

COMMISSION OF INQUIRY ON HORMONE RECEPTOR TESTING

The Honourable Madam Justice Margaret A. Cameron, Commissioner

Reply to Submissions
to the Commission of Inquiry on Hormone Receptor Testing
by Dr. Kara Laing, et al.

December 15, 2008

Submitted By:
Curtis, Dawe
Solicitors for Dr. Kara Laing, et al.
Eleventh Floor, Fortis Building
139 Water Street
P.O. Box 337
St. John's, NL
Canada A1C 5J9
Peter N. Browne, Q.C.
Lead Counsel
Tel: (709) 722-5181

Jane C. Hennebury
Associate Counsel
Tel: (709) 722-5181

1. Dr. Kara Laing et al. present the following points in Reply to the submissions made by other parties with standing at the Commission of Inquiry.

Reply to the Submission on Behalf of the Members of the Breast Cancer Testing Class Action

Page 53, Paragraph (m)

2. With respect to the comments at page 53, paragraph (m) of this Submission, Dr. Kara Laing et al. state that it is erroneous to infer that Dr. Cook “misled the consultants” in relation to the ethics consult which occurred in June 2006.
3. Dr. Cook testified that he did not receive a copy of the ethics consult report and therefore did not review the statement that alleged “there were no mistakes or technical errors at the route of this problem”. Dr. Cook and others present at the consult were questioned extensively on their recollections surrounding the meeting. Dr. Cook gave evidence as follows:

Q: Now, Doctor, the idea that there were no mistakes or technical errors at the route of this problem, from your perspective as of June, 2006, would you have maintained that that was an accurate statement?

A: I wouldn't have made that statement. I don't know where that came from at that meeting.

Q: Certainly your recollection of the meeting was you didn't offer that opinion yourself?

A: No.

Q: And I'm not suggesting you did at all.

A: No, I don't know where that came from.

Q: Was the cause or causes of the problem discussed at the meeting, do you know?

A: We would have used a word like “system” describing concerns that we had, how the tissue was prepared, how it was tested, how it was

interpreted. So we were looking in terms of discussion at “system”. This is what the – I guess of anything, that would be the terms that we were using and probably discussing what was going on with the rest of Canada with the testing and the United States, and the problems with the testing in general.¹

4. Mr. Rick Singleton’s testimony supports Dr. Cook’s evidence on this issue. He did not recall the specifics of what Dr. Cook had said during the consult but his general sense of the overall discussion was that there was a systemic problem.²
5. Mr. Singleton, in providing this overall impression, explained to the Commission that his thinking about potential mistakes or errors was related to a specific error by a specific individual – “something identifiable as the specific cause or causes of the problem”.³
6. Dr. Kara Laing et al. submit that by the time of the consult in 2006 and continuing until today, as evidenced by testimony at the Inquiry, no physician was in a position to identify exactly what had caused the problem. There were multiple possibilities and Dr. Cook’s general description of a “system” problem would have captured the complexity of the issue for the purposes of the consult.

¹ Evidence of Donald Cook, 07/07/2008, pages 95-96, lines 4-9.

² Evidence of Rick Singleton, 19/06/2008, pages 278-279, lines 19-17.

³ Evidence of Rick Singleton, 25/09/2008, pages 80-81, lines 15-18.

7. More importantly, Mr. Singleton pointed out that the whole focus of the consult was about how to deal with disclosure involving deceased patients versus a discussion about what had caused the ER/PR problems. Mr. Singleton stated:

A: Yes, simply to say that the discussion, the direction of our discussion that day was really about the issues of communicating with families and what needs to be taken into consideration in communicating with families, that this information is available and may be available, and so that was the direction. We didn't, you know, spend much time on -- ... These were the kinds of things that we were talking about that day, you know, that type of information is there, it's part of the person's health record and, you know, who should be informed, or at least have the information offered to them. That's what we were—most of our discussion was about. It wasn't about the kind of history of how it all came about.⁴

8. Dr. McCarthy was also present at the ethics consult. She recalled that Drs. Cook and Denic did speak to the possible issues in the lab that may have contributed to the problem. She too did not recall anyone stating that there were no mistakes or technical errors at the root of the problem.⁵
9. Similarly, Mr. Boone stated that he did not hear anyone at the consult say that no mistakes had been made.⁶
10. In light of the above evidence, it cannot and should not be inferred that Dr. Cook misled the ethics consultants.

⁴ Evidence of Rick Singleton, 19/06/2008, pages 287-288, lines 23-24.

⁵ Evidence of Joy McCarthy, 19/09/2008, page 299, lines 2-16.

⁶ Evidence of Dan Boone, 29/10/2008, pages 185-187, lines 24-1.

Page 52-53, Paragraph (j)

11. Regarding the allegation at pages 52-53, paragraph (j) of the Class Action's Submission, Dr. Kara Laing et al. deny the characterization of Dr. Elms' conduct following the changed test result for Mrs. Deane. After speaking with Dr. Rorke and possibly Dr. McCarthy to inform them of the positive result, Dr. Elms went to his supervisor, Dr. Cook, to inform him of the situation. Contrary to what is stated in the Submission, Dr. Elms did not approach Dr. Cook *because* he thought there might be a complaint. That was simply one piece of information he relayed in addition to the facts surrounding Mrs. Deane's case. Dr. Elms explained that he felt Dr. Cook should be aware since he was Clinical Chief. Dr. Cook appreciated that Dr. Elms had informed him.⁷
12. When asked why Dr. Elms had spoken to him about Mrs. Deane's changed result, Dr. Cook testified that Dr. Elms kept him updated since the conversion could have resulted in a change in treatment for the patient and it was a significant event.⁸
13. As a result, it is incorrect to state that Dr. Elms' conduct is an illustration of the culture of "don't get caught".

⁷ Evidence of Ford Elms, 02/09/2008, pages 133-135, lines 8-18.

⁸ Evidence of Donald Cook, 03/07/2008, pages 35-36, lines 16-3.

Page 30-31, Paragraph 72

14. With respect to the comment at pages 30-31, paragraph 72 of the Class Action's Submission that it was Dr. Cook who suggested that Dr. Ejeckam look into the problems with staining in 2003, Dr. Kara Laing et al. state that it was in fact a consensus decision made by the pathologists who attended the Tuesday and Wednesday conferences at the Health Sciences Centre.⁹

Page 35, Paragraph 87

15. Dr. Kara Laing et al. deny the allegation contained at page 35, paragraph 87 that Dr. Cook was "forced to disclose" Dr. Ejeckam's memos to Dr. Williams in July of 2005. At that time, the leadership team was still trying to identify the nature and extent of the problem. Dr. Cook had a discussion with Dr. Ejeckam around July 12, 2005 to get information on what exactly had been done in 2003 when the 8 stains were stopped and re-started. Following that discussion Dr. Cook asked Dr. Ejeckam to photocopy one of his 2003 memos and Dr. Cook proceeded to submit all three 2003 memos to Dr. Williams. The positivity percentage rates and Dr. Ejeckam's memos were pieces of information to help determine what the situation was. Dr. Cook was attempting to gather as much information as possible for Dr. Williams.¹⁰

⁹ Evidence of Gershon Ejeckam, 03/06/2008, pages 203-204, lines 6-23.

¹⁰ Evidence of Donald Cook, 03/07/2008, pages 141-145, lines 23-21.

Page 41, Paragraphs: 103-106

16. Dr. Kara Laing et al. deny the assertion contained at page 41, paragraphs 103 and 106 that Dr. Cook did not discuss the issues with ER/PR staining with Dr. Ejeckam in 2003. As outlined in our Submissions at paragraph 187, Dr. Cook spoke with Dr. Ejeckam following his memo in June 2003. There was a concern about available resources and the management structure and frustration in trying to get things done. Dr. Cook then spoke with Mr. Gulliver and was reassured that measures would be taken to improve the situation and address Dr. Ejeckam's concerns.¹¹
17. Therefore, the comment at page 41, paragraph 105 that Dr. Cook was "the only person" who could have taken action is inaccurate. In hindsight, Dr. Cook could have followed-up with Dr. Ejeckam but at the time he felt that an understanding had been reached with Mr. Gulliver.¹²

Page 19, Paragraph 43

18. With respect to the reference to UK NEQAS at page 19, paragraph 43, Dr. Kara Laing et al. deny that this external proficiency program was available for ER/PR testing in the early 1980s. Rather, it is our understanding that UK NEQAS only began the specific module for breast cancer hormonal receptors in 1997. It

¹¹ Evidence of Donald Cook, 02/07/2008, pages 260-262, lines 4-22.

¹² Evidence of Donald Cook, 02/07/2008, page 264-265, lines 12-13.

began as a small activity but grew quickly. However, it was likely only by about 2000 that the UK external quality assurance effort began to be noticed on a global scale.

Page 20, Paragraph 98

19. Regarding the Class Action's analysis at page 20, paragraph 48, Dr. Kara Laing et al. state that it is inappropriate to draw any conclusions about Dr. Khalifa's actions from such data. The calculations performed by Dr. Hutton for the Class Action have not been verified or accepted as accurate. As the Commission is aware, Dr. Hutton was not called as a witness and was not cross-examined on his "statistics" contained in Exhibit P-1841.

Page 33, Paragraph 79

20. As for the suggestion at page 33, paragraph 79 that nobody thought of the clinical effect of poor staining in breast cancer cases in 2002-2003, Dr. Kara Laing et al. state that this is obviously incorrect. Once the poor staining issues were noticed and discussed by pathologists in late 2002/early 2003, action was taken by agreeing to have Dr. Ejeckam investigate and ultimately stop certain IHC stains, including ER and PR. Pathologists are of course aware that their diagnoses impact clinical considerations.

21. We have already addressed the issue relating to Dr. Khalifa and the 30% value in our Submissions at paragraphs 198-200 but it is important to highlight again the fact that cut-off values were not well-defined even up to 2000, as demonstrated by Dr. Santeusano et al. in their article.¹³ This group of renowned scientists compared four different antibodies, including 1D5 which was in use in NL during Dr. Khalifa's time here, and used 30% as the cut-off value because they found that it possessed the best sensitivity/specificity ratio.¹⁴

22. Furthermore, in the 2007 recommendations for improved standardization of immunohistochemistry written by experts in the field, they comment that "There is no universal IHC scoring system...There is also no universal cut-point threshold for a positive result that can be applied across all clinical situations and types of specimens".¹⁵

23. Finally, in response to paragraphs 35, 39 and 41 at pages 16-17 of the Class Action's Submission addressing the issue of conducting clinical trials when evaluating new antibodies, we reference a 2001 article by Dr. Eric D. Hsi entitled "A Practical Approach for Evaluating New Antibodies in the Clinical Immunohistochemistry Laboratory".¹⁶

¹³ Applied Immunohistochemistry & Molecular Morphology, vol. 8, no. 4, December 2000, 275-284 at 281. [Tab 1]

¹⁴ *Supra* at 275.

¹⁵ Exhibit 1767 (page 7).

¹⁶ Arch Pathol Lab Med, vol. 125, February 2001, 289-294. [Tab 2]

Dr. Hsi states:

New antibodies introduced in clinical laboratories are generally commercially available antibodies with well-characterized specificities. For ASRs [Analyte Specific Reagents], a category that includes most IHC reagents, one may presume that good manufacturing practices have been followed and that there is a high likelihood that the antibody will perform as advertised.¹⁷

The article continues as follows:

Common sense dictates that when the antibody in question has a long and established history of use in diagnostic immunohistochemistry, such as the antibodies for S100 or CD30, one does not need to go to great lengths to validate the antibody. A small panel of 10 positive and 10 negative test cases may be sufficient, depending on the results.¹⁸

24. In his testimony, Dr. Dabbs acknowledged that certain antibodies, including 1D5, are “very robust” and would give “very comparable” results.¹⁹
25. As a result of the above, and further to paragraph 194 of our Submissions, Dr. Kara Laing et al. submit that Dr. Khalifa’s correlation exercise was sufficient to evaluate the 1D5 antibody and it was not necessary to conduct a clinical trial.

¹⁷ *Supra* at 290.

¹⁸ *Supra* at 293.

¹⁹ Evidence of David Dabbs, 15/09/2008, p. 203, lines 6-13.

Reply to the Submission by Eastern Regional Integrated Health Authority

Page 134, Paragraph 285(c)

26. There is a statement found at page 134, paragraph 285(c) attributed to Dr. Kwan that is incorrect. Dr. Kwan did not express the view that a large percent of the changes appeared to be due to technical change at the meeting on July 24, 2005. His evidence is as follows:

Q: "He feels happier at this meeting that a large percentage due to technological change"...The comment attributed to you is right here. What do you recall then, Doctor, about this, about the meeting overall and what's attributed to you?

