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Alterations in phenotypic biochemical markers in bladder epithelium during tumorigenesis

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ABSTRACT **Phenotypic biochemical markers of oncogenesis and differentiation were mapped in bladder biopsies to investigate changes that occur in bladder tumorigenesis and to identify markers for increased bladder cancer risk. Touch preparations from biopsy specimens from 30 patients were obtained from tumors, the adjacent bladder epithelium, and random distant bladder epithelium. Markers, including DNA ploidy, epidermal growth factor receptor (EGFR), and oncoproteins, were quantified in individual cells by using quantitative fluorescence image analysis. Cluster analysis revealed the markers fell into three independent groups: (*i*) G-actin and EGFR; (*ii*) ploidy, cytology, and p185 (HER-2/neu oncoprotein) (ERBB2); and (*iii*) p300, a low-grade tumor antigen. Each marker displayed a gradient of abnormality from distant field to adjacent field to tumor. Different patterns for each marker suggested a developmental sequence of bladder cancer oncogenesis; G-actin was altered in 58% of distant biopsies (vs. 0/6 normals, $P < 0.001$), ploidy and cytology were altered in <20% of distant fields and ≈80% of tumors, and the other markers were intermediate. Patterns of EGFR and p185 suggest low- and high-grade tracks diverge early ($P < 0.05$ by Mann–Whitney *U* test for EGFR and ANOVA for p185). In conclusion, this study shows that a sequence of phenotypic changes accompanies development and progression of bladder cancers. Biochemical alterations in cells of the bladder field are often detectable before abnormal pathology, and markers previously thought to be limited to tumors were found in the field. The hierarchy of expression may be useful in identifying high-risk patients, assessing completeness of response to therapy, and monitoring and predicting recurrence.**

Decreasing future deaths from bladder cancer will require a strategy of prevention, based in part upon identification of individuals at risk for invasive metastatic disease well before it becomes symptomatic. Recognition that clinical cancer is the end-point of the underlying disease of carcinogenesis (1) suggests that appropriate intervention with individuals determined to be at risk could prevent development of life-threatening disease.

The concept of "field cancerization" or "field disease," introduced in 1953 (2), suggests that the whole field of tissue is exposed to carcinogen and is at increased risk for developing cancers, even though individual lesions are of clonal origin. The field disease theory as applied to bladder cancer has provided a central organizing theory for understanding these multifocal, frequently recurrent tumors (3–5). The normal bladder, or any other solid organ, represents a complex ecosystem of interacting epithelial and stromal cells, and years are required for the progressive subversion of growth and differentiation controls (6–8) to result in eventual emergence of cells capable of at least partial autonomous growth. Identifying specific biochemical markers for the changes resulting from the process of carcinogenesis offers an attractive approach to identifying individuals at risk for bladder cancer. The genotypic and phenotypic characteristics of a tumor represent the end product of a complex evolutionary process driven by genetic instability (9) and epigenetic factors, which tend to obscure the mechanisms and processes that were originally responsible for producing the tumor. In the presence of field disease, a tumor-bearing bladder contains cells that range the phenotypic spectrum from normal to carcinoma, and this range of phenotypes provides a model system to study markers that reflect the emergence of a malignant phenotype.

Biochemical markers of the malignant phenotype were mapped quantitatively in the bladder in individual cells obtained from biopsy specimens taken from the tumor, the adjacent epithelium, and distant epithelium to chart the sequence of biochemical changes that occur during bladder tumorigenesis. This approach models the mapping of histopathologic premalignant lesions of the bladder to predict risk (10, 11). Altered histopathology is a relatively late event in carcinogenesis, but biochemical manifestations of carcinogenesis are detectable earlier (12). Because quantitative rather than qualitative differences in gene expression and protein levels probably are responsible for most of the differences between malignant and normal phenotypes (13), quantitative fluorescence image analysis was used to quantify a panel of phenotypic markers in single cells from the biopsy specimens. Because of its visual morphologic component, quantitative fluorescence image analysis can link conventional morphologic assessment with quantitative biochemical markers at the single-cell level (14–16). The markers included quantitative fluorescence image analysis cytology (14), a combination of visual morphologic classification and identifying cells with DNA in excess of 5C (2C = amount of signal equivalent to the diploid chromosome complement), which is a marker for genetic instability; the p300 tumor-related antigen detected by the M344 monoclonal antibody (17); the differentiation-related proteins epidermal growth factor receptor (EGFR) (18, 19) and G-actin (the globular monomeric precursor protein to actin filaments); and p185, the product of the HER-2/*neu* oncogene.

