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INTRODUCTION

We are investigating multiple markers in human breast and bladder cancers. Our aim is to identify markers that are clinically relevant and that contribute to our understanding of the disease process in individual patients. Good markers accurately assess the malignant potential of a cancer in an individual patient. Thus, they help identify those cancers that will recur, and they may be used to predict more accurately time to recurrence, response to treatment, and overall prognosis. Therapy and patient management may then be optimized to the individual patient.

Relevant markers reflect the underlying pathobiology of individual tumors (1,2). As a tissue undergoes transformation from benign to malignant, the cells lose their differentiated phenotype. Markers of differentiation may be lost, new markers, frequently characteristic of the fetal or less well differentiated phenotype, may be expressed, and cellular proliferation may increase. But underlying all manifestations of malignancy are changes at the genetic level; oncogenes may be activated, anti-oncogenes may lose their functionality or be lost, and other genes, including their control regions, may undergo mutations that alter their activity or create variant end-products. As a generalization, the more the cellular phenotype, cellular proliferation and cellular genotype depart from normal, the more advanced is the tumor in its biological evolution and the more likely it is that the patient has a poor prognosis.

We use three studies to illustrate our investigation of potential tumor markers. Breast cancers are labeled *in vivo* with 5-bromodeoxyuridine (BrdUrd) to give a direct measure of the tumor labeling index. Bladder cancers are analyzed immunocytochemically using an antibody against proliferating cell nuclear antigen (PCNA) to give an indirect measure of cellular proliferation. Finally, the techniques of molecular genetics are used to detect allelic loss in breast cancers.

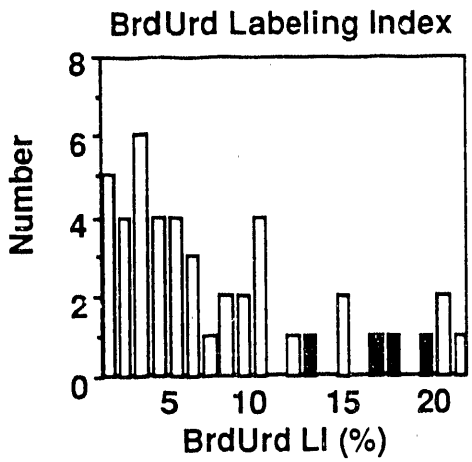
In Vivo Incorporation of BrdUrd to Measure S-phase Fraction in Breast Cancers

To date, most information about S-phase fraction come from either *in vitro* labeling experiments, in which a portion of the tumor is incubated *in vitro* with DNA precursors (either tritiated thymidine or BrdUrd) to give cellular DNA labeling index, or else indirectly using flow cytometry to estimate the fraction of the cells having an S-phase DNA content. A third approach utilizes *in vivo* labeling of tumor DNA with BrdUrd, which is given to the patient prior to removal of the tumor (3, 4). In this approach, the BrdUrd labeling is a direct measure of those cells in DNA synthesis immediately prior to surgery. Thus, it avoids uncertainties associated with *in vitro* incubation or with analysis of DNA histograms.

Patients with breast cancer at UCSF are accessed successively into the study. Whenever possible, BrdUrd is administered to patients (with their informed consent) in order to label their tumors *in vivo*. BrdUrd (200 mg BrdUrd per sq meter) is administered intravenously as a short infusion about one hour before the blood supply to the tumor is interrupted by the surgery. Following surgery the tumor is fixed in either ethanol or formalin, and then is embedded in paraffin following normal pathologic procedures.

The BrdUrd labeled cells are detected immunocytochemically in sections cut from paraffin-embedded tissues. The DNA is partially denatured and the DNA containing BrdUrd is identified using the IU-4 monoclonal antibody (Lawrence Livermore National Laboratory) and immunoperoxidase staining. Two thousand tumor nuclei are counted. The BrdUrd labeling index is expressed as the percent of counted nuclei that stain positively.

FIGURE 1.



The distribution of BrdUrd labeling index for the first 45 patients in our study is shown in Figure 1. The dark boxes indicate the four patients in this series who had either recurrence or died within one year of the primary treatment. Note that, of the ten cases that had very high labeling indices (> 10%), four had recurrence. No early recurrences occurred in the other 35 cases with low labeling indices.

TABLE 1. Patients with Early Death or Recurrence

Case #	Age	Stage	Grade	ER	LI(%)	Status
029	42	III	2	+	12.2	Dead (<1 yr)
086	57	I	3	-	16.5	Recur (11 mo)
137	35	I	3	-	17.2	Recur (1 yr)
146	44	IIa	3	-	19.2	Dead (<1 yr)

Table 1 gives clinical and marker information for the four women who had early recurrent disease. It is significant that three of these had a low clinical stage and would not have been considered to be in an unusually high risk group (patients 086, 137, and 146) even though they all had histopathologic grade 3 tumors.

