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Cellular F-Actin Levels as a Marker for Cellular Transformation: Correlation with Bladder Cancer Risk[1]

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ABSTRACT

Previous findings in cultured cells that differentiated cells had markedly higher F-actin levels than undifferentiated cells (Cancer Res., *50:* 2215–2220, 1990) suggested that quantitative F-actin measurements in urinary cells might provide diagnostic or prognostic information by identifying those individuals with cells tending towards a lower degree of differentiation. The feasibility of such an approach was investigated using a risk stratification schema. Bladder wash samples were obtained from 163 symptomatic patients being evaluated for bladder cancer and 41 asymptomatic controls without hematuria or other symptoms consistent with bladder cancer. F-actin levels were evaluated by flow cytometry using a fluorescent phalloidin probe. The risk of bladder cancer was stratified according to biopsy, either DNA ploidy by flow cytometry or quantitative fluorescence image analysis cytology, previous bladder cancer history, and hematuria. A strong correlation between the presence of cells with abnormally low F-actin content in cells obtained by bladder wash from 38 patients and biopsy-proved bladder transitional cell carcinoma ($P < 0.001$) was observed. A strong correlation was also observed between the presence of cells with low F-actin content and risk of bladder cancer assessed by either stratification schema ($P < 0.0001$). The correlation was more consistent with the stratification by quantitative fluorescence image analysis cytology because of the 37% false-positive rate of ploidy analysis by flow cytometry among the control patients. Further evidence that low F-actin was correlated with cellular abnormality was obtained from simultaneously labeling cells for F-actin and with M344 antibody, a monoclonal antibody against a low-grade bladder tumor-related antigen. These studies showed that the F-actin content of the M344-positive cells was lower than that of the M344-negative cells. These results suggest that F-actin could be an early and sensitive marker for bladder cancer detection and risk prognostication.

INTRODUCTION

Bladder cancer develops as multiple foci in both time and place in a bladder that may contain wide areas of partially altered cells existing along a continuum from normal to precancerous (1, 2). Carcinogenesis is a multistep process that may proceed in parallel at different rates in many cells within the bladder, and long times may transpire between initiation and its attendant oncogenic activation and the appearance of macroscopic tumors (3) or the classic markers of abnormal cytology, histology, or altered DNA ploidy (4). Random bladder biopsies to detect concurrent severe dysplasia or carcinoma *in situ* does provide a means to specifically sample the urothelium and identifies some patients with advanced premalignant lesions who are likely to develop recurrences rapidly (5). Yet the observation that hematuria can precede positive biopsies by at least 8 years (6) suggests both a need for and the possibility that markers could detect elevated risk well before an abnormal histology is detectable.

Actin, one of the major components of cytoskeleton, is important in a variety of cell functions, including regulation of cell shape, motility, secretion, intracellular transport, endocytosis and exocytosis, and cell division (7, 8). The phenotypic changes resulting from transformation are reflected in distinct alterations in the cytoskeleton (9, 10), and in a previous study using model systems of differentiable transformed cells we demonstrated that quantitative measurements of F-actin content provided a marker for transformation that reflected the level of differentiation of individual cells (11). This result indicated that F-actin might be a general marker that could reflect alterations in several oncogenic pathways and therefore might well be a useful biochemical marker for delineating the extent and progression of field disease well before morphological abnormalities become evident. A marker for bladder cancer risk would be particularly useful in evaluating patients with unexplained hematuria and no previous history of bladder cancer.

Our previous study stimulated us to investigate the possibility of using F-actin as an early marker in clinical samples and to compare it with ploidy and cytological markers. F-actin, DNA ploidy, and proliferation index were measured by flow cytometry in 163 clinical bladder wash samples from symptomatic patients being evaluated for bladder cancer and from 41 urological patients asymptomatic of bladder cancer. The bladder wash specimens also were analyzed by QFIA[3] cytology for highly abnormal hyperdiploid cells and abnormal visual cytology (12, 13). The individuals were stratified by bladder cancer risk using either QFIA cytology or flow cytometry DNA results, in combination with a previous history of bladder cancer and hematuria to group patients according to a steadily decreasing risk for recurrence. Markers sensitive to early changes that predict an unfavorable biological potential should parallel such stratification. The use of risk stratification in this way can provide early information on the value of markers in the absence of long-term follow-up data.

