

Back to the Drawing Board on Immunohistochemistry and Predictive Factors

Donald Earl Henson

The search for predictive biomarkers has been a recipient of funding largess. Potentially, biomarkers have the power to provide diagnostic, therapeutic, and prognostic information for personalized medicine. However, immunohistochemistry, a popular technique for evaluating biomarker expression, may contain procedural flaws that jeopardize its promise. In this issue of the *Journal*, McCabe et al. (1) used immunohistochemistry to show that the association between biomarker expression and outcome changes depending on the concentration of the biomarker antibody. They found that variations in antibody concentration could even reverse the relationship between biomarker expression and outcome. These observations have profound implications for the interpretation of immunohistochemical studies on biomarkers as predictors of outcome or response to therapy.

To demonstrate this effect, the authors addressed some of the inherent variables associated with the application of immunohistochemistry. Three biomarkers were investigated—HER2, p53, and the estrogen receptor (ER). They used an automatic quantitative method to assess biomarker expression that eliminated subjective interpretation. As a supplementary control, they also used cell lines known to contain a wide range of concentrations of the biomarker protein HER2. Also, the authors investigated the relation between multiple cutpoints and outcome, at several antibody concentrations, by use of tissue microarrays.

The results of this study arise from the fact that three variables are involved: cutpoints, biomarker protein concentration, and concentration of antibody. According to the authors, the explanation for the observation is that the concentration of the antibody in the assay may not be sufficient to span the range of expression of the biomarker protein. Part of the range of expression may also be obscured by either insensitivity or antibody saturation of the assay. However, the work of McCabe et al. reveals far more—that the dilution of antibody can reverse the relationship between biomarker expression and outcome. This reversal can occur only if the predictions of outcome made by the biomarker are nonlinear and follow a U-shaped curve. A biomarker with a U-shape curve is associated with an adverse outcome at both high and low concentrations in the tumor. Thus, p53 and HER2 are nonlinear prognostic biomarkers, but ER is linear. These results have implications for the design of prognostic systems that incorporate new biomarkers.

The authors conclude, perhaps correctly, that biomarker proteins that have a broad range of expression in tumors may require more than one antibody concentration to assess expression accurately. More importantly, they observed that the less sensitive conventional immunohistochemical methods did not reveal the relationship between antibody dilution and outcome. These methods are largely qualitative and usually designed to give an all-or-none response. The results reported by McCabe et al. may account for many of the discrepancies noted in publications on

the frequency of HER2 and p53 expression in various tumors (2). In some studies, p53 expression was associated with outcome, but in others, there was no relationship (3).

These results were not unexpected. Previous reports have described changes in the relation between protein expression, detection technique, and outcome. For instance, the duration of storage of slides used for immunohistochemistry can affect the patterns of outcome, because staining quality declines with storage (4). This change in staining quality makes clinical studies over time difficult. Studies on endometrial tumors, however, have shown that the proportion of p53-labeled cells and the staining intensity varies according to antibody concentration (2). Investigators have emphasized the importance of antibody concentration in the interpretation of staining characteristics (5,6).

Furthermore, other variations have been noted with immunohistochemical analyses. A disconnect between staining results and genetic changes has been observed. Abnormal protein expression is not always associated with gene expression. For example, abnormal expression of the p53 protein detected by immunohistochemistry has been observed in cancer cells in the absence of p53 mutations (7,8). Of four antibodies directed against the tumor suppressor protein PTEN, three produced predominant cytoplasmic staining and the fourth produced predominant nuclear staining, suggesting that antibodies directed at different epitopes provide conflicting information (9).

For more than 20 years, investigators have commented on the vagaries of immunohistochemistry and the need for standardization of the techniques used, as well as on the careful interpretation of stained slides (10,11). However, when one considers all the variables inherent in the procedure, standardization seems to be complex and difficult to implement (12–17). Debate continues about the lack of inter- and intralaboratory reproducibility, optimal methods for antigen retrieval, variations in antibody affinity, selection of antibodies, detection systems, diagnostic thresholds, proper controls, and source and quality of reagents. Consider the impact when these variables are added to others that relate to the storage of the specimen, duration of fixation, type of fixative, and conditions of tissue processing (18–20). For so many variables, especially ones that are difficult to control, standards that all laboratories can accept and sustain seem impossible to realize.

Despite a failure to achieve full concordance in nearly all inter- and intralaboratory quality-control trials and lack of formal laboratory standardization (11,21,22), immunohistochemistry is

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fully embedded in medical practice. Pathologists and other physicians have accepted the inter- and intralaboratory variation inherent in reporting the results of immunohistochemistry testing. After all, the variation is probably no greater than the diagnostic process in the clinic.

As a contributor to translational research, immunochemistry serves as a bridge between laboratory and bedside. In the practice of oncology, it can individualize therapy, for instance in breast cancer. For pathologists, it can confirm a diagnostic opinion that is based on morphology or provide an adjunctive test for corroborating or expanding tumor classification (23). The recognition of unusual or rare types of cancer is often substantiated by specific protein expression patterns revealed by immunohistochemistry. Immunohistochemistry can detect specific tissue or cell-type differentiation markers, which often reflect histogenesis, as well as functional proteins such as p53.

What are the consequences of this disconcerting report that the association between outcome and biomarker expression can vary by the concentration of the biomarker antibody? In all likelihood, published results that have associated protein expression detected by immunohistochemistry with outcome will be considered problematic. However, of the three biomarker antibodies tested, McCabe et al. found the concentration effect with antibodies directed at HER2 and p53 but not at the ER. Expression of ER was always associated with increasing outcome, regardless of antibody concentration. Nevertheless, in this regard, it should be noted that some women with ER-positive breast cancers, as assessed by immunohistochemistry, do not respond to tamoxifen. Generalizations therefore may be premature until additional studies investigating other antibodies, detection methods, and biomarkers are published.