BrdUrd administered *in vivo* has many advantages for estimating the S-phase fraction in tumor. It is a direct and reliable method in which the whole tumor is labeled. It does not depend on tissue incubation *in vitro*, and so it is much less

susceptible to artefacts, such as penetration, that can compromise *in vitro* labeling. Furthermore, unlike estimates based on flow cytometry, it is known that the count is based only on morphologically identifiable tumor cells and so is not contaminated with debris or non-malignant cells.

PCNA Labeling Index in Bladder Cancer

We are investigating the use of an antibody against PCNA (otherwise known as cyclin) as an alternative approach to analysis of cellular proliferation (5). Unlike labeling with BrdUrd, it does not require that the tumor is labeled while the cells are still alive, and, unlike labeling with KI-67, it does not require a cryostat section but can be used with sections cut from paraffin-embedded specimens.

PCNA is a 36 kd auxiliary protein to DNA polymerase ∂. This protein is necessary for DNA synthesis and for cell proliferation. It is synthesized late in the G1 phase and during the S phase. But it has a short half-life and disappears rapidly in the G2 phase. It is not normally found in mitotic cells or in non-cycling cells. Thus, PCNA labeling normally relates closely to the S-phase fraction.

We compare PCNA and BrdUrd labeling indices in bladder tumors. Fresh pieces of tissue obtained at transurethral resection are incubated in BrdUrd solution containing fluorodeoxyuridine and hyperbaric (3 atmosphere) oxygen. After 30 minutes incubation, the tissue is fixed in ethanol and embedded in paraffin. Sequential sections are cut and stained immunocytochemically. The first section is stained for BrdUrd using IU4 antibody (Caltag). The second section is stained for PCNA using 19A2 IgM antibody (American BioTech). In each case, the labeling index is based on counting 2000 tumor nuclei.

FIGURE 2.

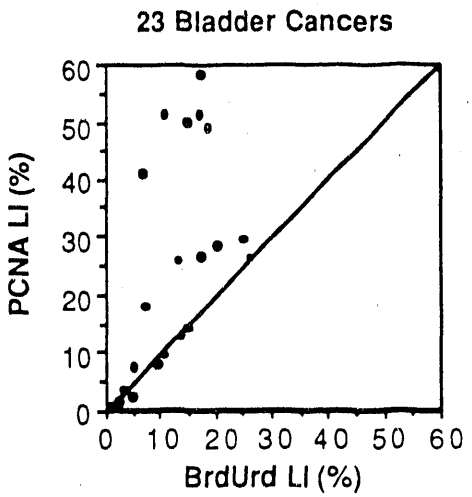
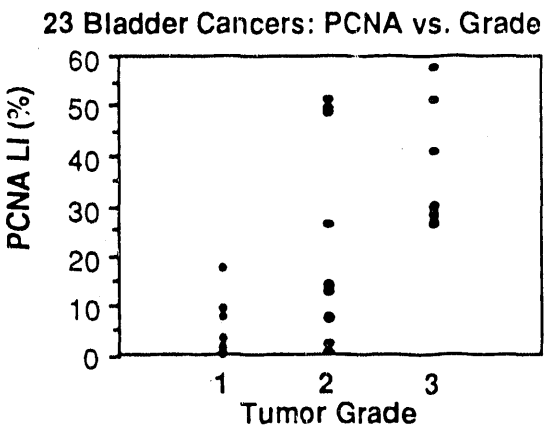


Figure 2 compares the BrdUrd and PCNA labeling indices. Most of the tumors lie close to the line of equality between BrdUrd and PCNA. However, 6 of the 23 tumors have a PCNA labeling index that is considerably greater than the corresponding BrdUrd labeling index.

FIGURE 3.



In Figure 3, the PCNA labeling index is plotted against tumor grade for the 23 bladder tumors. The results show a strong correlation between tumor grade and labeling index. All grade 1 tumors have low (<10%) labeling indices, all grade 3 tumors have

high (>10%) labeling indices, and the grade 2 tumors have the full range of labeling indices.

We conclude from these studies that PCNA is a promising marker for cellular proliferation. It shows generally good correlation with the BrdUrd labeling index, but in about 25% of the cases this correlation fails with the PCNA index being much greater than the BrdUrd index. We have not been able to follow these patients for sufficient length of time to assess the significance of a high PCNA labeling index or to know whether the divergence between the PCNA and BrdUrd labeling indices is in itself a marker of tumor behavior.

