Anti-P-glycoprotein Antibody C219 Cross-reactivity With c-erbB2 Protein: Diagnostic and Clinical Implications

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It has been more than two decades since the discovery of P-glycoprotein, the prototype of the transmembrane multidrug resistance transporters. The expression of P-glycoprotein in normal and malignant tissues has been studied extensively. Because many drug-resistant or poor-prognosis cancers overexpress the MDR1 gene, which encodes P-glycoprotein, a number of clinical correlative studies have been conducted. Many, but not all, of these studies reported that overexpression of P-glycoprotein was associated with an adverse chemotherapy outcome, whereas low expression or undetectable P-glycoprotein was associated with good prognosis (1,2), in keeping with the proposed function of P-glycoprotein as a drug efflux “pump” that expels anticancer drugs from resistant cancer cells (3). The results of these clinical correlative studies in various cancer types have spurred ongoing trials that attempt to modulate P-glycoprotein function in vivo with different inhibitors (4). Thus far, because such trials have not conclusively “reversed” the adverse outcome of P-glycoprotein-expressing cancers, there has been a resurgence of interest in examining whether the disparate results of different clinical correlative studies of various cancer types can be attributed to differences in the diagnostic tools, experimental reagents, and methodology used or in the criteria applied for the interpretation of results. These deliberations led to a North American and European consensus conference involving 15 institutions—the 1994 “St. Jude Methods to Detect P-glycoprotein-Associated MDR [i.e., multidrug resistance] Workshop”—at which recommendations were forged and published (5) to guide future clinical research on multidrug resistance.

Relevant to the increased awareness of potential problems in detecting P-glycoprotein, Liu et al. (6) report in this issue of the Journal that the monoclonal antibody C219 cross-reacts with the c-erbB2 protein (also known as p185c-erbB2) because it contains a peptide sequence that is homologous to the C219 epitope of P-glycoprotein. The implication of this finding is that immunologic detection of P-glycoprotein with the C219 antibody may be confounded by the overexpression of c-erbB2 protein in clinical and research tumor samples. Briefly, these investigators found, by means of immunoblot and immunohistochemical analysis, that the C219 antibody (Signet Laboratories, Inc., Dedham, MA) cross-reacts with p185c-erbB2, whereas the monoclonal anti-p185c-erbB2 antibody, c-neu-Ab3 (OncoGene Science, Inc., Uniondale, NY), does not recognize P-glycoprotein. Comparison of peptide sequences revealed that the C219 antibody recognizes an epitope in P-glycoprotein that shares sequence homology with p185c-erbB2. Enzyme-linked immunosorbent assays also demonstrated that the C219 antibody recognizes synthetic peptides derived from the C219 epitope in P-glycoprotein as well as from the C219 epitope-homologous region in p185c-erbB2. Their findings are consistent with the conclusion of Childs et al. (7), who undertook a systematic study of the epitope sequence of C219 by synthesizing a complete set of peptides that vary in a single amino acid from the native binding sequence. Childs et al. observed that the first (valine), second (glutamine), and final (aspartate) amino acids of the epitope are the least tolerant of sequence change. The sequence of p185c-erbB2 would be predicted to be recognized by C219 but with a lower efficiency than observed for P-glycoprotein. Nevertheless, because several investigators [Ro et al. (8), Verelle et al. (9), and Schneider et al. (10)] have reported on the immunodetection of P-glycoprotein in breast carcinoma, a tumor that can also express the p185c-erbB2 protein, Liu et al. have urged that caution be exercised in interpreting such results. They also have recommended that more than one antibody be used to assess P-glycoprotein expression, especially in p185c-erbB2-expressing tumors such as breast carcinoma.

The report of Liu et al. (6) raises several interesting questions: 1) Is c-erbB-2 gene amplification and/or overexpression clinically relevant to the outcome of breast cancer? 2) What is the appropriate methodology for the detection of P-glycoprotein...
in clinical samples of cancers? 3) How should antibody cross-reactivity be investigated?

In 1981, Shih et al. (11) identified a novel transforming gene, "neu," which is partially homologous to the epidermal growth factor receptor gene, in a chemically induced neuroblastoma. The neu gene encodes a 185-kd transmembrane oncoprotein, p185 (12,13). The human equivalent of neu was cloned from a complementary DNA library (and called "HER-2") (14), from genomic DNA (and called "c-erbB2") (15), and from a breast cancer cell line (and called an "erbB-related gene") (16). This p185 protein has tyrosine kinase activity (14,15,17). Although the precise function of p185 is unknown, structural and functional similarities to the epidermal growth factor receptor and animal experiments showing anti-p185 antibody inhibition of the growth of neu-activated xenografts (18) suggest that p185 is a growth factor receptor, for which a ligand has been identified (19,20).

