Prognostic Factors in Breast Cancer

College of American Pathologists Consensus Statement 1999

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• ***Background.*—Under the auspices of the College of American Pathologists, a multidisciplinary group of clinicians, pathologists, and statisticians considered prognostic and predictive factors in breast cancer and stratified them into categories reflecting the strength of published evidence.**

***Materials and Methods.*—Factors were ranked according to previously established College of American Pathologists categorical rankings: category I, factors proven to be of prognostic import and useful in clinical patient management; category II, factors that had been extensively studied biologically and clinically, but whose import remains to be validated in statistically robust studies; and category III, all other factors not sufficiently studied to demonstrate their prognostic value. Factors in categories I and II were considered with respect to variations in methods of analysis, interpretation of findings, reporting of data, and statistical evaluation. For each factor, detailed recommendations for improvement were made. Recommendations were based on the following aims: (1) increasing uniformity and completeness of pathologic evaluation of tumor specimens, (2) enhancing the quality of data collected about existing prognostic factors, and (3) improving patient care.**

***Results and Conclusions.*—Factors ranked in category I included TNM staging information, histologic grade, histologic type, mitotic figure counts, and hormone receptor status. Category II factors included c-*erb*B-2 (Her2-*neu*), proliferation markers, lymphatic and vascular channel invasion, and p53. Factors in category III included DNA ploidy analysis, microvessel density, epidermal growth factor receptor, transforming growth factor-α, bcl-2, pS2, and cathepsin D. This report constitutes a detailed outline of the findings and recommendations of the consensus conference group, organized according to structural guidelines as defined.**

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CATEGORY 1

Tumor Size

Tumor size is one of the most powerful predictors of tumor behavior in breast cancer.[1–6] The frequency of nodal metastases in patients with tumors smaller than 1.0 cm is 10% to 20%,[1,7] and node-negative patients with tumors smaller than 1.0 cm have a 10-year disease-free survival rate of about 90%.[7–9] Precise assessment of tumor size is necessary to properly stratify patients, particularly since screening mammography has resulted in a steadily increasing proportion of pT1 cancers.

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Variation issues

- Interobserver variation in measurement of tumor size due to the confounding effects of desmoplastic stromal reaction, coexistent ductal carcinoma in situ, and tumor multicentricity.[10]
- Measurement of tumor size by gross examination versus microscopic examination.[11] Tumors such as lobular carcinoma may be considerably larger than visually apparent, while those with an extensive stromal reaction may be smaller than the gross examination would suggest.
- Inclusion of ductal carcinoma in situ in the measurement of tumor size. The size of the invasive component alone determines prognosis.[8]
- Reporting the size of the tumor in 1 versus 3 dimensions.

Recommendations

- The tumor should be measured in at least 2 dimensions, and the single greatest dimension of the invasive tumor is used for determining stage.
- The size of the tumor, as measured by gross examination, must be verified by microscopic examination. If there is a discrepancy between gross and microscopic measurements, the microscopic measurement of the invasive component takes precedence and should be used for tumor staging.

- For pT1 lesions or those with an extensive in situ component, measurement of tumor size on the histologic slide is more accurate than gross measurement.
- For tumors with both invasive and in situ components, only the invasive component is included in the tumor measurement for staging purposes.
- When 2 or more distinct invasive tumors are present, each is measured and reported separately; they are not combined into a single larger measurement.

Nodal Status

Axillary lymph node status has repeatedly been shown to be the single most important predictor of disease-free survival and overall survival in breast cancer.[12–15] Only 20% to 30% of node-negative patients will develop recurrence within 10 years, compared with about 70% of patients with axillary nodal involvement. The absolute number of involved nodes is also of prognostic importance; patients with 4 or more involved nodes have a worse prognosis than those with fewer than 4 involved nodes.

Variation Issues

- The extent of axillary dissection.[16,17]
- Method of finding lymph nodes in axillary dissection specimens (eg, palpation alone vs use of clearing solutions).
- Submission of each node in its entirety versus representative sampling of large nodes.
- Single- versus multiple-level sectioning.
- Reporting the number of involved lymph nodes.
- Reporting the level of nodal involvement versus only reporting the status of the apical or highest node.
- Reporting the size of the largest involved node versus the size of the largest nodal metastasis.
- The significance of tumor extension into the soft tissue adjacent to the lymph node is controversial. Early studies suggested that perinodal extension conferred an increased risk of breast cancer recurrence,[18] but more recent studies show no significant differences in prognosis when patients are controlled for extent of axillary involvement[19] or when extranodal tumor is microscopic.[20]

Recommendations

- The pathologist responsible for specimen examination has the discretion to choose the best method of finding lymph nodes.
- Grossly uninvolved lymph nodes should be entirely submitted for histologic examination, whereas representative sections of grossly positive nodes may be submitted. Small nodes may be submitted intact, but larger nodes should be sectioned for proper fixation and examination.
- The pathology report should clearly state the total number of lymph nodes examined, the total number of involved nodes, and the greatest dimension of the largest metastatic focus.
- For axillary dissections, 1 microscopic slide from each block is sufficient for routine examination (sentinel lymphadenectomies are discussed under ''Sentinel Lymphadenectomy'').
- The presence of extranodal tumor extension (regardless of extent) should be included in the pathology report, but more studies are needed to determine the significance of microscopic extranodal extension.

