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Breast Cancer Diagnosis: Past, Present and Future

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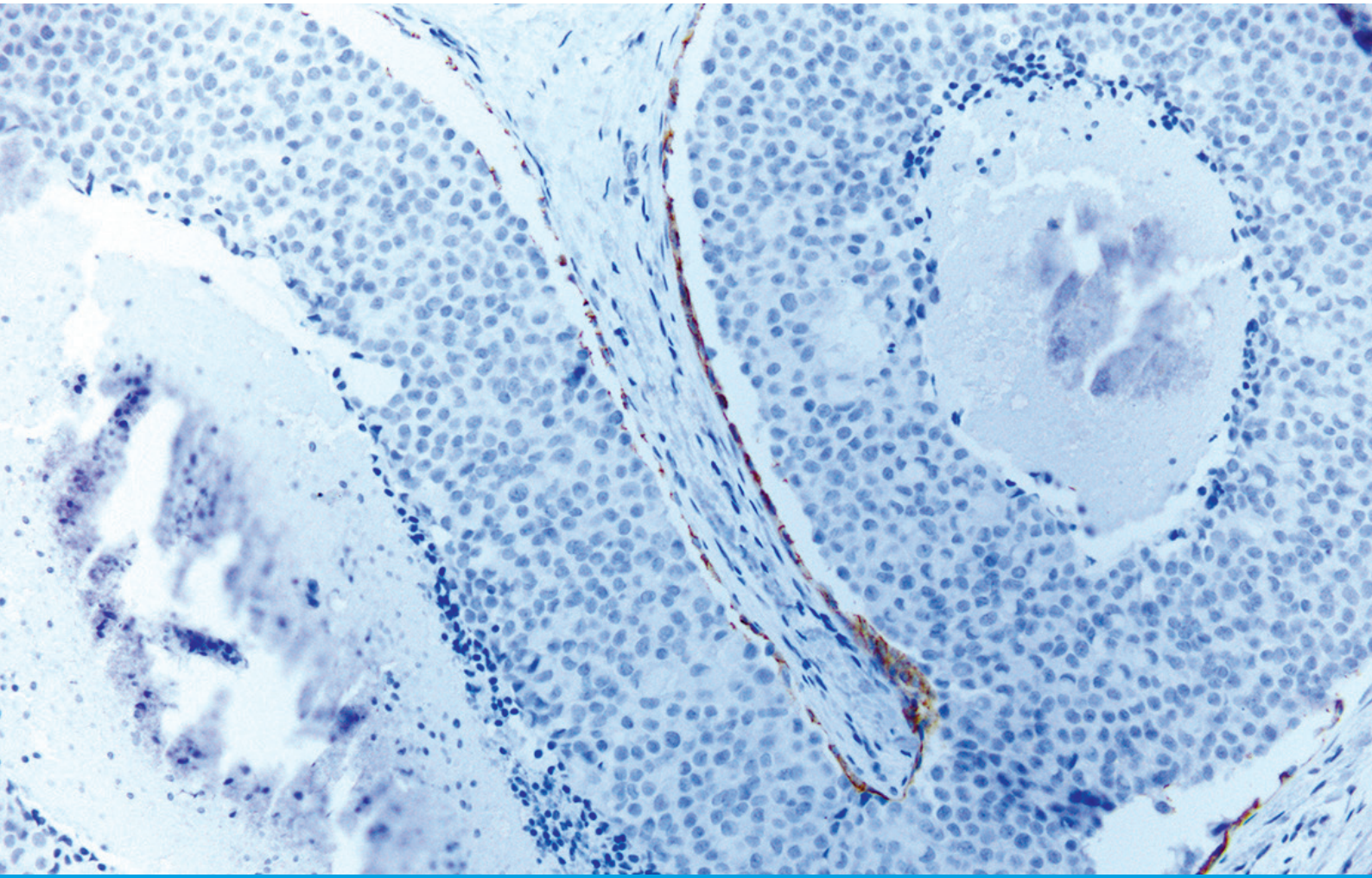

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Ping Tang MD, PhD

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Ping Tang, MD, PhD Obtained her medical degree from West China University of Medical Science in Chengdu, Sichuan, China. She then spent three years as a visiting research fellow at Harvard Medical School. In 1995, she received her PhD on Cancer Biology from University of Texas MD Anderson Cancer Center in Houston, TX. After spending three years of post-doctoral training at the same institution, she moved on to her 4 year AP-CP pathology residency training at North Shore University Hospital in Long Island and I year fellowship training at Yale New Haven Hospital, CT. She was recruited to the Department of Pathology and Laboratory Medicine at the University of Rochester Medical Center in 2003.

Currently Dr. Tang is the professor of pathology, and director for Breast Pathology and Breast Pathology Fellowship Program at University of Rochester Medical Center. Her current research interests focus on panels of IHC predicting markers for high risk ductal carcinoma in situ and bone metastasis of breast carcinoma. Dr. Tang has co-authored almost 70 journal articles that have appeared in a variety of journals, including Human Pathology, Archives of Pathology and Laboratory Medicine, Cancer Investigation, Ann Surgical Oncology, Histopathology, etc. She has been invited to speak at a variety of International and National meetings on the topic of breast cancer and breast pathology, and was served as a faculty member for CAP training courses for ER,PR,HER2 testing.

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Christine Desmedt received her bio-engineer degree in Cells and Genes Biotechnology from the Catholic University of Leuven, Belgium, in 2000. Since 2000 she is working at the Jules Bordet Institute, an autonomous comprehensive cancer centre devoted entirely to the fight against cancer. For two years she worked as a clinical monitor for the Breast European Adjuvant Studies Group (Br.E.A.S.T), co-coordinating the monitoring activities of external groups for the conduct of breast cancer trials. In 2003, she joined the Breast Cancer Translational Research Laboratory of this Institute, headed by Christos Sotiriou, where she started a PhD entitled "Multimarker approach for improving breast cancer treatment tailoring". In 2004 she earned a master in bio-medical sciences at the Free University of Brussels and she defended successfully her PhD in 2008. Since then she is acting as the Translational Research Coordinator of the lab, conducting research projects and assisting the head of the lab in the scientific and administrative management of the lab. Her projects involve identification and validation of prognostic and predictive markers in breast cancer, as well as a better characterization of breast cancer development and metastasis, with a strong interest in multifocal and lobular breast cancer. She received grants from the MEDIC Foundation, the "Fonds National de la Recherche Scientifique", the Fondation Lambeau-Marteau, and the Fonds Heuson. She is also actively involved in several EU-projects. Christine Desmedt has received awards from the AACR and ASCO

David G. Hicks, MD

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David G. Hicks, MD, is currently the director of Surgical Pathology at the University of Rochester Medical Center. Dr. Hicks is a western New York native and earned his medical degree from the University of Rochester School of Medicine and Dentistry. His postgraduate training includes an internship in internal medicine and a residency in anatomic pathology at the Hospital of the University of Pennsylvania in Philadelphia. He has held faculty positions at Cleveland Clinic Foundation, Roswell Park Cancer Institute and the University of Rochester Medical Center.

Dr. Hicks' innovative approaches to improving efficiency and quality in surgical pathology have been highlighted in publications and invited lectures sponsored by national pathology organizations. Dr. Hick's current research interests focus on the molecular genetic profiling of clinical samples from patients with cancer. Much of his recent work has involved a search for new molecular genetic markers to help better understand the prognosis and guide therapeutic management of breast cancer. Dr. Hicks participated in the ASCO/CAP ER/PgR Guideline panel and served as co-chair of the 2013 ASCO/CAP HER2 Testing Guideline. He has co-authored a text book on Breast Pathology, authored or co-authored numerous book chapters and more than 160 peer-reviewed articles that have appeared in a variety of journals. Dr. Hicks is active in both the College of American Pathologists and the United States & Canadian Academy of Pathology and has presented numerous work shops and platform presentations for both organizations.

Reviewed by Professor Giuseppe Viale, M. D. F. R. C. Path, Director of the Division of Pathology at the European Institute of Oncology,Milano, Italy

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Summary:

Breast cancer is a leading cause of cancer-related morbidity and mortality among women worldwide, with more than 522,000 estimated deaths due to breast cancer expected annually. Despite this significant global disease burden, there has been an encouraging decline in mortality from breast cancer over the last several decades, which has been attributable to a number of complex factors including public education and important therapeutic advances based on an evolving understanding of tumor biology (1). What has emerged is the realization that "breast cancer" consists of a group of heterogeneous tumor types with varied morphology, biology and response to therapies (2). In recent years, profiling of breast carcinomas using immunohistochemistry (IHC) and other advanced biomarker assays has assumed an increasingly important role in breast cancer diagnosis and treatment. IHC analysis for various biomarkers can be used as a tool to aid in the diagnosis of breast cancer and can also provide important prognostic and predictive information related to tumor biology and disease subtypes. More recently, with the emergence of the application of genomics in medicine, IHC analysis has been used as a surrogate for the molecular classification of breast cancer tumors, and helps to stratify patients into clinically meaningful subsets. It has also played a role in the search for new prognostic and predictive markers, and therapeutic targets. This review will discuss the evolution of our understanding of breast cancer, the development of clinically meaningful biomarkers and ancillary testing as well as the potential and pitfalls of the application of these biomarkers in breast cancer diagnosis and treatment planning.

A Historical perspective

Historically, breast cancer has been thought of as a single disease with a "one size fits all" approach to treatment, which worked well for some patients but not at all for others. With our increasing understanding of the molecular alterations that drive disease progression and an ever expanding menu of therapeutic options, our approach to the diagnosis and treatment of breast cancer has been profoundly changed. A major clinical task for treatment planning of breast cancer patients has been the identification of which patients are more likely to develop a recurrence of their disease so that the most appropriate treatment regimen can be employed. This challenge is directly related to the fact that "breast cancer" is a biologically diverse disease (or group of diseases) (3). Currently, the main source of information that can be used to assess the recurrence risk and the clinical course of the disease comes from the careful evaluation of the primary resected tumor from a patient. A number of validated patient factors and tumor-related features, including patient age, menopausal status, tumor size (4), histologic type (5), histologic grade (6), measures of proliferation (7), lymphovascular invasion (8), lymph node staging (9) and evidence of distant metastasis, are used clinically and represent the starting point for initial decisions about tumor staging, diagnosis and treatment. In addition to these pathologic metrics, prognostic and predictive biomarker testing for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2) play a critical role in decisions on adjuvant therapy. Some of the more important pathologic metrics in breast cancer are commented on briefly below.

Tumor size

The size of the primary tumor (greatest diameter measured in centimeters) is used in assessing the patient's tumor burden and has been shown to be an independent prognostic factor; it is significantly correlated with the number of involved lymph nodes and risk for recurrence (4, 10). After lymph node staging, tumor size is the second most important traditional prognostic factor that is routinely used to make decision on adjuvant therapy. The tumor size is determined by clinical examination, imaging and pathological evaluation, and used for tumor staging.

Nodal status

Nodal status is the most robust and reliable pathologic prognostic factor for breast cancer patients. There is a direct linear relationship between the number of involved lymph nodes and the risk of distant recurrence (9, 11). The use of sentinel lymph node biopsy has become the standard of care for axillary staging, replacing axillary dissection, which has a higher potential for morbidity (lymphedema) (12). The Z11 trial demonstrated a subgroup of patients with low axillary disease burden who did not benefit from axillary lymph node dissection (ALND) at short-term follow-up when treated with adjuvant whole-breast radiotherapy and systemic therapy (13).

Histologic grade

Histologic grading is routinely used by pathologists to help stratify breast cancer patients into favorable (low-grade or well differentiated tumors) and unfavorable (high-grade or poorly differentiated tumors) outcome groups (Figure 1). A widely used system for the histologic grading of breast cancers is the Elston and Ellis modification of the Scarff-Bloom-Richardson score (Nottingham grading system), based on the degree of tubular formation, nuclear atypia/pleomorphism and the mitotic index (6) (Figure 1). Studies have validated and confirmed the prognostic significance of histologic grading, as long as the morphologic criteria used for grading are strictly followed (14, 15). Numerous studies (16) have shown that Nottingham grading provides important clinical information in breast cancer and is significantly correlated with the underlying tumor characteristics, prognosis and tumor biology (Table1).

Immunohistochemistry: Diagnostic marker applications in breast pathology

The diagnostic evaluation in breast pathology for cancer and other benign processes remains grounded in the careful evaluation of tissue morphology from high quality hematoxylin and eosin (H&E) stained sections. However, with advancements

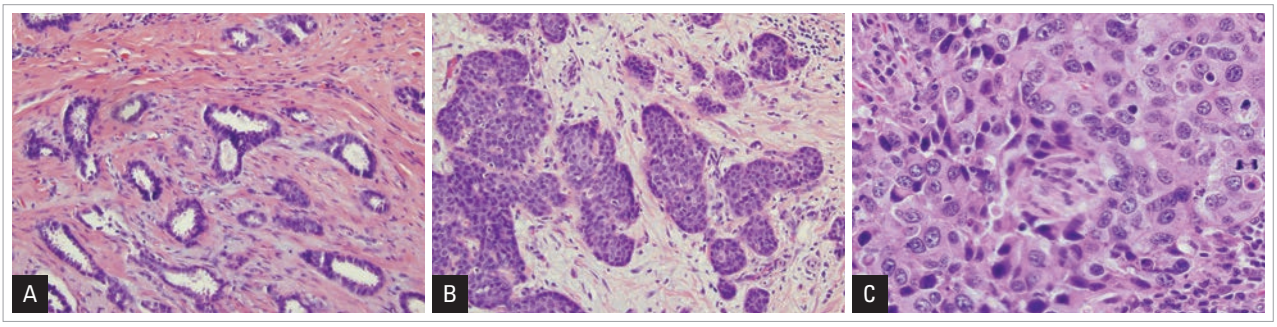


Figure 1: Examples of grades 1-3 breast carcinomas
Histologic grading is routinely used by pathologists to help stratify breast cancer patients into favorable (low-grade or well differentiated tumors) and unfavorable (high-grade or poorly differentiated tumors) outcome groups. Based on the degree of tubular formation (1-3), nuclear atypia/pleomorphism (1-3) and mitotic index (1-3), breast cancer can be histologically graded as grade 1 (3-5/9), grade 2 (6-7/9) and grade 3 (8-9/9). The morphologic features for each histologic grade are shown here: **(1A)** grade 1, **(1B)** grade 2, and **(1C)** grade 3 (H&E stained sections).

Table 1: Breast Cancer Histologic Grade and Tumor Characteristics

Histologic Grade	ER, PR, HER2	Proliferation	TP53	DNA copy number changes	Gene expression profiling
Low-grade	Typically ER/PR(+) & HER(-)	Low proliferative index	Normal function (p53 IHC negative)	Fewer copy number changes; most common changes are losses on 16q and gains on 1q	Most likely Luminal-A, some luminal-B profile
High-grade	Typically low or negative ER and more likely HER2(+) or triple negative (negative for ER, PR and HER2)	High proliferative index	Loss of function (p53 IHC positive)	More frequent, extensive, and complex chromosomal alterations. Gains are often on 8q, 17q, and 20q and losses are on 17p, 1p, 19p, and 19q	Luminal-B, HER2 enriched and Basal-like profiles

ER – estrogen receptor, PR – progesterone receptor, HER2 – human epidermal growth factor receptor-2

in our understanding of tumor biology and the development of new treatment options, pathologists have become increasingly important members of the multidisciplinary patient care team. Pathologists not only provide a pathologic diagnosis, but also deliver prognostic and predictive information about the patient's tumor. IHC analysis has assumed a critical role in clarifying the diagnosis in challenging cases and resolving differential diagnoses. Moreover, IHC testing of ER, PR, HER2 and other markers can provide information useful for planning targeted therapies in the clinical setting. Furthermore, IHC can be used as a surrogate for the molecular classification of breast cancer into different molecular subtypes, and aid in the identification of new prognostic and therapeutic targets. Below, we will discuss each of these areas in detail. While IHC and other ancillary studies have proven to be useful for diagnosis and treatment planning, it is important to remember that the results of these assays should always be interpreted within the clinical and morphologic context for each patient's lesion.

Differential diagnosis between in situ and invasive breast cancer

Most of the time in situ breast carcinoma can be easily differentiated from invasive breast cancer with a careful microscopic evaluation of an H&E stained section. Occasionally, however,

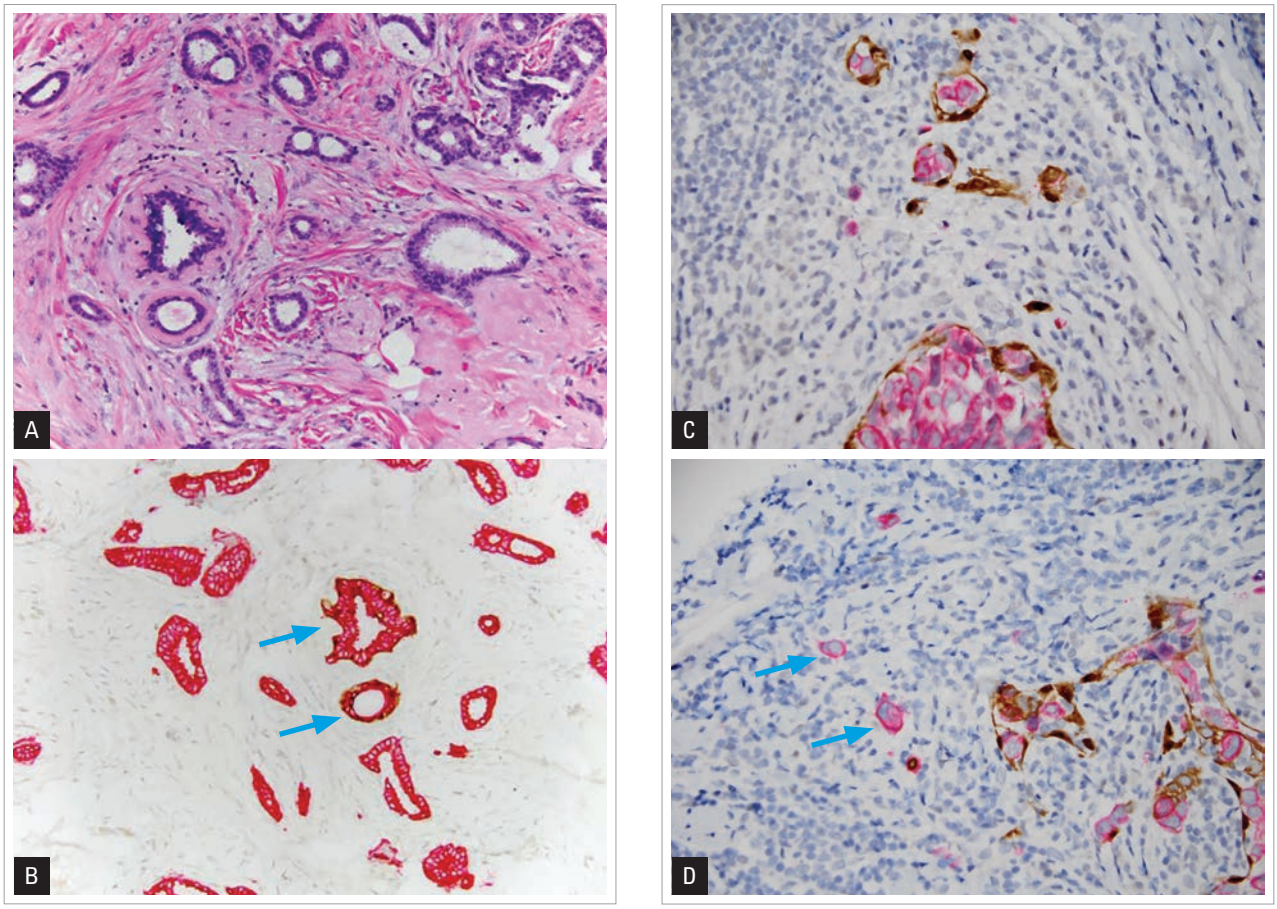


Figure 2: H&E and IHC for invasive ductal carcinoma and microinvasive carcinoma
The loss of peripheral myoepithelial cells is the hallmark of diagnosing invasive breast carcinoma. **2A** shows a well differentiated invasive ductal carcinoma H&E stain. **2B** shows IHC analysis for cytokeratin and p63 (CK8/18 red chromogen, CK5/15, and p63 brown chromogen), which demonstrates the loss of myoepithelial markers around the neoplastic glands. The two arrows point at the two benign glands in the center that retain myoepithelial cells.
Microinvasive carcinoma is defined as invasive carcinoma of ≤1mm in size. It's often associated with high grade DCIS with marked desmoplastic changes. **2C** and **2D** are examples of IHC analysis for CK8/18, CK5/15, and p63, showing a few single cells with loss of myoepithelial markers (arrow).

this distinction can be quite challenging. The fundamental difference between in situ and invasive disease is tumor cell invasion beyond the duct space and basement membrane into the surrounding stroma with loss of peripheral myoepithelial cells associated with the infiltrating carcinoma. In this regards, the ability to highlight the myoepithelial cell layer by IHC analysis for myoepithelial markers has become an important adjunct for establishing the correct diagnosis. The most commonly used myoepithelial markers include p63, calponin, smooth muscle myosin heavy chain (SMMHC), smooth muscle actin (SMA), high molecular weight cytokeratins (HMW CKs) (Figure 2a, b); collagen IV and laminin have also been used by some as well (18). For microinvasive carcinoma (≤1 mm), the addition of keratin staining may be helpful as well to highlight the microscopic foci of invasive tumor cells and will complement the lack of an associated myoepithelial cell layer with microinvasion (Figure 2c, d). Immunostains for collagen IV and laminin may be problematic since in situ lesions may show variable loss of their expression, which can be carried over to this minute invasive foci with partial staining (19). A number of these antibodies are quite sensitive for myoepithelial cells, including SMMHC, SMA, calponin and p63; however, they have varying specificities and may react with other cells types such as myofibroblasts. Consequently, the use of more than one antibody such as p63 (nuclear reactivity) and SMMHC or HMWCKs (cytoplasmic reactivity), which are complimentary, can help to improve the specificity for the identification of the myoepithelial cell component.

