Journal of Cellular Biochemistry, Supplement 16I:93–110 (1992)

Intermediate Endpoint Biomarkers for Chemoprevention

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Abstract The understanding of intermediate endpoint biomarker expression in relation to the sequential events in bladder tumorigenesis establishes a useful approach for evaluating chemopreventive agents. Biomarkers may be genotypic or phenotypic and function as biomarkers of susceptibility, exposure, effect, or disease. This paper reviews several years of research on biomarkers and their use in monitoring chemoprevention therapy. In initial animal experiments, mice were dosed with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (OH-BBN) while co-administering *N*-(4-hydroxyphenyl)retinamide (4-HPR). 4-HPR did not statistically reduce tumor incidence, but did affect tumor differentiation and, consequently, nuclear size and DNA ploidy. These results suggest that nuclear size and ploidy may function as intermediate endpoint biomarkers of effect for oncogenesis and that epigenetic as well as genetic mechanisms may be primary in the oncogenic process. Early biomarkers of effect which occur prior to genetic effects or chromosome aberration may portend a higher probability of being modulated by differentiating agents such as retinoids. *In vitro* studies demonstrated that RPMI-7666 cells cultured with a phorbol ester tumor promoter (12-*O*-tetradecanoyl-phorbol-13-acetate) could be redifferentiated with 13-*cis*-retinoic acid and dimethyl sulfoxide (DMSO). F-actin, a cytoskeletal biomarker with a presumed function in the epigenetic mechanisms of carcinogenesis, could also be normalized in HL-60 cells treated with 4-HPR or DMSO.

A clinical evaluation of F-actin in patients with varying degrees of risk confirmed the value of F-actin as a differentiating biomarker useful for bladder cancer risk assessment. The clarification of when the phenotypic changes of F-actin occur in the oncogenic process was achieved when a variety of biochemical changes were mapped in the patients with bladder cancer. These studies confirmed that G-actin, a reciprocal form of F-actin, is increased relatively early in bladder cancer oncogenesis when multiple biomarkers are quantitated in the field, adjacent area, and the tumor. Comparison of each individual biomarker's expression from field, adjacent to tumor, and tumor, and subsequent cluster analysis of these biomarkers, indicated that the possible sequence of phenotypic expression of biomarkers in bladder cancer oncogenesis is from G-actin, to p300 antigen, to epidermal growth factor receptor (EGFR), to p185 (*neu* oncogene product), to DNA aneuploidy and, finally, to visual morphology. To date, a battery of three biomarkers, G-actin, M344, and DNA, with routine cytology has been used to monitor eleven patients receiving Bacillus Calmette-Guerin (BCG) immunotherapy and eight patients clinically free of bladder cancer (negative cytology and biopsy) who were treated with the differentiation agent, DMSO. These results indicate that G-actin may be a useful biomarker for evaluating the efficacy of chemopreventive agents.
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Key words: bladder cancer, chemoprevention, F-actin, G-actin, intermediate biomarker, intermediate endpoint biomarker

The assessment of individual risk for cancer is rapidly being transformed by improved understanding of the molecular basis of tumorigenesis and by refinements in molecular techniques and instrumentation [1]. Intermediate endpoint biomarkers describing individual alter-

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ations in cells are replacing epidemiologic descriptors applicable to groups [2–6]. Biomarkers for key steps in carcinogenesis may provide surrogate endpoints applicable to the design of chemoprevention programs [7–9]. Intermediate endpoint biomarkers relevant to chemoprevention trials may include those related to exposure, susceptibility, effect, and disease [7,10–12]. Understanding biomarkers as they relate to the events that occur in the oncogenic process is primary to applying them in chemoprevention strategy [9]. Selection of biomarkers should consider the underlying biology, where possible, with an understanding of the sensitivity, specificity, and validity of available methods [13,14]. Table I compares the major methodological approaches to measuring molecules.

Integrating intermediate endpoint biomarkers into clinical trials requires consideration of several factors—availability of adequate samples, invasiveness of the sample collection method, and appropriate methods for sample preparation and transportation [14]. In the context of this forum, our goal is to determine which cohorts of patients who are at risk for recurrent bladder cancer are suitable candidates for evaluating chemopreventive agents. Biomarkers are useful for stratifying patients at risk for recurrence and for demonstrating the efficacy of chemopreventive agents.

During the course of this conference, significant discussions have been devoted to the classification of biomarkers in relation to their functional status. Classification may also be studied in relation to molecular class, including DNA [13–15], RNA, or protein gene products with their associated lipid or carbohydrate functional groups [10,11]. Each class has certain advantages for the study of intermediate endpoint biomarkers. For example, the stability of DNA in paraffin-embedded tissues facilitates retrospective studies but has the disadvantage that genes may not be transcribed or translated. RNA is ideal for determining if a gene is transcribed, but there is no certainty that it reflects the functionality of the gene products. Since many of the subtleties of carcinogenesis are related to quantitative differences in normal gene products, and these products are the functional entities in growth regulation, proliferation and metastasis [3], our personal bias is to concentrate on the functional proteins.

QUANTITATIVE FLUORESCENCE IMAGE ANALYSIS FOR BIOMARKER RESEARCH

Carcinogenesis is the disease process with cancer as the endpoint, and the most effective control of cancer could be treating the early stages when reversibility may be possible [16]. Such a system is analogous to preventing myocardial infarcts by treating the underlying coronary artery disease by lowering cholesterol and lipoprotein levels through diet or drug therapy. Evaluating the effects of sustained low-level insults presents a primary problem in toxicological and biomarker research, particularly when complicated by the marked genetic polymorphism or previous exposures that exist in human populations. These exposures and genetic differences, whether endogenous or exogenous, may markedly influence individual risk assessment. Nowhere is this more true than in the urinary tract. Most of the carcinogens entering the body exit through the urinary tract, and individuals and populations vary widely in the expression of enzymes that metabolize such compounds [17,18]. In return, the study of the urinary tract benefits the researcher because cells from most of the tract are exfoliated into the urine where they are accessible by noninvasive means.

A sensitive method employed in recent years is quantitative fluorescence image analysis (QFIA) which, theoretically, can detect as few as 300 molecules of fluorescent probe per cell. Exfoliated urinary cells or cells obtained by fine needle aspiration (FNA) can effectively function as a microcuvette for analysis of xenobiotic substances, DNA alterations, or changes in gene products (*i.e.*, proteins). Just as multiple biomarker profiles can be obtained on serum samples for diagnostic purposes, similar measurements can be made at the single cell level. Automated image analysis systems can screen samples and identify potentially abnormal cells which exceed defined size-brightness thresholds or cell frequencies. Artifacts which bypass machine rejection can be eliminated visually by designing a human observer-interactive system [19]. In the future, neural networks using artificial intelligence may replace this human observer [20].

The QFIA approach employs a TV camera attached to a fluorescence microscope and an

TABLE I. Methods of Detecting Molecules and Mutations With the Approximate Lower Limits of Detection

Nucleic Acids	Lower Limit
NA/Rad	3×10^4 molecules
DNA/Southern/Rad	1×10^6 molecules
DNA/PCR	10 molecules
RNA/cDNA/PCR	100 molecules
Mutations	
DNA/PCR/SSCP	1/100 relative abundance
Proteins in Solution	
ELISA/RIA	pg–ng
Electrophoresis/Blot/Rad	1–10 pg
Electrophoresis/Blot/Enzyme	10–100 pg
Photometric	1 μM
in 1 ml	1 nmol
in 1 μL	1 pmol
Proteins in Cells	
Fluorescence	300 mcle
Enzyme	Not quant.