Micrometastasis

Several retrospective studies found that the prognosis of patients with isolated micrometastases in axillary lymph nodes (defined as <2 mm in diameter) is the same as that for patients with negative nodes,[21–23] while others have suggested that such patients have a worse prognosis.[24–26] Microscopic foci of metastatic tumor can be found in 9% to 13% of ''node-negative'' breast cancers by serial step-sectioning (hematoxylin-eosin stain only). This percentage increases to 15% to 20% of cases if immunohistochemistry (IHC) is used[24–28]; however, the prognostic significance of a histologically inapparent focus detected only by IHC is still controversial.[25–27]

Variation issues

- Use of single hematoxylin-eosin–stained sections, serial step sections, IHC, or molecular analyses, such as polymerase chain reaction (PCR) technology to detect and report micrometastases.
- Micrometastases are traditionally defined as histologically detected tumor foci measuring less than 2.0 mm, but histologically inapparent foci demonstrated by IHC staining and isolated keratin-positive cells may also be classified as micrometastases. The significance of micrometastasis detected only by IHC has not yet reached complete consensus.
- Patients with nodal metastases found only by IHC stains are grouped together with those with conventional micrometastases as N1a.

Recommendations

- A single microscopic section from each lymph node block is considered sufficient for evaluation.
- Any histologically confirmed focus of tumor that measures less than 2 mm in greatest dimension is classified as a micrometastasis.
- Unless clinical trials confirm the significance of micrometastases found only by IHC, detection and reporting of micrometastases should be based on staining with hematoxylin-eosin. If IHC stains are performed and tumor cells are detected only by that method, this finding should be clearly stated in the report.
- There are insufficient data to recommend routine IHC or molecular evaluations such as PCR to detect lymph node metastases, apart from research protocols.
- Cytokeratin-positive cells only, in the absence of a histologically identified tumor cell nest, should be classified separately.
- There are insufficient data to recommend changing pathologic tumor stage (''upstaging'') based only on finding rare cytokeratin-positive cells in axillary lymph nodes.

Sentinel Lymphadenectomy

Sentinel lymph node biopsy has emerged rapidly as a potential alternative to axillary dissection for staging breast cancer[29] and is sensitive and specific in predicting axillary status.[30–33] Axillary dissection is generally considered a staging procedure, but may have therapeutic benefit for some patients; however, chemotherapy and radiation, which may reduce axillary metastases,[34–36] confound the magnitude of this benefit.

Sentinel lymph node biopsy without axillary dissection is attractive because it may reduce the morbidity associ-

ated with axillary dissection, but the procedure has not yet been shown to have disease-free and overall survival rates equivalent to those of axillary dissection. The reported false-negative rates range from 0% in smaller, single institution studies to 11.4% (range 0% to 28.6%) in a large multi-institution study.[37] Several studies have used more intensive pathologic evaluation of sentinel nodes to maximize detection of micrometastases.[38]

Both occult and nonoccult metastases are more likely to be identified in sentinel nodes than in nonsentinel nodes. While the presence of occult metastases may have prognostic significance, it is not clear whether identifying occult metastases, particularly those smaller than 1 or 2 mm in greatest dimension, has clinical significance with regard to guiding current therapy.

Because sentinel lymph nodes have been shown to be highly predictive, it is reasonable to preserve their predictive utility by following some of the protocols used in published studies. Because surgeons may elect to perform a completion axillary dissection on patients with positive sentinel nodes, an intraoperative assessment of the node may help avoid a second anesthetic procedure.

Variation Issues

- Wide variation in methods used to evaluate sentinel lymph nodes, including the variable use of IHC, PCR, or both.
- Variations in the number of sections and levels examined histologically.
- Variations in the intraoperative examination of sentinel nodes. False-negative rates of up to 25% for frozen section detection of micrometastases in sentinel nodes have been reported.
- Cytokeratin antibodies used for detecting metastases.

Recommendations

- Sentinel lymph nodes should be sectioned as close to 2 mm as possible and entirely submitted for histologic examination (regardless of node size).
- A single microscopic section from each lymph node block is considered sufficient for evaluation. There are currently insufficient data to recommend routine serial step-sectioning of sentinel lymph nodes.
- Routine cytokeratin staining of histologically negative sentinel lymph nodes should not be considered standard until clinical trials demonstrate its clinical significance.
- For the intraoperative assessment of sentinel lymph nodes, careful gross examination with cytologic evaluation (imprint cytology) is preferable to frozen section examination, since the latter may consume significant amounts of nodal tissue.

Histologic Grade

Histologic grade is an important determinant of prognosis that also allows risk stratification within a given tumor stage.[39–42]

Variation Issues

- Different grading systems (eg, Nottingham combined histologic grade, nuclear grade).
- Grading of special types of cancers. Most of the special types of breast carcinoma are associated with a favorable prognosis, but this is generally true only for tumors with low-grade cytology. This latter statement is not the case for medullary carcinoma, which has a better prognosis than grading would suggest.
- Effect of specimen type on grading. Some studies have suggested that undergrading of tumors can occur when grading is performed on limited samples obtained by needle biopsy.[43]

Recommendations

- All invasive breast carcinomas with the exception of medullary carcinoma (as defined below) should be graded.
- The grading system used must be specified in the report, and the Nottingham combined histologic grade (Elston-Ellis modification of Scarff-Bloom-Richardson grading system) is recommended.[44]
- Grading of large-core needle biopsies may be done when there is sufficient tissue.