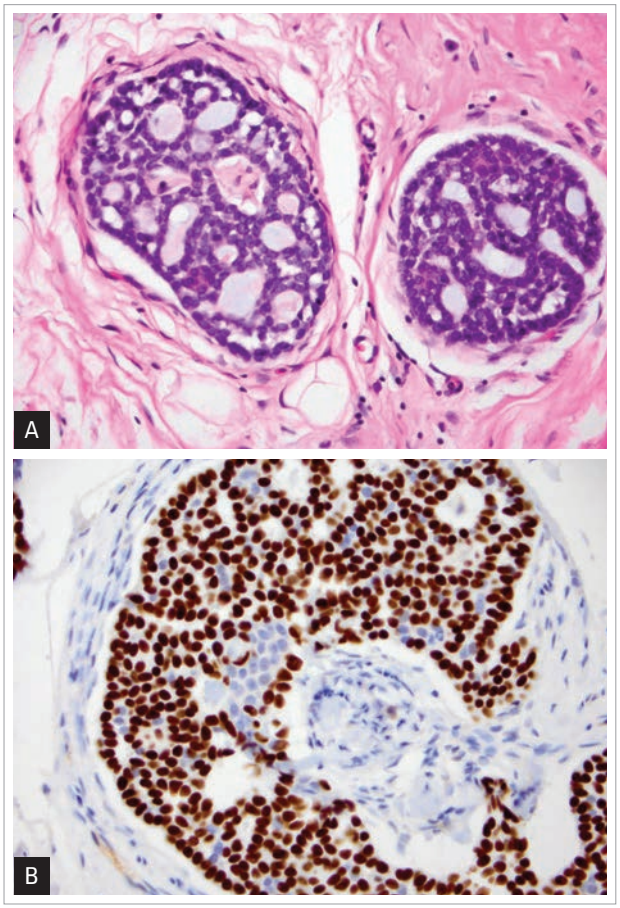


Figure 3: H&E and IHC analysis for adenoid cystic carcinoma and metaplastic carcinoma
Figures **3A** and **3B**: show that adenoid cystic carcinoma (ACC) consists of epithelial cells and myoepithelial cells (3A - H&E), in which the myoepithelial cells are part of the tumor and are positive for the myoepithelial marker p63 (3B). Note the p63 positive cells are part of the tumor, not at the periphery of the gland as we would see in DCIS.
(continues)

Exceptions:

1. Invasive lesions with myoepithelial markers

Both adenoid cystic carcinoma (ACC) and metaplastic carcinoma (MC) consist of tumor cells that are positive for myoepithelial markers; so the presence of these cells is not indicative of an in situ lesion. In these cases a careful evaluation of their expression patterns, i.e. the location and topographic distribution of myoepithelial marker positive tumor cells, is critical for making a correct diagnosis. ACC consists of epithelial cells (forming true glandular spaces) and myoepithelial cells (forming pseudo glandular spaces), which are positive for myoepithelial markers (20) such as SMA, p63, calponin and HMW CK (Figure 3a, b). Metaplastic carcinoma consists of glandular epithelial tumors with differentiation of squamous and/or mesenchymal elements, which can be highlighted with p63, high molecular weight keratins and other myoepithelial markers (Figure 3c, d).

2. Benign lesions without myoepithelial markers

Microglandular adenosis (MGA) is a benign breast lesion forming small round glands with an open lumen and a thickened basement membrane; it lacks myoepithelial cells and frequently presents as an infiltrative lesion. Thus, it is necessary to differentiate it from invasive breast cancer, namely, tubular car-

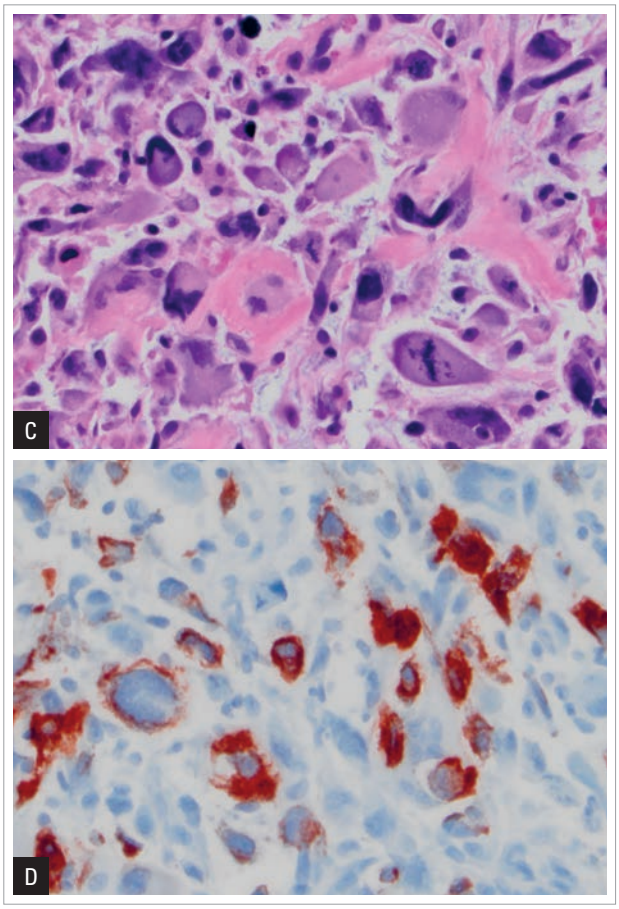


Figure 3: *(continued)*
Figures **3C** and **3D**: show that metaplastic carcinoma can consist of tumor cells with marked pleomorphism **(3C - H&E)** and mesenchymal differentiation. These neoplastic cells are highlighted with CK5/6, a high molecular weight keratin **(3D - CK5/6)**, supporting the diagnosis of metaplastic carcinoma. Note the CK5/6 positive cells are part of the tumor and do not show the same topographic distribution and localization that would be expected for myoepithelial cells.

cinoma. For suspected cases of MGA, the assessment of ER, PR, p63 and S100 expression is recommended to help make the important distinction between MGA and tubular carcinoma. MGA should be negative for ER, PR and p63, is often strongly positive for S100 (Figure 4a-d); while tubular carcinoma should be ER, PR positive and negative for p63 and S100. (21)

Differential diagnosis of lobular lesion vs. ductal lesions

Since lobular and ductal carcinoma have different clinical behaviors and different clinical implications, the differentiation between these two lesions is clinically important, especially when considering the choice of imaging detection (MRI vs. mammogram), potential patterns of recurrence and surgical management options. Ductal carcinoma tends to be unifocal, with distant metastasis to liver, lung and brain, while lobular carcinoma tends to be multifocal, bilateral with distant me-

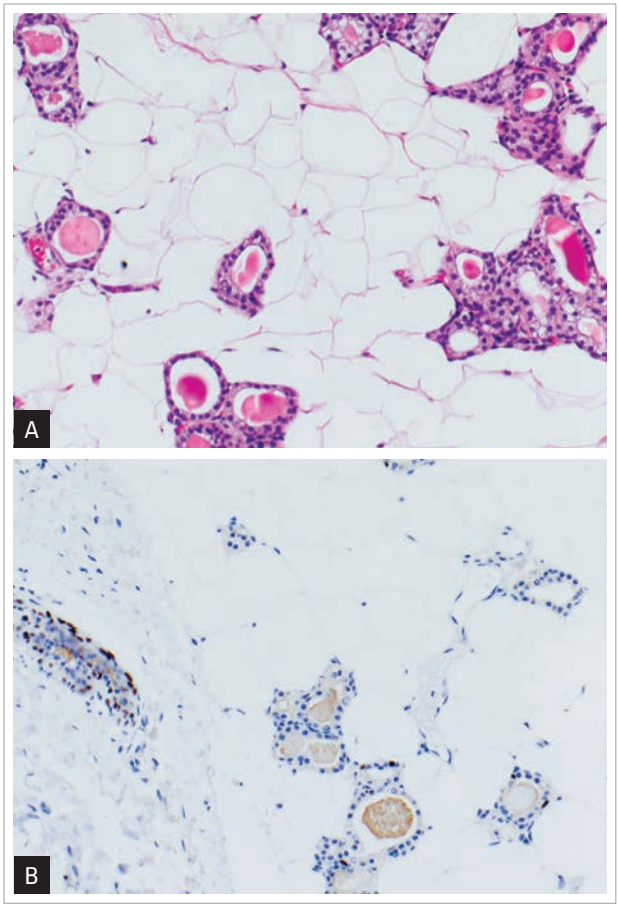
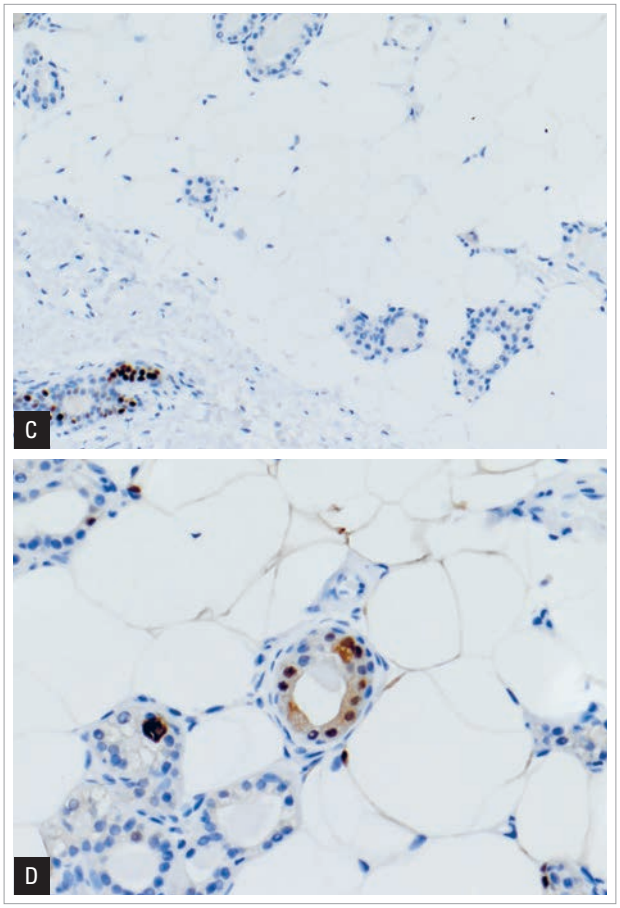


Figure 4: H&E and IHC for microglandular adenosis
Microglandular adenosis (MGA) is a benign breast lesion forming small round glands with an open lumen and a thick basement membrane; it lacks myoepithelial cells and frequently presents as an infiltrative lesion **(4A)**. Thus, it is necessary to differentiate it from invasive breast cancer, namely, tubular carcinoma. Assessment of ER, PR, p63 and S100 expression should be helpful in distinguishing tubular carcinoma from MGA. MGA should be negative for p63 **(4B)**, ER **(4C)**, and is often positive for S100 **(4D)**.



tastases to serosal surfaces, the gastrointestinal (GI) tract and organs in the gynecologic (GYN) systems. Although both ductal and lobular in situ carcinomas are considered to be non-obligate precursors for their invasive counterparts, the rates of developing into invasive carcinoma is much lower for lobular lesions compared to ductal lesions.

E-cadherin

One of the most consistent molecular changes in lobular lesions is the loss of expression of E-cadherin, a cell-to-cell adhesion protein, which contributes to the discohesiveness of these tumor cells. E-cadherin is a calcium-dependent transmembrane protein that plays a functional role in intracellular adhesion and cell-polarity. E-cadherin binds the actin cytoskeleton through interactions with the catenin complex, including p120 alpha, beta and gamma catenin, and its loss affects cellular adhesion, motility and possibly cellular proliferation. E-cadherin expression is lost in both in situ and invasive lobular lesions, but is retained in ductal lesions. It has become an important diagnostic marker for differentiating ductal vs. lobular lesions, especially for those cases with ambiguous morpholgy (22). Before the discovery of E-cadherin, the differentiation between lobular and ductal lesions was entirely based on morphologic features, namely, the single file growth pattern and loss of cohesiveness in neoplastic lobular lesions on H&E slides. The use of E-cadherin IHC analysis is especially helpful in the diagnosis of two variants of lobular carcinoma in situ (LCIS), pleomorphic lobular carcinoma in situ (23, 24) (Figure 5a, b) and solid LCIS

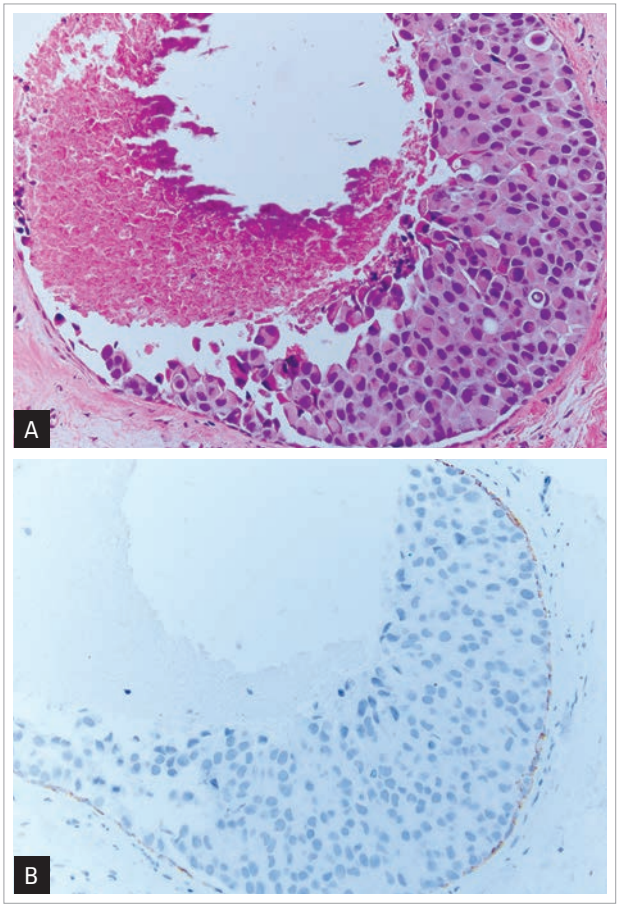
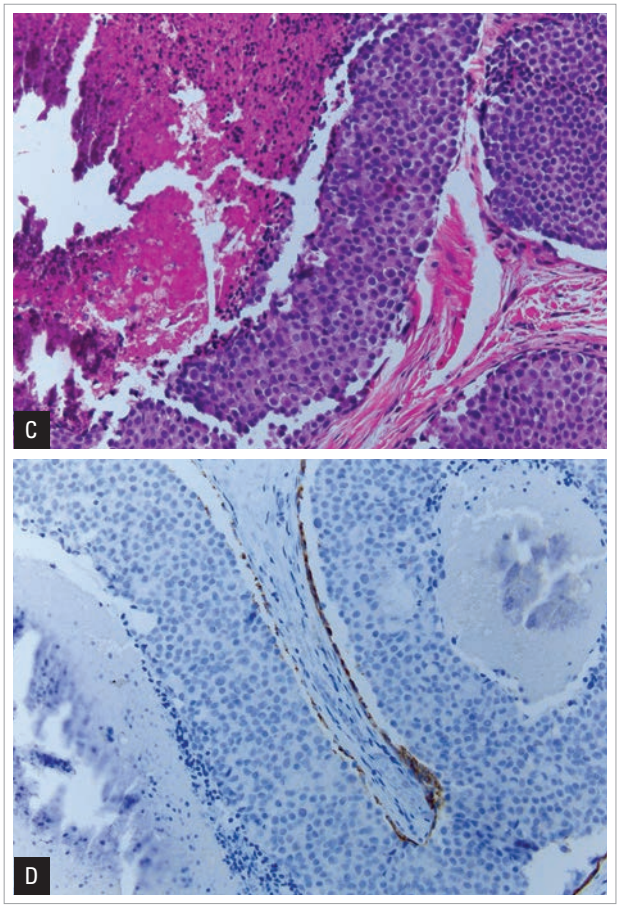


Figure 5: E-cadherin for lobular carcinoma in situ
One of the most consistent molecular changes in lobular neoplastic lesions is the loss of expression of E-cadherin. The use of E-cadherin IHC analysis is especially helpful in the diagnosis of two variants of lobular carcinoma in situ (LCIS), pleomorphic lobular carcinoma in situ and solid LCIS with central necrosis (Florid LCIS). Example of H&E **(5A)** and loss of E-cadherin stain **(5B)** for pleomorphic lobular carcinoma in situ. Example of H&E **(5C)** and loss of E-cadherin stain **(5D)** for florid lobular carcinoma in situ. Note E-cadherin is positive for myoepithelial cells surrounding the in situ carcinoma **(5B, 5D)**.



with central necrosis (Florid LCIS, 25) (Figure 5c, d). Both lesions have more genetic changes compared to classic LCIS, and seem to have a more aggressive clinical course.

P120 catenin (P120)

While the loss of E-cadherin expression provides evidence for a lobular neoplastic lesion (Figure 6a and b), it can be difficult to interpret in some cases, especially for invasive lesions with sparse single cells. P120, which is part of the e-cadherin/catenin membrane complex, demonstrates membrane staining for ductal neoplastic lesions. In lobular carcinomas, the loss of e-cadherin leads to the release of P120 from the membrane complex, resulting in diffuse cytoplasmic staining for lobular lesions (26). Therefore, the combination of loss of e-cadherin membrane expression along with diffuse cytoplasmic staining for P120 can be a helpful adjunct in the diagnosis of in situ and invasive lobular carcinomas. (Figure 6c and d)

Exception

Occasionally, E-cadherin can show aberrant expression in lobular lesions (27), although these lesions carry an E-cadherin gene mutation and protein dysfunction (Figure 7a-d). Thus, interpretation of the staining results in such cases must be correlated with H&E morphology to help ensure a correct diagnosis. For such cases, evaluation of P120 may be helpful and the

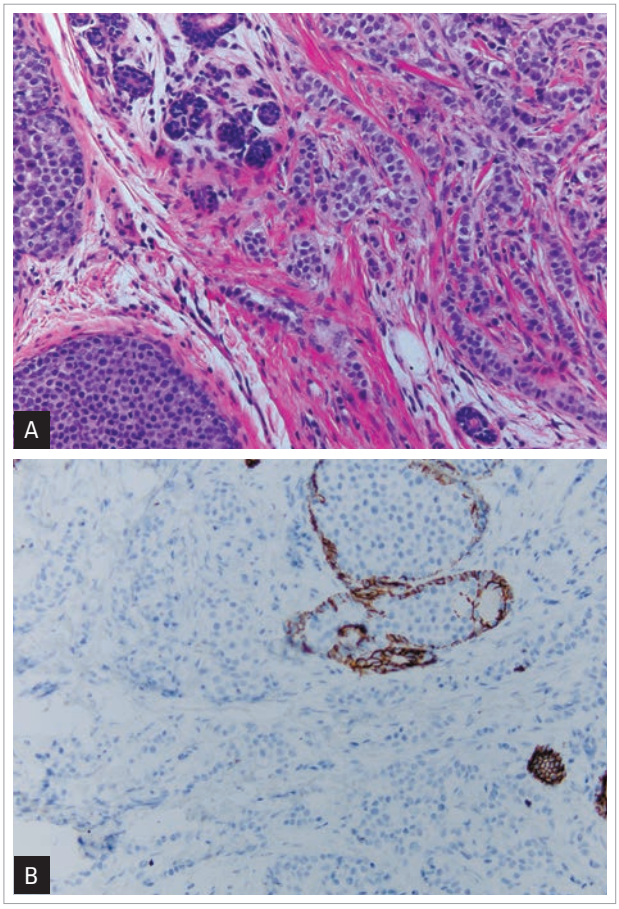
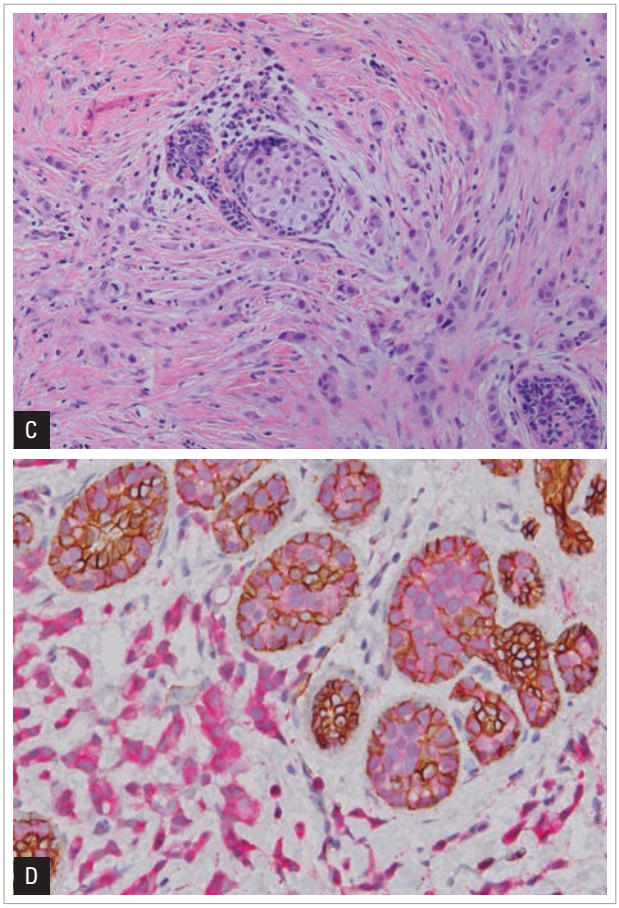


Figure 6: E-cadherin and p120 for invasive lobular carcinoma
P120 is part of the e-cadherin/catenin membrane complex and demonstrates membrane staining for ductal neoplastic lesions. In lobular carcinomas, the loss of e-cadherin leads to the release of P120 from the membrane complex, resulting in diffuse cytoplasmic staining for lobular lesions. **6A** and **6B** show an example of invasive lobular carcinoma (A-H&E) with loss of expression for E-cadherin (B). **6C** and **6D** show another example of invasive lobular carcinoma (6C-H&E) with strong cytoplasmic stain of p120 (6D, red chromogen). This double stain highlights E-cadherin (brown) in residual duct spaces that are partially involves by lobular carcinoma in situ (loss of e-cadherin and cytoplasmic staining for p120).



lesion should not be reclassified as a ductal lesion solely based on the presence of E-cadherin expression.

Differential diagnosis of usual ductal hyperplasia (UDH) versus atypical ductal hyperplasia (ADH)/low-grade ductal carcinoma in situ (LG-DCIS)

UDH and ADH/LGDCIS are biologically distinct intraductal epithelial proliferative lesions with different clinical implications in terms of the relative risk for the subsequent development of carcinoma. Because UDH and ADH/LG-DCIS carry different implications for clinical management and subsequent cancer risk, the ability to accurately distinguish between these two diagnostic possibilities is important. UDH consists of a heterogeneous proliferation of mixed populations of cells, including epithelial cells, myoepithelial cells, or even apocrine metaplastic cells with an architectural pattern of irregular "slit-like" spaces. It is not currently considered as a precursor lesion, and has a minimal increased risk (1.5-2 fold over the general population) for subsequent development of invasive carcinoma (28). On the other hand, ADH/LG-DCIS is a proliferation of a low-grade monotonous population of cells showing rigid punched out space, with a significantly higher risk (3-5 fold over the general population) of subsequent invasive carcinoma development (29).