A: Well, I mean, I can vaguely remember, such a long time ago now, that we are thinking that we have a problem, and I thought that somebody in the meeting said, well, this is probably due to technology changes we have at that time...At least I was at that time given the impression that it may be technological change that we have within the laboratory, but I may be wrong. That was the impression at that time. ...

Q: Doctor, the idea that "you would feel happier at this meeting that a large percentage due to technological change", does that relate to the idea, that, well, look, sometimes newer equipment is just better at doing it?

A: Well, it may be, and I think that change in technology – you have new equipment doing things different, and now you get better results than we had before. Maybe Mount Sinai have much better equipment than we have, I don't know. I mean, and it seems to me that was some sort of topic of that, and if that's so, that is easy to correct.²⁰

27. So Dr. Kwan was simply reacting to the suggestion at the meeting on July 24, 2005 that the changes were probably due to advances in technology.

Reply to the Submissions on Behalf of the Canadian Cancer Society, NL Division

²⁰ Evidence of Alan Kwan, 09/29/2008, pages 105-108, lines 22-13.

Page 11, Paragraph 24

28. Dr. Kara Laing et al. wish to elaborate on Dr. Dankwa's testimony about registering tumor reports which is referenced at page 11, paragraph 24 of the Cancer Society's Submissions.
29. Dr. Dankwa explained that cancer diagnoses had previously been sent automatically to the Cancer Registry. He became aware around 1996 or 1997 that personal patient information was being forwarded without consent despite the fact that the practice was not mandated. This awareness came about as a result of a confrontation by a patient who was not pleased that his/her information had been forwarded. So Dr. Dankwa felt he could not continue to release tumor reports until there was consent. He was prepared to provide numbers of tumor diagnoses, but got the impression that the Registry wanted identifiable patient information as well.²¹
30. Dr. Dankwa recalled speaking with Ms. Bertha Pause around 2000, who was then the Director of the NCTRF, and she promised that she would address his concerns. Unfortunately, however, the issue was not addressed so he continued not releasing the reports to the Registry. Dr. Dankwa told the Commission that efforts are still underway to resolve this issue.²²

²¹ Evidence of Kweku Dankwa, 07/11/2008, pages 373-374, lines 5-25.

²² Evidence of Kweku Dankwa, 07/11/2008, pages 375-378, lines 1-2.

All of which is respectfully submitted in Reply this ^{5th} day of December, A.D., 2008.



PETER N. BROWNE, Q.C.

Curtis, Dawe

Solicitors for Dr. Kara Laing, et al

11th Floor, Fortis Building

139 Water Street

St. John's, NL A1C 5J9

TO: Commission of Inquiry on Hormone
Receptor Testing
50 Tiffany Lane
St. John's, NL A1A 4H7
Attn: Madam Justice Margaret A. Cameron, Commissioner
CC: Ms. Sandra Chaytor, Q.C., Commission Co-Counsel
Mr. Bernard Coffey, Q.C., Commission Co-Counsel

AND TO:

White, Ottenheimer & Baker
10 Fort William Place
Baine Johnston Centre
P.O. Box 5457
St. John's, NL A1C 5W4
Attn: Daniel W. Simmons

Ches Crosbie Barristers
169 Water Street
St. John's, NL
A1C 1B1
Attn: Mr. Chesley Crosbie, Q.C.

Stewart McKelvey
Barristers & Solicitors
Cabot Place, 100 New Gower Street
P.O. Box 5038
St. John's, NL A1C 5V3
Attn: Mr. Daniel Boone

Benson, Myles
Barristers & Solicitors
Suite 900, Atlantic Place
P.O. Box 1538
St. John's, NL A1C 5N8
Attn: Mr. Mark Pike, Q.C.

McInnes Cooper
Barristers & Solicitors
10 Fort William Place
P.O. Box 5939
St. John's, NL A1C 5X4
Attn: Mr. David Eaton, Q.C.

Martin, Whelan, Hennebury & Stamp
Barristers & Solicitors
15 Church Hill
P.O. Box 5910
St. John's, NL A1C 5X4
Attn: Ms. Jennifer Newbury

Department of Justice
P.O. Box 8700
Confederation Building
St. John's, NL A1B 4J6
Attn: Mr. Rolf Pritchard & Ms. Jacqueline Brazil, Q.C.

Immunohistochemical Analysis of Estrogen Receptors in Breast Carcinomas Using Monoclonal Antibodies That Recognize Different Domains of the Receptor Molecule

Giuseppe Santeusano, M.D., Alessandro Mauriello, M.D.,
Luca Ventura, M.D., Fabrizio Liberati, M.D., Alfredo Colantoni, M.D.,
Rosa Lasorella, M.D., and Luigi Giusto Spagnoli, M.D.

Estrogen receptor (ER) analysis was performed in 46 primary breast carcinomas using four monoclonal antibodies (MABs) to ER (AER311, ER1D5, LH1, and LH2), each of which recognizes a distinct domain of the receptor protein. ER was expressed as the percentage of positively stained tumor cells. Statistical analysis was performed using the SPSS/PC+ program to set the cut off of positivity and the prognostic value of each MAB. A positivity >30% for each MAB possessed the best sensitivity/specificity ratio and was used as the cut-off value. Multivariate discriminant analysis showed that MABs AER311, ER1D5, and LH2 had significant prognostic value. Fourteen tumors showed positivity for these three MABs; 17 were positive for one or two of the three MABs, and 15 were negative for all three MABs. Survival analysis showed that patients with tumors negative for all three of these MABs had progression of the disease within 8 years from the diagnosis of the tumor, whereas all patients with tumors positive for all three MABs were alive 13 years after surgery. A significant correlation ($P = 0.0006$) between tumor grading and ER status was found; 71% of the tumors that were positive for all three MABs were grade 1, whereas tumors negative for all three MABs were mostly grades 2 and 3. No significant relationship was observed between ER status and tumor size. A significant correlation ($P = 0.008$) between lymph node status and ER was found; breast tumors positive for all three MABs were in the majority (92.9%) of cases pN0, whereas 67% of tumors negative for all three MABs were pN1. Results from the present study suggest

that the use of a panel of MABs that target distinct epitopes within domains of the ER protein could offer a better approach for assessing the ER status in breast cancer patients, because it enables the recognition of breast tumors with intact or structurally defective ER proteins.

Key Words: Estrogen receptors—Monoclonal antibodies—Immunohistochemistry—Breast cancer.

Applied Immunohistochemistry & Molecular Morphology 8(4): 275-284, 2000.

The availability of monoclonal antibodies (MABs) to estrogen receptors (ER) (1) has allowed the cloning and sequencing of the receptor protein leading to a better understanding of its structure and function (2). Human ER is a protein composed of 595 amino-acid residues, with a molecular weight of approximately 66 kd, that is divided into six distinct domains with specific functions denoted from the NH₂ to the COOH terminal regions by six letters from A to F (3). The amino terminal domain A/B is implicated in transactivating function (Taf1) and is involved in the activation of estrogen-responsive genes. C is the DNA-binding domain. The D domain is involved in the nuclear localization of receptors. E is the hormone binding domain and is also implicated in another transactivating function (Taf2). F is the carboxyl-terminus domain and appears to have a modulatory function in regulating the transcriptional response to estrogens and antiestrogens (4).

The availability of MABs to ER that target specific epitopes within domains over the entire length of the receptor protein offers the opportunity to study the expression of such domains in breast tumors (5). This ability is potentially very useful in that discordant results of ER status observed in some breast carcinomas by using

Manuscript received December 7, 1999; accepted May 4, 2000.

From Università degli Studi di Roma "Tor Vergata," Facoltà di Medicina e Chirurgia, Dipartimento di Biopatologia e Diagnostica per Immagini, Sezione di Anatomia Patologica, Rome (G.S., A.M., L.V., A.C., R.L., L.G.S.); Università degli Studi di Perugia, Facoltà di Medicina e Chirurgia, Cattedra di Anatomia Patologica presso Ospedale di Terni, Terni (F.L.), Italy.

This study was supported in part by a grant from Banca Nazionale del Lavoro, Sede Centrale di Roma.

Address correspondence and reprint requests to Prof. Giuseppe Santeusano, M.D., Università degli Studi di Roma "Tor Vergata," Facoltà di Medicina e Chirurgia, Dipartimento di Biopatologia e Diagnostica per Immagini, Sezione di Anatomia Patologica, Via della Ricerca Scientifica, 1, 00133 Rome, Italy. E-mail: santeusano@med.uniroma2.it

MABs, which recognize different domains (6–19), seem owing to the presence of ER variants (6,20). Additionally, the failure of some ER-positive breast tumors to respond to endocrine therapy, the development of endocrine resistance, and progression in human breast cancer could be related to the presence of such altered receptors.

We performed a retrospective immunohistochemical study of ER in paraffin sections from 46 primary breast carcinomas using four MABs to ER, each of which recognizes a distinct domain of the receptor protein. The main purpose of this study was to ascertain whether this approach could provide information of the ER status beyond that obtained by using single MABs to ER.

MATERIAL AND METHODS

Materials

We used formalin-fixed, paraffin-embedded tumor specimens from 46 patients who had operable primary breast carcinoma. None of the patients had received prior adjuvant systemic therapy. All cases were selected adopting the following criteria: (a) all tumors were assayed for ER by the dextran-coated charcoal (DCC) method, (b) at least one paraffin block of the primary tumor and one paraffin block containing nonneoplastic breast tissue from the same patient were available, (c) all patients were treated by total mastectomy with axillary dissection followed by hormonal manipulation, which was based on original ER DCC results, with tamoxifen alone or combined with chemotherapy or radiotherapy, (d) follow-up was available.

Clinical data on all patients included patient age, tumor size, histologic classification, tumor grading (21), axillary lymph node status, pTNM (22), and ER status assessed by DCC analysis. Patient ages ranged from 35 to 79 years (seven premenopausal, four perimenopausal, 35 postmenopausal). All tumors used were primary, infiltrating carcinomas of the ductal type in 40 cases and of the lobular type in six cases. Nine patients had node-negative disease and 37 had node-positive disease. After surgery, ER status was assayed by the DCC ligand-binding method using a standard cut-off point of 5 fmol/mg cytosol protein to distinguish positive from negative tumors; 22 tumors were ER positive and 24 were ER negative by DCC assay. Adjuvant chemotherapy was given to node-positive patients, either pre- or postmenopausal, with negative ER status; postmenopausal patients with positive ER status received tamoxifen. The patients were followed-up from 6 months to 14 years. All patients had complete clinical follow-up at 6-month intervals for the first 36 months and at 1-year intervals thereafter. Before carrying out the present study, the follow-up of all cases was updated. Twenty-one patients (mean age, 56.61 ± 2.23) had progression of the disease (local

recurrence, metastasis, or death) and 25 (mean age, 58.16 ± 1.81) were stable (alive and without local recurrence or metastasis at this time).

Immunohistochemistry

For each case, we sought one paraffin block containing both tumor and normal breast; when normal breast was absent, one paraffin block from the same breast specimen containing nonneoplastic breast tissue was used in parallel for positive control. Serial sections (4 μ thick) were cut from paraffin blocks, placed on silane-coated slides, and dried overnight at $+60^\circ\text{C}$.

Antibodies

The following MABs were used according to the manufacturer's instructions: LH2, clone CC4-5 (YLEM, Avezzano, Italy), produced using as antigen for immunization a recombinant ER fusion protein—the location of the epitope recognized by this MAB is on the A/F domain (23); LH1, clone ER-P31 (YLEM), produced using as antigen for immunization a synthetic peptide (24)—the epitope recognized by this MAB is on the F domain of the C-terminal (23); ER1D5, clone 1D5 (YLEM), produced using as antigen for immunization a recombinant ER fusion protein—the location of the epitope recognized by this MAB is in the A/B domain at the N-terminal (25); this MAB reacts not only with wild-type (WT) ER but also with several truncated forms that are translated from splice variant mRNA. AER311, an antibody raised against the SDS-denatured calf uterus ER (kindly provided by Dr. Ciro Abbondanza, Cattedra di Patologia Generale, II Università di Napoli, Naples, Italy), which recognizes an epitope in the E/F domain at the C-terminal (5) and reacts only with WT ER, with the exception of the DNA-binding truncated protein (11).