Histologic Type

Variation Issues

- Variations in the classification of invasive lobular carcinoma.[45] Many breast cancers have a lobular-type, single-file, or targetoid growth pattern, but only those with very-low-grade nuclei and low cell density are associated with a better prognosis than ordinary breast cancer or other subtypes of invasive lobular carcinoma. Lobular carcinomas with these classic features represent only about 4% of invasive breast cancers.
- Distinguishing tubular carcinoma from low Nottingham combined histologic grade carcinomas. While some authors suggest that tumor grade is more meaningful than terminology, pure tubular carcinoma (>90% pure) has been shown to have a particularly favorable prognosis.
- Identification of pure mucinous carcinoma. The production of abundant extracellular mucin alone is insufficient to confer the favorable prognosis of pure mucinous carcinoma (>90% pure).
- Lack of adherence to diagnostic criteria for medullary carcinoma. Medullary carcinoma is a rare variant of breast cancer (about 0.5% of cases) associated with a better prognosis than ordinary invasive breast cancer, particularly for node-negative patients. However, this improved prognosis is seen only when one finds the complete constellation of diagnostic features.
- Variations in terminology of ordinary breast cancers (ductal vs ordinary vs no special type).

Recommendations

- Classic invasive lobular carcinoma is diagnosed only when the tumor exhibits a single-file growth pattern, a monotonous population of small cells with very-low-grade nuclei, and low cell density.
- Tumors with a diffuse infiltrative growth pattern that do not fulfill the criteria for classic invasive lobular carcinoma should be reported primarily by histologic grade with the suffix ''with lobular features'' (or ''lobular variant''). Such tumors are identified separately because this growth pattern may be associated with extensive intramammary growth and distinctive patterns of metastasis.
- A diagnosis of pure mucinous carcinoma requires the presence of low-grade nuclei and extracellular mucin in at least 90% of the tumor. Tumors with less extensive

mucin production should be reported primarily by histologic grade with the suffix ''with mucinous features.''
- Pathologists must rigidly adhere to strict diagnostic criteria for a diagnosis of medullary breast carcinoma. These criteria include a sharply circumscribed tumor border; high histologic grade with patternless syncytial sheets of large, undifferentiated tumor cells; a substantial and diffuse lymphoplasmacytic infiltrate between cellular nests; and scant fibrous stroma.

Mitotic Figure Count

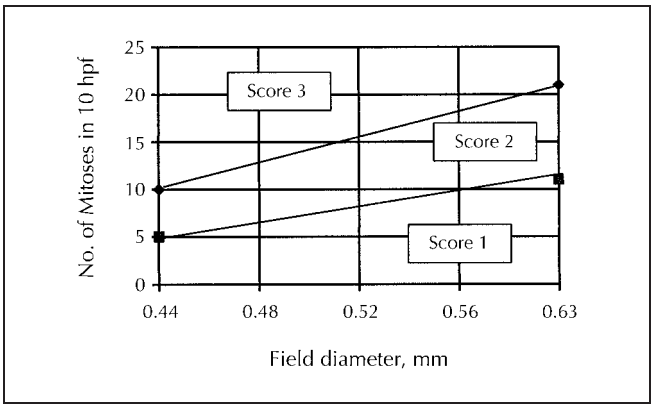
Mitotic index, defined as the number of mitotic figures in a given area of tumor, is an accurate means of estimating tumor cell proliferation and represents an integral part of the Nottingham combined histologic grade.[46–49] High mitotic rates have been correlated with poor clinical outcome.[50]

Variation Issues

- Variation in mitotic count by area of tumor selected for counting and by counting method.
- The assignment of points for mitotic index in the Nottingham combined histologic grade varies according to microscope field size.
- Criteria for identifying an acceptable mitotic figure.
- Variation in reporting mitotic figures, such as per high-power field (HPF), per 10 HPFs, per 50 HPFs, or by mitotic index.

Recommendation

- The mitotic figure count is reported as the number of mitotic figures found in 10 consecutive HPFs in the most mitotically active part of the tumor.
- Only clearly identifiable mitotic figures (eg, cells in prophase, metaphase, or anaphase) should be counted; hyperchromatic, karyorrhectic, or apoptotic nuclei should not be counted.
- The mitotic figure count should be specified in the pathology report (in addition to the histologic grade).
- For the purposes of grading, the HPF size must be determined for each microscope used in evaluating breast cancers, and the counts should be adjusted accordingly. Using a micrometer to measure the field diameter of the microscope is recommended. The appropriate point score for purposes of grade is then obtained by plotting the actual mitotic count against microscope field diameter (see Figure).



The field diameter (x axis) varies among microscopes, influencing the cutoff values (y axis) used in assigning mitotic score in the Nottingham combined histologic grade. Adapted from Sloane et al.[167]

Hormone Receptor Status

Estrogen receptor and progesterone receptor determinations are established procedures in the routine management of patients with breast cancer, primarily as predictive factors for response to therapeutic and adjuvant hormonal therapy.[51–61] Their predictive power in this setting is primarily based on studies conducted during the past 2 decades using ligand-binding assays. Recently, however, IHC has become the preferred method for determining the estrogen receptor and progesterone receptor status of breast cancer.[52] However, while the correlation between ligand-binding assay and IHC results is quite high, there have been relatively few clinical studies specifically demonstrating the predictive abilities of IHC assays for estrogen receptor and, especially, progesterone receptor.

Variation Issues

- Selection of antibody (6F11, H222, H226, D547, D75, 1D5, etc).
- Validation of immunohistochemical detection of progesterone receptor.
- Analysis done on primary tumor versus nodal metastasis.
- Absence of invasive tumor on stained block.
- Improper fixation or processing.
- Nuclear counterstain too strong—obscures low-level staining.
- Nuclear counterstain too weak—possible false-positive result.
- No uniform control tissues with known reactivity.
- Variations in antigen-retrieval methods.
- Comparability of antibodies and detection systems.
- Different scoring systems used (positive vs negative, H score, proportion score, intensity score). Some studies suggest that as few as 1% positive tumor cells may be associated with significant clinical responses to therapeutic and adjuvant hormonal therapy.[51]
- Heterogeneous staining.