IHC analysis for differential cytokeratin staining can be helpful in difficult cases, especially in cases of UDH with the presence of rare foci of necrosis or mitosis. Since UDH consists of mixed

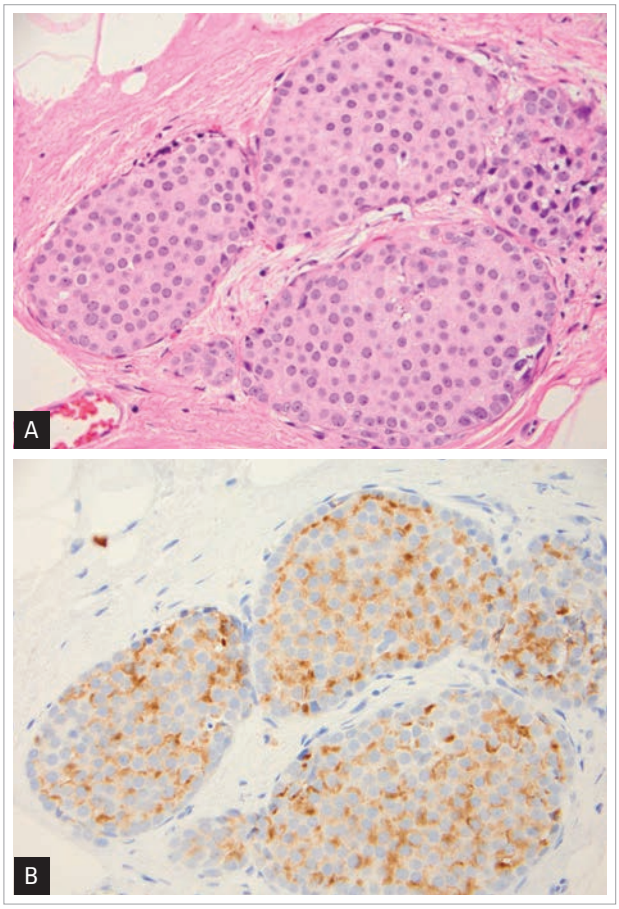
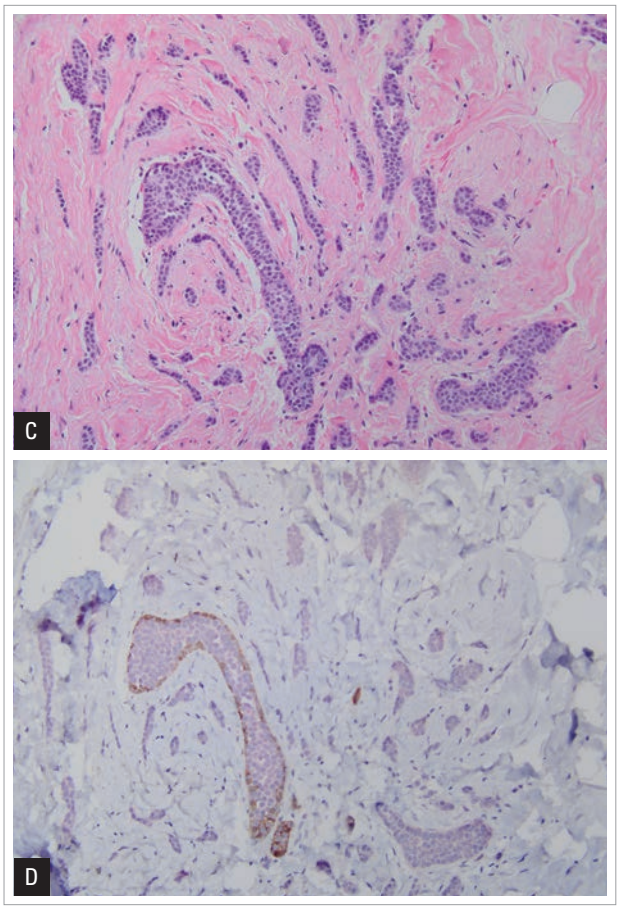


Figure 7: Aberrant expression of E-cadherin for in situ and invasive lesions
The interpretation of the E-cadherin immunohistochemistry staining results must be correlated with H&E morphology **(7A and 7C)**. E-cadherin can show aberrant expression (marked reduced membrane staining or some degree of cytoplasmic staining) in lobular lesions **(7B and 7D)**.



populations of different cell types, they show a mixed phenotype for low (CK7, CK8, CK18, luminal cytokeratins) and high molecular weight keratins (CK5, CK14, or CK17, basal cytokeratins) and for ER, demonstrating a heterogeneous or mosaic staining pattern (30). This admixture of different cell types, highlighted by IHC, is consistent with a polyclonal hyperplasia in UDH (Figure 8a, b). In contrast, the monomorphic cells of ADH are typically negative for high molecular weight keratins (CK5, CK14, or CK17) and instead show restricted luminal cytokeratin (CK7, CK8, CK18) expression along with high levels of ER expression, which is consistent with the clonal nature of these proliferative lesions (Figure 8c,d). Although differential cytokeratin staining can be helpful in the evaluation of difficult intraductal proliferative lesions, these results must be carefully interpreted in the context of the morphologic findings. In addition, this differential staining is not useful for distinguishing ADH from LGDCIS, as both are clonal proliferations that share similar patterns of cytokeratin expression and molecular alterations. The major morphologic and immunophenotypic features that are helpful in making this important diagnostic distinction are summarized in Table 2.

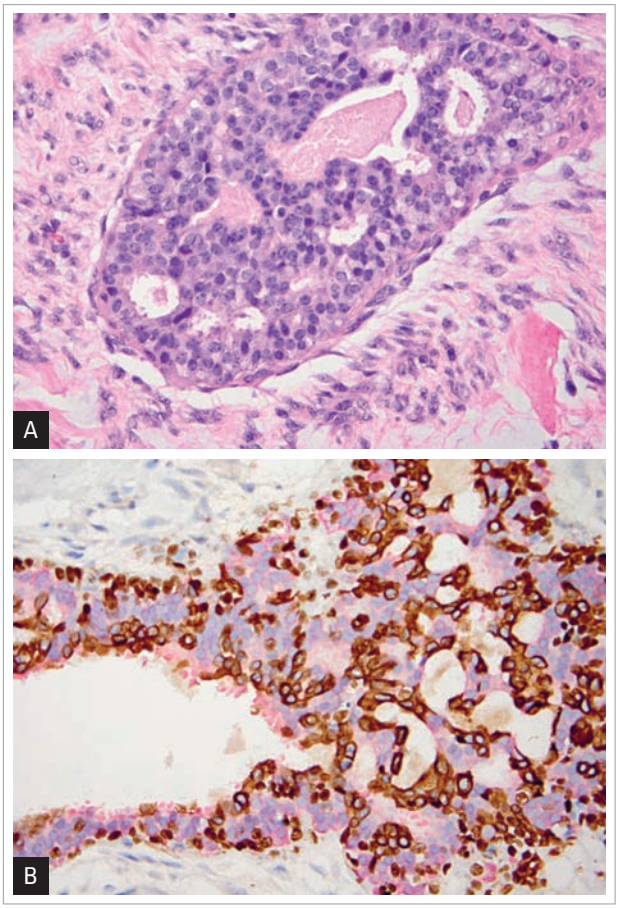


Figure 8: Differential cytokeratin staining in usual ductal hyperplasia, atypical ductal hyperplasia/low grade DCIS, and basal-like DCIS

Figures 8A and 8B: Usual Ductal Hyperplasia (UDH) consists of mixed populations of different cell types, and shows a mixed phenotype for low (CK8/18 - red) and high molecular weight keratins (CK5/17 and p63 - brown). This admixture of different cell types, highlighted by IHC, is consistent with a polyclonal hyperplasia in UDH.

Figures 8C and 8D: Atypical Ductal Hyperplasia (ADH) is typically negative for high molecular weight keratins such as CK5/17 and p63, and instead shows restricted luminal cytokeratin (8/18) expression, which is consistent with the monoclonal nature of these proliferative lesions. Normal myoepithelial cells can be seen at the periphery of this lesion.

Figures 8E and 8F: Basal-like DCIS will have diffuse CK5, 14 or 17 staining or patterns similar to those of UDH. These lesions, however, are usually high nuclear grade, with abundant mitoses and necrosis.

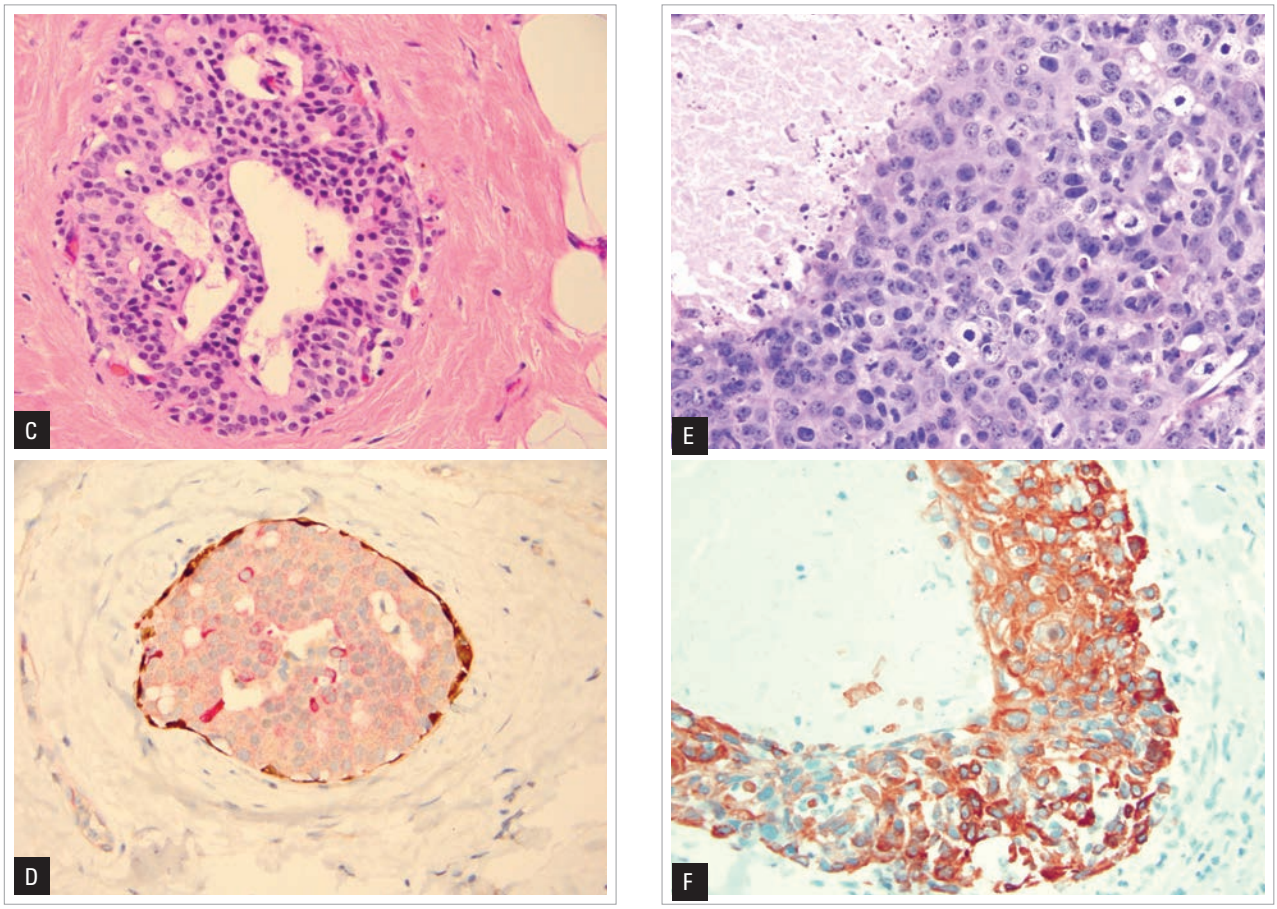


Table 2: Differential diagnosis for intraductal epithelial proliferative lesions

Features	Usual Epithelial Hyperplasia	Atypical Ductal Hyperplasia	Low-grade ductal carcinoma in situ
Cell populations	Heterogeneous(mixed cell population)	Variable (partially heterogeneous)	Monotonous (uniform, monomorphic population)
Cellular cytology	Variation in cell size, shape and orientation	Admixture of monotonous and heterogeneous cells	Monotonous, uniform small round atypical nuclei
Cell borders	Overlapping, borders poorly defined	Two populations, overlapping and well defined borders	Non-overlapping, evenly spaced, well defined borders
Architectural pattern	Solid, fenestrated, irregular 'slit-like' spaces	Solid, fenestrated, micropapillary, admixture of luminal space patterns	Solid, fenestrated, micropapillary, rigid/round punched out spaces ('cookie-cutter')
Clonality	Polyclonal	Monoclonal	Monoclonal
Basal cytokeratins: HMWCK (CK5/6, CK14, CK17, 34-beta-E12)	Diffuse &/or mosaic ('checkerboard') staining pattern	Usually negative, may be partially variable	Negative
Luminal cytokeratins (CK7, CK8, CK18)	Mosaic ('checkerboard') staining pattern	Usually diffusely positive, may be partially variable	Diffusely positive
Estrogen receptor	Variable, patchy expression	Usually diffusely positive	Diffusely positive

ER – estrogen receptor, PR – progesterone receptor, HER2 – human epidermal growth factor receptor-2

Exception

Basal-like DCIS can sometimes mimic UDH; it can have positive CK5, CK14 or CK17 staining or patterns similar to those of UDH. The neoplastic cells, however, are usually high nuclear grade, with abundant mitoses, necrosis and are negative for ER and PR (Figure 8e, f).

IHC evaluation of papillary lesions

Papillary lesions of the breast are characterized by an epithelial proliferation arising within the ductal/lobular system, supported by fibrovascular cores, with or without an intervening myoepithelial cell layer. They consist of a broad range of benign, atypical, in situ and even invasive lesions; and it is one of the most problematic areas in diagnostic breast pathology. Thus, IHC analysis has been routinely used as an aid in the diagnosis of these lesions. (Table 3)

Intraductal papilloma (IP)

IP is a benign papillary lesion characterized by fibrovascular cores lined by myoepithelial and epithelial cell layers. Morphologic evaluation on H&E slides is often sufficient for making the correct diagnosis of IP. The presence of myoepithelial cells and their distribution can be a helpful diagnostic feature. IHC analysis for myoepithelial markers such as p63, calponin, or smooth muscle myosin heavy chain can be very useful in confirming their presence in difficult cases. These markers will highlight the presence of myoepithelial cells along the fibrovascular cores, around ducts trapped in sclerosis and at the periphery of the lesions (Figures 9a, b, c, d), In sclerosing papilloma, there are often small ducts entrapped in a fibrotic stroma, mimicking invasive ductal carcinoma. IHC analysis for myoepithelial cells markers such as p63, HMWCKs or SMMHC can highlight the associated myoepithelial cell layer (Figure 9c, d).

Table 3: Differential diagnosis for papillary lesions

Features	Intraductal papilloma	Papilloma with atypia	Papillary DCIS	Encapsulated papillary carcinoma	Solid papillary carcinoma
Papillae architecture	Thick, 'club-like', may be infarcted	Thick or thin	Thin, delicate	Thin, delicate	Multinodular, solid growth
Myoepithelial cell markers; pheriphery	Present, surrounding lesion	Present, surrounding lesion	Present, surrounding lesion	Absent	May be absent, very focal or present
Myoepithelial cell markers within papillae	Present, often prominent	Usually present except in areas involved by ADH/ LGDCIS	Absent	absent	Absent
Foci of apocrine metaplasia	Frequent, may be prominent	May be present	Absent	Absent	Absent (may show mucinous features)
Basal cytokeratins: HMWCK (CK5/6, CK14, CK17, 34-beta-E12)	Diffuse &/or mosaic ('checkerboard') staining pattern in areas of hyperplasia	Usually present except in areas involved by ADH/ LGDCIS	Absent	Absent	Absent
Luminal cytokeratins (CK7, CK8, CK18)	Present, mosaic ('checkerboard') staining pattern in areas of hyperplasia	Present, prominent in areas involved by ADH/ LGDCIS	Diffusely present,	Diffusely present	Diffusely present
Estrogen receptor	Variable, patchy expression	Variable, patchy expression, prominent in areas involved by ADG/ LGDCIS	Diffusely positive	Diffusely positive	Diffusely positive
Neuroendocrine markers (synaptophysin, chromogranin, CD56)	Negative	Negative	Negative	Negative	~60-70% express neuroendocrine markers

ER – estrogen receptor, PR – progesterone receptor, HER2 – human epidermal growth factor receptor-2

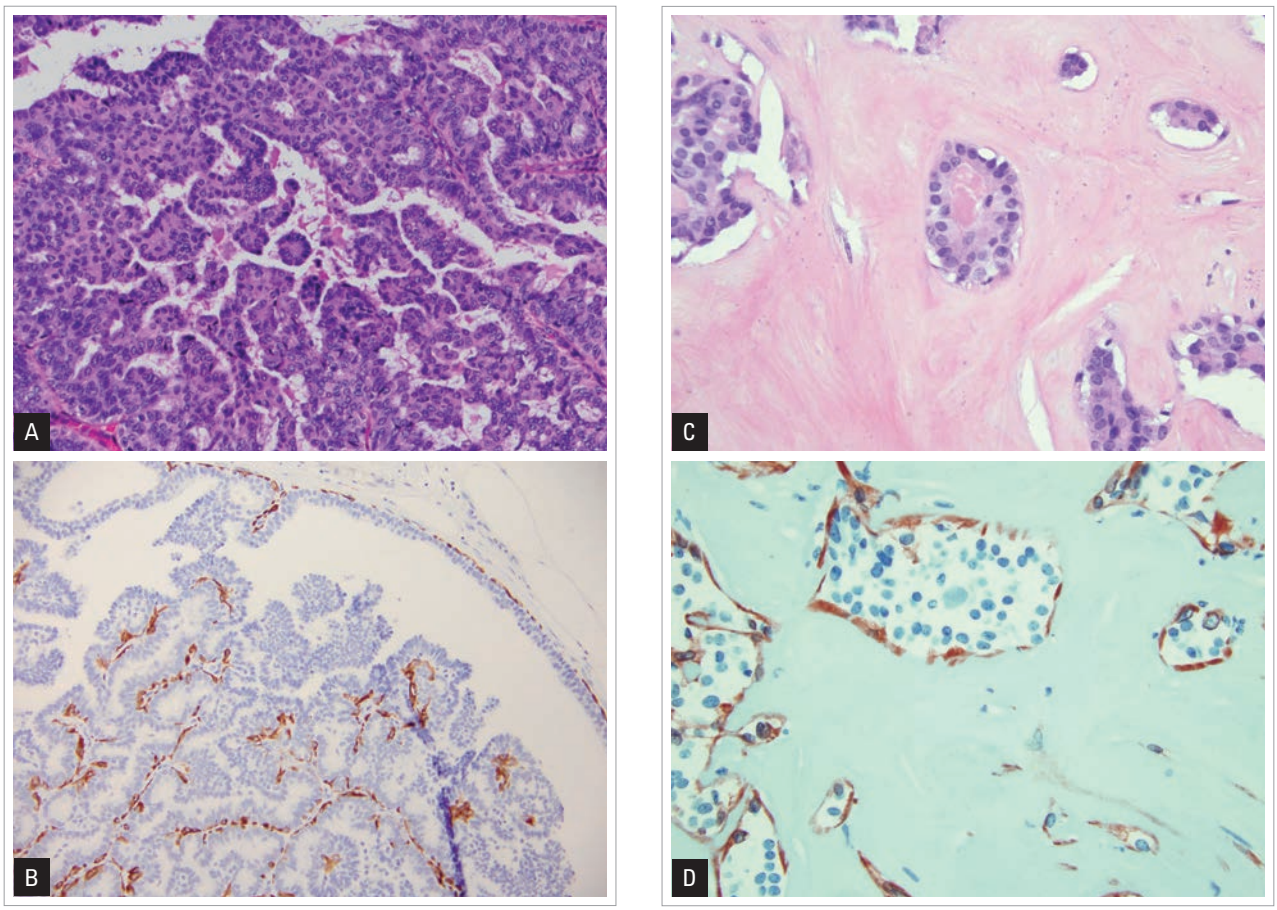


Figure 9: Calponin for intraductal papilloma
Intraductal papilloma (IP) is a benign papillary lesion characterized by fibrovascular cores lined by myoepithelial and epithelial cell layers. Morphologic evaluation on H&E slides is often sufficient for making the correct diagnosis of IP **(9A)**. The presence of myoepithelial cells and their distribution can be a helpful diagnostic feature. IHC analysis for myoepithelial markers (calponin) will highlight the presence of myoepithelial cells along the fibrovascular cores and at the periphery of the lesions **(9B)**. In sclerosing papilloma, there are often small ducts entrapped in a fibrotic stroma, mimicking invasive ductal carcinoma. IHC analysis with myoepithelial cells can highlight the associated myoepithelial cell layer **(9C & 9D)**.

Papilloma with ADH or DCIS

Intraductal papillomas may contain areas of atypia that would be diagnostic of ADH or DCIS when found elsewhere in the breast. These lesions are characterized by the presence of a focal population of monotonous cells with cytologic and architectural features of low-grade breast neoplasia. Myoepithelial cells are typically scant or absent in areas showing atypia. CK5 can be helpful to highlight the presence of florid UDH within an IP (Figure 10a, b). In contrast, atypical foci will show a lack of staining for high molecular weight cytokeratins (CK5/6) with restricted expression of luminal cytokeratins (CK7/8/18) (Figure 10c, d), along with a uniform high expression of ER (31). An IP with atypical foci can be classified as papilloma with ADH or papilloma with LG-DCIS depending on the size of the atypical proliferation; (<3 mm IP with ADH, ≥3 mm IP with DCIS) (5)

Intraductal papillary carcinoma (IPC)

IPC is a malignant non-invasive neoplastic epithelial proliferation with papillary architecture. These lesions are considered to be a de novo in situ papillary malignant process without a recognizable benign component in their background. They consist of slender fibrovascular cores covered by a single layer of monotonous neoplastic cells without the presence of associated

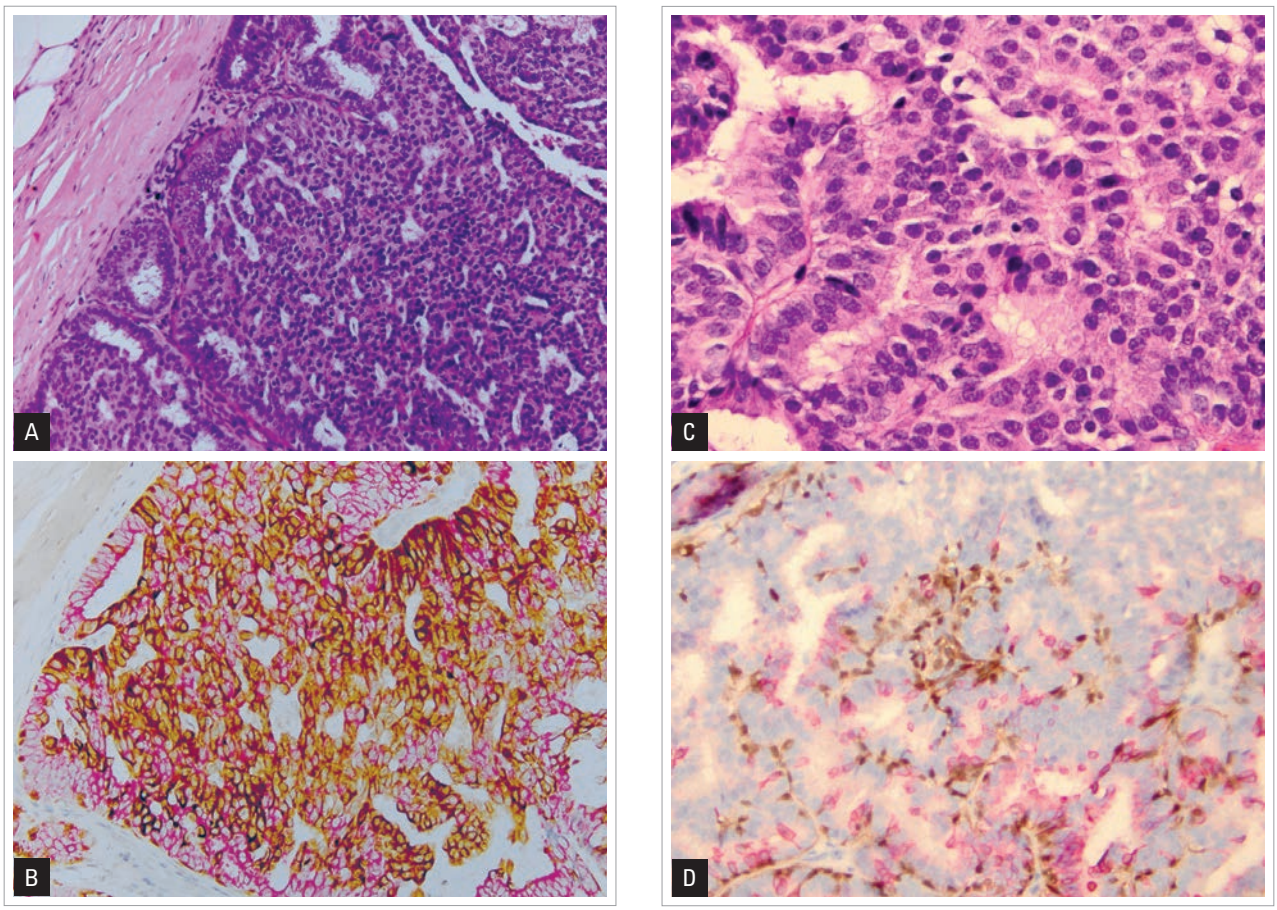


Figure 10: IHC for intraductal papilloma with usual ductal hyperplasia or atypical ductal hyperplasia
10A (H&E) and 10B (Differential cytokeratin stain). Occasionally CK5/14 can be useful to help highlight the presence of florid usual ductal hyperplasia (UDH), or occasionally, squamous metaplasia, within an intraductal papilloma.
10C (H&E) and 10D (Differential cytokeratin stain). Intraductal papillomas (IP) may contain areas of atypia that would be diagnostic of atypical ductal hyperplasia (ADH) or DCIS when found elsewhere in the breast. Atypical foci in IP will show a lack of staining for high molecular weight cytokeratins (CK5/6) with restricted expression of luminal cytokeratins (CK7/8/18) and myoepithelial cells are usually scant or absent in areas showing atypia.