NA: nucleic acid
Rad: radiometric
SSCP: single strand conformational polymorphisms
PCR: polymerase chain reaction

image analysis system that measures features of cells. The optics and electronics are optimized so that the system is quantitative, *i.e.*, light intensity is proportional to fluorophore concentration. The system is programmed and standardized to make biochemical and immunochemical measurements with multicolored fluorescent probes. Fluorescence offers several advantages over absorption dyes. The probes can be used at lower concentrations and are thus more selective for the molecules of interest; the relationship between light intensity and concentration is linear, not logarithmic; and unlike absorption probes, the binding between probe and molecule is frequently stoichiometric. Figure 1 shows the results obtained by QFIA measurement of the *neu* oncogene protein (p185) as compared to a parallel ELISA. The result shows a linear relationship between mean fluorescence per cell and p185 protein content. This method allows the p185 content of individual cells selected by morphology (or other criteria) to be measured in a heterogeneous cell population. Combinations of biomarkers on single cells may yield significantly more information than a single biomarker. Measurement of multiple fluorescence probes on single cells is now possible with increased speed and careful attention to cellular detail. The IBAS (Roche Biomedical Laboratories, Inc., Elon College, NC)

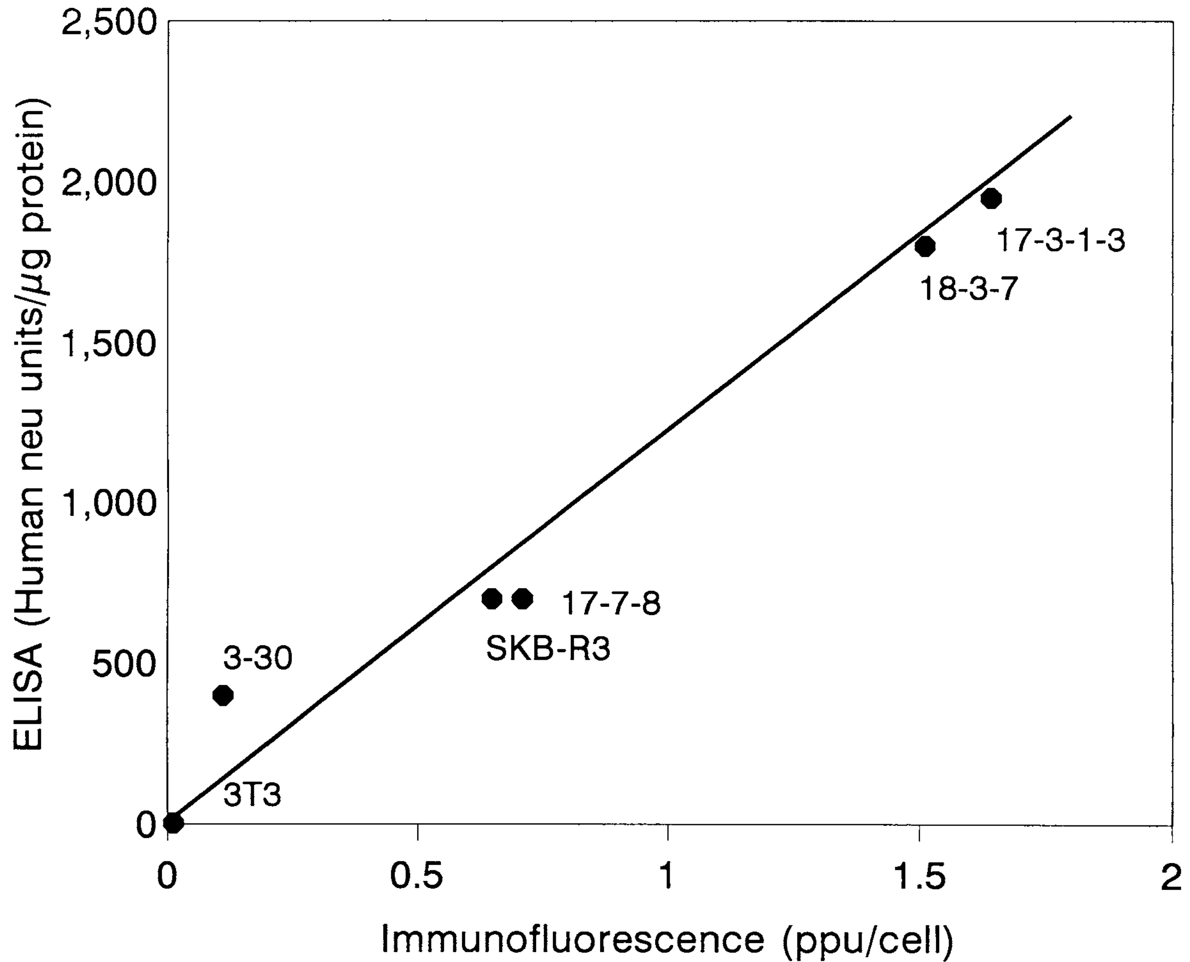


Fig. 1. Comparison of ELISA and immunofluorescence. The indicated cell lines were grown to high density and split. One part of the sample was analyzed by immunofluorescence using the TA1 antibody; the mean immunofluorescence of 200 cells was measured. The remainder of the sample was analyzed by ELISA against a standard of purified p185 protein. The 17–3–13, 18–3–7, 3–30 and 17–7–8 lines are 3T3 cells transfected with plasmids containing the intact human p185 protein, or truncated versions (3–30) with promoters yielding different levels of expression. SKB-R3 is a breast tumor line.

ppu/cell = phosphoparticle unit/cell

imaging system currently used in our laboratory has been programmed to scan a single slide with 10,000 cells within 20 minutes using multiple biomarkers.

Our laboratory is building on the concept introduced by Ploem, West and others [21–23] that early alterations in premalignant cells can be detected. Two different approaches have been employed to extract this information: equilibrium binding studies with fluorescent probes with known stoichiometry, and studies with absorption dyes which frequently are NOT stoichiometric [5,13,14]. Under appropriate fixation conditions, equilibrium binding can usually be achieved. Figure 2 demonstrates such a reaction in which two dyes bind stoichiometrically to DNA. Note the plateau which occurs prior to the quenching or significant dye–dye interactions. Determination of multiple biomarkers on single cells using antibodies is more complex. The sensitivity of the assays will reflect the purity of reagents, saturation of the

