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Role of the Pathologist in Biomarker Studies

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Abstract Cancer chemoprevention is defined as intervention by chemical agents prior to invasion to inhibit or slow the carcinogenic process. Using surrogate endpoint biomarkers in chemoprevention studies may reduce the size, length and cost of clinical prospective randomized trials in high-risk populations. Intermediate biomarkers are measurable alterations in the tissues at risk and include differentiation, genetic composition, biochemical expression, and proliferation. Assessment is possible because invasive epithelial neoplasms are known to begin as intraepithelial proliferations with a spectrum of cellular abnormalities extending to carcinoma *in situ*. Genetic heterogeneity begins in the intraepithelial phase; a stochastic accumulation of genetic errors characterizes the progression of clonal evolution within the tumor through the process of invasion and metastasis. Pathologic features associated with this process include tumor classification as well as whether it is intraepithelial or invasive. If the process is intraepithelial, the grade and extent of the intraepithelial lesion are reported. If the neoplasm is invasive, tumor size, extent, degree of differentiation (histologic and nuclear grade), mitotic rate, vascular invasion, and lymph node involvement are evaluated. In assessing biomarkers relevant to chemoprevention, and without complete regression of the neoplasm with the chemopreventive agent or agents, measurable parameters along with histopathologic features are applicable. Three methods readily applicable for this purpose that can be applied to paraffin-embedded, formalin-fixed tissue include quantitative pathology, immunohistochemistry, and molecular biologic applications. These methods require some consistency in handling and processing the tissues under study; results may deteriorate due to a number of processing variables, including time to fixation, time in fixative, and fixative type. Quantitative pathology, including static image analysis and flow cytometry, can determine total DNA content. Using static image analysis, very small tumors can be studied. In addition, adjacent intraepithelial and invasive components of a tumor may be studied from a single slide. Steroid receptors, oncogenes, and other proteins detectable through immunohistochemical or molecular biologic methods can be quantitated by this technique as well. Cell cycle synthetic function is assayable by both methods. Flow cytometry can calculate the total percentage of cells in S-phase, or the tumor cell S-phase fraction based on the percentage of cells detected between the G_0, G_1 peak and the G_2 + M peak. A similar approach is generally not applicable with current image analysis equipment; however, cell cycle related proteins such as MIB-1 (Ki-67 associated) can be quantified. Immunohistochemical methods can employ a wide variety of monoclonal antibodies to detect oncogene related proteins, including HER-2/*neu* (c-*erb*B-2) and p53. Molecular biologic methods, including *in situ* hybridization, polymerase chain reaction, and *in situ* PCR, can have many applications when applied to paraffin-embedded tissues, including detection of viral DNA, identification and measurement of apoptosis, and defining gene deletions. © 1995 Wiley-Liss, Inc.

Key words: Immunohistochemistry, molecular pathology, quantitative pathology

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Cancer chemoprevention is defined as the use of chemical agents to intervene prior to invasion to inhibit or slow the carcinogenic process [1]. The assessment of this interventional process can include surrogate endpoint biomarkers to reduce the size, length, and cost of clinical prospective randomized trials in high-risk populations. These intermediate biomarkers are defined as measurable alterations in the tissues at risk and include differentiation, genetic composition, biochemical expression, and growth and proliferation. Assessment is possible because invasive epithelial neoplasms are known to begin as intraepithelial proliferations with a spectrum of cellular abnormalities extending to carcinoma *in situ*. Genetic heterogeneity begins in the intraepithelial phase; a stochastic accumulation of genetic errors characterizes the progression of clonal evolution within the tumor through the process of invasion and metastasis.

Currently, cohorts for Phase II chemoprevention studies include patients with both intraepithelial and invasive lesions. Intraepithelial changes considered for study include cervical intraepithelial neoplasia, colonic adenomas, bronchial atypical metaplasia and dysplasia, dysplastic leukoplakia of the oral cavity, prostatic intraepithelial neoplasia, and dermal actinic keratosis, as well as dysplastic nevi. Among invasive neoplasms, superficial bladder carcinoma and breast carcinoma have been identified [2]. Currently, no single molecular or genetic marker defines neoplasia; however, a limited set of biologic markers are available to characterize most neoplasms under study.

