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*Invited Review*

# Immunohistochemical markers of prognostic value in surgical pathology

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**Summary.** Immunohistochemical methods have enjoyed rapid growth in application and utility since their initial adaptation to paraffin sections 20 years ago. Initial applications were directed primarily to the identification of cell and tumor sub-types (lineage related markers). More recently immunohistochemical markers have been described that show great promise in determining tumor prognosis, at a very early stage of tumor development, independent of stage and grade. This review surveys the recent use of immunohistochemistry in prognosis, and offers some speculation as to areas of future promise.

**Key words:** Immunohistochemistry, Prognostic markers, Micrometastases, Proliferation markers, Oncogenes, Tumor suppressor genes

## Introduction

Increasingly, the goal of the surgical pathologist extends beyond a simple diagnosis to provision of information that defines the clinical outcome, based on specific clinical and pathologic parameters. Staging criteria, for instance, are used to evaluate virtually every type of solid tumor and are important not only in predicting prognosis, but also in selecting therapy. However, tumor stage and grade provide only general estimates of outcome for a particular patient, and current clinical and pathologic staging parameters do not identify precisely those individuals destined to relapse. Importantly stage and grade provide information on what a tumor has done, not on what it will do. Immunohistochemical markers provide the potential to predict future behavior independent of orthodox staging criteria.

These considerations are motivating efforts to identify (enzymes, oncogenes, tumor-suppressor genes or gene products), whose presence (or absence) may predict the future biologic behavior of a particular tumor. Such studies represent a fundamental shift in the means by which tumor behavior is defined, a change from outcome-based analysis to one founded upon tumor biology.

In this context, the immunohistochemical analysis of tumors is undergoing a profound shift in emphasis, from attempts to define tumor histogenesis to methods designed to reveal the biologic potential of tumors, thus, providing a more scientific basis for tumor management. (Table 1).

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## Microinvasion and pseudoinfiltration: staining for basement membrane and basal cells

Microinvasion of a carcinoma is generally defined as focal infiltration of malignant cells through the underlying basement membrane. In routine H&E sections it may be difficult to localize and define the basement membrane. In this respect, antibodies to basement membrane components assist in the evaluation of basement membrane integrity; the two most commonly used target either collagen type IV or laminin (Birembaut et al., 1985). With an intact basement membrane, invasion is by definition, not present. In many normal tissues, however, the basement membrane may appear to be discontinuous, and may be better evaluated when studied by immunohistochemical procedures. Microinvasion should only be diagnosed when the basement membrane is grossly disrupted. Benign 'pseudoinfiltrative' lesions that can be extremely difficult to distinguish from infiltrating carcinoma are particularly common in the breast and prostate. A basement membrane that circumscribes all suspicious cell groups suggests a benign process or at worst, in situ malignancy.

In addition many types of normal glandular epithelial cells are circumscribed by a basal cell layer. In the case of breast tissue, the basal cell layer is composed of myoepithelial cells, that are readily identified with antibodies to muscle-specific actin (Hedrick and Epstein, 1989). The presence of myoepithelial cells around "infiltrating" tubular elements suggests a benign process (such as sclerosing adenosis or radical scar). However, their absence is not diagnostic of malignancy, as breast myoepithelial cells may often be discontinuous in

distribution and thereby not captured in the solitary plane of every section.

In the prostate, basal cells can be distinguished from the luminal cells by virtue of expression of a different subset of cytokeratin intermediate filaments. Several antibodies have been described that react with the unique, higher molecular weight cytokeratins expressed by some basal cells, contrasting with the lower molecular weight cytokeratins expressed by luminal cells (Hedrick and Epstein, 1989; Grizzle et al., 1996). In addition basal cells of the prostate displays some 'stem cell-like' features such as expression of bcl-2 (Grizzle et al., 1996). These immunoreactivity patterns may be used to distinguish benign from malignant prostatic epithelial lesions, because basal cells can be identified in nearly all benign glandular proliferations of the prostate, but are never present at the periphery of malignant glands.

**Table 1.** Immunohistochemical markers in cancer prognosis and therapy.

| |
|---|
| *Tumor staging* |
| Microinvasion |
| Detection of bone marrow micrometastases |
| Detection of lymph node micrometastases |
| *Tumor-associated antigens* |
| Tn |
| Carcinoembryonic antigen (CEA) |
| Glycosilation antigens |
| Blood-group antigens |
| *Tumor cell proliferation* |
| Ki-67 |
| Proliferating cell nuclear antigen (PCNA) |
| Topoisomerase II |
| *Oncogenes/growth factors and receptors* |
| Her-2/*neu* |
| *ras* |
| N, c-*myc* |
| c-*kit* |
| INT-2/HST-1 |
| EGFR (epidermal growth factor receptor) |
| TGF-α, β (transforming growth factor) |
| *Tumor-suppressor gene products* |
| RB (retinoblastoma gene) |
| p53 |
| DCC (gene deleted in colon cancer) |
| NF-1 (neurofibromatosis type 1) |
| Wilm's tumor gene |
| NM23 |
| *Metastatic potential* |
| Laminin receptor |
| Type IV collagenase |
| Cathepsin D |
| OA519 |
| *Predictors of response to therapy* |
| Estrogen receptor (ER) |
| Progesterone receptor (PR) |
| Androgen receptor (AR) |
| p52 |
| MDR (multidrug resistance) |

## Occult micrometastasis

Following pioneering studies at the Ludwig Institute and Royal Marsden Hospital in London, England, (Redding et al., 1983) several groups employed immunohistochemical methods to identify otherwise occult micrometastases of carcinoma within bone marrow and lymph nodes (Cote et al., 1988, 1995). While initial studies focused on breast carcinoma, many other tumors are now under investigation (Lindemann et al., 1992; Bretton et al., 1994; Cote et al., 1995). These studies take advantage of the ability of antibodies to distinguish cells of differing histogenesis; in this case, epithelial (carcinoma) cells from the normal constituents of the bone marrow and lymph nodes.

### *Bone marrow*

A variety of antibodies with specificity for epithelia have been employed to detect metastatic carcinoma cells

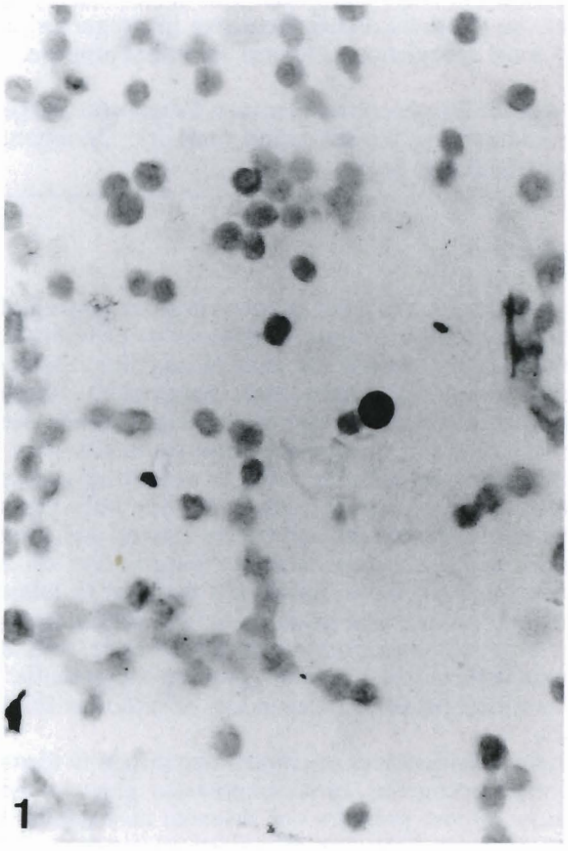


**Fig. 1.** Bone marrow from a patient with stage I breast carcinoma. This patient had no clinical or pathological evidence of metastatic spread of tumor. Using a cocktail of anti-cytokeratin monoclonal antibodies, rare positive cells were seen in the bone marrow, consistent with carcinoma. Note that the surrounding normal bone marrow elements show no evidence of immunoreactivity. x 200

in bone marrow (Redding et al., 1983; Cote et al., 1988), exploiting the fact that the normal bone marrow compartment contains no epithelial elements (Cote et al., 1988). By such means, occult 'marrow' micrometastases have been detected in patients with carcinomas of colon, prostate, ovary, and lung, as well as neuroblastoma and other tumors (Redding et al., 1983; Cote et al., 1988, 1995; Lindemann et al., 1992; Bretton et al., 1994).