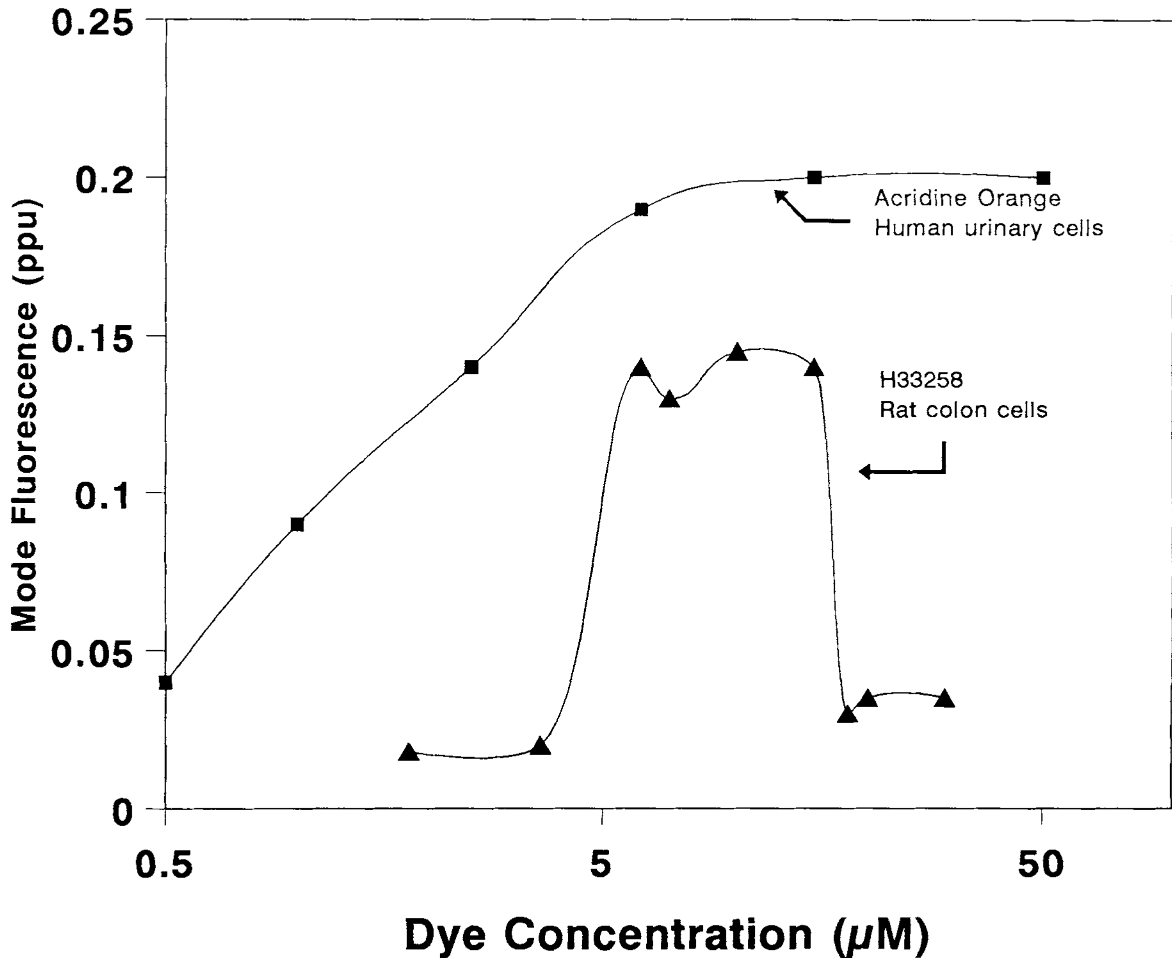


Fig. 2. Saturation of dyes. The mean immunofluorescence of 100–200 cells labeled in an excess of dye at the indicated concentration was determined. Both dyes show saturation at the plateau, but H33258 exhibits dye–dye interactions at high concentrations that lead to the drop in fluorescence.

binding sites, and the subtraction of background fluorescence. Once these conditions are established, it should be possible to extend the methods to other anatomic sites with many different reagents.

DNA as an Intermediate Endpoint Biomarker

Original experiments in our laboratory evaluated the use of QFIA with concurrent cytology as an intermediate endpoint indicator for chemoprevention studies [24]. Table II summarizes the design of a study of *N*-(4-hydroxyphenyl) retinamide (4-HPR) in the *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (OH-BBN)-induced rat bladder carcinogenesis model. The difference in total number of bladder tumors between the treated and untreated groups following conventional histopathology was only significant at p = 0.10 [25]. However, when QFIA was performed on bladder wash samples, highly significant differences were seen in the cytologic characteristics of cells from the animals treated with 4-HPR, as defined by nuclear size and DNA determinations. Exfoliated cells scored as visually suspicious had a mean DNA content of 10.9C in the carcinogen control group compared to 5.9C in the OH-BBN group treated with 4-HPR. (Note: The normal nondividing cell has a DNA complement of 2.0C.) In addition, the mean nuclear area was reduced from 419 to 270 μm^2 in cytologically suspicious cells from rats treated with

TABLE II. Experimental Design: Number of Test Animals by Carcinogen and Retinoid Treatment Status [25]

		Retinoid (4-HPR)		
		+	–	Total
Carcinogen	+	36	107	143
(OH-BBN)*	–	40	40	80
	Total	76	147	223

* 2×/week for 8 weeks, ig

4-HPR. 4-HPR treatment resulted in a mean shift in DNA content of atypical bladder cells which was significant but less marked: 4.4C vs. 3.7C (Tables III and IV).

This study demonstrates that QFIA effectively detected cytological, biochemical, and morphometric changes in exfoliated bladder wash cells. This approach may be useful for monitoring the efficacy of chemopreventive agents. In the study described, the effect of retinoids on cellular biomarkers was clearly statistically significant, while conventional tumor counting only achieved significance at $p = 0.10$. In contrast to the chronic low dose exposure in humans, OH-BBN was administered to mice in high doses over a relatively short interval, affecting DNA primarily through mutations. The main function of retinoids may be to influence epigenetic mechanisms of carcinogenesis through cytoskeletal effects. Currently, it is our opinion that chemopreventive agents such as 4-HPR would be more effective in correcting intermediate endpoint biomarker changes which occur early in bladder cancer tumorigenesis rather than those which occur later.

Tumor-associated Antigens

Tumor-associated antigens potentially offer additional diagnostic biomarkers. The p300 tumor-associated glycoprotein antigen synthesized by diploid low-grade tumors [26] was tested in our laboratory for its sensitivity and specificity alone and in combination with DNA ploidy and cytology. Voided urine samples from 69 asymptomatic controls, urine specimens and bladder washings from 49 cancer patients, and 195 symptomatic controls were collected. Two thresholds were devised based on receiver-operating characteristic (ROC) plots, one at optimal sensitivity and the other at optimal specificity. High-grade and low-grade transitional cell carcinomas (TCCs) were detected with equal efficiency (78%, $p < 0.001$ vs. symptomatic controls), though samples from low-grade tumor patients tended to contain more positive cells. Subjects being monitored for recurrence, but without current detectable cancer, were intermediate between controls and cancer cases, suggesting that this biomarker also responds to dysplasia, or "field disease." Interestingly, among symptomatic controls, positive cells were found in elevated numbers only in outlet obstruction patients ($p = 0.025$ vs. other symptomatic controls) and were not significantly different from patients with previous TCC ($p = 0.95$). This evidence further supports the hypothesis that under conditions of prolonged exposure, urine can act as a bladder carcinogen. When combined with DNA ploidy measurements and fluorescence cytology, the sensitivity of this method was 88% for low-grade tumors and 95% for high-grade tumors.

The power of combining multiple biomarker profiles on single cells by measuring both qualitative (cytology) and quantitative (QFIA, M344) differences is further emphasized with data (Table V) obtained from a Chinese cohort exposed to benzidine [27]. Two of the three individuals in the exposed group and one in the unexposed group were identified to have cancer one year prior to its detection by the more conventional Papanicolaou (Pap) cytology.

Actin as a Biomarker for Cellular Differentiation and Transformation

Actin is a major component of the cytoskeleton and may reflect important biochemical changes associated with epigenetic mechanisms of carcinogenesis [28]. Actin is a ubiquitous structural and functional protein in eukaryotic cells. It influences cell morphology, cell–cell interactions, motility, intracellular transport, endocytosis, exocytosis, and cellular division. Mounting data also suggest that it may indirectly influence gene regulation [29,30].

Total actin may be quantitated using specific molecular probes in either its filamentous form,

TABLE III. The Effect of OH-BBN and Retinoid (4-HPR) on the Nuclear Area (μm^2) of Cytologically Suspicious, Atypical and Normal Cells Detected During Automatic Scan of Bladder Wash Samples by Texture Analyzing System (TAS) Image Analysis [25]

Treatment		Cytological Classification								
		Suspicious			Atypical			Normal		
OH-BBN	4-HPR	Mean Nuclear Area ± SD	10/90th %tile	n	Mean Nuclear Area ± SD	10/90th %tile	n	Mean Nuclear Area ± SD	10/90th %tile	n
+	+	270 ± 131^{1}	156/467	16	189 ± 79	133/230	100	150 ± 32^{2}	120/190	544
+	–	419 ± 148^{1}	241/624	14	183 ± 49	136/245	329	145 ± 51^{2}	120/177	1036
–	+	392	241/543	2	191 ± 38	145/244	290	135 ± 20^{3}	115/157	345
–	–	425	368/481	2	197 ± 47	143/259	172	137 ± 19^{3}	118/163	418

Test of statistical significance of differences:
[1] p = 0.0101.
[2] p = <0.05.
[3] Not significant.