MATERIALS AND METHODS

Materials. Fluorescein phalloidin was obtained from Molecular Probes Inc., Eugene, OR. Propidium iodide was prepared at a concentration of 2 mg/ml in 1.12% sodium citrate. RNase (Sigma Chemical Co, St. Louis, MO) was prepared at a concentration of 10 mg/ml in PBS and boiled for 15 min before use. Acridine Orange and Hoechst 33258 (Polysciences, Inc., Warrington, PA) were used to prepare 2.0 mM stock solution in deionized, distilled water. All stock solutions were at pH 7.4.

Patient Population and Sample Collection. Clinical bladder wash samples and associated patient information, including previous tumor

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[3] The abbreviations used are: QFIA, quantitative fluorescence image analysis; MCN, mean channel number.

history, were obtained from the urology clinic at Oklahoma Memorial Hospital, Oklahoma Veteran Administration Hospital, and Cytodiagnostics Inc. Most of the symptomatic patients exhibited hematuria and were being evaluated for primary or recurrent bladder cancer. Of the total 204 cases, 53% had a previous history of bladder cancer, and 20% were asymptomatic controls without hematuria or a previous cancer history with diagnoses such as interstitial cystitis, benign prostatic hyperplasia, prostatitis, or stones. Standard procedures were employed to collect bladder wash samples. Samples were fixed with an equal volume of 50% ethanol fixative, giving a final concentration of 25% ethanol. If necessary, samples were stored at 4°C and analyzed within 2 weeks after collection. Samples were split, with one aliquot being analyzed for QFIA cytology and the remainder by flow cytometry. Patient history was unknown to the persons performing the analyses. For the 204 cases, bladder wash samples were collected and analyzed; 142 were from males and 62 from females. The mean age of all patients was 68.7.

Flow Cytometry and DNA Ploidy Measurements. Cells were diluted to 1 million cells/ml and washed in phosphate-buffered saline by 2 cycles of centrifugation. The pellet was resuspended in 0.5 ml phosphate-buffered saline, 100 μl of fluorescein-labeled phalloidin were added, and the cells were incubated for 30 min at room temperature in the dark. The cells were washed as above, resuspended in 1 ml RNase (10 mg/ml), and incubated for 30 min, at which time 10 μl of propidium iodide (2 mg/ml) were added and the cells incubated for 30 min. Cells were placed on ice in the dark until analyzed.

The flow-cytometric analysis was described previously in detail (11). Briefly, an Epics 541 flow cytometer (Coulter Electronics Corp., Hialeah, FL) with a single 5-W argon laser operating at 200 mW at 488 nm was used to measure DNA and F-actin simultaneously at a flow rate of 2000 to 4000 cells/s. Optics were aligned using DNA beads purchased from Coulter Immunology, Inc., and checked with chicken red cells. To diminish the influence of cell size and debris on F-actin quantification, a data analysis program was developed to gate out large clumps of cells using 2 gating algorithms to collect data only from objects falling within discrete values of size and brightness. The first gate was set on the histogram of 90° light scattering (granularity) *versus* forward-angle light scattering (cell size). The second gate was set on the histogram of log-integrated green fluorescence (F-actin) *versus* integrated red fluorescence (DNA). The MCN (channel number being a relative intensity unit in flow cytometry) was used to represent the F-actin level of a particular cellular population. At the beginning of these experiments, RPMI 7666 cells (untransformed lymphoblast cell line, ATCC 114-CLL) were used as a quality control sample for F-actin and chicken red blood cells for DNA, but the F-actin quantification proved so reproducible, the coefficient of variation in peak channel number of 5 analyses on different days being approximately 1%, that the peak of normal urothelial cells was used instead as an internal quality control for F-actin labeling. The peak channel number consistently fell within channels 140–150.