METHODS

Patient Population and Sample Preparation. The patient population consisted of 30 patients with transitional cell carcinoma and 6 noncancer controls. The characteristics of the patient tumors are shown in Table 1. The controls were urologic subjects undergoing cystoscopy–biopsy for conditions other than cancer, including cystitis and benign pros-

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Abbreviations: EGFR, epidermal growth factor receptor; IGL, integrated grey level; AGL, average grey level.

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tatic hyperplasia. To obtain single cells for image analysis, "touch preparations" were made in the operating room from biopsy specimens of a tumor, the field immediately adjacent to the tumor (adjacent field), and random distant epithelium. The surface of the tissue was touched to a polylysine-coated slide, and the remainder of the tissue was submitted for routine pathologic diagnosis. The cells on the slide were fixed with 0.5% paraformaldehyde solution for 15 min, drained, and sprayed with Carbofix E cytology fixative (Stat Lab; Medical Products, Lewisville, TX) containing 1.5% (vol/vol) polyethylene. Separate forceps were used for each piece of tissue to prevent cross-contamination.

Triple-Label Immunofluorescence. The touch preparation slides were dewaxed with 95% (vol/vol) ethanol for 15 min, followed by 10 dips each in 70% and 50% ethanol and soaking in acetone for 5 min. The slides were triple-labeled for DNA by using 15 μM Hoechst 33258 (20), for p185 by using the TA1 antibody (21) directly conjugated to Texas red, and for p300 by using the M344 mouse monoclonal antibody (17) and a three-stage sequence using biotin-conjugated goat antibody to mouse IgG1 as secondary antibody and fluorescein-labeled avidin (22). With 14 of the tumors and 5 of the controls a second triple-labeled slide was prepared, labeling for DNA as above, for EGFR by using AB-1 mouse monoclonal antibody (Oncogene Science) and the same visualization system as was used with M344, and for G-actin by using DNase I (Molecular Probes) directly conjugated to Texas red (23). A corresponding negative control slide omitting primary antibodies was also prepared. All reagent concentrations were optimized to achieve saturation of cellular binding sites (22, 24). Each batch of reagent was individually titrated to determine the appropriate concentration that produced saturation and, hence, proportionality between immunofluorescence and the target biomolecule. Labeling was performed in an automated slide labeling device (Fisher Code-On). Cell lines known to be high and low expressers for each quantitative marker were also included as controls with each batch of slides.

Marker Quantitation by Image Analysis. Markers were quantified on 50–100 cells per slide by using the Zeiss IBAS image analysis system equipped for quantitative fluorescence. In tumor biopsies, regions of tumor cells were specifically analyzed, but cells were randomly selected in nontumor control specimens. Infiltrating lymphocytes were specifically excluded. The images of labeled cells were captured and the intensity of each pixel (image composing the dot) was converted to a digital grey level between 0 (black) and 256. Immunofluorescence was measured as the integrated grey level (IGL) or average grey level (AGL); AGL is the average grey level of the pixels composing an image and is proportional to the average concentration of the target molecule within a cell, while IGL is the integrated value—i.e., AGL multiplied by the area of the cell image—and is proportional to the total content of marker in each cell. The 18-3-7 line transfected with an expression vector containing *HER-2/neu* and the A431 line served as positive controls for p185 and EGFR, respectively. A large number of identical slides were prepared from a single batch of each cell line for use throughout the study. A positive and negative control slide for each marker was included and analyzed with each batch of patient samples. The IGL or AGL was corrected for background fluorescence by subtracting the mean IGL or AGL of approximately 100 cells on the negative control slide.

Scoring of Qualitative Markers. Cytology was scored by a trained cytologist (R.B.B.) and confirmed by a pathologist (K.W.M.). The presence of cells with "suspicious" morphology resulted in labeling of the sample as "positive," as did the presence of any cells with >5C DNA. The p300 marker was scored visually by two independent observers. A sample was considered positive if any positive cells were noted when the cells were labeled quantitatively at saturating concentrations of M344 antibody.

RESULTS

Distributions of Quantitative Markers. We first investigated how marker levels were distributed in normal tissues, in low- and high-grade tumors, in the fields adjacent to the tumors, and in random biopsy sites distant from any tumor. Fig. 1 shows the distributions of all the samples by biopsy site as scatter diagrams. With the exception of G-actin, there was a clear progression in the markers from the distant biopsy site to the adjacent field to the tumor. The normal tissues are not plotted on this scatter diagram because the values were all very low (see Fig. 3).