TABLE IV. The Effect of OH-BBN and Retinoid (4-HPR) on the Nucleic Acid Content (in C Units) of Cytologically Suspicious, Atypical and Normal Cells Detected During Automatic Scan of Bladder Wash Samples by TAS Image Analysis [25]

Treatment		Cytological Classification								
		Suspicious			Atypical			Normal		
OH-BBN	4-HPR	Mean Ploidy ± SD	10/90th %tile	n	Mean Ploidy ± SD	10/90th %tile	n	Mean Ploidy ± SD	10/90th %tile	n
+	+	5.9 ± 2.1^{1}	3.7/9.9	16	3.7 ± 1.0^{2}	2.5/4.7	100	2.6 ± 0.7^{4}	120/190	544
+	–	10.9 ± 3.9^{1}	5.6/16.9	14	4.4 ± 1.4^{2}	3.0/6.0	329	3.1 ± 0.7^{4}	120/177	1036
–	+	9.5	6.5/12.6	2	4.5 ± 1.0^{3}	3.3/5.8	290	3.1 ± 0.6^{5}	115/157	345
–	–	13.9	10.7/18.3	2	4.9 ± 1.3^{3}	3.3/6.2	172	3.1 ± 0.8^{5}	118/163	418

Test of statistical significance of differences:
[1] $p < 0.01$.
[2] $p < 0.01$.
[3] $p < 0.01$.
[4] $p < 0.01$.
[5] $p > 0.05$, NS.

TABLE V. Concordance of QFIA and Papanicolaou Cytologies and QFIA and p300 Among Subject Groups [27]

Tests		Test Subjects			
		Exposed		Non-exposed	Total
		Hx of CA	No Hx of CA	Controls	
QFIA	Pap				
+	+	1	1	0	2
+	–	1	2	0	3
–	+	0	0	0	0
–	–	15	16	10	41
QFIA	p300				
+	+	2	2	0	4
+	–	1	1	0	2
–	+	4	0	0	4
–	–	9	13	10	32

QFIA: + = QFIA risk category 1 or 2
– = QFIA risk categories 3, 4 or 5
Pap: + = Pap cytology positive or suspicious
– = Pap cytology negative or atypical
p300/M344: + = >3 positive cells/slide
p300/M344: – = 0–3 positive cells/slide
Hx: history

F-actin, using phalloidin [31], or in its globular form, G-actin, with DNase I [32]. Control of the ratio of G- to F-actin is undoubtedly a complex process but it is, in part, regulated by protein kinase activity which may in turn be regulated by tumor promoting agents such as phorbol esters [33].

1. *In vitro* model for evaluating the effect of chemopreventive agents on cellular F-actin. In initial experiments, abnormal F-actin levels were found to differ quantitatively between the untransformed lymphoblast cell line (RPMI-7666, RPMI) and the transformed promyelocytic leukemia cell line (HL-60) [31]. The F-actin levels were significantly higher in the untransformed RPMI cell line compared to the HL-60 cells when the F-actin level was quantitated using flow cytometry with fluorescein-labeled phalloidin (Table VI). Incubation of RPMI cells with a tumor promoting agent (a phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate) induced cellular dedifferentiation with a corresponding decrease in F-actin. It is postulated that the phorbol ester interfered with protein kinase C reactions which effectively regulated the G- and

TABLE VI. Mean F-actin Content of Untransformed and Transformed Cells [31]

Cell Line	F-actin (MCN)[1]	P vs. RPMI
RPMI	162 ± 7.4[2]	
Daudi	106 ± 2.3	<0.005
HL-60	117 ± 6.5	<0.005

Cells were collected after 3-day culture, fixed with 25% ethanol, incubated at 4°C overnight, double labeled with fluorescein-phalloidin and propidium iodide, and analyzed by flow cytometry.

[1] MCN = mean channel number
[2] Mean ± SD of five independent experiments

TABLE VII. F-actin Levels in HL-60 Cells as a Function of Cell Differentiation Induced by DMSO or Retinoic Acid (RA) [31]

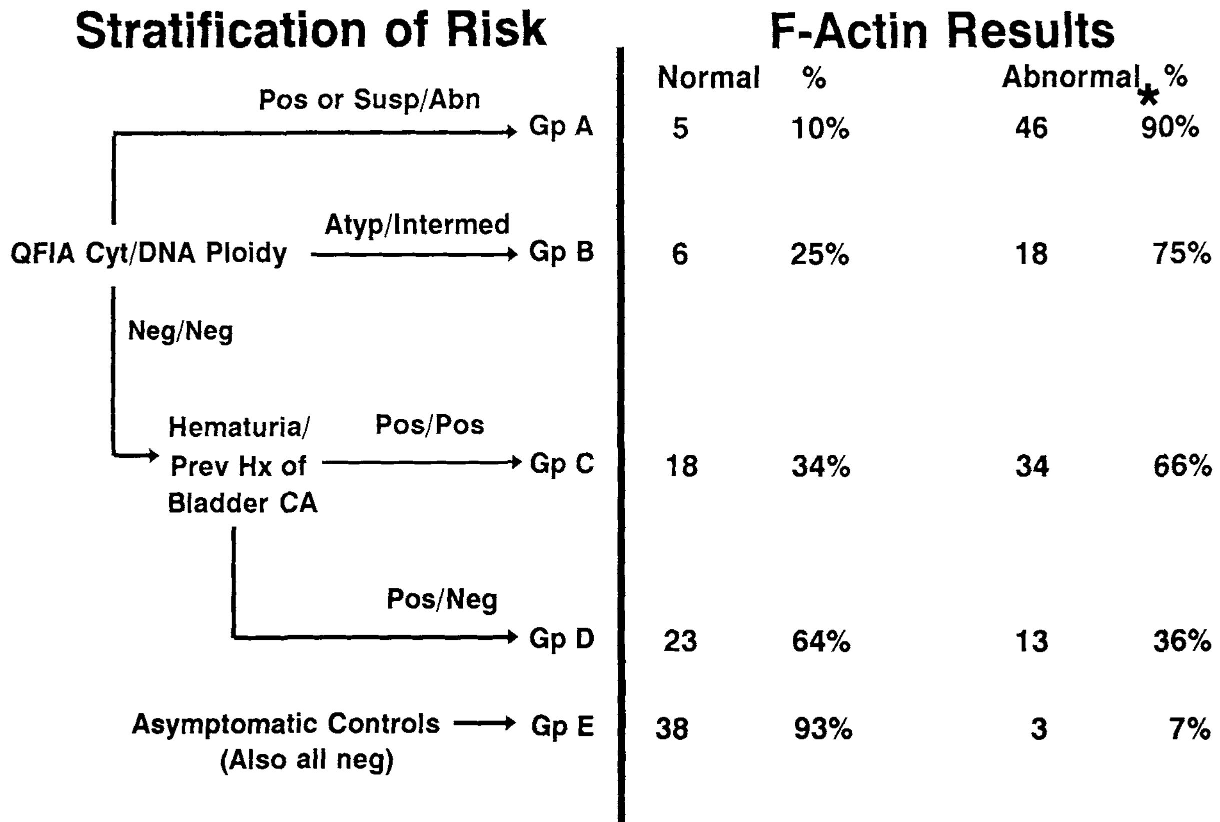
	F-actin Amount (MCN)[1]	Low F-actin Population (%)	PI (%)
Control	118 ± 7.2[2]	36 ± 2.5	36 ± 5.3
ETOH (10 μl)	119 ± 8.5	38 ± 3.5	34 ± 2.3
DMSO (% v/v)			
0.1	136 ± 1.3[3]	21 ± 1.0[4]	36 ± 3.0
1.25	151 ± 1.3[4]	10 ± 4.3[4]	17 ± 1.1[3]
RA (μM)			
0.1	139 ± 9.5	19 ± 1.9[4]	31 ± 1.2
0.5	146 ± 7.4[3]	15 ± 1.4[4]	20 ± 3.4[3]
1.0	166 ± 6.3[4]	12 ± 2.4[4]	19 ± 2.0[3]
2.0	155 ± 2.3[4]	12 ± 2.7[4]	16 ± 1.9[3]
4.0	151 ± 3.8[3]	15 ± 0.8[4]	19 ± 0.6[3]
RPMI Cells	162 ± 7.4	7 ± 2.1	18 ± 2.8

Exponentially growing cells were seeded at 1 to 2 × 10^5 cells/ml in the presence of the indicated concentrations of RA and DMSO or ethanol solvent. Cells were collected after culture for 6 days. The low F-actin population consisted of those cells with F-actin channel numbers below 120. The PI, or proliferation index, was the proportion of cells located in S and G2+M compartments.