With regard to breast carcinoma, immunohistochemical methods have demonstrated occult micrometastases in bone marrow in 25 to 30% of low stage (operable) patients (Fig. 1). Several studies have addressed the clinical significance of this finding, and the presence of occult micrometastases correlates with other known predictors of prognosis, including lymph node status and stage (Redding et al., 1983; Cote et al., 1988). In addition, the presence of bone marrow micrometastases has independent significance in identifying a population of patients at increased risk for disease progression (Cote et al., 1988). For example, one cohort of breast cancer patients followed for a median of 9.5 years, revealed overt progression in 85% of patients who had occult bone marrow micrometastases at the time of surgery; by contrast dissemination occurred in only one-third of those with no evidence of bone marrow micrometastases. A similar increased risk for disease progression has been observed in patients with colon and lung carcinoma (Lindemann et al., 1992; Bretton et al., 1994; Cote et al., 1995).

### *Occult lymph node metastases*

Regional lymph node metastases are the single most important prognostic factor for most tumors, an observation made more remarkable by the fact that routine histopathologic examination of lymph nodes understates the prevalence of such metastases. For example, Gusterson and Ott calculated that a pathologist has only a 1% chance of identifying a metastatic focus of breast cancer of a diameter of three cells in cross-section within a histologic section of a lymph node (Gusterson and Ott, 1990). Others have identified a false-negative rate of 8-30% (for the detection of metastatic carcinoma) within lymph nodes evaluated for metastatic breast carcinoma by conventional histologic means alone (Gusterson and Ott, 1990; International (LUDWIG) Breast Cancer Study Group, 1990; Dearnaley et al., 1991; Neville, 1991; Bretton et al., 1994; Cote et al., 1995) (Fig. 2). Similar data exist for carcinomas of colon, lung, and prostate, and for melanoma. There is, however, surprising disagreement regarding the prognostic importance of these occult tumor deposits, particularly with reference to breast cancer. Much of this disagreement stems from the fact that studies have involved too few patients to address the issue with statistical power. More recent studies of larger series of cases provide compelling evidence of the prognostic significance of micrometastases, and establish significant lymph nodal involvement beyond that detected by routine histologic screening (Dearnaley et al., 1991).

Furthermore, comparative analyses of methods for detection of micrometastases suggest that immunohistochemical methods are far superior to histologic examination of lymph node serial sections including specific examples of patients with stage I melanoma (Cochran et al., 1988; Freeman et al., 1995), Dukes B colon carcinoma (Greenson et al., 1994), and early stage non-small cell lung carcinoma (Chen et al., 1993).

## "Tumor" antigens

Various antigenic changes have been described in association with malignant transformation and tumor progression. These changes may be classified into four general categories: (1) expression of new 'tumor-specific' antigens; (2) changes in the cellular distribution of antigens; (3) increase or decrease in the level of antigen expression, including changes associated with differentiated cellular functions; and (4) alterations in the biochemical nature of antigens, particularly changes in


**Fig. 2.** Section of an axillary lymph node from a patient with breast carcinoma. No tumor was identified in any axillary lymph nodes from this patient after routine histologic analysis. However, using a cocktail of anti-epithelial monoclonal antibodies, a cluster of carcinoma cells was seen in this lymph node in the medullary sinus. x 200

glycosylation patterns of glycoprotein and glycolipid antigens.

1. *"Tumor-specific"* antigens include the protypic 'oncofetal' antigens alpha feto protein (AFP) and carcinoembryonic antigen (CEA), as well as mutated oncogene and tumor-suppressor gene products, such as p53 protein. For virtually all such antigens, however, tumor specificity is not absolute (Old, 1980). It is well recognized that both CEA & AFP occur in 'non-cancer' states, and mutations in the p53 gene have been described in premalignant lesions from the prostate and breast. Furthermore, the immunohistochemical detection of mutated p53 protein is not based on the mutation itself, but rather on the increased accumulation of the abnormal protein that results from the mutation.

2. *The microanatomic distribution* of antigens in malignant cells is often different from their normal counterparts. Epithelial differentiation antigens, particularly cell surface antigens, such as CEA, B72.3, and EMA, typically have an apical or luminal distribution in normal cells, but a more random pattern in malignant cells, often with a cytoplasmic expression. Although these changes are characteristic of malignant cells, they are not specific for malignancy (Kuhajda et al., 1983; Springer et al., 1985).

3. Increased *antigen expression* is a feature of some tumor cells. Well-characterized examples exist for epithelial tumors, including Tn, CEA, OC125, and others (Kuhajda et al., 1983; Springer et al., 1985). Again, these changes are relative and not tumor-specific. Many of the antigens that were originally thought to be tumor-specific are not, but early assay methods were too insensitive to detect low level antigen expression on normal cells. Most serum tumor markers belong to this class of antigens (i.e., low level expression by some normal cells, increased expression by tumor cells).

Just as some antigens have increased expression on neoplastic cells, other antigens have decreased expression by malignant cells. The most important examples are those cell products (antigens) that are associated with the differentiated function of the cell of origin, such as GCDFP-15 (breast), thyroglobulin, and calcitonin. Well differentiated breast carcinomas will often express GCDFP-15, but poorly differentiated breast carcinomas will do so less often (Wick et al., 1989). Luminal epithelial antigen (LEA 135) provides another example: it is readily detectable on normal breast epithelium expression and progressively diminished in more advanced malignancies (Imam et al., 1993). A similar situation exists with regard to the expression of thyroglobulin by thyroid carcinomas; decreased expression of thyroglobulin is associated with a poorer prognosis (Ordonez et al., 1991).

Dedifferentiation of epithelial tumors may also be associated with changes in structural components that are antigenic. For example, normal adrenal cortical cells express cytokeratins, but not vimentin (at detectable levels), while adrenal cortical carcinomas consistently show vimentin expression, but generally do not express cytokeratin (Cote et al., 1990). Interestingly, adrenal cortical adenomas usually demonstrate expression of both cytokeratins and vimentin.

In addition, *changes in the pattern of glycosylation* of blood group-related antigens occur in many neoplasms (Hakomori and Kannagi, 1982). Changes can take several forms. For example, the major blood group antigens (A, B, H) are not expressed by normal colonic epithelium, but are expressed (according to the blood type of the individual) by the majority of colonic adenocarcinomas (Cordon-Cardo et al., 1986). In contrast, normal urothelium (generally) expresses ABH antigens, while malignant urothelial cells show loss of ABH expression. However, Lewis-type blood group antigens (Lex and Ley), which are not found on normal urothelium, are expressed by the majority of urothelial tumors (Sheinfeld et al., 1992). These changes are consistent and have been used to aid in the diagnosis and screening of urothelial carcinomas. The identification of Lex by exfoliated urothelial cells significantly improves the detection of urothelial tumors and can be used as an early marker of bladder cancer recurrence (Sheinfeld et al., 1992). Finally, alteration in blood group antigen expression has been described in other epithelial tumors and has even been found to predict the outcome of some tumor types. The range of biological markers investigated in this context far exceeds the scope of this review, and the interested reader is referred elsewhere for additional bibliography (Taylor and Cote, 1994; Grogan et al., 1995; Yang and Shamsuddin, 1996). However, it should be noted that in spite of the plethora of 'antigen' changes described in tumor cells, the diagnostic utility of the majority of such changes remains limited.

### Tumor cell proliferation

Cell proliferation correlates with clinical outcome in many tumors, and logically so. However, quantitating proliferation by counting of mitotic figures is not easily learned or reproducible between different pathologists. Attempts to find more objective methods have included measurement of tritiated thymidine or bromodeoxyuridine (BrdU) incorporation into tumor cell DNA (Meyer et al., 1983) and measurement of the proportion of cells in the synthesis (S) phase of the cell cycle (S-phase fraction) by flow cytometry (Clark et al., 1989; Koss et al., 1989).