Amplification of the erbB-2 gene has been found in breast, ovary, stomach, salivary gland, and non-small-cell lung carcinomas (21–25). Amplification and/or overexpression of the c-erbB2 gene appears to be associated with poor prognosis, particularly in breast carcinoma, in keeping with the results of in vitro studies that suggest that p185erbB2 may play a role in the malignant progression of breast carcinoma (21,26). The 20%–30% incidence of c-erbB2 gene amplification in breast carcinoma has been shown to be associated with oncoprotein overexpression. While c-erbB2 overexpression is not observed in normal, hypoplastic, or dysplastic breast tissues, it is present in over one half of the early stage ductal carcinoma in situ (DCIS) lesions, in approximately one quarter of the late stage infiltrating ductal carcinoma (IDC) lesions with a DCIS component, and in only approximately 10% of the IDC lesions without DCIS (27,28). Overexpression of c-erbB2 is particularly common in highly necrotic comedo-type ductal carcinomas (27). The reason for this pattern of overexpression of c-erbB2 is unknown. Slamon et al. (26) first showed the prognostic significance of activation of the c-erbB2 gene in breast carcinoma. Overexpression of the c-erbB2 gene was associated with poor prognosis in patients with lymph node-positive breast cancer (21,26,29). Since these initial reports, many, but not all, investigators have retrospectively reported a similar association in patients with breast carcinoma (28). Subsequently, an association with poor clinical outcome was identified for c-erbB2 gene activation in patients with lymph node-negative breast cancer (28). As yet, a large prospective series is not available in which the prognostic significance of c-erbB2 amplification and/or overexpression is examined in patients stratified for important clinical prognostic factors, such as lymph node status, estrogen receptor status, progesterone receptor status, tumor size, and lymphatic and vascular invasion, or for other reported variables, such as age, menstrual status, histologic grade, nuclear grade, DNA ploidy, and S-phase fraction (30). Therefore, although overexpression of c-erbB2 appears to have clinical significance, the verdict is not yet in for how it "stacks up" against the other conventional prognostic variables. The ability to identify accurately "good-risk" versus "poor-risk" subsets of patients with breast carcinoma may have therapeutic implications, since intensive chemotherapy appears to be particularly beneficial for those with "poor-risk" disease (31). In addition, determination of c-erbB2 overexpression may help pinpoint which subset of "poor-risk" patients should be subjected to lymph node dissection, a procedure that has considerable long-term morbidity. Furthermore, tumor-inhibitory monoclonal anti-c-erbB2 antibody therapy or c-erbB2-targeting gene therapy may be applicable in the future for patients with overexpression of c-erbB2 (32).

The detection and interpretation of P-glycoprotein in clinical samples have been problematic for "service" laboratories. Testing has remained in the realm of the research laboratory because the MDR1 gene is rarely amplified in clinical samples, and messenger RNA (mRNA) expression does not necessarily correspond to protein expression. Initial P-glycoprotein levels may be low at diagnosis, and the number of P-glycoprotein-positive tumor cells may be few. Therefore, clinical studies of the expression of P-glycoprotein and the reversal of multidrug resistance in different cancer types have not yielded consistent results. Many of the discrepancies may be caused by the difficulty in detecting P-glycoprotein or the presence of other biochemical and pharmacologic factors that contribute to drug resistance. The Memphis MDR workshop (5) recognized that, although most laboratories can reliably detect high levels of expression of P-glycoprotein and MDR1 mRNA, lower levels of expression have proved to be more difficult to quantify. Furthermore, the detection of P-glycoprotein and MDR1 mRNA is more reliable in leukemia and lymphoma than in solid tumors, and accurate measurement of low levels of P-glycoprotein expression under most conditions remains an elusive goal. Therefore, certain consensus recommendations were established as guidelines for clinical research on multidrug resistance. Briefly, the workshop participants recommended the following: the use of tissue-specific controls, antibody controls, and standardized negative and positive control cell lines for calibrating detection methods; the use of two or more standardized anti-P-glycoprotein antibodies that recognize different epitopes to improve the reliability of the immunologic detection of P-glycoprotein; the use of carefully controlled methodology for sample fixation and antigen preservation; the application of multiparameter analysis for clinical assays of P-glycoprotein and MDR1 mRNA expression; the reporting of staining intensity and the percentage of positive cells for publishing immunostaining data; and avoiding the use of arbitrary minimal cutoff points for analysis that might compromise the reliability of conclusions.