Recommendations

- Hormone receptor analysis should be performed routinely in all primary breast carcinomas using IHC or ligand-binding assay. Immunohistochemistry is preferred for smaller tumors.
- When both the primary tumor and a nodal metastasis are available, analysis is preferably performed on the primary breast tumor. Analysis by IHC may be performed on large-core needle biopsy specimens when there is sufficient tissue.
- The primary antibody and substrate (paraffin vs frozen sections) used should be reported with the assay result. The name of the reagent kit used and the commercial supplier should be recorded in a diagnostic comment. If a fixative other than formalin is used, the fixative should be specified.
- Controls should be included in each assay. A tissue control (with positive cancer and adjacent benign epithelium) is recommended.
- The percentage or proportion of cells expressing the antigen should be specified in the pathology report. Avoid

using the terms ''positive'' and ''negative'' unless their definitions have been substantiated in clinical studies.
- There are insufficient data to recommend including an intensity score.

CATEGORY II

c-*erb*B-2 (Her2-*neu*)

*erb*B-2 (Her2-*neu*) gene amplification, which usually results in overexpression of the encoded transmembrane protein p185, occurs in about one third of breast cancers.[62–66] *erb*B-2 altered breast cancer is associated with high histologic grade, reduced survival,[67–73] lower responsiveness to methotrexate-based treatment regimens[67,68] and hormone receptor modulators such as tamoxifen,[74–76] and higher responsiveness to doxorubicin-based regimens.[67,70,71,77] Thus, *erb*B-2 analyses are requested to obtain prognostic (outcome independent of treatment) and predictive (outcome dependent on treatment) data.[78] Both molecular and immunohistochemical methods are used to detect *erb*B-2 alterations, but these methods are not standardized, and it is unclear which method or reagents are superior for prognostic or predictive value.[65,79]

Normal cells and the majority of breast cancers carry 2 copies of the *erb*B-2 gene on chromosome 17 and express low levels of p185.[80,81] Since this transmembrane receptor has homology to other family members (epidermal growth factor receptor [EGFR], *erb*B-3, and *erb*B-4), clinical tests must be specific for *erb*B-2 (as opposed to cross-reacting with homologous family members) and specific enough to detect amplification (increased gene copy number) or protein overexpression above the normal level. Neither amplification nor overexpression is equivalent to protein activation, which is the functionally activated form of the receptor. A monoclonal antibody that selectively recognizes activated *erb*B-2 has been reported,[82] but most reagents do not discriminate between active and nonactive forms of the *erb*B-2–encoded receptor.

The 2 most common assay systems use fluorescence in situ hybridization (FISH) and IHC. Numerous commercial reagents have been used for each system with variable results. Research data comparing these assays, or comparing data with other methods such as Southern blot or quantitative PCR-based methods, have shown significant associations, but not complete concordance.[61,65,66,78,79,83]

Commercial kits for FISH and immunohistochemical testing have now been approved by the Food and Drug Administration (FDA) and are commercially available. While clinical use of the anti-*erb*B-2 drug Herceptin (Genentech, Inc, South San Francisco, Calif) requires *erb*B-2 testing on tumors from potentially eligible patients,[84–86] the best method or reagent to select patients for Herceptin therapy is unknown. Besides the uncertainty regarding which methods or reagents are superior, a large proportion of *erb*B-2 testing uses nonstandardized in-house methods or conditions and various commercial reagents.

Commercially available controls include cell lines with high, moderate, and normal levels of *erb*B-2. While sections from fixed embedded pellets of cell lines are optimal controls, they are not widely used. Related issues, such as fixation,[87] antigen retrieval, and storage,[70,88] are not yet defined.

Method Variation Issues

Immunohistochemistry—

- Many commonly used primary reagents
  - Monoclonal reagents (eg, CB-11, others)
  - Polyclonal reagents (21N SAT, others)
  - Cocktail reagents (Mab-1/Pab 1, Zymed Laboratories, South San Francisco, Calif)
  - Hercept test (Dako Corporation, Carpinteria, Calif).
- Variables that may affect *erb*B-2 analysis (long-term storage, conditions of storage, fixation, optimization of reagents, etc) have not been extensively studied.
- Antigen-retrieval techniques.
- Antibodies against external, transmembrane, or internal domain (external domain may be suboptimal due to clipping in vivo).
- Reagent concentrations and optimization.
- Controls, use of fixed embedded cell lines with high, low, and no gene amplification (as marketed for kits, now commercially available).
- Automated immunostainer versus manual staining.

Fluorescence In Situ Hybridization—

Vysis, Inc, Her-2 Kit (Downers Grove, Ill)

- Centromeric (Ch 17) and gene (Her-2/*erb*B-2) probes.
- Data given as ratio of gene copy/centromeric copy.
- Direct labeled probes.
- Controls provided.
- Laboratory certification required.
- FDA-approved for prognosis.

Oncor Kit (Oncor/Ventana, Tucson, Ariz)

- Gene (*erb*B-2) probe only.
- Data given as an average number of signals per cell.
- Indirect labeled probe.
- Controls provided.
- Unclear utilization with automated staining systems.
- Laboratory certification required.
- FDA-approved for prognosis.

Traditional Molecular Genetic Techniques

- Southern blot, quantitative PCR, other.