A promising approach is immunohistochemical assessment of proliferative potential by the detection of nuclear proteins related to DNA replication (Lloyd et al., 1985). The principle advantage of this method over flow cytometric techniques, is that it permits concurrent morphologic examination of the tissue, distinguishes normal from malignant cells, and allows evaluation of heterogenicity in the tumor population.

Two extensively studied proliferation-related markers

are Ki67 and PCNA (proliferating cell nuclear antigen, or 'cyclin' (Fig. 3) (van Dierendonck et al., 1991). Ki67 is a nuclear antigen that is present in cells in the active phases of the cell cycle (i.e. $G_1$, S, $G_2$, and mitosis), but is absent in $G_0$ (resting) cells. PCNA shows a somewhat broader distribution in the cell cycles. Interestingly, human autoantibodies reacting to PCNA have been described.

There is a strong correlation between thymidine labeling and the proportion of cells exhibiting Ki67 or PCNA expression (van Dierendonck et al., 1991). As expected, the proportion of cells showing Ki67 or PCNA reactivity is greater than that identified by thymidine labeling; this is because these antigens are present in a broader range of cells ($G_1$, S, $G_2$, and mitosis) than is detected by labeling with thymidine (S- phase only). Also, there appears to be less intra-specimen variability in growth fraction estimates when assessed by Ki67 or PCNA versus thymidine labeling. Immunohistochemical assessment of proliferation is highly correlated with S-phase fraction analysis obtained by flow cytometry, particularly in aneuploid tumors. Discrepancies observed in diploid cell populations, have been attributed to the relatively slow decrease in Ki67 and PCNA expression in cells as they enter into $G_0$ (i.e., long half life); these cells do show only faint staining relative to the replicating cell population.

Ki67 and PCNA immunoreactivity also correlates well with morphologic features of cell proliferation, particularly mitotic index and tumor grade, and with other prognostic markers. For example, there is a strong inverse association between proliferation rate as assessed by Ki67/PCNA immunoreactivity and estrogen/progesterone expression. Conversely, there is a positive association between increased proliferation rate and p53 gene abnormalities. Finally, in patients with breast, prostate, colon, lung, liver, and gastric carcinomas, as well as some lymphomas and sarcomas, there is now substantial evidence that increased proliferation, as assessed by immunohistochemical analysis using Ki67 or PCNA, identifies patients whose tumors are more likely progress, and have a significantly shorter disease-free and overall survival (Sahin et al., 1991). Preliminary evidence suggests that antibody to topoisomerase II, which is more closely confined to S phase and $G_2$, may show an even better correlation with prognosis (Holden et al., 1994, 1995; personal observations, 1996).

The technical advantages of using proliferation-related markers such as Ki67 and PCNA in tissue sections include use of routinely processed (formalin paraffin) material (Fig. 3), applicability to very, small specimens, and direct evaluation of normal versus neoplastic cells, that is not possible by flow cytometry. As noted for other 'prognostic markers' standardized staining and antigen retrieval procedures must be employed for reliable results (Taylor and Cote, 1994; Shi et al., 1996; Taylor et al., 1996).

## Oncogenes, growth factors, and receptors

Many of the molecules that play a role in regulation of the cell cycle are now readily demonstrable by immunohistochemical methods. These include growth factors, receptors, cyclins and cyclin dependent kinases and the products of cellular proto-oncogene and tumor suppressor genes (Table 1). Not surprisingly, changes in compression of these molecules correlate with tumor progression.

### *HER-2/Neu*

HER-2/*neu* (or c-*erb* B-2) is a proto-oncogene first isolated from chemically induced rat ganglioneuroblastomas. The gene codes for a protein (molecular weight 185kD) that shows homology with the epidermal growth factor receptor (EGFR) and displays tyrosine kinase activity. Related genes includes erb B-3 and B-4. A putative ligand for erb B-2, termed heregulin, was described by Holmes et al. (1992). It binds to and activates the receptor and is similar to proteins in the epidermal growth factor (EGF) family: more recently it has been proposed that heregulin binds erb B-3 rather

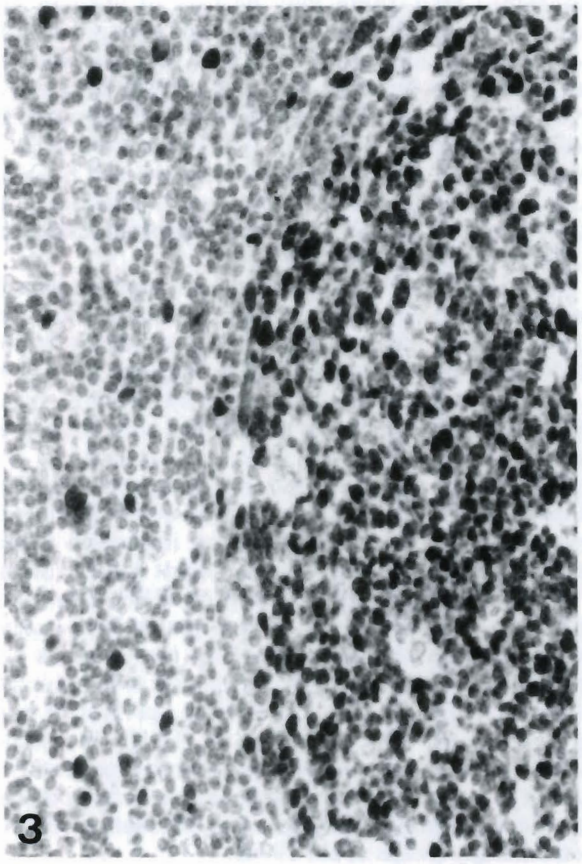


**Fig. 3.** Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) in normal lymph node. Note the density of PCNA-positive cells in the hyperplastic germinal center, where the majority of cells are proliferating. x 200

than erb B-2.

Amplification of the gene coding for HER-2/ *neu* has been described in breast, ovarian, prostate, gastric, salivary gland, lung, colon, and squamous cell carcinomas (Slamon et al., 1989; McCann et al., 1990). Overall, approximately one third of primary breast and ovarian cancers demonstrate amplification of the gene, correlating with an increased rate of recurrence and decreased survival (Slamon et al., 1989). The amplification of the HER-2/*neu* gene is accompanied by increased expression of the protein gene product, which can be detected in frozen or paraffin sections by immunohistochemical techniques (Slamon et al., 1989).

The correlation between gene amplification and immunohistochemical reactivity for HER-2/*neu* on frozen sections is so close that immunohistochemistry has become the standard method for analysis of HER-2/*neu* expression (Fig. 4). However, there are several important caveats. About 10% of breast cancers show immunohistochemical reactivity for HER-2/*neu* protein, but show no gene amplification, a finding attributed to increased gene expression (increased mRNA). This increased expression of the protein in the absence of gene amplification does appear to be prognostically important. Also, there may be loss of antigen after routine (formalin/paraffin) fixation and processing, necessitating the use of effective and standardized antigen retrieval methods (Shi et al., 1996; Taylor et al., 1996). Assessment of the localization of immunohistochemical reactivity is critical; when over-expressed, the protein accumulates at the cell membrane and is seen as a crisp membrane stain; cytoplasmic staining is not reliably associated with gene over-expression (Slamon et al., 1989; McCann et al., 1990).

While HER-2/*neu* over expression/amplification has been described in several tumor systems, it has been most extensively studied in the breast, occurring in 25 to 30% of primary breast carcinomas. Staining is restricted to cancer cells and is not seen in normal breast epithelium. HER-2/*neu* is associated with high grade tumors and there is an inverse association with expression of estrogen and progesterone receptors (Slamon et al., 1987). While there is some controversy, over-expression is thought to be an important adverse prognostic factor in node-positive breast cancer (Slamon et al., 1989). The relationship of HER-2/*neu* over expression to prognosis in node-negative breast cancer was, until recently, less clear, but new studies have reported that HER-2/*neu* over expression is associated with poorer disease-free and overall survival in node-negative patients also (Allred et al., 1992). In addition, HER-2/*neu* over-expression may identify patients who will not respond to conventional adjuvant chemotherapy, but who may gain advantage from alternative forms of chemotherapy (Muss et al., 1994).