QFIA Cytology and DNA Measurements. Samples were analyzed by QFIA cytology as described previously (12, 13). Briefly, this technique uses an automated image analysis system (Leitz TAS, Rockleigh, NJ) attached to a fluorescence microscope (Leitz Orthoplan) and DNA binding dye, Hoechst 33258 (10 μM) or Acridine Orange (15 μM), to screen the sample for cells that have >5C DNA and a visually atypical or suspicious morphology. The system operates by scanning the slide using a thresholding algorithm to identify potentially abnormal objects ("alarms"). The threshold is set to eliminate the majority of normal cells. The scan is continued until 2000 total cells have been examined or 40 alarms have been found. After completing the scan, the system returns to each alarm, which is then categorized by a trained cytologist according to standard morphological criteria. The availability of a visual image for each alarm eliminates artifacts, such as cell clusters or multinuclear cells, and focuses attention on the diagnostically significant cells. It should be noted than an "alarm" is not necessarily an abnormal cell. The DNA content of each alarm cell was calculated with reference to a standard fluorescent phosphor particle (NBS 1022) that had been standardized against normal human urothelial cells. The overall cytological impression of the sample (normal, atypical, suspicious, and positive) was also recorded.

Simultaneous Measurement of DNA, F-Actin, and M344 Antibody Binding by QFIA. For one case (male, age 56 years), bladder biopsies from the tumor were collected at cytoscopy. The pieces of tissue were placed into saline immediately and single cells were liberated by agitation. The cell solution was fixed as described for bladder washes and triple-labeled for F-actin, DNA, and with the M344 monoclonal antibody against a tumor antigen expressed preferentially by low-grade tumors (14). The F-actin was labeled with rhodamine-labeled phalloidin at 1:10 (w/v) dilution. DNA was labeled with aqueous Hoechst 33258 (15). The M344 antibody was detected with a 3-stage biotin-avidin assay using Lucifer Yellow-labeled avidin as the reporter fluorophore using a procedure virtually identical to that previously reported for Ki-67 with Texas Red as the reporter fluorophore (11). The 3 labels were evaluated using the same image analysis instrumentation as was used for QFIA cytology. The rhodamine (F-actin) and Hoechst 33258 (DNA) were quantified as described previously (11). Briefly, the slide is scanned as described above, exciting Hoechst 33258 fluorescence, except that a lower threshold is used to detect all cells. After identification of 40 cells, the system returns to each cell. The operator then switches to the rhodamine excitation wavelength (I2 excitation filter block) and measures the actin content of the cell. Finally, the operator switches to the Lucifer Yellow excitation wavelength (H2 excitation filter block) and visually scores the cell as positive or negative for M344 labeling.

Risk Stratification. Qualitative and quantitative F-actin data from cytometry were compared with the bladder cancer risk, which was stratified on the basis of biopsy, where available, and either QFIA cytology or flow cytometry DNA results, hematuria, and previous bladder cancer history for those patients who were either biopsy-negative or had no concurrent biopsy results.

The F-actin histogram was defined as "normal" if the F-actin MCN was ≥95 and ≤55% of cells had channel number below 100. F-actin was "abnormal" if F-actin MCN was <95 or >55% of cells had a channel number below 100. The flow cytometry DNA results were scored as "positive" if an aneuploid (a distinct nondiploid peak) population was discernible in the DNA histogram or if the proliferation index was ≥15%, "suspicious" if the proliferation index was <15% but ≥10%, and "normal" if the proliferation index was <10% (16, 17). Proliferation index was defined as the percent of cells between 2 and 4C and includes cells in G2+M and S phases of the cell cycle as well as any artifacts due to, for example, the formation of cell doublets or possible alterations in binding stoichiometry of a subpopulation of cells.

QFIA cytology was scored as "positive" if visually suspicious cells were seen or if more than 2 of 500 visually atypical cells with >5C DNA were detected; "intermediate" if 1–2 of 500 visually atypical cells with >5C DNA were detected, and "normal" if no cells with >5C DNA were detected. The sensitivity of this schema is approximately 70–80% with a specificity of 93–95% (13). Some small, minimally aneuploid tumors are found in the "intermediate" category together with a large number of people without apparent disease, but who express risk factors for bladder cancer, *e.g.*, among smokers, people with bladder carcinogen exposure, or early premalignant changes.

