immunophenotype as the HPV-related Poorly Differentiated Squamous Cell Carcinomas Which They Resemble Morphologically

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Abstract

Basal-like carcinomas (BLCs) of the breast share discriminatory morphologic features with poorly differentiated high-risk human papilloma virus (HPV)-related squamous cell carcinomas of the oropharynx, penis, and vulva. Because HPV E7 protein inactivates the retinoblastoma (Rb) protein, diffuse p16 expression is a surrogate marker for these high-risk HPV-related carcinomas. HPV E6 protein also inactivates p53, further compromising the G₁-S cell cycle checkpoint. The Rb/p16/p53 immunohistochemical profile of BLC of the breast has not been well characterized. Tissue microarrays containing 71 invasive ductal carcinomas (IDCs) of the breast were immunolabeled for p16, Rb, p53, and Ki-67. The cases included 4 distinct groups of IDCs having surrogate immunohistochemical profiles corresponding to categories defined by gene expression profiling (17 luminal A, 7 luminal B, 14 HER-2+, and 21 BLC), along with 12 unclassifiable triple negative carcinomas (UTNCs). Twenty-five of the 71 IDC were Rb negative/p16 diffuse positive (Rb -/p16 +). These included 15 of 21 BLC and 9 of 12 UTNC, but only 1 of 14 HER-2 positive cases and none of the 17 luminal A or 7 luminal B cases ($P < 0.01$, BLC or UTNC vs. others). Six of the Rb -/p16 + IDC also had a significant ductal carcinoma in situ component. The ductal carcinoma in situ in 4 of these 6 cases showed the same Rb -/p16+ phenotype as the associated IDC. BLC and UTNC had the highest Ki-67 indices of the 5 groups, even when matched for grade. The Rb -/p16+ phenotype and the Rb -/p16+/p53 overexpressing phenotype correlated with increased proliferation within the BLC group. In conclusion, BLC and UTNC, but not HER-2, luminal A, or luminal B carcinomas, frequently demonstrate an Rb -/p16+ phenotype, similar to the HPV-related squamous cell carcinomas that BLC resemble morphologically. This subset may represent a more homogenous group than BLC as defined currently.

Keywords

breast; carcinoma; basal-like; retinoblastoma; p16

Basal-like carcinomas (BLCs) are one of 5 distinct groups of invasive breast carcinomas defined by gene expression profiling (GEP).^{11,14,15} These groups are luminal A and luminal B (defined by significant expression of estrogen-related and luminal epithelium-related genes), HER-2 positive (defined by a low expression of estrogen-related genes and high expression of HER-2-related genes), normal breast-like (defined by high expression of adipose tissue/myoepithelium-related genes and low expression of luminal epithelium-related genes), and basal-like (defined by low expression of estrogen receptor (ER) and HER-2-related genes and high expression of myoepithelium-related genes).

BLC typically affect young patients, demonstrate rapid clinical growth, preferentially metastasize hematogenously to lung and brain as opposed to lymph nodes, are associated with *BRCA1* gene mutations, and are associated with poor outcome.^{8,11,14,15,26,37} BLC comprise approximately 15% to 20% of all breast carcinomas, and the majority of ER -, progesterone receptor (PR) -, HER-2 - breast carcinomas [(“triple-negative carcinoma”) (TNC)]. Although BLCs are currently defined by GEP, an ER -, PR -, HER-2 - immunophenotype with expression of cytokeratin 5/6 (CK 5/6) and/or epidermal growth factor receptor (EGFR) is a useful immunohistochemical (IHC) surrogate for BLC. It is estimated that this IHC phenotype is 100% specific and 76% sensitive for BLC as defined by GEP.³¹

The morphologic features which distinguish breast carcinomas with a BLC GEP from other breast carcinomas have been described by multiple groups. Several of the more distinctive features include a pushing, noninfiltrative border; a ribbonlike architecture surrounding central necrosis; cellular, solid tumor areas with minimal desmoplasia; small basaloid cells and larger polygonal cells with sharp borders resembling poorly differentiated squamous cell carcinoma (SCC); and a high proliferative index.^{16,27} Interestingly, all the above morphologic features also distinguish poorly differentiated human papilloma virus (HPV)-related SCCs of the oropharynx, penis, and vulva^{18,46} from non-HPV-related SCCs arising in these sites. HPV is usually not involved in the pathogenesis of the better differentiated, more keratinizing SCCs of the latter sites (Fig. 1).

Diffuse expression of p16, which normally inhibits cyclin-dependent kinases and thus blocks proliferation, is an excellent immunochemical surrogate for SCCs harboring high-risk HPV. The explanation stems from the fact that HPV E7 protein inactivates the retinoblastoma (Rb) tumor suppressor, a potent suppressor of the G1-S cell cycle transition. Rb inhibits p16 transcription through a negative feedback loop between p16 and Rb; as a result, p16 levels are paradoxically high in these highly proliferative tumors, resulting in diffuse, strong p16 immunoreactivity. This phenomenon has been termed “abrogated response to cellular stress,” as expression of p16 is normally a protective reaction to cellular stress which inhibits proliferation and initiates senescence.¹⁷ Diffuse p16 immunoreactivity is useful diagnostically for delineation of HPV-related high-grade intraepithelial neoplasia.^{2,23,24,32,41} HPV E6 protein also inactivates p53 in these cancers, further compromising the G1-S cell cycle checkpoint.