Interrelationships Among Markers. The markers were investigated for clustering and statistical independence to determine which gave information independently of other markers and which were related to each other. Cluster analysis, a statistical technique for determining which variables track each other, showed that the variables fell into three relatively independent groups; G-actin and EGFR in one cluster, cells with more than 5C DNA and p185 in a second cluster, and the p300 (M344) marker in the third. Visual morphology, being a qualitative variable not amenable to cluster analysis, was placed into the second cluster because of the close association with DNA ($P < 0.05$ by χ^2 analysis).

The measurement of multiple parameters on the same cells can be used to delineate markers that are coexpressed in the same cells and those that are expressed in different cells or that are unrelated to each other. Fig. 2 shows the mean p185 and G-actin content of cells as a function of the DNA content of the cell stratified by biopsy sites. The association between p185 with DNA content and biopsy sites is clearly evident; the p185 content increases with increasing aneuploidy in all sites (height of bars increases from front to back) and from distant biopsy to tumor biopsy (height of bars increases from right to left). The pattern for EGFR was almost identical to that of p185 (not shown). In contrast, G-actin shows a much

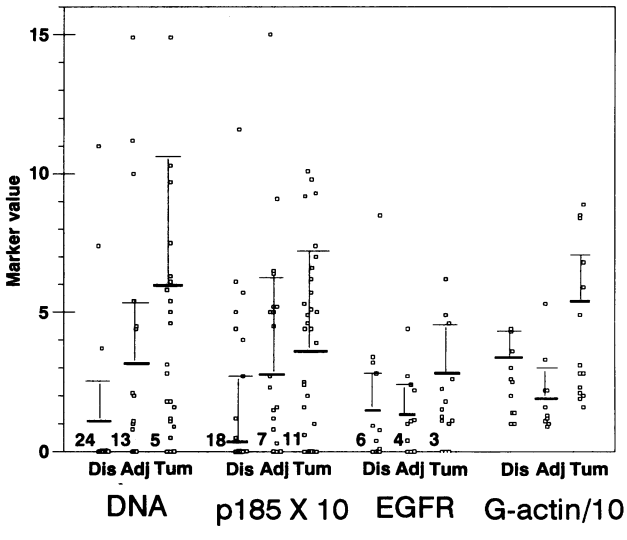

FIG. 1. Scatter diagram of four quantitative markers by biopsy site (Dis, distant; Adj, adjacent; Tum, tumor). The units for each marker are as follows: DNA = percentage of cells over 5C, p185 = mean IGL multiplied by a scaling factor of 10, EGFR = percentage of cells exceeding 2 units (see Fig. 3), and G-actin = mean AGL multiplied by a scaling factor of 0.1. The small numbers above and to the left of some of the values plotted on the *x*-axis refer to the number of observations lumped at or near zero. The thick bars represent the mean of all observations; the thin bars represent 1 standard deviation.

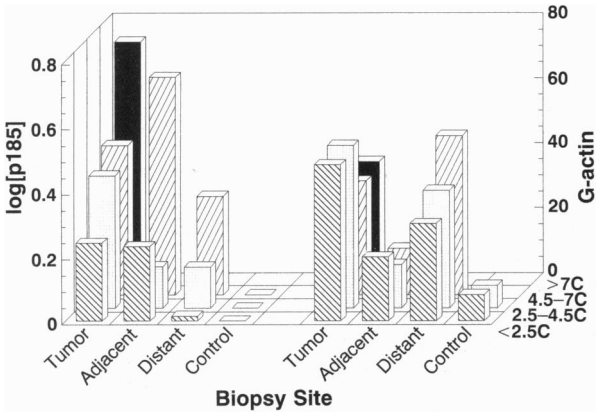

FIG. 2. Relationships between DNA ploidy and p185 or G-actin as a function of biopsy site. The cells were stratified by DNA ploidy and the mean marker contents for all the cells from all the patients were plotted as a function of ploidy.

less marked relationship to aberrant ploidy, certainly in the tumors and adjacent fields. Interestingly, the adjacent fields actually show less G-actin than the distant biopsy sites.

Analysis of Marker Distributions and Positive–Negative Scoring. Data from all of the several hundred cells in each of the groupings of patients were pooled and the distributions were plotted as cumulative frequency distributions as shown in Fig. 3 for EGFR. Criteria for positive and negative were derived from such plots, considering the different statistical distribution functions (gaussian vs. log normal), biology, and how marker measurements in normal tissues and cells derived from cancer-containing bladder are distributed.