[1] MCN = mean channel number
[2] Mean ± SD of three independent experiments
[3] $p < 0.05$ compared with control
[4] $p < 0.01$ compared with control

F-actin ratios. HL-60 cells can be induced to differentiate, and incubation of HL-60 cells with dimethylsulfoxide (DMSO) or retinoids corrected the F-actin defect in the HL-60 cells and the phorbol ester-treated RPMI cell lines (Table VII). These *in vitro* studies indicate that DMSO might also induce differentiation in bladder transitional cells.

2. Actin in monitoring for recurrence and chemoprevention in bladder cancer patients. When considering the use of F-actin as a biomarker for monitoring the effectiveness of DMSO or other potential chemopreventive agents, it was necessary to verify abnormal F-actin in bladder transitional cells from patients with malignancy. F-actin levels were quantitated in bladder wash cells from 163 symptomatic (hematuria) subjects and 41 asymptomatic (no hematuria) bladder cancer patients [34]. Flow cytometry was used to quantitate DNA and F-actin simultaneously with fluorescent probes and a dual labeling program. The risk for bladder cancer was stratified according to clinical and laboratory biomarkers as shown in Figure 3. A strong correlation between low F-actin content and the risk for bladder cancer was observed. Correlation of DNA changes in the risk stratification schema



*F-actin defined by flow cytometry. Abnormal if mean F-actin < MCN 95 or > 55% of cells had MCN < 100.
MCN(mean channel number) is a unit of fluorescence intensity

Fig. 3. Correlation of abnormal low F-actin content in bladder wash cells from 204 urologic patients stratified by risk of bladder cancer defined by QFIA cytology, hematuria and previous bladder cancer history [36]. QFIA cytology was "positive" when visually suspicious cells or >2/500 cells were visually atypical and contained >5C DNA, "intermediate" with 1–2/500 atypical cells with >5C DNA and "negative" otherwise. F-actin was measured by flow cytometry and defined "abnormal" if mean F-actin content <95 mean channel number (MCN), where MCN is a unit of fluorescence intensity, or 55% of cells had a MCN <100.

was also investigated by DNA ploidy as defined by a reference aneuploid cell population peak. Proliferation rate, as defined by proliferation index (percent of cells in S and G2+M compartments of greater than 15% as a positive biomarker), was determined in the same patients. Aneuploidy had a low sensitivity (54%) and high specificity (95%), while propidium iodide showed a high sensitivity (80%) and low specificity (63%) [34]. A strong correlation was also observed between abnormal F-actin in bladder wash transitional cells from 38 subjects with positive biopsies for bladder cancer. These results indicate that F-actin could be an early and sensitive biomarker for individual bladder cancer risk assessment. G-actin is a reciprocal biomarker to F-actin; G-actin is elevated in voided urine specimens and has important advantages because of the degenerative properties of urine which artificially lower the F-actin content of normal exfoliated cells.

Based on the above results, it was hypothesized that G-actin should yield results similar to F-actin in voided urines providing that the actin alteration in bladder tumorigenesis is a result of actin polymerization. A newly developed QFIA technique was employed for G-actin analysis. G-actin was labeled with a Texas Red-conju-

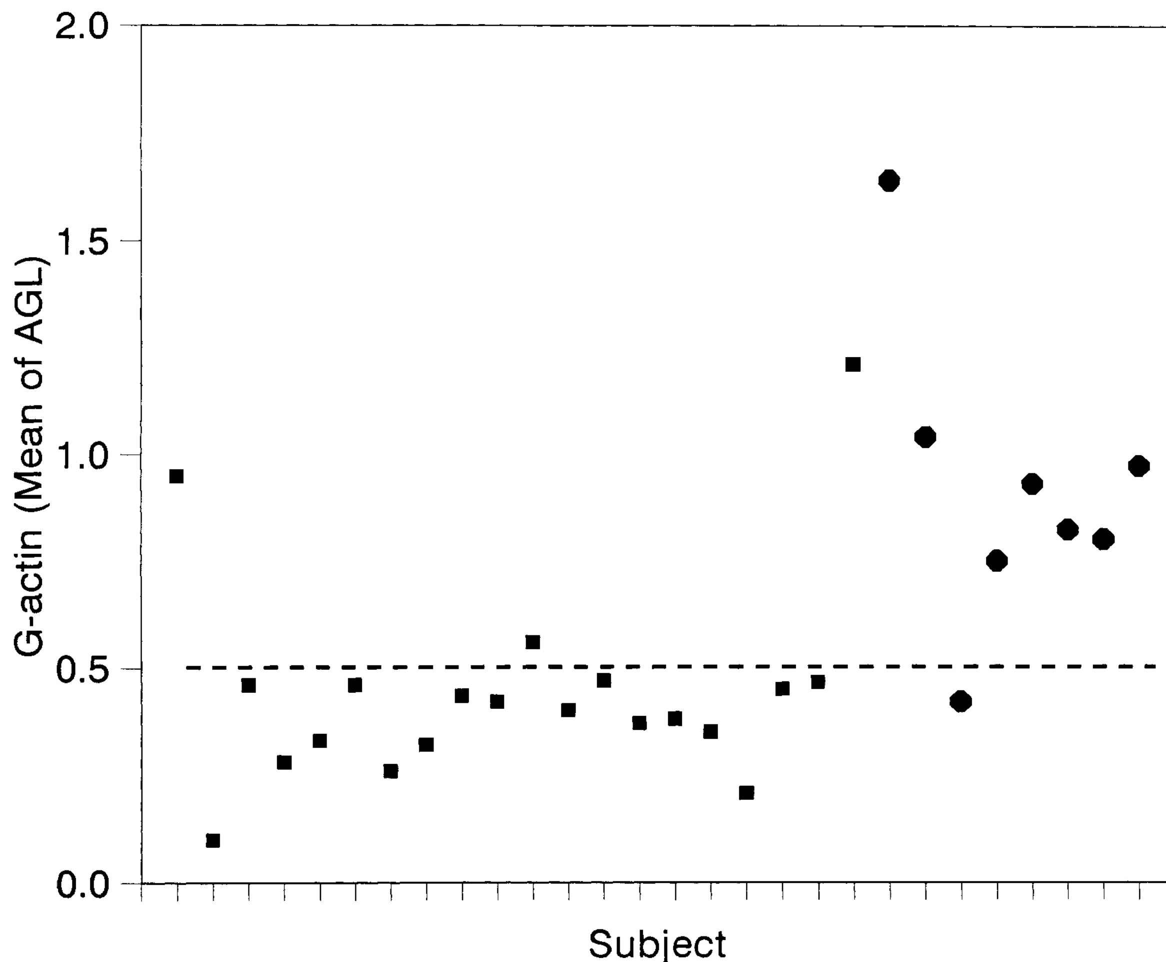


Fig. 4. G-actin content [mean of average grey level (AGL)] measured by image analysis (IBAS) in voided urine cells from 8 bladder cancer patients and 19 asymptomatic controls. For each sample, 100 cells were randomly selected to measure the G-actin fluorescence intensity. Each assay was normalized against replicate aliquots of a single batch of cells from a cell line (5637 cells).

gated DNase I, the nuclei were stained with Hoechst 33258, and the biomarkers were quantitated on the IBAS. The mean G-actin content was approximately twice as high in the cancer cases as compared to the controls. G-actin was elevated in 9 of 10 bladder cancers and 2 of 20 controls (Fig. 4).

