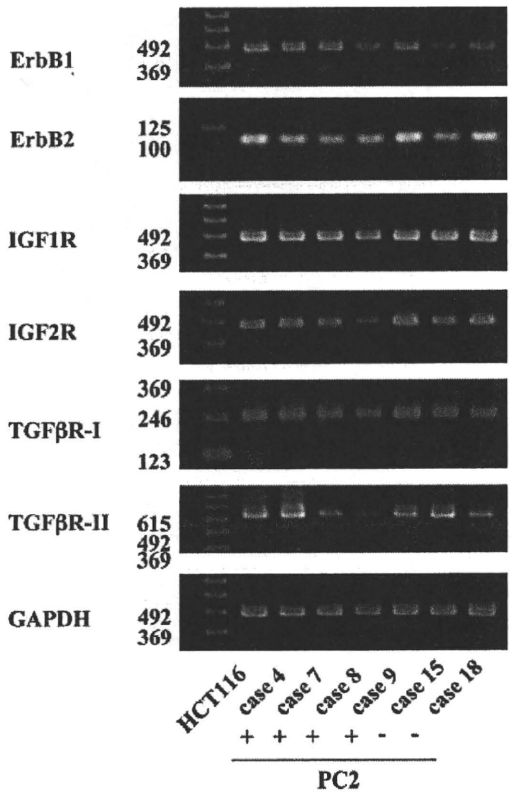

FIG. 3. RT-PCR examination in HCT116, four PC2-positive APPAs and two PC2-negative APPAs resulted in an amplification of the predicted 473-, 98-, 447-, 485-, 252-, and 784-bp bands corresponding to the human ErbB1, ErbB2, IGF1R, IGF2R, TGFβR-I, and TGFβR-II, respectively. There was no difference in the expression of the six receptor tyrosine kinases in PC2-positive and PC2-negative cases.

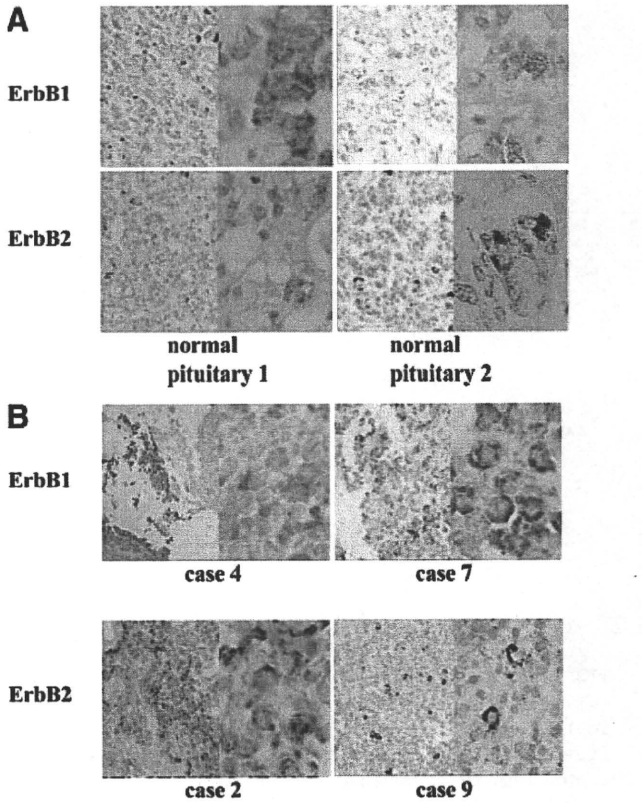

FIG. 4. Immunohistochemical staining pattern of ErbB1 and ErbB2 in normal pituitary anterior lobes (A) and ErbB1 and ErbB2 in APPA (B), respectively. For all figures, the *left part of each panel* is at a lower magnification, ×100, and the *right part* is at a higher magnification, ×250, with nuclear staining by hematoxylin. A, In normal pituitary, ErbB1 immunoreactivity was scattered, with a strong signal in a few cells among weak immunoreactivity in a great majority of cells. ErbB2 immunoreactivity was also present as a scattered pattern but was observed in only a few cells. Both immunoreactivities were observed only in the cytoplasmic region of immunopositive cells. B, In APPAs, immunohistochemical staining of ErbB1 staining was positive in the great majority of tumor cells in all 19 examined cases, showing a diffuse pattern with strong immunoreactivity. ErbB2 was also positive in all of APPAs examined but did not always present in a homogeneous pattern. Some signals were diffuse with a heterogeneous pattern of varying strength of staining (case 2), whereas other staining was completely scattered with various numbers of immunopositive cells (case 9). All of the immunoreactivities of both ErbB1 and ErbB2 were observed only in cytoplasmic regions. No membrane located receptors were identified.

amplification of the predicted 473-, 98-, 447-, 485-, 252-, and 784-bp bands corresponding to human ErbB1, ErbB2, IGF1R, IGF2R, TGFβR-I, and TGFβR-II, respectively (Fig. 3). There was no difference in expression of six receptor tyrosine kinases between PC2-positive and -negative APPAs. RT-PCR revealed that most APPAs might express receptor tyrosine kinases ubiquitously (Supplemental Table 2).

To determine the location of receptor tyrosine kinase expression, further examinations using IHC was performed. ErbB1 and ErbB2 were expressed in the two normal anterior pituitaries and 19 APPAs. In normal anterior pituitary, ErbB1 was scattered with a strong immunoreactivity in a few cells among weak immunoreactivity in a great majority of tumor cells. Noticeably, ErbB1 could be detected only in cytoplasmic regions. ErbB2 immunoreactivity was also present in a scattered pattern and only in cytoplasmic regions (Fig. 4A).

Positive staining for ErbB1 was present in all 19 examined APPAs. A typical pattern was a diffuse pattern with strong immunoreactivity in tumor cytoplasmic regions (Fig. 4B). Case 4 (Fig. 4B), in which both normal pituitary cells and adenoma cells are observed, revealed a different pattern of immunopositivity between both types of tissue. ErbB2 immunopositivity was also detected only in cytoplasmic regions of all APPAs and was present in a heterogeneous pattern (Fig. 4B). Neither ErbB1 nor ErbB2 was identified in membrane regions of adenoma cells.