Given the overlap of distinguishing morphologic features between BLC of the breast and HPV-related SCCs of the oropharynx, penis, and vulva, one might hypothesize that similar genes might be inactivated in each cancer. Indeed, inactivating *p53* mutations have been reported to be more frequent in BLC than in other subtypes of breast cancer,⁸ which parallels the inactivation of p53 by HPV E6 protein in SCCs. However, the Rb/p16 status of BLC of the breast has not been assessed. In this study, we examine the Rb/p16 status of BLC of the breast, and other unclassifiable triple negative breast carcinomas, in comparison to luminal and HER-2-positive breast carcinomas. We correlate expression of these markers with proliferative activity, as well as p53 and HPV status.

Materials and Methods

Institutional Review Board Approval

This study was approved by the Institutional Review Board of the Johns Hopkins Medical Institutions.

Case Selection and Tissue Microarray Construction

We reviewed cases of invasive ductal carcinoma (IDC) resected and processed at our institution between the years 2001 and 2007. All of these cases were processed uniformly, in that they were sectioned in a fresh state, and fixed overnight in 10% neutral buffered formalin before processing to ensure 24 to 33 hours of formalin fixation. IDCs showing processing artifacts (eg, incomplete sections in which fat did not stick to the hematoxylin and eosin-stained slides), of small size (<1 cm), or treated with neo-adjuvant chemotherapy, were excluded from consideration.

Tissue microarrays (TMAs) were constructed as previously described.⁴⁷ Each TMA contained 99 tissue cores, each 1.4mm in diameter. These were arranged as 9 rows and 11 columns. Column 6 consisted of unrelated control tissue, leaving 90 cores on the array for carcinoma samples. For each carcinoma case, 5 areas were identified on the hematoxylin and eosin slides, punched from the corresponding donor blocks, and placed on the array. Therefore, each array contained 18 different IDCs. Among the 5 samples of each case, we attempted to include normal tissue and carcinoma in situ in 1 sample if possible, leaving 4 to 5 cores of IDC per case.

Immunohistochemistry Methods and Scoring

IHC for ER, PR, and HER-2 were previously performed on all cases as part of a routine panel applied to any IDC at The Johns Hopkins Hospital. The slides were reviewed by 2 of the authors (A.P.S. and P.A.) to confirm the reported interpretation. IHC labeling was performed using standard methods. IHC labeling for ER and PR were performed on the Benchmark XT autostainer (Ventana Medical Systems Inc, Tucson, AZ) using I-View detection kit. The antibodies, dilutions, and sources were as follows: ER, monoclonal antibody; 1:1 dilution, Ventana, catalog No. 760-2596; PR, monoclonal antibody, 1:60 dilution, DAKO, catalog No. M3569. IDCs demonstrating weak, moderate, or strong nuclear labeling for ER or PR in greater than 1% of cells were considered ER-positive or PR-positive, respectively.¹⁹ HER-2 IHC was performed using the DAKO Herceptest kit according to the manufacturer's standard protocol. IDCs were scored using established criteria as 0 or 1⁺ (negative), 2⁺ (equivocal), and 3⁺ (positive). Fluorescence in situ hybridization analysis for HER-2 amplification was performed on all cases with 2⁺ (equivocal) IHC results using the Path Vysion kit (Des-Plaines, IL). To qualify as HER-2 positive for this study, a case had to demonstrate either a 3⁺ (strong positive) IHC score or a HER-2 fluorescence in situ hybridization amplification ratio of greater than 4. Cases with equivocal ratios (1.8-2.2), or low level amplification (ratios 2.2-4.0) were excluded from this study due to their uncertain clinical significance.

Because its labeling is often focal, CK 5/6 IHC was performed on whole sections of IDCs which were negative for ER, PR, and HER-2 to identify BLC cases. CK 5/6 labeling was performed using the Benchmark XT autostainer (prediluted monoclonal antibody, DAKO, catalog No. M7237). Cases were scored on the basis of percentage of positive cells into 1+ (1% to 25%), 2+ (26% to 50%), 3+ (51% to 75%), and 4+ (76% to 100%) categories. For this study, cases demonstrated convincing membranous or cytoplasmic labeling in >25% of neoplastic cells were considered positive. Cases with equivocal or less extensive labeling, which was difficult to distinguish from biotin artifact were excluded from the study, so as to include only unequivocal cases in the BLC category. IHC for EGFR was performed on TMAs of cases negative for ER, PR, and HER-2 to identify additional BLC cases. EGFR labeling was

performed using the monoclonal antibody from Zymed, catalog No. 28005, at 1:50 dilution, and the capillary action HRP/DAB detection system (catalog No. 2402, Signet Laboratories Inc, Dedham, MA). Any strong membranous labeling for EGFR was considered a positive result. Cases were scored on the basis of percentage of positive cells into 1+ (1% to 25%), 2+ (26% to 50%), 3+ (51% to 75%), and 4+ (76% to 100%) categories. In general, positive cases demonstrated labeling in 10% to 50% of neoplastic cells (Table 1).

IHC for p16 and Rb was performed on TMAs and on whole sections from donor blocks of selected cases (see Results). IHC for Rb was performed manually using the G3-245 mouse monoclonal antibody (BD Biosciences, Pharmingen, San Jose, CA) at a dilution of 1:2000, after 20 minutes of steaming in 10mM citrate buffer. Labeling was visualized using the DAKO LSAB kit (catalog No. K0690). For Rb, only nuclear labeling was analyzed; cytoplasmic nonspecific labeling (likely related to biotin artifact) was ignored. The percentage of neoplastic cell nuclei labeling in each case (mean of the 4 to 5 cores) was assessed. Cases were considered negative for Rb when no neoplastic cell nuclei demonstrated labeling in sections in which stromal nuclei did label, the latter serving as an internal control. IHC for p16 was performed on the Benchmark XT autostainer using the monoclonal antibody 16PO4, at a dilution of 1:100 (Cell Marque Inc, Hot Springs, AR). Positive p16 labeling was defined as the presence of nuclear and/or cytoplasmic reactivity. The percentage of cells showing nuclear and/or cytoplasmic labeling was recorded (mean of the 4 to 5 cores). For this study, diffuse p16 labeling was defined as labeling for p16 in greater than 95% of neoplastic cells.