These risk factors were combined to categorize patients by risk as shown in Table 1. Capital letters are used to denote stratification using QFIA cytology, lower-case letters to denote stratification using DNA ploidy measured by flow cytometry. The test for linear trends in proportions method was used to test the statistical significance (18). The hypothesis is that there should be a strong correlation between appropriate objective measurements of risk.

RESULTS

Defining the Pattern of F-Actin and DNA by Flow Cytometry. Figure 1 shows the 3-dimensional histograms of DNA *versus* F-actin from a control bladder wash (A), and a typical bladder wash from a patient with transitional cell carcinoma (B). The individual DNA and F-actin histograms are also shown. In the control bladder washes, a single F-actin peak at MCN 140,

indicating a high F-actin content, was seen together with a normal-appearing DNA histogram. In contrast, in the bladder cancer case, the F-actin peak was shifted to the region of MCN 75, indicating lower F-actin content. In general, control bladder washes showed only a single F-actin peak in the region of channel number 120–150, whereas abnormal samples frequently showed in addition, or exclusively, a second peak at a channel number from less than 100. The DNA histogram from the bladder cancer case showed a high fraction of cells with DNA in excess of the G0+G1 cells together with a distinct aneuploid population of cells.

Comparison of F-Actin with Risk. The highest risk group was obviously those patients in whom cancer could be proven by biopsy. Table 2 compares the F-actin results with histological findings among the 38 cases for whom concurrent biopsy results were available. Of the patients with biopsy-proven cancer, 94% showed an abnormal F-actin result ($P < 0.001$). Of the 3 patients with negative (for cancer) biopsy and abnormal F-actin, 2 had a previous history of carcinoma *in situ* and the third had squamous metaplasia.

A large group of patients did not have concurrent biopsy results, but other objective parameters were used to stratify this group according to risk. Table 3 lists F-actin as a positive-negative variable in relation to bladder cancer risk as assessed by clinical and QFIA cytological criteria. Fig. 2 shows the mean F-actin content as a continuous variable in relation to risk. Of the 51 cases in the cytologically positive group (A), 90% had an abnormal F-actin histogram. Among the cytologically intermediate risk group cases (B), 75% had abnormal F-actin contents. After stratifying the group that was negative by QFIA cytology with hematuria according to their previous history of bladder cancer (*i.e.*, separating Group C from D), 34 (66%) of Group C had abnormal F-actin, compared with 18 (34%) who had normal F-actin, whereas 13 of Group D had abnormal F-actin and 23 had normal F-actin. Two of the 13 individuals with abnormal F-actin were clinically diagnosed with low-grade transitional cell carcinoma. No recurrent or new bladder cancer

Table 1 *Definitions of levels of risk for bladder cancer*

Definitions based upon QFIA cytology and DNA ploidy by flow cytometry are referred to with capital and lower case letters, respectively, to show that, although analogous, the difference between the 2 methods will lead to a somewhat different stratification. Criteria for scoring QFIA cytology and flow cytometry are described in "Materials and Methods." The difference between the 2 methods was most significant for the control group of asymptomatic individuals with no history or suspicion of bladder cancer. All were negative to QFIA cytology, but a significant fraction had positive DNA ploidy measurements, usually the proliferation index, thereby necessitating division of the controls into e^+ and e^- groups when stratified using flow cytometry.

Group	Hematuria	QFIA cytology/DNA ploidy	Previous history of bladder cancer
Patient groups			
A/a		Positive/positive	
B/b	Yes	Intermediate/suspicious	
C/c	Yes	Negative/negative	Yes
D/d	Yes	Negative/negative	No
Control group			
E	No	Negative/—	No
e^+	No	—/Positive or suspicious	No
e^-	No	—/Negative	No

was found among any of the individuals with normal F-actin content. The difference between the groups with and without a previous bladder cancer was highly significant ($\chi^2 = 7.33$, $P < 0.01$). Only 7% of the controls (Group E) had abnormal F-actin. The χ^2 value of 70 shows that the trend of increasing abnormal F-actin results with increasing risk is highly significant ($P < 0.0001$).