Immunostaining

Paraffin sections were deparaffinized, rehydrated in graded alcohol to distilled water, and then immersed in 0.01 mol/L citrate buffer (pH 6) solution and subjected to antigen retrieval (AR), irradiated using a standard microwave oven (Miele Supratronic M750, Italy) at 700 W for 15 minutes (3×5 minutes). Microwaved sections were then incubated for another 20 minutes in the hot buffer before processing for immunocytochemistry. Four serial sections from each paraffin block were incubated with MABs to ER and immunostained with the streptavidin-biotin-peroxidase (YLEM) technique followed by diaminobenzidine (DAB) chromogen solution. All steps of the immunostaining procedure were 30 minutes long and were followed by washes in buffered solution. Finally, sections were weakly counterstained with hematoxylin

and mounted on Eukit. In all cases, positive, negative, and internal controls and controls for general tissue reactivity were used; in fact, a paraffin section of a breast tumor known to be ER positive was used as positive control, whereas a negative control was obtained by omitting the primary antibody. Moreover, as internal control, we looked for the occurrence of immunostaining in the nonneoplastic breast tissue; if this was not available, we used sections of nonneoplastic breast tissue from the same case. Finally, MABs to cytokeratin AE1/AE3 (YLEM) and vimentin (clone V9, YLEM) were used as overall indicators of breast tissue immune reactivity.

Interpretation of Immunostaining Results

For each case, ER results were first examined by simultaneously viewing the immunostained slides on a dual-head microscope using a $\times 10$ objective to select the same neoplastic areas that were localized with a permanent pen for the counting of positive nuclei. Positive cells were counted in adjacent fields in the areas showing neoplastic infiltration; the stained sections were scored independently by two pathologists (G.S. and F.L.) using a Nikon E600 Eclipse microscope (Nikon, Italy), by counting an average of 3,500 neoplastic cells at $\times 40$ magnification. Variations in staining intensity were observed between tumor cells and between different areas within the same section, a phenomenon that might be attributed to the heterogeneity of the tumor-cell population. Although the intensity of staining was noted, it was not incorporated into the final score, because there was disagreement between the two observers.

Statistical Analysis

The statistical analysis was performed using the SPSS/PC + program (26). The positivity for each MAB was evaluated by analyzing different values of positivity, ranging from 10 to 50%, with 10% increases. For each value of positivity, the specificity and sensitivity were analyzed according to the following indices of Galen and Gambino (27):

$$\text{Sensitivity (positivity in disease)} = \frac{[(TP)/(TP + FN)] \times 100}$$

$$\text{Specificity (negativity in health)} = \frac{[(TN)/(TN + FP)] \times 100}$$

where TP is true positive, TN is true negative, FP are false positive, and FN is false negative.

Moreover, multivariate discriminant analysis, including stepwise analysis with the Wilks' lambda method (26), was applied to include variables that contained information concerning group differences. The χ^2 test was

used to evaluate the relationship between ER results and histopathologic features (26). Last, survival analysis was performed according to the Kaplan-Meier method (26); differences between survival curves were analyzed by log-rank method (26). For all statistical analyses, a *P* value < 0.05 was considered to be significant.

RESULTS

Positive immunostaining was seen in the nuclei of both malignant and nonmalignant epithelial breast cells; a small percentage of cases showed scanty and focal cytoplasmic immunostaining with all MABs used (Figs. 1 and 2). In the nonneoplastic breast tissue surrounding the tumor, the percentage of positive nuclei ranged from 30 to 100% for all MABs. The percentage varied depending on the presence of normal ducts and lobules, duct ectasia, cysts, and intraductal hyperplasia. In normal ducts and lobules, the percentage of positivity ranged from 30 to 100%; however, differences in the percentages of positivity were often found in the same breast specimen; in fact, some ducts and lobules exhibited lesser or greater positivity percentages. In some breast tissue from postmenopausal patients, a marked reduction in the percentage of positivity in the lobules and ducts was observed, whereas there was greater positivity in the ducts with intraductal hyperplasia. Last, in ectatic ducts and even more so in cysts with apocrine metaplasia, we observed greater reduction and, at times, absence of nuclear positivity. In general, there were no marked differences in staining intensity between nonneoplastic, hyperplastic, and neoplastic breast tissue; however, in some elderly patients, the carcinomatous cells often exhibited greater staining intensity compared with nonneoplastic cells.

Table 1 provides a summary of ER immunostainings and histologic grading, size of the tumors, lymph node status, and follow-up. Some tumors showed differences of percentage of positivity for each MAB used; moreover, $< 5\%$ of neoplastic cells were positive in 18 cases immunostained with AER311 MAB, in 13 cases with LH2 MAB, in 10 cases with ER1D5 MAB, and in 13 cases with LH1 MAB.

As shown in Tables 2–5, a positivity $> 30\%$ for each MAB possessed the best sensitivity/specificity ratio. Using as cut off of positivity the value $> 30\%$, AER311 and ER1D5 MABs were positive in 23 tumors and negative in 23; LH2 and LH1 MABs were positive in 22 tumors and negative in 24. The multivariate discriminant analysis has shown that MABs AER311, ER1D5, and LH2 had significant prognostic value (Table 6) in that they discriminated patients with disease progression from those with stable disease; on the contrary, LH1 and ER DCC assays did not show significant prognostic value. Of all MABs, AER311 had the highest discriminant

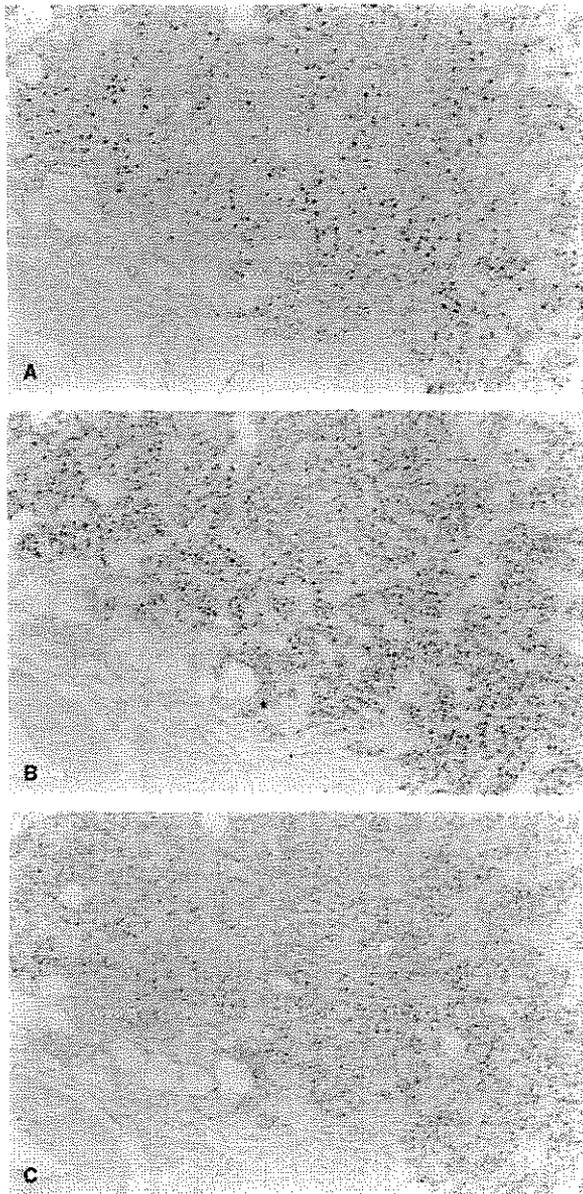


FIG. 1. Infiltrating breast carcinoma immunostained with MABs to ER AER311 (A), ER1D5 (B), and LH2 (C). Streptavidin-biotin-peroxidase, DAB chromogen, hematoxylin counterstain, original magnification $\times 20$.

value in that it had the highest Wilks lambda coefficient. At the cut-off value of positivity $>30\%$, there were 14 tumors showing positivity for these three MABs that had significant prognostic value, whereas 17 tumors were positive for one or two of three MABs, and 15 were tumors negative for all three MABs. Within the group of tumors positive for one or two MABs, eight were positive for two MABs (four for AER311 and ER1D5, three for AER311 and LH2, one for LH2 and ER1D5) and nine were positive for only one MAB (two for AER311, four for LH2, and three for ER1D5).

A significant correlation ($P = 0.0001$) was also observed between ER results and the outcome of disease; all 14 patients with tumors positive for all three prognostically significant MABs were stable; on the contrary, all 15 patients with tumors negative for these three MABs had progression of the disease. It is worth noting that in the group of patients with tumors positive for one or two MABs, the presence of positivity for two MABs in a tumor indicates a tendency toward stabilization of the disease compared with tumors positive for only one

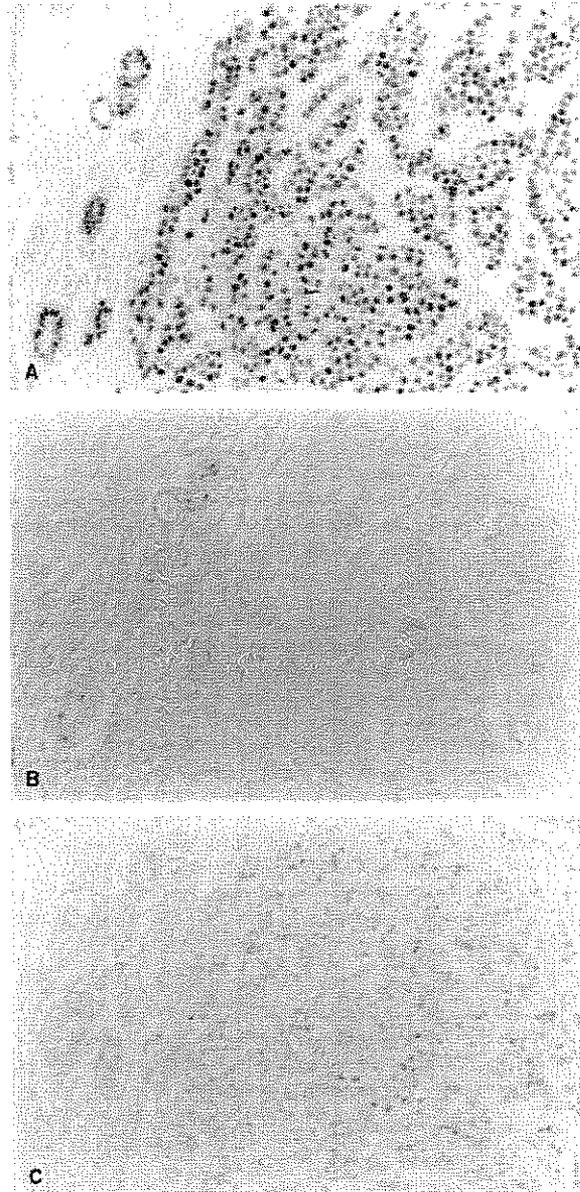


FIG. 2. Infiltrating breast carcinoma immunostained with MABs to ER AER311 (A), ER1D5 (B), and LH2 (C). Streptavidin-biotin-peroxidase, DAB chromogen, hematoxylin counterstain, original magnification $\times 40$.