Interpretation Variation Issues

- Interpretation of staining pattern (membranous vs cytoplasmic).
- Scoring of breast cancers (invasive component only should be scored).
- Internal negative control (benign breast and other cell types).
- Positive controls (cell lines with no, low-level, and high-level gene amplification, fixed and embedded in cell blocks optimal).

Reporting Variation Issues

Immunohistochemistry—

Scoring System

- Percent positive, intensity, cutoff points, 0 to 3+ Dako Hercept system, in situ versus invasive, concentric versus partial membrane staining, cytoplasmic versus membranous.
- Report comment.
- Reporting of primary reagent, batch number, method.
- Reporting of institutional experience with assay (percent positive, cutoff point, etc).

- Reporting of scoring system used.
- Statement of slide quality and controls.
- Statement regarding reproducibility, sensitivity, and specificity.

Fluorescence In Situ Hybridization—

Scoring System

- Signal copy number versus signal-centromere ratio.
- Gene copy heterogeneity.

Report Comment

- Kit type, batch number.
- Reporting of scoring system.
- Statement of slide quality and controls.
- Statement regarding laboratory certification.
- Statement regarding laboratory experience, reproducibility, sensitivity, and specificity.

Statistical Variation Issues

Immunohistochemistry—

- Estimated percentage, raw data versus positive/negative versus 0 to 3+, cutoff points, manipulated data.

Fluorescence In Situ Hybridization—

- Range versus median (how to deal with gene copy heterogeneity).
- Oncor FISH (Oncor/Ventana), ≤4 versus >4.
- Vysis FISH (probe-centromere ratio).

Other Issues

- Herceptin trials—what method is best for determining eligibility?
- Hercept test—highly variable data reported. Reproducibility issues need to be addressed.

Recommendations (General)

- The prognostic and predictive value of *erb*B-2 (Her2-*neu*) in invasive breast cancer is compelling and may warrant *erb*B-2 testing as a routine part of the diagnostic workup. *erb*B-2 testing of primary invasive cancers should be recommended prior to utilization of Herceptin, an anti–*erb*B-2 novel therapeutic agent, and may assist in predicting response to systemic agents.
- The predictive value of *erb*B-2 in some patient groups will require further validation through randomized clinical trials.
- Since many significant issues relating to the translational application of *erb*B-2 testing are unresolved (eg, testing methods, reagents, interpretation, and controls), a conservative approach is warranted.
- It is unclear whether FISH assays are superior to IHC, or whether FISH should be considered an adjunct or replacement.
- Given the current lack of standardization and comparability data, specific reagents or methods for *erb*B-2 testing cannot yet be recommended. It should be recognized that *erb*B-2 testing is a work in progress, and data to resolve these important issues are not available.

Recommendations (Specific)

- The method and primary reagent should be reported with the assay result. The name of the reagent kit used and the commercial supplier should be recorded in a diagnostic comment.
- Controls should be included in each assay. A tissue control (with strongly positive cancer and adjacent benign epithelium) is recommended. Fixed embedded cell lines with normal, slightly amplified, and significantly amplified *erb*B-2 are strongly recommended as companion assay controls and for assay development.
- Only the invasive component of a tumor (not in situ disease) should be scored.
- For IHC, membranous reactivity only should be considered positive.
- *erb*B-2 staining should not be observed in adjacent stroma or inflammatory cells, nor should benign epithelium show membranous reactivity. If staining is observed in benign components, the assay may be considered indeterminate.
- Reporting should include an estimate of the percentage of immunopositive invasive cancer cells. If a separate scoring system or cutoff point is also used to define positivity, it must be defined in a diagnostic comment.
- Variance in methodology (including any changes to FDA-approved or supplier-recommended protocols) should be recorded in a diagnostic comment.
- Indeterminate cases may warrant confirmatory testing using another method.
- Laboratory *erb*B-2 data and their correlation with histologic grade should be reviewed on a regular, ongoing basis.

p53

Nearly one third of breast cancers have mutations of the tumor suppressor gene *p53,* which are associated with high histologic grade and clinical aggressiveness.[89–95] Since mutations usually result in prolonged half-life and protein accumulation, immunohistochemical detection of p53 can be used as a surrogate for mutational analysis.[90,96–98] Immunostaining should be considered a screening method for *p53* mutation, as some cases have neither protein overexpression nor an increased half-life.[92,93]

p53 mutations in breast cancers appear to cluster in exons 5 through 9. Studies of mutation based on genetic sequencing have been limited because of the molecular complexity of this large gene, but newer high-throughput sequencing technologies are being developed. Other methods to detect p53 abnormalities include PCR-based amplification, with screening for mutations using single-strand conformational polymorphism assays or sequencing. Sequencing studies of breast cancer are often limited to the exon sequences 5 through 9 because of the mutational hot spots that have been identified there.

Immunohistochemical assays generally detect overexpression of the gene, which is often related to conformational alterations and a prolonged half-life of the encoded protein.[99,100] Given the diverse functions of the *p53* gene and the location and type of genetic abnormalities (including gene loss and point mutation), the specific genetic lesion may be shown to have prognostic importance.

While most *p53* abnormalities occur as spontaneous somatic events, patients with germline *p53* mutations (Li-Fraumeni syndrome) also have an increased incidence of breast cancer.[101–103] Recent evidence suggests a relationship between *BRCA1* and *p53* in hereditary breast cancer, such that *p53* acts as a cancer cofactor in these patients.[104]

p53 appears to be a useful prognostic marker, particularly in node-negative breast cancer patients,[105] and may also help identify patients likely to respond to chemotherapy or radiotherapy.[106–108] However, consensus as to the

need for routine p53 immunostaining has not occurred. Some studies report antigenic degeneration with time, therefore storage and fixation issues may be relevant.[88] Given the current clinical consensus that at least some node-negative breast cancer patients at high risk should be treated with chemotherapy, the issue of prognostic markers in this patient set is particularly relevant. Patients with p53-immunopositive cancers may develop autoantibodies against p53, which have been used by some to detect or follow cancers.[109] Array-based technologies that can screen for mutations in some regions of the gene have become commercially available, but these applications have not yet been widely adopted.