As seen in Fig. 2, the controls comprise a relatively homogeneous group clustered around the mean of 118, whereas the other groups are more broadly distributed with an increasing proportion of low values with increasing risk. The overall difference of mean F-actin content among the 5 groups was highly significant by analysis of variance (variance ratio, 11.9; $P < 0.0001$). Pairwise comparison by Tukey's Studentized range test indicated that the mean F-actin content of Groups D and E were significantly different (both $P < 0.001$) from the QFIA-

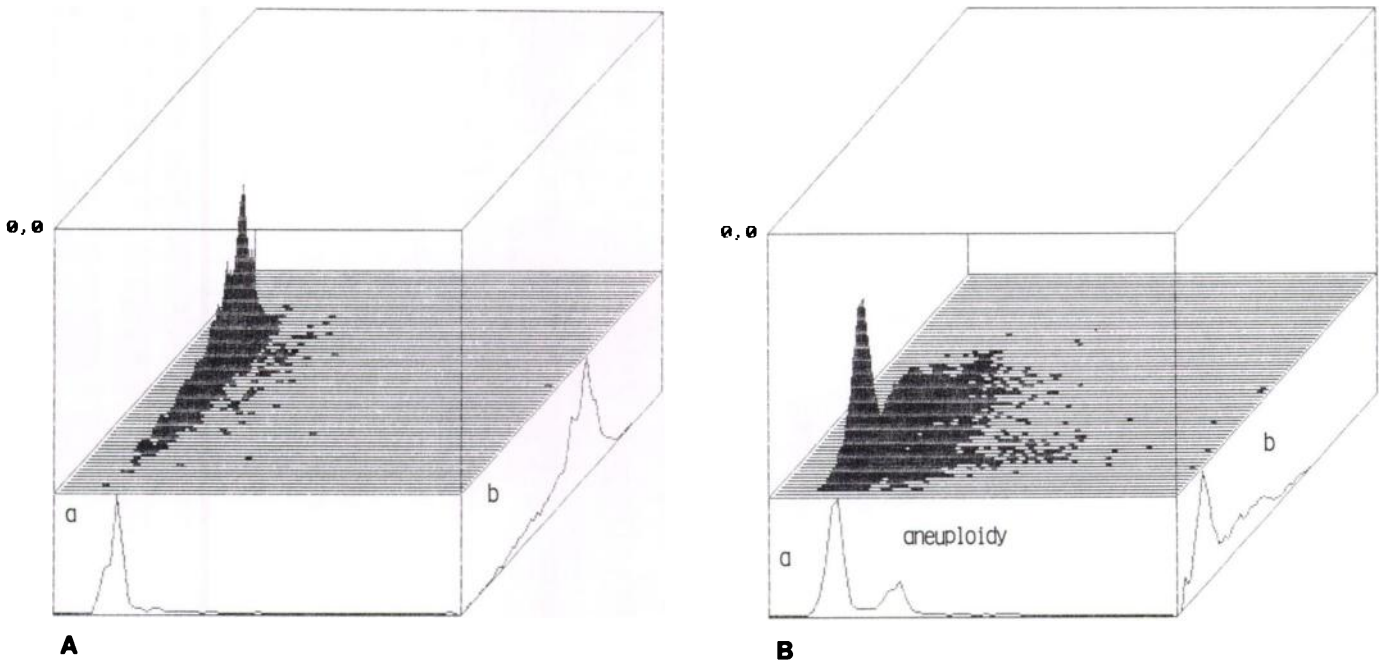

Fig. 1. Three-dimensional flow cytometry histograms of F-actin and DNA parameters. In each case, *side a* is the DNA single-parameter histogram, and *side b* is the F-actin single-parameter histogram. *A*, bladder wash specimen from an asymptomatic control; *B*, bladder wash specimen from a cytologically and biopsy-positive case.