TABLE 1. Relationship between histologic grading, tumor size, lymph node status, outcome, ER by DCC, and ER by MABs

Case no.	Age (y)	Histologic grading	pT	pN	Outcome	Follow-up (y)	ER-DCC (fmol)	AER311 (% + cells)	LH2 (% + cells)	ER1D5 (% + cells)	LH1 (% + cells)
1	56	3	1	1	P	1	<5.0	<5	29	<5	<5
2	44	2	3	1	P	1	<5.0	<5	18	<5	5
3	36	2	2	1	P	1	<5.0	<5	28	<5	<5
4	58	3	1	0	P	1	95.6	72	<5	5	<5
5	63	3	2	1	P	1	<5.0	<5	<5	9	8
6	51	3	1	0	P	2	91.6	65	26	5	<5
7	57	2	3	1	P	2	<5.0	<5	<5	<5	<5
8	40	3	2	1	P	2	<5.0	<5	26	<5	<5
9	61	2	1	1	P	2	11.2	27	<5	18	5
10	64	2	1	1	P	2	<5.0	<5	5	5	<5
11	60	3	2	1	P	2	<5.0	<5	<5	5	5
12	65	2	1	0	P	3	<5.0	<5	38	8	<5
13	58	3	1	0	P	3	<5.0	<5	<5	28	5
14	57	2	1	1	P	3	<5.0	<5	8	<5	<5
15	42	2	1	0	P	3	<5.0	<5	36	5	5
16	79	2	1	0	P	3	<5.0	<5	<5	<5	5
17	64	3	1	0	P	4	<5.0	<5	<5	5	5
18	69	3	3	1	P	6	24.2	28	<5	36	48
19	59	3	2	0	P	7	<5.0	<5	<5	30	19
20	58	1	2	0	P	8	7.5	<5	17	20	<5
21	48	3	4	1	P	9	24.2	16	40	48	40
22	58	1	1	0	S	13	40.4	52	10	72	65
23	61	1	2	0	S	13	<5.0	39	78	65	80
24	39	1	1	1	S	13	11.2	56	71	95	86
25	59	2	1	1	S	13	29.2	18	39	<5	<5
26	64	2	1	0	S	13	166.7	51	86	84	71
27	63	2	1	1	S	13	108.7	<5	<5	47	38
28	69	2	2	0	S	13	149.7	<5	<5	38	37
29	50	1	2	0	S	13	<5.0	52	77	10	10
30	59	1	1	0	S	13	<5.0	52	82	72	77
31	35	1	1	0	S	13	<5.0	61	85	94	75
32	40	2	1	0	S	13	25.5	57	20	75	90
33	68	2	2	0	S	13	<5.0	66	75	72	81
34	70	1	2	0	S	13	12.4	53	83	91	75
35	59	2	1	1	S	13	110	38	<5	37	47
36	47	2	2	0	S	13	135.5	71	48	<5	<5
37	57	1	1	0	S	13	150.5	58	74	84	87
38	62	1	1	0	S	13	<5.0	54	71	91	81
39	61	1	2	0	S	13	<5.0	55	76	83	71
40	60	2	2	0	S	13	<5.0	38	71	86	85
41	63	2	2	0	S	13	21.5	54	78	82	77
42	59	1	1	0	S	13	35.5	58	81	75	82
43	60	2	1	0	S	13	60.5	51	9	71	65
44	61	2	1	1	S	13	35.6	29	40	<5	<5
45	63	1	2	0	S	13	<5.0	53	66	73	73
46	66	2	1	0	S	13	15.6	56	72	10	8

P, progression; S, stable; y, years; CC4-5 (clone LH2), ER1D5 (clone 1D5), LH1 (clone ER-P31).

TABLE 2. Sensitivity and specificity of MAB AER311 using various cut-off points of positivity

Cut off ^a	Cases positive			Cases negative			Sensitivity	Specificity
	Total	Stable	Progression	Total	Stable	Progression		
>10%	28	23 (82.1%)	5 (17.9%)	18	2 (11.1%)	16 (88.9%)	92.0	76.2
>20%	26	22 (84.6%)	4 (15.4%)	20	3 (15.0%)	17 (85.0%)	88.0	81.0
>30%	23	21 (91.3%)	2 (8.7%)	23	4 (17.4%)	19 (82.6%)	84.0	90.5
>40%	20	18 (90.0%)	2 (10.0%)	26	7 (26.9%)	19 (73.1%)	72.0	90.5
>50%	5	3 (60.0%)	2 (40.0%)	41	22 (53.7%)	19 (46.3%)	12.0	90.5

^a Percentage of positive cells.

TABLE 3. Sensitivity and specificity of MAB LH2 using various cut-off points of positivity

Cut off ^a	Cases positive			Cases negative			Sensitivity	Specificity
	Total	Stable	Progression	Total	Stable	Progression		
>10%	29	20 (69.0%)	9 (31.0%)	17	5 (29.4%)	12 (70.6%)	80.0	57.1
>20%	26	19 (73.1%)	7 (26.9%)	20	6 (30.0%)	14 (70.0%)	76.0	66.7
>30%	22	19 (86.4%)	3 (13.6%)	24	6 (25.0%)	18 (75.0%)	76.0	85.7
>40%	17	17 (100%)	0	29	8 (27.6%)	21 (72.4%)	68.0	100
>50%	16	16 (100%)	0	30	9 (30.0%)	21 (70.0%)	64.0	100

^a Percentage of positive cells.

MAB. Seven (87%) of 8 patients with tumors positive for two MABs (four tumors were positive for AER311 and ER1D5 and three tumors were positive for AER311 and LH2) were stable, whereas 4 (44.4%) of nine patients with tumors positive for one MAB (two tumors were positive for ER1D5 and two for LH2) had stabilization of the disease. Survival analysis shows that patients with tumors negative for all three of these MABs had progression of the disease within 8 years from the original diagnosis, whereas all patients with positive tumors for all three MABs were alive 13 years after surgery (Fig. 3).

Table 7 shows the relationship between ER results obtained with AER311, ER1D5, and LH2 MABs and tumor grading, tumor size, and lymph node metastases. A significant correlation ($P = 0.0006$) between tumor grading and ER status was found; tumors that were positive for all three MABs were grade 1 in 71% of cases, whereas tumors negative for all three MABs were mostly grades 2 and 3. Furthermore, no significant relationship was observed between ER status and tumor size. Last, a significant correlation ($P = 0.008$) between lymph node status and ER was found; in fact, breast tumors positive for all MABs were pN0 in the majority (92.9%) of cases, whereas ER-negative tumors were pN1 in the majority (66.7%) of cases.

DISCUSSION

In the present study of 46 primary breast carcinomas, the analysis of ER status using MABs against different domains of the ER protein showed differences in the percentage of staining for each antibody used; these findings support the notion that some domains of the ER

protein were missing. Similar results were previously noted in studies in which two MABs that recognize different domains in the NH₂ or the COOH terminal regions of the ER protein were compared (6–19). Moreover, in the study performed by Elias et al. (6), the discrepancy observed between reactivity of ER1D5 MAB (which targets an epitope in the A/B region) and H222 MABs (which targets an epitope in the E region) in breast tumors was interpreted as the presence of “outlaw variants.” Furthermore, Saccani-Jotti et al. (15) found some breast tumors strongly positive for ER1D5 MAB and negative for H222 MAB, suggesting that MABs directed against different parts of the receptor protein may also be of value in the investigation of variant forms of ER. Also, Traish et al. (28), who developed MABs to specific domains of ER protein, demonstrated the presence of structurally defective ER in human breast, suggesting that MABs to ER domains could provide a means of identification of dysfunctional ER in breast tumor biopsies.

In the present study, <5% of neoplastic cells were positive in 18 cases immunostained with AER311 MAB, in 13 cases with LH2 MAB, in 10 cases with ER1D5 MAB, and in 13 cases with LH1 MAB. These results document the occurrence of the lack of the carboxyl and amino terminal domains in some breast carcinomas and confirm the results of Hori et al. (16), who found a number of breast cancers lacking the carboxyl terminal of ER protein.

Because we used MABs that target different domains of the ER protein, we hypothesize that alterations of the receptor protein could have resulted in absence of the domain recognized by MABs. This alteration may be a

TABLE 4. Sensitivity and specificity of MAB ER1D5 using various cut-off points of positivity

Cut off ^a	Cases positive			Cases negative			Sensitivity	Specificity
	Total	Stable	Progression	Total	Stable	Progression		
>10%	26	20 (76.9%)	6 (23.1%)	20	5 (25.0%)	15 (75.0%)	80.0	71.4
>20%	24	20 (83.3%)	4 (16.7%)	22	5 (22.7%)	17 (77.3%)	80.0	81.0
>30%	23	20 (87.0%)	3 (13.0%)	23	5 (21.7%)	18 (78.3%)	80.0	85.7
>40%	19	18 (94.7%)	1 (5.3%)	27	7 (25.9%)	20 (74.1%)	72.0	95.2
>50%	17	17 (100%)	0	29	8 (27.6%)	21 (72.4%)	68.0	100

^a Percentage of positive cells.

TABLE 5. Sensitivity and specificity of MAB LH1 using various cut-off points of positivity

Cut off ^a	Cases positive			Cases negative			Sensitivity	Specificity
	Total	Stable	Progression	Total	Stable	Progression		
>10%	23	20 (87.0%)	3 (13.0%)	23	5 (21.7%)	18 (78.3%)	80.0	85.7
>20%	22	20 (90.9%)	2 (9.1%)	24	5 (20.8%)	19 (79.2%)	80.0	90.5
>30%	22	20 (90.9%)	2 (9.1%)	24	5 (20.8%)	19 (79.2%)	80.0	90.5
>40%	19	18 (94.7%)	1 (5.3%)	27	7 (25.9%)	20 (74.1%)	72.0	95.2
>50%	17	17 (100%)	0	29	8 (27.6%)	21 (72.4%)	68.0	100

^a Percentage of positive cells.

consequence of abnormalities of receptor protein at DNA or mRNAs level, rather than intratumoral heterogeneity of ER expression, different antibody affinities, loss of a portion of the protein molecule owing to proteolysis, or masking of the epitope target by either protein conformation changes or by binding of heat-shock protein. This hypothesis is also sustained by studies of Huang et al. (11), who also reported discordant results using ER1D5 and AER311 MABs in assessing the ER status in breast tumors. They also found significant correlation between expression of certain ER variant mRNAs, encoding truncated ER proteins assessed by polymerase chain reaction analysis, and inconsistent ER results in breast carcinomas assessed by immunohistochemistry using ER1D5 and AER311 MABs (20). This observation supports a growing body of evidence suggesting that ER variant mRNAs may be translated in vivo into altered ER-like proteins (29,30), thus determining the discrepant results of ER analysis when two or more MABs that recognize different domains of ER are used. The difference in target epitope specificity of MABs to ER may be important in view of the many different ER mRNA variants that have been described in normal and human breast cancer biopsy samples and cell lines (31,32), any of which may be translated into a variety of truncated or abnormal proteins (33–35) that may fail to produce any biologic function. The lack of response to endocrine therapy, for example, the development of tamoxifen resistance, in some patients with ER-positive tumors could be attributed to the presence of structural alteration of the ER protein.

The use of a panel of MABs that target distinct epitopes within domains of the ER protein, including AER311, which targets an epitope within the E/F hormone-binding domain, and ER1D5, which targets an epi-

tope in the A/B of estrogen-responsive genes domain, could offer a better approach for assessing the ER status in breast cancer patients. This approach would allow the recognition of breast tumors with structurally defective ER proteins and would be helpful in cases that are initially negative for ER. Using a single MAB that recognizes a restricted domain within the ER protein may give negative results in tumors with defective domains with disappearance or distortion of their transcriptional function. These observations may have great clinical relevance, permitting the identification of that group of ER-positive tumors that exhibit no response to hormonal therapy, thereby reducing the proportion of apparently ER-negative responders. Therefore, in the evaluation of ER status in breast cancer specimens, if the "intact" ER is the predominant receptor species, then the tumor should be considered positive. However, using MABs that react with different domains of ER protein, any discrepancy in positivity observed should be considered as indicative of the possible presence of ER variants and should be further investigated using antibodies to progesterone receptors (PR) and pS2.

Although immunohistochemistry, compared with biochemical methods, offers greater advantages in evaluating ER status, it is influenced by a number of factors that must be considered if results are to be reliable. Among these factors, fixation and tissue processing are most important. Furthermore, the MABs used with particular attention to the ER domain recognized, the method of AR, and the sensitivity of the detection system are also key factors (36,37). Moreover, in evaluating staining results, the scoring method and the cut-off point for positivity that is chosen to define a tumor as ER positive are also important factors. At present, there is neither a standard scoring method for evaluating immunostaining results nor a uniformly agreed cut-off value that defines ER positivity and that can be considered as the standard for all histopathology laboratories (38–40).

In the present study, we observed that a cut-off value of positivity >30% for all MABs used best separates patients with tumor stability from those with tumor progression. Moreover, multivariate discriminant analysis revealed that among MABs and DCC assay, AER311, LH2, and ER1D5 MABs have independent prognostic

TABLE 6. Multivariate discriminant analysis: discriminant coefficient (Wilks lambda) and relative statistical significance of the three MABs (AER311, ER1D5, and LH2) that had significant prognostic value

Discriminant variables	Wilks' lambdas	P
AER311	0.45	<0.0001
LH2	0.29	<0.0001
ER1D5	0.35	<0.0001

Canonical discriminant function: $P < 0.0001$.