Discussion

In this study, nine of 19 APPAs (47.4%) were immunopositive for PC2, which is in accordance with previous studies (3, 4). PC2 expression in pituitary tumor cells seems to

lead to further processing of ACTH or β-lipotropin, resulting in the production of αMSH, CLIP, β-endorphin, and γ-lipotropin. Four D2DR-positive tumors did not show immunoreactivity for PC2, suggesting that PC2 expression does not always indicate that the tumor originated from the intermediate lobe (14). Furthermore, PC2-positive tumors may not always respond as expected because of the clinical effects of a D2DR agonist (15).

Traditionally, plasma αMSH levels were thought to be very low to undetectable (2–5 pg/ml) in healthy human controls (16–18). In recent years, however, plasma levels of αMSH have been shown to be controlled by other factors, *i.e.* food intake and energy metabolism in the hypothalamus (19) and UV radiation exposure to the skin (20). Peripheral αMSH might also increase to a detectable level under some kinds of stress or by adipocytokine administration. Several reports indicated that human baseline peripheral αMSH may be approximately 20 pg/ml using the αMSH RIA kit (ALPCO Diagnostics, Salem, NH) without plasma extraction (20, 21). In our study, plasma αMSH levels were undetectable in normal control subjects. A conflicting result may occur because our procedure contained a plasma extraction step because αMSH is a small peptide. Plasma αMSH was not detected in PC2-negative cases (except one) or in normal controls, and αMSH was detectable in only a few PC2-positive cases. We believe that it may be clinically worthwhile to measure αMSH and propose that PC2 expression in tumor cells can be determined via a significant elevation of peripheral αMSH. However, αMSH concentration in the human pituitary accounts for only 2% of the total MSH (22). In fact, we could not determine whether αMSH detected in plasma originated from pituitary cells or other tissues. To evaluate levels of αMSH originating from the pituitary, measurement of αMSH concentration in the inferior petrosal sinus or cavernous sinus, as a comparison for peripheral concentrations, may be required (5).

Our study revealed that 36.8% of cases of PC2-positive tumors were categorized as macroadenomas, and some tumors were morphologically aggressive. PC2-positive APPAs may be characterized by their large size, and a possible relationship between PC2 expression and tumor growth may exist. The bioactivity of tumor-produced ACTH may be one of the determinants of tumor growth in CD. The ACTH/cortisol ratio in PC2-positive tumors is significantly higher than that in PC2-negative tumors, suggesting that they produce low biological activity peptides, which might be detected by the ACTH assay, *e.g.* pro-ACTH or CLIP. As a result of the lower bioactivity of ACTH, a PC2-positive APPA might slow the development of clinically apparent CD. This implies that a diagnosis of CD is made after a substantially longer time, therefore allowing for increased tumor growth.

PCs are expressed in various tumor cell lines and human primary tumors (23–25), and they may play an important role during tumor development and progression. For example, furin, a well-studied member of the PC family, has been reported to be associated with enhanced invasion and proliferation in head, neck, breast, colon and small cell lung cancers (26). Additionally, PC inhibitors may potentially induce decreased tumor growth and tumorigenicity (27). Using human pituitary adenoma cell lines, Kobayashi *et al.* reported that PC2 as well as 7B2, PC1 and TGFβ1 regulate anterior pituitary cell proliferation and hormone secretion (28). Our data showing PC2 expression in pituitary macroadenomas also supports the relationship between PC2 expression and tumor cell proliferation or survival.

Ki-67 LI analysis results contradict a correlation between PC2 expression and tumor growth. Ki-67 LI is a marker of neoplasm cellular proliferation, which may reflect the aggressive behavior of these tumors. In two previous reports describing Ki-67 expression in APPAs, Ki-67 LI in macroadenomas was higher than that in microadenomas (29, 30). In this study, we did not find high scores of Ki-67 in PC2-positive APPAs, and LI distribution was not associated with PC2 expression. Whereas a relationship between these two factors may be uncovered with a greater number of APPA patients, this observation indicates that PC2-positive APPA growth may occur, not as a result of accelerated proliferation but because of increased cell survival.

Next, immunohistochemical examination of cell proliferation-related proteins including p-Akt and p-ERK in the APPAs revealed a positive relationship between PC2 and p-Akt expression in tumor cells. Akt is activated by phosphatidylinositol 3 phosphates and regulates many intracellular processes, including suppression of apoptosis and anoikis and induction of cell cycle progression (31). Musat *et al.* reported the overexpression and activation of the Akt pathway in pituitary adenomas and speculated that cell-cycle changes observed in such tumors are secondary to these alternations (32).

Results of quantitative real-time PCR revealed that Akt mRNA levels were varied and had no significant relation to PC2 expression. Akt mRNA was detected by real-time PCR, even in cases that did not show p-Akt expression by IHC. Therefore, PC2 expression may not be associated with mRNA expression but with Akt phosphorylation, which is an important step in the Akt signal pathway.

Phosphorylation of Akt is a major signaling process located downstream of ligand-stimulated growth factor receptor tyrosine kinases. We examined expression of six

receptor tyrosine kinases including ErbB1, ErbB2, IGF1R, IGF2R, TGFβR-I and TGFβR-II and detected all examined receptors in APPAs. Several reports have shown receptor tyrosine kinase expression in either normal or neoplastic pituitary cells, including ErbB1 (33–35), ErbB2 (35, 36), TGFβR-II (37), and receptors for fibroblast growth factor (38) and vascular endothelial growth factor (39). Our data obtained using RT-PCR confirm observations made by other investigators.

IHC studies, however, did not identify receptor tyrosine kinase expression in plasma membrane, revealing that even if the above receptor mRNA was detected using RT-PCR, assumptions concerning functionality cannot be made. We could not find any apparent differences in either the distribution or location of receptor tyrosine kinases among normal pituitary cells, PC2-positive or PC2-negative APPAs. Therefore, other factors besides expression of receptor tyrosine kinases in neoplastic cells may determine tumor characteristics. Nevertheless, activity of receptor tyrosine kinases, alteration in the percentage of cells expressing these kinases, or interference from another kind of receptors may be relevant.

