USE OF IMMUNOHISTOCHEMISTRY AS AN ADJUNCT IN THE DIAGNOSIS OF LIMITED ADENOCARCINOMA OF THE PROSTATE CANCER

Jonathan Epstein

- The use of immunohistochemistry for basal cell markers and AMACR for the diagnosis of limited adenocarcinoma of the prostate should be used as an adjunct to the H&E diagnosis as there is false positive and negative staining with these markers.
- Examples of labeling of prostate adenocarcinoma with basal cell markers include: aberrant scattered HMWCK staining of cancer cells; rarely the retention of a basal cell layer in carcinoma; and diffuse expression of p63 in some prostate cancers.
- Basal cell markers are useful in distinguishing mimickers, such as adenosis, atrophy, radiation changes, and sclerosing adenosis, from prostatic adenocarcinoma recognizing that in some cases the staining pattern between mimickers and prostate carcinoma overlap.
- Basal cell stains are helpful but are associated with pitfalls in the diagnosis of HGPIN and its distinction from carcinoma, PIN-like ductal adenocarcinoma, and intraductal carcinoma.
- Although the vast majority of prostate adenocarcinomas label with antisera to PSA, there are some that are negative where the P501S (prostein) and PSMA (prostate specific membrane antigen) may be positive.
- Other examples of useful immunohistochemistry include stains for CD68 to distinguish prostatic xanthoma and nonspecific granulomatous prostatitis from high grade prostate cancer.

Use of Basal Cell Markers and AMACR to Diagnose Limited Carcinoma

The most commonly used antibody to label basal cells in benign mimickers of prostate cancer, is high molecular weight cytokeratin (34ßE12, cytokeratin 5/6). High molecular weight cytokeratin immunoreactivity in benign glands is localized to the cytoplasm of basal cells and is negative in prostate cancer. More recently, antibodies to p63 have been shown to label the nuclei of basal cells in benign prostatic lesions.

Several studies comparing high molecular weight cytokeratin and p63 have showed p63 to be slightly superior. One study demonstrated that ck5/6 was superior to 34ßE12, although only a minority of pathologists use ck5/6. The use of a double cocktail combining HMWCK and p63 can increase the sensitivity of basal cell detection with a decrease in staining variability.

The use of high molecular weight cytokeratin or p63 in a focus with only a few atypical glands is not as diagnostic, since benign glands may not show uniform positivity with these markers. Negative staining for basal cell markers is most diagnostic when more than a few glands are present for evaluation and the morphologic features are very suspicious for carcinoma. Rather than used to establish a diagnosis of cancer, we use these antibodies to help verify a suspicious focus as cancer. If we favor, although are not
sure, that a focus is benign and the basal cell stains are negative, we will diagnose it as atypical rather than as cancer. In a small focus of atypical glands on prostate biopsy, negative staining for high molecular weight cytokeratin should not necessarily lead to a definitive malignant diagnosis in all cases, as almost half these biopsies on follow-up sampling are benign. If we are confident the focus is benign and stains performed at an outside institution are negative in a small focus of glands, we will still diagnose the focus as benign since certain mimickers of prostate cancer may not react with these antibodies.

Alpha-methylacyl-CoA-racemase (AMACR), an enzyme involved in the beta-oxidation of branched-chain fatty acids, is significantly up-regulated in prostate cancer. Antibodies have been developed against its gene product, P504S protein. By immunohistochemistry, the majority of prostate cancers are positive for AMACR, the sensitivity varying amongst studies from 82%-100%. Often the staining is fine dot-like and luminal. Although the data is somewhat conflicting, some studies have shown relative decrease AMACR immunoreactivity in foamy gland, atrophic, and pseudohyperplastic prostate cancers. AMACR staining of PIN and mimickers of prostate cancer is discussed in chapters 5 and 7, respectively. As negative staining for basal cell markers especially in a small focus of atypical glands is not necessarily diagnostic of prostate cancer, positive staining for AMACR can increase the level of confidence in establishing a definitive malignant diagnosis.

Different cocktails have been investigated combining antibodies for AMACR and basal cell specific markers. One combination is with antibodies to p63 which label basal cell nuclei of benign glands and AMACR which stains cytoplasm of cancer. Although these authors have reported that this cocktail is essentially equal to each antibody used separately, in our experience a problem with this cocktail is that in some cases stains for p63 show some background staining of the cytoplasm in benign glands, which can be confused with AMACR immunoreactivity. With small foci of atypical glands, the lesion may not survive sectioning to do separate stains for basal cell markers and AMACR on different slides. A triple stain cocktail using a brown chromogen for both high molecular weight cytokeratin and p63 and a red chromogen for AMACR optimizes the preservation of tissue for immunohistochemistry and has been shown to be better than basal cell markers by themselves.


Abrahams NA, Ormsby AH, Brainard J. Validation of cytokeratin 5/6 as an effective substitute for keratin 903 in the differentiation of benign from malignant glands in prostate needle biopsies. Histopathology 2002;41:35-41.


Kunju LP, Chinnaiyan AM, Shah RB. Comparison of monoclonal antibody (P504S) and polyclonal antibody to alpha methylacyl-CoA racemase (AMACR) in the work-up of prostate cancer. Histopathology 2005;47:587-96.


Use of Basal Cell Markers in Radiated Prostate

In addition to radical prostatectomy, external beam radiation and or interstitial radiotherapy (brachytherapy) are currently among the most common options available for the management of localized prostate cancer with a curative intent. Within the nonneoplastic prostatic glands, radiation results in glandular atrophy, squamous metaplasia, and cytologic atypia. Though one may find vascular radiation changes and stromal fibrosis, the stromal atypia characteristic of radiation in other organs is not usually seen. The degree of cytologic atypia in non neoplastic glands and degree of stromal fibrosis appear to be higher after brachytherapy compared to external beam radiation. Furthermore, the marked epithelial atypia tend to persist for a longer time (up to 6 years) following brachytherapy.

The distinction between irradiated nonneoplastic prostatic glands and carcinoma is best made on the architectural pattern of the glands. Within the radiated normal prostate, glands maintain their normal architectural configuration. In contrast to carcinoma, the nonneoplastic glands are separated by a modest amount of prostatic stroma. On higher magnification, there is piling up of the nuclei within irradiated normal prostate as well as an occasional recognizable basal cell layer. Multilayered cells in radiated benign glands frequently appear slightly spindled resembling urothelial metaplasia. The finding of scattered markedly atypical nuclei within well-formed acini is typical of radiated benign glands and rare in prostate carcinoma. Prostate carcinomas that are sufficiently differentiated to form glands rarely manifest the degree of atypia seen with radiation, and if present would be more uniformly present in all cells. Radiated nuclei showing atypia also have a degenerative, hyperchromatic smudgy appearance as opposed to malignant prostatic nuclei that usually contain prominent nucleoli, although occasional nucleoli can be seen in benign prostate glands with radiation affect. Irradiated nonneoplastic glands often are atrophic, in contrast to gland-forming prostatic adenocarcinomas that typically have abundant cytoplasm.

Radiated adenocarcinoma of the prostate may show either no recognizable difference from nonradiated cancer or the effects of radiation damage. In order to diagnose either pattern of cancer, the key feature is that architecturally the findings are inconsistent with benign glands. The presence of closely packed glands with a haphazard
infiltrative growth pattern is typical of adenocarcinoma without treatment effect and cannot be attributed to radiation change. Similarly, the presence of numerous infiltrating individual epithelial cells is diagnostic of carcinoma with treatment affect. Cancers not showing any treatment effect have typical prostate cancer nuclei with prominent nucleoli and glands with a modest amount of cytoplasm. Cancers with radiation effect typically demonstrate individual cells with abundant vacuolated cytoplasm or single cells with indistinct cytoplasm. Nuclei lack apparent nucleoli and are either large with bizarre shapes or pyknotic with smudged chromatin.

It has been demonstrated that high molecular weight cytokeratin immunohistochemistry can aid in the diagnosis of irradiated prostate by identifying basal cells within benign radiated glands. Expression of alpha-methylacyl-coenzyme A racemase (P504S) is usually maintained in radiated adenocarcinoma.


Immunohistochemistry for Selected Mimickers of Prostate Adenocarcinoma

**Adenosis**

**Adenosis**

**Cancer**

**Lobular**

Haphazard growth pattern
Small glands share features with admixed larger glands. They may have:

- Pale-clear cytoplasm
- Occasionally amphophilic cytoplasm
- Medium sized nucleoli
- Occasionally large nucleoli
- Blue mucinous secretions rare
- Blue mucinous secretions common
- Corpora amylacea common
- Corpora amylacea rare
- Basal cells present
- Basal cells absent

Features Shared in Adenosis and Cancer

- Crowded glands
- Crystalloids
- Medium sized nucleoli
- Scattered poorly formed glands and singles
- Minimal infiltration at periphery
- AMACR immunoreactivity

Atrophy

Post-atrophic hyperplasia (PAH) also often appears basophilic at low power. It consists of acini that are small and mostly round that are arranged in a lobular distribution. Often these acini appear to be surrounding a somewhat dilated “feeder” duct. Many of these lesions frequently resemble normal appearing resting breast lobules, and are referred to by some authors as lobular atrophy. The lesions appear hyperplastic since the close packing of multiple small acini suggests that there is an increase in their number compared to normal tissue. PAH glands have a much higher proliferation rate than nonatrophic benign glands. Although the glands may appear infiltrative, they appear invasive as a patch not as individual glands infiltrating in between larger benign glands. The basophilic appearance of glands of atrophy is due to their scant cytoplasm and crowded nuclei such that at low magnification one is merely seeing a nuclear outline of the gland.
When there are concerns as to whether a focus represents PAH or adenocarcinoma, immunohistochemistry with antibodies to high molecular weight cytokeratin or p63 can be performed to resolve the issue, as PAH uniformly labels with basal cell markers. As opposed to partial atrophy (see below), PAH uncommonly expresses racemase.