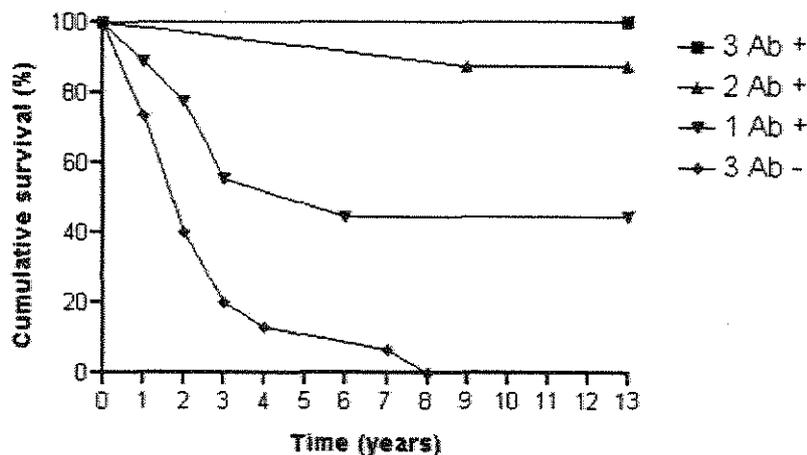


FIG. 3. Survival analysis according to the Kaplan-Meier method. Patients with tumors negative for all three MABs had progression of the disease within 8 years from the diagnosis of the tumor; all patients with tumors positive for all three MABs were alive 13 years after surgery.

value, and among these three MABs, AER311 has the highest discriminating power. At the positivity value of >30%, 14 tumors were positive for three MABs; this group of tumors should represent the estrogen-dependent phenotype tumors. In addition, 15 tumors were negative for these three MABs; these tumors, which show lack of both amino and carboxyl terminals, appear to have an hormone-independent phenotype. From a clinical point of view, recognition of "true" ER-negative tumors is most important in that aggressive therapies could be considered for patients with ER-negative tumors. Seventeen tumors that were positive for one or two MABs may represent that group of tumors in which there are ER variants.

Our results also indicate that ER-negative tumors are mostly grades 2 and 3 and more commonly have lymph node metastases. All patients with tumors negative for all three MABs showed progression of the disease within 8 years from the time of the diagnosis of the tumor, whereas all patients with tumors positive for all three MABs were alive 13 years after surgery. It has been

hypothesized that the expression of variants of ER mRNAs may have a role in the development of endocrine resistance and progression of human breast cancer (33,41), but in the present study the presence of ER variants does not seem predictive of tumor progression into more aggressive stages.

Results from this study indicate that MABs that target different domains of the ER protein such as AER311, ER1D5, and LH2 should be used along with immunocytochemistry to detect ER in paraffin sections from breast tumors in that they improve the assessment of receptor status. This approach, at the cut-off positivity value of >30% of tumor cells, appears to offer better accuracy in the evaluation of receptor status, because MABs directed toward certain ER domains may give false negative results, if changes in the ER protein resulted in absence or conformational alteration of the domain targeted.

The present study deals only with specimens from primary tumors and not from recurrences and does not include PR analysis of the patient population. Further studies including primary and recurrent tumor specimens

TABLE 7. Relationship between ER results (using MABs AER311, ER1D5, LH2) and tumor grading (G), tumor size (pT), and lymph node status (pN)

Histopathologic features	Total cases	Immunohistochemical results				P
		3 MABs positive (14 cases)	2 MABs positive (8 cases)	1 MAB positive (9 cases)	3 MAB negative (15 cases)	
Grade						
1	13	10 (71.4%)	2 (25.0%)	0	1 (6.7%)	0.0006
2	22	4 (28.6%)	5 (62.5%)	6 (66.7%)	7 (46.7%)	
3	11	0	1 (12.5%)	3 (33.3%)	7 (46.7%)	
Tumor size						
pT1	26	7 (50%)	5 (62.5%)	7 (77.8%)	7 (46.7%)	0.25
pT2	16	7 (50%)	2 (25.0%)	1 (11.1%)	6 (40.0%)	
pT3	3	0	0	1 (11.1%)	2 (13.3%)	
pT4	1	0	1 (12.5%)	0	0	
Lymph node status						
pN0	29	13 (92.9%)	6 (75.0%)	5 (55.6%)	5 (33.3%)	0.008
pN1	17	1 (7.1%)	2 (25.0%)	4 (44.4%)	10 (66.7%)	

P < 0.05 is statistically significant.

along with PR evaluation will be necessary to confirm whether breast tumors in which the "intact" ER is the predominant receptor type are likely to be estrogen dependent and tamoxifen sensitive and whether the presence of ER variants may contribute to tamoxifen resistance and to progression of human breast cancer. □

Acknowledgment: We thank Prof. Giovanni Battista Bolis (Cattedra di Anatomia Patologica, Università di Perugia, Servizio di Anatomia Patologica, Ospedale di Terni, Terni, Italy) and Prof. Gaetano De Marco (Servizio di Anatomia Patologica, Ospedale di Taranto, Taranto, Italy) for providing some of the breast paraffin blocks used. We also thank Angela Ortenzi for expert technical assistance.

REFERENCES

- Greene GL, Nolan C, Engler JP, Jensen EV. Monoclonal antibodies to human estrogen receptor. *Proc Natl Acad Sci USA* 1980;77:5115-9.
- Green S, Walter P, Kumar V, et al. Human estrogen receptor cDNA: sequence, expression, and homology to v-erb-A. *Nature* 1986;320:134-9.
- Kumar V, Green S, Stack G, et al. Functional domains of the human estrogen receptor. *Cell* 1987;51:941-51.
- Montano MM, Muller V, Trobaugh A, Katzenellenbogen BS. The carboxy-terminal F domain of the human estrogen receptor: role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists. *Mol Endocrinol* 1995;9:814-25.
- Abbondanza C, de Falco A, Nigro V, et al. Characterization and epitope mapping of a new panel of monoclonal antibodies to estradiol receptor. *Steroids* 1993;58:4-12.
- Elias JM, Hyder DM, Miksicek RJ, Heiman A, Margiotta M. Interpretation of steroid receptors in breast cancer. A case with discordant receptor results using ER1D5 and H222 antibodies. *J Histochem* 1995;18:331-5.
- Bevitt DJ, Milton ID, Piggot N, et al. New monoclonal antibodies to estrogen and progesterone receptors effective for paraffin section immunohistochemistry. *J Pathol* 1997;183:228-32.
- Hendricks JB, Wilkinson EJ. Comparison of two antibodies for evaluation of estrogen receptors in paraffin-embedded tumors. *Mod Pathol* 1993;6:765-70.
- Pertschuk LP, Feldman JG, Kim Y-D, et al. Estrogen receptor immunocytochemistry in paraffin embedded tissues with ER1D5 predicts breast cancer endocrine response more accurately than H222 in frozen sections of cytosol-based ligand-binding assays. *Cancer* 1996;77:2514-9.
- Goulding H, Pinder S, Cannon P, et al. A new monoclonal antibody for the assessment of estrogen receptor status on routine formalin-fixed tissue samples. *Hum Pathol* 1995;26:291-4.
- Huang A, Pettigrew NM, Watson PH, et al. Immunohistochemical assay for oestrogen receptors in paraffin wax sections of breast carcinoma using a new monoclonal antibody. *J Pathol* 1996;180:223-7.
- Bier B, Bankfalvi A, Grote L, et al. Wet autoclave pretreatment for immunohistochemical demonstration of oestrogen receptors in routinely processed breast carcinoma tissue. *Histochem J* 1995;27:148-54.
- Pelicer EM, Sundblad A. Evaluation of antibodies to estrogen receptors (letter to editor). *Appl Immunohistochem* 1993;2:141.
- Mauri FA, Veronese S, Giraldo S, et al. ER1D5 and H222 (ER-ICA) antibodies to human estrogen receptor protein in breast carcinomas: results of multicentric comparative study. *Appl Immunohistochem* 1994;2:157-63.
- Saccani Jotti G, Johnston SR, Salter J, Detre S, Dowsett M. Comparison of new immunohistochemical assay for estrogen receptor in paraffin wax embedded breast carcinoma tissue with quantitative enzyme immunoassay. *J Clin Pathol* 1994;47:900-5.
- Hori M, Iwasaki M, Yoshimi F, Asato Y, Itabashi M. Determination of estrogen receptor in primary breast cancer using two different monoclonal antibodies, and correlation with its mRNA expression. *Pathol Int* 1999;49:191-7.
- Wong SY, Purdie A, Sewell HF, et al. Immunohistochemical assessment of ER-P31, a mouse anti-oestrogen receptor protein monoclonal antibody in human breast cancers: comparison with ER-ICA (Abbott) and radioligand assays. *Tum Biol* 1991;12:16-23.
- Barbareschi M, Losi L, Capotanio G, et al. Expression of estrogen receptors in breast cancer detected by various methods: lack of correlation. *Pathologica* 1997;89:397-404.
- Nedergaard L, Christensen L, Rasmussen BB, Jacobsen GK. Comparison of two monoclonal antibodies for the detection of estrogen receptors in primary breast carcinomas. *Pathol Res Pract* 1996;192:983-8.
- Hunag A, Leygue ER, Snell L, Murphy LC, Watson PH. Expression of estrogen receptor variant messenger RNAs and determination of estrogen receptor status in human breast cancer. *Am J Pathol* 1997;150:1827-33.
- Bloom HJG, Richardson WW. Histological grading and prognosis in breast cancer. *Br J Cancer* 1957;11:359-77.
- Sobin LH, Wittekind Ch, eds. *International Union Against Cancer, TNM classification of malignant tumors*, 5th ed. New York: Wiley-Liss, 1997.
- Mitchel K, ed. *Novocastra Laboratories product catalogue 1997*. Blayson on Tyne, UK: Colden Offsel Limited, 1997:6.
- Wilkinson L, Angus B, Akiba R, et al. ER-P31: A new monoclonal antibody recognising the oestrogen receptor effective in immunohistochemistry. *J Pathol* 1988;155:349A.
- AL Saati T, Clamens S, Cohen-Knafo E, et al. Production of monoclonal antibodies to human estrogen receptor protein (ER) using recombinant ER (RER). *Int J Cancer* 1993;55:651-4.
- Norusis MJ. *SPSS for Windows. Advanced statistics*. Chicago, IL: SPSS, Inc., 1993.
- Galen RS, Gambino SR. *Beyond normality: the predictive value and efficiency of medical diagnoses*. New York: Wiley, 1975:10-4.
- Traish AM, Al-Fadhi S, Klinge C, et al. Identification of structurally altered estrogen receptors in human breast cancer by site-directed monoclonal antibodies. *Steroids* 1995;60:467-74.
- Castles CG, Fuqua SAW, Klotz DM, Hill SM. Expression of a constitutively active estrogen receptor variant in the estrogen receptor negative BT-20 human breast cancer cell line. *Cancer Res* 1993;53:5934-9.
- Pink JJ, Wu SQ, Wolf DM, Bilimoria MM, Jordan VC. A novel 80 kDa human estrogen receptor containing a duplication of exons 6 and 7. *Nucleic Acid Res* 1996;24:962-9.
- Murphy LC, Dotzlaw H. Variant estrogen receptor mRNA species detected in human breast cancer biopsy samples. *Mol Endocrinol* 1989;3:687-93.
- Fuqua SA, Wolf DM. Molecular aspects of oestrogen receptors variants in breast cancer. *Breast Cancer Res Treat* 1995;35:233-41.
- Murphy LC, Hilsenbeck SG, Dotzlaw H, Fuqua SAW. Relationship of clone 4 oestrogen receptor variant messenger RNA expression to some known prognostic variables in human breast cancer. *Clin Cancer Res* 1995;1:155-9.
- Fuqua SA, Fitzgerald SD, Allred DC, et al. Inhibition of oestrogen receptor action by a naturally occurring variant in human breast tumors. *Cancer Res* 1992;52:483-6.
- Fuqua SA, Fitzgerald SD, Chamness GC, et al. Variant human breast tumor oestrogen receptor with constitutive transcriptional activity. *Cancer Res* 1991;51:105-9.
- Batalon AJ, Mathieu M-C, Le Doussal V. Optimization of heat-induced epitope retrieval for estrogen receptor determination by immunohistochemistry on paraffin sections. *Appl Immunohistochem* 1996;4:259-63.

37. Riera J, Simpson JF, Tamayo R, Battifora H. Use of cultured cells as a control for quantitative immunocytochemical analysis of estrogen receptor in breast cancer. The Quiegel method. *Am J Clin Pathol* 1999;111:329-35.
38. Bamed DM, Harris WH, Smith P, Millis RR, Rubens RD. Immunohistochemical determination of oestrogen receptor: comparison of different methods of assessment of staining and correlation with clinical outcome of breast cancer patients. *Br J Cancer* 1996;74:1445-51.
39. Elledge RM, Osbourne CK. Oestrogen receptor and breast cancer. *BMJ* 1997;314:1843-4.
40. Clark GM, Harvey JM, Osbourne CK, Allred DC. Oestrogen receptors status determined by immunohistochemistry is superior to biochemical ligand-binding (LB) assay for evaluating breast cancer patients. *Proc Am Soc Clin Oncol* 1997;16:129A.
41. Johnston SRD, Saccani-Jotti G, Smith IE, et al. Changes in estrogen receptor, progesterone receptor, and pS2 expression in tamoxifen-resistant human breast cancer. *Cancer Res* 1995;55:3331-8.