Investigating PC2 expression by analyzing peripheral αMSH before pituitary surgery may provide an avenue to predict CD characteristics. αMSH levels may be an important indicator for activation of the phosphatidylinositol 3/Akt cascade in PC2-positive APPAs, which may ultimately result in tumor growth acceleration.

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American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Immunohistochemical Testing of Estrogen and Progesterone Receptors in Breast Cancer

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Abstract

Purpose—To develop a guideline to improve the accuracy of immunohistochemical (IHC) estrogen receptor (ER) and progesterone receptor (PgR) testing in breast cancer and the utility of these receptors as predictive markers.

Methods—The American Society of Clinical Oncology and the College of American Pathologists convened an international Expert Panel that conducted a systematic review and evaluation of the literature in partnership with Cancer Care Ontario and developed recommendations for optimal IHC ER/PgR testing performance.

Results—Up to 20% of current IHC determinations of ER and PgR testing worldwide may be inaccurate (false negative or false positive). Most of the issues with testing have occurred because of variation in preanalytic variables, thresholds for positivity, and interpretation criteria.

Recommendations—The Panel recommends that ER and PgR status be determined on all invasive breast cancers and breast cancer recurrences. A testing algorithm that relies on accurate, reproducible assay performance is proposed. Elements to reliably reduce assay variation are specified. It is recommended that ER and PgR assays be considered positive if there are at least 1% positive tumor nuclei in the sample on testing in the presence of expected reactivity of internal (normal epithelial elements) and external controls. The absence of benefit from endocrine therapy for women with ER-negative invasive breast cancers has been confirmed in large overviews of randomized clinical trials.

INTRODUCTION

In 2008, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) decided to pursue an investigation of whether a guideline for estrogen receptor (ER) and progesterone receptor (PgR) testing would be necessary and beneficial for patients with breast cancer. The two organizations had published a joint guideline on human

epidermal growth factor receptor 2 (HER2) testing in 2007.[1,2] A new Expert Panel was convened to address this issue in 2008, and a document reflecting their expert and evidence-based opinions was developed and approved by both organizations. This version of that document is abbreviated from the original approved document, which is available online and includes introductory sections dealing with ER physiology and measurement, history of ER testing, and discussion of the current issues related to ER and PgR testing for patients with breast cancer.

GUIDELINE QUESTIONS

The overall purpose of this guideline is to improve the accuracy of hormone receptor testing and the utility of ER and PgR as prognostic and predictive markers for assessing in situ and invasive breast carcinomas. Therefore, this guideline addresses two principal questions regarding ER and PgR testing. Findings are listed in Table 1.

1. What is the optimal testing algorithm for determining ER and PgR status?
    - **1.1** What are the clinically validated methods that can be used in this assessment?
2. What strategies can ensure optimal performance, interpretation, and reporting of established assays?
    - **2.1** What are the preanalytic, analytic, and post-analytic variables that must be controlled to ensure that assay results reflect tumor ER and PgR status?
    - **2.2** What is the optimal internal quality management regimen to ensure ongoing accuracy of ER and PgR testing?
    - **2.3** What is the regulatory framework that permits application of external controls such as proficiency testing and on-site inspection?
    - **2.4** How can internal and external control efforts be implemented and their effects measured?

The Panel also reviewed a few special questions.

1. Should immunohistochemistry (IHC) of ER/PgR be performed in ductal carcinoma in situ (DCIS) or recurrent breast cancer specimens?
2. Does PgR expression in breast cancer correlate with or influence the choice of endocrine therapy?

PRACTICE GUIDELINES

ASCO/CAP's practice guidelines reflect expert consensus based on the best available evidence. They are intended to assist physicians and patients in clinical decision making and to identify questions and settings for further research. With the rapid flow of scientific information in oncology, new evidence may emerge between the time an updated guideline was submitted for publication and when it is read or appears in print. Guidelines are not continually updated and may not reflect the most recent evidence. Guidelines address only the topics specifically identified in the guideline and are not applicable to interventions, diseases, or stages of diseases not specifically identified. Furthermore, guidelines cannot account for individual variation among patients and cannot be considered inclusive of all proper methods of care or exclusive of other treatments. It is the responsibility of the treating physician or other health care provider, relying on independent experience and knowledge of the patient, to determine the best course of treatment for the patient. Accordingly, adherence to any guideline is voluntary, with the ultimate determination

regarding its application to be made by the physician in light of each patient's individual circumstances and preferences. ASCO/CAP guidelines describe the use of procedures and therapies in clinical practice and cannot be assumed to apply to the use of interventions in the context of clinical trials. ASCO and CAP assume no responsibility for any injury or damage to persons or property arising out of or related to any use of ASCO/CAP's guidelines or for any errors or omissions.

METHODS

Panel Composition

The ASCO Clinical Practice Guidelines Committee (CPGC) and the CAP Council on Scientific Affairs (CSA) jointly convened an Expert Panel (hereafter referred to as the Panel) consisting of experts in clinical medicine and research relevant to hormone receptor testing, including medical oncology, pathology, epidemiology, statistics, and health services research. Academic and community practitioners, a patient representative, and experts from the US National Cancer Institute (NCI) and international organizations were also part of the Panel. Representatives from the US Food and Drug Administration (FDA) and the US Centers for Medicare and Medicaid Services served as ex-officio members. The opinions of Panel members associated with official government agencies like the US National Cancer Institute represent their individual views and not necessarily those of the agency with which they are affiliated. The Panel members are listed in Appendix Table A1 (online only). Representatives of commercial laboratories and assay manufacturers (Appendix Table A2, online only) were invited as guests to attend the open portion of the 2-day meeting held at ASCO headquarters in Alexandria, VA, in December 2008. The planning, deliberations, and manuscript drafting were led by a six-member steering committee composed of two ASCO representatives (Drs Hayes and Wolff), two CAP representatives (Drs Hammond and Schwartz), and two additional experts in testing and evaluation of ER (Drs Allred and Dowsett).