McCabe et al. have demonstrated that the dilution of antibody is critical for evaluating biomarker expression. Although antibody dilution may be one variable, biomarker concentration in tissue is likely to be another. At the very least, the results reported by McCabe et al. warrant consideration, especially before the integration of predictive biomarkers evaluated through immunohistochemistry into formal prognostic systems or their use as indicators for specific therapy.

REFERENCES

- (1) McCabe A, Dolled-Filhart M, Camp RL, Rimm DL. Automated quantitative analysis (AQUA) of in situ protein expression, antibody concentration, and prognosis. *J Natl Cancer Inst* 2005;97:1808–15.
- (2) McCluggage WG, Connolly LE, McGregor G, Hyland PL, Hall PA. A strategy for defining biologically relevant levels of p53 protein expression in clinical samples with reference to endometrial neoplasia. *Int J Gynecol Pathol* 2005;24:307–12.
- (3) Elledge RM, Allred DC. Prognostic and predictive value of p53 and p21 in breast cancer. *Breast Cancer Res Treat* 1998;52:79–98.
- (4) Mirlacher M, Kasper M, Storz M, Knecht Y, Durmuller U, Simon R, et al. Influence of slide aging on results of translational research studies using immunochemistry. *Mod Pathol* 2004;17:1414–20.
- (5) McCormick D, Yu C, Hobbs C, Hall PA. The relevance of antibody concentration to the immunohistochemical quantification of cell proliferation-associated antigens. *Histopathology* 1993;22:543–7.
- (6) Camp RL, Dolled-Filhart M, King BL, Rimm DL. Quantitative analysis of breast tissue microarrays shows that both high and normal levels of HER2 expression are associated with poor outcome. *Cancer Res* 2003;63:1445–8.
- (7) Ambros RA, Sheehan CE, Kallakury BV, Ross JS, Malfetano J, Paunovich E, et al. MDM2 and p53 protein expression in the histologic subtypes of endometrial carcinoma. *Mod Pathol* 1996;9:1165–9.
- (8) Stewart RL, Royds JA, Burton JL, Heatley MK, Wells M. Direct sequencing of the p53 gene shows absence of mutations in endometrioid endometrial adenocarcinomas expressing p53 protein. *Histopathology* 33:1998:440–5.
- (9) Pallares J, Bussaglia E, Martinez-Guitarte JL, Dolcet X, Llobet D, Rue M, et al. Immunohistochemical analysis of PTEN in endometrial carcinoma: a tissue microarray study with a comparison of four commercial antibodies in correlation with molecular abnormalities. *Mod Pathol* 2005;18:719–27.
- (10) Lambkin HA, Mothersill CM, Kelehan P. Variations in immunohistochemical detection of p53 protein overexpression in cervical carcinomas with different antibodies and methods of detection. *J Pathol* 1994;172:13–8.
- (11) Rudiger T, Hofler H, Kreipe HH, Nizze H, Pfeifer U, Stein H, et al. Quality assurance in immunochemistry: results of an interlaboratory trial involving 172 pathologists. *Am J Surg Pathol* 2002;26:873–82.
- (12) Taylor CR. The total test approach to standardization of immunochemistry. *Arch Pathol Lab Med* 2000;124:945–51.
- (13) Taylor CR. Report from the Biological Stain Commission: FDA issues final rule for classification/reclassification of immunohistochemistry reagents and kits. *Biotech Histochem* 1998;73:175–7.
- (14) Taylor CR. An exaltation of experts: concerted efforts in the standardization of immunochemistry. *Hum Pathol* 1994;25:2–11.
- (15) DeLellis RA, Sternberger LA, Mann RB, Banks PM, Nakane PK. Immunoperoxidase techniques in diagnostic pathology. Report of a workshop sponsored by the National Cancer Institute. *Am J Clin Pathol* 1979;71:483–8.
- (16) O'Leary T. Standardization in immunochemistry. *Appl Immunohistochem* 2001;9:3–8.
- (17) Mengel M, Hebel K, Kreipe H, von Wasielewski R. Standardized on-slide control for quality assurance in the immunohistochemical assessment of therapeutic target molecules in breast cancer. *Breast J* 2005;11:34–40.
- (18) Jacobs TW, Prioleau JE, Stillman IE, Schnitt SJ. Loss of tumor marker-immunostaining intensity on stored paraffin slides of breast cancer. *J Natl Cancer Inst* 1996;88:1054–9.
- (19) Bertheau P, Cazals-Hatem D, Meignin V, de Roquancourt A, Verola O, Lesourd A, et al. Variability of immunohistochemical reactivity on stored paraffin slides. *J Clin Pathol* 1998;51:370–4.
- (20) Shi SR, Cote RJ, Taylor CR. Antigen retrieval techniques: current perspectives. *J Histochem Cytochem* 2001;49:931–8.
- (21) Rhodes A, Jasani B, Anderson E, Dodson AR, Balaton AJ. Evaluation of HER-2/neu immunohistochemical assay sensitivity and scoring on formalin-fixed and paraffin-processed cell lines and breast tumors: a comparative study involving results of laboratories in 21 countries. *Am J Clin Pathol* 2002;118:408–17.
- (22) 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.
- (23) Muro-Cacho CA. The role of immunochemistry in the differential diagnosis of soft-tissue tumors. <http://www.moffitt.usf.edu/pubs/ccj/v5n1/departments5.html>.