p53 (Ventana, monoclonal antibody, catalog No. 760-2542) and Ki-67 (Ventana, monoclonal antibody, catalog No. M7240) IHC was also performed on the Benchmark XT autostainer. For p53 and Ki-67, only nuclear labeling was scored. For p53, we regarded labeling of >30% of nuclei to be aberrant overexpression (which correlates well but not perfectly with p53 mutation),^{38,40,45} labeling of less than 30% of nuclei as negative for aberrant overexpression, and labeling of approximately 30% of nuclei to be equivocal. The latter cases were excluded from statistical analysis of p53 expression. When assessing proliferation activity, we reasoned that the mean Ki-67 index might be artificially low due to sampling of central hypoxic regions of the IDC, whereas the highest Ki-67 index of a single sample core might better reflect proliferation at the IDC's leading edge. Therefore, for Ki-67, we recorded both the mean labeling index of the 4 to 5 sample cores, and the highest labeling index of an individual sample core from each case.

Case Characterization

The IHC characteristics of the cases on the TMAs are summarized in Table 1. Cases on the TMA were categorized into one of 5 categories based upon accepted and previously validated IHC surrogate profiles.^{8,31} Seventeen cases were immunoreactive for ER and negative for HER-2, and therefore were considered luminal A tumors (luminal A cases 1 to 17). Of note, all luminal A cases demonstrated nuclear-labeling for ER in at least 70% of invasive carcinoma cells. Of 21 HER-2–positive cases, 7 were immunoreactive for ER and/or PR, though to a lesser degree than the luminal A cases. These 7 cases were therefore considered luminal B tumors (luminal B cases 1 to 7), with the 14 remaining HER-2–positive cases considered HER-2–positive tumors (HER-2 cases 1 to 14).

Among the 33 cases which were negative for ER, PR, and HER-2 (triple negative carcinomas, or TNC), 8 were immunoreactive for CK 5/6 and EGFR, 9 were immunoreactive for CK 5/6 but not EGFR, and 4 were immunoreactive for EGFR but not CK 5/6. On the basis of published criteria, all 21 of these cases were considered BLCs (BLC cases 1 to 21). The remaining 12 cases, which were negative for ER, PR, HER-2, CK 5/6, and EGFR were considered unclassifiable triple negative carcinomas (UTNC cases 1 to 12). Of note, IDC considered

“normal breastlike” by GEP remain poorly defined and lack a validated IHC surrogate profile, so such a category was not included in our study.

HPV In Situ Hybridization

In situ hybridization for HPV was performed as previously described.⁴ Probes for wide spectrum HPV (which covers HPV 6, 11, 16, 18, 31, 33, 35, 45, 51, and 52) and HPV 16 were applied to the BLC and UTNC TMAs.

Statistical Methods

Data were analyzed using Student *t* test, the Fisher exact test, or analysis of variance.

Results

Patient Demographics, Tumor Grade, and Stage

Details of age, ethnicity, grade, and lymph node status of all subtypes of carcinoma are summarized in Table 2. Several trends are apparent from this data. First, luminal A cancers occurred in older patients and were more likely to be of lower grade (Elston grades 1 to 2) (ages of luminal A vs. BLC $P=0.0035$, vs. UTNC $P=0.021$, vs. luminal B $P=0.040$, vs. HER-2 $P=0.055$) (grade of BLC, UTNC, and HER-2 each vs. luminal A $P<0.001$, grade of luminal B vs. luminal A $P=0.21$). Second, a higher percentage of BLC and UTNC occurred in African American patients (combined TNC vs. all others $P=0.046$). Both of these trends are consistent with the published literature.^{8,14,15}

p16 IHC

The mean p16 labeling index for the 5 groups was as follows: BLC, 82.7% (± 36.4); UTNC, 92.1% (± 27.4); HER-2, 37.6% (± 38.2); luminal A, 27.1% (± 30.1); and luminal B, 23.7% (± 24.7).

Seventeen of 21 BLC demonstrated a diffuse, strong positive p16 labeling pattern, along with 11 of 12 UTNC. Three of the 14 HER-2 cancers showed diffuse positive p16 labeling, but none of the 17 luminal A or 7 luminal B cases did (Fig. 2).

Rb IHC

The mean Rb labeling indices for the 5 groups were as follows: BLC 15.2% (± 28.2); UTNC, 12.5% (± 29.9); HER-2, 58.9% (± 33.0); luminal A, 19.6% (± 24.5); and luminal B, 40.7% (± 33.7).

Fifteen of 21 BLC demonstrated complete loss of Rb expression, along with 9 of the 12 UTNC and 1 of the 14 HER-2 cases. None of the 17 luminal A or 7 luminal B cancers showed complete loss of Rb expression (Table 3).

Diffuse p16 Expression Correlates With Rb Loss

Because diffuse p16 expression may be effected by Rb inactivation, we correlated p16 expression with Rb expression. All 25 cases (15 BLC, 9 UTNC, and 1 HER-2) demonstrating loss of Rb expression demonstrated diffuse p16 expression. Only 2 HER2 cancers, 2 BLC, and 2 UTNC with diffuse p16 expression had intact Rb expression. Therefore, overall, the Rb negative/p16 diffuse positive immunophenotype (Rb $-$ /p16 $+$) was identified in 15 of 21 BLC and 9 of 12 UTNC, but only 1 of 14 HER-2 cancers and none of the luminal A or luminal B cancers ($P<0.01$).