Gross clinical and pathologic features, including conventional histopathology, are applied in most studies to determine architectural and nuclear changes. Alterations identifying intraepithelial neoplasia in humans include seven features: increased nuclear size; nuclear hyperchromasia (increased staining); abnormal nuclear shape; nuclear pleomorphism; disordered or loss of differentiation (disordered maturation); increased mitotic activity; and abnormal mitotic figures [1]. Early changes in epithelia identifiable as precursors to neoplasia remain relatively poorly defined as to biologic behavior. For example, in studies of cervical intraepithelial neoplasia, moderate dysplasia (CIN 2) is accepted as a precursor to squamous cell carcinoma of the cervix. Although some lesions progress to invasive carcinoma, regression has been reported in more than one-third of the cases studied, and conventional histopathologic studies are unable to define the subset of cases that regress [3].

Chemoprevention studies have four general categories of biomarkers—histopathologic features, markers of cellular proliferation, measurements of cellular differentiation and/or loss, and determination of alterations in cell growth regulators, including oncogene expression and evidence of genetic instability or loss [4]. The pathologist's role in chemoprevention studies traditionally involves proper identification of the specimen received; when a histopathologic or cytopathologic interpretation is indicated, the pathologist provides appropriate gross description as well as proper fixation and preparation, including paraffin embedding, sectioning, and staining. From prepared slides, interpreting the findings includes the recognition of neoplastic or other pathologic processes, as well as proper diagnosis. Neoplasia, if identified, is classified according to accepted terminology. The pathologic features identified in epithelial tumors include tumor classification as well as whether it is intraepithelial or invasive. If the neoplasm is intraepithelial, its type, grade and extent of the intraepithelial process are reported. If invasive, tumor type, degree of differentiation, including histologic and nuclear grade, and mitotic rate are noted. Documentation includes tumor size (diameter and depth of invasion and/or thickness), as well as the extent of the neoplastic process and involvement of adjacent structures. The presence or absence of vascular space involvement by the tumor must be noted. In addition to tumor type, other tumor features including histologic grade, nuclear grade, and mitotic count, recorded as the number of mitotic figures identified in 10 high-power fields, may also be applicable. Other features may be noted, such as the character of the tumor's infiltrative margins (pushing or finger-like), the presence or absence of tumor necrosis, degree and type of inflammatory cell infiltrate, adequacy of excision, and the presence or absence of tumor at the surgical margins. In all invasive tumors, the assessment of regional lymph node involvement and evaluation of regional or distant metastasis need to be documented.

In assessing biomarkers relevant to chemoprevention, the definitive biomarker would be the

absence of development of a neoplasm. What are the minimum, necessary and sufficient markers to define neoplasia? Without complete tumor regression, relevant histopathologic features would depend upon whether or not the tumor was intraepithelial or invasive as well as on its tumor type. Regressive features associated with chemoprevention are assumed to reverse progressive pathologic features of human neoplasia; for example, deeply invasive tumors were preceded by more superficial invasion and intraepithelial neoplasia. It is also assumed that the pathologic features identified in chemoprevention animal model studies will be reflected in the human tissues and neoplasms under study. It is further assumed that changes related to chemopreventive agents will be quantifiable by current pathology methods.

Intraepithelial changes of regression related to a chemopreventive agent may be observed clinically and cytologically if the area under study is readily accessible and visible—for example, in the cervix or oral cavity. Within malignant tumors, effects of chemopreventive agents may include changes in tumor size and dimension. This is usually the most reliable finding of response to antitumor treatment in experimental animals and in a clinical setting, and would also indicate response to chemopreventive agents. Various non-interventional techniques can evaluate changes in tumor size, including radiographs, ultrasound, and nuclear magnetic resonance. Non-interventional assessment of the cellular features of the tumor can be obtained using washings, cytologic brushings, or fine needle aspiration for cytologic sampling. Directed incisional or needle biopsy is appropriate for histopathologic evaluation. Improvements in needle biopsy devices and localization permit biopsies and fine needle aspirates to be performed on tumors of 1 cm or less in diameter. Assessment of regression applicable to observational clinical-pathologic features include some or all of the findings summarized in Table I.

Identified morphologic changes can be classified in a quantitative manner to reflect the response identified, as described in Table II.

The assessment of histopathologic features and changes within a neoplastic process can be complemented by measurement and assessment of genetic markers. Methods to measure these markers include applying quantitative pathology, immunohistochemistry, and molecular biology. These methods require consistency and quality control in handling and processing the tissues under study because a number of handling and processing variables can cause results to deteriorate, including time from excision to fixation, time in fixative, and fixative type. Inappropriate fixatives, air drying, and excess heat can prevent assessment of most markers. Pathologists assume responsibility for these aspects of study.