Method Variation Issues

Molecular PCR/Sequencing—

- Primers/conditions.
- Technology/equipment.
- Which exons/introns.
- Controls.
- Lack of FDA approval.
- Guidelines for when sequencing should be ordered.
- LiFraumeni syndrome (tumor, germline).
- Screening for somatic mutations, breast cancers.
- Screening for mutations, *BRCA1* patients.

Molecular Single-Strand Conformational Polymorphism Screening—

- Microdissection/macrodissection.
- Primers/conditions.
- Technology/equipment.
- Which exons/introns.
- Controls.
- Guidelines for when it should be used.
- Should apparent positives be submitted for sequencing?
- Sensitivity/specificity.

Immunohistochemistry—

- Reagents.
- Fixation effects (frozen, fixed, fixative).
- Antigen degeneration with storage (for formalin-fixed tissue, for tissues fixed with alcoholic formalin or other).
- Manner of storage to prevent or slow down degeneration.
- Antigen retrieval.
- Controls.

Interpretation Variation Issues

Molecular PCR sequencing—

- Reproducibility.
- Predictive value (sensitivity, specificity), particularly if limited sequencing is performed.
- Separation of artifact or polymorphism from true mutation.
- Reporting of concordant controls.
- Statement of method (PCR/sequencing [which exons/introns], array-based, etc).

Molecular Single-Strand Conformational Polymorphism Screening—

- Reproducibility.
- Sensitivity/specificity.
- Confirmation by sequencing.
- Reporting of technology, strengths, and weaknesses.

Immunohistochemistry—

- Sensitivity/specificity and controls—should laboratory report experience and ability to detect controls using panels of cell lines and same reagents?
- What is true positive (any staining, focal staining, cut points, etc)?
- Pattern of reactivity (nuclear only).
- Pattern of positivity (normal epithelium and other cells should be negative, an internal negative control).

Reporting Variation Issues

- Scoring system—percent positive, cutoff points, in situ versus invasive.
- Report comment.
- Reporting of primary reagent, batch number, method.
- Reporting of institutional experience with assay (percent positive, cutoff point, etc).
- Reporting of scoring system used.
- Statement of slide quality and controls.
- Statement regarding reproducibility, sensitivity, specificity.
- Disclaimers regarding non–FDA-approved methods.
- Distribution of reactivity, in situ, invasive, metastases (should this be described?).

Recommendations

- Although *p53* gene alterations in breast cancer have been associated with poor prognosis, there is not yet consensus that p53 testing should be performed routinely in clinical practice.
- Utility as a predictive marker has been reported, but extensive validation studies have not yet been performed.
- Several methods can be used to screen for or define p53 alterations in human tissue samples, but consensus regarding optimal methodology or reagents does not exist for either molecular or immunohistochemical assays.
- Immunohistochemical studies of p53 provide only surrogate data for *p53* mutation analysis, and sensitivity and specificity of IHC are affected by many factors. Confirmation by molecular genetic methods may be appropriate in some cases.
- In patients with suspected familial *p53* mutation, laboratory analysis (molecular sequencing) for germline alterations is appropriate in conjunction with a molecular genetic workup of patients or their family members.

Lymphatic or Vascular Channel Invasion

Peritumoral vascular invasion (either blood vessel or lymphatic channel) is predictive of local failure and reduced overall survival.[110–112] Although some studies have found no correlation with clinical outcome, this may be a reflection of differences in distinguishing true vascular space invasion from retraction artifact.

Method Variation Issues

- Assessment of intratumor versus peritumoral vascular channel invasion.[113]

- Distinction of true vascular channels from artifactual retraction spaces. A variety of special stains (eg, elastic stains, type IV collagen, laminin, CD31, CD34, and factor VIII) have been used to identify vascular spaces.[114,115]

Reporting Variation Issues

- Terminology—lymphatic channel versus vascular versus blood vessel.

Recommendations

- Vascular invasion is assessed in peritumoral breast tissue in routinely processed tissue.
- There is no consensus on the need for special stains to identify vascular spaces.
- It is not necessary to distinguish lymphatic channels from blood vessels. Such cases should be classified simply as vascular invasion.

Additional Proliferation Markers: MIB-1

Ki-67 is a labile, nonhistone nuclear protein that is not expressed in resting (G0) cells, but can be detected in the G1 through M phases of the cell cycle. Studies of its use as a marker of cell proliferation have shown that the percentage of Ki-67–positive cells (as detected by anti–Ki-67 stains of frozen sections) can be used to stratify patients into good and poor prognostic groups.[116–120] The monoclonal antibody MIB-1 recognizes Ki-67 but can be used in formalin-fixed, paraffin-embedded tissue sections. Several studies have suggested that MIB-1 may have greater predictive value than anti–Ki-67.[121–123]

Reactivity of Common Immunohistochemical Proliferation Antibodies

Ki-67: All phases except G0 and early G1.
MIB-1: All phases except G0 and early G1.
Proliferating cell nuclear antigen (nonhistone nuclear protein cofactor for DNA polymerase delta): Increases in G1, maximal in S-phase, and decreases in G2/M.