There are also reports of an association with a poorer prognosis in patients with ovarian carcinoma (Slamon et al., 1989; McCann et al., 1990), while the significance of HER-2/*neu* staining significance in salivary gland tumors, squamous cell carcinomas of the head and neck, gastric carcinomas, and prostate carcinomas remains uncertain (McCann et al., 1990). HER-2/*neu* gene amplification has not yet been identified in non-small cell lung carcinoma or colon cancer.

### Other oncogenes

Although antibodies to many oncogene products have been developed, immunohistochemical analyses remain at an early stage, with the exception of lymphomas (see below).

The *ras* oncogene is a good case in point. Once thought to be perhaps the single most important genetic event in cancer, mutations in the *ras* gene and increased expression of the *ras* product have been associated with a wide variety of tumor types (Ghosh et al., 1986). For some types of tumors (e.g., pancreatic carcinoma) mutations in the *ras* gene are detected in the majority of examples studied. The primary event is generally a point mutation, which takes place at a few characteristic sites.

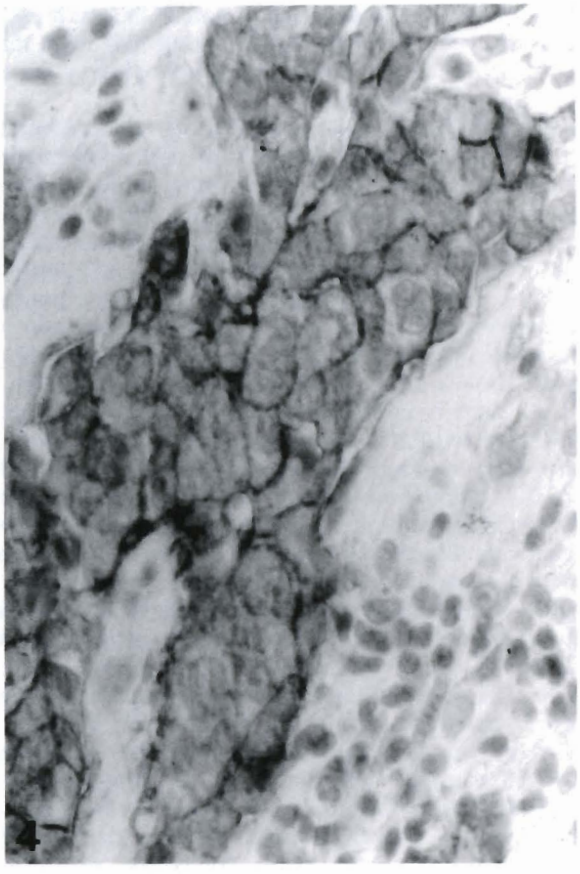

**Fig. 4.** Infiltrating ductal carcinoma of the breast demonstrating over-expression of Her-2/*neu* oncoprotein. Over-expression of the protein is associated with amplification of the Her-2/*neu* gene. The presence of membrane reactivity (as seen here) indicates protein (and thus gene) over-expression; cytoplasmic reactivity is not related to Her-2/*neu* expression. x 400

Immunohistochemical analysis of *ras* has been compromised by the failure to develop antibodies that reliably distinguish mutated forms from the wild-type *ras* gene product, which comprises a 21-kD protein with GTPase activity. Attention has consequently been focused on the immunohistochemical analysis of the amplification of the *ras* gene product. Despite early reports to the contrary, this has not proved to be of prognostic value in most cases (Wick et al., 1989).

Alterations of the *myc* family of proto-oncogenes (c- and N-*myc*) also have been associated with increased cellular proliferation. In the case of small cell lung carcinoma, both c-*myc* and N-*myc* amplification have been associated with poor prognosis (Funa et al., 1987). N-*myc* amplification may also identify small cell lung carcinomas that will show a poor response to chemotherapy (Funa et al., 1987); but clinical utility is not established.

*Epidermal growth factor receptor* (EGFR) belongs to a family of growth factor receptors and shares structural and functional characteristics with the HER-2/*neu* (erb B-2) gene product. Binding of EGFR by its ligand (EGF) produces proliferative activity in cells. EGF is one of a family of polypeptide growth factors and has significant homology with transforming growth factor-alpha (TGF-α) (Carpenter and Cohen, 1979). EGFR is present in a wide variety of tissues, with predominant (but not exclusive) expression in epithelial cells (Damjanov et al., 1986). Expression of EGFR on the cell surface allows for signal transduction in response to binding of available external ligand, while loss of such expression indicates loss of responsiveness to the ligand. Cytoplasmic localization, conversely, identifies cells which maintain the capacity to bind any ligand available internally (for example, by autocrine mechanisms, or following induction of membrane localization by physiologic stimuli), but which do not respond to external (circulating) ligand (Damjanov et al., 1986).

Increased expression of EGFR has been demonstrated by immunohistochemistry in a wide variety of tissues, and may be related to tumor progression in certain instances (e.g., breast, esophageal, adrenal cortical, lung, bladder, thyroid, and gastric carcinomas) (Sainsbury et al., 1987; Sugiyama et al., 1989). In the case of gastric carcinomas, those tumors which express both EGFR and EGF have a worse prognosis than those with neither EGFR or EGF expression, or those with EGFR expression only, suggesting an autocrine drive for proliferation (Sugiyama et al., 1989).

### Oncogenes in lymphoma

The bcl-2 proto-oncogene is located distal to a cluster of break points on chromosome 18, such that the t(14;18) translocation frequently results in juxtaposition of bcl-2 with the immunoglobulin heavy chain gene on chromosome 14. In such circumstances the bcl-2 gene appears to be deregulated, synthesizing excess amounts of a 25-kD protein that localizes to the inner mitochondrial membrane. In some instances, neoplastic cells show abnormalities of heavy chain gene function and of immunoglobulin production. Functionally, bcl-2 protein prolongs the survival of cells by interfering with normal programmed cell death (apoptosis), whereby redundant lymphoid cells in the immune response are deleted (Swerdlow et al., 1993). Immunohistochemical studies of normal lymph nodes using antibody to bcl-2 protein (clone 124) reveal expression in normal memory cells, and other long-lived lymphoid cells (Korsmeyer, 1992). This is illustrated particularly well in reactive follicles, where long-lived follicular mantle B-cells typically express bcl-2, reflecting the presumptive inhibition of apoptosis by high levels of bcl-2, while rapidly proliferating germinal center cells show little bcl-2 expression (stain 'negative' for bcl-2), reflecting a high rate of apoptic cell death in the absence of bcl-2. While this is the typical appearance in normal reactive centers, neoplastic follicles characteristically differ by virtue of the intense staining for bcl-2 within cells of both the mantle zone and neoplastic follicle center. The presence of the characteristic t(14:18) translocation in many follicular center cell lymphomas that exhibit abnormal bcl-2 expression has led to the hypothesis that this translocation and the consequent abnormal activation of bcl-2 may be a seminal event leading to neoplastic transformation in this subset of B-cell tumors. Indeed, there is experimental evidence in support of a role of elevated bcl-2 activity in lymphomagenesis in that increased bcl-2 expression, accompanied by c-*myc* activation, induces aggressive B-cell lymphomas in transgenic mice (McDonnel et al., 1989).

While some investigators are convinced of the role of bcl-2 in lymphoma and its close association with the t(14;18) translocation, changes in bcl-2 compression may arise by other means in certain instances. Also the change may not be diagnostic of follicular center cell malignancy, in light of the findings that t(14;18) translocation may occur early in normal B-cell development, and may be detected (albeit infrequently, 1 in $10^5$ cells) in normal lymph nodes. Finally, bcl-2 expression in non-Hodgkin's lymphoma overall may be without prognostic significance (Pezzella et al., 1992).