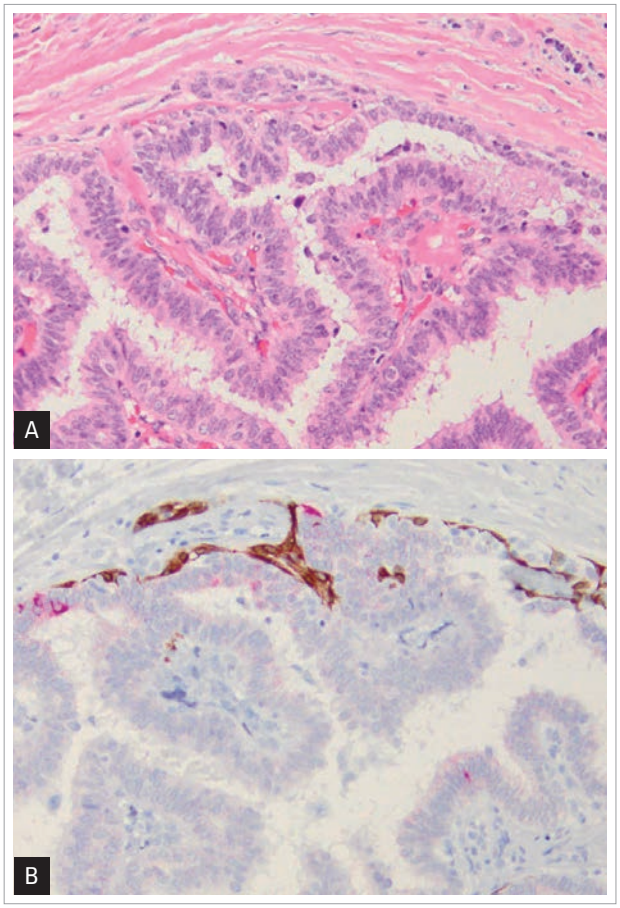
myoepithelial cells; however, the myoepithelial cells are retained at the periphery of the lesions, but often in a more attenuated form (Figure 11a, b). The neoplastic cells are usually low to intermediate nuclear grade, and are most often strongly positive for ER and PR as well as for luminal cytokeratins (CK7/8/18).

Encapsulated papillary carcinoma (EPC)

EPC is a variant of IPC, characterized by fine fibrovascular cores covered by low to intermediate nuclear grade neoplastic cells and surrounded by a fibrous capsule. These lesions lack myoepithelial cells both at the periphery of the lesion and within the fibrovascular cores (32, 33) (Figure 11c, d, e) Although it is currently staged and managed as Tis (5), some authors consider it to be a slow growing invasive lesion or a lesion in transition from in situ to invasive (34).

Solid papillary carcinoma (SPC)

SPC is a distinctive variant of papillary carcinoma with a solid growth pattern and inconspicuous fibrovascular cores. These lesions will typically have a single large, expansile mass or multiple solid closely opposed nodules, and may show spindle cell morphology and/or mucin production. The morphologic features of these tumors can suggest neuroendocrine differentiation and about 60-70% of these lesions express one or more



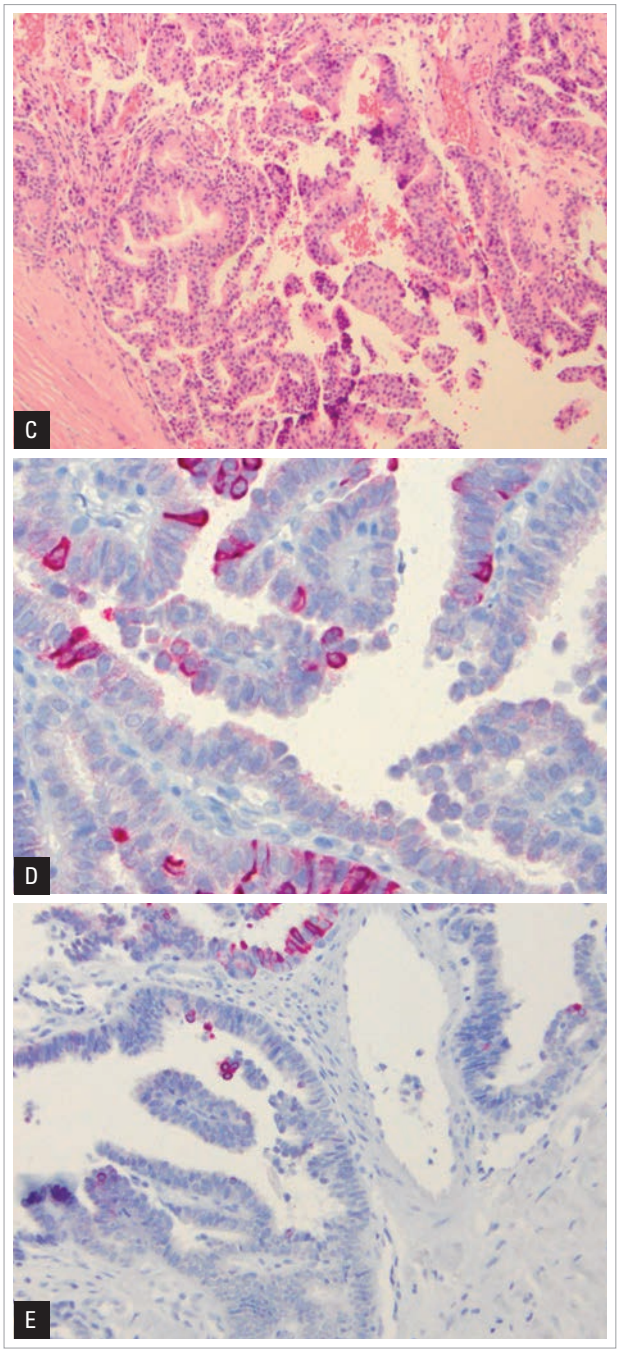


Figure 11: Cytokeratins and myoepithelial cell markers for intraductal papillary carcinoma and encapsulated papillary carcinoma
11A and 11B: Intraductal papillary carcinoma (IPC) **(11A)** consists of slender fibrovascular cores covered by a single layer of monotonous neoplastic cells without the presence of associated myoepithelial cells; however, the myoepithelial cells are retained at the periphery of the lesions **(11B)**, but often in a more attenuated form (CK 7/8/18, CK5/6 and p63).
11C - 11E: Encapsulated papillary carcinoma (EPC) **(11C)** is a variant of papillary carcinoma that lacks myoepithelial cells both within fibrovascular core **(11D)** and at the periphery of the lesion **(11E)**. (CK 7/8/18, CK5/6 and p63).

neuroendocrine markers such as chromogranin, synaptophysin, or CD56 (35). The tumor cells are also strongly positive for ER and PR, and negative for HER2 (Figure 12a-d). Mitoses are consistently present but not numerous. It should be differentiated from UDH, which is positive for CK5 (36, 37). Regardless of the presence or absence of peripheral myoepithelial markers, they are staged as tumor in situ (Tis) as long as they have a smooth nodular border. The differentiation between in situ and invasive SPC can be difficult at times, especially if there is a lack of staining of myoepithelial markers at the periphery of the lesion. The lesion may be considered invasive SPC only if there is the presence of a geographic jigsaw pattern with ragged and irregular margins in the absence of myoepithelial cells.

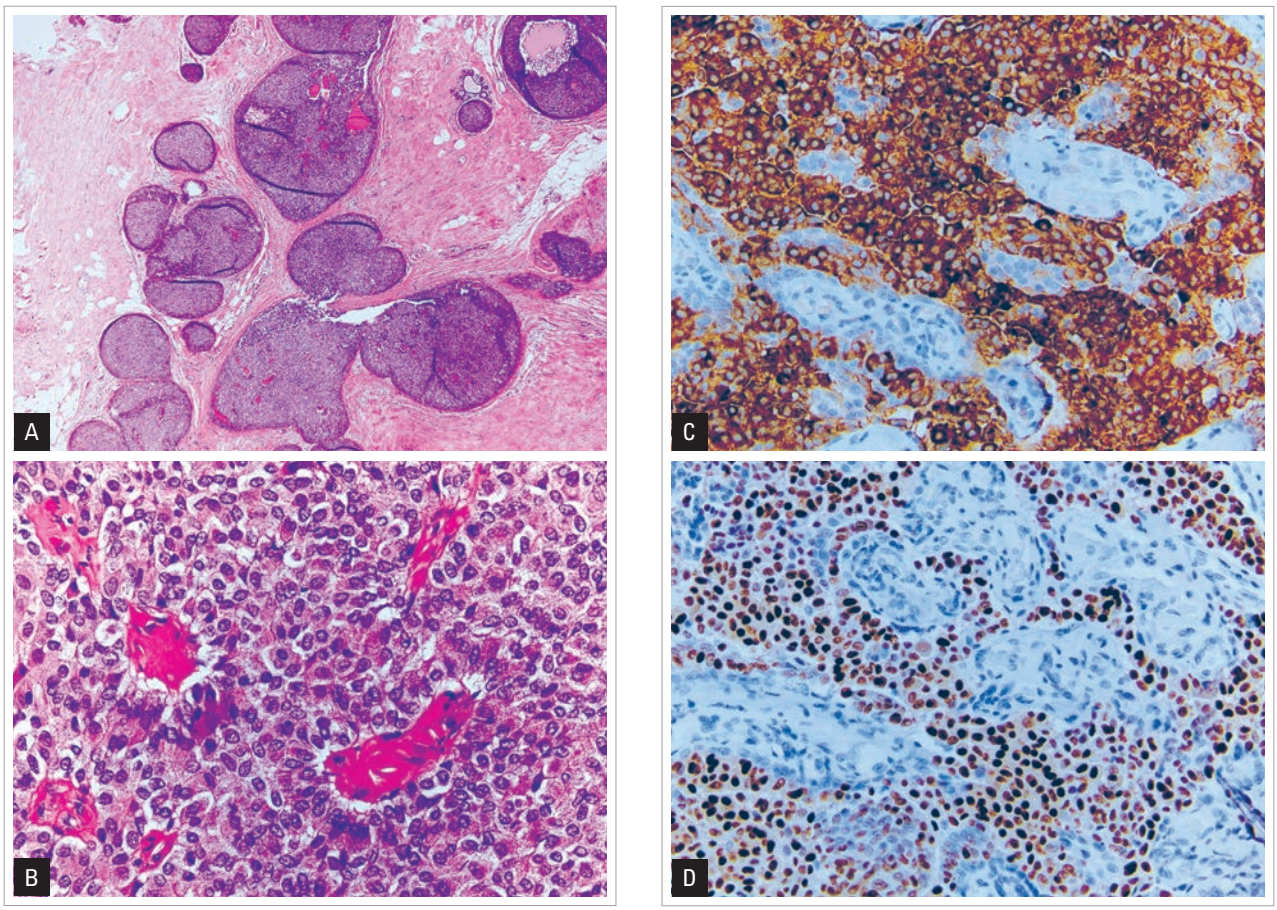


Figure 12: H&E and IHC for solid papillary carcinoma
Solid papillary carcinoma (SPC) is a distinctive variant of papillary carcinoma with a solid growth pattern and inconspicuous fibrovascular cores **(12A and 12B)**. About 60-70% of the cases express one or more neuroendocrine markers such as synaptophysin **(12C)**. The tumor cells are also strongly positive for ER **(12D)**.

Exceptions

An adenomyoepithelial lesion is a tumor of proliferating myoepithelial cells surrounding epithelium-lined spaces. It can be in lobulated, tubular, papillary or mixed patterns. When the papillary pattern predominates, it is very difficult to differentiate from intraductal papilloma with myoepithelial hyperplasia. IHC analysis will highlight their dual epithelial and myoepithelial cell populations. The myoepithelial cells can be demonstrated with markers such as p63, SMMHC,or calponin, but they should be negative for desmin (38, 39) (Figure 13a-d). These myoepithelial cells are ER and PR negative (or weakly positive) and HER2 negative (40). When adenomyoepithelial lesions are encountered, the demonstration of the myoepithelial cell component by markers such as p63, calponin and SMMHC can be very helpful in confirmation of the diagnosis.

IHC analysis of spindle cell lesions

Although many types of mesenchymal lesions, such as nodular fasciitis, vascular lesions, myofibroblastoma, fibromatosis, etc, can occur in breast, the two most common spindle cell lesions in breast are spindle cell metaplastic carcinoma and high grade phyllodes tumors. Often IHC analysis is required to make a diagnosis of these lesions. (Table 4)

Metaplastic carcinoma

Metaplastic carcinoma with spindle cell morphology can be very challenging to differentiate from other spindle cell lesions of the breast. These tumors may be predominantly spindle cell proliferations or may contain mixed epithelial or heterologous elements, including extracellular matrix production. The spindle cell component may be deceptively benign in appearance or

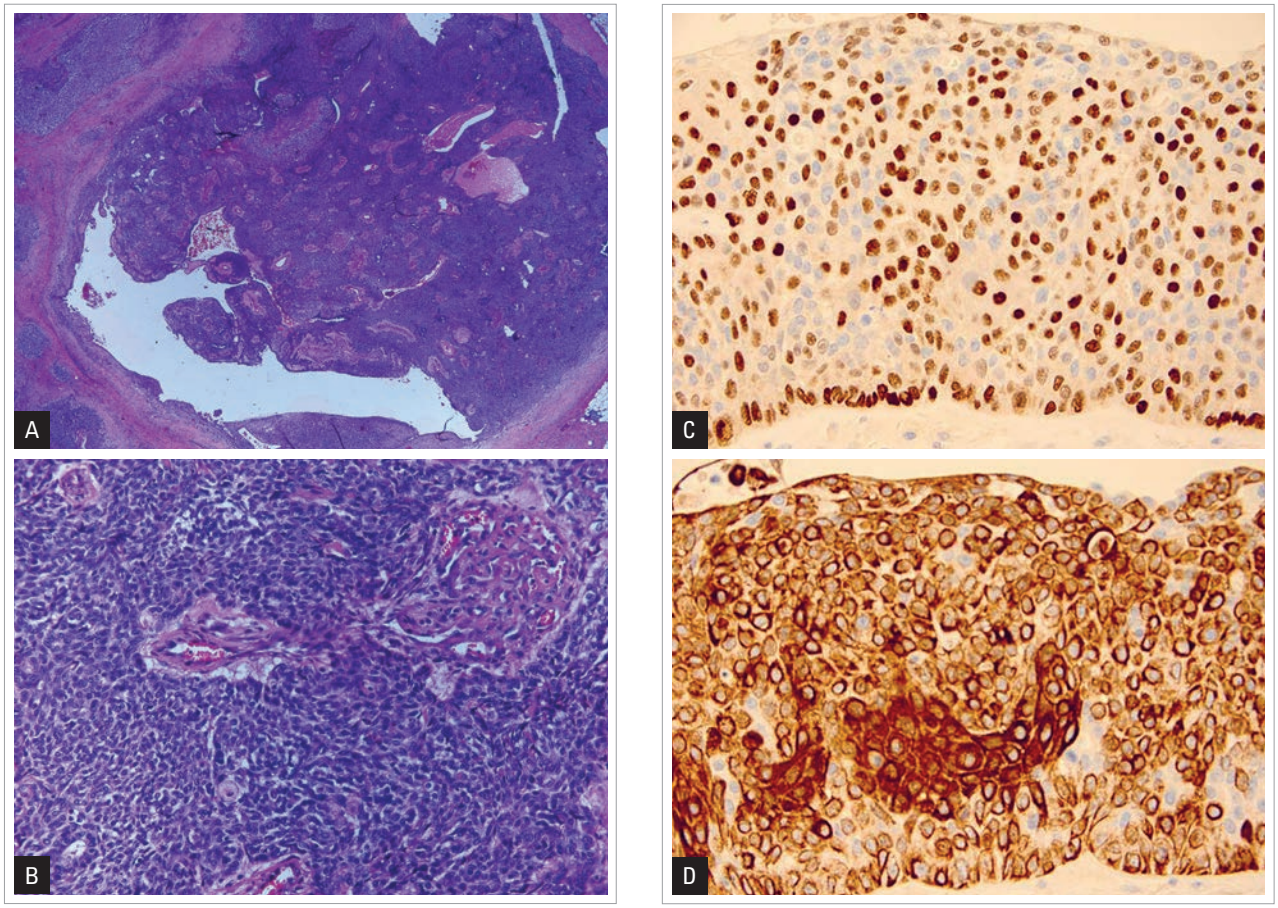


Figure 13: H&E and IHC analysis for adenomyoepithelioma
An adenomyoepithelial lesion with papillary architecture **(13A, 13B - H&E)** can be very difficult to differentiate from intraductal papilloma with myoepithelial hyperplasia. IHC analysis can be helpful in making this distinction and will highlight the dual epithelial and myoepithelial cell populations in these lesions. The proliferating myoepithelial cell component of these lesions are positive for myoepithelial markers **(13C - p63; 13D - CK5)** and are ER and PR negative.

Table 4: Immunohistochemical evaluation of spindle cell lesions of breast

Diagnosis	Immunohistochemical profile	Morphologic features
Spindle cell metaplastic carcinoma	Positive for cytokeratin and myoepithelial marker expression (multiple cytokeratin's may be needed P63, CK5/6, 34betaE12 and CAM5.2 most helpful); negative for ER, PR and HER2	May be pure spindle cells or mixed spindle and epithelial morphology; may be low grade or high grade.
Phyllodes tumor (stromal component)	No specific markers or panels are characteristic; low-grade phyllodes positive for CD34, Bcl-2; high-grade phyllodes stroma may be focally positive for cytokeratin	Mixed and distinct benign glandular elements and stroma, stromal component can be low, intermediate or high grade; high grade lesions may contain heterologous elements
Fibromatosis	Positive for beta-catenin (nuclear), actin &/or desmin (+/-); negative for ER, PR, Bcl-2 and CD34	Pure spindle cell morphology, long sweeping fascicles, bland cytology
Myofibroblastoma	Positive for CD34, vimentin, Bcl-2, CD99; SMA (+/-), desmin (+/-), ER, PR, AR (+/-); negative for cytokeratin, EMA, S100, beta-catenin	Plump spindle cells in groups and fascicles, well circumscribe, uniform cellularity, rare variant may appear epithelioid
Angiosarcoma	Positive for CD31, CD34, factor VIII, FLI1; may be focally positive for cytokeratin	May be low or high grade, infiltrative proliferation of anastomosing vascular spaces

ER – estrogen receptor, PR – progesterone receptor, HER2 – human epidermal growth factor receptor-2

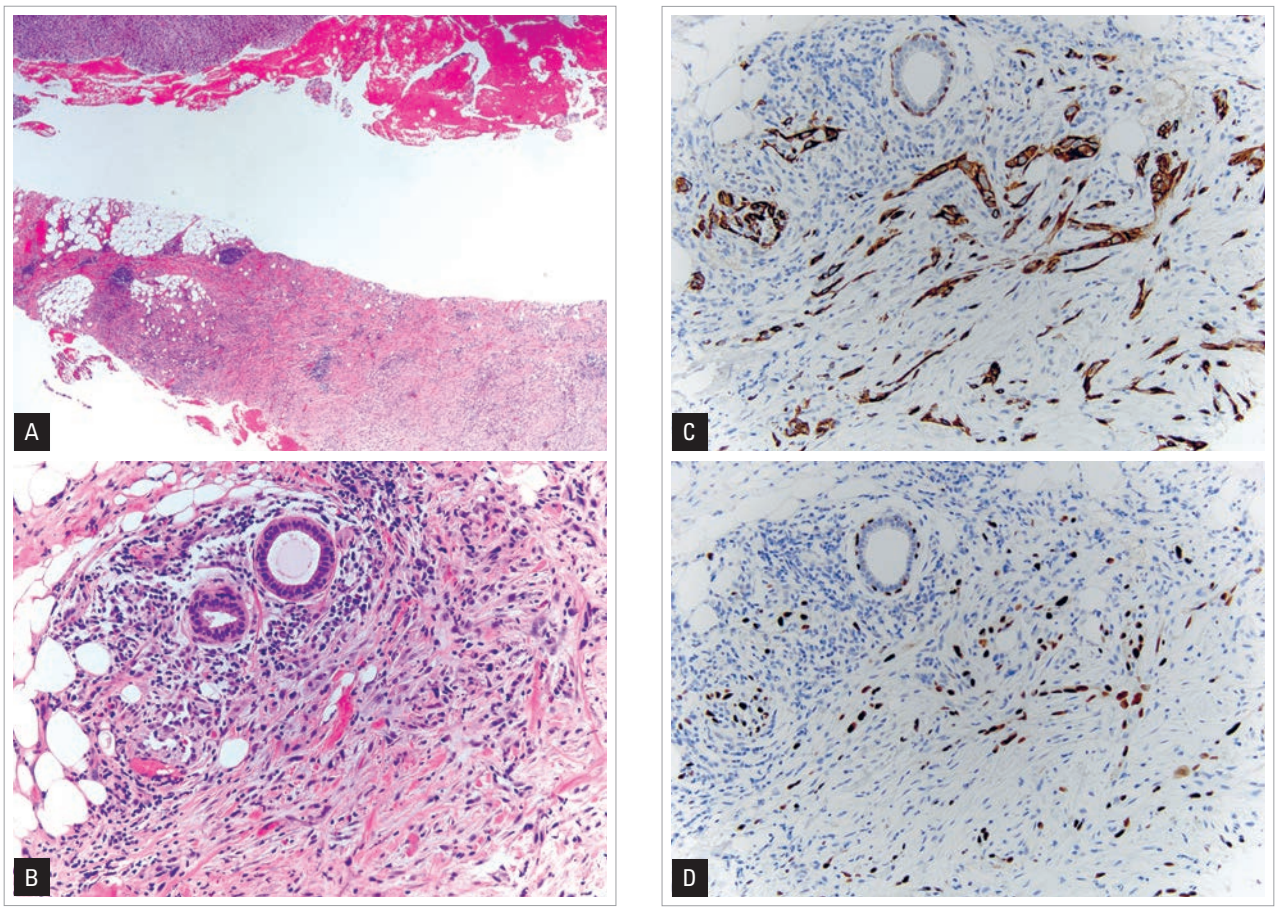


Figure 14: H&E and IHC for metaplastic carcinoma
Metaplastic carcinoma with spindle cell morphology can be very challenging to differentiate from other spindle cell lesions of the breast **(14A, 14B)**. IHC analysis with a panel of keratin markers is essential to help make the correct diagnosis. These markers include high molecular weight cytokeratins such as CK5/6 **(14C)**. P63 **(14D)** expression has been shown to be present in >90% of spindle cell metaplastic carcinomas.