This process is illustrated with EGFR (Fig. 3). The EGFR distribution was only slightly skewed from a normal gaussian distribution. Cells from control tissues were all below 2 EGFR units, which defined one threshold value (threshold 1) representing the upper limit of normal cells. However, the high-grade tumors appeared to have a larger proportion of very high-expressing cells than did low-grade tumors or the field samples. A second arbitrary threshold was drawn at the 90th percentile of all the cells except high-grade tumor cells (5 EGFR units, threshold 2). All the adjacent field and tumor samples showed significant ($P < 0.05$ by χ^2) increases over the normal field in cells expressing between 2 and 5 EGFR units.

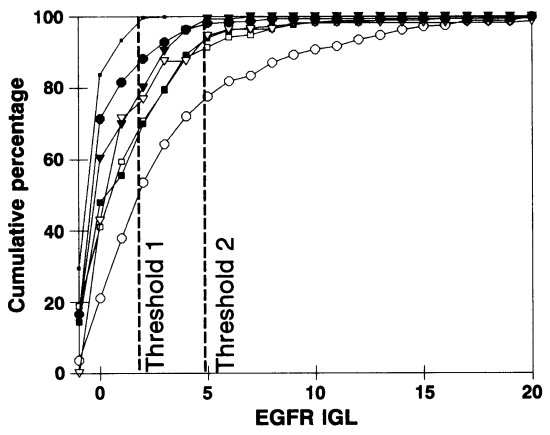

FIG. 3. Cumulative distributions of EGFR as measured by IGL for touch preparation cells. Filled symbols represent low-grade tumor-related cells; open symbols represent high-grade tumor-related cells. Squares = distant fields, triangles = adjacent fields, circles = tumors, and small squares = normal tissue.

Little, if any, p185 expression was found in the normal controls, but in all the abnormal samples an approximately log normal distribution was seen, with a progressive shift to higher mean expression from low-grade to high-grade and from field to tumor. A schema based upon comparison of mean values was therefore derived to score samples. If the mean of the sample significantly (t test at $P < 0.05$) exceeded that of the low-expressing cell line, the sample was scored as positive.

Tumor cells contained about 5- to 10-fold more G-actin than normal. These differences in means were highly statistically significant, $P < 0.001$ comparing cells from tumor and distant field by using the t test and $P < 0.01$ for cells from adjacent field compared with cells from noncancer control bladders. Samples were scored as positive if the mean of all the cells significantly (by t test) exceeded the mean of cells from normal control tissues.

As shown in Table 1, the tumors ranged from grade 1 to grade 4, with the majority being grade 2. Every tumor was abnormal for G-actin, and 26/30 were abnormal for the p300 marker, without regard to grade. Cells with abnormal DNA content were seen in 50% of the grade 1 tumors, 79% of the grade 2 tumors, and 88% of the grade 3 tumors. Also included was one sample that was scored negative by histopathology but was abnormal by all markers except for p185. In the adjacent field samples for which a pathologic diagnosis was available, 16 were scored negative or inflammatory, and 6 showed some abnormal histopathologic findings, while the distant biopsies were mostly negative (21/23). Roughly half of the histopathologically negative adjacent field biopsies were associated with abnormal measurements in one or more of the biochemical parameters, and 10–60% of distant field biopsies were abnormal, depending upon the marker.

Fig. 4 summarizes the results after each parameter was stratified into a discontinuous classification schema without respect to tumor grade. Each marker showed a statistically significant ($P < 0.01$) progression from distant field to adjacent field to tumor tissue, as shown by the increasing height of the bars from front to back. With the exception of p300, for which one of the six controls was positive, none of the markers was positive in the control bladders. Every marker was positive in some fraction of the distant field biopsies, and the markers became progressively more abnormal in the adjacent field biopsies and tumors. A large increase between distant and adjacent field was observed for both p300 and DNA ploidy ($P < 0.01$ and 0.05, respectively, by χ^2). A consistent distribution pattern for the markers was also seen, with some, such as G-actin, being consistently more abnormal than others, such as p185, regardless of the site.