Literature Review and Analysis

ASCO/Cancer Care Ontario (CCO) Systematic Review—ASCO and CAP commissioned a systematic review of the literature on hormone receptor testing published since 1990. That review conducted by ASCO and CCO is being published separately (manuscript in preparation) and served as the primary source of the evidence for this guideline. Articles were selected for inclusion in the systematic review if they met the following prospective criteria. Studies comparing IHC in paraffin-embedded female breast cancer sections with another assay and comparative studies whose objectives were to improve or validate the quality of IHC studies that linked test performance to clinical outcome were specifically sought. Systematic reviews, consensus statements, and practice guidelines from 1990 onward were included if they addressed hormone receptor testing in female breast cancer using IHC in paraffin-embedded sections or gene expression signatures for ER and PgR. A cutoff date of 1990 was chosen because this was the time that IHC began to come into common use. Additional details of the literature search strategy are provided in the Systematic Review (manuscript in preparation).

ASCO/CAP Expert Panel literature review and analysis—The Panel reviewed all data from the systematic review, as well as additional studies obtained from personal files.

Consensus Development Based on Evidence

The entire Panel met in December 2008, and additional work on the guideline was completed through e-mail and teleconferences of the Panel. The purpose of the Panel meeting was to refine the questions addressed by the guideline, draft guideline

recommendations, and distribute writing assignments. All members of the Panel participated in the preparation of the draft guideline document, which was then disseminated for review by the entire Panel. The guideline was submitted to *Journal of Clinical Oncology* and *Archives of Pathology & Laboratory Medicine* for peer review. Feedback from external reviewers was also solicited. The content of the guidelines and the manuscript were reviewed and approved by the ASCO CPGC and Board of Directors and by the CAP CSA and Board of Governors before publication.

Guideline and Conflict of Interest

The Expert Panel was assembled in accordance with ASCO's Conflict of Interest Management Procedures for Clinical Practice Guidelines ("Procedures," summarized at http://www.asco.org/guidelinescoi). Members of the Panel completed ASCO's disclosure form, which requires disclosure of financial and other interests that are relevant to the subject matter of the guideline, including relationships with commercial entities that are reasonably likely to experience direct regulatory or commercial impact as the result of promulgation of the guideline. Categories for disclosure include employment relationships, consulting arrangements, stock ownership, honoraria, research funding, and expert testimony. In accordance with the Procedures, the majority of the members of the Panel did not disclose any of these types of relationships. Disclosure information for each member of the Panel is published adjunct to this guideline.

Revision Dates

At biannual intervals, the Panel Co-Chairs and two Panel members designated by the Co-Chairs will determine the need for revisions to the guidelines based on an examination of current literature. If necessary, the entire Panel will be reconvened to discuss potential changes. When appropriate, the Panel will recommend revised guidelines to the ASCO CPGC, the CAP CSA, the ASCO Board, and the CAP Board for review and approval.

Definition of Terms

See Appendix (online only) for definitions of terms used throughout this document.

Summary of Outcomes Assessed

The primary outcome of interest was the correlation between hormone receptor status, as tested by various assays and methods, and benefit from endocrine therapy, as measured by prolongation of disease-free, progression-free, or overall survival or, in selected instances, response rates. Other outcomes of interest included the positive and negative predictive values, accuracy, and correlation of assays used to determine hormone receptor status, including (but not necessarily limited to) specific assay performance, technique, standardization attempted, quality assurance, proficiency testing, and individual or institutional training. Finally, improvement in assay results based on any of these interventions was examined.

Literature Search

The ASCO/CCO systematic review identified 337 studies that met the inclusion criteria.

RECOMMENDATIONS

What Is the Optimal Testing Algorithm for the Assessment of ER and PgR Status?

Summary and recommendations—The Panel reviewed the literature on ER and PgR testing and discussed its implications for patients diagnosed with breast cancer. The purpose of both tests is to help determine likelihood of patients responding to endocrine therapy.

Therefore, the optimal threshold to define clinical benefit should be based on thresholds that are clinically validated against patient outcome in patients treated with endocrine therapy compared with those who were not.

What Are the Clinically Validated Methods That Can Be Used in This Assessment?

Table 2 shows significant correlations between ER levels determined by IHC and clinical outcome in patients with less advanced disease treated with adjuvant hormonal therapy. Table 3 lists the assays that are currently considered to be clinically validated. A thorough discussion of these topics appears in the unabridged version of this guideline.

Laboratory concordance with standards—In the case of IHC assays of ER and PgR assays, there is no gold standard assay available. The Panel agreed that a relevant standard would be any assay whose specific preanalytic and analytic components conformed exactly to assays whose results had been validated against clinical benefit from endocrine therapy (clinical validation). Currently, there are several assay formats that meet this criterion as models against which a laboratory can compare its testing. Examples include the ER and PgR methods described in the publications by Harvey et al[6] and Mohsin et al[10] and the FDA 510(k)-cleared ER/PR pharmDx assay kit (Dako, Glostrup, Denmark). ER can also be determined by evaluation of RNA message, either by individual assay or as part of a multigene expression assay, such as a multigene array or as a multigene quantitative polymerase chain reaction. For example, the 21-gene recurrence score (RS) assay includes ER and PgR as one of the genes in the signature.[11] However, comparison between measures of ER/PgR protein by local IHC and of mRNA by central reverse transcription polymerase chain reaction showed a discordance rate of 9% and 12%, respectively,[12] and there are no published correlations of the individual measures of ER and PgR mRNA from the 21-gene signature with clinical outcome. As a result of this lack of published data correlating the ER and PgR individual measures within the 21-gene RS directly with clinical outcome, the Panel concluded it was premature to recommend these individual measures for assay standardization and validation.

As discussed later, a laboratory performing ER testing should initially validate its proposed or existing assay against one of these clinically validated assays and demonstrate acceptable concordance. Details of acceptable validation methods are described in a separate publication.[3] To be considered acceptable, the results of the assay must be initially 90% concordant with those of the clinically validated assay for the ER- and PgR-positive category and 95% concordant for the ER- or PgR-negative category. Table 3 lists details of clinically validated assays including reagents, thresholds, and publications.