Partial atrophy, another variant of atrophy, is the most common mimicker of prostate. Partial atrophy may still retain the lobular pattern of PAH, or have more of a disorganized diffuse appearance. Partial atrophy lacks the basophilic appearance of fully developed atrophy (simple atrophy, PAH) as the nuclei are more spaced apart. The presence of crowded glands with pale cytoplasm may lead to an overdiagnosis of low-grade adenocarcinoma. At higher power, however, the glands have benign features characterized by undulating luminal surfaces with papillary infolding. Most carcinomas have more straight, even luminal borders. In addition, the glands are partially atrophic with nuclei in areas reaching the full height of the cytoplasm. The nuclear features in partial atrophy tend to be relatively benign without prominent nucleoli, although nuclei may appear slightly enlarged with small nucleoli. As with adenosis, partial atrophy typically has a patchy basal cell layer and may express racemase.

Sclerosing Adenosis

Adenocarcinomas of the prostate composed of an admixture of glands, poorly formed glandular structures, and single cells would be assigned a high Gleason score (7 or 8). Prostatic adenocarcinomas with these scores are only rarely seen as limited foci within a TURP. The finding of only one or several small foci of a cellular lesion suspicious for high-grade carcinoma should prompt a consideration of sclerosing adenosis. Furthermore, although sclerosing adenosis may be minimally infiltrative at its perimeter, the lesion is still relatively circumscribed in contrast to high-grade prostate adenocarcinoma.

The glandular structures in sclerosing adenosis resemble those seen in ordinary adenosis. They are composed of cells with pale to clear cytoplasm and relatively benign-appearing nuclei. In many of the glandular structures, a basal cell layer can be identified on H&E-stained sections. This contrasts to carcinoma, where basal cells are absent. Sclerosing adenosis contains a dense spindle cell component that is typically lacking in adenocarcinomas. Usually, adenocarcinomas of the prostate show no apparent stromal response or at most a hypocellular fibrotic reaction. A rather unique feature of sclerosing adenosis is the presence of a hyaline sheath-like structure around some of the glands. The glands in ordinary adenocarcinoma lack such a collarette and have a “naked” appearance as they infiltrate the stroma.

Sclerosing adenosis contains a basal cell layer around most of the glandular structures as well as among the individual cells and cords of cells. The basal cells within sclerosing adenosis, however, are distinctive in their immunophenotypical staining and differ from ordinary basal cells. Ordinary basal cells of the prostate show no myoepithelial cell differentiation. They lack staining for muscle specific actin and ultrastructurally do not show contractile elements. Within sclerosing adenosis, the basal cells show muscle specific actin positivity consistent with myoepithelial cell differentiation. The dense spindle cell component in sclerosing adenosis also shows partial staining with keratin and muscle-specific actin consistent with myoepithelial cell
differentiation. Ultrastructural examination of several of these cases has verified their myoepithelial differentiation. There is no known association between sclerosing adenosis and adenocarcinoma of the prostate.


Pitfalls with Basal Cell Markers:
Aberrant Staining; Cancers with Retention of Basal Cells; p63+ Cancer
Uncommonly, one can see occasional cancer cells that are positive for antibodies to high molecular weight cytokeratin and less likely p63, yet as long as these cells are not in a basal cell distribution, these cells represent aberrant expression of the antigen in cancer.

Rare lesions with the appearance of prostate cancer show high molecular weight cytokeratin staining in a basal cell distribution either from retention of basal cells by early invasive cancer or from high grade PIN outpouching. The lack of adjacent PIN in some cases and the large ratio of small atypical glands to PIN glands argue against high grade PIN outpouching as the sole explanation. In cases with adjacent high grade PIN, a comparison of the proximity and number of the small, atypical, infiltrative appearing glands to high grade PIN is helpful. The diagnosis of prostate cancer in the face of positive high molecular weight cytokeratin basal cell staining should be made with extreme caution, only in the face of unequivocal cancer on the H&E stain.

There are also uncommon prostate adenocarcinomas that diffusely express p63, yet not HMWCK. Many of these tumors have distinctive morphology composed of atrophic glands lined by hyperchromatic nuclei that are often slightly spindled and minimally multilayered.


Use of Basal Cell Markers in the Differential Diagnosis of HGPIN

PINATYP

A common scenario where it is difficult to distinguish acinar adenocarcinoma from high grade PIN is when there are a few atypical glands immediately adjacent to high grade PIN. The differential diagnosis is whether these small glands represent tangential sectioning or outpouching of the high grade PIN glands or a small focus of carcinoma adjacent to the high grade. We refer to these foci at PINATYP. A diagnosis of carcinoma can be rendered only if the small atypical glands are too numerous or too far away from the high grade PIN glands to represent outpouching or tangential sectioning from the PIN glands. In cases of PINATYP, the lack of basal cells in the small atypical glands can be construed as evidence that these glands represent infiltrating cancer only if there are more than a few such glands. As high grade PIN glands can have discontinuous basal cells, one can envision tangential sections off PIN glands in which all cells would appear negative for basal cell markers, such that a few negative small atypical glands adjacent to PIN is not diagnostic of cancer. Some cases may have the appearance of PINATYP yet
will be entirely negative for basal cell markers; these foci may be diagnostic of cancer if there are a sufficiently large number of glands that are not immunoreactive. One may also see classic high grade PIN where some of the glands show the expected patchy basal cell layer and other identical glands are negative for the basal cell markers; these cases we would still diagnose as high grade PIN. Racemase does not differentiate between high grade PIN and cancer, as both typically express this antigen.


PIN-like Ductal Adenocarcinoma
A more recently described variant of ductal adenocarcinomas closely resembles high grade prostatic intraepithelial neoplasia (HGPIN) and is composed of simple glands with flat, tufting or micropapillary architecture. PIN-like ductal adenocarcinoma differs from HGPIN by the presence of cystically dilated glands, a greater predominance of flat architecture, and less frequently prominent nucleoli. Verification often requires the immunohistochemical documentation of the absence of basal cells in numerous atypical glands. Although usual ductal adenocarcinoma is considered comparable to Gleason score 8, PIN-like ductal adenocarcinoma was accompanied by Gleason score 6 acinar carcinoma and behaved similar to Gleason score 6 acinar cancer.


Intraductal Carcinoma
Intraductal carcinoma of the prostate (IDC-P) in radical prostatectomy specimens is described as an atypical glandular lesion that spans the entire lumen of prostatic ducts or acini while the normal architecture of ducts or acini is still maintained. Rarely, IDC-P may be identified on biopsy material in the absence of infiltrating carcinoma. Our definition of IDC-P on needle biopsy was derived to identify objective morphological criteria that either architecturally or cytologically clearly exceed those seen in high grade PIN. It is critical to distinguish between high grade PIN and IDC-P, as the former is typically not treated with definitive therapy and recent data has questioned whether high grade PIN on needle biopsy even requires immediate rebiopsy within the first year following its diagnosis. Both entities share cytological features such nuclear enlargement,
hyperchromasia, and enlarged nucleoli. Although dense cribriform (more solid than luminal areas) and solid patterns are not architectural patterns associated with high grade PIN, loose cribriform and micropapillary patterns overlap between the two entities. To establish the diagnosis of IDC-P in the latter two patterns, other cytological features such as markedly enlarged nuclei (6 times larger than those in adjacent non-neoplastic cells) and non-focal comedonecrosis are required. Whereas, it has been accepted that classic high grade PIN can contain a rare gland with focal necrosis, more extensive necrosis is not acceptable. IDC-P also tends to show more prominent nuclear pleomorphism, as opposed to typical high grade PIN with its uniformly enlarged nuclei. Cases which do not satisfy the strict criteria for IDC-P on needle biopsy yet appear more atypical either architecturally or cytologically than usual high grade PIN can be diagnosed as borderline between IDC-P and high grade PIN with a strong recommendation for repeat biopsy.

Infiltrating cribriform acinar adenocarcinoma (Gleason pattern 4 or Gleason pattern 5 with comedonecrosis) closely mimics cribriform IDC-P. Most cases of IDC-P would be diagnosed as cribriform carcinoma if immunohistochemistry demonstrating basal cells had not been performed. In some cases, the contour and branching pattern of normal duct architecture distinguishes IDC-P from infiltrating carcinoma. Ultimately, the presence of a basal cell layer either identified on routine hematoxylin and eosin prepared slides or with immunohistochemistry rules out infiltrating acinar prostate adenocarcinoma. Despite the presence of comedonecrosis, Gleason pattern 5 adenocarcinoma is ruled out also by the identification of a basal cell layer. Although there are extremely rare cases of early small foci of non-cribriform carcinoma of the prostate with focal retention of basal cell layer, this has never been described in cribriform, solid, or micropapillary prostate acinar carcinoma.


Prostate Adenocarcinoma vs. Urothelial Carcinoma

Even in poorly differentiated prostatic carcinomas, there is typically relatively little pleomorphism or mitotic activity compared to poorly differentiated urothelial carcinoma. Poorly differentiated prostate cancers may have enlarged nuclei and prominent nucleoli, yet there is little variability in nuclear shape or size from one nucleus to another. High-grade urothelial carcinomas often reveal marked pleomorphism with tumor giant cells. A subtler finding is that the cytoplasm of prostatic adenocarcinoma is often very foamy and pale imparting a “soft” appearance. In contrast, urothelial carcinomas may demonstrate hard glassy eosinophilic cytoplasm or more prominent squamous differentiation. The findings of infiltrating cords of cells or focal cribriform glandular differentiation are other features more typical of prostatic adenocarcinoma than urothelial carcinoma. Urothelial cancer tends to grow in nests, even when poorly differentiated. Although the above distinction between urothelial carcinoma and prostatic
adenocarcinoma on H&E stained sections is valid for almost all cases, we have seen rare cases where prostate adenocarcinoma has had marked pleomorphism identical to urothelial carcinoma. Consequently, in a poorly differentiated tumor involving the bladder and prostate without any glandular differentiation typical of prostate adenocarcinoma, the case should be worked up immunohistochemically.