Marker Distributions in Low- and High-Grade Tumors. High-grade tumors contained more EGFR ($P < 0.05$ by the Mann–Whitney U test), significantly ($P < 0.05$) larger numbers of high-expressing cells exceeding threshold 2, and more abnormal DNA morphology ($P < 0.05$ by χ^2) than did low-grade tumors or field samples. High-grade tumors produced more p185 ($P = 0.04$ by ANOVA), although the positive–negative classification was not significantly different for low- and high-grade samples. The percentage of samples positive for p300 in the fields adjacent to low- to high-grade tumors (14/17 vs. 2/7) was statistically different at $P < 0.05$ by χ^2.

DISCUSSION

Until a recent paper showing that multiple bladder tumors from five patients were clonal in origin (25), the heterochronotropicity (multiple occurrence in time and space) of bladder tumors had been deemed to be due to "field disease," which was defined as the presence of areas of premalignant altered urothelium that continued to progress at different rates,

Table 1. Summary of positive findings for tumor markers by biopsy site and pathologic diagnosis

	Group 1 (n = 36)					Group 2 (n = 19 from group 1)			
		No. of positive samples					No. of positive samples		
Pathologic diagnosis	n	Visual morphology	DNA	p300	p185	n	G-actin	Threshold 1*	Threshold 2†
Tumor (n = 30)									
Negative	1	1	1	1	0	0	0	0	0
TCC grade 1	6	4	3	5	5	2	2	2	0
TCC grade 2	14	9	11	13	7	6	6	3	2
TCC grades 3 and 4	9	8	8	7	7	6	6	6	6
Adjacent field (n = 24)									
Unknown	2	0	1	1	1	1	1	1	0
Negative‡	16	7	7	10	6	6	4	5	3
Atypical	5	0	3	4	3	4	2	1	1
Dysplasia	1	1	0	1	1	0	0	0	0
Distant field (n = 27)									
Unknown	4	0	1	0	1	2	1	0	0
Negative‡	22	3	2	8	6	9	6	5	4
Hyperplasia	1	1	0	1	0	0	0	0	0
Noncancer controls									
Negative	6	0	0	1	0	5	0	0	0

The definition of positive and negative findings is the same as used in Fig. 4. TCC, transitional cell carcinoma.
*Positive if a sample contains >5% cells with >2 EGFR units (threshold 1).
†Positive if a sample contains >5% cells with >5 EGFR units (threshold 2).
‡Includes samples with inflammatory diagnosis (five adjacent fields and one distant field).

thereby leading to recurrent and multiple tumors. Field disease was first described in terms of histopathologically defined lesions that could be physically mapped within the bladder (10) and when present were a very strong risk factor for recurrence (3, 4, 10, 11). That field disease exists seems unquestionable. The questions, therefore, seem to be (*i*) what mechanisms produce field disease, and (*ii*) is the seeding of tumor cells to produce additional tumors (25) a general phenomenon.

The differential expression of the biochemical markers and their statistical clustering suggest that a definite sequence of phenotypic changes accompanies bladder tumorigenesis. Alteration in the cytoskeleton reflected by a shift from a high level of microfilament actin to a high level of globular actin (G-actin) seems to represent an early common marker for altered differentiation that persists during cytologic dedifferentiation (26–29). The decreased G-actin content of adjacent fields compared with distant fields, however, is unexplained. The p300 marker, previously thought to be expressed mainly in low-grade tumor cells (17), is also seen to be expressed in most adjacent low-grade and some high-grade fields as well. The current data suggest that this protein represents a neoantigen related to altered differentiation (29) that may disappear during further dedifferentiation. This antigen may provide a marker for premalignant high-grade disease as well as for low-grade tumors.

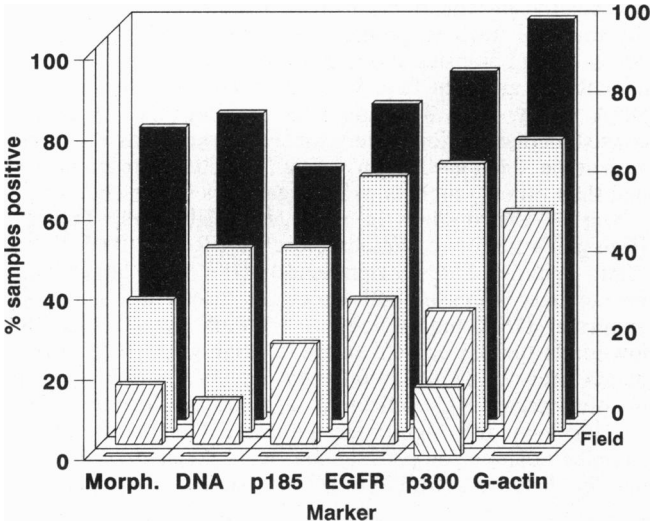

FIG. 4. Progression of biochemical markers over a cross-section of lesions using cells obtained from touch preparations of control biopsies from individuals with no evidence of cancer (front row; n = 6), from a random distant field (second row; n = 27), from the field adjacent to a tumor (third row; n = 24), and from transitional cell carcinoma of the bladder (back row; n = 30). Markers were scored by positive/negative criteria. Multiple χ^2 analysis of the progression from normal tissue to distant field to adjacent field to tumor showed the observed progressions were significant to at least $P < 0.01$.