Definition of positive and negative ER and PgR tests—The Panel deliberated carefully about recommending a universal cut point to distinguish "positive" and "negative" ER levels by IHC. The original cut point established for the ligand-binding assays (LBAs) in the 1970s was based primarily on the odds of response in the metastatic setting to a variety of endocrine treatments being used at the time in many centers.[18] Cytosol protein 10 fmol/mg was generally accepted as the optimum clinically useful cut point, and the FDA-approved kits using radiolabeled LBAs specified this value. Even then, the odds of responding for patients with ER levels less than 10 fmol/mg tissue were greater than 0, and others suggested that lower levels, such as more than 3 fmol/mg, might be appropriate.[19,20]

When IHC assays replaced LBAs in the early to mid-1990s, relatively few clinical studies were performed to establish optimum cut points for these assays. Instead, most studies simply compared the two and assumed that the IHC level corresponding to the previously determined LBA cut point was also valid. However, some early studies demonstrated that

IHC was equivalent or superior to LBA in predicting benefit from adjuvant endocrine therapy.[6,10] Others showed significant correlations between ER levels determined by IHC and clinical outcome in patients with less advanced disease treated with adjuvant hormonal therapy (Tables 2 and 3).

Overall, the most comprehensive breast cancer studies have consistently shown that IHC is equivalent or superior to LBA in predicting response to hormonal therapy and that levels as low as 1% positive-staining carcinoma cells are associated with significant clinical response (Tables 2 and 3). Therefore, given the substantial impact of tamoxifen and other endocrine therapies on mortality reduction and their relatively low toxicity profile, the Panel recommended that the cutoff to distinguish "positive" from "negative"cases should be ≥ 1% ER-positive tumor cells. The Panel recommended considering endocrine therapy in patients whose breast tumors show at least 1% ER-positive cells and withholding endocrine therapy if less than 1%. We recognize that these recommendations will result in a slight increase in the application of endocrine therapy in some practices. We also recognize that it is reasonable for oncologists to discuss the pros and cons of endocrine therapy with patients whose tumors contain low levels of ER by IHC (1% to 10% weakly positive cells) and to make an informed decision based on the balance.

The percentage of stained tumor cells may provide valuable predictive and prognostic information to inform treatment strategies. Eight studies described the relationship between hormone receptor levels and patient outcomes.[5,7,17,21–25] Overall survival,[7,23,24] disease-free survival,[24] recurrence/relapse-free survival,[22,23] 5-year survival,[21] time to treatment failure,[7] response to endocrine therapy,[7,25] and time to recurrence[17] were all positively associated with ER levels. Overall survival,[7] time to treatment failure/progression,[5,7] response to endocrine therapy,[7,25] and time to recurrence[17] were positively related to PgR levels. These studies suggest that patients with higher hormone receptor levels will have a higher probability of positive outcomes and may influence oncologists' and patients' treatment decisions.

Although some studies suggest that the predictive role of PgR may not be as important clinically as ER,[5,13,26] other studies have shown that PgR status provides additional predictive value[10] independent of ER values,[25,27] especially among premenopausal women.[9,22] Again, predictive validity for PgR has been demonstrated with as few as 1% of stained tumor nuclei cells in retrospective studies.[10,25] Among patients who received adjuvant endocrine therapy, the best cutoff for both disease-free (adjusted $P = .0021$) and overall (adjusted $P = .0014$) survival was a total PgR Allred score of greater than 2, which corresponds to greater than 1% of carcinoma cells exhibiting weakly positive staining.[10] For patients with metastatic breast cancer who received first-line endocrine therapy on relapse, a correlation was found between PgR receptor status and response to endocrine therapy at a 1% staining threshold ($P = .044$) or response to tamoxifen therapy at 10% ($P = .021$) and 1% staining thresholds ($P = .047$). Furthermore, patients with carcinomas exhibiting ≥ 1% PgR staining levels had better survival after relapse ($P = .0008$).[25]

Reporting Results

Taking these issues into consideration, the Panel recommends that ER and PgR results be reported with three required result elements and two optional result elements (Table 1). The three required elements are as follows.

1. The percentage/proportion of tumor cells staining positively should be recorded and reported; all tumor containing areas of the tissue section on the slide should be evaluated to arrive at this percentage. The percentage can be arrived at either by estimation or by quantification, either manually by counting cells or by image

analysis. Image analysis holds promise for improving inter- and intraobserver reproducibility, but controversy exists about how imaging should be implemented at this time. Standards of system performance have not yet been developed. If the sample is a cytology specimen, at least 100 cells should be counted or used to estimate the percentage of hormone receptor–positive tumor cells, particularly if the tumor specimen is limited and if the positive staining seems to involve only a minority of tumor cells.

2. The intensity of staining should be recorded and reported as weak, moderate, or strong; this measurement should represent an estimate of the average staining of the intensity of the positively stained tumor cells on the entire tissue section relative to the intensity of positive controls run with the same batch. Intensity is provided as a measure of assay quality over time and also allows for optional composite scoring.

3. An interpretation of the assay should be provided, using one of three mutually exclusive interpretations. The reader should provide an interpretation of the assay based on the following criteria.

    - Receptor positive (either ER or PgR). The Panel recommends a cutoff of a minimum of 1% of tumor cells positive for ER/PgR for a specimen to be considered positive. There is no agreement about a range for receptor equivocal, so this term should not be used.
    - Receptor negative. Tumors exhibiting less than 1% of tumor cells staining for ER or PgR of any intensity should be considered negative based on data that such patients do not receive meaningful benefit from endocrine therapy. The sample should only be considered negative in the presence of appropriately stained extrinsic and intrinsic controls. Any specimen lacking intrinsic elements (normal breast epithelium) that is negative on ER and/or PgR assay should be repeated using another tumor block or another tumor specimen and reported as uninterpretable rather than as negative.
    - Receptor uninterpretable. The Panel agreed that there are no absolute assay exclusions. Nevertheless, a result should be considered uninterpretable if a sample did not conform to preanalytic specifications of the guideline, was processed using procedures that did not conform to guideline specifications or the laboratory's standard operating procedure, or the assay used to analyze the specimen was not validated and controlled as specified in the guideline. Examples of circumstances that may lead to uninterpretable results include testing of needle biopsies or cytology samples fixed in alcohol, use of fixatives other than 10% neutral buffered formalin ([NBF] unless that fixative has been validated by the laboratory before offering the assay), biopsies fixed for intervals shorter than 6 hours or longer than 72 hours, samples where fixation was delayed for more than 1 hour, samples with prior decalcification using strong acids, and samples with inappropriate staining of internal assay controls (including intrinsic normal epithelial elements) or extrinsic assay controls. These conditions are not absolute because they depend on which conditions have been validated by the laboratory and which are subject to the judgment of the circumstances by the pathologist. The reason for an uninterpretable result should be specified (eg, fixation for < 6 hours), and an alternative potential sample for retesting should be suggested, if appropriate.