Approximately 95% of poorly differentiated prostatic adenocarcinomas show PSA and PSAP staining although it may be focal. While some studies claim superiority of PSA over PSAP in staining prostatic carcinoma, other articles have demonstrated poorly differentiated prostatic carcinomas that lacked PSA staining but still maintained their immunoreactivity with antibodies to PSAP. In our own hands, PSA has in general been more sensitive. Monoclonal antibodies to PSAP have lower sensitivities than their polyclonal counterparts. We have compared PSA staining in a group of poorly differentiated prostatic adenocarcinomas with “poor” PSA staining to newer prostate specific markers including prostate specific membrane antigen (PSMA), p501S (Prostein) and NKX 3.1. Completely negative staining was seen in 15% (PSA), 12% (PSMA), 17% (P501S) and 5% (NKX 3.1) of the cases. Five per cent of the cases were negative for all four markers combined. A similar 5% rate of “false negativity” is found when combining PSA and PSAP stains. Therefore, the lack of immunoreactivity to prostate specific markers in a poorly differentiated tumor within the prostate, especially if present in limited amount, does not exclude the diagnosis of a poorly differentiated prostatic adenocarcinoma.

In a poorly differentiated tumor occurring in the bladder and the prostate where the differential diagnosis is between high-grade prostatic adenocarcinoma and urothelial carcinoma, focal strong staining for either marker can be used reliably to make the diagnosis of prostatic adenocarcinoma, since PSAP and PSA false positivity have not been convincingly described in urothelial carcinomas.

In general, various cytokeratins (CK7, CK20, high molecular weight cytokeratin) show strong positivity in cases of urothelial carcinoma involving the prostate. Although CK7 and CK20 are more frequently seen in urothelial carcinoma as compared to adenocarcinoma of the prostate, they may also be positive in adenocarcinoma of the prostate, such that in our experience they are not that helpful in this differential diagnosis. We and others have found high molecular weight cytokeratin to be positive in more than 90% of urothelial carcinomas. In contrast, high molecular weight cytokeratin is only rarely (8%) expressed, and usually in a very small percentage of cells, in adenocarcinoma of the prostate. P63 is another useful marker in differentiating high grade urothelial from prostatic adenocarcinoma. Using tissue microarrays, we found p63 to have a greater specificity albeit lower sensitivity for urothelial carcinoma compared to high molecular weight cytokeratin (100% specificity and 83% sensitivity). Other markers that also appear highly specific but only of modest sensitivity for urothelial carcinoma include uroplakin and thrombomodulin (49%-69 % sensitivity).


Ellis DW, Leffers S, Davies JS, Ng AB. Multiple immunoperoxidase markers in benign


Breast Cancer: Old and the New Approaches to Predictive Markers

Allen M. Gown, M.D.
Medical Director and Chief Pathologist
PhenoPath Laboratories
Seattle, Washington
Breast Cancer: Old and New Approaches to Predictive Markers

- Estrogen receptors
- HER2
- Cell proliferation
- Molecularly defined breast cancers: old wine in new bottles?
The “Old” - Most Important Information

- Age, menopausal status
- Tumor histologic type
- Tumor size
- Nodal status
- Tumor grade
Histologic Approaches to “Phenotyping” Breast Cancers

- Microscopic description of tumors
- Size and shape of nuclei
- Interface with connective tissue
- Presence of “angiolympathic space invasion”
- Frequency of mitotic figures
- Grade - total, nuclear
Evolution of ER Assays in Breast Cancer

Direct ▶ DCC ▶ EIA ▶ IHC-Frozen ▶ IHC-Paraffin ▶ ?PCR
Methods to Detect and Distinguish Between Bound Steroid and Excess Unbound Hormone

- Sedimentation in sucrose gradients
- Electrophoresis
- Isoelectric focussing (gels)
- Precipitation with protamine sulfate
- Absorption onto hydroxylapatite

Too costly, time consuming, not scalable
Methods to Detect and Distinguish Between Bound Steroid and Excess Unbound Hormone

- Dextran coated charcoal (DCC)
- Enzyme immunoassay
A. Aliquots (100 µl) of cytosol prepared from a frozen powder of human breast cancer were incubated with 100 µl of buffer containing increasing amounts of [³H] estradiol, in the presence (open circle) or absence (closed circle) of a 200-fold excess of diethylstilbestrol. After adsorption of unbound steroid on dextran-coated charcoal, the specific binding (closed box) was obtained by the difference in soluble radioactivity in the presence and absence of competitor. B. The titration data from A were plotted according to the method of Scatchard, the free steroid concentration being calculated by the difference between the bound radioactivity and that originally present. The binding capacity is calculated from the intercept on the abscissa, and the dissociation constant (0.21 nM) from the slope of the line.
Inherent Problems with All Steroid Binding Assays

- ER is labile protein that may lose binding capacity during storage and processing (preanalytical)
- Does not detect receptor that is already occupied by endogenous hormone (or exogenous agent)
Enzyme Immunoassay (EIA)

Prognostic Value of Estrogen and Progesterone Receptors Measured by Enzyme Immunoassays in Human Breast Tumor Cytosols

John A. Foekens,1 Henk Portengen, Wim L. J. van Putten, Harry A. Peters, Hendrik L. J. M. Krijnen, Jana Alexieva-Figusch, and Jan G. M. Klijn

Division of Endocrine Oncology (Biochemistry and Endocrinology) and Department of Statistics [W. L. J. v. P.], Dr. Daniel den Hoed Cancer Center, 3008 AE Rotterdam, The Netherlands

N = 205

DCC v. EIA

- Concordance > 90%
- Spearman rank correlations: ER Rs = 0.94; PR Rs = 0.88
- Equivalent prognostic power (clinical outcome)
Enzyme Immunoassay vs. Dextran Coated Charcoal: Advantages of EIA

- Rapid and easy to perform
- Requires less tissue
- Measures bound and unbound receptors
- Does not involve use of radioactive substances
Advantages of Degradative Techniques for ER Assessment

- Inherently quantitative
- Can be standardized, in kit form
- Can be tested using known standards (e.g., lyophilized reference samples)
Disadvantages of Degradative Techniques for ER Assessment

- Requires fresh tissue
- Requires fresh tissue
- Requires fresh tissue
Optimal Method of ER Testing: IHC?

Estrogen Receptor Immunocytochemistry in Paraffin Embedded Tissues with ER1D5 Predicts Breast Cancer Endocrine Response More Accurately than H222Sp γ in Frozen Sections or Cytosol-Based Ligand-Binding Assays

Louis P. Pertschuk, D.O.1,2
Joseph G. Feldman, Dr. P.H.3
Yong-Doo Kim, M.D.1,2
Lorraine Braithwaite, A.B.2
Frank Schneider, M.D.1
Albert S. Braverman, M.D.4
Constantine Axiotis, M.D.1,2

Cancer 77:2514-19, 1996
### B. Correlation between ER1D5 in paraffin section and ER by DCC

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<th>Negative</th>
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<td>34</td>
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<td>Total</td>
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<td>36</td>
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Kappa = 0.204 ± 0.082; \( P < 0.02 \)
Paradigm Shift: Quantitative Degradative Methods Bad, IHC Good

Cancer 77:2419-22, 1996

Paraffin Section Immunocytochemistry for Estrogen Receptor
The Time Has Come

Clive R. Taylor, M.D., Ph.D.

Department of Pathology and Laboratory Medicine, University of Southern California Medical Center, Los Angeles, California.
<table>
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<td>Correlation with hormone response/survival</td>
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<td>Directly evaluates malignant cells not normal</td>
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*Taylor CR, Cancer 77:2419-22, 1996*
<table>
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<tr>
<th>FEATURE</th>
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| False Negatives | 1. Improper tissue handling  
2. Tumor not present  
3. Competition by endogenous hormone | 1. Improper tissue handling  
2. Technical failure (e.g., poor epitope retrieval) |
| False Positives | 1. Contamination by normal breast  
2. Nonspecific binding of ligand to non-receptor molecules | |
Potential Problems with Immunohistochemistry

- Pre-analytical variables (e.g., specimen type, fixative content and duration, processing)
- Analytical variables (e.g., antibody, epitope retrieval, detection system)
- Interpretive variables (e.g., scoring system, dichotomization cutoff)
Allred Score

Proportion Score (PS)

- 0 = negative
- 1 = 1/100
- 2 = 1/10
- 3 = 1/3
- 4 = 2/3
- 5 = 1

Intensity Score (IS)

- 0 = negative
- 1 = weak
- 2 = intermed
- 3 = strong

Total Score = PS + IS (score from 0 to 8)

from Mohsin, SK 2003
Defining ER Positivity

Estrogen Receptor Status by Immunohistochemistry Is Superior to the Ligand-Binding Assay for Predicting Response to Adjuvant Endocrine Therapy in Breast Cancer

Jennet M. Harvey, Gary M. Clark, C. Kent Osborne, and D. Craig Allred

Best Cutpoint: IHC score >2 (p<0.0001)
Automated quantitative analysis of estrogen receptor expression in breast carcinoma does not differ from expert pathologist scoring: a tissue microarray study of 3,484 cases

Dmitry A. Turbin · Samuel Leung · Maggie C. U. Cheang · Hagen A. Kennecke · Kelli D. Montgomery · Steven McKinney · Diana O. Treaba · Niki Boyd · Lynn C. Goldstein · Sunil Badve · Allen M. Gown · Matt van de Rijn · Torsten O. Nielsen · C. Blake Gilks · David G. Huntsman
Optimal cut-point for Ariol using X-tile software was 0.4%.