overtly high grade and pleomorphic. IHC analysis with a panel of keratin markers is essential for determining the correct diagnosis. These markers include HMW CK markers (34betaE 12, CK5/6, CK14, AE1/3), which often have variable or focal staining. Low molecular weight (LMW) CK markers are usually negative for metaplastic carcinoma (41, 42). P63 expression has been shown to be present in >90% of metaplastic carcinoma, and can be a very useful marker for differentiation of metaplastic spindle cell carcinoma from other spindle cell lesions of the breast (43, 44). The demonstration of p63 and HMWCK expression in a spindle cell lesion of the breast is an important diagnostic adjunct in confirming a diagnosis of metaplastic carcinoma (Figure 14a-d)

Phyllodes Tumor

Phyllodes tumors are biphasic fibroepithelial lesions that histologically resemble fibroadenomas with an intracanalicular pattern, characterized by hypercellular stroma and elaborate leaf-like structures. Depending on the degree of stromal cellularity, mitosis, atypia, overgrowth, and nature of the tumor borders, phyllodes tumors are further divided into benign, borderline and malignant categories. CD34 IHC stain may be positive for phyllodes tumors, but the amount of staining is inversely associated with the grade of the phyllodes tumors, being positive in benign or borderline phyllodes tumors, and negative in malignant phyllodes tumors (45) (Figure 15a-d). A phyllodes tumor with extensive stromal overgrowth may be difficult to distinguish from a spindle cell metaplastic carcinoma in a limited biopsy specimen. A careful

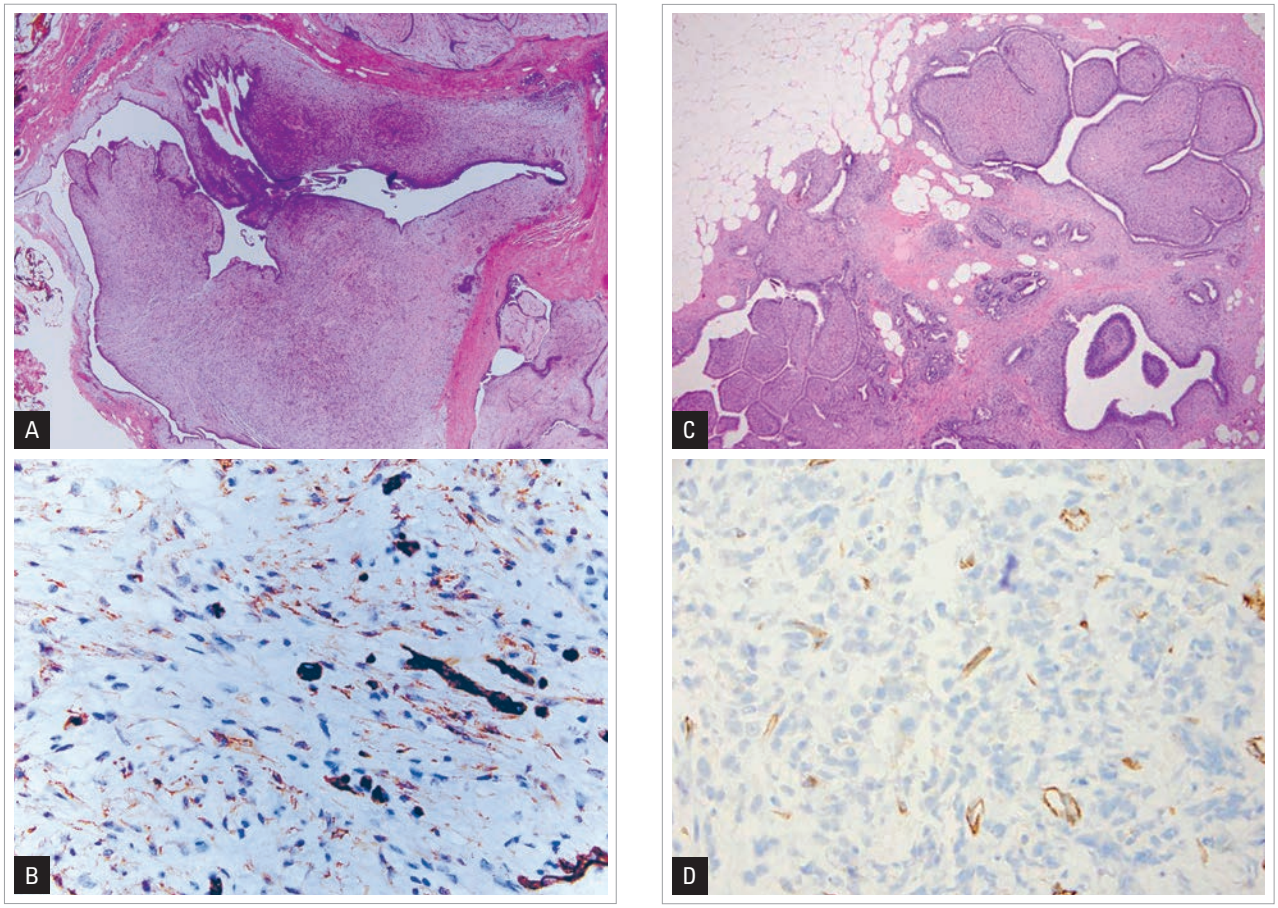


Figure 15: H&E and IHC for phyllodes tumors
Phyllodes tumors (PT) histologically resemble fibroadenomas with an intracanalicular pattern, characterized by a hypercellular stroma component and elaborate leaf-like benign glandular structures **(15A, 15C)**. Based on the degree of stromal cellularity, mitosis, atypia, overgrowth, and nature of the tumor borders, phyllodes tumors are further subdivided into benign, borderline, and malignant tumors categories. CD34 IHC stain may be positive for stromal cells in PT **(15B)**, with the amount of staining often inversely associated with the grade of the tumor (CD34 negative phyllodes tumor, **15D**). Spindle cell metaplastic carcinoma may be in the differential diagnosis, especially in a limited core biopsy sample showing only the stromal component. IHC analysis for a panel of keratin markers (p63, C5, CK14, 17) should be helpful in its differentiation from metaplastic carcinoma; however, high-grade phyllodes tumors may show focal weak cytokeratin and p63 expression

morphologic examination looking for a biphasic fibroepithelial component (phyllodes tumor) and examination for HMWCK expression (metaplastic carcinoma) can help make this distinction.

Exceptions

A recent report demonstrated focal and patchy keratin expression in stromal cells of high grade phyllodes tumors using CK7, 34betaE12, AE1/3, and CK14 stains. Therefore caution should be applied before rendering a diagnosis of metaplastic carcinoma based solely on an immunohistochemical result, especially in very limited core biopsy specimens (46).

IHC analysis in identification of a metastasis of a breast primary

Besides frequent metastases to regional lymph nodes, breast cancer also metastasizes to distant organs such as liver (Figure 16a) lung, brain, bone, GI tract, and organs in the GYN system. Comparison of the original breast primary with the site of metastatic recurrence is the single most important thing to help make a determination for the origin of the primary. In addition to comparing morphology, comparing immunophenotypic patterns of expression is also important, since breast cancer tends to retain both its morphologic appearance and biomarker

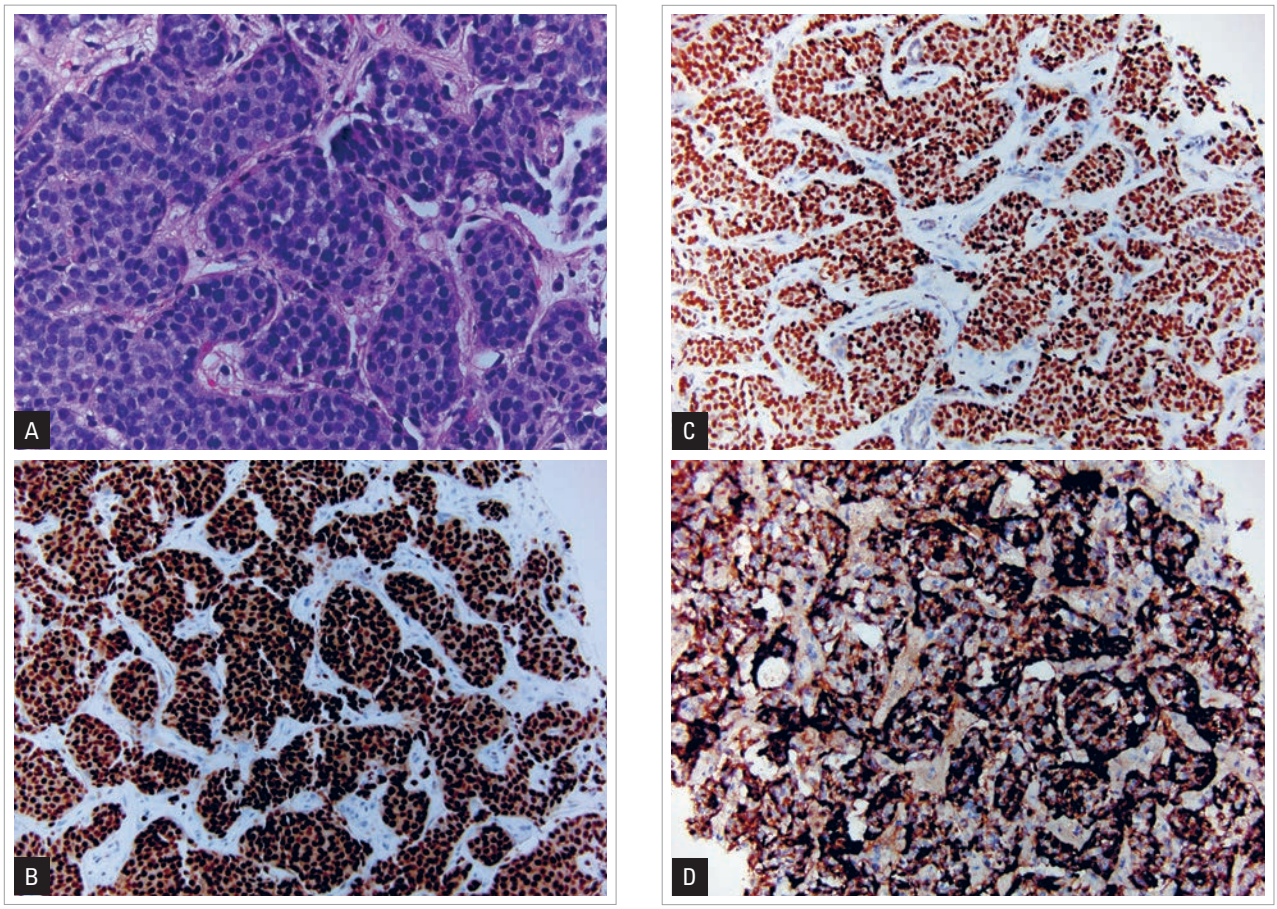


Figure 16: H&E and IHC for metastatic breast carcinoma in the liver
When the primary lesion is not available for review and comparison, IHC analysis with markers showing relative specificity for a breast primary can be very helpful. **Figure 16A** is an example of a metastasis to the liver from a primary lobular carcinoma diagnosis 15 years earlier. IHC analysis with markers having relative specificity for breast expression include: ER **(16B)**, GATA3 **(16C)**, GCDFP15 **(16D)** as well as mammaglobin (not shown).

status throughout its progression. Sometimes the primary lesion is not available or the metastasis presents before the primary lesion has been discovered; in such cases, IHC analysis with markers known to show breast expression may be helpful. These markers include: mammaglobin, gross cystic disease fluid protein-15 (GCDFP15), GATA3 and ER. The selection of which marker may be helpful should be based on the morphologic content as well as the differential diagnosis, and selected on a case by case basis. (Table 5)

Estrogen receptor

75-80% of breast cancers are positive for ER, and a strong ER expression is indicative of breast primary; however, lack of expression does not exclude a breast origin (Figure 16b). The expression of ER is inversely correlated with nuclear grade; therefore a low grade metastatic lesion that is ER negative would be unlikely to be from a breast primary.

GCDFP-15

GCDFP-15 has shown a 98% specificity and 58% sensitivity for lesions of a breast origin. (47) (Figure 16d). It tends to be strongly expressed in lobular and apocrine lesions; however in other carcinomas, GCDFP-15 expression can be focal.

Mammaglobin

Mammaglobin is a secretory protein expressed in over 50% of breast cancers (48), and its expression is not correlated with tumor grade, tumor stage or hormonal receptor status. It

Table 5: Immunohistochemical evaluation of breast carcinoma versus other solid tumors

Differential diagnosis	% of Tumors Positive	% of Tumors Positive	% of Tumors Positive	% of Tumors Positive
Breast carcinoma vs	Pax-8 (0%)	WT1 nuclear (3%)	GATA3 (85%)	
GYN primary	Pax-8 (> 85%)	WT1 nuclear (85%)	GATA3 (5%)	
Breast carcinoma* vs	ER (80%)	TTF-1 (~2%)	GCDFP-15 (30-60%)	Mammaglobin (70-80%)
Lung adenocarcinoma	ER (5-10%)	TTF-1 (~60%)	GCDFP-15 (5-10%)	Mammaglobin (< 2%)
Breast carcinoma vs	CK7 (90%)	GATA3 (85%)	Chromogranin (20%)	Synaptophysin (15%)
Carcinoid tumor	CK7 (20%)	GATA3 (0%)	Chromogranin (85%)	Synaptophysin (95%)
Breast carcinoma vs	Cytokeratin (100%)	GATA3 (85%)	S100 (30%)	Melan-A (0%)
Melanoma	Cytokeratin (<5% focal)	GATA3 (0%)	S100 (95%)	Melan-A (85%)

* ER negative breast carcinomas are less likely to express GCDFP and mammoglobin (~20%),

has been reported to be more sensitive, but less specific than GCDFP-15 for breast lesions (49). A recent study from (50) showed that while mammaglobin may be more sensitive than GCDFP-15 for non-triple negative breast cancer (59% vs 43%), it is less sensitive if the tumor is triple negative (7% vs. 18%).

GATA3

In the original study by Miettinen at al with over 2500 tumors, over 90% of breast cancer tumors were positive for GATA3; other tumors that are likely positive for GATA3 include urothelial carcinoma, germ cell tumors, cutaneous basal cell carcinoma and benign skin adnexal tumors (51). Later, Deftereos et al showed that GATA3 is positive in 100% of non-triple negative breast cancers, but only 60% of triple negative breast cancer, 0% of metaplastic carcinoma, and 100% of pleomorphic lobular carcinoma and apocrine carcinoma (50). GATA3 may be especially useful in the identification of breast as the origin if the tumor is also CK7+/ER+/CK20- (52). GATA3, PAX8 and WT1 are good markers for separating breast cancer from ovarian cancer (53) (Figure 16c).

IHC analysis for the diagnosis of axillary lymph node metastases and benign glandular inclusions

The routine application of IHC analysis for cytokeratin (CK) markers in sentinel lymph nodes is not recommended by the College of American Pathologists (CAP); however, many pathologists will use these stains for lymph node evaluation, especially in cases of invasive lobular carcinoma, which can be subtle and difficult to detect on routine H&E stained sections (Figure 17a,b).

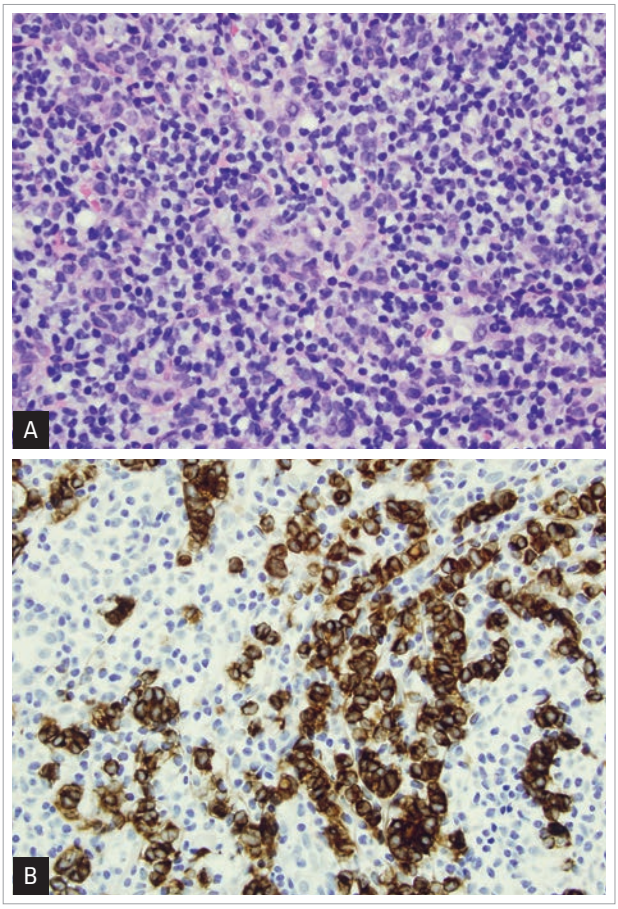


Figure 17: pan-CK for metastatic lobular carcinoma
Lymph node metastases from invasive lobular carcinoma can be subtle and difficult to detect on routine H&E staining **(17A)**. IHC analysis for epithelial markers like cytokeratin **(17B)** can be very helpful in identifying metastatic lobular carcinoma cells admixed with lymphocytes

The two most common benign inclusions in axillary nodes are benign epithelial inclusion cysts and benign nevus inclusions, both of which can potentially be misinterpreted as metastatic breast carcinoma, especially on frozen section. These inclusions

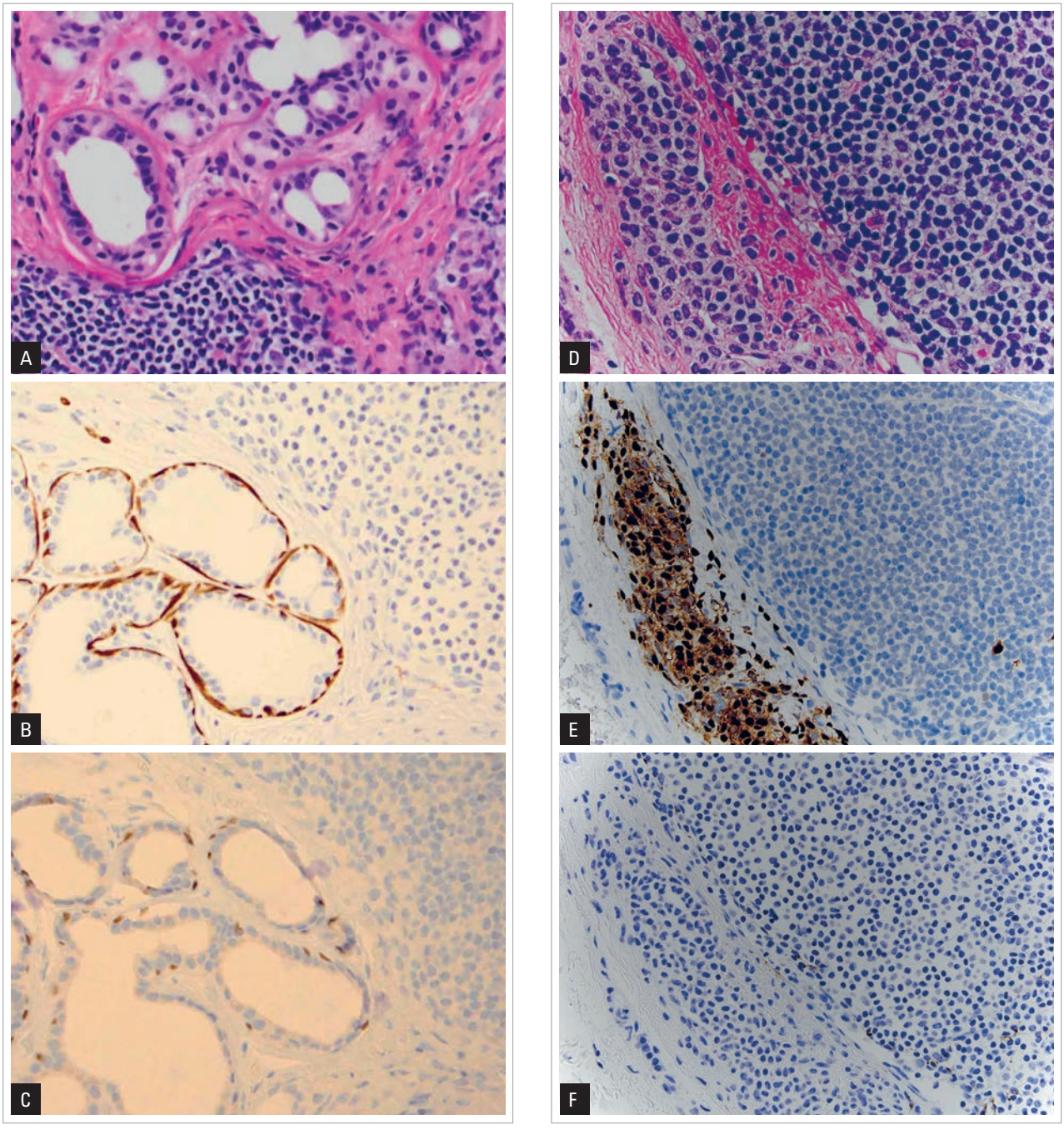


Figure 18: H&E and IHC for benign inclusions in lymph nodes
The two most common benign inclusions in axillary nodes are benign epithelial inclusion cysts **(18A)** and benign nevus inclusions **(18D)**, both of which may be confused with metastatic carcinoma of the breast in certain cases. In addition to comparison of the lymph node with the morphology of the primary breast tumor, IHC staining for myoepithelial cells **(18B - calponin; 18C - p63)**, melanoma marker **(18E - S100)** and cytokeratin **(18F)** should be helpful in the differential diagnosis.

are typically located in the capsule of the node and demonstrate uniform benign cytologic features. Morphologic comparison of the lymph node with the primary breast carcinoma is critically important for the evaluation of a potential benign lymph node inclusion. IHC analysis for myoepithelial markers (Figure 18 a-c) (54, 17) and for melanocytic markers (Figure 18d-f) may be helpful for cases where there is diagnostic uncertainty after morphologic evaluation. (55).

Prognostic and Predictive Factor Testing in Breast Cancer

ER, PR and HER2 status not only provide prognostic information, but are also critical predictive markers for currently available anti-hormonal and anti-HER2 therapies. Thus, accurate, reliable and reproducible evaluation of hormonal receptors and HER2 in breast cancer is critically important to help ensure appropriate treatment planning. Breast cancer treatment and ultimately patient outcomes are predicated on the ability of IHC and other ancillary methodologies to provide an accurate assessment of the expression of these biomarkers in formalin–fixed, paraffin-embedded breast tumor tissue. It is important to remember that the utilization of IHC as a predictive test is fundamentally different from diagnostic classification used by the pathologist in practice. The risk of harm to patients and the potential consequences from assay variability is higher with predictive tests and therefore the testing must be carefully controlled with appropriate quality assurance in place. Consequently, specification of tissue quality and standardization of all pre-analytic, analytic and post-analytic variables are important components for ensuring quality testing (Table 6). All laboratories performing IHC assays for breast cancer biomarkers should closely follow quality control and quality assurance measures outlined in published guidelines (56, 57, 58). In addition, the use of FDA approved tests (class I versus class II/III) is recommended as per published guidelines (57).