While bcl-2 expression is observed in 80-90% of cases of follicular center cell lymphomas, it may also be observed in 20% or more of diffuse lymphomas, including some T-cell lymphomas, a small number pf Ki-1 positive anaplastic large cell lymphomas, and within the Reed-Sternberg cells in some cases of Hodgkin's disease (Doussis et al., 1993). As noted above, while many lymphomas show elevated bcl-2 levels, not all of these show of demonstrable translocation of t(14;18). In these cases, it has been postulated that other disregulatory mechanisms may be in effect, exemplified by the fact that latent membrane protein (LMP-1) of Epstein-Barr virus (EBV) can induce expression of bcl-2 in EBV-infected cells. Abnormalities of a number of other oncogenes and tumor-supressor genes (e.g., c-*myc*, bcl-1, p53 and Rb) also have been

observed in malignant lymphomas, but the immunohistochemical data are limited (Koch et al., 1992). Bcl-2 expression may be reliably demonstrated in formalin-paraffin sections, again with appropriate standardized protocols and antigen retrieval techniques.

## Tumor-suppressor genes and gene products

The existence of tumor-suppressor genes (also known as "anti-oncogenes") was first suggested by Knudson in 1971 (Knudson, 1971), who explained the epidemiology of retinoblastoma by postulating 'multiple hits' (double deletion) of both alleles of a recessive retinoblastoma (Rb) gene that normally acts to inhibit the formation of tumors. The primary characteristics of tumor-suppressor genes are that a) they encode normal cellular products involved in growth control, and b) since the abnormality is recessive, both the alleles must be inactivated for loss of function (i.e., loss of tumor-suppression) to occur. This is in contrast to the abnormalities described for oncogenes (positive growth factors), which are dominant, and thus require mutation in only one allele.

Several putative tumor-suppressor genes have been identified, including the Rb gene, p53, DCC (deleted in colon cancer), the Wilms tumor gene, and the neurofibromatosis type I gene (NF1), (Knudson, 1971; Nowell and Croce, 1986). NM23 may also be an example of a tumor-suppressor gene (Leone et al., 1991). The two best characterized are the Rb and the p53 genes; the gene products for both have been identified and studied by immunohistochemical methods. Both p53 and Rb gene products are thought to be involved in growth control through regulation of transcription; wild-type p53 protein has been shown specifically to activate transcription, which may then lead to production of products that control cell growth (Farmer et al., 1992). Both are nuclear phosphoproteins, and there is evidence that phosphorylation may inactivate the Rb protein. In addition, a variety of viral oncoproteins, including SV40 T antigen, E1A from adenovirus, and E6 from human papilloma virus, bind to and neutralize Rb and/or p53 proteins. More recently, a cellular oncoprotein (MDM2) has been described, which binds to and may neutralize p53 protein (Oliner et al., 1992).

### *Rb*

The Rb gene is located on chromosome 13q14 and spans a region of more than 200,000 bases, including 27 exons. Rb protein has a molecular weight of 105,000 daltons, and a number of antibodies have been developed which recognize specific parts of this protein (Xu et al., 1991). The Rb gene is the only tumor suppressor for which there is direct clinical evidence of suppression of tumor formation. Alterations in this gene have been described in a number of human tumors, including retinoblastoma, osteosarcoma, other sarcomas, leukemias, lymphomas, and certain carcinomas, including those derived from breast, lung, prostate, bladder, kidney, and testis (Reissmann et al., 1989; Horowitz et al., 1990; Cordon-Cardo et al., 1992). Rb gene alterations also have been associated with advanced tumor grade and stage in a variety of tumors (Cordon-Cardo et al., 1992). In the case of breast carcinoma, alterations in the Rb gene have been associated with other signs of progression, such as loss of hormone receptor expression. Furthermore, there is growing evidence that Rb alterations may identify primary tumors which are at higher risk of developing metastases (Drobnjak et al., 1993).

It is now clear that genetic alterations in the Rb gene correlate with loss of expression of Rb protein as determined by immunohistochemical methods (Xu et al., 1991). In fact, it has been suggested that immunohistochemical analysis of Rb protein may detect Rb gene inactivation more rapidly, specifically, and reliably than direct screening of DNA for gene abnormalities (Borg et al., 1992). Assessment of Rb gene loss by immunohistochemistry is based upon loss of detectable nuclear staining for Rb protein. This is in contrast to the immunohistochemical staining pattern seen in tumors with altered p53, in which staining is increased due to accumulation of 'mutant' protein (see below).

### *p53*

The p53 protein was originally described as a nuclear protein (MW, 53,000 daltons) expressed in methylcholanthrene-induced mouse sarcomas, but not in normal mouse cells (DeLeo et al., 1979). Originally thought to be a cellular oncoprotein specifically expressed by tumor cells, it is now included in the tumor-suppressor family. p53 protein is expressed by all normal cells, but the half life of the wild-type (`normal') protein is so short (6-30 minutes) that it does not accumulate to levels high enough to be detected by standard immunohistochemical techniques; mutant p53 protein, by contrast, has an extended half-life, accumulates, and is readily detectable in the cell nucleus, hence mutation is indicated by 'positive staining' (Fig. 5).

The p53 gene is located on chromosome 17p13.1. Alterations of the p53 gene, identified by cytogenetic, molecular, and immunohistochemical methods, are extremely common in human cancer and have been described in a bladder, colon, lung, breast, and other carcinomas, as well as astrocytomas, leukemias, sarcomas, and mesotheliomas (Cote et al., 1991; Esrig et al., 1993, 1994; Nigro et al., 1989; Taylor and Cote 1994). Loss of p53 suppressor function usually results from complete loss of one allele associated with a point mutation in the second allele; the mutant p53 gene continues to be expressed, but lacks the regulatory activity of the wild-type p53. Mutant p53 protein has several important properties: it can bind to and neutralize residual wild-type p53 proteins (i.e., it has 'anti-suppressor' or oncogenic effects), and it is a metabolically stable protein, with a long half life (hence accumulation and detection).

There is increasing evidence that the immunohistochemical detection of p53 protein in the nucleus identifies cells that have mutations in the p53 gene (Esrig et al., 1993). However, a small percentage of tumors having p53 gene mutations do not show detectable p53 nuclear accumulation by immunohistochemistry for reasons that are unclear.

Data suggest that the immunohistochemical analysis of p53 may not only be more sensitive than molecular methods in detecting p53 gene alterations, but immunohistochemistry may detect important alterations in p53 function that do not involve mutations at the gene level (Esrig et al., 1993). In addition, when p53 nuclear reactivity is detected by immunohistochemistry, significant heterogeneity in the staining pattern (percentage of reactive tumor cell nuclei) is observed, even within the same tumor type. While some of the variation may be due to differences in fixation, there is now evidence that this may be due to a property of the mutant p53 protein (Esrig et al., 1993). The heterogeneity in the staining pattern is apparently due (at least in part) to different sites of mutation; resulting in differing half lives for the expressed proteins.

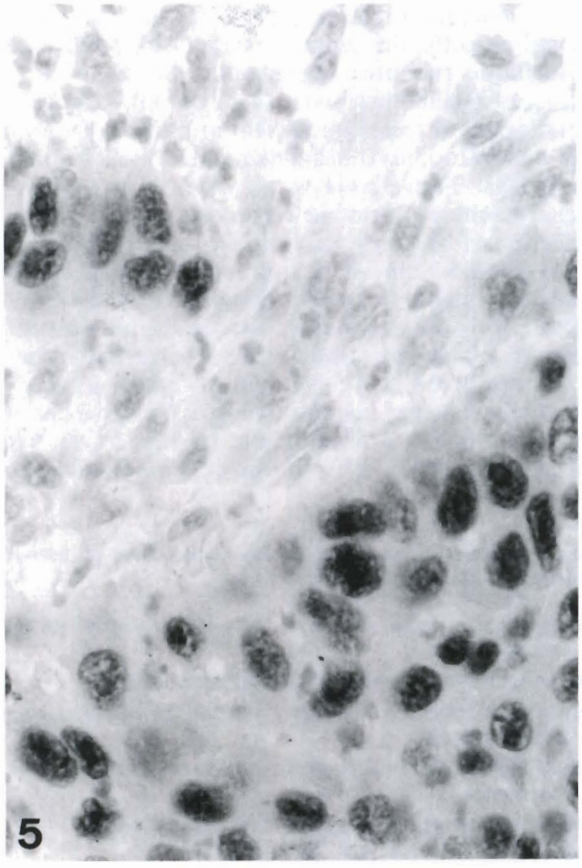


**Fig. 5.** Transitional cell carcinoma of the bladder showing nuclear immunoreactivity with the anti-p53 antibody Ab 1801. Note that the tumor cells show heterogenous expression of the antigen, and the surrounding normal stroma cells show no detectable immunoreactivity. Point mutations in the p53 gene result in a protein product which has a significantly longer half-life than the wild type p53 protein. The mutated p53 protein accumulates in the nucleus at high enough levels to be detected by standard immunohistochemical techniques; this is the basis for the immunohistochemical detection of p53 mutations. x 400

Another possible source of heterogenous staining is that some cells in a tumor may have a mutant p53 gene, while others do not. While this mechanism has been suggested for some tumors (particularly astrocytomas), it may be uncommon, as p53 is apparently an early event in most of the common tumors, such as breast, prostate, and bladder carcinoma (Esrig et al., 1993).