GENETIC BIOMARKERS: DNA PLOIDY ANALYSIS

Quantitative pathology methods for DNA ploidy analysis include static image analysis and flow cytometry. Both methods can quantitate tumor nuclear DNA to determine DNA content.

Flow cytometric analysis has been employed extensively to quantitate total DNA content in fresh and paraffin-embedded tumors. The diagnostic and prognostic value of this technique has been demonstrated for a variety of tumors. Recent trends in early breast tumor diagnosis has resulted in smaller tumors being identified. In addition, improved needle biopsy devices, and the ability to needle biopsy non-palpable breast tumors employing stereotactic methods, has greatly reduced the applicability of flow cytometric techniques due to the small size of needle biopsy specimens.

Because of problems of both methodology [5–7] and interpretation [8,9] of flow cytometric DNA histograms generated from paraffin-embedded tissue, image cytometry has been proposed as an alternative approach. Static image analysis has some advantages over flow cytometry in that very small tumors, or very small samples of tumor, can be studied with both intraepithelial and invasive components of a single tumor from a single slide. Total DNA quantitation by image cytometry has been performed on paraffin-extracted cell nuclei [10] and thin sections. The advantage of thin sections, particularly in the context of Phase II chemoprevention trials, is that tissue structure is preserved, and small foci of tumor cells can be examined. The disadvantage of thin sections is that cut nuclei, rather than whole nuclei, are measured. Algorithms designed to estimate DNA content of whole nuclei from measurements made on thin sections have been described [11,12]. However, these al-

TABLE I. Morphologic Features Expected With Regression of Intraepithelial and Invasive Epithelial Neoplasms

Clinical features:
- Reduction in size or extent of the intraepithelial or invasive neoplastic process
- Change in the external appearance of the neoplasm tending toward normal

Architectural changes:
- Loss of cellular disarray
- Cellular stratification within stratified epithelium
- Orderly arrangement of basal cells in relation to the basement membrane
- Return of normal functional cells and differentiation for the epithelium
- Partial or complete return of normal architectural features and stromal epithelial relationships (histologic response)*

Nuclear changes:
- Loss of nuclear hyperchromasia and pleomorphism
- Reduction in nuclear size
- Orderly nuclear maturation
- Mitotic activity limited to the basal layer
- Normal mitotic figures
- Degeneration of neoplastic cells, including apoptosis

Stromal changes:
- Inflammatory cell infiltrate involves the neoplastic cells
- Loss of stromal desmoplasia*
- Loss of stromal vascularity about and within the tumors (loss of angiogenesis)*
- Regression of the infiltrative stromal component of the neoplasm* including lymphocytic infiltrate within the tumor, foreign body giant cell reaction involving the tumor cells with loss of tumor cells, calcification associated with tumor cell necrosis, tumor necrosis

* Changes identified in invasive neoplasms

TABLE II. Classification of Histologic Response Within Epithelial Tumors

A.	Complete response, no tumor is identified.
B.	Partial response, a lowering of any of the grading indicators.
C.	No response, no changes are identified between the primary and subsequent tissues.
D.	More severe disease is observed by an increase in any of the grading indicators.

gorithms have not been tested thoroughly using human tumor tissue. The DNA histogram produced from tissue sections must be interpreted with great caution; tissue section correction does not resolve the problem [13]. Thin sections and paraffin-extracted nuclei from breast tumors were used for total DNA content analysis (ploidy assessment) by image cytometry; although all tissue was sectioned at 5 µm for Feulgen staining, software corrections of 4.5 µm, 5.0 µm, 5.5 µm, 6.0 µm, 6.5 µm, 7.0 µm, and 7.5 µm were examined using the CAS 200 image cytometer. Results were compared to those obtained using flow cytometry of paraffin-extracted nuclei. As Table III demonstrates, concordance between flow cytometry and image analysis of tissue sec-

TABLE III. Concordance by Ploidy Between Flow and Image Cytometry Using Nuclear Extract and Tissue Sections [13]

Ploidy Status	Nuclear Extract (%)	Tissue Section (No correction %)
Diploid	100	90
Tetraploid	81	27
Aneuploid	100	77

tions varied considerably within each ploidy group, depending on the degree of software thickness correction. The best overall concordance was observed using no correction or a software correction of 6.5 µm. Better concordance was observed for diploid and aneuploid tumors than for tetraploid tumors [13].