A Practical Approach for Evaluating New Antibodies in the Clinical Immunohistochemistry Laboratory

Eric D. Hsi, MD

• Paraffin section immunohistochemistry (IHC) is widely used in diagnostic surgical pathology. Today, it is difficult to imagine the practice of surgical pathology without IHC. The availability of automated immunostainers with reagent rental contracts makes this technology commonplace. One potential danger is that many laboratories are now offering immunostains without significant prior knowledge or experience in IHC. As part of its mission, the Cell Markers Committee of the College of American Pathologists offers this manuscript as a basic guide to introducing new antibodies in the clinical IHC laboratory. Issues relating to regulatory developments, antibody selection, staining optimization, and test validation are addressed.

(*Arch Pathol Lab Med.* 2001;125:289–294)

Paraffin section immunohistochemistry (IHC) has now become indispensable in the practice of surgical pathology. We routinely rely on IHC to assist us in the diagnosis and classification of neoplasms. A recent 4-month survey of cases accessioned in the Department of Anatomic Pathology at the Cleveland Clinic showed that approximately 13% of cases made use of IHC. No longer limited to large university-based or reference laboratories, IHC is also widespread in community hospitals. The reasons for this widespread use include the proliferation of automated immunostainers from many vendors, which makes IHC possible without prior technical expertise (thus not requiring significant increases in personnel), and the large variety of primary antibodies that recognize fixation-resistant epitopes.

Despite the widespread use of IHC, there is a surprising lack of standardization among IHC laboratories. Occasional articles have appeared detailing potential problems and putting forth proposals for improving the quality of stains or standardizing IHC.^{1–3} A recent review showed that the governmental regulations pertaining to IHC primarily address general laboratory operations.⁴ Such regulations require compliance with guidelines regarding standard laboratory manual formats; reagent labeling and storage practices; equipment maintenance; adequate record keeping; quality control programs (use of appropriate positive

and negative controls); and quality assurance programs (such as participation in proficiency testing programs approved by the Clinical Laboratory Improvement Amendments '88).⁵ There is little guidance or regulation concerning the desired diagnostic performance requirements of a particular stain. The National Committee for Clinical Laboratory Standards (NCCLS) consensus guidelines provide more details about both technical and theoretical aspects of IHC, and the reader is referred to this document for further information.⁶

A recent occurrence familiar to those who deal with IHC on a daily basis is the implementation of the Food and Drug Administration (FDA) ruling on the classification of immunohistochemical reagents and kits.⁷ This ruling has led to the classification of the great majority of immunohistochemical reagents as "Analyte Specific Reagents" (ASR).^{8,9} Under this ruling, most immunohistochemical staining reagents were reclassified as class I medical devices, exempting them from premarket notification. This classification is allowed because IHC staining results are incorporated into the diagnostic report by a pathologist as one part of the entire diagnostic evaluation. The IHC results are not stand-alone results. A few IHC stains, such as estrogen and progesterone receptor stains, are considered class II devices. These stains have no routine morphologic correlates but do have substantial and accepted scientific validation. Class III devices would include stains that are not considered part of the surgical pathology diagnostic process and would result in a stand-alone report to a physician. Such tests require premarket notification and specific FDA approval.

The FDA ruling essentially allows IHC laboratories to continue operating as they had been prior to the ruling. Manufacturers must label the reagents they sell for diagnostic use (but for which they have not sought FDA clearance) as ASRs. Manufacturers have the responsibility of following good manufacturing practices to ensure that antibodies are of consistent high quality and have the specificities that are claimed. However, a disclaimer is required in all surgical pathology reports in which IHC is used that states that the individual laboratory (not the manufacturer of reagents) has the ultimate responsibility for assuring the quality of staining.¹⁰ Thus, the laboratory director has the duty to provide a high-quality immunostain and to document its performance.

With this as background, it is the intention of this article to guide the reader in what we feel is an appropriate and practical approach for evaluating new antibodies in the modern automated IHC laboratory, with brief theoretical

Accepted for publication September 8, 2000.

From the Departments of Anatomic and Clinical Pathology, Cleveland Clinic Foundation (for the Cell Markers Resource Committee of the College of American Pathologists), Cleveland, Ohio.

Reprints: Eric D. Hsi, MD, Head, Section of Hematopathology, Department of Clinical Pathology, L-11 Cleveland Clinic Foundation, 9500 Euclid Ave, Cleveland, OH 44195 (e-mail: hsie@ccf.org).

discussions related to these recommendations. It is beyond the scope of this article to review the many different staining systems available today, although some may be specifically mentioned by way of example.

SELECTION OF PRIMARY ANTIBODIES FOR A NEW TEST

The first question to be answered is whether one wants to offer a new antibody in the laboratory. The reasons for offering a new antibody include fiscal reasons (to eliminate sending the test out to a reference laboratory or to make the most use of on-site equipment that otherwise would be underused) and standard of practice issues (demands from professional staff). To assess the former reason, one needs to have a prediction of test volume, the anticipated cost per test, and the projected reimbursement rates. The latter reason would include factors such as a demand from clinicians that a certain stain be available in-house and a demand from one's own pathology staff that certain antibodies with great diagnostic significance be available on-site, such as antibodies against cytokeratin, S100 protein, or leukocyte common antigen.

New antibodies introduced in clinical laboratories are generally commercially available antibodies with well-characterized specificities. For ASRs, a category that includes most IHC reagents, one may presume that good manufacturing practices have been followed and that there is a high likelihood that the antibody will perform as advertised. Unless a laboratory has a developmental focus in which new antibodies are evaluated in clinical practice, prior peer-reviewed literature likely exists that gives a realistic review of the performance of the antibody in a particular diagnostic setting. This literature should give the user a good basis from which to start, since antibody dilutions, antigen retrieval methods, and diagnostic performance (sensitivity and specificity) are then available. In the absence of this literature, one may often call the manufacturer's technical service representatives for this information, although details on diagnostic utility will not be available. Note that a primary antibody labeled as an ASR will not have the package insert many users have been accustomed to, which lists the manufacturer's claims of specificity, uses, and recommendations.

Many vendors now offer their ASR antibodies as pre-diluted reagents "optimized" for immunohistochemical staining. This is becoming a common practice that may limit the flexibility in performing a stain. The concern is that the antibody may be diluted such that it works when optimally fixed tissues are available for use in the manufacturer's own immunostaining system. However, the dilution may be too dilute for cases in which suboptimally processed tissue is evaluated or too dilute for use in a competitor's staining system. When possible, undiluted primary antibody is preferable.

One should note whether the antibody is polyclonal or monoclonal and also the species and isotype of the primary antibody. Polyclonal antibodies may contain good high-affinity clones, allowing for great dilution (often over 1:1000) but may also have problems with increased background, requiring extra blocking steps. Knowing the species and isotype will help avoid problems that occur when an inappropriate, suboptimal secondary antibody is used. An example in which the isotype might make a difference is the primary antibody of the LeuM1 clone (anti-CD15), an immunoglobulin M (IgM) monoclonal antibody that

has been shown to have superior performance when an anti-IgM is used as the secondary antibody.¹¹

Oftentimes, several different antibodies against a particular antigen are available from different sources. Selecting a particular antibody clone should be an informed choice, since antibodies to the same antigen may have very different characteristics. One may find assessments in the literature to assist in this decision. For example, some antibodies to CD5 that are immunoreactive in paraffin sections are not as useful in the diagnosis of B-cell lymphomas as others may be.^{12,13} Antibodies to the pan-B-cell antigen CD79a have been shown to have variable cross-reactivity with acute promyelocytic leukemias that appears to be clone dependent.^{14,15} Comparison of multiple p53 antibodies in fixed tissues has demonstrated differences in reactivity that affect the prognostic significance of immunoreactivity in breast cancer¹⁶; the pAb1801 and DO7 antibodies have been found to be more effective than other antibodies tested.

A more recent example of the importance of antibody selection is the reliability of immunostaining of HER-2/neu in tissue from breast cancer patients. Press and colleagues¹⁷ demonstrated variation in the performance of HER-2/neu antibodies; some antibodies showed surprisingly poor results in breast cancers with known HER-2/neu amplification status. A rabbit polyclonal HER-2 antibody is currently used in FDA-approved testing for expression of this protein in breast cancer. However, its reliability has been called into question because of possible false-positive reactions.^{18,19} A comprehensive review of the reactivities of different antibody clones with various antigens is beyond the scope of this manuscript. Suffice it to say that it is critical for the user to be familiar with the recent literature regarding specific antibodies so as to avoid, or at least to be aware of, potential problems specific to individual clones.

CONTROL MATERIAL

Selection of positive and negative control tissue is critical to the evaluation and validation of new antibodies. The lack of standardized tissues makes it important for the immunohistochemist to select tissues that reflect the intended diagnostic use of the antibody, as well as the processing conditions prevailing in the laboratory (including the fixative used and the duration of fixation). This requirement is made abundantly clear to the author on a daily basis. Unlike many laboratories, the Cleveland Clinic often uses Hollande's fixative for many tissues due to its good performance in detailing nuclear morphology. However, this practice can cause problems when IHC is attempted using staining protocols that have been optimized for formalin-fixed tissues. Thus, we must procure positive and negative control tissues fixed in Hollande's fixative, as well as tissues fixed in neutral buffered formalin. Positive control material may be easy to find if the antibody specificity is present in normal tissues; alternately, it may be difficult to find when the antigen is expressed only in certain tumors. An example of the latter is the anaplastic lymphoma kinase (ALK) protein recognized by the antibody ALK1. It is present only in a fraction of anaplastic large cell lymphomas.²⁰ Thus, if one wants to obtain control tissue that reflects the diagnostic use (diagnosis of anaplastic large cell lymphoma), one must obtain control material evaluated first in other ac-

credited laboratories or using cell lines known to overexpress this protein.

OPTIMIZING IMMUNOREACTIVITY

Numerous factors can be manipulated to optimize staining. Among the most commonly altered factors are antibody dilution, duration of primary antibody incubation, choice and concentration of secondary reagent, antigen retrieval technique, incubation temperature, choice of detection system, and the addition of amplification steps. *Checkerboard* approaches to optimizing antibody staining have been advocated to test new antibodies; in a checkerboard approach, each variable is systematically altered while all other variables remain constant.^{6,21} However, the great number of variables just listed would require the processing of an unrealistic number of slides. Because most automated stainers have limited temperature control and have optimized secondary reagents and detection chemistries as part of the automation process, one is left primarily with the variables of antibody dilution, primary incubation time, and choice of antigen retrieval technique. This dramatically reduces the number of test slides that need to be run to evaluate an antibody. As a practical note, it is reasonable to expect that an immunostain robust enough for diagnostic use should demonstrate adequate staining when these 3 variables are systematically optimized. Generally, the lower the dilution, the better the staining for a given incubation time. However, highly concentrated antibodies may result in a prozone effect, leading to decreased intensity of staining. In addition, non-specific staining can become a problem at higher antibody concentrations. Longer incubation times may permit greater dilutions of primary antibodies. However, because some automated stainers operate in a batch mode, it may not be possible to vary incubation times. If prolonged incubations are required, these incubations are best done "off-line." Optimizing the antibody dilution allows one to use the primary antibody most efficiently.

Perhaps the most significant advance in IHC in the last 10 years has been the discovery of heat-induced epitope retrieval (HIER).^{22,23} We will focus most of our attention on this development and its impact on IHC. Heat-induced epitope retrieval allows antibodies that might previously have reacted only in frozen tissues to react in fixed tissues. It has greatly expanded the number of antibodies that can now be applied to paraffin-embedded tissue.^{24,25} Increased sensitivity also results in cost savings for the laboratory, since HIER enables more tests per vial of antibody.