To further substantiate the reciprocal relationship between F- and G-actin, a double-labeling program and a staining technique has been developed. Treatment of HL-60 cells with 1 μM retinoic acid at 37°C for 30 minutes resulted in reverse quantitative expression, *i.e.*, a decrease in G-actin and an increase in F-actin (Fig. 5). These studies support the potential for using G-actin as a biomarker on exfoliated cells in voided urine.

3. Actin as a potential intermediate endpoint biomarker for chemoprevention trials. As summarized above, previous studies indicated abnormal G-actin and F-actin levels in the majority of patients with bladder cancer as well as in patients in remission (*i.e.*, negative cytology and negative biopsy). DMSO is currently instilled into the bladder for treatment of pain in patients with interstitial cystitis, but its mechanism of action remains an enigma [35]. A pilot study was designed and initiated to evalu-

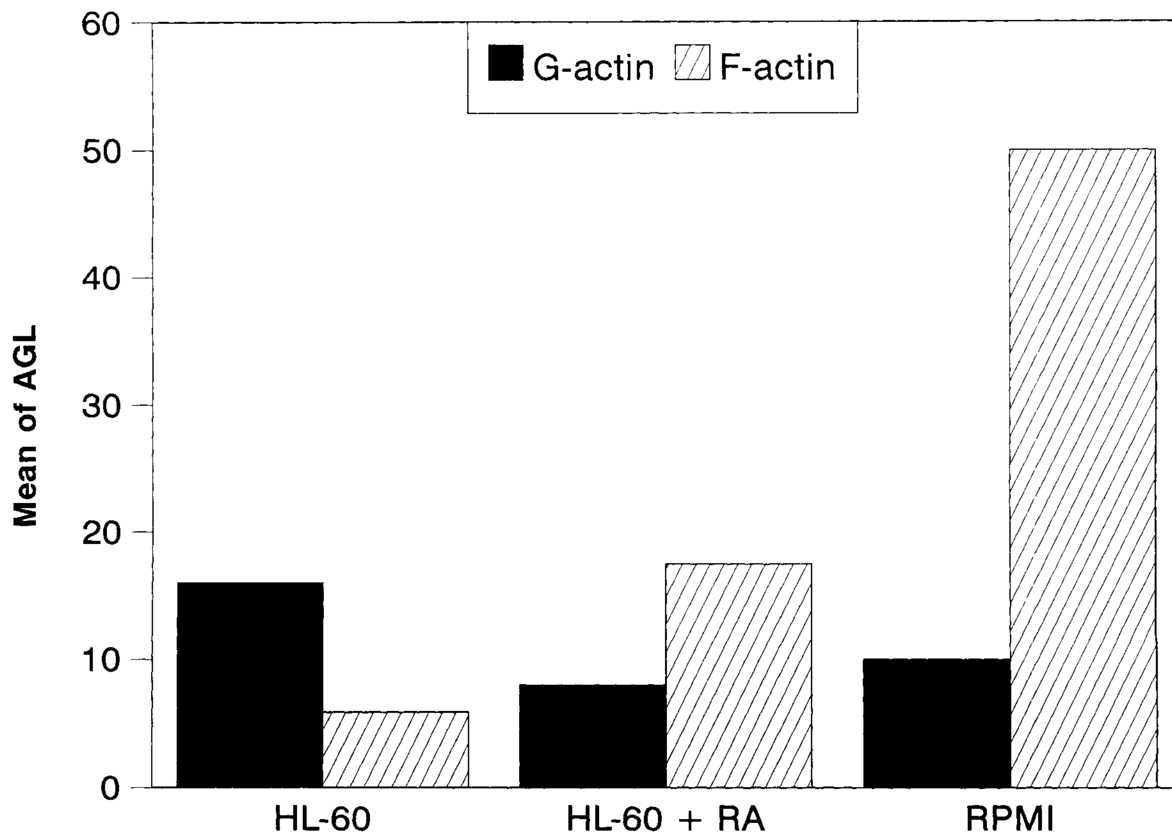


Fig. 5. Comparison of G- and F-actin levels of undifferentiated HL-60 cells and HL-60 cells differentiated by 1 μM retinoic acid. Also shown are the actin levels in the differentiated RPMI line. G- and F-actin were each measured (mean of AGL) by image analysis (IBAS) on 500 randomly selected cells.

ate the effect of intravesical DMSO on bladder epithelium of patients previously treated for bladder cancer and who were at high risk for recurrence. Included were patients with Ta, G1 disease with multiple recurrences or Ta/T1, G2, G3 or carcinoma *in situ* (CIS), who were tumor-free after intravesical Bacillus Calmette-Guerin (BCG) immunotherapy or transurethral resection (TUR) surgery. The majority of patients in the protocol had been previously treated with BCG until remission was achieved. Patients were considered disease-free if they had negative cytologies, biopsies, and cystoscopic examinations. No patients had previous radiation or history of muscle invasion or TCC of the prostate. A control group was not included in the Phase I–II study. All patients had baseline biomarker profiles which included G-actin, F-actin, M344, and visual QFIA cytology, including fluorescent cytology and determinations of cells with >5C DNA content. DMSO was instilled into the bladder through a catheter previously used to obtain the bladder wash sample. The DMSO was instilled in the bladder (50 ml, ≈ 50% w/w aqueous solution) for 15 minutes while the patient rotated his position.

The effect of DMSO on G- and F-actin was evaluated in 8 patients. DMSO normalized the F- and G-actin in 7 of the 8 patients evaluated to date. Figure 6a demonstrates the effect of DMSO on a single patient following BCG administration. Figure 6b shows a patient in whom the DMSO had no effect on the G-actin. Two of the 8 patients were discovered to have recurrent tumors in less than 3 months following therapy. The duration of response remains undetermined, and longitudinal follow-up with a randomized trial will be necessary to determine if DMSO administration following primary treatment with BCG or TUR will reduce tumor

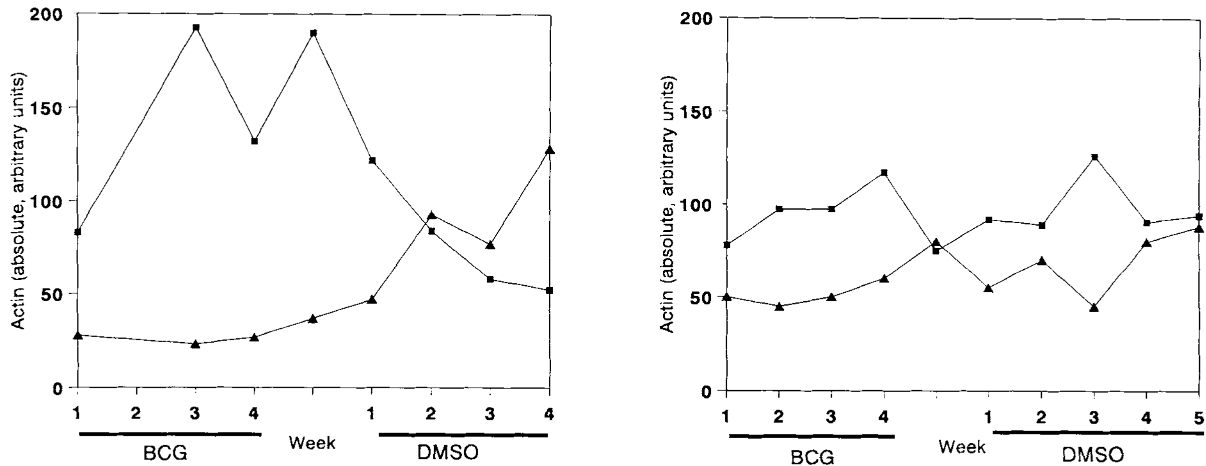


Fig. 6. Two examples of longitudinal monitoring for therapeutic response of BCG and DMSO with F-actin and G-actin content. F-actin (▲) was labeled with fluorescein-phalloidin and quantified by flow cytometry, arbitrarily setting the mean channel number of the control HL-60 cells to 90. G-actin (■) was labeled with DNase I-Texas Red and measured by image analysis using the IBAS, arbitrarily setting the absolute grey mean of the control 5637 cells to 100. Both units are arbitrary, absolute units proportional to the F-actin content by unknown proportionality constants. The F- and G-actin content generally was not affected by BCG immunotherapy. However, in several cases, DMSO produced a change in actin content indicative of a positive differentiation response, *i.e.*, a decline of G-actin and increase of F-actin content followed DMSO therapy. Figure 6a plots a case with a positive response as compared to a negative response illustrated in Figure 6b.