Because of the importance of p53 alterations in human cancer and the ease of detecting p53 mutations by molecular or immunohistochemical techniques, p53 alterations have been the focus of intense examination. As with Rb alterations, p53 alterations are associated with tumors of high histologic grade and tumors with a high proliferative index (again, suggesting a relationship between tumor-suppressor genes and control of cell growth). In the case of breast tumors, p53 alterations are associated with loss of hormone receptor expression and hormone-independent tumor growth (as are Rb alterations), again suggesting that tumors which lose normal growth controls may do so because they become independent of normal growth regulators (Cattoretti et al., 1988; Drobnjak et al., 1993). There is growing evidence that, at least for some types of tumors, p53 alterations identify patients with shorter disease-free interval and poorer overall survival (Esrig et al., 1993, 1994; Hamada et al., 1995; Lazaris et al., 1995). In the case of breast and bladder carcinoma, the association between p53 alterations and prognosis appears strongest in the earliest stage tumors, thus emphasizing the potential value of p53 examination in assessing the future behavior of the tumor. It appears that in many tumors, p53 alterations are an early event in tumorigenesis and can be detected in situ carcinomas (in the case of breast and bladder carcinoma) (Cattoretti et al., 1988; Walker et al., 1991; Esrig et al., 1993, 1994), or even in dysplastic lesions (in prostate and colon carcinoma). Thus, tumors which are at highest risk for progression may be identified even prior to becoming invasive.

One final concern relates to the possible instability of p53 in cut sections. Some investigators have reported loss of p53 staining in stored cut sections (Jacob et al., 1996). We have confirmed this finding. However, the problem can be remedied by use of appropriate antigen retrieval methods (Stein et al., unpublished results), again emphasizing the importance of optimizing and standardizing the total stain procedure (Taylor and Cote, 1994, 1996).

### Invasion and metastases

Metastasis is a multi-step process involving tumor cell interactions with the extracellular matrix and disruption of basement membranes, as noted earlier. Once in the stroma, tumor cells interact with specific glycoproteins

in the matrix via receptors, such as laminin receptor, present on the plasma membrane of many tumor cells. Invasive cells then secrete, or induce the secretion of, proteolytic enzymes that degrade the components of the extracellular matrix (e.g., laminin, heparin) and the underlying basement membrane (e.g., Type IV collagen, laminin, and heparin sulfate proteo-glycan), thereby facilitating migration away from the primary site. In keeping with this postulate there is an increased expression of a 67-kd laminin receptor, at both the mRNA and protein levels in several human cancers, including colon and breast carcinomas associated with metastatic phenotype. Using immunohistochemical methods with an affinity-purified antibody against a cDNA-derived laminin receptor peptide, cells from benign breast tissue may be seen to express low levels of laminin receptor, primarily cell membrane-associated. Further progression from in-situ ductal and in situ lobular carcinomas to their infiltrating counterparts is associated with an increase in the percentage of cells that express laminin receptor (Castronovo et al., 1990; D'Errico et al., 1991; Liotta et al., 1991). Detection of increased expression of the laminin receptor may therefore be a measurable marker of the metastatic phenotype in cells still at their primary site.

Other studies of the mechanism of metastasis has shown that invasive tumor cells have increased levels of proteolytic activity. Particular interest has focused on Type IV collagenase, of which two forms (72 kD and 92 kD isozymes) have been described. Evidence indicates that levels of the 72 kD form correlate with invasiveness in a variety of human tumors, including hepatomas, melanomas, sarcomas, and in various carcinomas, e.g., those of gastric, colorectal, and breast origin (Castronovo et al., 1990; D'Errico et al., 1991; Liotta et al., 1991).

## Immunohistochemical approaches for predicting response to therapy

Analysis of cell surface receptors may provide information as to how a tumor will respond to certain types of therapy. The responsiveness of breast and prostate carcinoma to hormonal manipulation is the classic example. More recently, a trans-membrane protein, encoded by the so-called, multi-drug resistance (MDR) gene, has been found to identify cells which are resistant to specific types of chemotherapy. This protein acts as a "pump" to remove toxins (including certain chemotherapeutic agents) before they can act to kill the cell. Finally, the expression of certain proteins (principally oncogene products) has been associated with resistance to some types of chemotherapy, either by direct or indirect mechanisms. All of these proteins can be assessed by immunohistochemical methods.

### Estrogen and progesterone receptors

The observation that certain tumors, principally those derived from reproductive organs (breast, prostate, endometrium, and ovary), are under the growth regulation of steroid sex hormones (estrogens and androgens) has had a fundamental impact upon the therapeutic approach to these tumors. Seminal work by Beatson (1986), Huggins and Hodges (1941), and Jensen and colleagues (1982) demonstrated that the growth of certain tumors (breast and prostate carcinoma, in particular) is dependent upon the presence of circulating hormones (estrogens, androgens), and that removal of the source of these hormones (by oophorectomy or orchidectomy) could result in dramatic tumor remissions. The growth regulation of the tumors was found to be mediated through the expression of specific receptors for the circulating hormones; tumors expressing high levels of the receptor tended to respond to hormone ablation therapy, while tumors which expressed low levels of the receptor tended to be unresponsive to such therapy (i.e., these tumors demonstrate hormone-independent growth) (Jensen et al., 1982). These observations paved the way for rational, patient-specific cancer treatment, based on specific biologic characteristics of the tumor cells.

Until recently, the primary method for determination of hormone receptor expression was based on the binding of labeled hormones (e.g., estradiol, progestins, or androgen) to receptors present in tissue homogenates (the so-called, cytosol-based assays) (Jensen et al., 1982). These assays provide a 'quantitative' measurement of hormone receptor content in tissues. Prediction of response to hormone ablation therapy by this method is imperfect, however, as sampling error frequently results in the contamination of the cytosol-extract by non-neoplastic stromal and epithelial elements, which may obscure the true receptor content of the tumor cells. Also, cytosol-based assays are technically exacting, require biologically active hormone receptor (which is present only in fresh or frozen tissue) and may be affected by the presence of endogenous

**Table 2.** Practical advantages of immunohistochemical assays for detection of steroid hormone receptors.

| |
|---|
| • Use on small samples, even those too small for routine biochemical methods |
| • Use on fine-needle aspiration and cytology preparations |
| • Use on both frozen and routinely processed formalin-fixed, paraffin-embedded tissue |
| • Allows assessment of different populations of cells (such as in situ versus invasive components) |
| • Direct assessment of heterogeneity of receptor expression |
| • Distinguishes tumor cells from other cell populations |
| • Determines if the specimen was appropriately collected |
| • Produces a permanent histologic record |
| • Allows use of internal positive and negative controls in most tissue samples to assess quality of each test |
| • Results obtained more quickly than with standard assays |
| • More predictive of prognosis than standard biochemical assays |
| • More predictive of response to hormonal therapy than standard biochemical assays |

hormones (which compete with the radiolabeled ligands for the receptor). Direct determination of receptor content in histologic preparations of tissues resolves some of these difficulties (Table 2).