Although the exact mechanisms by which HIER unmasks antigens is not known, the leading hypothesis is that HIER probably works by chelating calcium.^{26,27} In addition to cross-linking proteins by methylene bridge formation, formalin fixation leads to the formation of calcium coordinate compounds with neighboring hydroxymethyl, carboxyl, and phosphoryl moieties. These reactions mask epitopes by steric hindrance. Calcium chelation destroys these complexes, leading to antigen unmasking. Both citrate and EDTA have calcium-chelating properties that vary according to pH. EDTA chelates at an alkaline pH, whereas citrate chelates at both alkaline and acidic pHs.²⁷ Antigen retrieval at very low pH probably occurs due to hydrogen ion dissociation of the calcium complexes.²⁶ Optimal HIER also depends on the maximum temperature achieved and the duration for which this temperature is maintained, rather than on the exact method by which the

slides are heated. For example, MIB-1 staining was shown to be equivalent when antigen retrieval was performed at 100°C for 20 minutes, 90°C for 20 minutes, 80°C for 50 minutes, and 70°C for 10 hours.²⁸

Most antibodies show increased staining when HIER is used; however, each antibody's performance may be slightly different, with some performing better in acidic citrate-based buffers and many performing better in alkaline EDTA-based buffers. A recent study by Pileri et al²⁹ systematically evaluated a large series of antibodies under multiple antigen retrieval conditions, including HIER with several buffers, protease digestion, and no antigen retrieval. These authors demonstrated a marked increase in sensitivity without loss of specificity for the great majority of antibodies using HIER; furthermore, EDTA was often superior to citrate for antigen retrieval. As illustrated in this study, however, HIER is not superior for all antibodies and can occasionally result in loss of immunoreactivity. Some antibodies, such as Epstein-Barr virus LMP-1 or CD21, perform best when enzyme digestion is used as the method of antigen retrieval. There appears to be no way to predict the behavior of a particular antibody, and results are empiric. Several studies have been published looking at a fairly broad range of antibodies, and these papers can serve as guides for the immunohistochemist.^{24,29-31}

Some authors have recommended developing extensive test batteries for optimizing HIER.²¹ However, practicalities (both financial and operational) of a busy clinical laboratory supervene and mandate a limited approach that will likely be successful in the majority of cases. Although the heat required for HIER can be generated by several appliances, including steamers, microwave ovens, pressure cookers, and autoclaves, we recommend antigen retrieval using microwave pressure cookers. These cookers enable rapid attainment of higher temperatures and uniform heating, and the self-contained cookers do not require refilling of solution during heating.^{25,32-34} Two different solutions, a pH 6.0 10 mmol/L citrate solution and a 1 mmol/L EDTA pH 8.0 solution, can be tested, along with protease digestion. For HIER, a standard heating at 100°C for 15 to 20 minutes (shorter if higher temperatures are achieved) is generally recommended, with a 20-minute cool-down period. These 3 choices (2 HIER treatments and a protease protocol) span the greatest variation in antigen retrieval while keeping the number of antigen retrieval protocols to a minimum. Applying these conditions at 5 different primary antibody dilutions (the manufacturer's recommended dilution plus 2 serial twofold dilutions above and below this concentration) using positive control materials should allow one to rapidly determine the optimal working conditions for the new antibody. In general, secondary antibodies and detection steps are kept constant. If supersensitive detection methods are used, one may use larger dilution intervals (perhaps serial fivefold or 10-fold dilutions). The slides should be interpreted carefully to ensure that the proper staining pattern (eg, membrane, cytoplasmic, or nuclear) is demonstrated in the appropriate cells with little or no background staining (Table). This latter point cannot be overemphasized. In this author's experience with consultation cases, occasional laboratories produce poor-quality staining, leading to misinterpretation of cytoplasmic staining (probably background) for other expected patterns, such as membrane or nuclear reactivity. Immunostaining protocols that produce

Sample Staining Protocol Worksheet for Evaluating a New Antibody*

Antibody/Specificity: X Vendor: Company Catalog #: 327 Lot #: 1
Date: 4/1/00 Positive Control Tissue: Tonsil

Dilution	Antigen Retrieval	Primary Incubation Time, min	Detection System	Detection Amplification	Formalin-Fixed Tissue	B5-Fixed Tissue	Hollandes-Fixed Tissue
1:10	10 mmol/L citrate, pH 6 20 min	32	ABC, DAB	No	4+	3+	2+
1:20	10 mmol/L citrate, pH 6 20 min	32	ABC, DAB	No	4+	3+	2+
1:40	10 mmol/L citrate, pH 6 20 min	32	ABC, DAB	No	4+	3+	1+
1:80	10 mmol/L citrate, pH 6 20 min	32	ABC, DAB	No	3+	2+	0
1:160	10 mmol/L citrate, pH 6 20 min	32	ABC, DAB	No	2+	2+	0
1:10	1 mmol/L EDTA pH 8, 20 min	32	ABC, DAB	No	4+	4+	3+
1:20	1 mmol/L EDTA pH 8, 20 min	32	ABC, DAB	No	4+	4+	3+
1:40	1 mmol/L EDTA pH 8, 20 min	32	ABC, DAB	No	4+	4+	3+
1:80	1 mmol/L EDTA pH 8, 20 min	32	ABC, DAB	No	4+	4+	2+
1:160	1 mmol/L EDTA pH 8, 20 min	32	ABC, DAB	No	3+	3+	±
1:10	Protease, 8 min	32	ABC, DAB	No	2+	2+	1+
1:20	Protease, 8 min	32	ABC, DAB	No	2+	1+	1+
1:40	Protease, 8 min	32	ABC, DAB	No	1+	0+	±
1:80	Protease, 8 min	32	ABC, DAB	No	0	0+	0
1:160	Protease, 8 min	32	ABC, DAB	No	0	3+	0

* Sample worksheet for the optimization of a monoclonal antibody with a recommended working dilution of 1:40 demonstrating optimal reactivity at 1:40 using EDTA-based heat-induced epitope retrieval. These conditions could then be used on a validation series of cases mimicking the intended diagnostic situation for which the antibody will be used. Scoring is based on the intensity of staining of the appropriate cellular elements with proper subcellular localization, as well as the proportion of cells expected to be immunoreactive, on a scale of 0-4+. 0 indicates negative; 1+, weakly positive in a small percentage of the cells expected to be positive; 2+, weakly positive in the majority of the cells expected to be positive; 3+, moderately positive in the majority of cells expected to be positive; and 4+, strongly positive in all cells expected to be positive. ABC indicates avidin-biotin complex; DAB, 3,3'-diaminobenzidine.

optimal staining with the correct cellular localization must be developed.

With the increased sensitivity for detecting antigens comes the increased ability to detect a false signal due to nonspecific staining. In particular, it has been shown that endogenous biotin activity can be retrieved by HIER, causing false-positive, granular cytoplasmic reactions.³⁵ This phenomenon was responsible for the erroneous report of inhibin staining in hepatocellular carcinomas that was subsequently shown to be due to endogenous biotin.^{36,37} This source of false-positive staining can be eliminated by adding a biotin-blocking step.^{35,38} Other potential causes of false-positive staining include endogenous peroxide in peroxidase-based systems and endogenous phosphatase in alkaline phosphatase-based systems. Both of these sources can be minimized by blocking with hydrogen peroxide and levamisole, respectively.^{39,40}

DETECTION SYSTEMS

Numerous methods are available for detecting bound primary antibodies. The most commonly used methods are indirect enzyme labeling using peroxidase or alkaline phosphatase. Examples include peroxidase-antiperoxidase (PAP), alkaline phosphatase-antialkaline phosphatase (APAAP), labeled streptavidin-biotin peroxidase (LSAB), and avidin-biotin complex (ABC) methods. Peroxidase methods seem to be favored for use in most commercial automated immunostainers in clinical laboratories. New amplification techniques are also becoming available, including dextran polymer conjugates and the tyramide system; these techniques can potentially increase sensitivity by factors of 100 or more.^{31,41,42} These newer, catalyzed, signal amplification techniques and catalyzed, reporter deposition techniques have the potential to offer extreme sensitivity, which most users are not accustomed to. A novel amplification system that makes use of mutually attractive antibodies based on species specificities has also

recently been described.⁴³ This system reportedly increases sensitivity up to 200-fold and avoids use of avidin-biotin detection. A drawback is the additional time required for the multiple antibody incubation steps. Whether these newer methods will be practical and affordable for routine use in the automated clinical laboratory remains to be seen.

Chromogen selection can also be important. With the alkaline phosphatase system, one may use 5-bromo-4-chloro-3-indonyl phosphatase/nitroblue tetrazolium (BCIP/NBT) to produce a blue-purple product or fast red/naphthol AS-TR phosphate to give a red product. Peroxidase systems generally use 3,3'-diaminobenzidine (DAB) (brown) or 3-amino-9-ethylcarbazole (AEC) (red) chromogens. The choice of chromogen may be influenced by personal preference (red vs blue vs brown), sensitivity issues (peroxidase/DAB tends to be more sensitive than phosphatase/fast red), safety issues (DAB is a carcinogen), or technical issues (AEC is soluble in organic solvents and cannot be used with xylene-based mounting medium). We have found DAB to be a reliable chromogen that produces crisp, well-localized reactions that are permanent. Because DAB is a carcinogen, technicians must handle this chromogen carefully to eliminate the possibility of skin or mucous membrane contact.

FORMAL VALIDATION

Once one has decided to bring a new antibody into the clinical laboratory and has worked out the optimal staining conditions, the antibody should be validated on a set of clinical cases designed to test the diagnostic utility (sensitivity and specificity) of the antibody. In many cases, the literature provides a guide as to this utility. Although NCCLS guidelines require that antibodies undergo a validation process in individual laboratories, the details of this validation are left to the laboratory director.⁶ Thus, one is left balancing the ideal situation of running a com-

prehensive validation study versus the reality of the complexity and expense of carrying out such a study for every new antibody.

Common sense dictates that when the antibody in question has a long and established history of use in diagnostic immunohistochemistry, such as the antibodies for S100 or CD30, one does not need to go to great lengths to validate the antibody. A small panel of 10 positive and 10 negative test cases may be sufficient, depending on the results. However, when one is dealing with a newly available antibody with little or no track record in the literature, the laboratory director may wish to undertake a more detailed study with at least 20 positive and 20 negative cases that mimic the diagnostic setting in which the antibody will be used. However, even this approach may be impractical, particularly when one is dealing with a rare entity. As mentioned earlier, an example of this would be the use of the ALK1 antibody on anaplastic large cell lymphoma tissue. A single institution may not be able to find 20 cases of anaplastic large cell lymphoma to test. In this case, it might be reasonable to collaborate with other institutions already offering the stain (sample exchanges) or to monitor the performance of the antibody prospectively after a more limited validation study is performed.

THE NEW USE OF IHC PROGNOSTIC AND THERAPEUTIC MARKERS

A new application of IHC is worth discussing briefly. In contrast to the previous use of IHC results, which entailed use in the context of clinical, histologic, and other immunophenotypic features to arrive at a diagnosis, IHC is now being used to determine whether specific therapies are warranted. Antibodies are being used to evaluate the expression of relevant proteins for which there may be no histologic correlate, perhaps qualifying these antibodies as class II or III medical devices. For example, monoclonal antibodies to CD20 and CD25 are now used for the therapy of lymphoid malignancies expressing these markers, and our laboratory is often being asked to confirm antigen expression before therapy is started.^{42,44-47} Anti-estrogen therapy for breast cancer is being routinely used in cases that have been shown to be positive for estrogen receptors by IHC. Immunohistochemistry has been shown to be a reliable and valid method for assessing estrogen receptor status and predicting clinical outcome.^{48,49} Issues regarding its accuracy and reproducibility are still discussed and center around interpretive criteria, as well as technical issues.⁴⁹⁻⁵³ Encouraging data are emerging from the UK National External Quality Assessment Scheme for Immunocytochemistry (UK NEQAS-ICC).⁵¹ Using fixed breast tumor tissue from multiple different laboratories, these authors found that, in a central laboratory, routine IHC procedures could be 90% to 100% efficient at demonstrating estrogen receptor expression. This assay was performed using the 1D5 clone with pressure cooker antigen retrieval. Most participating laboratories used either the 1D5 or 6F11 clones with HIER. Thus, variations in tissue processing in different laboratories did not seem to be a limiting factor in accurately determining the estrogen receptor status of breast cancers. A companion study showed that there was good concordance among different laboratories when tumors expressed high or moderate levels of estrogen receptors.⁵² However, the study also revealed that considerable interlaboratory variation still exists for the staining of tumors that expressed low levels of

estrogen receptors, leading to significant false-negative rates (30-60%).

Herceptin has become available for the treatment of HER-2/neu-positive breast cancer.⁵⁴⁻⁵⁶ Since there are no good histologic correlates for the presence or absence of this marker, we are relying completely on our technical abilities to detect it. This reliance puts even more pressure on the IHC laboratory to be sure that the results that are being generated are accurate. Inadequacies in the uniform quality of staining have been made abundantly clear in recent controversies regarding HER-2/neu IHC in breast cancer.^{17,19,57} Issues relating to the uniformity of staining procedures (including specimen processing, antigen retrieval, detection systems, and antibody selection) and interpretation must be resolved before one can confidently base therapeutic decisions solely on this single test result.⁵⁸ National proficiency testing programs, such as those conducted by the College of American Pathologists, may be helpful in this regard.