The poor results observed for total DNA content analysis on tissue sections are related, in part, to the lack of a reliable method for determining the true thickness of the section. The section correction algorithm employed by the CAS 200 [11] calculates the DNA content of a whole nucleus from the estimated fraction of a cut nucleus contained in the section. This calculation is based on the tissue section thickness keyed into the software by the operator. The selected section thickness is chosen quite arbitrarily. The section thickness set on the microtome is not suitable for correction, as microtomes tend to underestimate the actual section thickness. In our study, poor correlation was observed between results of flow cytometry and image analysis of tissue sections corrected for the thickness set on the microtome [13].

The foregoing discussion shows that nuclei extracted from paraffin are preferable to tissue sections for DNA quantitation using image analysis. In Phase II chemoprevention trials, however, small sample size precludes the use of a nuclear extract. When tissue section image analysis must be used to establish total DNA content, we advocate interpretation of uncorrected DNA histogram data using a binary classification scheme [14]. Histograms are classified as DNA-diploid or DNA-aneuploid. The DNA-tetraploid classification is avoided because of poor concordance (range: 18–36%) between flow cytometry and image analysis of tissue sections in this group. Using this binary classification scheme, we demonstrated excellent agreement between image cytometry of bladder washings and corresponding biopsies from patients with transitional cell carcinoma of the bladder [15].

CHROMOSOME-SPECIFIC ANEUPLOIDY

Chromosome-specific aneuploidy can be detected using non-fluorescent chromosome-specific DNA probes and *in situ* hybridization techniques. Specific α-satellite DNA probes can target numerical chromosome abnormalities in tissue biopsies of individuals enrolled in chemoprevention trials. This technique can be performed on paraffin sections of tissue biopsies and applied to malignant and premalignant lesions. Application of chromosome-specific probes to tissue sections is difficult because thin sections often do not encompass the whole nucleus. Through the use of quantitative image analysis, however, hybridization signals can be enumerated automatically with greater statistical power in a large number of nuclei.

IMMUNOHISTOCHEMICAL TECHNIQUES

Immunohistochemical methods, primarily performed on formalin-fixed, paraffin-embedded tissue, can be applied to a widening variety of proteins, antigens, and other tumor-related substances. Of these, steroid receptors and oncogene-related proteins are of special interest. Immunohistochemical methods employ a wide variety of mono- and polyclonal antibodies to detect oncogene-related proteins, including p53.

p53

Missense point mutations of the p53 gene are generally accepted as a common events in human neoplasia. A strong correlation has been observed between p53 mutations and nuclear accumulation of the p53 protein. Several monoclonal antibodies recognize the p53 protein in formalin-fixed, paraffin-embedded tissue. The major advantage of using immunohistochemistry to evaluate p53 oncoprotein aberrations is its high resolution at the single cell level. Immunostaining permits quantitation of the proportion of

positive tumor cells in each lesion, as well as subclassification of p53-positive lesions according to staining pattern. The identification of distinct p53 staining patterns [16] raises the issue of uniform reactivity of antibodies to p53 oncoprotein. For instance, the antibody Pab 1801 is known to cross-react with granular cytoplasmic structures present even in genetically p53-negative cells [17]. We investigated the relationship between expression of the monoclonal antibodies Pab 1801 and BP53-12 in 38 cases of squamous carcinoma of the vulva. Immunostaining was quantitated by image cytometry. Good correlation was observed between the positive nuclear area of p53 immunostaining for the two antibodies ($r = 0.731$; $p < 0.001$).

Quantitative p53 scores tended to correlate with the histologic type of vulvar carcinoma. Currently three types of vulvar squamous cell carcinoma can be subclassified by histologic features: basaloid type; warty type; and well-differentiated, keratinized type. The warty and basaloid types are often associated with human papillomavirus, usually type 16. These tumor types are typically found in younger women and are commonly associated with vulvar intraepithelial neoplasia (VIN). Well-differentiated carcinoma is more commonly found in older women and is not associated with human papillomavirus or VIN. Quantitative p53 studies identify a higher score in well-differentiated squamous cell carcinomas, as compared to basaloid or warty types. This was consistent whether the Pab 1801 or BP53-12-1 antibodies were used.