The Rb Negative/p16 Diffuse Positive Immunophenotype (Rb – /p16+): Correlation of TMA Results With Whole Section Results

Complete loss of expression of the Rb protein observed on the limited tumor samples arrayed on the TMAs could be artifactual if expression within the IDC was heterogeneous, and the TMA sampled only the negative areas. To help exclude this possibility, whole sections from the donor blocks of 21 cases of TNC (BLC and UTNC), which were negative for Rb on the TMA, were labeled for Rb. As a control, whole sections from 4 TNC (BLC and UTNC) cases with intact Rb expression on the TMA were also labeled for Rb. The results on the whole sections mirrored those on the TMA: all 21 cases that lacked Rb expression on the TMA were also negative on whole section; the 4 cases with intact Rb expression on TMA were positive on whole section as well.

Similarly, diffuse expression of p16 protein observed on the limited samples arrayed on TMA could be an artifact if expression within the IDC was heterogeneous. To exclude this possibility, whole sections from the donor blocks of 14 cases of BLC and UTNC that were diffusely positive for p16 on the TMA were labeled for p16. Once again, the results on whole sections mirrored those on the TMA. All 14 cases, which showed diffuse p16 expression on the TMA had the same labeling pattern on whole sections, whereas 2 of 2 cases with focal p16 expression on the TMA also showed focal expression on whole section.

The Rb– /p16+ Phenotype Frequently Occurs at the Ductal Carcinoma In Situ Stage in BLC and UTNC

On reviewing all hematoxylin and eosin slides from the 24 cases of TNC (BLC and UTNC) with the Rb – p16+ phenotype, a significant ductal carcinoma in situ (DCIS) component (> 10% of the tumor) was identified in 6 cases. Whole sections from donor blocks of these 6 cases were labeled for p16 and Rb, along with the myoepithelial markers p63 and smooth muscle myosin heavy chain, to help delineate the Rb/p16 phenotype of the DCIS adjacent to the IDC. In 4 cases, the DCIS component demonstrated the same Rb–/p16+ phenotype as the associated IDC (Fig. 3). In 2 other cases, the DCIS component of the Rb–/p16+ IDC retained Rb expression and did not diffusely label for p16 (DCIS-IDC discordance) (Fig. 4).

The Rb– /p16+ Phenotype Correlates With Higher Ki-67 Indices

BLC and UTNC had the highest proliferation indices of the 5 groups. The mean Ki-67 index for the BLC group was 67.5% (71.1% using the highest sample from each case); the mean for the UTNC group was 61.5% (65.3% using the highest sample). HER-2-positive cases had intermediate proliferation indices: the mean Ki-67 index for the HER-2 cases was 36.5% (39.7% using the highest sample), whereas the mean for the luminal B cases was 35.1% (36.4% using the highest sample). Using analysis of variance, these groups demonstrated a statistically significant difference in mean Ki-67 indices ($P < 0.001$). As expected, luminal A cases, which included more cancers of lower grade, had the lowest Ki-67 indices. The mean Ki-67 index average for the luminal A cases was 8.6% (8.6% using the highest sample) (Table 4) (luminal A vs. all others $P < 0.001$).

Even when matched for grade, BLC and UTNC had higher Ki-67 indices than HER-2+ cases. Elston grade 3 BLC had a mean Ki-67 index of 66.4% (70.3% using the highest sample). Elston grade 3 UTNC had a mean Ki-67 index of 61.5% (65.3% using the highest sample), whereas Elston grade 3 HER-2 cancers had a mean Ki-67 index of 36.5% (39.7% using the highest sample) ($P < 0.01$ for BLC or UTNC vs. HER-2).

The Rb–/p16+ phenotype correlated with high Ki-67 indices within the BLC category. The mean Ki-67 index for the 15 BLC with the Rb –/p16+ phenotype was 75.2% (80.3% using the highest sample). In contrast, the mean for the 6 BLC without the Rb –/p16+ phenotype was

48.3% (48.3% using the highest sample) ($P = 0.019$ and 0.002) (Table 5). This difference was not seen in the UTNC group; the mean for the 9 UTNC with Rb -/p16+ phenotype was 59.2% (64.2% using the highest sample), whereas the mean for the 3 UTNC without the Rb -/p16+ phenotype was 68.3% (68.3% using the highest sample). However, using the combined 33 TNC (21 BLC and 12 UTNC), the 24 with the Rb -/p16+ phenotype had higher Ki-67 indices (mean 69.2%, 74.3% using the highest sample) compared with the 9 without the Rb -/p16+ phenotype (mean and highest 55.0%). This difference was at borderline statistical significance using the mean Ki-67 index ($P = 0.06$), but was significant using the highest samples' Ki-67 index ($P < 0.01$).

HPV In Situ Hybridization

Given the known association of diffuse p16 immunoreactivity with high-risk HPV infection in cancers, we performed in situ hybridization using a probe for HPV16 and a wide spectrum HPV probe on all BLC and UTNC (33 cases). No case demonstrated detectable HPV DNA.

p53 IHC

Ten of 21 BLC aberrantly overexpressed p53, with 1 additional equivocal case. Eight of 12 UTNC aberrantly overexpressed p53, along with 8 of 14 HER-2 cases, 2 of 17 luminal A cases, and 4 of 7 luminal B cases. The p53 expression pattern between the each of the BLC, UTNC, and HER-2 groups compared with the luminal A cancers was significant ($P < 0.05$), but there was no significant difference between the 3 former groups (Table 3). Luminal B cancers also overexpressed p53 more frequently than luminal A cancers ($P = 0.03$).