Allelic Loss in Breast Cancers

The third marker approach we are investigating is the use of molecular genetic techniques to detect genetic loss in breast cancers. Allelic loss can be detected in tumors from patients whose normal cells show allelic heterozygosity. This approach depends on using DNA probes for loci that are heterozygous by RFLP and Southern blot analysis. DNA from the tumor is compared with DNA from normal cells from the patient. Informative patients are those whose normal DNA shows differences between the maternal and paternal alleles. Allelic loss is then detected in such heterozygous patients when analysis of tumor DNA shows that one of the two bands seen for the normal DNA is missing.

We are studying allelic loss in breast cancers. Probes, obtained from the American Type Culture Collection, map to the q21-23 region of chromosome 1 and to the p13 region of chromosome 17. For each case, DNA from both the primary tumor and skin fibroblasts is analyzed by Southern blot. About half of the cases are informative for each of these probes. Ten tumors in 36 informative cases (28%) show allelic loss for the chromosome 1q locus, and 19 tumors in 34 informative cases (56%) show allelic loss for the chromosome 17p locus.

We compare allelic loss with BrdUrd labeling index for the same tumors. For the chromosome 1q21-23 locus, there is no significant difference in the means of the labeling indices between 26 tumors with no loss and 10 tumors with a loss. However,

for the 17p13 locus, there is a significant difference ($p<0.05$) in labeling index between tumors with no loss and tumors with allelic loss. All tumors without loss have a low (<8%) labeling index. In contrast, tumors with allelic loss at the 17p13 locus span the range of labeling indices from low through very high. All tumors with a high (>8%) labeling index also show allelic loss at the 17p13 locus.

These results are particularly interesting in that the 17p13 locus has been shown to be closely associated with the p53 anti-oncogene as described by Vogelstein (6). It is hypothesized that loss of heterozygosity at this locus is likely to be associated with loss of functionality of this protein, presumably by mutation of the remaining allele.

When analysis of the data is expanded to include clinical stage, only one of the six stage 3 breast cancers fail to show allelic loss for chromosome 17. BrdUrd labeling index seems to relate more to allelic loss than to clinical stage for stage 1 and 2 tumors. For the tumors with allelic loss, the stage 1 tumor and the stage 2 tumor with the highest labeling indices are from cases 086 and 146 respectively. These are two of the patients who had extremely poor clinical outcome, as reported in Table 1.

Summary

We have explored the utility of different markers as diagnostic and prognostic probes in human cancers. We find that *in vivo* BrdUrd labeling is useful for breast cancer patients and that those with a high BrdUrd labeling index have a poor prognosis. PCNA analysis provides an alternate labeling approach for cell cycle analysis. In bladder cancer it relates generally to BrdUrd labeling index but on occasions it is notably greater. The basis for and significance of this latter finding are not known. Allelic loss analysis opens up the prospect of a whole new spectrum of cancer markers that may relate directly to the underlying genetic and biological lesions. In breast cancers, we find that loss of the allelic marker for the chromosome 1q21-23 locus is unrelated to the BrdUrd labeling index. However, breast cancers that show no loss at the 17p13 locus invariably have a low BrdUrd labeling index, and those that have a high BrdUrd labeling index and poor clinical outcome invariable show loss of the 17p13 allele. As 17p allelic loss correlates with, but is not identical to, the labeling

index, it satisfies the criteria for a potentially useful marker for breast cancer prognosis.

We have presented three potential markers that can be used to supplement clinical, pathologic, and cytometric data already available on these cancer patients. The aim is to identity markers that complement existing knowledge by providing additional information about the clinical nature of individual cancers. Thus, all these markers correlate with existing markers, but there will be situations in which they deviate from it and prove to be more powerful than such existing markers. We are particularly interested in identifying markers that differentiate early stage tumors between those that do well and are cured by initial treatment and those that recur and have a poor ultimate prognosis. Whenever possible, we are using archival material on patients who have been followed and so have known clinical outcomes. However, markers such as BrdUrd labeling index and allelic loss analysis require live or frozen tissue. Thus patients who are analyzed in these studies must be followed prospectively to determine their actual clinical outcome (disease-free survival and overall survival). This is a lengthy but necessary procedure and may take many years before the clinical outcome is known for a relatively indolent disease such as breast cancer. In the meantime, potentially useful markers are evaluated against currently used predictors of outcome, such as tumor size, nodal status, and, for node negative patients, labeling index. Our studies are providing new insights into the underlying biology of breast and bladder cancers and we anticipate them leading to improved diagnosis, treatment, and overall management of patients with these cancers.

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