Two optional report elements are recommended by the Panel, but not required.

1. A cautionary statement may be added to negative ER and PgR interpretations when the histopathology of the tumor is almost always associated with ER-positive and PgR-positive results. These include tubular, lobular, and mucinous histologic types or tumors with a Nottingham score of 1. The cautionary statement should indicate that although the patient's tumor tested as ER negative, tumors with the same histologic type or Nottingham score almost always test positive.
2. Using the percentage and intensity measurements provided, the pathologist may also provide a composite score such as the H score, Allred score, or quick score (Table 3). Because each of these is somewhat differently calculated and may lead to confusion across institutions, scoring is not required.

Appropriate populations to be tested—The Panel developed consensus that ER and PgR status should be determined on all newly diagnosed invasive breast cancers. For patients with multiple synchronous tumors, testing should be performed on at least one of the tumors, preferably the largest. The Panel acknowledges that all newly diagnosed DCISs are also commonly being tested for ER and PgR. This practice is based on the results of a retrospective subset analysis of the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-24 clinical trial comparing tamoxifen versus placebo after lumpectomy and radiation, which has thus far been reported only in abstract form. There was a significant 40% to 50% reduction in subsequent breast cancer (ipsilateral and contralateral) restricted to patients with ER-positive DCIS at 10 years of follow-up, and a full manuscript has recently been submitted for peer review (personal communication from NSABP, September 2009). Because the results are scientifically reasonable and consistent with previous studies of invasive/metastatic breast cancer, the Panel sees value in assessing ER in patients with DCIS. However, because there are unlikely to be any validation studies, the Panel leaves it up to patients and their physicians to decide on testing, rather than making a formal recommendation. Breast recurrences should also always be tested to ensure that prior negative results of ER and/or PgR were not falsely negative and to evaluate the specimen for biologic changes since the previous testing.

What Strategies Can Ensure Optimal Performance, Interpretation, and Reporting of Established Assays?

Summary and recommendations—The Panel considered those strategies that would ensure optimal performance of ER/PgR testing, interpretation, and reporting and was heavily influenced by the previous experience with the implementation of the elements included in the ASCO/CAP HER2 testing guideline. This guideline included measures to improve standardization of preanalytical variables, type of fixative and duration of tissue fixation, antibodies and controls, and assay interpretation.

What Are the Preanalytic, Analytic, and Postanalytic Variables That Must Be Controlled to Ensure That the Assays Reflect the Tumor ER and PgR Status?

Preanalytic standardization: tissue handling—The warm and cold ischemic times are widely accepted as important variables in the analysis of labile macromolecules such as proteins, RNA, and DNA from clinical tissue samples. Warm ischemia time is the time from the interruption of the blood supply to the tumor by the surgeon to the excision of the tissue specimen; cold ischemia time is the time from excision to the initiation of tissue fixation. Numerous studies have documented the progressive loss of activity of these labile molecules after the surgical interruption of blood flow, leading to tissue ischemia, acidosis, and enzymatic degradation.[28–30] The contribution to this macromolecular degradation by the warm ischemic interval is currently under study. The standardization of the time between tissue removal and the initiation of fixation is an important step to help ensure that

differences in levels of protein expression for clinically relevant targets such as ER are biologically meaningful and are not an artifact related to the manner in which the tissue was handled.

The breast resection specimen should be fixed as quickly as possible in an adequate volume of fixative (optimally 10-fold greater than volume of the specimen). The time of tissue collection (defined as the time that the tissue is handed from the surgical field) and the time the tissue is placed in fixative both must be recorded on the tissue specimen requisition to document the time to fixation of the specimen. The pathologist should effectively communicate this priority to all members of the breast care management team so processes are put in place to make sure these times are routinely recorded. It is the responsibility of the surgeon and operating room staff or the radiologist and his/her staff obtaining the specimen to document the collection time, and it is the responsibility of the pathologist and laboratory staff to document the fixation start time. Every effort should be made to transport breast excision specimens with a documented or suspected cancer from the operating room to the pathology laboratory as soon as they are available for an immediate gross assessment. The time from tumor removal to fixation should be kept to ≤ 1 hour to comply with these recommendations.

On receipt in the pathology laboratory, these specimens should be oriented and carefully inked for surgical margin assessment and then carefully sectioned at 5-mm intervals and placed in 10% NBF. Gauze pads or paper towels should be placed in between tissue slices to assist with the penetration of formalin into all areas of the tissue sample if the specimen will be further sectioned and placed into tissue cassettes at a later time. If gross tumor is easily identifiable, a small portion of tumor and fibrous normal breast tissue can be included together in a cassette and placed immediately into fixative at the time of the initial gross evaluation. This will initiate good tissue fixation and also ensure that normal breast elements are available as an internal positive control that have been handled and fixed in a manner that is identical to the tumor tissue. In situations where excision specimens are obtained remotely from the grossing laboratory, the pathologists should work with personnel in the remote operating suites to ensure that the sample is bisected through the tumor and promptly placed in NBF before transport. The time to insertion of tumor sample into fixative and the time of removal of the tumor from the patient should be noted on the specimen requisition by the remote personnel. Although less optimal than immediate gross examination of the fresh sample by the pathologist, this process is preferable to storage of the sample in the refrigerator unfixed or in fixative without sectioning.