Table 2 *Comparison of F-actin with biopsy results*[a]

Bladder wash samples were fixed in 25% EtOH. Cells were labeled with fluorescein-phalloidin for F-actin and propidium iodide for DNA. The F-actin content was defined as "normal" if the F-actin mean channel number ≥95 and ≤55% of cells had channel number below 100, or "abnormal" if the F-actin mean channel number was <95 or >55% of cells had channel number below 100, and compared with concurrent biopsy results for the 38 patients for which such data were available. The biopsy was considered "negative" if there was no transitional cell carcinoma, *i.e.*, "normal," "negative," benign prostic hyperplasia, or cystitis.

	Biopsy		
	Negative (%)	TCC[b] (%)	Total
F-actin			
Normal	16 (94)	1 (6)	17
Abnormal	3 (14)	18 (86)	21
Total	19	19	38

[a] $\chi^2 = 19.0$, $P < 0.001$.
[b] TCC, transitional cell carcinoma.

Table 3 *Abnormal low F-actin content in bladder wash cells from 204 urological patients stratified by risk defined by QFIA cytology, hematuria, and previous bladder cancer history*[a]

F-actin levels were determined as described in Table 2. QFIA cytology was "positive when visually suspicious cells or >2 of 500 cells were visually atypical and contained >5C DNA, "intermediate" with 1–2 of 500 atypical cells with >5C DNA, and "negative" otherwise.

	F-actin content	
Group	Normal (%)	Abnormal (%)
A	5 (10)	46 (90)
B	6 (25)	18 (75)
C	18 (34)	34 (66)
D	23 (64)	13 (36)
E	38 (93)	3 (7)
Total	90	114

[a] $\chi^2 = 70$, $P < 0.0001$.

positive group (Group A). The mean F-actin content of the asymptomatic control group (Group E) was also significantly higher than those of Group B and C as well ($P < 0.05$). In contrast to the finding in Table 3, there was no significant difference ($P > 0.05$) between groups C and D, respectively. This difference probably reflects that the positive-negative schema, which contains a second parameter describing the percent of cells below Channel 100, is more sensitive to the presence of a minor population of cells with low F-actin content than is the mean F-actin schema.

Table 4 shows the relationship between F-actin and risk as assessed from flow cytometry. The correlation is highly significant, as shown by the χ^2 of 72. However, the results are somewhat clouded by the high proportion of DNA ploidy-positive results in the control group (15 of 41, 37%) and an apparent higher F-actin negative rate among the DNA ploidy-positive patients (19%). It was necessary to subdivide the control group into those who were positive and those who were negative. None of the controls was positive by QFIA cytology. Nevertheless, the results confirm those with QFIA cytology; F-actin levels show a high correlation with risk for bladder cancer.

Comparison of F-Actin and M344 Labeling. To obtain further evidence that cells with low F-actin occurring in a human bladder affected by the carcinogenic process are indeed abnormal, the correlation among DNA ploidy, F-actin, and the reactivity of M344 antibody against low-grade bladder cancers and dysplasias was determined by QFIA (Table 5). There was no significant difference in mean ploidy between M344-positive and M344-negative cells. However, cells that were positive for the marker detected by the M344 antibody showed much lower F-actin values than did negative cells. This was true in comparing both the average intensity, or when normalized to cell size to account for the slightly larger sizes of the positive cells (integrated intensity).

DISCUSSION

Recent studies of cancer biology have suggested that cancers develop as cells progressively acquire genetic mutations that lead to acquisition of both enhanced growth potential due to oncogenic activation and loss of growth inhibition due to loss of tumor suppressor genes (19–22). Cancer also seems to involve a generalized genetic instability, with a propensity to undergo genetic rearrangements, chromosomal or gene duplication, or deletion (23, 24). Instability drives further progression by randomly generating clones that then compete with normal cells and other abnormal clones (25). Genetic changes are reflected in alterations of the differentiation program, as shown by the finding of epidermal growth factor receptors in the outer cell layer of urothelial dysplasias, where they are normally found only on the surfaces of basal layer cells (26).