It has been recognized previously that several anti-P-glycoprotein antibodies show cross-reactivity. For instance, the C219 antibody cross-reacts in immunobLOTS with an approximately 200-kd protein that migrates electrophoretically in the same position as myosin, and it immunostains cardiac and skeletal muscle fibers (33). The C219 antibody also cross-reacts with a 190-kd protein in bovine and rat brain capillaries (34). Another anti-P-glycoprotein antibody, C494 (35), has been shown to cross-react with pyruvate carboxylase, a mitochondrial enzyme in rat, bovine, and human tissues, as does yet another anti-P-glycoprotein antibody, JSB-1 (36). Rao et al. (35) have shown that the approximately 130-kd nonmembranous pyruvate carboxylase contains the epitope-specific sequence recognized by the C494 antibody. For these reasons, Rao et al. (35) recommended that the immunologic detection of P-glycoprotein should employ at least two antibodies that are directed against different epitopes in P-glycoprotein. C219 and C494 are fully
characterized anti-P-glycoprotein antibodies that have been mapped to single-amino-acid resolution (37). They are directed against separate internal (i.e., the part of the protein that is inside the cell) linear epitopes in P-glycoprotein. While the C494 antibody is known to be specific for the product of the human MDR1 gene, which confers multidrug resistance, the C219 antibody recognizes products of both the human MDR1 gene and the MDR3 gene, which is not related to multidrug resistance (37). C219 also recognizes products of the hamster pgp1, pgp2, and pgp3 genes and products of the mouse mdr1, mdr2, and mdr3 genes (38). Other anti-P-glycoprotein antibodies are less well characterized, i.e., the internally binding JSB-1 antibody (39) and the externally binding MRK-16 (40, 41), UIC2, HYB-241, and 4E3 antibodies (42–44). Of these antibodies, only the discontinuous epitope of the MRK-16 antibody has been mapped (41). Because C219 and C494 are internally binding, their epitope-binding ability is less sensitive to conformational changes, glycosylation status, and environmental effects (e.g., high salt in the media) than observed for externally binding antibodies. Therefore, C219 and C494 are well suited for immunoblotting and immunostaining of clinical samples, in which the P-glycoprotein may be degraded, and when other spurious variables may be present.

Finally, the series of experimental studies carried out by Liu et al. (6) to characterize the cross-reactivity of the C219 antibody to the c-erbB2 protein, involving a detailed examination of the epitope, provides a paradigm for the investigation of the cross-reactivity of antibodies applied to the immunologic detection of clinically important multidrug resistance proteins or other proteins and tumor markers utilized in the clinical setting.

References

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Can p53 Status Resolve Paradoxes Between Human and Non-Human Retinoblastoma Models?

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The cloning of the retinoblastoma gene (RB) stands as a landmark in modern cancer research as it was the first human cancer susceptibility gene isolated (1), it immediately validated the two-hit hypothesis for tumorigenesis (2), and it has served the scientific community for the past 11 years as a paradigm for the concept of a tumor suppressor gene. In addition, subsequent work on the functional properties of the RB protein has provided clues that begin to link the biology of DNA tumor viruses, the eukaryotic cell cycle, the regulation of RNA polymerases, and pathways of mammalian carcinogenesis (3). Despite these remarkable advances, however, several unanswered questions remain. In particular, a fundamental paradox comes to mind when considering the study of Schlamp et al. (4), which is reported in this issue of the Journal. Why are humans the only known animal species that spontaneously develop retinoblastoma tumors? A dramatic confirmation of this surprising observation is the demonstration that RB(+/−) gene knockout mice (the counterpart to individuals with human familial retinoblastoma) never develop retinal tumors, although these heterozygous mice eventually succumb to pituitary tumors with a high penetrance (5,6). While a satisfactory (i.e., reductionist) model that can incorporate these findings is still lacking, experimental data over the past two decades have suggested several interesting leads. The first clue was provided in 1973 by the generation of retinoblastoma-like tumors in rats following a single intraocular injection of human adenovirus (7). Although this outcome was subsequently replicated in baboons (8), the implications of these findings had to wait until the late 1980s when the binding of adenovirus proteins to cellular RB protein and p53 protein was shown to be essential for the viral transformation of animal cells (9,10). A similar result was obtained in 1990 by investigators studying the effect of the ectopic expression in mice of a simian virus 40 large T antigen (Tag) transgene. Unexpectedly, the transgene was expressed in retinal tissues, giving rise to the development of multifocal retinal tumors (11). A subsequent Tag transgene driven by a photoreceptor-specific promoter confirmed these findings, with the development of mouse retinal tumors that were indistinguishable from human retinoblastoma tumors (12). Since Tag binds (and presumably inactivates) both the RB and p53 proteins, a more elegant study examined the effect of targeting RB protein alone using the RB-specific viral oncoprotein E7 (13). In these mouse models, the targeted inactivation of RB protein alone in retinal cells resulted in retinal cell apoptosis, while the inactivation of RB using the same viral E7 transgene in a p53(−/−) mouse resulted in multifocal retinal tumors (14,15). In summary, these findings consistently show that mouse retinal cells require the simultaneous loss of both RB and p53 activity for malignant clonal expansion. Thievery, however, lies at least one dilemma when considering these animal studies as models for human retinoblastoma. Inactivation of the