No difference in prognostic significance of ER positivity by Ariol vs. pathologist.
Quantitative analysis of estrogen receptor heterogeneity in breast cancer

Gina G Chung¹, Maciej P Zerkowski², Sriparna Ghosh¹, Robert L Camp³ and David L Rimm³

AQUA (automated image analysis technology)

Made “gold standard array” of breast cancer cases with known ER scores of 0, 20, 50, 70, 80, 100%

“Gold standard array” served as means of normalizing each whole section AQUA score
RT-PCR Performed on Deparaffinized, Formalin-Fixed Tissue

A Multigene Assay to Predict Recurrence of Tamoxifen-Treated, Node-Negative Breast Cancer

Soonmyung Paik, M.D., Steven Shak, M.D., Gong Tang, Ph.D., Chungyeul Kim, M.D., Joffre Baker, Ph.D., Maureen Cronin, Ph.D., Frederick L. Baehner, M.D., Michael G. Walker, Ph.D., Drew Watson, Ph.D., Taesung Park, Ph.D., William Hiller, H.T., Edwin R. Fisher, M.D., D. Lawrence Wickerham, M.D., John Bryant, Ph.D., and Norman Wolmark, M.D.
16 Genes in OncoTypeDX™

**Proliferation**
- Ki67
- STK15
- Survivin
- CCNB1 (cyclin B1)
- MYBL2

**HER2**
- GRB7
- HER2

**Estrogen**
- ER
- PGR
- BCL2
- SCUBE2

**Invasion**
- MMP11 (stromolysin 3)
- CTSL2 (cathepsin L2)

**GSTM1**

**CD68**

**BAG1**

**Reference**
- ACTB (β-actin)
- GAPDH
- RPLPO
- GUS
- TFRC

*Paik S et al., NEJM 351:2817-26, 2004*
Calculation of Recurrence Score

**Weighting Factors**

- GRB-7 (HER-2) 0.47
- ER 0.34
- Cell proliferation 1.04
- Invasion 0.10
- CD68 0.05
- GSTM1 0.08
- BAG1 0.07

Paik S et al., NEJM 351:2817-26, 2004
Quantitative RT-PCR
ER Analysis

The Oncotype DX assay uses RT-PCR to determine the RNA expression of the genes below. These results may differ from ER, PR, or HER2 results reported using other methods or reported by other laboratories. The ER, PR, and HER2 Scores are also included in the calculation of the Recurrence Score.

ER Score = 10.0 Positive

The ER Score positive/negative cut-off of 6.5 units was validated from a study of 761 samples using the 1D5 antibody (immunohistochemistry) and 607 samples using the SP1 antibody (immunohistochemistry). The standard deviation for the ER Score is less than 0.5 units.

Clinical Experience:
For ER positive breast cancer, the magnitude of tamoxifen benefit increases as the ER Score increases from 6.5 to ≥12.5.

Please note: The Average Rate of Distant Recurrence reported on Page 1 based on the Recurrence Score was determined in patients who received 5 years of tamoxifen treatment and takes into account the magnitude of tamoxifen benefit indicated by the ER Score.
Estrogen- and Progesterone-Receptor Status in ECOG 2197: Comparison of Immunohistochemistry by Local and Central Laboratories and Quantitative Reverse Transcription Polymerase Chain Reaction by Central Laboratory


N = 776

- Allred scores on TMAs
- ER using 1D5 and PR using PgR 636
- ER RT-PCR score isolated from 16 gene assay
## Concordance IHC and RT-PCR

<table>
<thead>
<tr>
<th></th>
<th>IHC Positive (Central)</th>
<th>IHC Negative (Central)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RT-PCR Positive</strong></td>
<td>404 (99%)</td>
<td>50 (14%)</td>
</tr>
<tr>
<td><strong>RT-PCR Negative</strong></td>
<td>5 (1%)</td>
<td>310 (86%)</td>
</tr>
</tbody>
</table>

Badve SS et al., J Clin Oncol 26:2573-81, 2008
<table>
<thead>
<tr>
<th>Method</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER via IHC</td>
<td>p = 0.091</td>
</tr>
<tr>
<td>ER via RT-PCR</td>
<td>p = 0.014</td>
</tr>
<tr>
<td>Oncotype Dx RS</td>
<td>p &lt; .0001</td>
</tr>
</tbody>
</table>

Badve SS et al., J Clin Oncol 26:2573-81, 2008
### RT-PCR v. IHC for ER

#### Table 2: ER Concordance

<table>
<thead>
<tr>
<th></th>
<th>Central IHC ER+</th>
<th>Central IHC ER-</th>
<th>Total Oncotype DX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oncotype DX ER+</td>
<td>501 (99%)</td>
<td>20 (20%)</td>
<td>521</td>
</tr>
<tr>
<td>Oncotype DX ER-</td>
<td>7 (1%)</td>
<td>79 (80%)</td>
<td>86</td>
</tr>
<tr>
<td>Total Central IHC</td>
<td>508</td>
<td>99</td>
<td>607</td>
</tr>
</tbody>
</table>

**CONCORDANCE** 96%  
Kappa: 83%  
95% CI (94%, 97%)  
95% CI (77%, 89%)

*Baehner FL et al, San Antonio Breast Cancer Symposium, 2007*
- Of the 7 discordant pairs that are ER positive by IHC but ER negative by RT-PCR, 6 (86%) are within 1 unit of the 0.5 cutoff.
- Of the 20 discordant pairs that are ER negative by IHC but ER positive by RT-PCR, 17 (85%) are within 1 unit of the 0.5 cutoff.
## IHC v. PCR

<table>
<thead>
<tr>
<th>FEATURE</th>
<th>PCR</th>
<th>IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlation with hormone response/survival</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Correlation with prognosis</td>
<td>?</td>
<td>Yes</td>
</tr>
<tr>
<td>Specimen requirements</td>
<td>Paraffin</td>
<td>Paraffin</td>
</tr>
<tr>
<td>Retrospective studies feasible</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Suitable for FNAs</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Quantitative</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Turnaround time</td>
<td>Days</td>
<td>Hours</td>
</tr>
<tr>
<td>Can evaluate tumor heterogeneity</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Directly evaluates malignant cells not normal</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Taylor CR, Cancer 77:2419-22, 1996*
RT-PCR

Degradative technique

Lose granularity, heterogeneity

Can get false positives from normal breast
Promise of RT-PCR

- Can be performed on deparaffinized, formalin fixed sections as easily as IHC
- Quantifiable
- More reproducible? Better precision?
- Better predictor of relapse/response?
- Bad tissue more forgiving of mRNA than protein?
- How to validate?
Problems With All Methods

- Do not speak to functionality of estrogen receptor
- Do not speak to activation of downstream pathways
- Do not identify the subset of patients responding to hormone receptor targeted therapy
“Not everything that can be counted counts, and not everything that counts can be counted.”

Albert Einstein
HER2
Why Determine HER-2 Status?

- Predictor of outcome
- Predictor of negative response to chemotherapy (CMF)
- Predictor of positive response to chemotherapy (anthracyclines)
- Predictor of resistance to tamoxifen
- Predictor of response to trastuzumab (Herceptin™) and lapatinib
HER-2 Gene Amplification and Protein Overexpression

Her-2 gene → HER-2 mRNA → HER-2 protein

Normal Breast Epithelial Cell → Breast Cancer Cell (with HER-2 alterations)
HER2 Gene Amplification = Protein Overexpression

- Initial study of 187 frozen breast cancer specimens by Slamon D et al., 1989 showed 10% Western blot positive but Northern blot negative.
- Almost all proved to be false negative.
- mRNA studies on FISH analysis.
- "Single copy" overexpressers exceedingly rare.
Overexpression/Amplification: Binary or a Spectrum?

IHC Score
- 0
- 1+
- 2+
- 3+

FISH Count
- 2
- 2
- 4
- 10
- >20

Increasing mRNA, receptors on surface
HER-2 IHC Scoring System (Revised*)

- Negative
- Equivocal
- Positive

*in conjunction with ASCO-CAP Guidelines
Positive (3+)
Strong, membranous ("chicken wire") signal in >30% of tumor cells
2+ Equivocal
1+ Negative
HER-2 IHC Testing May Not Be Accurate
Diagnostic Evaluation of HER-2 as a Molecular Target: An Assessment of Accuracy and Reproducibility of Laboratory Testing in Large, Prospective, Randomized Clinical Trials

Michael F. Press,1,2 Guido Sauter,5 Leslie Bernstein,1,3 Ivonne E. Villalobos,2 Martina Mirlacher,5 Jian-Yuan Zhou,2 Rooba Wardeh,2 Yong-Tian Li,2 Roberta Guzman,2 Yanling Ma,2 Jane Sullivan-Halley,3 Angela Santiago,2 Jinha M. Park,4 Alessandro Riva,6 and Dennis J. Slamon4

N = 2,600

Assessment for entry into BCIRG clinical trials

Overall 77.5% agreement in community lab HER2 IHC vs. central lab FISH
<table>
<thead>
<tr>
<th></th>
<th>FISH-NEG</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF</td>
<td>96.4%</td>
<td>93.9%</td>
<td>83.3%</td>
<td>21.9%</td>
<td></td>
</tr>
<tr>
<td>POS</td>
<td>3.6%</td>
<td>6.1%</td>
<td>16.7%</td>
<td>78.1%</td>
<td></td>
</tr>
</tbody>
</table>

IHC Scoring of Outside Labs

Press MF et al., 2005

Clin Cancer Res 11:6598-607, 2005
Discordance Between IHC and FISH

- IHC 3+/FISH-negative or IHC 0,1/FISH-positive
- Alleged to be 4% in Reddy JC et al., Clin Breast Cancer 7:153-7, 2006, but actual numbers are 14% of HER-2 3+ cases FISH-negative
Concordance Between Central and Local Laboratory HER2 Testing from a Community-Based Clinical Study

Jolina C. Reddy,1 James D. Reimann,1 Steven M. Anderson,2 Pamela M. Klein1
### Table 3: HER-First Concordance: Central IHC Versus Central FISH Results (n = 1289)