recurrence or replace maintenance BCG immunotherapy. More importantly, the biomarkers G-actin and F-actin appear to be useful intermediate endpoint biomarkers and useful for evaluating a select class of chemopreventive agents which induce cellular differentiation.

Biochemical Mapping of Intermediate Endpoint Biomarkers in Bladder Cancer Tumorigenesis

The malignant phenotype typically develops over a period of years, *e.g.*, fifteen to twenty years for bladder cancer. Charting the phenotypic changes that accompany tumorigenesis is difficult when followed over time. However, a tumor-bearing bladder will typically contain all the cell types that evolve over time in different areas within the bladder. The evolution of the malignant phenotype can be mapped by quantitating biomarkers using QFIA in biopsies obtained from different areas [36].

Touch preps were made from biopsies from the tumor, the area adjacent to the tumor, and random samples from 30 patients with bladder cancer and 6 noncancer controls [36]. The biomarkers investigated are shown in Figure 7. Each biomarker showed a clear progression from distant field to adjacent field to tumor, but differences in the rate indicate a sequence to the change. G-actin was altered in 56% of the field and 100% of cells derived from the tumor but not in cells derived from normal bladder epithelium. Epidermal growth factor receptor (EGFR) and HER-2/*neu* protein were associated with conversion to high-grade tumors. Ploidy and morphologic changes occurred late. The biomarkers clustered into three groups: G-actin and EGFR; ploidy, cytology and HER2/*neu*; and p300 low-grade tumor antigen. The expression of G-actin early in tumorigenesis indicates that epigenetic mechanisms may be important in bladder cancer. Oncogenesis and quantitation of multiple biomarker cell profiles may improve the diagnosis and prediction of individual cancer risk.

Multiple biomarker measurements on single cells provide the advantage of evaluating the interrelationship between phenotypic biomarkers which represent different biochemical path-

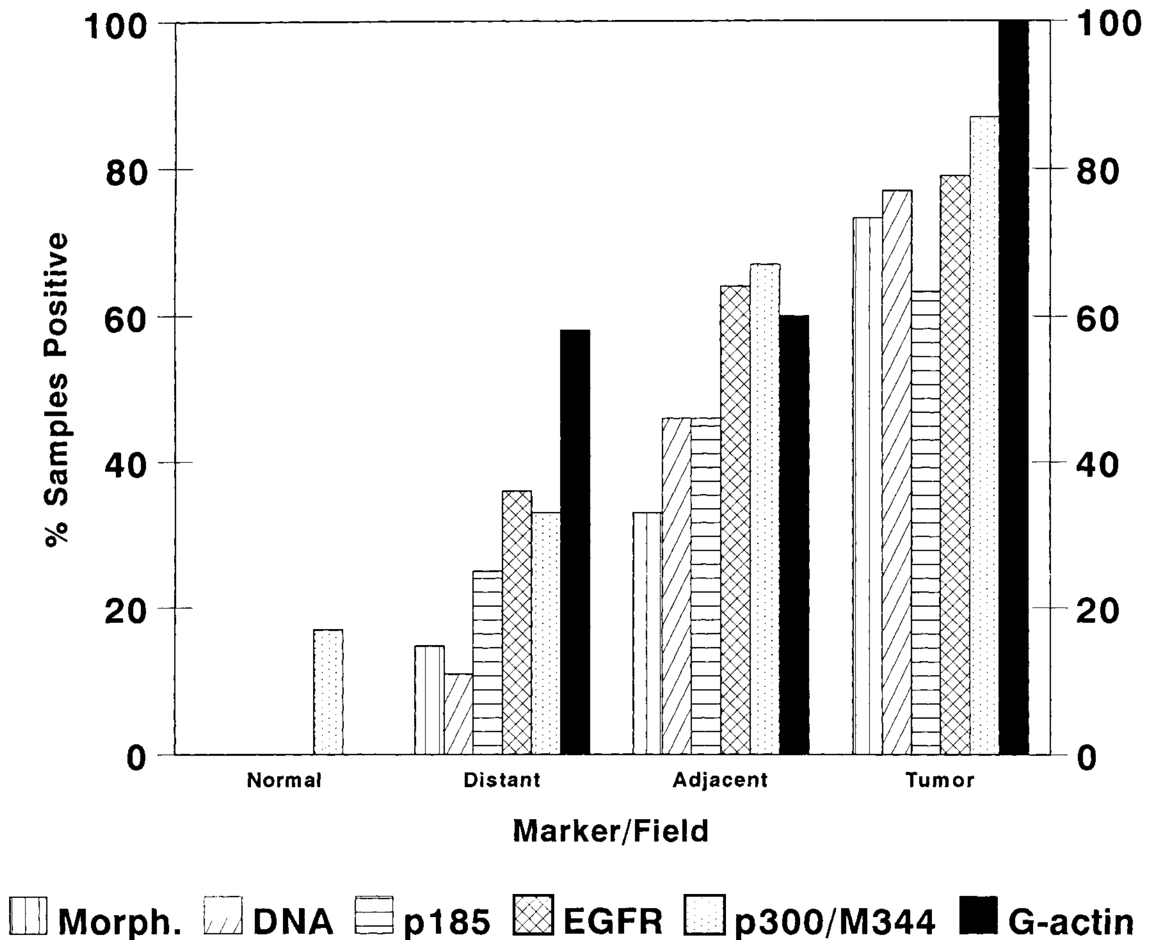


Fig. 7. The progression of biochemical biomarkers from control (n = 6), random distant field (n = 27), adjacent field (n = 24) and tumor (n = 30) of TCC with biomarkers scored by positive/negative criteria. Cytology was scored by a trained cytologist (RAB) and confirmed by a pathologist. The presence of cells with "suspicious" morphology labeled the sample as "positive" as did the presence of any cells with >5C DNA. The p300 biomarker was scored visually by two independent observers. A sample was considered positive if any positive cells were noted. EGFR and G-actin were approximately normally distributed while p185 showed a lognormal distribution. For p185, the mean integrated grey level (IGL) of cells on the negative control slide was calculated and was subtracted from the IGL of each cell on the sample slide. If the adjusted mean IGL significantly (Student's t-test) exceeded the adjusted IGL of a low-expressing cell line (3T3 SW480), the sample was labeled as positive. For EGFR, a histogram of normal cells from control patients was constructed, and the presence of cells above the upper limit of normal was used as an indicator of positive. For G-actin, a sample was labeled as positive if the mean IGL was significantly higher ($p < 0.05$ by Student's t-test) than the mean of the control patients.

ways. In the study, the interrelationship between DNA ploidy (>5C DNA) as a biomarker for genetic instability and either G-actin, EGFR or p185 was investigated. A strong correlation was found between DNA content and either EGFR or p185, but not G-actin. These data further support the concept that G-actin alteration is an early event preceding genetic instability, reflected by the increasing DNA content [36].