Preanalytic standardization: type of fixative—Only 10% NBF should be used as the fixative for breast tissue specimens. Higher or lower concentrations of NBF are not acceptable. This recommendation is based on published literature regarding the expected or characteristic immunoreactivity for ER in breast cancer, which has been accrued over many years and has been clinically validated with patient outcomes in numerous clinical trials.[31] In addition, FDA approval for assay kits analyzing ER and HER2 explicitly states that formalin fixation should be used and that the FDA approval for the kits is not applicable if an alternative fixative is used. If the laboratory uses a formalin alternative for fixation, the assay must be validated against NBF fixation, and the laboratory director assumes responsibility for the validity of these assay results.

Preanalytic standardization: duration of tissue fixation—Breast tissue specimens must be fixed in 10% NBF for no less than 6 hours and for not more than 72 hours before processing.[32,33] Further information about the need for standardization of tissue fixation appears in the unabridged version of this guideline.

Analytic standardization: antibody selection for ER testing—The selection of antibodies for ER and PgR IHC testing should be restricted to those reagents that have well-established specificity and sensitivity and have been clinically validated, demonstrating good correlation with patient outcomes in published reports. Alternatively, the results of laboratory-selected antibodies should be at least 90% concordant with those of the clinically validated assay for the ER- and PgR-positive category and 95% concordant with those for the ER- or PgR-negative category that have been correlated with clinical outcomes of endocrine treatment. The Panel determined that the antibodies for ER that have met these criteria are clones 1D5, 6F11, SP1, and 1D5+ER.2.123, whereas the antibodies for PgR include clones 1A6, 1294, and 312 (Table 3). There a single FDA 510(k)-cleared ER/PgR kit. Published reports have demonstrated that each of these antibodies is equivalent or superior to LBAs in terms of correlation with outcome and/or benefit from endocrine therapy (Tables 2 and 3). Antibodies sold as research use only or investigational use only or developed by the testing facility may not be used in ER and PgR testing. Use of research use only, investigational use only, and laboratory-developed antibodies in an assay is not compliant with these guidelines.

Analytic standardization: control samples for ER and PgR IHC assays—Positive and negative controls should be included with every ER and PgR IHC assay batch run. Batch controls are used to monitor assay performance over time and to detect a loss of sensitivity or assay analytic drift. Acceptable batch controls include cell lines with defined receptor content varying from high positive to negative and including at least one intermediate level of receptor content. Other acceptable external controls include endometrial tissue with known receptor content. On-slide external controls and internal normal epithelial elements should be used to help ensure that all reagents were dispensed onto the slide containing a test sample and that the assay is performing properly. The internal positive control must display a heterogeneous staining pattern of the luminal cells, with a mixture of a variable number of cells exhibiting weak, moderate, and intense immunoreactivity. If the assay only highlights a few cells among the normal breast epithelium with a homogeneous staining pattern, then the risk of a false-negative assessment of the tumor ER and/or PgR is higher as a result of an insufficient sensitivity of the reaction to detect the tumor cells with a weak to moderate immunoreactivity. The normal breast tissue also represents a useful built-in negative control of the staining because the myoepithelial cells and the stromal cells must invariably show a negative result. In some specimens, there are no internal control elements (normal breast epithelium); in this case, the pathologist must exercise judgment as to whether the assay can be interpreted based on the level of ER and/or PgR positivity of the tumor cells, the histologic type of the tumor, the fixation status of the tumor, and the status of external controls.

To ensure that there has not been analytic drift because of subtle differences in technique or dilution, controls with intermediate reactivity or controls covering a spectrum of expression should be scored and recorded daily (percent positive tumor cells and intensity of staining) using laboratory standard scoring system or image analysis. It is not appropriate to use a single strong positive control tissue to evaluate assay performance.

If an external or internal control does not produce the expected reaction, the result of patient testing must not be reported. Instead, the assay should be repeated with the standard reagents under the standard conditions until acceptable ER and/or PgR reactivity of control material is achieved. No patient material should be reported until controls react appropriately.

If the particular histologic type of breast cancer is unlikely to be ER negative (tubular, mucinous, or lobular morphology or Nottingham score of 1), the tumor should also be subjected to confirmatory testing, such as sending the same specimen to a reference

laboratory for retesting or by repeating the assay on another block or on a separate breast cancer specimen.

Postanalytic standardization: interpretation of IHC assays for ER and PgR— The interpretation of ER and PgR assays should include an evaluation of both the percentage of positive tumor cell nuclei and the intensity of the staining reaction. The level of expression of ERs in different breast tumors demonstrates a broad dynamic range that can vary by several hundred–fold. There is still no consensus about what level of expression constitutes the equivocal range for ER/PgR, and this terminology should not be used in the report. Table 4 lists interpretation guidelines.

Postanalytic standardization: reporting of ER and PgR by IHC—The elements to be reported are listed in Tables 5 and 6. The staining of normal breast elements, if present within the specimen, should also be reported as an additional check on the IHC assay performance.

Postanalytic standardization: ER and PgR IHC assay internal quality control and validation—A comprehensive quality control program for ER/PgR IHC analyses should include all aspects of the total test including periodic trend analysis to help ensure an appropriate and expected number of ER-positive breast cancers in the patient population served by the laboratory. Table 7 lists specific suggestions; additional suggestions are provided in a separate publication.[3]

What Is the Regulatory Framework That Allows for Increased Scrutiny?

The Clinical Laboratory Improvement Act of 1998 (CLIA 88) provides stringent quality standards for highly complex tests, which include all predictive cancer factor assays. This legislation also requires application of external controls to assure compliance with CLIA standards. These external controls include required successful performance on external proficiency surveys (or alternative external assessment of assay accuracy) and on-site biennial inspection of laboratories performing highly complex tests with defined criteria and actions required when performance is deemed deficient. On-site inspections may be performed by the Centers for Medicare and Medicaid Services or its agents or by various deemed private accreditors, including CAP, The Joint Commission, and COLA (formerly known as Commission on Office Laboratory Accreditation).

The FDA regulates medical devices as a result of the 1976 Medical Devices Amendments Act. ER and PgR testing reagents and kits, which have potentially high impact on patient mortality and morbidity, have been the subject of several guidance documents and reports referencing FDA opinion on the subject.[34]

After review of the legislation and applicable regulations, the Panel agreed that the current regulatory framework provided sufficient justification for the guideline recommendations without modification, just as it had for the previously published ASCO/CAP HER2 guideline. Other countries such as Australia and New Zealand have similar requirements.