Immunohistologic detection of receptor expression in tissue sections can theoretically be achieved either indirectly, by utilizing the biologic binding activity of the receptor for the steroid with subsequent detection of the bound steroid molecule by an anti-steroid antibody, (Taylor et al., 1981) or directly and more accurately, by immunologic reactivity of the receptor protein with an anti-receptor antibody (Press and Green, 1988).Both methods discriminate between receptor sites confined to tumor cells and those localizing to non-neoplastic components. Furthermore, the exact proportion of tumor cells showing reactivity is made evident. The ability to assess morphologically receptor expression at the cellular level is important. A tumor in which only 10% of the tumor cells are positive, but strongly so, would be expected to respond differently to hormonal manipulation from another tumor in which all of the tumor cells are weakly positive, yet both tumors could give identical results on a cytosol-based assay. With the availability of monoclonal antibodies that recognize epitopes on estrogen, progesterone, and androgen receptor proteins (anti-receptor antibodies), this direct approach has supplanted all others (Figs. 6, 7), and has proved superior to the standard cytosol (biochemical) assays (Kinsel et al., 1989; Pertschuk et al., 1996; Taylor, 1996).

Immunohistochemical assays can be used on extremely small tissue specimens, including cytologic preparations of cells, (e.g., fine needle aspirates [FNA]), and on core needle biopsy specimens. This is particularly important in evaluating receptor content of metastatic disease, in which a formal biopsy may not be possible or desirable.

Immunohistochemical detection of estrogen receptors initially was restricted to frozen tissue, but techniques now allow use of routinely processed, formalin-fixed paraffin-embedded tissue. Comparative studies of these cytosol assays with the immunohistochemical anti-

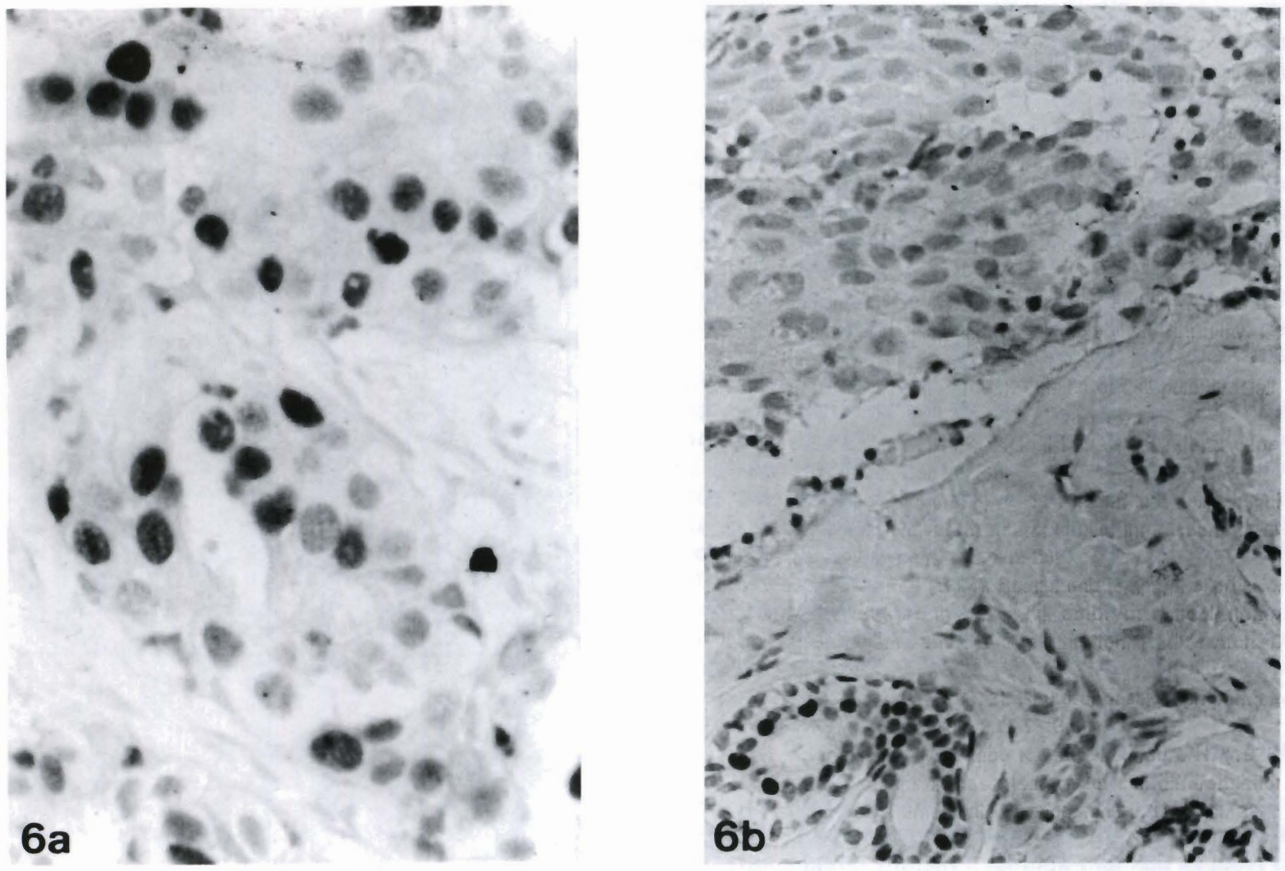


**Fig. 6.** Breast carcinoma assessed for estrogen receptor expression using the anti-receptor monoclonal antibody. **A.** This tumor shows intense homogenous nuclear reactivity with the anti-estrogen receptor monoclonal antibody. The immunoreactivity demonstrates specific nuclear localization; any cytoplasmic reactivity would be discounted. In this case, use of the immunohistochemical assay allows for assessment of estrogen receptor expression in both the infiltrating and in situ component of this tumor. **B.** In this section from a different tumor, the infiltrating ductal carcinoma (right side of picture) does not show positive nuclear reactivity. The presence of significant amounts of non-tumor tissue which expresses hormone receptor (as indicated by the arrow) could lead to a false positive result when using biochemical assays, which do not allow for the morphologic evaluation of the tissue being tested. x 400

receptor assays (King et al., 1985; McCarty et al., 1986; Parl and Posey, 1988) reveal high concordance, ranging from 77% to more than 90%. Discordance, when present, is usually limited to borderline cases and often rests upon heterogeneity of receptor expression, tissue sampling errors (scant tumor cells available for the assay), or the presence of endogenous hormone which binds to the receptor, thus making it unavailable for assay (this may occur in premenopausal patients, who can have substantial levels of circulating hormone, or in patients being treated by endocrine therapy) (Toi et al., 1989) and, therefore, lead to "false"-negative biochemical test results. "False"-positive biochemical results have been attributed to the binding of the labeled hormone ligand to non-receptor proteins, or to the presence of receptor-positive normal tissues admixed with receptor-negative carcinoma. Available data now overwhelmingly support the superiority of paraffin section IHC for ER over both the cytosol method, and the frozen section IHC method in which receptor may be lost or diffused during freezing (Pertschuk et al., 1996; Taylor, 1996).

The results of immunohistochemical assays for both estrogen receptor and progesterone receptor expression correlate with prognosis (disease-free survival and overall survival) in patients with breast cancer (Reiner et al., 1990). They also correlate with other putative prognostic factors, such as tumor grade, ploidy, and stage (Helle et al., 1989; Toi et al., 1989), and with response to hormonal treatment (Pertschuk et al., 1985, 1996; McClelland et al., 1986; Coombes et al., 1987; Allred et al., 1990). Furthermore, correlation of the paraffin IHC method for both prognosis and hormonal responsiveness is superior to the cytosol and frozen section methods (Pertschuk et al., 1996).

While immunohistochemical assays do not provide the quantitative endpoint of the biochemical assays, the use of semiquantitative measures, which take into account receptor heterogeneity and intensity of immunoreactivity, show an excellent correlation with prognosis and response to therapy (Pertschuk et al., 1996; Taylor 1996). Also, identification of a significant decrease in receptor-positive tumor cells by immunohistochemistry is associated with increasing proliferative cell fractions, increased Her-2-*neu* oncogene amplification, and EGFR expression (Helle et al., 1989).