PROLIFERATIVE BIOMARKERS

The standard method for assessing tumor proliferation in the clinical laboratory setting employs flow cytometry. However, flow cytometric estimation of S-phase fraction from formalin-fixed tissue suffers from various technical limitations [5]. In the context of Phase II chemoprevention trials, small sample size prohibits the use of flow cytometry. DNA image cytometry is also not applicable for estimating proliferation due to the relatively small number of tumor cells currently analyzed using this technique (150 cells by image cytometry compared to an average 25,000 cells by flow cytometry). Therefore, proliferation is best assessed using immunohistochemical markers (MIB-1) or AgNOR quantitation.

PCNA and Ki-67 (MIB-1)

A variety of monoclonal antibodies are available for studies of tumor proliferation. Proliferating cell nuclear antigen (PCNA) has been used in several studies for the immunohistochemical detection of proliferating cells. There is growing evidence, however, that a deregulated expression of this antigen can occur in tumor cells [18]. Thus, it has been suggested that PCNA be used with caution in studies of tumor proliferation [19]. Another proliferation-associated antigen detectable in paraffin-embedded tissue is Ki-S1, which displays a cell cycle-expression similar to that of Ki-67. Very few studies of tumor proliferation have employed the Ki-S1 antibody, although one study suggests that Ki-S1 positivity is significantly correlated with the S-phase fraction in breast carcinomas. Immunohistochemical analysis of proliferation can also be accomplished with the Ki-67 monoclonal antibody. Unfortunately, this antibody requires fresh frozen sections and cannot be used for retrospective studies that employ formalin-fixed, paraffin-embedded tissue. Recently, however, a Ki-67 equivalent murine monoclonal antibody, designated MIB-1, was generated which can detect tumor proliferative activity in routinely processed tissue [20]. Immunohistologic detection with this antibody requires antigen retrieval by microwave oven heating [21].

The study of proliferative activity in gynecologic tumors has recently attracted the interest of investigators in search of a biological indicator of tumor aggressiveness that might be more reliable than the classical prognostic indicators. In most studies, results of Ki-67 immunostaining are expressed as the number of positive cells per total number of tumor cells counted. Recently, however, we hypothesized that the pattern of distribution of Ki-67-positive cells may be as important as the Ki-67 count [22]. In a study of Ki-67 expression in vulvar carcinoma, we observed two patterns of Ki-67 immunoreactivity: a diffuse distribution of Ki-67-positive nuclei within the tumor mass, and a localized distribution of Ki-67-positive nuclei staining in predominantly basilar components of the tumor aggregates. The diffuse pattern of Ki-67 reactivity tended to be associated with a shorter survival time compared to the localized pattern.

Nucleolar Organizer Regions

Nucleolar organizer regions (NORs) are loops of ribosomal DNA that occur in the nucleoli of cells. NORs can be visualized by a one-stage argyrophil (AgNOR) method, which stains NOR-associated proteins. This technique has proven useful in various fields of tumor histopathology, particularly in the study of non-Hodgkin's lymphomas (NHLs). Evaluation of AgNORs is usually accomplished by counting the silver-stained nuclear dots that are approximately 2–3 μm in diameter. Unfortunately, the AgNOR dot count appears to be influenced by sampling method.

AgNOR quantitation by image cytometry has proven useful. Recently, we proposed a method of AgNOR quantitation that is independent of AgNOR dot counting, less subjective and tiring, and invariant to sampling technique [23]. This method is based on image quantitation of the ratio of AgNOR area to nuclear area, which we have termed NOR percentage nuclear area (NPNA). We have demonstrated that NPNA values derived from touch imprints and tissue sections can be compared directly.

Weeks *et al.* [24] hypothesized that NPNA best reflects the proliferative status of a cell. We recently confirmed this hypothesis by simultaneous demonstration and quantitation of Ki-67 and AgNORs in formalin-fixed, paraffin-embedded tonsil tissue [25]. Because uncertainty continues over whether or not AgNORs reflect ploidy or cell proliferation, we chose to use tonsil tissue for these studies. Tonsil provides a convenient source of proliferative and non-proliferative cells in an undeniably diploid tissue. Quantitative analysis of dual stained paraffin sections revealed a significant difference in NPNA between Ki-67-negative and -positive cells [25].