Seven of the 15 BLC with the Rb-/p16+ phenotype overexpressed p53, along with 6 of 9 UTNC. There was no correlation in the BLC or UTNC groups between p53 overexpression and presence of the Rb -/p16+ phenotype.

Similar to the Rb -/p16+ phenotype, p53 overexpression correlated with increased proliferation in the BLC group but not the UTNC group. The mean Ki-67 index for the 10 BLC with p53 overexpression was 74.5% (76% using the highest score); the 10 BLC without p53 overexpression had a mean Ki-67 of 58.8% (63.1% highest) (1 equivocal case was excluded) ($P = 0.089$, 0.15 , respectively). In contrast, the 8 UTNC with p53 overexpression had a mean Ki-67 of 58.8% (63.1% highest), and the 4 UTNC without p53 overexpression had a mean Ki-67 of 67% (69.5% highest).

IDCs with the Rb-/p16+/p53 overexpression immunophenotype demonstrate evidence of inactivation of both p53 and Rb, similar to the functional effects of HPV E6 and E7 proteins in poorly differentiated HPV-related SCCs (though in the latter low p53 protein levels are the IHC correlate of p53 inactivation). We therefore compared the proliferation rates of TNC with and without this phenotype. Once again, we found a statistically significant increase in proliferation rate only in the BLC group. There were 7 BLC cases with the Rb -/p16+ immunophenotype that showed aberrant p53 overexpression. The mean Ki-67 index in these cases was 86.4% (88.6% using the highest sample core). The mean Ki-67 index for all remaining BLC cases¹⁴ was 58% (62.5% highest) ($P = 0.01$, 0.01 , respectively) (Table 5). In contrast, the 6 UTNC cases that had the Rb -/p16+ immunophenotype and aberrant p53 overexpression had a mean Ki-67 index of 56.7% (62.5% highest), whereas the mean Ki-67 index for the remaining UTNC cases⁶ was 63.3% (68% highest). The latter difference was not statistically significant.

Discussion

To summarize our results, we have shown that BLCs and other UTNC of the breast frequently demonstrate a distinctive immunophenotype characterized by loss of expression of the Rb protein and diffuse immunoreactivity for the p16 protein (Rb -/p16+). This immunophenotype is similar to that of the HPV-related SCCs of several specific sites (oropharynx, vulva, and penis), which BLC resemble morphologically. In latter sites, the diffuse immunoreactivity for p16 is thought to reflect the consequences of HPV E7 protein inactivation of Rb. In BLC and UTNC, Rb expression is frequently absent, but we show herein that HPV is not detectable by in situ hybridization. We demonstrate functional consequences of inactivation of the Rb protein in BLC, in that loss of this mediator of G₁ cell cycle arrest is associated with the higher proliferation rates seen in BLC and UTNC than high-grade HER-2-positive IDCs. Moreover, Rb-/p16+ BLC have a higher proliferation rate than those BLC lacking this immunophenotype. This difference was magnified when we considered those Rb -/p16+ BLC with aberrant p53 overexpression, a useful though clearly imperfect surrogate for p53 mutation in these cancers. The latter cancers mimic even more closely HPV-related SCCs at the molecular level, as HPV E6 and E7 protein inactivate p53 and Rb, respectively. Finally, we demonstrate that the Rb-/p16+ phenotype often occurs at the stage of DCIS.

Our data are concordant with most of the prior literature regarding expression of p16, Rb, and p53 in breast carcinomas. Aberrant p53 overexpression and p53 mutations have been correlated with high-grade IDCs (including BLC), so our finding of frequent aberrant p53 overexpression in BLC, UTNC, and HER-2 IDC is not unexpected.^{8,25,39} Most (but not all) prior studies have shown that loss of Rb protein or overexpression of p16 protein is associated with breast carcinomas having poor prognostic factors.^{1,9,12,13,20,21,29,30} *Importantly, virtually all of these studies occurred before the entity of BLC was established by gene expression profiling in 2001.* Since the recognition of the entity of BLC, the p16 and Rb status of BLC has not been systematically assessed. One study³⁴ indicated that BLC associated with *BRCA1* gene inactivation express *lower* levels of p16 than carcinomas associated with inactivation of *BRCA2*, which were more frequently ER positive. In addition, a review of BLC and *BRCA1*-associated tumors also states that BLC are characterized by *lower* levels of p16 than the typical breast carcinoma.⁴³ In contrast, Gauthier et al,¹⁷ in the setting of a study of DCIS, examined their published GEP data and noticed that BLC show high levels of p16 transcripts and low levels of Rb transcripts. Analyzing a subset of primary tumors representing each of the 5 molecular subtypes of breast cancer, they showed that p16 IHC labeling correlated well with mRNA levels in BLC, though they did not statistically compare their p16 results in BLC with those of the other different molecular subtypes of breast cancer. They hypothesized that “loss of functional p16/Rb signaling may play a defining role in the biology of this tumor subtype.” Our results correlate perfectly with their hypothesis.

We suspect that the Rb-/p16+ phenotype is biologically distinctive, as supported by several observations. First, we have shown that this phenotype is almost always restricted to TNC (BLC and UTNC). Second, we demonstrated that this phenotype correlates with higher proliferation rates among BLC. Such correlation makes biologic sense, in that inactivation of Rb results in inactivation of the G₁-S cell cycle checkpoint, and promotes unblocked entry into the cell cycle. Indeed, another type of carcinoma in which Rb is consistently inactivated, small cell neuroendocrine carcinoma, is characterized by strikingly high Ki-67 indices compared with other carcinomas.³ It is now generally recognized that BLC defined by either gene expression or IHC are heterogeneous, and one challenge is to identify a more homogenous subset to which targeted therapy can be applied.^{5,22,35,36,42,44} We suspect that the Rb-/p16+ immunophenotype may define a more homogenous subgroup of BLC. Some cases, which otherwise considered UTNC but which share this phenotype may also belong to this group. Although our Ki-67 proliferation index results support this assertion, correlation with other

clinical parameters, such as response to chemotherapy and outcome, and association with *BRCA1* gene inactivation, would be required to substantiate this possibility.