Immunohistochemistry: prognostic and predictive factor testing for ER and PR

ER is a nuclear transcription factor with one DNA-binding domain and two AF (activation function) domains. Expression of ER plays a major role in tumor development in ER positive tumors and drives disease progression for these tumors; thus ER positive breast cancer is eligible for anti-estrogen therapy (59, 60, 61). Clinically, ER expressing invasive breast cancers are usually better differentiated, have a more indolent course and favorable prognosis. There is a direct correlation between the

Table 6: Standardization of IHC Testing: Addressing Sources of Variability

Pre-analytic Variables	Analytic Variables	Post-analytic Variables
Standardize time to fixation (limiting cold ischemic time, ideally < 60 min after removal from the patient)	Standardize assay validation	Standardize interpretation criteria (pathologist training, certification, reproducibility and competency)
Standardize tissue sectioning (thinly slice tissue 2-3 mm)	Standardize automated equipment maintenance and calibration	Rigorous quality assurance and quality control program
Standardized type of fixation (10% neutral buffered formalin)	Standardize type of antigen retrieval	Participation in laboratory accreditation program
Standardize time in fixative (minimum 6-8 hours, maximum 72 hours in formalin)	Standardize test reagent (utilize FDA approved or cleared reagents whenever possible for breast biomarkers)	Participation in proficiency testing program
Standardize processing embedding and sectioning	Standardize use of control materials (cell line controls, on-slide positive and negative controls)	
	Standardize use of control materials (cell line controls, on-slide positive and negative controls)	

ER – estrogen receptor, PR – progesterone receptor, HER2 – human epidermal growth factor receptor-2

likelihood of response to hormonal therapies and the levels of expression. However, even tumors expressing very low levels of ER show significant benefit from hormonal therapy above that of entirely negative tumors (59).

ER and PR expression is routinely tested in breast cancers for their prognostic and predictive information. These receptors are expressed in about 75-80% and 65% of all breast tumors, respectively. The American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines recommend that ER and PR should be considered positive if ≥1% of tumor cells shows nuclear staining of any intensity (58). These guidelines also emphasize standardization and quality assurance that must be followed to help ensure testing accuracy. Allred scoring and H-scoring are two commonly used systems for ER and PR evaluations (59, 60).

The intensity of ER and PR stains should be included in the pathology report as weak, moderate or strong. (Figure 19a-c). The evaluation of normal breast tissue as an internal positive control is an integral part of the IHC evaluation for ER and PR expression in breast cancer. Normal breast elements should show heterogeneous ER expression in 10-20% or higher of cells, and when present suggests that the tissue is adequate for hormone receptor evaluation. A positive internal control is especially important in ER and PR negative cases. If the internal normal breast epithelial cells are not stained properly with ER

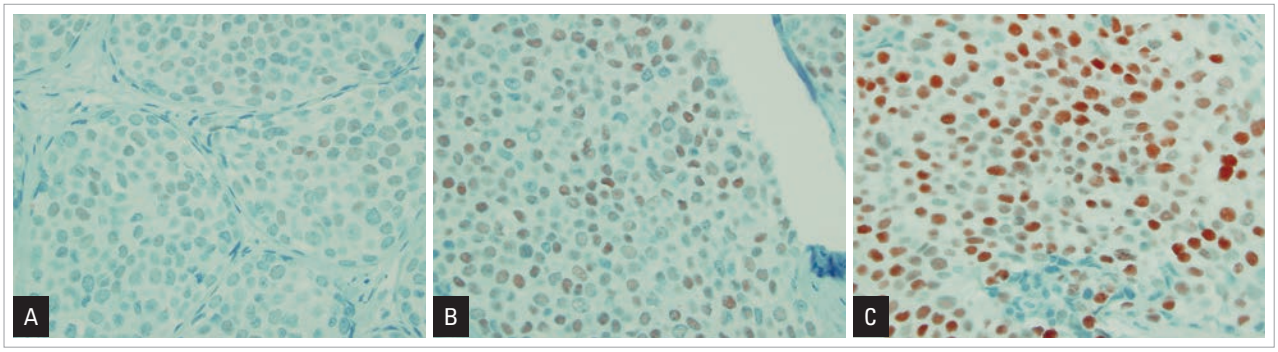


Figure 19: ER and PR staining intensity
The intensity of ER and PR stains should be included in the pathology report as weak **(19A)**, moderate **(19B)** and strong **(19C)** as per ASCO/CAP guidelines.

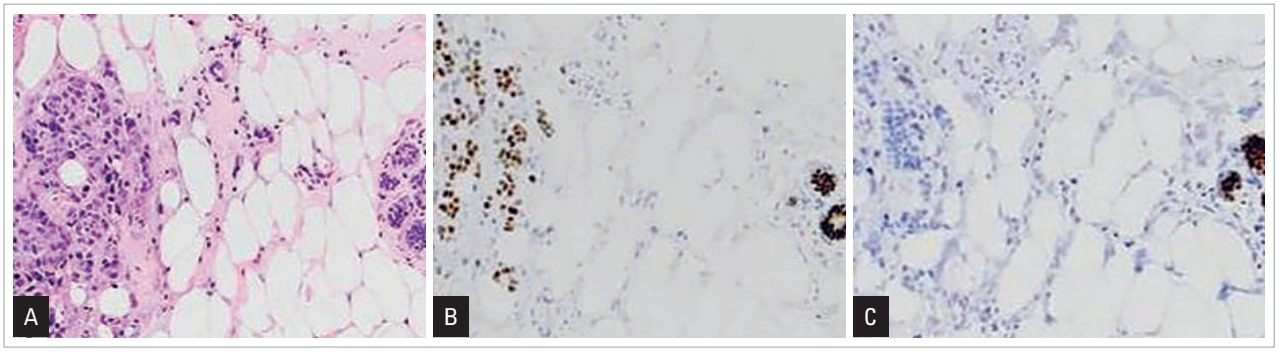


Figure 20: Internal controls for ER and PR
Normal breast tissue that is present within the resection specimen from a breast cancer can serve as an important internal control and is an integral part of IHC evaluation for ER and PR expression. Normal breast elements should show expression in 10-20% or higher of cells. In this example, invasive tumor cells are seen on the left hand side of the image and normal breast elements are seen on the right hand side. **(20A)**. The tumor cells are positive for ER as are the internal control cells **(20B)**. This invasive carcinoma is negative for PR, a feature that is associated with a more aggressive clinical course, and the normal breast elements show the expected expression of PR **(20C)**. A positive internal control is especially important in the evaluation of a breast cancer that is negative for ER and PR.

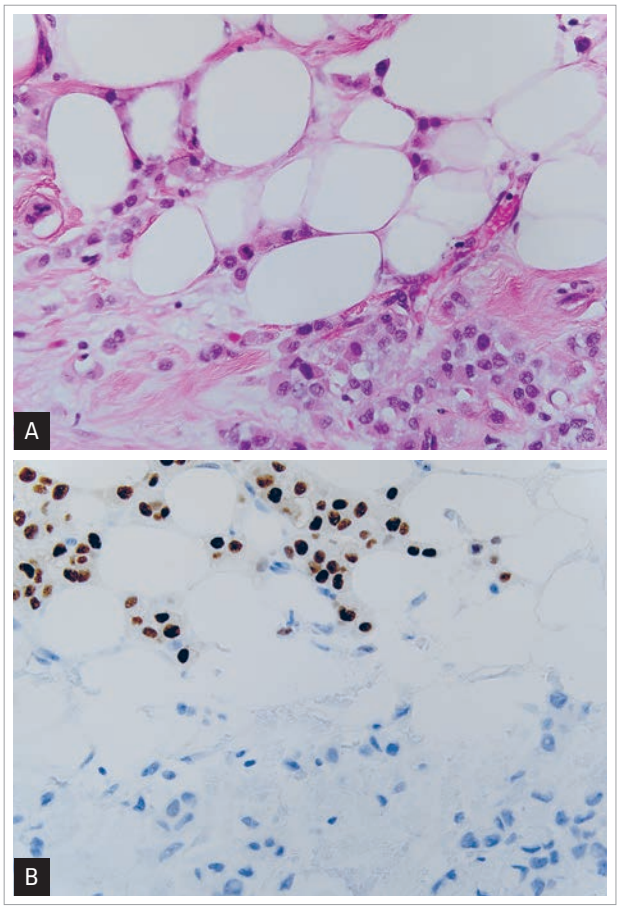


Figure 21: ER and PR heterogeneity
Intratumoral heterogeneity for ER expression is a well-known phenomenon in invasive carcinoma of the breast. Breast cancer can show a broad dynamic range of ER and PR expression ranging from uniform strong nuclear expression throughout the tumor to patchy weak expression in 1-10% of the invasive carcinoma. According to the ASCO/CAP guidelines, ER expression in >1% of invasive tumor cells is considered ER-positive and the patient would be considered a candidate for targeted ER-therapy. The invasive carcinoma shown here **(21A)** demonstrates intratumoral heterogeneity for ER expression **(21B)**. Due to the intratumoral heterogeneity of breast cancer, negative ER and PR staining in the core biopsy specimen should prompt a repeat test in the surgical specimen of the patient to help ensure that the patient is not denied the potential benefit from endocrine therapy.

and/or PR, the test should be repeated and signed out as indeterminant if an absence of staining in normal breast cells persists in a repeated test (Figure 20a-c). Also due to the marked intratumoral heterogeneity of breast cancer, a negative ER and PR in the core biopsy specimen should prompt a repeat test in the surgical specimen of the patient (Figure 21a,b).

PR is also a transcription factor, largely regulated by ER (62) and to some degree by growth factors, and is expressed in 55-65% of invasive carcinomas. The consensus opinion of a number of investigators is that while the predictive role of PR may not be as useful clinically as ER (63), the assessment of this receptor provides useful information. The loss of PR expression in ER-positive tumors is associated with a worse prognosis and decreased response to tamoxifen therapy (64, 65, 66) Given that PR is regulated by an active ER pathway, PR is coexpressed with ER in most cases. A recent study showed that a higher expression of PR in ER-/PR+ tumors is associated with favorable relapse-free survival and disease-specific survival, indicating the important prognostic significance of PR testing in breast cancer patients (63, 67).

Immunohistochemistry: prognostic and predictive factor testing for HER2

HER2 is a member of a family of transmembrane tyrosine kinase receptors that play an important role in the regulation of cellular signaling that affects cell growth, differentiation and survival (68, 69). Over-expression of HER2 in 10-20% of invasive breast cancers has an important bearing on prognosis, as HER2-positive breast cancer is associated with an aggressive clinical course and poor outcome (69, 70). Because of the role that HER2-overexpression plays in driving aggressive tumor biology and because of its location on the cell surface, this molecular alteration (HER2 gene amplification with protein over-expression) was felt to be an ideal therapeutic target. This important insight into the underlying biology in this subset of human breast cancers led to the development of the drug trastuzumab, a humanized monoclonal antibody that directly targets the HER2 receptor by binding with high affinity to an extracellular epitope of the molecule (71, 72). Along with the development of trastuzumab, the first IHC based companion diagnostic test was developed that measured expression levels of the HER2 protein in formalin-fixed paraffin-embedded breast cancer tissue and defined tumor cells as being negative (scored as 0 or 1+), equivocal (2+) or positive (3+), based on the degree of staining seen at the membrane of the tumor cells (HercepTest, Dako, Figure 22a-c). Both the test and the drug trastuzumab received co-approval from the FDA in 1998 for identifying HER2-positive patients who were candidates

for therapy in the metastatic setting. Since that time, targeting HER2 over-expression with the drug trastuzumab in breast cancer has proven to be remarkably successful in clinical trials, which have demonstrated significant improvements in disease free survival and overall survival in the metastatic setting, adjuvant setting and more recently correlating with an excellent pathologic response to therapy in the neoadjuvant setting (73, 74, 75). In light of the demonstrated clinical benefit from trastuzumab, other HER2-targeted drugs, including lapatinib (76), pertuzumab (77) and the antibody-drug conjugated ado-trastuzumab emtansine (T-DM1, 78), have been developed and approved for the treatment of HER2-positive metastatic breast cancer. These new HER2-targeted drugs are now being tested in the adjuvant and neoadjuvant setting. In an unprecedented move, on September 30, 2013, the FDA granted accelerated approval to the drug pertuzumab for use in combination with trastuzumab and docetaxel as neoadjuvant treatment of patients with HER2-positive locally advanced or inflammatory breast cancer (79), thereby expanding the role of neoadjuvant treatment for HER2-positive disease. Given the continued expansion of options for targeting the HER2 pathway in breast cancer, accurate and reliable HER2 testing to help ensure that the right patients receive the right treatment is now more critical than ever (80, 81).

Improving the quality of HER2 testing: the ASCO/CAP guidelines

In 2007, a joint Expert Panel assembled by ASCO and CAP met to develop and publish guidelines with the aim of improving the quality, consistency and reliability of HER2 testing in clinical samples from breast cancer patients (82). This collaboration was triggered by the substantial therapeutic benefit observed in the initial randomized adjuvant clinical trials of HER2-targeted therapy (83) as well as prospective sub-studies from two of the adjuvant HER2 trials, which suggested that up to 20% of the HER2 testing being performed in the community at that time was inaccurate. The goals of producing these guidelines was to provide practical, 'real-world' recommendations to help standardize pre-analytic, analytic and post-analytic factors involved in testing, in the hope that this would reduce discrepancies in testing results between laboratories. The panel decided, based on clinical trials' results, that the available evidence supported the use of either an immunohistochemistry (IHC) or fluorescence in situ hybridization (FISH) methodology for the iden-

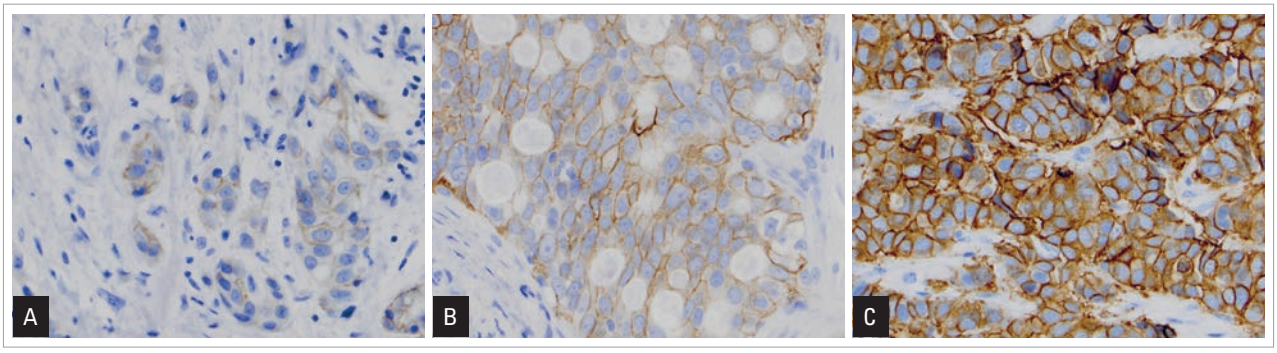


Figure 22: HER2 immunohistochemistry
Immunohistochemistry utilizes antibodies against HER2 to assess the level of protein expression at the membrane of invasive tumor cells in formalin fixed paraffin embedded sections (DAKO Hercep Test, x400 original magnification). The interpretation of HER2 results must be semi-quantitatively evaluated in order to be useful for treatment planning. Breast tumors with either absent or partial weak membrane staining **(22A)** will typically demonstrate a normal HER2 gene status by FISH and are considered HER2 negative (scored as 0 or 1+). This pattern of staining will show a good concordance with an absence of gene amplification in the majority of cases and these patients are unlikely to benefit from HER2-targeted therapy. Breast tumors with evidence of circumferential membrane staining that is either weak/moderate **(22B)** or heterogeneous in its distribution (>10% of tumor cells) should be scored as equivocal (scored as 2+). This pattern of staining has shown poor concordance with the HER2 gene status by FISH and is considered inconclusive. Breast tumors with an equivocal HER2 IHC result need to undergo reflex testing by FISH to resolve the HER2 status for clinical decisions on adjuvant treatment. Breast cancers with diffuse intense circumferential membrane staining (so called "chicken-wire" pattern) in >10% of invasive tumor cells in a clustered pattern should be considered HER2 positive by IHC (scored as 3+). In the majority of cases, this staining pattern is seen diffusely throughout the invasive tumor **(22C)**. Tumors with this staining pattern show a good concordance with HER2 gene amplification by FISH in the majority of cases and will be the most likely to benefit from HER2 targeted therapy.

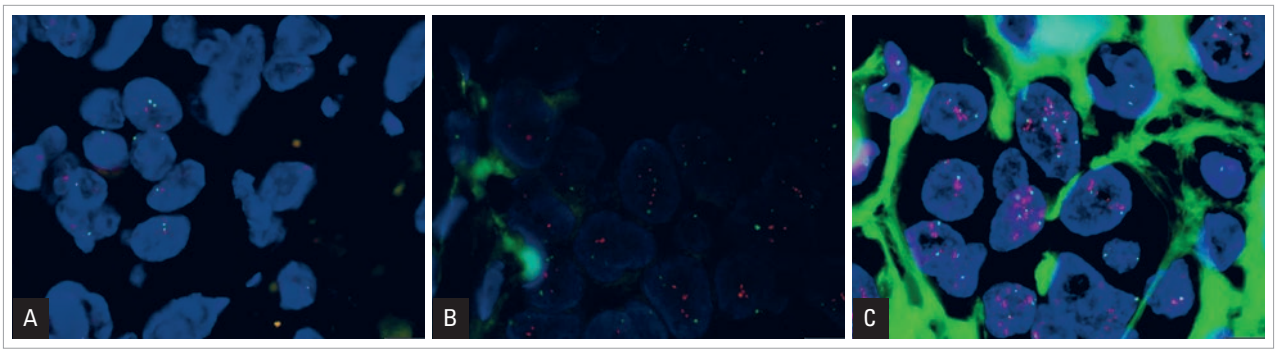


Figure 23: HER2 Fluorescence In Situ Hybridization
Fluorescence in situ hybridization (FISH) assay for HER2 quantitatively measures the level of HER2 gene amplification in breast cancer tumor cell nuclei. This image shows the appearance of the dual-colored FISH assay (DAKO HER2 FISH pharmDXTM, x1000 original magnification). Invasive tumor cell nuclei are highlighted by the blue fluorescence of a DNA counter stain (4',6-diamidino-2-phenylindole [DAPI]). **(23A)** shows a non-amplified breast cancer with roughly equal numbers of HER2 (red signals) and CEP17 (green signals) with a calculated HER2/CEP17 ratio of less than 2. These patients are unlikely to benefit from HER2-targeted therapy. **(23B)** shows a FISH equivocal breast cancer where the average HER2 copy number is increased (> 4 but < 6) with a calculated HER2/CEP17 ratio of < 2. An equivocal FISH result requires additional testing (HER2 IHC, testing another block from the patient's tumor, repeat FISH with an alternative chromosome 17 reference probe) to try and resolve the HER2 status for clinical decisions on adjuvant treatment. **(23C)** shows a HER2-amplified breast cancer with an increased number of HER2 gene signals (red signals) relative to CEP17 (green signals) resulting in a calculated HER2/CE17 ratio of greater than 2. These patients will be the most likely to benefit from HER2 targeted therapy.

tification of patients with HER2-positive disease who should be considered for HER2-targeted therapy. Criteria for "HER2-positive" and "HER2-negative" assay results were defined for each methodology (Figures 22a-c and 23a-c showing HER2 IHC and FISH) and a "HER2-equivocal' category was established to trigger additional reflex testing using the alternative methodology, in order to provide clinicians and patients with additional information for treatment planning.

In 2012, ASCO and CAP convened an Update Committee to conduct a comprehensive review of the published literature on HER2 testing since 2006 and to update the guideline recommendations, as appropriate, in light of new findings, publications and ongoing testing challenges that had arisen since the original 2007 publication (57). A number of important changes were made in this guideline update. To address concerns over false-negative HER2 testing results, the 2013 update has recommended changes to the testing algorithm and pathologist interpretation criteria as well as added new language on reflex and/or repeat testing when there is an apparent histopathologic discordance with the test result (84, 85, 86). The guideline panel felt that the core biopsy was an acceptable sample for the initial HER2 analysis at the time of breast cancer diagnosis. Nevertheless, repeat testing on the excision may be necessary if a HER2 result is negative on the core in certain circumstances, including high tumor grade, limited invasive tumor on the core biopsy, resection specimen containing a high grade component not seen on the core, or indeterminant results due to issues related to pre-analytic variables (87, 88). In addition, the core biopsy alone may not be adequate for the evaluation of HER2 in cases demonstrating intratumoral heterogeneity for HER2 over-expression (Figure 24a-d). The 2013 guideline update advocates interpreting the HER2 results in the context of the clinical and morphologic features of the patient's breast cancer and further recommends that pathologists and oncologists should exercise clinical judgement in respect to which patients will require additional testing before the HER2 status can be assuredly determined (87, 88). Such an approach, driven by clinical judgement and a careful deliberation of all of the data for each individual patient, will help to avoid false negative evaluation of the HER2 status and enable the best possible treatment recommendations for patients with breast cancer (87, 88). For metastatic disease, the 2013 HER2 guideline update has placed new emphasis on the importance of performing a repeat biopsy, if clinically possible, upon the recurrence of breast cancer for HER2 analysis to help ensure accurate assessment of tumor histology, biology and facilitate appropriate further treatment planning (Figure 25a,b).