SUMMARY

In summary, this article briefly reviews the important developments in diagnostic IHC, with an emphasis on recommendations for testing new antibodies in the automated IHC laboratory. Because a modest degree of standardization is now possible, at least within an individual laboratory, systematic approaches to antibody evaluation must become routine. Ultimately, the performance of a particular immunostain is, as it should be, the responsibility of the medical director of the laboratory. This review, along with the cited references, should serve as a helpful guide for practicing pathologists dealing with immunohistochemical stains on a daily basis.

References

1. Herman GE, Elfont EA. The taming of immunohistochemistry: the new era of quality control. *Biotech Histochem*. 1991;66:194-199.
2. Taylor CR. Quality assurance and standardization in immunohistochemistry. A proposal for the annual meeting of the Biological Stain Commission, June, 1991. *Biotech Histochem*. 1992;67:110-117.
3. Wold LE, Corwin DJ, Rickert RR, Pettigrew N, Tubbs RR. Interlaboratory variability of immunohistochemical stains. Results of the cell markers survey of the College of American Pathologists. *Arch Pathol Lab Med*. 1989;113:680-683.
4. Fetsch PA, Abati A. Overview of the clinical immunohistochemistry laboratory: regulations and troubleshooting guidelines. *Methods Mol Biol*. 1999;115:405-414.
5. Health Care Financing Administration (1992) Clinical and Laboratory Improvement Amendments of 1988; Final Rule. 57 *Federal Register* 7001-7288 (1992).
6. National Committee for Clinical Laboratory Standards. Quality assurance for immunocytochemistry; approved guideline. 1999;19:18-68. NCCLS document MM4A.
7. Classification/reclassification of immunochemistry reagents and kits. 63 *Federal Register* 30132-30142 (1998) (21 CFR part 864. Doc. No. 94P-0341).
8. Taylor CR. FDA issues final rule for classification and reclassification of immunochemistry reagents and kits. *Am J Clin Pathol*. 1999;111:443-444.
9. Taylor CR. Report from the Biological Stain Commission: FDA issues final rule for classification/reclassification of immunochemistry (IHC) reagents and kits. *Biotech Histochem*. 1998;73:175-177.
10. Swanson PE. Labels, disclaimers, and rules (oh, my!). Analyte-specific reagents and practice of immunohistochemistry. *Am J Clin Pathol*. 1999;111:445-448.
11. LeBrun DP, Kamel OW, Dorfman RF, Warnke RA. Enhanced staining for Leu M1 (CD15) in Hodgkin's disease using a secondary antibody specific for immunoglobulin M. *Am J Clin Pathol*. 1992;97:135-138.
12. Ben Ezra JM, Kornstein MJ. Antibody NCL-CD5 fails to detect neoplastic CD5+ cells in paraffin sections. *Am J Clin Pathol*. 1996;106:370-373.
13. Diaz de Leon E, Alkan S, Huang JC, Hsi ED. Utility of an immunohistochemical panel (CD5, CD10, CD20, CD23, CD43, and cyclin D1) in paraffin-embedded tissues for the differentiation of B-cell non-Hodgkin's lymphomas of small lymphocytes. *Mod Pathol*. 1998;11:1046-1051.
14. Bhargava P, Kallakury BVS, Ross JS, Azumi N, Bagg A. CD79a is heterogeneously expressed in acute non-lymphoblastic leukemias (ANLL) and normal megakaryocytes and is antibody clone dependent. *Mod Pathol*. 2000;13:143a.

15. Arber DA, Jenkins KA. Paraffin section immunophenotyping of acute leukemias in bone marrow specimens. *Am J Clin Pathol.* 1996;106:462-468.
16. Horne GM, Anderson JJ, Tiniakos DG, et al. p53 protein as a prognostic indicator in breast carcinoma: a comparison of four antibodies for immunohistochemistry. *Br J Cancer.* 1996;73:29-35.
17. Press MF, Hung G, Godolphin W, Slamon DJ. Sensitivity of HER-2/neu antibodies in archival tissue samples: potential source of error in immunohistochemical studies of oncogene expression. *Cancer Res.* 1994;54:2771-2777.
18. Jacobs TW, Gown AM, Yaziji H, Barnes MJ, Schnitt SJ. Specificity of Herceptin in determining HER-2/neu status of breast cancers using the United States Food and Drug Administration-approved scoring system. *J Clin Oncol.* 1999;17:1983.
19. Roche PC, Ingle JN. Increased HER2 with U.S. Food and Drug Administration-approved antibody. *J Clin Oncol.* 1999;17:434.
20. Falini B, Pileri S, Zinzani PL, et al. ALK+ lymphoma: clinico-pathological findings and outcome. *Blood.* 1999;93:2697-2706.
21. Shi SR, Cote RJ, Yang C, et al. Development of an optimal protocol for antigen retrieval: a 'test battery' approach exemplified with reference to the staining of retinoblastoma protein (pRB) in formalin-fixed paraffin sections. *J Pathol.* 1996;179:347-352.
22. Shi SR, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry: past, present, and future. *J Histochem Cytochem.* 1997;45:327-343.
23. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J Histochem Cytochem.* 1991;39:741-748.
24. Cuevas EC, Bateman AC, Wilkins BS, et al. Microwave antigen retrieval in immunocytochemistry: a study of 80 antibodies. *J Clin Pathol.* 1994;47:448-452.
25. Chan JK. Advances in immunohistochemical techniques: toward making things simpler, cheaper, more sensitive, and more reproducible. *Adv Anat Pathol.* 1998;5:314-325.
26. Morgan JM, Navabi H, Jasani B. Role of calcium chelation in high-temperature antigen retrieval at different pH values. *J Pathol.* 1997;182:233-237.
27. Morgan JM, Navabi H, Schmid KW, Jasani B. Possible role of tissue-bound calcium ions in citrate-mediated high-temperature antigen retrieval. *J Pathol.* 1994;174:301-307.
28. Taylor CR, Chen C, Shi RS, Yang C, Cote RJ. A comparative study of antigen retrieval heating methods. *CAP Today.* 1995;9:16-22.
29. Pileri SA, Roncador G, Ceccarelli C, et al. Antigen retrieval techniques in immunohistochemistry: comparison of different methods. *J Pathol.* 1997;183:116-123.
30. Cattoretti G, Pileri S, Parravicini C, et al. Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. *J Pathol.* 1993;171:83-98.
31. von Wasielewski R, Werner M, Nolte M, Wilkens L, Georgii A. Effects of antigen retrieval by microwave heating in formalin-fixed tissue sections on a broad panel of antibodies. *Histochemistry.* 1994;102:165-172.
32. Cattoretti G, Suameijer A. Antigen unmasking of formalin-fixed paraffin-embedded tissues using microwaves: a review. *Adv Anat Pathol.* 1995;2:2-9.
33. Miller K, Ajjeraa KM. Antigen unmasking in formalin-fixed routinely processed paraffin wax-embedded sections by pressure cooking: a comparison with microwave oven heating and traditional methods. *Adv Anat Pathol.* 1995;2:60-64.
34. Taylor CR, Shi SR, Cote RJ. Antigen retrieval for immunohistochemistry: status and need for greater standardization. *Appl Immunohistochem.* 1996;4:144-146.
35. Bussolati G, Gugliotta P, Volante M, Pace M, Papotti M. Retrieved endogenous biotin: a novel marker and a potential pitfall in diagnostic immunohistochemistry. *Histopathology.* 1997;31:400-407.
36. McCluggage WC, Maxwell P, Patterson A, Sloan JM. Immunohistochemical staining of hepatocellular carcinoma with monoclonal antibody against inhibin. *Histopathology.* 1997;30:518-522.
37. Iezzoni JC, Mills SE, Pelkey TJ, Stoler MH. Inhibin is not an immunohistochemical marker for hepatocellular carcinoma. An example of the potential pitfall in diagnostic immunohistochemistry caused by endogenous biotin. *Am J Clin Pathol.* 1999;111:229-234.
38. Wood GS, Warnke R. Suppression of endogenous avidin-binding activity in tissues and its relevance to biotin-avidin detection systems. *J Histochem Cytochem.* 1981;29:1196-1204.
39. Li CY, Ziesmer SC, Lazzano-Villareal O. Use of azide and hydrogen peroxide as an inhibitor for endogenous peroxidase in the immunoperoxidase method. *J Histochem Cytochem.* 1987;35:1457-1460.
40. Ponder BA, Wilkinson MM. Inhibition of endogenous tissue alkaline phosphatase with the use of alkaline phosphatase conjugates in immunohistochemistry. *J Histochem Cytochem.* 1981;29:981-984.
41. Sabattini E, Bisgaard K, Ascani S, et al. The EnVision++ system: a new immunohistochemical method for diagnostics and research. Critical comparison with the APAAP, ChemMate, CSA, LABC, and SABC techniques. *J Clin Pathol.* 1998;51:506-511.
42. King C, Payne S, Walker F, Murray GI. A highly sensitive detection method for immunohistochemistry using biotinylated tyramine. *J Pathol.* 1997;183:237-241.
43. Mangham DC, Isaacson PG. A novel immunohistochemical detection system using mirror image complementary antibodies (MICA). *Histopathology.* 1999;35:129-133.
44. McLaughlin P, Hagemeyer FB, Grillo-Lopez AJ. Rituximab in indolent lymphoma: the single-agent pivotal trial. *Semin Oncol.* 1999;26:79-87.
45. Grillo-Lopez AJ, White CA, Varns C, et al. Overview of the clinical development of rituximab: first monoclonal antibody approved for the treatment of lymphoma. *Semin Oncol.* 1999;26:66-73.
46. Saleh MN, LeMaistre CF, Kuzel TM, et al. Antitumor activity of DAB389IL-2 fusion toxin in mycosis fungoides. *J Am Acad Dermatol.* 1998;39:63-73.
47. LeMaistre CF, Saleh MN, Kuzel TM, et al. Phase I trial of a ligand fusion-protein (DAB389IL-2) in lymphomas expressing the receptor for interleukin-2. *Blood.* 1998;91:399-405.
48. Barnes DM, Harris WH, Smith P, Millis RR, Rubens RD. Immunohistochemical determination of estrogen receptor: comparison of different methods of assessment of staining and correlation with clinical outcome of breast cancer patients. *Br J Cancer.* 1996;74:1445-1451.
49. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol.* 1999;17:1474-1481.
50. Nicholson RI, Leake R. Quality control for the immunohistochemical demonstration of estrogen and progesterone receptors. *J Clin Pathol.* 2000;53:247.
51. Rhodes A, Jasani B, Balaton AJ, Miller KD. Immunohistochemical demonstration of estrogen and progesterone receptors: correlation of standards achieved on in house tumors with that achieved on external quality assessment material in over 150 laboratories from 26 countries. *J Clin Pathol.* 2000;53:292-301.
52. Rhodes A, Jasani B, Barnes DM, Bobrow LG, Miller KD. Reliability of immunohistochemical demonstration of estrogen receptors in routine practice: interlaboratory variance in the sensitivity of detection and evaluation of scoring systems. *J Clin Pathol.* 2000;53:125-130.
53. Barnes DM, Millis RR, Beex LV, Thorpe SM, Leake RE. Increased use of immunohistochemistry for estrogen receptor measurement in mammary carcinoma: the need for quality assurance. *Eur J Cancer.* 1998;34:1677-1682.
54. Baselga J, Tripathy D, Mendelsohn J, et al. Phase II study of weekly intravenous trastuzumab (Herceptin) in patients with HER2/neu-overexpressing metastatic breast cancer. *Semin Oncol.* 1999;26:78-83.
55. Baselga J, Norton L, Albanell J, Kim YM, Mendelsohn J. Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. *Cancer Res.* 1998;58:2825-2831.
56. Cobleigh MA, Vogel CL, Tripathy D, et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol.* 1999;17:2639-2648.
57. Jacobs TW, Gown AM, Yaziji H, Barnes MJ, Schnitt SJ. HER-2/neu protein expression in breast cancer evaluated by immunohistochemistry. A study of interlaboratory agreement. *Am J Clin Pathol.* 2000;113:251-258.
58. Tubbs RR, Stoler MH. The quality of Her-2/Neu predictive immunohistochemistry: something FISHy? *Mod Pathol.* 2000;13:1-3.