Variation Issues

- Selection of antibody (Ki-67 in frozen sections vs MIB-1 in fixed tissue).
- Effect of tissue fixation.
- Selection of appropriate area for staining (eg, center or periphery of tumor, area of highest tumor density, area of highest tumor reactivity).
- Which control tissues are used.[124]
- Variations in antigen-retrieval methods.
- Effects of preoperative therapy, menopausal status, or phase of menstrual cycle.
- Interpretation of multifocal tumors.
- Definition of a ''positive'' result (eg, >1%, >10%, >20%).
- Visual analyses.
- Counting total cells and percent positive.
- Point-counting methods.
- Visual estimations.
- Computerized image analysis.
- Percent area of staining versus percent of positive nuclei.
- Setting of staining thresholds.
- Is assessment of cell proliferation an independent prognostic factor?
- Reference intervals not determined (should different reference intervals be made for diploid vs aneuploid tumors, for different histologic types, for different grades?).
- Should reference intervals be discrete or continuous variables?

Recommendations

- Assessment of cell proliferation should be performed routinely in the evaluation of breast cancers. Mitotic figure count is listed as a category I factor and, thus, should be done in all cases. Assessment of other proliferation markers, such as MIB-1 or Ki-67, is considered optional.
- MIB-1 staining of fixed tissue sections is preferable to Ki-67 staining of frozen sections for routine analysis.
- The terms MIB-1 and Ki-67 are not synonymous and should not be used interchangeably.
- Analysis may be performed on large-core needle biopsy specimens when there is sufficient tissue.
- Reference intervals and performance characteristics must be determined by each individual laboratory.

DNA Analysis: Phase Fraction

Automated DNA analysis by flow cytometry or image analysis allows for accurate assessment of cell proliferation by measuring the number of cells actively synthesizing DNA (S-phase fraction). Image analysis is slower than flow cytometry, and the cell preparation takes longer and requires more technical expertise, but improvements in precision and speed of static or image cytometry may result in DNA analysis being done predominantly by these techniques rather than by flow cytometry.[125–129] However, most published studies that correlate DNA analysis with other prognostic factors and clinical outcomes have used flow cytometry.

The published literature supports an association between high S-phase fraction and increased risk of recurrence and mortality for patients with both node-negative and node-positive invasive breast cancer.[130] Wenger and Clark[131] recently reviewed a decade of experience with S-phase fraction determined by flow cytometry and concluded that it has clinical utility for patients with breast cancer. Remvikos and coworkers[132] noted that tumor responsiveness to neoadjuvant chemotherapy was directly related to S-phase fractions in 50 premenopausal women; however, S-phase fractions in other studies of adjuvant therapies have not been predictive of response to chemotherapy. Dressler and associates[133] analyzed tumors from node-negative patients enrolled in a large, randomized, intergroup study comparing cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) therapy with observation and found that chemotherapy was equally effective for patients with either low or high S-phase fractions. Muss and colleagues[134] evaluated S-phase fractions in tumors from node-positive patients enrolled in a Cancer and Leukemia Group B study designed to study dose intensification of cyclophosphamide, doxorubicin, and 5-fluorouracil (CAF). Although the dose-intensity hypothesis was confirmed, S-phase fraction did not predict response to therapy either alone or in combination with other predictive factors. Additional retrospective and prospective clinical trials with well-defined treatment regimens that also measure S-phase fraction will be required to address these issues.

Variation Issues

- DNA flow cytometry is performed on fresh tissue spec-

imens, frozen biopsy samples, needle aspirates taken directly from the tumor, and paraffin-embedded tissues.
- DNA flow cytometry can be performed on material originally fixed in formalin or a formaldehyde–acetone–acetic acid mixture, but results have been poor with tissue fixed in Bouin solution and unsatisfactory when mercury-based fixatives were used.
- A major limitation of single-parameter DNA flow cytometry is the variable admixture of stromal elements that produce DNA histograms that are composites of normal and malignant cells.[129] This problem is greatest with DNA diploid tumors when, because of complete overlap between the 2 populations, the measured S-phase fraction represents a composite of normal host cells and tumor cells.
- The DNA Cytometry Consensus Conference[130] found a lack of standardized methods and suboptimal measurement of S-phase fraction.
- Variations in reporting methods used when S-phase populations of DNA diploid and DNA aneuploid cells overlap. Most studies have used the S-phase fraction from the aneuploid population, but some studies report total S-phase fraction, and a weighted average of the 2 S-phase fractions may also be valid.
- Paraffin-embedded tissue contains a considerable amount of debris and clumps.
- Different laboratories use different cutoff points to classify S-phase fractions.

Recommendations

- S-phase fraction correlates with clinical outcomes of patients with primary breast cancer, but standardization and quality control must be improved before it can be considered in category I and used routinely.
- Samples from solid tumors should, in general, contain at least 20% tumor cells, and, particularly if S-phase fraction is to be determined, a minimum of 10 000 events should be analyzed.
- Combined staining with fluorescein-labeled anticytokeratin antibodies allows the DNA content of epithelial cells to be separated from that of other elements, which may further improve the prognostic significance of the S-phase fraction in breast cancer.
- Each laboratory should establish its own distribution of S-phase values and interpret individual results in the context of these distributions rather than by comparison with published cutoff points established by other laboratories.
- The optimal separation of patients into different risk groups by S-phase fraction has not been established, but the use of 3 rather than 2 risk groups may lessen the significance of misclassified tumors with near-borderline values.
- S-phase fraction cannot be determined for a significant percentage of paraffin-embedded specimens. For small or paraffin-embedded specimens, proliferation should be measured by alternative means, such as MIB-1.
- Additional studies are needed to resolve the issue of reporting S-phase fractions when diploid and aneuploid populations overlap.
- Cutoff points should be calibrated to the clinical outcome of patients whose tumors have been analyzed for S-phase fractions. At a minimum, the cutoff points should produce a similar distribution of S-phase values across laboratories.