Several conclusions are suggested from the study. First, a sequence of phenotypic changes accompanying the development of bladder cancer occurs, and biochemical changes are appar-

ent prior to abnormal pathology. Cancer pathology in biomarkers initially thought to be limited to the tumor was also found in the field. Second, an appreciation for the hierarchy of biomarker expression provides the framework for defining which biomarkers to include in bladder cancer chemoprevention trials, which biomarkers or combination of biomarkers are ideal for bladder cancer screening, and how to interpret the biochemical profiles as they relate to the effectiveness of bladder cancer therapy. Finally, the results help to distinguish those biomarkers which are more likely to be prognostic in relationship from those which should be employed in chemoprevention trials.

Other Intermediate Endpoint Biomarkers for Bladder Cancer Chemoprevention Studies

In addition to DNA, actin, and p300, the potential of several other biomarkers to be intermediate endpoint biomarkers for bladder cancer chemoprevention has also been evaluated in this laboratory. The biomarkers include EGFR [37] and *neu* oncogene product p185 [38]. Biomarkers have been evaluated either independently or in combination with each other, using strategies as stated above. These studies are currently underway and will be reported later.

DISCUSSION

Intermediate endpoint biomarkers are important for defining individual cancer risk, evaluating the effectiveness of conventional or chemopreventive therapy [34,39,40], identifying individuals at high risk for cancer recurrence, and defining candidates for chemoprevention [8]. The selection of biomarkers to achieve these objectives is dependent on many considerations including when they are expressed in tumorigenesis, the stability of the antigenic epitope, the required method of sample collection and preservation, and the consistency of analysis.

A major distinction must be made between biomarkers of disease and biomarkers of effect. With the former, the objective is to obtain a high specificity and sensitivity and a high positive predictive value against the proven histopathologic diagnosis as the indicator for disease. In contrast, biomarkers for effect or biomarkers for disease that occur before abnormal morphology is found on biopsy, which might typically be used for chemoprevention trials, will be less specific with a lower positive predictive value if histologic diagnosis is the defined endpoint. The gold standard for biomarkers in a chemoprevention setting is a high correlation between the number of patients with a positive biomarker and those that will develop disease [41]. Because cancer is a multistep process and not all patients at risk will have lesions which undergo the final step, it is useful to evaluate the sequence of oncogenic biomarker expression during oncogenesis [33]. This may be accomplished by following patients who are at risk for incident disease (*i.e.*, first-time tumor). However, this approach has disadvantages because of the time requirement for establishing biomarker validity. Monitoring patients in remission whose disease is expected to recur may shorten the time for identifying those biomarkers which may be clinically useful. Although the treatment of bladder cancer patients who are in remission with chemopreventive agents may provide a clue to drug efficacy, the final test for the predictability of a biomarker is its correlation with prevention of incident cases in a population at risk. Occupationally exposed cohorts at high risk for disease are ideal candidates for this purpose [27,42,43].

In animal studies, increased DNA (>5C) per cell was identified as a candidate intermediate endpoint biomarker. In fact, the use of cells with >5C DNA has become a hallmark as a biomarker for bladder cancer detection, improving the sensitivity for detection of low-grade tumors 10–20% depending on the population under investigation [19,44]. Unlike many cancers, exfoliated cells from low-grade bladder cancers do not have cytologic features distinguishing them from those resulting from inflammation. Biomarkers are useful for subclassifying patients with atypical or dysplastic cells into a group at high risk for disease. The subclassification of atypical cells with more or less than 5C DNA, as mentioned above, has improved the detection of these tumors. However, subsequent studies have demonstrated that cells with >5C DNA are representative of a relatively late event in carcinogenesis and are associated with disease which has a poor prognosis and is likely to recur [15,36].

Bladder cells of rats treated with the carcinogen OH-BBN had decreased ploidy and nuclear size compared with carcinogen controls after administration of retinoids [25]. Although these results showed promise for cells with >5C DNA as a useful biomarker for measuring the effectiveness of chemopreventive agents, the markedly abnormal DNA within these cells will not be effectively normalized by conventional chemopreventive agents.

Additional studies outlined in this presentation suggest that F- and G-actin may be relatively early cytoskeletal differentiation biomarkers [31]. Changes in the F- and G-actin may reflect alterations in the epigenetic mechanisms important to carcinogenesis. Cytologic alterations in stress fibers confirm changes in actin associated with differentiation. Abnormal F- and G-actin are quantifiable by either flow cytometry or image analysis; however, F-actin may be a less useful biomarker than its reciprocal, G-actin. The latter is an elevated biomarker and is not artificially lowered by a hostile urine environment.

QFIA of G-actin yields quantitative data, providing that reactions occur stoichiometrically and that the instrumentation employed is carefully calibrated. Interactive systems, such as QFIA, which store video images, allow artifact rejection and facilitate the collection of quantitative biomarker data on single cells while simultaneously detecting rare events. This is the methodological approach which we have developed in our laboratories.

As demonstrated in the data presented here, QFIA technology has been extended to detect multiple biomarkers in single cells as well as to detect events as rare as 1 or 2 abnormal cells among 10,000 normal cells on a slide. The impact of this technological advance on biomarker analysis in chemoprevention trials remains to be determined. However, the use of QFIA to quantitate biomarker profiles on touch preps from single cells confirms our earlier observations that G-actin is an early biomarker for cellular dedifferentiation [31,36]. In previous studies, 7% of clinical samples from non-cancer patients had abnormal F-actin [34]. Analysis in a large population reveals that alterations are frequently associated with urinary stasis associated with bladder outlet obstruction, occupational exposure to carcinogens, and cigarette smoking, all known risk factors for bladder cancer. Biomarker profiles on single cells or samples are likely to improve the positive predictive value for defining an individual at risk for developing bladder cancer. In addition, biomarker combinations may predict where in the continuum of carcinogenesis an individual's premalignant lesions reside. The mapping of biochemical field disease as presented here elucidates three biomarkers which are independent for diagnosis: DNA, M344, and G-actin, in the order of late to early expression. Treatment of patients with immunotherapeutic agents such as BCG shows a hierarchy of tumor biomarker regression or progression depending on the success or failure of treatment. Preliminary observations suggest that an increasing number of cells expressing the p300 antigen portends recurrence, as reported by Fradet [45] in patients not receiving BCG therapy. Other biomarkers such as the Lewis^{X} antigen and 19A211 antigen may be similarly useful [46], but we have observed that the antibodies currently used to detect these biomarkers also have a high background level which, in theory, limits their value for detecting smaller bladder tumors.

In a study of eight patients receiving DMSO, the differentiation agent corrected the G-actin defect in 7 patients. Two of the 8 had known recurrence despite G-actin normalization; tumors recurred in both of the patients within three months, indicating that persistent clones of cells may have been present in the bladder which were unaffected by DMSO. The alternative is that DMSO, although effective in differentiating the cells, did not prevent tumor recurrence. The chemopreventive agent may correct the biomarker alteration which is related to the epigenetic mechanisms of carcinogenesis without influencing the critical mutagenic steps which are irreversible in the carcinogenic process. Extended clinical chemoprevention trials in patients who have previously treated bladder cancer who have a high probability of recurrence and are at low risk for metastasis establish the rationale for further biomarker evaluations [7,47].

The carcinogenic process alters the biochemistry of cells, and perhaps only a very few within any organ will progress to the final stages. Nevertheless, detection of altered cells is a bio-

marker for increased risk. Risk is potentially quantifiable by determining the profile of the degree of alteration and the number of altered cells. Chemoprevention is the process of halting or reversing this process, and a strategy of cancer prevention based upon detection of individuals at increased risk in conjunction with targeted chemoprevention will have much more impact on cancer prevention than a strategy based upon early detection of cancer itself. The use of QFIA is opening up new approaches to detecting individuals at risk by detecting changes in the biochemistry of individual cells.

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