We note that the correlation of the Rb-/p16+ and p53 overexpression immunophenotypes with higher Ki-67 indices was present and significant only within the BLC group, and not within the UTNC group. Several nonmutually exclusive explanations are apparent. First, the BLC group (21 cases) in this study was larger than the UTNC group (12 cases), making statistical comparisons more robust in the BLC group. Second, and perhaps more importantly, the BLC group is likely more homogeneous than the UTNC group. UTNC lacks a specific defining positive IHC marker (such as CK 5/6 or EGFR expression in BLC) and therefore is a diagnosis of exclusion. The UTNC category therefore likely is a heterogeneous “mixed-bag” of cases, which includes carcinomas related to and unrelated to BLC. Third, cell type may influence the effects of the Rb-/p16+/p53 overexpression phenotype. The high molecular weight CK 5/6 immunoreactivity seen in BLC may reflect early stages of squamous differentiation, making these cancers immunophenotypically closer to HPV-related squamous carcinomas (also CK 5/6 positive) that the BLC resemble. One can postulate that the effects of Rb and p53 inactivation are most distinctive and robust in cells showing squamous differentiation, resulting in higher Ki-67 indices.

Our observation that the Rb-/p16+ immunophenotype occurs at the DCIS stage of BLC is also concordant with the literature. Indeed, DCIS with a basal-like immunophenotype (ER -, PR -, HER-2 -, CK 5/6+, or EGFR+) has recently been described.^{7,10,28} It is known that BLC frequently have only a minimal DCIS component, and that pure DCIS with a basal immunophenotype (10% of DCIS) is less common than basal-like invasive carcinoma (20% of IDC). Therefore, it has been postulated that DCIS with the basal-like immunophenotype rapidly evolves toward IDC. Perhaps the frequent (4/6 cases, 67%) inactivation of Rb protein within these DCIS lesions, by promoting unchecked proliferation and further accumulation of genetic alterations, contributes to this rapid evolution to IDC. The data of Gauthier et al¹⁷ support this hypothesis. These authors found that DCIS demonstrating a high p16/high Ki-67 phenotype, which reflects an abrogated response to cellular stress (and likely reflects Rb inactivation), recur more frequently than DCIS demonstrating a high p16/ low Ki-67 index profile, which reflects a physiologic response to stress.

Finally, our study also highlights the often underappreciated power of morphology in elucidating the molecular pathogenesis of cancers. We chose to examine the Rb/p16 pathway and p53 in BLC of the breast because of their distinct morphologic similarities to HPV-related poorly differentiated SCCs of the oropharynx, penis, and vulva. On the basis of similar morphology, we hypothesized that similar genes may be inactivated in these neoplasms, albeit by different mechanisms (genomic or epigenetic alterations in breast cancer, HPV in SCCs). As surgical pathologists, it is gratifying that our study, triggered by analysis of morphology (“traditional pathology”), has recently¹⁷ been supported by analysis of GEP data (“molecular pathology”). Our findings also suggest that one may define a family of BLCs of certain anatomic sites in which distinctive basal-like morphology correlates with inactivation of Rb protein and diffuse p16 expression. Poorly differentiated SCCs of the oropharynx, penis, and vulva are established members of this family, and are linked by the presence of high-risk HPV, which inactivates Rb protein. Parenthetically, this correlation of morphology with HPV status does not hold in the cervix or anus, where virtually all SCCs are HPV positive and diffusely express p16 protein. Our study suggests that BLC of the breast is an additional member of this family, representing a non-HPV-related carcinoma in which basal-like morphology predicts inactivation of Rb protein and diffuse p16 expression. We note with interest that basaloid lung carcinomas have recently been demonstrated to more frequently harbor inactivation of Rb protein than other non-small cell carcinomas of the lung, suggesting that this carcinoma may represent another member of this family.⁶ We also note that aberrant diffuse p16 and p53

protein expression by IHC now has shown to be characteristic of 2 carcinomas, BLC of the breast (this study) and high-grade serous carcinoma of the ovary,³³ which both are associated with germline *BRCA1* gene mutations. This observation suggests that inactivation of *BRCA1* may mediate carcinogenesis in concert with alterations in the Rb/p16 and p53 pathways.

Acknowledgments

The authors thank Ralph H. Hruban, MD, for critically reviewing this manuscript.

Supported by NCI P50 CA 88843.

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FIGURE 1.

HPV-related squamous cell carcinomas of the oropharynx (HPV SCCs) mimic BLCs of the breast. HPV-related SCC of the tonsils feature ribbonlike, cellular areas of basaloid cells surrounding geographic areas of necrosis (A, B). In contrast, a non-HPV-related squamous cell carcinoma of the tonsils demonstrates more extensive keratinization and desmoplastic reaction, and less basaloid neoplastic cells (C). BLC of the breast also feature ribbonlike, cellular areas of basaloid cells surrounding geographic areas of necrosis (D, E). In contrast, most non-BLC IDC (such as this HER-2 case) feature more desmoplastic stroma and less basaloid neoplastic cells (F). BLC indicates basal-like carcinoma; HPV, human papilloma virus; IDC, invasive ductal carcinoma; SCC, squamous cell carcinoma.

FIGURE 2.