CATEGORY III

DNA Ploidy Analysis

Besides determining S-phase fraction, DNA analysis allows for identification of tumors with abnormal DNA profiles (aneuploidy). The terms ''DNA diploid'' and ''DNA aneuploid'' are used to describe cells containing apparently normal and apparently abnormal amounts of DNA, unless actual ploidy is established by cytogenetic studies. The degree of DNA content abnormality is given by the DNA index, which is the ratio of G0-G1 peak locations of the sample (tumor) cells and normal or reference cells. For a sample to be classified as DNA aneuploid, 2 distinct G0/G1 peaks must be present in the histogram.

DNA ploidy has not been shown to correlate with clinical outcomes of patients with primary breast cancer. The DNA Cytometry Consensus Conference[130] concluded that neither DNA index nor DNA ploidy status achieves independent prognostic significance using multivariate analyses.

Variation Issues

- Distinguishing hypodiploid tumors from near-diploid, hyperploid tumors. Even though the incidence of such tumors is quite low (2% to 4%), the clinical outcomes are different.[128]
- Stained nuclei from chicken or rainbow trout erythrocytes are useful for fluorescence calibration as standards for DNA content estimation with fresh or frozen tissues, but not with archival tissue.
- Methods used to isolate and prepare cells and to analyze histograms.
- Lack of consensus about cutoff points to define DNA diploidy and aneuploidy that can be applied to all laboratories.
- Effect of debris on cell cycle analysis of fixed tissues. A background subtraction algorithm to compensate for debris is found in the 2 most widely used programs for DNA histogram analysis (ModFit, Verity Software House, Topsham, Me; Multicycle, Phoenix Flow Systems, San Diego, Calif).

Tumor Angiogenesis

Growth and risk of metastasis for some breast cancers appears to depend on the growth of new blood vessels adjacent to the tumor. There have been several reports of a direct association between density of tumor microvessels and risk of metastasis.[135–138] Most of these studies have used IHC to assess vascular density. Weidner et al[135] counted microvessels in the most densely vascularized areas of 49 cases and found a correlation between the frequency of metastasis and the number and density of vessels. However, other reports have shown no such association.[139–141] Axelsson and colleagues[139] found that measurement of microvessel density was too variable to be clinically useful.

Variation Issues

- Selection of antibody (CD31, CD34, factor VIII–related antigen, type IV collagen).
- Differences in type of fixative used.
- Methods of counting vessels.
- Microvessel density in 1 hot spot.
- Mean microvessel density value of 3 hot spots.
- Highest microvessel density value in three hot spots.

- Quality control.
- Variation in estimation of microvessel density by different observers.[139,142]
- Effect of observer experience on selection of vascular hot spots.[143]
- Definition of increased vascularity.
- How is a positive result defined?
- Handling of heterogeneity of positivity.
- Biologic variation.

Epidermal Growth Factor Receptor

Epidermal growth factor receptor is a cell membrane receptor for the epidermal growth factor, which has been shown to have a stimulatory effect on the growth of some breast cancers.[144] The receptor binds both epidermal growth factor and transforming growth factor-α. Overexpression of EGFR can be demonstrated in some breast cancers and has been associated with absence of estrogen receptor[145,146] and poor response to tamoxifen.[147] Studies of the prognostic significance of EGFR expression have provided mixed results, with only some studies showing a correlation between EGFR and poorer disease-free survival.[144,145,147–151]

Transforming Growth Factor-α

Transforming growth factor-α is a growth factor closely related to EGFR and competes with epidermal growth factor for the EGFR. Transforming growth factor-α appears to have a promoting effect on the growth of some breast cancers.[152]

bcl-2

bcl-2 has been reported to be a marker of good prognosis and responsiveness to tamoxifen. In the studies of Elledge et al[153] and Visscher et al,[154] the presence of bcl-2 correlated with the presence of estrogen receptor and with longer disease-free survival than bcl-2–negative tumors. Better response to tamoxifen was seen in the study by Elledge et al. A study by Hellemans et al[155] showed no prognostic significance for bcl-2 expression in node-negative patients, but bcl-2 negativity correlated with reduced survival among node-positive patients.

pS2

pS2 is a cytoplasmic protein that is expressed only after estrogen stimulation and appears to function in some way as a growth factor. Since pS2 is only produced if there is a functioning estrogen receptor–related pathway, measurement of pS2 theoretically could serve as a more accurate predictor of tumor behavior or responsiveness to hormonal therapy.[156,157] Several studies have shown that pS2-positive tumors have a better prognosis and a better response to tamoxifen than pS2-negative tumors,[156,158,159] and that pS2-negative tumors have a poor prognosis.[159]

Cathepsin D

Cathepsin D is a lysosomal proteinase that is overexpressed in some breast cancers. Overexpression of cathepsin D is associated with several poor prognostic features, such as high histologic grade, large tumor size, and node positivity,[160] and has been reported to be associated with an increased risk of recurrence and reduced disease-free survival.[161,162] However, while some studies have suggested that cathepsin D is an independent prognostic factor in node-negative patients,[163] others show no prognostic significance among node-negative patients.[164,165] Cathepsin D is also a normal constituent of stromal cells and macrophages, and some studies have found that there is no prognostic significance to cathepsin D expression in tumor cells.[166]

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