<table>
<thead>
<tr>
<th>Central IHC Testing</th>
<th>Central FISH Testing, n (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FISH-Negative</td>
<td>FISH-Positive</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>200 (99)</td>
<td>3 (1)</td>
<td></td>
</tr>
<tr>
<td>1+</td>
<td>433 (97)</td>
<td>14 (3)</td>
<td></td>
</tr>
<tr>
<td>2+</td>
<td>192 (83)</td>
<td>39 (17)</td>
<td></td>
</tr>
<tr>
<td>3+</td>
<td>59 (14)</td>
<td>349 (86)</td>
<td></td>
</tr>
</tbody>
</table>

High concordance between immunohistochemistry and fluorescence *in situ* hybridization testing for HER2 status in breast cancer requires a normalized IHC scoring system

Allen M Gown¹,², Lynn C Goldstein¹,², Todd S Barry¹,², Steven J Kussick¹,², Patricia L Kandalaff¹,², Patricia M Kim¹,² and Christopher C Tse¹,²

¹PhenoPath Laboratories, Seattle, WA, USA and ²Immunohistochemistry and Molecular Pathology Research Institute of Seattle, Seattle, WA, USA

- N = 6604 cases
- >100 hospitals in 29 states
- All tissues fixed in formalin
HER2/CEP17 ratio = 1.4 (Not amplified)
Concordance Rates
Normalized v. Non-Normalized

- 0.1+ and Nonamplified:
  - Normalized: 99.2%
  - Non-normalized: 99.5%

- 3+ and Amplified:
  - Normalized: 94.7%
  - Non-normalized: 69.4%

N = 6604

Gown et al., Mod Pathol 21:1271-7, 2008
Normalized Score

3+ - 1+ = 2+
HER-2 Gene Amplification Detected by FISH

HER-2 gene
Chromosome 17

Non-Amplified Tumor
Ratio = 1.0

Amplified Tumor
Ratio = 3.5
Quantitative FISH Setup
HER-2 FISH Scoring System: HER-2/Chr 17

<1.8 = not amplified

>2.2 = amplified

Between 1.8 and 2.2 = equivocal*

2.2-5 low amplification >5 high amplification

*in conjunction with ASCO-CAP Guidelines
HER-2 signals = 3.1
Chr 17 signals = 1.8
Ratio = 1.723 (not amplified)
HER-2 signals = 18.6
Chr 17 signals = 2.1
Ratio = 8.87 (amplified)
Metasystems ratio = 2.3 (amplified)
### Distribution of HER2 FISH Ratios

**PhenoPath Laboratories 2003-2009**

<table>
<thead>
<tr>
<th>Is the Amount of HER2 Amplification of Significance?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Is HER2 FISH a binary or continuous variable?</td>
</tr>
</tbody>
</table>
N = 2,071 in HERA (largest adjuvant trastuzumab trial)

Impact of HER2 gene copy number on survival

Impact of IHC (2+ v. 3+) in context of FISH amplification
No Relationship Between HER2 Copy Number and Survival

Dowsett M et al., J Clin Oncol 27:2962-69, 2009
Considerations of test accuracy, reproducibility, and precision favor FISH. Protein not consistently analyzed in formalin fixed tissues owing to vagaries of fixation. FISH less dependent upon tissue fixation methods.
N = 978 metastatic breast CA

Two clinical trials of lapatinib v chemotherapy

HER2 assessed by IHC, FISH*, RT-PCR

*HVLab and ALab
HVLab

- High volume commercial reference laboratory
- PathVysion™ (Abbott)
- Assignment of FISH ratio by laboratory technician
- Reviewed by pathologist when requested

ALab

- Academic reference/research laboratory
- Pathysion™ (Abbott)
- Enumerations done by a licensed clinical laboratory scientist and confirmed by a board certified pathologist
- At least 20 nuclei counted
FISH-FISH Concordance

- More than one third of cases FISH-negative at HVLab were FISH-positive at ALab
- 70% of these discordants IHC 3+

Table 4. Comparison of FISH determinations at two different reference laboratories

<table>
<thead>
<tr>
<th></th>
<th>ALab</th>
<th></th>
<th>HVLab</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FISH amplified</td>
<td>FISH nonamplified</td>
<td>Total</td>
</tr>
<tr>
<td>FISH-positive</td>
<td>199</td>
<td>23</td>
<td>221</td>
</tr>
<tr>
<td>FISH-negative</td>
<td>6</td>
<td>35</td>
<td>43</td>
</tr>
<tr>
<td>Total</td>
<td>205</td>
<td>58</td>
<td>263</td>
</tr>
</tbody>
</table>

NOTE: Concordance rate: 234/263 = 89% (P < 0.0001; 95% CI, 85-93%).

Press MF et al., Clin Cancer Res 14:7861-70, 2008
ASCO-CAP Guidelines

Breast cancer specimen (invasive component)

HER2 testing by validated IHC assay for HER2 protein expression

Positive for HER2 protein expression IHC 3+ (defined as uniform intense membrane staining of > 30% of invasive tumor cells)

Equivocal for HER2 protein expression IHC 2+

Test with validated assay for HER2 gene amplification

Positive for HER2 gene amplification

Equivocal HER2 gene amplification (Patients with HER2/CEP17 ratio ≥ 2.0 were eligible for the adjuvant trastuzumab trials)

Negative for HER2 gene amplification

Negative for HER2 protein expression IHC 0 or 1+
16 Genes in OncoTypeDX™

**Proliferation**
- Ki67
- STK15
- Survivin
- CCNB1 (cyclin B1)
- MYBL2

**Estrogen**
- ER
- PGR
- BCL2
- SCUBE2

**Invasion**
- MMP11 (stromolysin 3)
- CTSL2 (cathepsin L2)

**Reference**
- ACTB (β-actin)
- GAPDH
- RPLPO
- GUS
- TFRC

**HER2**
- GRB7
- HER2

**GSTM1**

**CD68**

**BAG1**
The Oncotype DX assay uses RT-PCR to determine the RNA expression of the genes below. These results may differ from ER, PR, or HER2 results reported using other methods or reported by other laboratories.¹

The ER, PR, and HER2 Scores are also included in the calculation of the Recurrence Score.

Hercept Test: The HER2 positive cut-off of ≥11.5 units, equivocal range from 10.7 to 11.4 units, and negative cut-off of <10.7 units were validated from concordance studies of 755 samples using the HercepTest™ assay (immunohistochemistry) and another study of 568 samples using the PathVysion® assay (FISH). The standard deviation for the HER2 score is less than 0.5 units.⁴
HER2 assessment in a large Kaiser Permanente case-control study: comparison of FISH and quantitative RT-PCR performed by central laboratories
Baehner FL, Achacoso NS, Maddala T, Alexander C, Shak S, Quesenberry CP, Gown AM, Goldstein LC, Habel LA

HER2 assessment in a large Kaiser Permanente case-control study: comparison of FISH and quantitative RT-PCR performed by central laboratories

Table 4: HER2 Concordance 2x2 According to Mandated ASCO/CAP Guidelines

<table>
<thead>
<tr>
<th></th>
<th>Central FISH +</th>
<th>Central FISH -</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR by Oncotype DX +</td>
<td>55 (98%)</td>
<td>11 (3%)</td>
<td>66</td>
</tr>
<tr>
<td>RT-PCR by Oncotype DX -</td>
<td>1 (2%)</td>
<td>408 (97%)</td>
<td>409</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>419</td>
<td>475</td>
</tr>
</tbody>
</table>

\*Equivocal cases excluded by both assays.\(^2\)
- **CONCORDANCE** 97%, 95% CI (96%-99%). Kappa 89%, 95% CI (82%-95%).
- Concordance calculated as \((55+408)/475\).

San Antonio Breast Cancer Conference, 2007
Cell Proliferation
Assessment of Cell Proliferation in Breast Cancer

- Mitotic index
- Ki-67 antigen expression
- Expression of cell proliferation-related mRNAs
Ki-67 as prognostic marker in early breast cancer: a meta-analysis of published studies involving 12 155 patients

Meta-analysis of 46 published studies with DFS and/or OS

Cutoffs for “high” v. “low” according to individual authors
Ki-67 as prognostic marker in early breast cancer: a meta-analysis of published studies involving 12,155 patients

<table>
<thead>
<tr>
<th>Probability of relapse, all pts</th>
<th>1.93</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probability of relapse, node negative</td>
<td>2.31</td>
</tr>
<tr>
<td>Probability of relapse, node positive</td>
<td>1.59</td>
</tr>
<tr>
<td>Survival, all patients</td>
<td>1.95</td>
</tr>
<tr>
<td>Survival, node negative</td>
<td>2.54</td>
</tr>
<tr>
<td>Survival, node positive</td>
<td>2.33</td>
</tr>
</tbody>
</table>
Proliferation markers and survival in early breast cancer: A systematic review and meta-analysis of 85 studies in 32,825 patients

R. Stuart-Harris a,*,1, C. Caldas a, S.E. Pinder b, P. Pharoah c

- Thymidine or bromodeoxyuridine labelling (N=11)
- Mitotic index (N =20)
- PCNA (N=11)
- Ki-67 (N=43)
Proliferation markers and survival in early breast cancer: A systematic review and meta-analysis of 85 studies in 32,825 patients

R. Stuart-Harris $^{a,*,1}$, C. Caldas $^a$, S.E. Pinder $^b$, P. Pharoah $^c$
Proliferation markers and survival in early breast cancer: A systematic review and meta-analysis of 85 studies in 32,825 patients

R. Stuart-Harris a,*,1, C. Caldas a, S.E. Pinder b, P. Pharoah c

Ki-67 Index
MULTIVARIATE ANALYSIS

OS

DFS
All proliferation-related measurements proved in multivariate analysis* to be associated with shorter overall survival in early breast cancer.

Not certain whether they provide information in addition to prognostic indices such as Nottingham Prognostic Index or Adjuvant!Online.

* e.g., grade, ER, HER2, nodal status, etc.
Why is Ki67 index not included in routine clinical decision making regarding use of adjuvant chemotherapy?

- There is lack of clarity regarding how Ki67 measurements should influence clinical decisions.