APOPTOTIC BIOMARKERS

Apoptosis is a mode of cell death characterized by specific morphological, biochemical, and molecular changes. Cell death by apoptosis is common during embryogenesis, in normal tissue and organ involution, and at the end of the lifespan of completely differentiated cells. This mode of cell death is also of interest as a surrogate endpoint biomarker for Phase II chemoprevention trials. For instance, the synthetic retinoid fenretinide (4-HPR) has been shown to induce apoptosis in susceptible cells. Although apoptosis can be measured on strictly morphologic grounds, this method tends to suffer from subjectivity and interobserver variability. The key biochemical event in apoptosis is endonucleolysis, which results in cleavage of nuclear DNA into oligonucleosome-sized fragments. This fragmented DNA can be visualized by the typical "ladder" pattern on agarose gel electrophoresis. This method, however, cannot provide information regarding apoptosis in individual cells nor relate apoptosis to histologic architecture.

In Situ End-Labelling of Fragmented DNA

In situ end-labelling of fragmented DNA has been shown to be specific for apoptosis in formalin-fixed, paraffin-embedded tissue sections [26]. The assay is based on labelling of the 3'-OH termini of DNA breaks with digoxygenin-labelled-dUTP (dig-dUTP) by exogenous deoxynucleotidyl transferase (TdT). To visualize dig-dUTP, a standard peroxidase-antiperoxidase method is employed with diaminobenzidine as the chromogen. Staining is quantitated by image cytometry and expressed as a positive nuclear area (PNA).

BCL-2 Protein

The protein encoded by the *bcl*-2 proto-oncogene has been shown to provide protection from apoptosis. Immunohistochemical detection of BCL-2 protein can be performed on formalin-fixed, paraffin-embedded tissue. A survey of a number of human tissues identified BCL-2 protein expression in sites of stem cells and long-lived cells. BCL-2 protein has also been found in the cells of various malignant and premalignant conditions. For instance, we have reported the significance of immunohistochemical detection of BCL-2 protein in gastric epithelial dysplasia [27], keratoacanthoma, and well-differentiated squamous cell carcinoma [28]. BCL-2 is expressed in squamous intraepithelial neoplasia as well as squamous carcinoma and basal cell carcinoma of the skin; however, it is only weakly expressed in hypertrophic actinic keratosis. Chronic dermatitis, psoriasis vulgaris and seborrheic keratosis are not immunoreactive for BCL-2 [29]. The expression of *bcl*-2 is sufficiently consistent to distinguish trichoepitheliomas, which may histologically mimic basal cell carcinoma but are BCL-2-

negative, from basal cell carcinoma, which is BCL-2-immunoreactive. In Phase II chemoprevention trials, the expression of BCL-2 protein may define a phenotype resistant to apoptosis induced by chemopreventive agents. Furthermore, because BCL-2 protein is expressed in cells protected from apoptosis, BCL-2 protein expression can serve as a control on results obtained by *in situ* end-labelling of fragmented DNA for apoptosis.

DISCUSSION

The pathologist involved in surrogate endpoint biomarker studies has a broad complement of immunohistochemical and molecular biologic markers, supplemented by sophisticated instrumentation, to complement conventional histopathologic methods. The application of these methodologies to the study of surrogate endpoint biomarkers requires defining, developing, and applying methods of tissue handling, fixation, sampling, and processing that will permit the accurate and reproducible quantitative and semi-quantitative application of the assay systems required in such studies. It will also require identification of markers that are reproducible and applicable to a wide variety of human tissues and neoplasms. These markers must have sufficient sensitivity and specificity to the presence or absence of the marker under study, and also be able to quantitate variations in the expression or content of the marker under the influence of treatment.

The quantitative assessments necessary to define the cellular changes produced by chemopreventive agents will require development of consistent and reproducible criterion and terminology for the pathologic and cellular features observed. The subjective problems of attempting to define various degrees of atypia, or architectural disarray, is well recognized by surgical pathologists and cytopathologists in dealing with specific situations where comparison between cases is the issue. The interpretation of regressive or progressive changes in a given intraepithelial or invasive neoplastic process are not well defined. Morphologic histopathologic features, as well as cytologic criterion applicable to changes related to chemoprevention protocols in patients with intraepithelial or invasive neoplasms, will need to be tested against current criterion to define acceptable and reproducible criteria applicable to surrogate biomarker studies.

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