Prognosis, adjuvant treatment planning and predicting chemotherapy benefit

Although the assessment of patient suitability for hormonal or HER2-targed therapy is based on the assessment of specific biomarkers (ER and HER2 expression), the determination for which patients will benefit from chemotherapy is more challenging. For decades, traditional clinical tumor characteristics described above, such as histologic-grade and (p) TNM stage (T, tumor; N, nodes; M, metastases; AJCC pathologic (p) TMN tumor staging) have been used when considering chemotherapy. However, it has been repeatedly shown that this approach can lead to overtreatment that may cause significant toxicity, with many patients receiving therapy with little if any impact on outcome (89). If robust prognostic markers were available

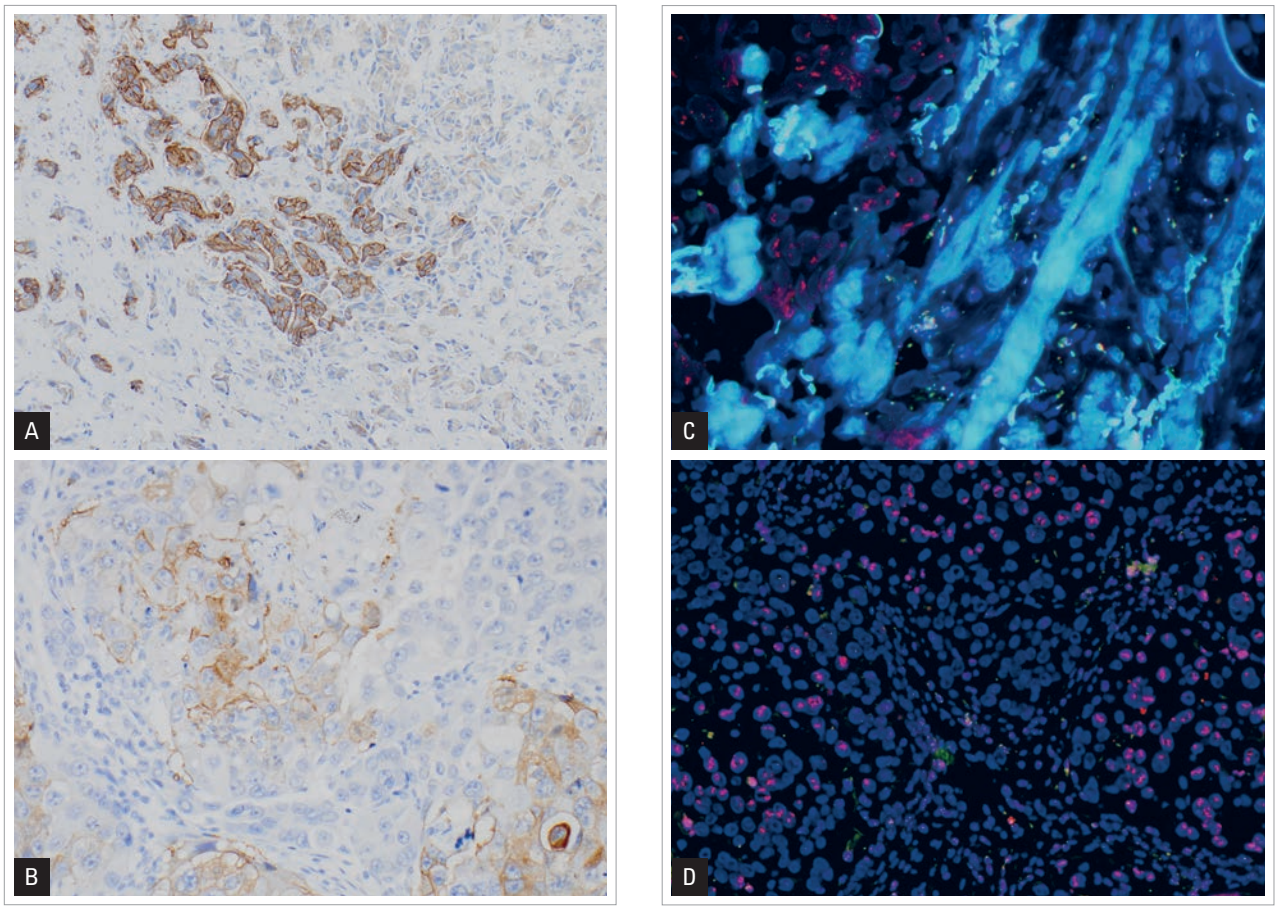


Figure 24: Intratumoral heterogeneity for HER2 over-expression
The majority of tumors that demonstrate HER2 gene amplification/protein over-expression show this alteration diffusely throughout all areas of the invasive tumor. Occasionally, one encounters heterogeneity of gene amplification with clusters of cells with HER2 over-expression or amplification amid cells having a normal HER2 protein and gene status, all within the same or different regions of a single tumor. The term 'HER2 intratumoral-heterogeneity' or genomic heterogeneity has been used to describe this coexistence of multiple tumor cell subpopulations with distinctive HER2 amplification/over-expression characteristics within the same cancer. These changes can be observed at the level of gene amplification and/or protein over-expression in ISH and IHC assays respectively. If the invasive tumor shows HER2 over-expression/gene amplification in >10% of the tumor in a clustered pattern, that carcinoma is considered "HER2-positive" and the patient is a candidate for therapy. **(24A)** and **(24B)** show intratumoral heterogeneity for HER2 protein over-expression in a clustered pattern and **(24C)** and **(24D)** show the same corresponding area of the tumor from the FISH slide during the low power scan, which shows clustered groups of amplified tumor cells adjacent to cells with a normal HER2 genotype. It can be very helpful to use the IHC slide as a guide to target these "hot-spots" with protein over-expression during FISH analysis in cases demonstrating intratumoral heterogeneity.

that could be used to help stratify accurately a patient's risk for recurrence, the information would be valuable for refining and augmenting treatment decisions. In the last decade, molecular analysis of clinical samples has provided a new conceptual approach to breast cancer diagnosis that has enhanced the potential for understanding tumor biology, clinical behavior and for guiding therapy. This approach was based initially on gene expression studies in clinical samples (90, 91, 92) and subsequently has been translated into quantitative real time polymerase reaction (17), immunohistochemical panels (93, 94, 95) and other molecular methodologies (96). Table 7 summarizes the predicted sensitivity and indications for the use of chemotherapy in breast cancer.

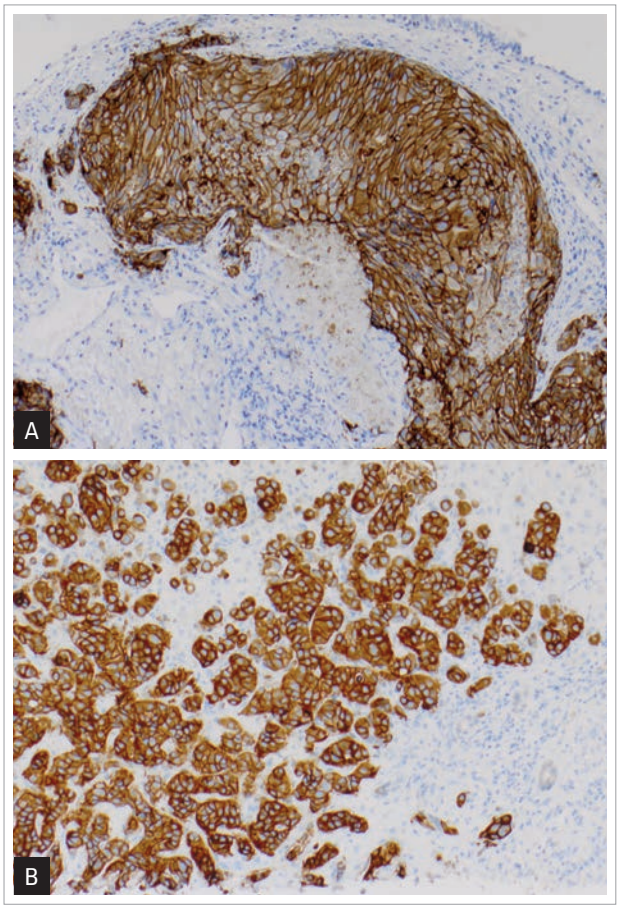


Figure 25: Metastatic breast cancer and HER2
HER2 positive breast cancers are aggressive, with a higher rate of recurrence and mortality for those patients who do not receive any adjuvant therapy. These tumors are more likely to spread early to major visceral sites, including brain, lung **(25A)**, liver **(25B)**, adrenals and ovaries. With the advent of HER2-targeted therapies, progressive visceral disease is significantly diminished. The HER2-alteration is an early and stable genetic change in these tumors; therefore, metastatic HER2-positive breast cancers usually also over-express HER2 at the site of the distant spread **(25A & 25B)**. The 2013 HER2 guideline update has placed new emphasis on the importance of performing a repeat biopsy upon the recurrence of breast cancer, if clinically possible, for HER2 analysis to help ensure accurate assessment of tumor histology, biology and facilitate appropriate further treatment planning. While for most patients, the HER2 results are similar between the primary tumor and recurrent disease, published reports have shown that the HER2 status may change from negative to positive in 9% to 16% of cases from early to later stage disease, which could potentially dramatically alter subsequent treatment recommendations.

Novel applications of Immunohistochemistry for breast cancer prognosis and prediction

A number of reports from the literature have suggested that the application of selective antibody panels and routine IHC can be used to profile breast cancer and predict clinical behavior, identifying subsets of patients with different outcomes (97). These studies have led to the development of IHC panels for the evaluation of newly diagnosed breast cancer patients and have shown potential to provide useful information for guiding clinical decisions about adjuvant treatment in a cost-effective manner.

IHC surrogates for the intrinsic molecular classification subtypes

Although individual molecular markers were introduced in the field of breast cancer management years ago, the concept of molecular classification was raised after the introduction of gene expression profiling (GEP) of breast cancer by Perou and Sorlie et al (90, 98, 99). Using unsupervised clustering analysis, these investigators identified multigene classifiers that could divide breast cancer into five intrinsic subtypes: luminal A, luminal B, normal breast-like, HER2 enriched, and basal-like subtypes, each unique in incidence, patterns of recurrence, survival and response to therapy (90, 98, 99). Because the application of GEP in daily practice is not economical or practical at the present time, many studies have investigated the use of immunohistochemical (IHC) surrogates as a substitute for determining the intrinsic molecular classification of invasive breast cancer. The most commonly used IHC surrogates are ER, PR, and HER2, dividing breast tumors into luminal, HER2 and triple negative (TN) subtypes (100). The addition of Ki-67, CK5, and epidermal growth factor receptor (EGFR) separates lumi-

Table 7: Indication for and predicted sensitivity to adjuvant chemotherapy

Tumor features	Histologic grade	Histologic type	Lymph nodes	Tumor size	ER	HER2	Oncotype DX®	MammaPrint®	Mammostrat®
Features favoring chemo	Grade-3	Ductal (NST)	Positive (> 4)	> 5 cm	ER(-)	(+)	High RS > 30	High risk	High risk index
Features against chemo	Grade-1	Lobular, tubular, mucinous histology	Negative	< 1 cm	ER(+) High	(-)*	Low RS < 18	Low risk	Low risk index

Chemo – chemotherapy, NST – no special type, ER – estrogen receptor, HER2 – human epidermal growth factor receptor-2, RS – recurrence score
* Indications for chemotherapy in HER2 negative breast cancers are dependent on other tumor features.

Table 8: Immunohistochemical Surrogates for the Molecular Classification

ER-Positive Breast Cancer			**ER-Negative Breast Cancer**		
Luminal-A	Luminal B (HER2 negative)	Luminal B (HER2 positive	"HER2-enriched"	Triple negative (basal-like)	Triple negative (nonclassified)
ER (+), PR (≥20%), HER2 (-), Ki-67 (<14%)	ER (+), HER2 (-), and PR (<20%) or Ki-67 (≥14%)	ER and/or PR (+)/ HER2 (+); or ER (+) PR (+) HER2 (+) and ER (+) PR (-) HER2 (+)	ER (–), PR (–), HER2 (+)	ER (–), PR (–), HER2 (–), CK5 (+) and/or EFGR (+)	ER (–), PR (–), HER2 (–), CK5 (-) and EFGR (-)
Molecular Apocrine BC (Vera-Badillo FE 2014; Farmer O 2005; Guedj M, 2012; Lakis S, 2014). A modified IHC surrogate panel may be ER (–), PR (–), AR (+) or HER2 (+) or GCDFP15 (+).					
Claudin low BC (Herschkowitz JI, 2007; Prat A, 2010) (CLBC) has low-to-absent expression of luminal markers and enrichment of epithelial-to-mesenchymal transition markers; defined by low expression of claudin 1, 3, 4, 7, and 8; are found in 77% of BLBC, 20% of HER2 positive cancers, and 3% of luminal cancers					

ER – estrogen receptor, PR – progesterone receptor, HER2 – human epidermal growth factor receptor-2

nal B from luminal A subtypes (101, 102, 103), and basal-like subtype from TN breast cancer (104, 105). More recently, biomarkers such as androgen receptor (AR) and p53 have been shown to further stratify these molecular subtypes (106, 107). Based on current available data, Table 8 summarizes the IHC surrogates for each of the molecular subtypes of breast cancer. The pathologic correlates, clinical features and therapeutic implications for each of these tumor subtypes are summarized in Table 9. The IHC surrogates have shown potential to add value in translational breast cancer research. However, much more work needs to be done before molecular classification can be used routinely in the clinical setting, especially in the standardization of IHC analysis and scoring for each biomarker, standardization of the definition for each classification, and the continued addition of newly discovered biomarkers.

Mammostrat® IHC-based multiplex assay

Gene expression profiling of clinical breast cancer specimens has begun to provide a molecular basis for the clinical, morphologic and biological diversity of breast cancer and has shown great promise for providing clinically applicable information for treatment planning. An alternative approach has been to translate the rich biologic multiplicity revealed by gene expression studies into new IHC tests with potential clinical applications and utility (108, 109). Investigators from Applied Genomics Inc (now owned by Clarient Diagnostic Services, Aliso Viejo CA) used gene expression data to select hundreds of novel protein targets for production of new antibodies. These new antibody markers have been screened against a wide range of formalin-fixed, paraffin-embedded tumor samples to help identify high-quality IHC reagents that may be useful in identifying

Table 9: Intrinsic molecular subtypes of invasive breast cancer: biologic and clinical features

Tumor features	Luminal A type	Luminal B type	HER2-Enriched	Basal-Like (triple negative)
Common patient characteristic	Older age, screen detected	Younger age	Younger age, may be more common in Asians	Younger age, African American and Hispanic women, BRCA1 carriers
Percentage of breast cancer	~55%	~15%	12-18%	10-15%
Histologic grade	Grade-1 or 2	Grade-2 or 3	Grade-2 or 3	Usually grade-3
Special histologic breast cancer types	Tubular, cribriform, papillary, mucinous, classic lobular carcinomas	Invasive carcinoma NST	Invasive carcinoma NST, apocrine carcinoma	Medullary, secretory, adenoid cystic, metaplastic carcinoma
Extensive associated DCIS	~15%	~25%	30-40%	~10%
Lymph-vascular invasion	~30%	~50%	~50%	~40%
> 4 positive lymph nodes	~10%	~20%	~30%	15%
Estrogen receptor	Positive: high expression	Positive: may be low expression	Typically negative	Negative
Progesterone receptor	Usually positive	May be low expression or negative	Typically negative	Negative
HER2	Negative	30-50% positive	Positive	Negative
Ki67 proliferative index	Low (< 10%)	Typically high (> 14%)	High (> 20%)	Typically very high (> 50%)
CK5/6 or EGFR	Absent or low	Absent or low	May be present	Positive 40-85%
TP53 positive by IHC	Absent or low	Absent or low	Frequent	frequent
Prognosis	Favorable, possible late recurrence	Less favorable (more aggressive)	Unfavorable (improved with HER2-targeted therapy)	Unfavorable (subset show good response to chemotherapy)
Time to recurrence	Late recurrence (may be > 10 years)	Earlier recurrence	Usually short (5-10 years)	Usually short (< 5 years)
Sites of metastatic recurrence	Bone (70%), liver or lung (20%), brain (<10%)	Bone (80%), liver or lung (30%), brain (10-15%)	Bone (60%), liver or lung (45%), brain (30%)	Bone (40%), liver or lung (35%), brain (25%)
Systemic therapy	Benefit from hormonal therapy; benefit from chemotherapy less clear	May see most benefit from both hormonal and chemotherapy	Significant benefit from chemotherapy + HER2-targeted therapy	Subset benefit from chemotherapy

ER – estrogen receptor, PR – progesterone receptor, HER2 – human epidermal growth factor receptor-2

clinically significant subsets of solid tumors. This approach was used to develop a five-antibody IHC panel that has been shown to be useful for defining the prognosis of early stage ER-positive breast cancer (Mammostrat®, Clarient Laboratory). The five antibodies measure diverse tumor biology that is independent of hormone receptors, HER2 and proliferation, including markers related to nutrient transport, cell cycle progression, hypoxia and embryonic differentiation (93). The staining results from this antibody panel can be used to calculate a risk index that can classify patients into low, intermediate and high risk groups in term of prognosis and disease recurrence. This Mammostrat® multi-protein antibody panel has been validated as prognostic in three independent institutional cohorts of breast cancer patients (93) as well as in archival tissue samples from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B14 and B20 clinical trials (110). Subsequently, Bartlett et al. (111, 112) confirmed the efficacy and prognostic significance of Mammostrat® in a validation study of 3837 cases from tamoxifen or exemestane treated node-positive or -negative patients who were enrolled in the TEAM trial.

IHC4 score

The IHC4 score was developed using a retrospective cohort from the ATAC endocrine therapy trial of 1125 ER positive breast cancer patients who did not receive adjuvant chemotherapy (113). Immunohistochemistry that incorporated semi-quantitative ER, PR, Ki-67 and HER2 results were used to calculate a risk score using weighting factors and an algorithm, known as the IHC4 score. The IHC4 score was found to provide prognostic information that was independent of traditional histopathologic variables and demonstrated prognostic utility in terms of clinical outcomes that was similar to the Oncotype DX recurrence score in a head to head comparison (113). These investigators went on to combine the IHC4 score with clinical and pathologic variables, including the pN and pT categories, histologic grade and patient age, which improved the prognostic accuracy of the IHC4 + C test (clinical parameters added to IHC4). In a subsequent study, the IHC4 + C score reclassified as low risk more than half of the patients stratified as intermediate risk by Adjuvant Online and the Nottingham Prognostic Index (114).

Pitfalls and potential limitations of the use of IHC for predictive factor testing and breast cancer profiling

The potential limitations and concerns associated with the use of IHC for predictive factor testing, breast cancer profiling and treatment planning are related to apprehension about the lack of reliability and reproducibility in the routine clinical setting due to poor assay standardization (116, 117). A wide variety of factors can impact the quality of tissue samples for immunohistochemical analysis, including tissue handling (118, 115), tissue fixation, antibody reagents, staining protocols and the pathologists' interpretation of the assay results (117). In reaction to these concerns, several national quality assurance programs have been introduced in the United States and abroad including ad hoc consensus conference recommendations (56, 117) as well as guideline recommendations from the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) for both HER2 and ER/PR testing (57, 58, 82). These published guidelines advocate for the standardization of all pre-analytical, analytical and post-analytical testing factors and mandatory proficiency testing (82, 57, 58,119). These efforts along with technical advances in automated staining platforms have resulted in a marked improvement in the quality, interlaboratory agreement and reliability for breast cancer testing (120, 121, 122, 123, 124) and have made the use of semiquantitative IHC assay results more realistic for helping to assess breast cancer risk (95).

Multigene assays for breast cancer prognosis and prediction

The completion of the Human Genome Project along with an explosion of exciting new genomic technologies has converged and brought us to a new crossroad in diagnostic prognostic/predictive assays that hold great promise to improve the care of breast cancer patients. Technological advances have been a major driving force in these efforts and have led to the application of genomic to clinical samples from breast cancer patients with surprising results. What has emerged is a new understanding of the molecular alterations underlying tumor heterogeneity and driving disease progression, as well as new insights into predictive markers for response to therapy. The ensuing discussion examines some of these new molecular approaches that are currently being used mainly in the United States for assessing prognosis and patient management. Most of these molecular approaches remain laboratory developed tests that are offered primarily in central reference laboratories, but this is also rapidly evolving.

Oncotype DX 21 gene recurrence score assay

New molecular methodologies and multigene assays for prognosis and treatment response have been developed to help address clinical decisions concerning the appropriateness of adjuvant chemotherapy therapy in breast cancer. This novel approach has begun to enter clinical practice with the introduction of assays, such as the Genomic Health, Inc. Oncotype DX® (ODX) test (Redwood City, CA). The ODX test is a validated 21 gene quantitative reverse transcriptase polymerase chain reaction (qRTPCR) assay that has been developed for use in formalin-fixed, paraffin-embedded breast cancer samples that are ER positive and node negative. More recent data has suggested that the results of ODX may also be relevant for similar patients with node-positive disease (125). This assay can be used to quantify the expressions of 16 cancer-related genes and five "house-keeping" genes that are used to check RNA integrity and to normalize expression levels (126, 127). ODX uses quantitative RTPCR results and an algorithm to calculate

a numerical recurrence score (RS), which gives the greatest weight to proliferation, including Ki-67, followed by HER-2, ER and PR. The ODX recurrence scores (ODXRS) are divided into low (< 18), intermediate (18 - 30), or high (> 30) recurrence risk categories. Four of these 16 genes (ER, PR, HER2 and Ki-67) measured as parts of the ODX panel are also routinely assessed by IHC (127) as part of the routine diagnostic evaluation of breast cancer (128). The ODX test has been shown to be prognostic (92) and predictive for chemotherapy benefit (126) in ER-positive breast cancer patients based on retrospective analysis of tissue samples from the NSABP B14 and B20 clinical trials. The test currently is used clinically to make decisions concerning which ER-positive breast cancer patients need or can be spared adjuvant chemotherapy based on the recurrence score for their tumor. The Oncotype Dx® assay is a proprietary laboratory developed test, offered by Genomic Health, Inc. (Redwood City, CA) that has not received FDA clearance.

MammaPrint

Another early breast cancer prognostic gene expression assay was the 70 gene classifier that was developed to distinguish patients with a good prognosis from patients at risk for developing early distant metastases (129). The 70 gene prognostic classifer (Agendia MammaPrint assay, Amsterdam, The Netherlands) is now commercially available and can be used to stratify patients into low risk and high risk categories for distant recurrence. This assay measures gene expression using microarray technology and originally required fresh or frozen tissue. The test subsequently has been modified for formalin-fixed, paraffin-embedded tumor samples and this new adaptation has received FDA approval as a prognostic assay in breast cancer. From the initial studies, in which 25,000 genes were examined, a system of supervised classification identified 70 genes that were correlated independently with prognosis and patient outcome. The functions of these 70 genes are related to cellular pathways that involve apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless potential to replicate, tissue invasion, metastasis and sustained angiogenesis (130). Validation studies of MammaPrint have shown that patients found to have a good prognostic gene signature had < 15% risk of recurrence at 10 years, while those associated with a poor prognostic signature had a 50% risk for distant metastases (91,129). These validation studies showed that the risk predicted by MammaPrint is most likely associated with early recurrence, which probably is explained by the strategy that was used to develop this risk classifier (131). Patients at risk for early relapse may, in fact, be appropriate candidates for adjuvant chemotherapy; a concept which is supported by a meta-analysis that showed that MammaPrint also appears to be predictive for chemotherapy benefit (132).

Intrinsic subtype classification of breast cancer and prediction analysis of microarrays (PAM50) assay

Using cDNA microarrays and unsupervised clustering analysis, breast cancers can be subdivided into distinct molecular subtypes based on similarities in the patterns of their global gene expression profiles (133, 99, 90). These so-called molecular "intrinsic subtypes" of breast cancer have significant prognostic significance and include two categories of hormone receptor-positive tumors (luminal subtypes, divided into luminal-A and luminal-B), a group of HER2 positive/hormone receptor negative tumors and a group of "basal-like" tumors that are negative for hormone receptors and HER2 (90). The intrinsic subtype concept has gained wide acceptance for both preclinical and translational research and now is considered a major classification framework for further exploration of the biology of breast cancer (134). Parker et al. (107) proposed a 50 gene set, PAM50, for standardizing the intrinsic subtype classification, which now is available commercially (Nanostring Technology, Seattle, WA). PAM50 is a qRTPCR assay that measures the expression levels of 50 genes and five control genes and has been validated for use on formalin fixed, paraffin embedded clinical samples. A PAM50 risk of recurrence (ROR) score has been devised (107) to translate the prognostic information associated with the different intrinsic subtypes into a clinically meaningful prognostic score. The ROR score is applicable to all subtypes of breast cancer and was shown to be superior to the ODX and IHC4 scores in a 1017 patient cohort treated with either tamoxifen or anastrozole in the ATAC trial (135). Furthermore, PAM50 is predictive for complete or near-complete response to neoadjuvant chemotherapy (136).The PAM50 assay has received FDA clearance for assessing a patient's risk of distant recurrence at ten year in postmenopausal women with node-negative (stage I or II) or node-positive (stage II) hormone receptor positive breast cancer.