- What is the optimal cutpoint?
Tested hypothesis that Luminal A v. Luminal B distinction could be performed by IHC analysis of cell proliferation

Determined optimal cutoff for Ki67 index

Determined outcome in patients treated only with tamoxifen as function of Ki67 index
Ki67 Index, HER2 Status, and Prognosis of Patients With Luminal B Breast Cancer

Maggie C. U. Cheang, Stephen K. Chia, David Voduc, Dongxia Gao, Samuel Leung, Jacqueline Snider, Mark Watson, Sherri Davies, Philip S. Bernard, Joel S. Parker, Charles M. Perou, Matthew J. Ellis, Torsten O. Nielsen

- N = 357 (formalin fixed paraffin embedded)
- qRT-PCR performed for 50 discriminatory genes (Parker et al., 2009)
- Validation set 4,046 BCCA breast cancer tissue microarrays
- Determined outcome in patients treated only with tamoxifen as function of Ki67 index
## Ki67 Index, HER2 Status, and Prognosis of Patients With Luminal B Breast Cancer

Maggie C. U. Cheang, Stephen K. Chia, David Voduc, Dongxia Gao, Samuel Leung, Jacqueline Snider, Mark Watson, Sherri Davies, Philip S. Bernard, Joel S. Parker, Charles M. Perou, Matthew J. Ellis, Torsten O. Nielsen

<table>
<thead>
<tr>
<th>Hormone Rec</th>
<th>HER2</th>
<th>Ki67</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>ER and/or PR positive</td>
<td>Neg</td>
</tr>
<tr>
<td>Luminal B</td>
<td>ER and/or PR positive</td>
<td>Neg</td>
</tr>
<tr>
<td>Luminal HER2</td>
<td>ER and/or PR positive</td>
<td>Pos</td>
</tr>
</tbody>
</table>
Breast Cancer Gene Expression Signatures and Cell Proliferation

<table>
<thead>
<tr>
<th>Signature Type</th>
<th>Number</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>70 gene signature</td>
<td>70</td>
<td>Cell cycle, angiogenesis, invasion and metastasis</td>
</tr>
<tr>
<td>76 gene signature</td>
<td>76</td>
<td>Cell cycle, proliferation, DNA repair, immune response and apoptosis</td>
</tr>
<tr>
<td>OncotypeDx RS</td>
<td>16</td>
<td>Proliferation, ER and HER2, invasion</td>
</tr>
<tr>
<td>Genomic grade index</td>
<td>97</td>
<td>Cell cycle and proliferation</td>
</tr>
</tbody>
</table>
Ki67 Index, HER2 Status, and Prognosis of Patients With Luminal B Breast Cancer

Maggie C. U. Cheang, Stephen K. Chia, David Voduc, Dongxia Gao, Samuel Leung, Jacqueline Snider, Mark Watson, Sherri Davies, Philip S. Bernard, Joel S. Parker, Charles M. Perou, Matthew J. Ellis, Torsten O. Nielsen

(B) Predicting Luminal B

%Ki67 IHC labeling index
Best cutoff: 13.25%
Sensitivity: 77% (95% CI 64%-87%)
Specificity: 78% (95% CI 68%-87%)
Ki67 Index, HER2 Status, and Prognosis of Patients With Luminal B Breast Cancer

Maggie C. U. Cheang, Stephen K. Chia, David Voduc, Dongxia Gao, Samuel Leung, Jacqueline Snider, Mark Watson, Sherri Davies, Philip S. Bernard, Joel S. Parker, Charles M. Perou, Matthew J. Ellis, Torsten O. Nielsen
Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications

- N = 78 cancers
- Hierarchical clustering
- Focus on 427 genes showing variations in expression 4-fold median abundance
Unsupervised ("Bottom Up")
What classes of breast cancer exist, based on molecular features of tumor differentiation and/or grade?

Supervised ("Top Down")
What differences in gene expression exist between 'good outcome' and 'bad outcome' breast cancers?

Candidate Gene
Selected genes of interest on basis of existing biologic knowledge combined into multivariate predictive model.
Hierarchical Clustering
• **Basal-like** - High expression of CK5, CK17, laminin, and fatty acid binding protein 7

• **ERBB2 (HER2) positive** - High expression of HER2 and GRB7 on 17q22.24

• **Normal breast-like** - High expression of genes of adipose tissue and non-epithelial cell types

• **Luminal** - A and B (and C?) variants A with higher expression of ER than B,C
Unique clinical outcomes for molecularly defined groups

IHC Analogues of Molecular Classification?

Modified from Sotiriou C and Pusztai L, NEJM 360:790-800, 2009
Gene expression profiling predicts clinical outcome of breast cancer


* Divisions of Diagnostic Oncology, Radiotherapy and Molecular Carcinogenesis and Center for Biomedical Genetics, The Netherlands Cancer Institute, 121 Plesmanlaan, 1066 CX Amsterdam, The Netherlands
‡ Rosetta Inpharmatics, 12040 115th Avenue NE, Kirkland, Washington 98034, USA
† These authors contributed equally to this work

Nature 415:530-6, 2002
van’t Veer et al, 2002
SUPERVISED ANALYSIS

Correctly identifies outcome in 83% of patients
Commercialization of 70 gene assay

- First fully commercialized microarray-based assay for breast cancer
- Received 510(k) clearance from FDA in 2007
- Prognostic for all women <61 with either ER-positive or ER-negative disease
- Requires fresh frozen tumor specimens
RT-PCR based assay on deparaffinized, formalin fixed tissue

Derived from 250 candidate genes, using NSABP B-20 dataset

Developed algorithm based on weighted expression of 16 genes (+5 reference)
Genes in Recurrence Score

**Proliferation**
- Ki67
- STK15
- Survivin
- CCNB1 (cyclin B1)
- MYBL2

**HER2**
- GRB7
- HER2

**Estrogen**
- ER
- PGR
- BCL2
- SCUBE2

**Invasion**
- MMP11 (stromolysin 3)
- CTSL2 (cathepsin L2)

**GSTM1**

**CD68**

**BAG1**

**Reference**
- ACTB (β-actin)
- GAPDH
- RPLPO
- GUS
- TFRC

OncotypeDx™
Calculation of Recurrence Score

Weighting Factors

- GRB-7 (HER-2) 0.47
- ER 0.34
- Cell proliferation 1.04
- Invasion 0.10
- CD68 0.05
- GSTM1 0.08
- BAG1 0.07
Lists of genes in different gene expression based predictors overlap only slightly if at all.

Selected using different patient cohorts, microarray platforms, mathematical methods.

Are they concordant with respect to predictions for individual patients?
<table>
<thead>
<tr>
<th>Type</th>
<th>Low</th>
<th>High</th>
<th>Good</th>
<th>Poor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal-like</td>
<td>53</td>
<td>53</td>
<td>0</td>
<td>53</td>
</tr>
<tr>
<td>Luminal A</td>
<td>123</td>
<td>36</td>
<td>62</td>
<td>36</td>
</tr>
<tr>
<td>Luminal B</td>
<td>55</td>
<td>50</td>
<td>1</td>
<td>46</td>
</tr>
<tr>
<td>HER2+ ER-</td>
<td>35</td>
<td>35</td>
<td>0</td>
<td>32</td>
</tr>
</tbody>
</table>

Fan C et al., NEJM 355:560-9, 2006
OncotypeDX RS v. 70 Gene Classification

Agreement in classification: 239/295* or 81%*

Fan C et al., NEJM 355:560-9, 2006

*collapsing low and intermediate RS scores
Meta-analysis of publicly available breast cancer gene expression and clinical data

- N = 2833

- Gene coexpression modules of proliferation, ER, and HER2 signaling used to dissect role of constituent genes of 9 prognostic signatures
• Identified 524 genes significantly associated with survival

• 71% of these genes were strongly correlated with proliferation

• 26% of these genes were associated with estrogen receptor

• 2.2% of these genes associated with HER2
Are We All Looking at the Same Thing?

- Supervised gene expression
- Unsupervised gene expression
- IHC (ER, PR, HER2, Ki67, p53)
- Histology (e.g., grade)
The Blind Men and the Elephant

19th century poem by John Godfrey Saxe based on South Asian source

It’s a high grade tumor!

It’s a HER2 amplified tumor!

It’s a luminal B!

It’s a high RS tumor!
Problems with Gene Expression Methodology

- Classification schemes based on small numbers of tumors
- Reproducibility (methodological)
- Complicated statistical methods
- Most require fresh tissue
- Still have unclassified tumors
Conclusions

- Breast marker studies ER, HER2, and cell proliferation are critical to predicting outcome and determining optimal treatment.
- IHC or FISH if properly performed can be accurate but ‘real world’ performance of these may not be accurate enough.
- Gene expression methods may prove to be surrogates for IHC, FISH assessment of ER, HER2 and cell proliferation.
Thank you for your attention

Questions?

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Helpful Markers for Diagnosis and Prognosis: What and When

Mesothelioma Versus Carcinoma: Tempest in a Pleural Teapot?

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Key words
• malignant mesothelioma
• metastatic adenocarcinoma
• pleural biopsy
• immunohistochemistry

Objectives
At the end of this lecture attendees who paid attention will be able to,
• define diffuse pleural mesothelioma
• apply traditional and contemporary diagnostic tools to distinguish mesothelioma from its mimics
• articulate the role of pathology in predicting prognosis in patients with diffuse pleural mesothelioma

Define diffuse pleural mesothelioma
Pleural malignant mesotheliomas are serosal neoplasms derived from multipotent mesothelial cells and characterized by a diffuse pattern of growth over the pleural surface.(8) The incidence of mesothelioma in men in the USA peaked in the late 1990’s or early 2000’s although recent analysis suggests that attributable deaths may not peak until 2010.(3, 44) Death rates in Europe and Australia may not peak until 2020.(34) Trends in incidence and death rates mirror trends in asbestos use that peaked in 1973 combined with a 20-40 year lag period in occupationally related mesotheliomas.(3) It is estimated that in North America about 90% of pleural mesotheliomas in men are asbestos related. The percentage of mesotheliomas attributable to asbestos is dramatically lower in women at about 20%.(38)

Histologically mesotheliomas are classified into three categories: epithelioid (epithelial), sarcomatoid (sarcomatous), and mixed. Epithelioid mesotheliomas are the most common, accounting for just over half of cases, and the remainder are about evenly split between sarcomatous and mixed tumors.(29) The differential diagnosis is broad and heavily dependent on the histologic type. Epithelioid mesotheliomas may be difficult to distinguish from benign mesothelial hyperplasia on one end of the spectrum, and from pleural involvement by carcinoma in obviously malignant tumors. Epithelioid hemangioendotheliomas and angiosarcomas may occasionally enter the differential diagnosis. A subset of sarcomatoid mesotheliomas may also be difficult to distinguish from benign pleural fibrosis, but when obviously malignant have a limited differential
diagnosis in the setting of diffuse pleural disease without a dominant soft tissue or parenchymal mass. Mixed tumors tend to be less problematic but are sometimes confused with either sarcomatoid carcinoma or synovial sarcoma.