### *Androgen receptors*

Prostate carcinomas can express androgen receptor, a finding grounded in the empiric observation by Huggins and Hodges that anti-androgen therapy could control the growth of prostate carcinomas, often in a dramatic fashion (Huggins and Hodges, 1941). However, approximately 25% of patients are unresponsive to such hormonal manipulation and measurement of androgen receptor levels has not routinely been performed in cases of prostatic carcinoma, due in part to the fact that prostate carcinomas are notoriously difficult to recognize and cytosolic extracts were impossible to standardize. Measurement of nuclear androgen receptor, unlike cytosolic receptor, may be predictive of response to endocrine therapy and prognosis is independent of both tumor grade and stage in predicting progression of disease (Gonor et al., 1984).

Monoclonal and polyclonal antibodies specific for the androgen receptor have been developed and used to the study receptor distribution in normal and malignant tissues (Ruizeveld de Winter et al., 1991). Both benign and malignant prostatic epithelia express nuclear androgen receptor. In the case of prostatic carcinoma, the heterogeneity in receptor expression observed within tumors, as assessed biochemically, has been confirmed by immunohistochemical techniques using anti-androgen receptor antibodies (Ruizeveld de Winter et al., 1990). To date, however, studies of the relationship of the immunohistochemical detection of androgen receptors with response to endocrine therapy and prognosis are limited.

### *Drug resistance*

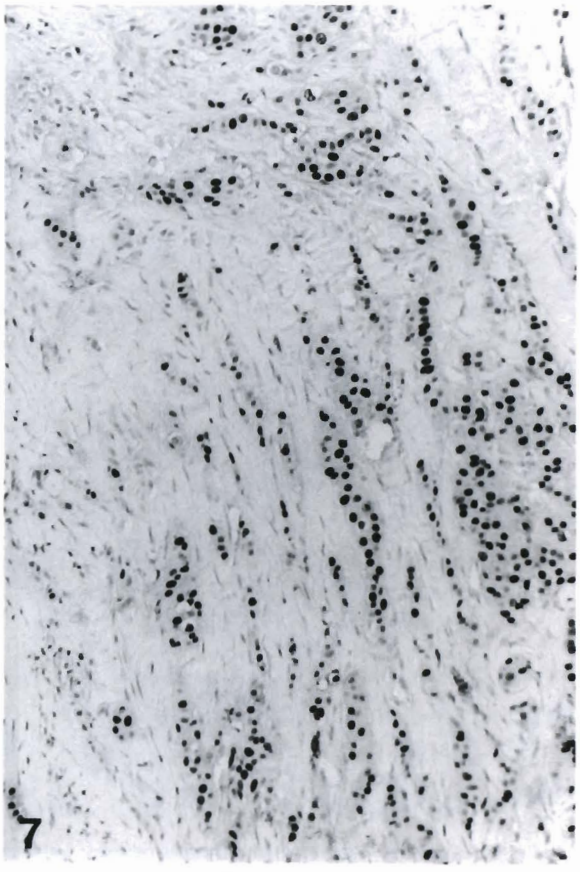


**Fig. 7.** Adenocarcinoma of the prostate showing positive nuclear immunoreactivity with anti-androgen receptor monoclonal antibody. This assay was performed on routinely processed, formalin-fixed, paraffin embedded tissue sections using the antigen retrieval (microwave) technique. x 100

P-glycoprotein

P-glycoprotein (P-170) is a trans-membrane protein of 170,000 MW that has been associated with both intrinsic and acquired resistance to certain chemotherapeutic agents, particularly anthracyclines and vinca alkaloids. P-170 is an energy-dependent "pump" which functions in drug efflux, reducing intracellular accumulation of chemotherapeutic drugs and, thus, conferring the so-called, "multi-drug resistance" (MDR) phenotype. P-glycoprotein is encoded by a family of so-called, "MDR" genes; however, only the protein encoded by the MDR 1 gene appears to induce the MDR phenotype (Kartner and Ling, 1989).

As studied by molecular or immunochemical means, the expression of P-glycoprotein has been observed in tumors which show a high degree of intrinsic drug resistance, such as renal, colon, and adrenal cortical carcinomas (Kartner and Ling, 1989; Cordon-Cardo et al., 1989, 1990). In these cases, high levels of expression of P-glycoprotein have also been observed in the normal tissues from which these tumors are derived. In fact, renal proximal tubular cells have become a standard positive control in the immunohistochemical analysis of P-glycoprotein expression (Cordon-Cardo et al., 1989, 1990).

Tumors responsive to chemotherapy generally show a low incidence of P-glycoprotein expression (Cordon-Cardo et al., 1989). Indeed, those solid tumors that are most responsive to systemic chemotherapy (such as seminomas and embryonal carcinomas) rarely show detectable expression of P-glycoprotein (Cordon-Cardo et al., 1989). In contrast, tumors from patients previously treated with chemotherapy frequently show elevation of P-glycoprotein, suggesting that the MDR phenotype is induced by exposure to chemotherapy (Schneider et al., 1989). It has been postulated that detection of elevated P-glycoprotein expression may identify tumors likely to be resistant to conventional chemotherapy and thus provide a rationale for the provision of alternative treatments for these patients. Immunohistochemical evaluation of P-glycoprotein expression appears to be the analytical method of choice, primarily because it allows the discrimination of P-glycoprotein expression in tumor cells from that in normal cells.

Only two MDR genes are known to be present in humans (MDR 1 and MDR 3), but only the MDR 1 gene product confers the MDR phenotype. Unfortunately, one of the most widely used antibodies to P-glycoprotein, C219, reacts with both the MDR 1 and MDR 3 gene product. Several antibodies specific for the MDR 1 gene product have now been described, however, including HYB-241, HYB-612, and C494. While most studies have used frozen tissue sections, because of perceived loss of P-glycoprotein reactivity after routine processing, our experience, shared with others, is that the method is applicable to routinely processed, paraffin-embedded material.

## Other predictors of drug resistance

As described above, the expression of certain oncogene products identifies tumors which are resistant to particular types of chemotherapy. Over expression of HER-2/*neu* by breast cancers is associated with some resistance to conventional chemotherapy regimens containing cyclophosphamide, methotrexate, and fluorouracil (CMF) (Allred et al., 1992). Similarly, increased expression of N-*myc* by small cell lung carcinomas and by neuroblastomas has been associated with lack of response to chemotherapy and rapid progression of disease (Funa et al., 1987; Esrig, et a., 1994). Whether or not over expression of HER-2/*neu* or *myc* itself contributes to the provocation of drug resistance, or is simply a manifestation of an increasingly aggressive phenotype remains unclear. In either case, these markers may have utility in the determination of the most effective course of treatment for these cancers.

The determination of drug resistance by the identification of specific cellular products (such as P-glycoprotein) in preserved (frozen or routinely processed) tissue specimens by immunohistochemistry represents a considerable simplification compared to the alternative "clonogenic" techniques, for the assessment of drug resistance, in which the response of cultured tumor cells to myriad chemotherapeutic agents is gauged by several means (Hamburger and Salmon, 1977). Most importantly, the immunohistochemical assays do not require viable tumor. Immunohistochemical analysis offers the added advantage that the entire population of cells within a representative portion of the tumor can be assessed, unlike the clonogenic assay, in which unknown clones of cells may be selected. Finally, the expression of the product on tumor cells can be distinguished from that on normal cells by immunohistochemical means; such is not the case in the culture assay.

## Therapy with monoclonal antibodies

Finally, phase III clinical trials are underway of antibodies employed for specific therapy of lymphomas and other solid tumors with encouraging results. Under these circumstances, selection of patients most likely to respond may be predicated upon the demonstration of the target antigen on the surface of lymphoma cells, by immunohistochemistry or flow cytometry (Lewis et al., 1995). Similarly the use of other therapeutic monoclonal antibodies such as anti Her/2*neu* in breast cancer may be predicted with the immunohistologic demonstration of the target antigen in biopsy material. In these instances, phenotype is more important than conventional histologic classification.

## Conclusion

The ability to specifically predict prognosis and the response of individual tumors to hormonal manipulation

or chemotherapeutic agents is likely to have a profound impact upon the way treatment decisions are made for patients with cancer. It is not difficult to envision the day when the selection of tumor therapy will be based upon defined information as to the characteristics of an individual tumor, as opposed to current methods that are generalizations based, at best, upon clinical trial outcomes of heterogenous tumor in heterogenous patients.

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