The clustering of EGFR with G-actin rather than DNA ploidy, p185, and morphology indicates it is differentiation related as well. Normal urothelium and low-grade tumor cells exhibit an EGF-mediated down-regulation of EGFR expression that is lost in high-grade tumor cells (30). The increase seen in high-grade tumors, but not their fields, suggests the loss of this regulatory function may occur at or near the stage where a macroscopic tumor emerges from a region of altered field and may represent a key event in progression. The markers of elevated p185, altered DNA ploidy, and visual morphology clustered together and appeared late, more strongly associated with high-grade than with low-grade tumors. The elevation in p185 in nearly 70% of all tumors and 82% of high-grade tumors exceeds the 36% found by Northern analysis and 21% found by immunohistochemistry (31). These apparent discrepancies are understandable; the normalization of mRNA levels to actin mRNA may depress the ratio if actin biosynthesis is increased in transformed cells (26), and immunofluorescence can be more accurately quantitative and sensitive than immunohistochemistry (22, 32).

These phenotypic differences between low- and high-grade tumors and their fields provide independent confirmation that high- and low-grade tumors develop by relatively independent paths (33, 34). The quantitative differences in p185 and EGFR levels suggest the possibility that abnormal HER-2/neu and EGFR in either the field or the tumor may be markers for elevated risk of progression. The major advantage of measuring phenotypic as opposed to genotypic markers is

that epigenetic effects are represented and a much smaller number of phenotypic markers is likely to be needed.

Four nonexclusive mechanisms may produce field disease: (*i*) hypersusceptibility of all the cells in a bladder resulting from inherited heterozygosity for a defective suppressor function (35, 36), (*ii*) widespread genotypic changes from carcinogenic exposure affecting a significant portion or all of the bladder epithelium that increases the pool of cells immediately susceptible to ultimate carcinogenic conversion (37), (*iii*) altered growth and differentiation resulting from alterations in the complex tissue ecology induced by cytokines secreted by tumor cells (38), and (*iv*) "seeding" of either tumor or dysplastic cells from a single precursor lesion (25). The findings in ref. 25 have probably been overinterpreted as disproving the field disease theory. That tumors can seed from exfoliated cells has long been suspected by surgeons, but whether this is an occasional or a common occurrence is not clear. Were seeding to occur very early during the dysplastic stage, field changes might represent seeding of premalignant cells with resulting alteration of the cytokine ecology of the bladder. Crucial experiments to distinguish among these possibilities could include demonstration of clonality of recurrent tumors separated in time or of dysplastic lesions from different sites within the bladder.

The action of the process of carcinogenesis is visible in the form of measurable biochemical changes in the bladder that emerge before characteristic altered cellular morphology. Measurements of profiles of phenotypic markers offer considerable promise in determining individual risk of bladder cancer, and quantitative fluorescence image analysis may offer an automated approach for multiple marker measurements at the cellular level that is analogous to the widely used automated serum profiles. Both the number of abnormal cells and the quantitative elevation in the marker are significant factors in risk assessment. The approach used in this study of identifying a differential distribution along the continuum from normal to malignant and of cluster analysis is a powerful one for selecting markers for analysis. We suggest that three markers, one from each cluster, namely G-actin, p300, and DNA ploidy, might be an effective tool for detecting elevated bladder cancer risk because this combination of markers embodies acceptable sensitivity and specificity both for detecting clinical cancer and for identifying the process of carcinogenesis. Generally, screening for positive markers in exfoliated cells is noninvasive, and identification of field changes may increase the sensitivity of testing for preclinical disease. Together these might make population screening for bladder cancer and the stratification of individuals labeled at high risk by epidemiologic risk factors (39) practical as well as simplifying monitoring of patients for therapeutic response and recurrence and targeting individuals who require aggressive interventions or chemoprevention (24). We suggest that by using quantitative phenotypic marker profiles the way is now open to investigate the cancer risk faced by an individual.

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