Apply traditional and contemporary diagnostic tools
Immunohistochemistry is helpful primarily in distinguishing epithelioid mesothelioma from metastatic carcinoma. Carcinomas occasionally involve the pleura in a diffuse manner that closely mimics mesothelioma, so-called pseudomesotheliomatous carcinoma.(1) An ever growing list of antibodies purports variable sensitivities and specificities for distinguishing mesothelioma from adenocarcinoma as recently reviewed by Ordóñez.(33) Squamous differentiation is rare in mesothelioma and therefore metastatic squamous cell carcinoma a less common consideration for which immunostains may nonetheless be helpful.(32) A small panel of two mesothelioma associated markers (e.g. calretinen, CK5/6, WT-1, mesothelin, podoplanin), two carcinoma associated markers (e.g. MOC-31, BG-8, Ber-EP4, B72.3, CEA) and tumor specific markers as indicated (e.g. TTF-1, napsin A, ER/PR) is usually sufficient.(33, 46) Molecular studies have limited utility in the differential diagnosis of mesothelioma, with the notable exception of synovial sarcoma which can occur as a primary pleural tumor and is associated with the characteristic t(X; 18) (SYT-SSX) translocation.(45) Recently several investigators have demonstrated differences in DNA methylation profiles that may prove helpful not only in distinguishing mesothelioma from other malignant tumors such as adenocarcinoma but also in separating benign from malignant mesothelial lesions.(5, 6, 21, 42)

No single diagnostic tool outperforms routine microscopic analysis when it comes to separating benign from malignant mesothelial proliferations. Invasion into the soft tissues of the chest wall or mediastinum or into the lung is the single most important histologic finding in distinguishing mesothelioma from mesothelial hyperplasia.(7) In this context immunohistochemical stains for cytokeratins can be helpful in highlighting the presence or absence of invasion. Aside from keratin staining to assess for invasion, special stains are of limited value in any individual case although immunoreactivity for epithelial membrane antigen (EMA), desmin, p53, Bcl-2, p-170, glucose transporter (GLUT)-1, X-linked inhibitor of apoptosis protein (XIAP), and cytoplasmic and nuclear staining for β-catenin occurs more frequently in malignant mesothelial lesions.(11, 25, 37) Malignant mesotheliomas also tend to have higher proliferation rates but with sufficient overlap to limit utility in any single patient.(40) Homozygous deletion of the 9p21 locus harboring p16/CDKN2A is the most consistently observed genetic abnormality in mesothelioma and offers a molecular strategy for separating benign from malignant mesothelial lesions using a commercially available FISH assay.(4, 27) Serum biomarkers (i.e. soluble mesothelin-related peptide [SMRP], megakaryocyte potentiation factor [MPF] and osteopontin) show promise (i.e. sensitivities 73%, 34% and 47% at a specificity of 95%, respectively) but are plagued by unacceptably high false positive rates and are not yet validated for standard practice.(35)

Role of pathology in predicting prognosis
Mesotheliomas are lethal tumors. Prognostic factors that may impact length of progression free and overall survival include performance status, disease stage, and non-epithelioid histology (see below).(12, 19) Cytotoxic chemotherapy may extend survival. A combination of cisplatin and pemetrexed is the current recommendation for front-line therapy in patients with unresectable disease.(35, 41) Multimodality therapy in which extrapleural pneumonectomy or pneumonectomy with pleural decortication is combined with chemotherapy and radiation offers the only hope of extended survival in a highly selected subgroup of patients.(15, 16, 18, 23, 26, 36, 39, 43) In patients undergoing multimodality therapy, epithelial histology, negative margins and negative extrapleural lymph nodes are all associated with prolonged survival.(13, 17, 31, 39)

Protein expression and molecular studies may play an increasingly important role in prognosis and patient selection for aggressive therapies.(28) Expression of cyclooxygenase-2, p21 and p27 and homozygous deletion of \(p16/\text{CDKN2A}\) have been linked to shorter survivals.(2, 10, 30) A novel prediction model predicated on three ratios of expression levels for four genes using a RT-PCR technique accurately predicted overall and cancer specific survival in a large number of patients undergoing debulking surgery at a single institution.(20) Adding other pathology based prognostic variables (i.e. histology and lymph node status) to the model separated patients into low, intermediate and high risk groups with median survivals of 31.9 months, 12.9 months, and 6.9 months respectively.

Effective targeted therapies are not currently available for mesothelioma. Epidermal growth factor receptor (EGFR) expression is common in mesothelioma and is associated with an epithelioid phenotype.(14) Unfortunately trials with tyrosine kinase inhibitors have not shown the same promise demonstrated in selected lung cancer patients likely because the sensitizing mutations associated with treatment response are absent in mesothelioma.(9, 22) Overexpression of vascular endothelial growth factor (VEGF) is also common in mesothelioma but to date targeted therapies using bevacizumab in combination with other chemotherapeutic agents have not proven effective.(24) Other candidate targets in the p53, retinoblastoma protein, and Wnt pathways show promise in in vitro and animal studies using gene therapy strategies.(28)

References


Intraductal Pancreatic Tumors
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Intraductal neoplasms of the pancreas have been increasingly recognized and studied over the past 10 years. Their clinical, radiographic, and histopathologic features are now well-described, and a number of variants have emerged. Because they represent clinically detectable precursors to invasive pancreatic carcinoma, intraductal neoplasms can serve a model for studying neoplastic progression in the pancreas, an illusive target given the usually occult nature of the more common invasive carcinoma precursor, pancreatic intraepithelial neoplasia (PanIN). There are both similarities and differences between the intraductal neoplasms and PanINs, and correlation of the morphologic variations with the molecular phenotypes has helped delineate novel pathways of tumorigenesis in the pancreas. Molecular subtyping has also opened the door for more sensitive preoperative diagnosis, both for intraductal neoplasms as well as for radiographically undetectable cancer precursors.

General features of intraductal neoplasms

Most intraductal neoplasms of the pancreas fall into one of several morphologic variants of intraductal papillary mucinous neoplasms (IPMNs). IPMNs are intraductal neoplasms composed of mucin-producing ductal epithelial cells, usually arranged in variably complex papillary masses that occur within the major pancreatic ducts, which are cystically dilated. IPMNs can be localized or they can diffusely involve the ductal system. They are defined to be macroscopically detectable and are therefore usually more than 1 cm in diameter; thus, the cystic dilatation of the ducts can be seen radiographically on CT scans or magnetic resonance cholangioapncreatography (MRCP). IPMNs are divided into main duct type, in which the main ducts of Wirsung or Santorini are dilated, branch duct type, in which the cystic changes are restricted to the secondary ducts, or combined type that involves ducts of both sizes.

The epithelium of IPMNs is usually composed of columnar cells containing cytoplasmic mucin, and mucinous secretions are common within the dilated ducts involved by the tumor. Spillage of mucin into the duodenum from the ampulla of Vater is a classic endoscopic finding in IPMNs. The epithelial cells can have varying degrees of cytoarchitectural atypia in different regions of the tumor. The most modest degree of atypia consists of mucin cells arrange in simple papillae or flat epithelium and containing uniform, basally oriented nuclei that lack atypia. Even this degree of atypia is regarded as low grade dysplasia, however. In areas of moderate dysplasia, the nuclei show full thickness pseudostratification and moderate atypia. Suprabasal mitotic figures can be found. The architecture may be mildly complex, but unsupported micropapillary tufts, cribriforming, and fusion of papillae into solid sheets is not found. High grade dysplasia (carcinoma in situ) is characterized by markedly atypical nuclei with loss of polarity, including apical nuclei, marked anisonucleosis, numerous mitotic figures, and severe
architectural complexity. IPMNs are to be graded based on the most severe dysplasia detected.

*Papilla subtypes in IPMNs*

Several different morphologic types of epithelium can occur in IPMNs. The most simple pattern is the gastric foveolar type, in which columnar cells with abundant mucin are found, resembling the foveolar cells of the gastric mucosa. Most gastric foveolar type IPMNs have low grade or moderate dysplasia and occur in predominantly in the branch ducts. Many have large areas of flat epithelium with minimal papilla formation. The intestinal type of IPMN has exuberant papillae which are usually architecturally simple and villiform. The nuclei are elongated and pseudostratified, resembling the appearance of villous adenomas of the large bowel. Rarely there remains abundant mucin in the cells and the dysplasia is only low grade; most intestinal type IPMNs have moderate or high grade dysplasia, and most are main duct or combined types. The pancreatobiliary type of IPMN is less common and is composed of markedly complex, branching papillae, with superimposed micropapillary formations and cribriform structures. The nuclei are large and more rounded than in the intestinal type, and there is less pseudostratification. Pancreatobiliary type IPMNs resemble the papillary tumors more common in the bile ducts, and almost all have high grade dysplasia. Areas resembling the gastric foveolar pattern, with lower grades of dysplasia, can be found associated with the pancreatobiliary (and sometimes the intestinal) type papillae, raising the possibility that the gastric foveolar type is a common precursor to the other patterns. Finally, some intraductal papillary neoplasms have oncocyctic epithelium and a markedly complex, arborizing, cribriform, or even solid architecture. In addition to intraepithelial lumina, there are intracellular lumina, and the nuclei are large and contain prominent nucleoli. These tumors were first reported as intraductal oncocyctic papillary neoplasms, but are now classified as the oncocyctic type of IPMN by the WHO. In addition to having morphologic differences, these four patterns of IPMNs have differences in immunolabeling and molecular findings (see below).