BLC of the breast. The tumor is composed of small basaloid cells forming nests (A). At higher power, high mitotic and apoptotic rates are evident (B). The tumor cells show loss of expression for Rb (note intact labeling of stroma) (C) and label diffusely for p16 (D). Virtually all carcinoma cells overexpress p53 (E) and label for Ki-67 (F). BLC indicates basal-like carcinoma; Rb, retinoblastoma protein.

FIGURE 3.

BLC of the breast with concordant Rb $-$ /p16 $+$ phenotype in DCIS. This BLC features a well-developed DCIS (A) and IDC (B) component. Both the DCIS (C) and IDC (D) show absence of Rb expression. Both the DCIS (E) and the IDC (F) show diffuse p16 expression. BLC indicates basal-like carcinoma; DCIS, ductal carcinoma in situ; IDC invasive ductal carcinoma; Rb, retinoblastoma protein.

FIGURE 4.

BLC of the breast with discordant Rb/p16 phenotype in DCIS. This invasive BLC at the edge of the image (A) is associated with a prominent lymphoid reaction, whereas central ducts represent DCIS. p63 IHC separates the DCIS, which is surrounded by p63-immunoreactive myoepithelial cells, from the invasive BLC (B). Rb protein is expressed in the DCIS but not the invasive BLC (C), while p16 is diffusely expressed in the invasive BLC but not the DCIS (D). BLC indicates basal-like carcinoma; DCIS, ductal carcinoma in situ; IHC, immunohistochemical; Rb, retinoblastoma.

TABLE 1

Patient Demographics and Basic Characteristics of the Cancers

Case	Age/Race	Grade/Stage	ER (% Intensity)	PR (% Intensity)	Her2 (IHC Score/FISH Ratio)	EGFR	CK5/6
Basal 1	64.5/B	3, pT1cN0	0	0	1+	1+	3+
Basal 2	60.7/W	3, pT2N1	0	0	2+/1.3	4+	2+
Basal 3	63.0/B	3, pT3N0	0	0	0	4+	3+
Basal 4	45.3/B	3, pT1N0	0	0	0	1+	2-3+
Basal 5	36.9/W	3, pT2N1mi	<1% weak	0	0	-	3+
Basal 6	60.7/W	3, pT2N2	0	0	0	-	3+
Basal 7	54.5/B	3, pT2N0	0	0	1+	-	2+
Basal 8	40.5/W	3, pT2N0	0	0	0	-	2+
Basal 9	57.5/B	3, pT2N1	0	0	0	1+	4+
Basal 10	53.2/W	3, pT2N1	0	0	2+/neg	-	3+
Basal 11	42.5/B	3, pT2N1	<1% weak	<1% weak	0	-	2+
Basal 12	41.2/B	3, pT1N0	0	0	0-1+	-	2+
Basal 13	35.3/W	3, pT2N0	0	0	0-1+	-	2+
Basal 14	48.6/B	3, pT2N1	0	0	1+	-	3+
Basal 15	43.1/W	3, pT2Nx	0	0	0	1+	2+
Basal 16	57.6/W	3, pT1N0	0	0	2+/neg	3+	2+
Basal 17	34.7/B	3, pT3N1	0	0	1+	2+	2+
Basal 18	67.8/O	3, pT1N1	0	0	0	2+	-
Basal 19	50.3/W	3, pT3N1	0	0	1+	3+	-
Basal 20	57.5/B	3, pT1N2	0	0	1+	4+	-
Basal 21	65.3/B	2, pT1N0	0	<1% weak	1+	2+	-
UTNC 1	46.2/W	3, pT2N0	0	0	0	-	-
UTNC 2	68.5/W	3, pT2N0	0	0	2+/1.1	-	-
UTNC 3	42.6/W	3, pT3N0	0	0	0	-	-
UTNC 4	52.4/W	3, pT2N0	0	0	0	-	-
UTNC 5	54.3/O	3, pT2N0	<1% weak	<1% weak	1+	-	-
UTNC 6	58.1/B	3, pT2N0	0	0	0	-	-
UTNC 7	54.3/B	3, pT2N1	0	0	0	-	-
UTNC 8	42.3/B	3, pT2N0	0	0	1+	-	-

Case	Age/Race	Grade, Stage	ER (% Intensity)	PR (% Intensity)	Her2 (IHC Score/FISH Ratio)	EGFR	CK5/6
UTNC 9	49.0/W	3, pT2N0	0	0	0	-	-
UTNC 10	60.7/W	3, pT1N1mi	0	0	0-1+	-	-
UTNC 11	46.6/B	3, pT2N0	0	0	1-2+/neg	-	-
UTNC 12	61.1/B	3, pT2N0	0	0	0	-	-
Lum A 1	47.0/W	3, pT2N2	90% str	30% str	2+/neg	NA	NA
Lum A 2	56.2/W	2, pT2N0	90% str	15% str	1+/neg	NA	NA
Lum A 3	92.5/B	2, pT1Nx	90% str	30% mod	1+	NA	NA
Lum A 4	67.0/W	1, pT2N0	95% str	60% mod	2+/1.6	NA	NA
Lum A 5	59.4/W	2, pT2N0	90% str	15% mod	1+	NA	NA
Lum A 6	51.0/W	2, pT2N0	90% mod-str	70% mod-str	2+/1.6	NA	NA
Lum A 7	94.4/W	3, pT2Nx	100% str	60% str	0	NA	NA
Lum A 8	79.4/W	1, pT2N0	95% str	90% str	0	NA	NA
Lum A 9	48.9/W	3, pT3N2	90% str	1% mod	2+/1.3	NA	NA
Lum A 10	43.7/W	3, pT3N3	80% str	20% mod	2+/1.0	NA	NA
Lum A 11	44.4/W	3, pT2N1	90% str	90% str	1+	NA	NA
Lum A 12	84.0/W	2, pT2N0	85% str	75% str	0	NA	NA
Lum A 13	58.3/B	1, pT2N1mi	90% str	80% str	0	NA	NA
Lum A 14	73.0/B	1, pT2N1mi	100% str	100% str	1+/neg	NA	NA
Lum A 15	50.8/W	2, pT2N0	70% str	70% str	1+	NA	NA
Lum A 16	76.6/B	2, pT3N3	100% str	80% str	0	NA	NA
Lum A 17	63.4/B	2, pT4N3	95% str	90% str	1+	NA	NA
Lum B 1	52.5/O	3, pT2N1	70% mod	40% mod	3+	NA	NA
Lum B 2	34.6/W	3, pT2N1	10% mod	10% str	2+/5.6	NA	NA
Lum B 3	59.2/W	2, pT2N0	20% str	30% str	3+	NA	NA
Lum B 4	80.1/W	2, pT2Nx	70% mod-str	10% str	2+ />4.0	NA	NA
Lum B 5	30.3/B	2, pT1N2	0	80% str	3+	NA	NA
Lum B 6	40.0/W	3, pT3N1	80% str	50% str	3+	NA	NA
Lum B 7	55.5/W	3, pT2N0	5% mod	0	3+	NA	NA
Her-2 1	55.8/B	3, pT2N0	0	0	3+	NA	NA
Her-2 2	63.3/W	3, pT2N0	0	0	3+	NA	NA
Her-2 3	49.2/W	3, pT2N1	0	0	3+	NA	NA
Her-2 4	47.7/W	3, pT2N0	0	0	3+	NA	NA