What Are the Optimal External Quality Assurance Methods to Ensure Ongoing Accuracy in ER/PgR Testing?

Summary and recommendations—The guideline is based on regulatory requirements of CLIA 88, published studies, previous CAP experience,[1,2] experience of other groups,[31] and the Panel's consensus.

Currently there are no regulatory requirements for proficiency testing of ER or PgR assays in the United States. CLIA regulations require alternative assessment schemes for ER and PgR as substitutes for mandated successful performance on external proficiency testing. However, proficiency testing can be used to meet the alternative assessment requirement if it is available. The current guideline will make successful performance in proficiency testing mandatory. There are mandatory requirements for successful performance in proficiency testing in Australia and New Zealand, which had been in place since 2001.

The guidelines also require enhanced levels of scrutiny at the time of laboratory inspection beyond those required by CLIA. The Panel recommends that ER and PgR testing be performed in a CAP-accredited laboratory or in a laboratory that meets the additional accreditation requirements set out within this guideline.

External quality assurance (laboratory accreditation)—Beginning in 2010, the CAP Laboratory Accreditation Program will require that every CAP-accredited laboratory performing ER and/or PgR testing participate in a proficiency testing program directed to these analytes. Other Centers for Medicare and Medicaid Services–approved certifying or accrediting organizations that wish to evaluate laboratory compliance with this guideline must bring their accreditation programs in conformance with this and other requirements.

The CAP Laboratory Accreditation Program will monitor performance in the required proficiency testing. Performance less than 90% (described in detail in the following section) will be considered unsatisfactory and will require internal or external response consistent with accreditation program requirements. Responses must include identification of the cause of the poor performance, actions taken to correct the problem, and evidence that the problem has been corrected. Competency of the laboratory personnel performing the ER/PgR testing, including the pathologists, is an important aspect of the laboratory proficiency. Competency of testing personnel and pathologists must be assured by the laboratory director of each facility in a manner consistent with CLIA. Competency assessments must be documented, and documentation shall be evaluated at the time of laboratory inspection accreditation. The checklist of requirements for laboratories is presented in Table 7.

Proficiency testing requirements—All laboratories reporting ER and/or PgR results must participate in a guideline-concordant proficiency testing program specific for each assay and method used. To be concordant with this guideline, proficiency testing programs must distribute specimens at least twice per year including a sufficient number of challenges (cases) to ensure adequate assessment of laboratory performance. For programs with ≥ 10 challenges per event, satisfactory performance requires correct identification of at least 90% of the graded challenges in each testing event. Laboratories with less than 90% correct responses on graded challenges in a given proficiency testing event are at risk for the next event. Laboratories that have unsatisfactory performance will be required to respond according to accreditation program requirements up to and including suspension of ER and/or PgR testing for the applicable method until performance issues are corrected. In some Canadian provinces and within the United Kingdom, the method of proficiency testing is different. In Canada, laboratories may participate in proficiency testing that uses sections of tissue microarrays offered by the Canadian Immunohistochemistry Quality Control (an academic program associated with the Canadian Association of Pathologists) or tumor samples or sections of cell blocks with characterized cell lines. Many Canadian laboratories also participate in CAP proficiency testing programs or European programs. The results may or may not be used for laboratory accreditation depending on the province. Laboratories receive unstained materials and must return those materials to a central laboratory for review and comment. The Australasian program developed by the Royal College of Pathologists of Australasia Quality Assurance Program consists of two components. Laboratories are sent

unstained sections from tissue microarray blocks and are required to stain these and return them for central review and scoring. In addition, laboratories are required to submit de-identified data on the ER/PgR and HER2 status of reported breast cancers for evaluation of acceptable performance. Enrollment and participation in these programs are mandatory.

How Can These Efforts Be Implemented and the Effects Measured?

Plans to ensure compliance with guideline—ASCO and CAP will provide educational opportunities (print, online, and society meetings) to educate health care professionals, patients, third-party payers, and regulatory agencies. In addition, CAP is producing a certificate program for pathologists that will assess their competency in following both the hormone receptor and the HER2 guideline recommendations. CAP will urge its members and participants in accreditation and proficiency testing programs to optionally append a statement to individual results or laboratory informational or promotional materials indicating that the laboratory's ER/PgR assays have been validated and performed in accordance with ASCO/CAP ER testing guidelines, provided that all of the guideline conditions are met.

ASCO and CAP will work to coordinate these recommendations with those of other organizations, such as the National Comprehensive Cancer Network, the Commission of Cancer of the American College of Surgeons, the American Joint Committee on Cancer, and patient advocacy organizations.

We are confident that these guidelines and measures developed for testing of ER, PgR, and HER2 will improve performance of laboratories using these and future predictive testing methods. CAP will actively review results of proficiency testing and laboratory accreditation activities and periodically publish performance results.

CAP will also work to include quality monitoring activities of ER and PgR testing in its programs designed for ongoing quality assessment, similar to its Q-tracks and Q-probes. In Australasia, participation in the programs is mandatory and linked to laboratory accreditation. In Australia and New Zealand, the laboratory accreditation is linked to funding of testing for laboratories ensuring compliance.

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APPENDIX

Definitions

Analyte-specific reagent

Antibodies, both polyclonal and monoclonal, specific receptor proteins, ligands, nucleic acid sequences, and similar reagents, which, through specific binding or chemical reaction with substances in a specimen, are intended for use in a diagnostic application for identification and quantification of an individual chemical substance or ligand in biologic specimens [21CFR864.4020(a)].

Research use only (RUO)

Products that are in the laboratory research phase of development (ie, either basic research or the initial search for potential clinical utility) and not represented as an effective in vitro diagnostic product (21CFR809.10).

Investigational use only (IUO)

A product being shipped or delivered for product testing before full commercial marketing (for example, for use on specimens derived from humans to compare the usefulness of the product with other products or procedures that are in current use or recognized as useful) (21CFR809.10).