*Other types of intraductal neoplasms*

In additional to IPMNs, other intraductal tumors have been recently characterized. One variant has a predominantly tubular architecture within the intraductal tumor nodules. These have been reported as intraductal tubular neoplasms or, for the cases that also contain papillary regions, intraductal tubulopapillary neoplasms (ITPNs). ITPNs differ from IPMNs in several respects. Most cases show little if any intracellular mucin in the epithelium. The nuclei are moderately atypical, but based on the highly complex architecture, almost all cases have high grade dysplasia. There can even be necrosis and stromal desmoplasia within the exophytic intraductal tumor. Some ducts are completely filled with tumor, making assessment for invasive carcinoma very difficult. The background usually shows little in the way of PanIN, and transitions to other patterns of intraductal neoplasia (i.e., IPMNs) are not found.
Intraductal tubular adenoma, pyloric gland type, analogous to the same tumor more common in the gallbladder, has been reported, but is now felt to be closely related to gastric foveolar type IPMN, which can also have small tubular glands at the periphery of the cystic ducts. This variant is not separated from IPMNs in the WHO classification.

Intraductal growth can be found in variety of non-ductal neoplasms, including neuroendocrine neoplasms, acinar cell carcinomas, and pancreatoblastomas. The intraductal growth is usually a focal feature, and these cases are not considered true intraductal neoplasms, which are ductal type by definition.

**Invasive carcinoma in IPMNs**

Invasive carcinoma is found in about 30-35% of IPMNs. Those with high grade dysplasia are more likely to have invasive carcinoma, which can be only microscopic in extent. Both high grade dysplasia and invasive carcinoma are more common in main duct or combined IPMNs, emphasizing the importance of this distinction in preoperative assessment. There are two different types of invasive carcinoma that commonly occur in association with IPMNs. About half of the cases have a tubular morphology, essentially indistinguishable from conventional pancreatic carcinoma. These cases usually arise in association with pancreatobiliary type IPMNs, although cases can also arise from intestinal type IPMNs. However, the other half of invasive carcinomas arising in IPMNs are colloid carcinomas composed of large pools of extracellular mucin in which strips, clusters and individual tumor cells are suspended. These colloid carcinomas almost always arise in association with intestinal type IPMNs. They have a much less aggressive clinical course, with a 5 year survival of 55% following resection. In contrast, IPMNs with tubular type invasive carcinoma have essentially the same dismal prognosis as convention pancreatic carcinoma, especially when the invasive component measures more than 2 cm. Invasive carcinoma can also arise in association with the oncocytic type of IPMN and with ITPNs, but the specific subtypes and biologic behavior of these carcinomas have not been well-characterized due to the rarity of these entities.

**Immunophenotype**

Immunohistochemistry is helpful to distinguish the types of IPMNs. Although all IPMNs and ITPNs express keratins (usually 7, 8, 18, and 19), and most also immunolabel for common glycoproteins (CEA, B72.3, CA19-9), there are other markers that are restricted to specific papilla types. The intestinal type of IPMN expresses intestinal markers such as CK20 and CDX2, which are usually not found in any other type. Also interesting is expression of MUC proteins. MUC1 (mammary type mucin, usually associated with tumors with an aggressive biology) is expressed in conventional ductal adenocarcinomas and also in pancreatobiliary type IPMNs and their associated tubular type invasive carcinomas. It is negative in intestinal type IPMNs and colloid carcinoma, which instead express MUC2, the intestinal type mucin that more commonly signifies a relatively indolent biology. These converse staining patterns demonstrate two different pathways of tumorigenesis – the more common pancreatobiliary pathway, typical of conventional ductal carcinoma and pancreatobiliary type IPMNs, and the uncommon
intestinal pathway found in intestinal type IPMNs and colloid carcinomas. Interestingly, gastric foveolar IPMNs have a “MUC-nul” phenotype, expressing neither MUC1 nor MUC2. All three types of IPMNs stain for a gastric foveolar-type mucin, MUC5AC, which has therefore not shown a correlation with morphology or biology. Another MUC protein, MUC6, is a pyloric type mucin expressed in normal pyloric glands of the stomach. Some expression is also seen in regions of gastric foveolar type IPMNs with pyloric type glands, but most cases are negative. In contrast, intraductal neoplasms with predominantly cuboidal rather than columnar cells strongly express MUC6. These include pancreatobiliary type IPMNs, oncocytic IPMNs, and IPTNs.

Molecular correlations

Ductal neoplasia in the pancreas is characterized by sequential accumulation of genetic abnormalities through the sequence of PanIN lesions to invasive carcinoma. Activating mutations in the KRAS oncogene are found in >95% of infiltrating ductal adenocarcinoma, and loss of function mutations (or promoter methylation) occur in p16/CDKN2A (95%), TP53 (60%), and MAD4/DPC4 (55%). Many other genes are also known to be altered with lower frequencies, and a genetic fingerprint of ductal carcinoma is emerging. Less information exists about intraductal neoplasms. However, many of the genes commonly altered in infiltrating ductal adenocarcinoma have been studied in IPMNs. KRAS mutations are common (30-45%) in IPMNs but are not as universal as in infiltrating ductal adenocarcinomas. They first occur at the level of low grade dysplasia, as they do in the PanIN sequence. Interestingly, they are not uniform among the different subtypes of intraductal tumors. Gastric foveolar and pancreatobiliary IPMNs have a higher frequency of KRAS mutations than intestinal type, and oncocytic IPMNs and IPTNs essentially never have mutations in this oncogene. TP53 and p16/CDKN2A mutations occur in IPMNs, at higher frequency in those with high grade dysplasia, but there are insufficient data to draw strong conclusions about varying rates among the morphologic subtypes. MAD4/DPC4 loss is very uncommon in IPMNs, especially in those of intestinal type, which essentially lack mutations in this gene, as do the colloid type invasive carcinomas that arise from them.

About 25% of IPMNs have biallelic inactivation of the Peutz-Jeghers gene (SKT11/LKB1). Mismatch repair gene abnormalities (and MSI) are uncommon in IPMNs. About 10% of IPMNs have mutations in PIK3CA mutations, which are very rare in conventional pancreatic carcinomas, and BRAF is rarely mutated.

These genetic alterations thus overlap with those of conventional carcinomas and PanINs and follow a similar pattern of gradual accumulation with increasing dysplasia, but the frequencies of abnormalities as well as some of the specific genes differ, supporting the concept that the molecular pathogenesis of intraductal neoplasms is not identical to that of conventional carcinomas.

Issues in the diagnosis of IPMNs
Due to the increased use of more sensitive cross sectional imaging, cystic lesions of the pancreas are being detected with greater frequency. Some of these are incidental benign cysts such as serous cystic neoplasms and retention cysts that do not require surgical resection. Other cases are IPMNs (usually branch duct type) or mucinous cystic neoplasms (MCNs), which can have high grade dysplasia or associated invasive carcinoma. However, small branch duct IPMNs are unlikely to have significant dysplasia, and clinical studies have shown that if they are managed conservatively, it is very rare for a patient to develop invasive carcinoma with close radiographic follow-up. However, this is the group (less than 3 cm cysts) that requires the most careful consideration about management. Preoperative distinction of purely benign cystic lesions from the group of “mucinous cysts” (IPMNs and MCNs) is one problem, and recognition of which IPMNs have a risk of high grade dysplasia or carcinoma sufficient to justify immediate resection is another. There are some radiographic criteria that are used to target patients for surgery, such as dilatation of the main pancreatic duct (main duct or combine type IPMN), size greater than 3 cm, and the presence of a solid mural nodule. Interval growth during follow-up can also be used to argue for surgery. But pathologic analysis of preoperative cyst fluid has become another strategy to identify high-risk patients. The goal is to identify all cases with high grade dysplasia or carcinoma, recognizing that some false positives may occur that would lead to resection of IPMNs with only low grade dysplasia.

Routine cytologic evaluation can help separate mucinous cysts from non-mucinous cysts. However, there are limitations to the use of cytology alone to recognize which mucinous cysts have high grade dysplasia or invasive carcinoma (and therefore require immediate resection). Therefore, a variety of ancillary studies have been proposed. The measurement of cyst fluid glycoproteins has been studied extensively over the years, and many different proteins have been examined. In the end, the level of CEA in the cyst fluid remains a useful parameter. Elevated levels are found in all mucinous cysts, but levels in excess of 2500 ng/ml are more predictive of malignancy. Cyst fluid proteomic profiling has also been used to separate mucinous from non-mucinous cysts, with CEA and CA72.4 emerging among a profile of proteins that accurately separates these lesions. MUC2 and MUC4 protein levels are more elevated in IPMNs with high grade dysplasia or carcinoma.

Detection of KRAS mutations has also been assessed as a diagnostic aid, both in cyst fluid aspirates and in pancreatic duct juice, but the presence of KRAS mutations in some IPMNs with low grade dysplasia coupled with the relatively low frequency of mutations in intestinal type IPMNs (even those with high grade dysplasia) introduces limitations to this technique. A commercial molecular assay involves the simultaneous assessment of DNA quantification, KRAS mutations, mutational amplitude, as well as LOH analysis for a panel of genetic loci corresponding to sites of common gene abnormalities in high grade dysplasia and carcinoma. The results correlate well with the presence of high grade dysplasia and carcinoma, although studies comparing this testing to other available modalities are still appearing. Probably, integration of various data from radiology, cytology, protein measurements, and possibly also molecular assays will provide the
most sensitive means to predict which intraductal neoplasms require surgery and which can be followed.
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