Case	Age/Race	Grade, Stage	ER (% Intensity)	PR (% Intensity)	Her2 (IHC Score/FISH Ratio)	EGFR	CK5/6
Her-2 5	45.0/B	3, pT2N0	0	0	3+	NA	NA
Her-2 6	53.2/W	3, pT3N0	0	0	3+	NA	NA
Her-2 7	70.3/W	3, pT2N2	0	0	3+	NA	NA
Her-2 8	46.5/B	3, pT3N1	0	0	3+	NA	NA
Her-2 9	53.2/B	3, pT2N3	0	0	3+	NA	NA
Her-2 10	63.6/W	3, pT2N0	0	0	3+	NA	NA
Her-2 11	70.4/W	3, pT1N1	0	0	3+	NA	NA
Her-2 12	54.7/W	3, pT1N1mi	0	0	2+/4.1	NA	NA
Her-2 13	57.7/W	3, pT2N0	0	0	3+	NA	NA
Her-2 14	55.0/W	3, pT2N1mi	0	0	2+/14.2	NA	NA

B indicates Black; EGFR, epidermal growth factor receptor; ER, estrogen receptor; FISH, fluorescence in situ hybridization; IHC, immunohistochemical; Lum A, luminal A; Lum B, luminal B; mod, moderate; NA, not analyzed; Neg, FISH result negative; O, other; PR, progesterone receptor; str, strong; W, white.

TABLE 2

Summary of Case Characteristics

	Age (Mean Years)	Ethnicity			Elston Grade			Nodal Metastases	
		White	African American	Other	1	2	3	Positive	Negative
BLC (n=21)	51.5	9	11	1	0	1	20	11	9
UTNC (n=12)	53.0	6	5	1	0	0	12	2	10
HER-2 (n=14)	56.1	10	4	0	0	0	14	7	7
Luminal B (n=7)	50.3	5	1	1	0	3	4	4	2
Luminal A (n=17)	64.1	12	5	0	4	8	5	8	7

Axillary nodes were not sampled in 1 BLC patient, 1 luminal B patient, and 2 luminal A patients. BLC indicates basal-like carcinoma; UTNC, unclassifiable triple negative carcinoma.

TABLE 3

p16, Rb, and p53 Results

	p16		Rb		p53		No. Rb – p16+ p53 +Cases/Total
	Mean (all Cases) (%)	No. Cases With Diffuse Expression/Total	Mean (all Cases) (%)	No. Cases With Loss/ Total	No. Cases With Overexpression/ Total	No. Rb – p16+ Cases/ Total	
BLC	82.7	17/21	15.2	15/21	10/21	15/21	7/21
UTNC	92.1	11/12	12.5	9/12	8/12	9/12	6/12
HER-2	37.6	3/14	58.9	1/14	8/13	1/14	1/14
Lum B	23.7	0/7	40.7	0/7	4/7	0/14	0/7
Lum A	27.1	0/17	19.6	0/17	2/17	0/17	0/17

p53+=overexpression.

BLC indicates basal-like carcinoma; Rb, retinoblastoma; UTNC, unclassifiable triple negative carcinoma.

TABLE 4

Proliferation Rates of the Different Tumor Types

	Ki-67 Mean of Cores (%)	Ki-67 Highest Core (%)
BLC	67.5	71.1
UTNC	61.5	65.3
HER-2	36.5	39.7
Lum B	35.1	36.4
Lum A	8.6	8.6

BLC indicates basal-like carcinoma; UTNC, unclassifiable triple negative carcinoma.

TABLE 5

Comparison of the Ki-67 Indices Within the BLC Group

	Ki-67 Mean of Cores (%)	Ki-67 Highest Core (%)
BLC (n=21)	67.5	71.1
Rb - /p16+ BLC (n = 15)	75.2	80.3
Non-Rb - /p16+ BLC (n = 6)	48.3	48.3
Rb - /p16+/p53+ BLC (n = 7)	86.4	88.6
Non-Rb - /p16+/p53+ BLC (n = 14)	58.0	62.5

p53+=overexpression.

BLC indicates basal-like carcinoma; Rb, retinoblastoma protein.