Future perspectives – Next Generation Sequencing

Next generation sequencing (NGS) permits, at high speed and relatively low cost, the simultaneous interrogation of the genomic alterations present in a panel of cancer genes, all coding genes (exome), or even the whole genome of individual patient's cancers. At the time of writing this White Paper, more than 1,200 primary breast tumors have been either whole genome or exome-sequenced by various initiatives, including the Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC)[137-143]. Several conclusions emerged from these studies with regard to the mutational landscape of breast cancer. First, it appeared that the most recurrently mutated genes were PIK3CA and TP53, both mutated in more than 30% of the population. These were followed by CCND1, FGFR1 and HER2 amplifications, present in approximately 15% of the cases [138, 140-143]. However, the majority of the cancer genes and new potential driver mutations (mutations expected to contribute to tumor development) were mutated at frequencies of less than 5%. Second, some cancer genes have been shown to be preferentially altered in a specific breast cancer molecular or histological subtype. Besides PIK3CA and TP53 mutations which were previously known to be enriched in estrogen receptor (ER)-positive and negative breast cancer, respectively, it soon appeared that some mutations were also enriched according to the breast cancer molecular subtype defined based on ER and HER2. For example, mutations in genes involved in the ER-dependent transcription program, such as GATA3 and FOXA1, appeared to be confined to the ER-positive disease. At the histological level, it has recently been demonstrated that the two most common histological subtypes of breast cancer, namely invasive ductal and lobular carcinoma, were characterized by a different mutational landscape, such as for example the enrichment for CDH1, TBX3, FOXA1, AKT1, HER2 and HER3 mutations in lobular tumors [138, 144]. Third, these studies highlighted the important genomic diversity among breast tumors. As an example, Stephens et al. demonstrated that 73 different combinations of mutations in cancer-related genes existed across the 100 primary tumors they sequenced [142]. Nevertheless, mutations could be grouped into the deregulation of similar pathway, like the common PIK3CA, PTEN and AKT1 mutations being part of the PI3K pathway. Fourth, with regard to clinical relevance, only few recurrently mutated genes were identified as potential new treatment targets (reviewed in [145]). These clinically-relevant alterations are at the basis of various genomically-driven clinical initiatives, which are carried out in the metastatic setting and aim at personalizing the treatment based on the genomic alterations present in a patient's tumor [146-149]. Finally, the application of NGS to pre and post-neoadjuvant chemotherapy samples has allowed identifying in some cases the genomic remodeling of the tumor in the residual disease, which was in some cases associated with the appearance of previously undetected targetable alterations [150-153]. In metastatic breast cancer, genomic alterations conferring resistance to endocrine and anti-PI(3)Kα therapy have also recently been identified, such as mutations in ESR1, the gene coding for ER [154-157] and PTEN bi-allelic alterations [158], respectively.

Finally, a promising area of these newer technologies concerns the genomic characterization of circulating tumor DNA (ctDNA). ctDNA is released both from tumor tissue and CTCs following apoptosis and necrosis of the tumor cells, and represents a tiny fraction of cell free DNA found in the blood stream. The characterization of ctDNA has several clinically relevant applications, which are all developing at a rapid pace and include non-invasive tumor genotyping [159], the monitoring of treatment response [160], the identification of resistance mutations, such as the ESR1 mutations [161] and the surveillance and identification of residual disease in early stage breast cancer [162].

Conclusions

The current clinical reality is that our ability to accurately predict which breast cancer patients are at increased risk for recurrence based on the currently established prognostic and predictive factors is, at best, limited; however, things are rapidly changing. Increasingly, clinical decisions regarding the suitability of adjuvant systemic therapy for individual breast cancer patients depends on a comprehensive evaluation of the underlying biology of each patient's tumor. Nonetheless, the most clinically relevant, practical, broadly available and cost-effective ancillary testing to help determine prognosis and guide treatment remains to be determined and continues to evolve.

New robust prognostic and predictive biomarkers would be valuable for helping to individualize treatment planning based on risk, enabling high risk patients to receive the most appropriate systemic therapies while avoiding unnecessary and potentially toxic treatments that may be of little or no benefit to patients with an excellent prognosis.

The application of new molecular technologies to clinical breast cancer samples has led to an overwhelming amount of new data on genetic alterations and molecular changes in tumor samples. All of this new information can be confusing and in some cases contradictory, resulting in a significant quandary about how to begin to apply this information to patient care. The job at hand is to begin sorting through this ever increasing mountain of new molecular data and start separating the clinically useful information from the 'molecular noise'. The pathology community has an unparalleled opportunity to play an important role in these efforts and must closely collaborate with basic research scientists, clinical colleagues and industry partners in order to help translate molecular differences among tumors into new clinical tools for patient management. This new molecular vocabulary is best interpreted in the morphologic and clinical context, taking into account existing validated clinical factors for each patient. The natural progression of these sorts of multidisciplinary studies will almost surely lead to the development of new validated diagnostic assay procedures and the identification of novel targeted therapeutic strategies for dealing with malignant disease. Moving forward, it will also be necessary to evaluate new prognostic assays prospectively in uniformly treated patient populations with clinical follow-up using standardized assay procedures and state of- the-art statistical methods to obtain level-I evidence of clinical utility. By uniting morphological, immunohistochemical and molecular methods, the pathology community has begun to provide a more relevant diagnosis for breast cancer patients that will include clinical implications and can help to better inform decisions about appropriate therapy. These exciting developments have led us closer to realizing and being able to provide personalized or "precision" cancer therapy for patients with breast cancer.

Acknowledgement

The authors and Dako thanks Mary C. Jackson for excellent assistance and preparation of the manuscript.

Stains Using Dako FLEX RTU Antibodies

Table of Dako antibodies referenced in this article

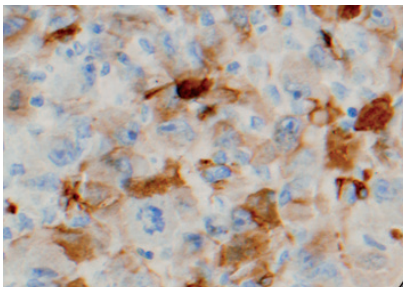
Antibody name	Clone	Dako RTU			Dako Concentrate
		GA[1)]	IR[2)]	IS[3)]	
Actin (Muscle)	HHF35	-	IR700	IS700	M0635
Actin (Smooth Muscle)	1A4	-	IR611	IS611	M0851
BCL2 Oncoprotein	124	-	IR614	IS614	M0887
Caldesmon	hCD	-	IR054	IS054	M3557
CD31, Endothelial Cell	JC70A	GA610	IR610	IS610	M0823
CD34 Class II	QBend 10	GA632	IR632	IS632	M7165
CD56	123C3	-	IR628	IS628	M7304
Chromogranin A	DAK-A3	-	-	-	M0869
Collagen IV	CIV 22	-	-	-	M0785
Cytokeratin	AE1/AE3	GA053	IR053	IS053	M3515
Cytokeratin 5/6	D5/16 B4	GA780	IR780	IS780	M7237
Cytokeratin 7	OV-TL 12/30	GA619	IR619	IS619	M7018
Cytokeratin 8/18	EP17/EP30	-	IR094	-	M3652
Cytokeratin 17	E3	-	IR620	IS620	M7046
Cytokeratin, High Molecular Weight	34βE12	GA051	IR051	IS051	M0630
E-Cadherin	NCH-38	GA059	IR059	IS059	M3612
Epithelial Membrane Antigen (EMA)	E29	-	IR619	IS629	M0613
Estrogen Receptor α	EP1	-	IR084	-	-
Gross Cystic Disease Fluid Protein-15	23A3	GA077	IR077	IS077	-
Ki-67	MIB1	GA626	IR626	IS626	M7240
Laminin	4C7	-	-	-	M0638
Mammaglobin	304-1A5	-	IR074	IS074	M3625
Myosin Heavy Chain (Smooth Muscle)	SMMS-1	-	IR066	IS066	M3558
p53 protein	DO-7	GA616	IR616	IS616	M3629
Progesterone Receptor	PgR 636	-	IR068	-	M3569
Progesterone Receptor	PgR 1294	-	-	-	M3568
S100	-	GA504	IR504	IS504	M0747
Synaptophysin	DAK-SYNAP	-	IR660	-	M7315

1) Dako Omnis - 2) Autostainer Link - 3) Autostainer Plus

Table of Dako breast cancer pharmDx products

Antibody name	Code no.
ER/PR pharmDx kit	SK310, K4071
HER2 CISH pharmDx kit	SK109
HER2 IQFISH pharmDx	K5731
HercepTest	K5204, K5207, SK001

Stains Using Dako FLEX RTU Antibodies



Actin (Muscle)
Clone HHF35
Rhabdomyosarcoma (FFPE) stained with IR700/IS700.

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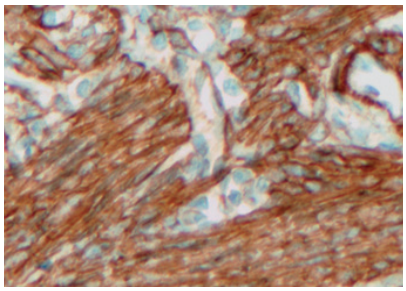
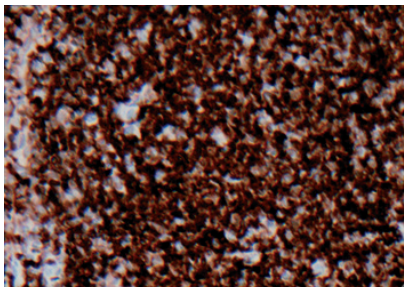
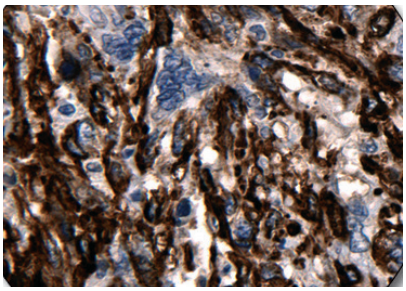


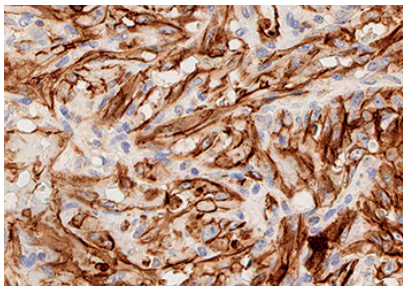
Actin (Muscle)
1A4
Uterine leiomyoma (FFPE) stained with IR611/IS611.



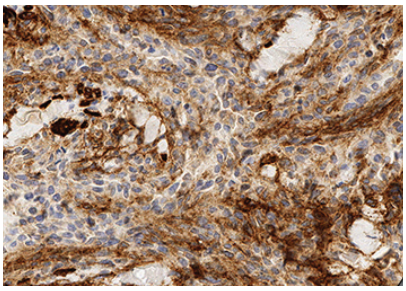
BCL2 Oncoprotein
124
Follicular lymphoma (FFPE) stained with IR614/IS614.



Caldesmon
hCD
Leiomyosarcoma (FFPE) stained with IR054/IS054.

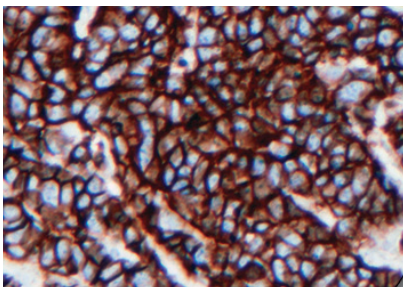


CD31, Endothelial Cell
JC70A
Angiosarcoma (FFPE) stained with FLEX Anti-CD31, Code GA610.

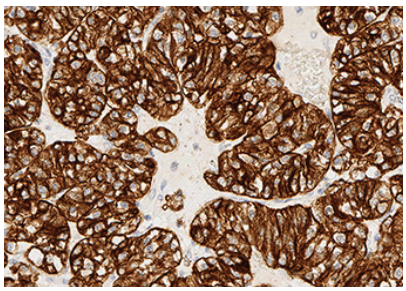


CD34 Class II
QBend 10
Angiosarcoma (FFPE) stained with FLEX Anti-CD34, Code GA632.

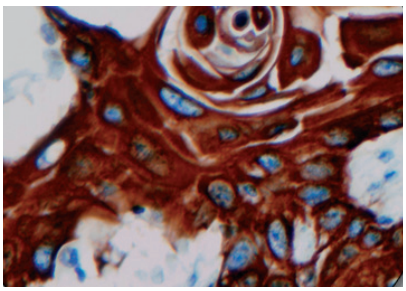
Stains using Dako FLEX RTU Antibodies



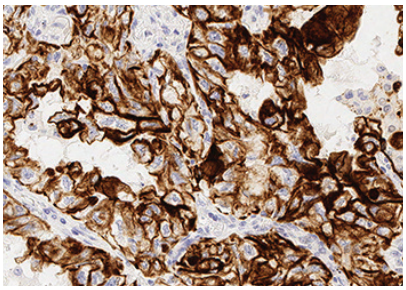
CD56
123C3
Small cell carcinoma of the lung (FFPE) stained with IR628/IS628.



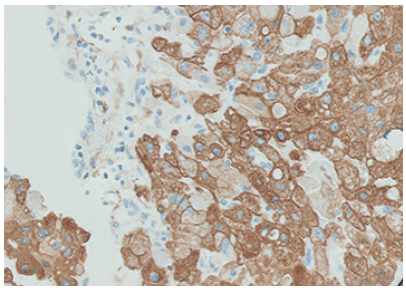
Cytokeratin
AE1/AE3
Adenocarcinoma (FFPE) stained with FLEX Anti-Cytokeratin, Code GA053.



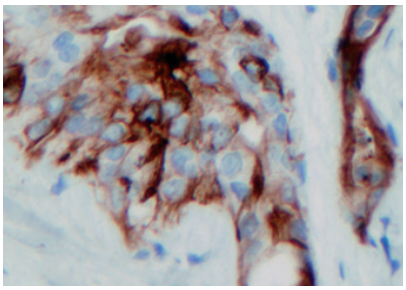
Cytokeratin 5/6
D5/16 B4
Squamous cell carcinoma of lung (FFPE) stained with IR780/IS780.



Cytokeratin 7
OV-TL 12/30
Ductal carcinoma (FFPE) stained with FLEX Anti-Cytokeratin 7, Code GA619.

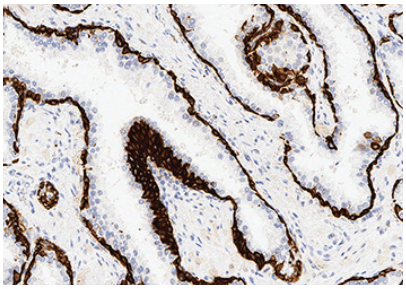


Cytokeratin 8/18
EP17/EP30
Hepatocellular carcinoma (FFPE) stained with FLEX Anti-Cytokeratin 8/18, Code IR094.



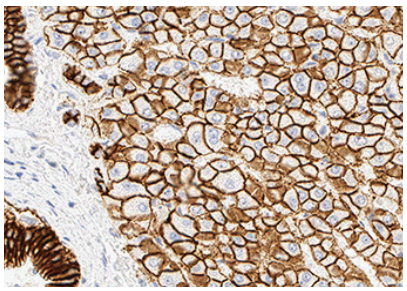
Cytokeratin 17
E3
Pancreatic adenocarcinoma (FFPE) stained with IR620/IS620.

Stains using Dako FLEX RTU Antibodies



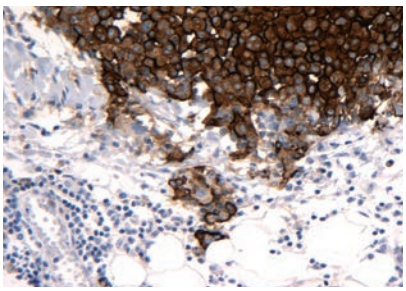
Cytokeratin, High Molecular Weight
34βE12

Normal prostate (FFPE) stained with FLEX Anti-Cytokeratin HMW, Code GA051.



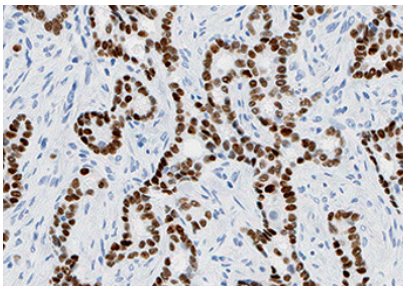
E-Cadherin
NCH-38

Poorly differentiated ductal carcinoma (FFPE) stained with FLEX Anti-E-Cadherin, Code GA059.



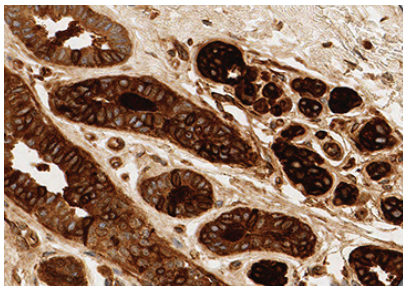
Epithelial Membrane Antigen (EMA)
E29

Breast ductal carcinoma (FFPE) stained with FLEX anti-EMA, Code IR629/IS629.



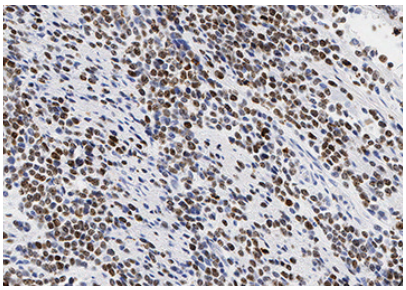
Estrogen Receptor α
EP1

Breast carcinoma (FFPE) stained with IR084.



Gross Cystic Disease Fluid Protein-15
23A3

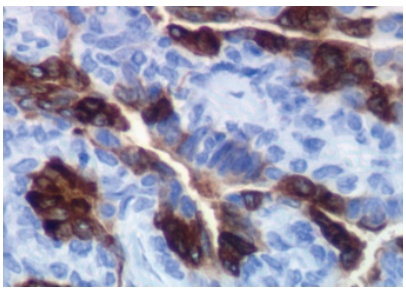
Breast hyperplasia (FFPE) stained with FLEX Anti-GCDFP-15, Code GA077.



Ki-67
MIB1

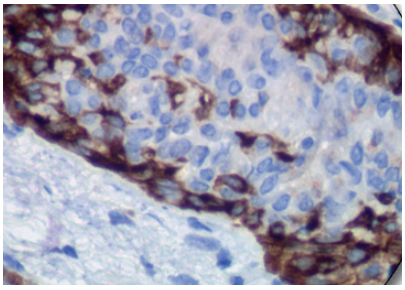
High grade lymphoma (FFPE) stained with FLEX Anti-Ki-67, Code GA626.

Stains using Dako FLEX RTU Antibodies



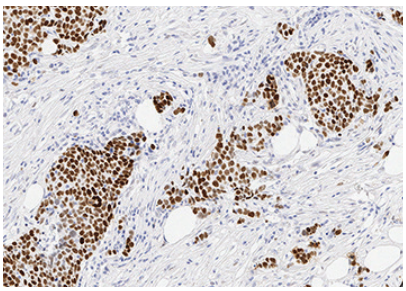
Mammaglobin
304-1A5

Breast hyperplasia (FFPE) stained with IR074/IS074.



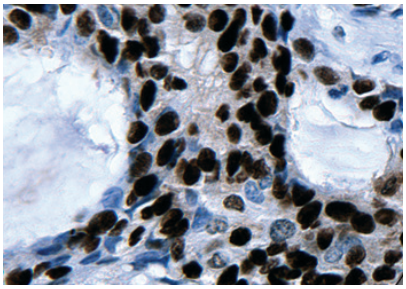
Myosin Heavy Chain (Smooth Muscle)
SMMS-1

Breast hyperplasia (FFPE) stained with IR066/IS066.



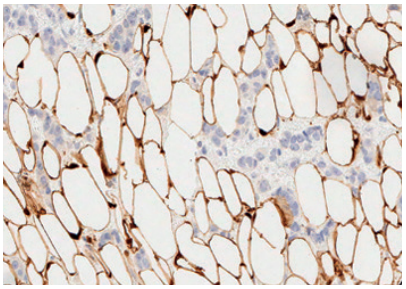
p53 protein
DO-7

Invasive transitional cell carcinoma (FFPE) stained with FLEX Anti-p53, Code GA616.



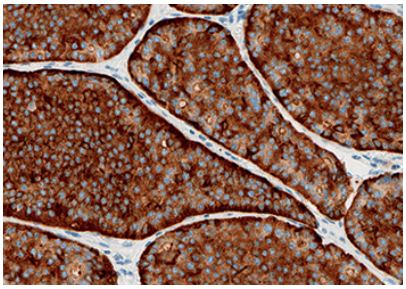
Progesterone Receptor
PgR 636

Breast ductal carcinoma (FFPE) stained with IR068.



S100
–

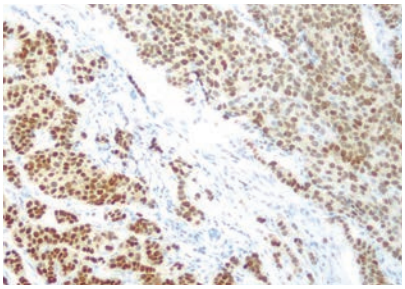
Breast carcinoma (FFPE) stained with FLEX Anti-S100, Code GA504.



Synaptophysin
DAK-SYNAP

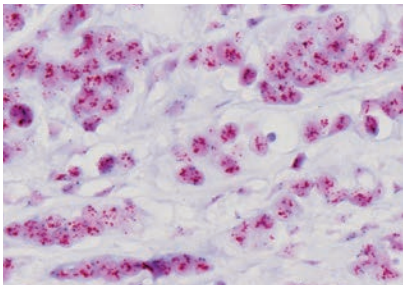
Small intestine carcinoid (FFPE) stained with IR660.

Stains Using Dako pharmDx Products



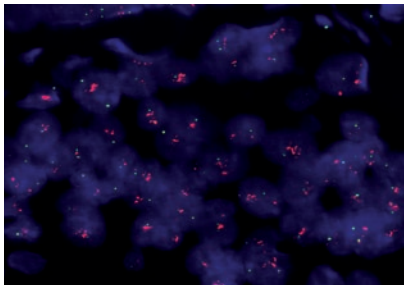
ER/PR pharmDx kit

Breast carcinoma (FFPE) stained with ER/PR pharmDx Kit, Code K4071.



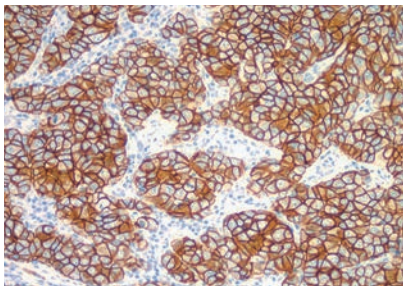
HER2 CISH pharmDx kit

Breast carcinoma (FFPE) with amplified *HER2* gene status stained with *HER2* CISH pharmDx Kit, Code SK109.



HER2 IQFISH pharmDx

Breast carcinoma (FFPE) stained with *HER2* IQFISH pharmDx, Code K5731.



HercepTest

Breast carcinoma (FFPE) stained with HercepTest, Code K5204.

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