Such changes are reflected in both protein and DNA markers. Genetic instability leads to the development of aneuploid populations of cells, whereas loss of growth control results in enhanced rates of cell division, which, in turn produces an increased number of cells with 2–4C DNA. Aneuploid cell populations and elevated proliferation index are detected easily by flow cytometry. In addition, aneuploidy also results in the appearance of a small number of cells with >5C DNA. The presence of such "rare-event" cells is a strong marker for cancer. Image analysis systems are best suited to detecting such rare-event cells indicative of aneuploidy (12, 13, 27). The cytoskeleton is a very sensitive indicator of the level of cellular differentiation, with decreased F-actin, which was characteristic of transformed cells, appearing to reflect altered differentiation rather than a property of transformation *per se* (11). In many of the patient bladder washes, a majority of the cells may show alterations in F-actin. The majority of these cells are not tumor cells, but must come from noncancerous areas of urothelium. In the triple-labeling experiment, the correlation between low F-actin levels and expression of the antigen recognized by M344 antibody shows that a decrease of F-actin is an early event in carcinogenesis that precedes development of aneuploidy. Thus, decreased F-actin may reflect the presence of cells that have

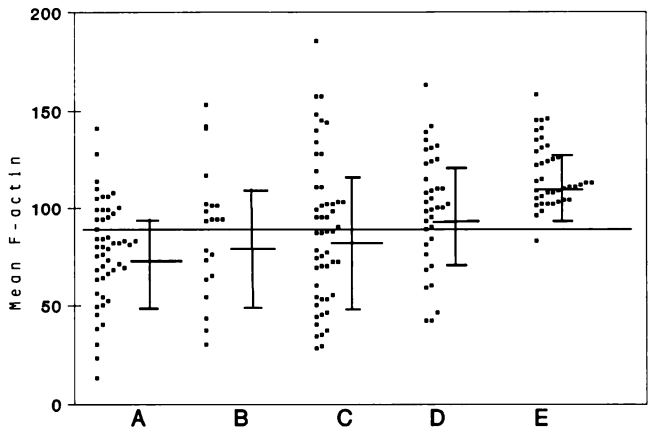

Fig. 2. Mean F-actin content (mean ± SD) in relation to bladder cancer risk strata. *A* to *D* are symptomatic with hematuria. *A* is QFIA cytology-positive, *B* is intermediate by QFIA cytology, *C* is QFIA cytology-negative with a previous bladder cancer history, and *D* is QFIA-negative but without a previous history of bladder cancer. *E*, asymptomatic control group.

Table 4 *Abnormal low F-actin content in bladder wash cells from 204 urological patients stratified by risk defined by flow cytometry, hematuria, and previous bladder cancer history*[a]

Cells were collected, fixed, and analyzed by flow cytometry as described in Table 2. Flow cytometry was scored "positive" if the DNA histogram contained an aneuploid peak or the proliferation index (the percentage of cells in G2+M and S phases) ≥15%, as "suspicious" if the proliferation index was ≥10% but <15%, and as "negative" if the proliferation index was <10%.

Group	F-actin content	
	Normal (%)	Abnormal (%)
a	14 (19)	61 (81)
b	3 (12)	22 (88)
c	14 (45)	17 (55)
d	21 (66)	11 (34)
e^+	12 (80)	3 (20)
e^-	26 (100)	0 (0)
Total	90	114

[a] $\chi^2 = 72$, $P < 0.0001$.

Table 5 *Comparison of F-actin and DNA content (mean ± SD) among M344-negative and -positive cells triple-labeled for DNA, M344 antigen, and F-actin*

Single cells were liberated from pooled biopsy specimens by agitation of the fresh tissue in saline. The sample was fixed as described for bladder washes and triple-labeled for F-actin with rhodamine-labeled phalloidin at 1:10 (w/v) dilution, DNA with aqueous Hoechst 33258, and M344, a monoclonal antibody against a tumor antigen expressed preferentially by low-grade tumors that was detected with a 3-stage biotin-avidin assay using Lucifer Yellow-labeled avidin as the reporter. The rhodamine (F-actin) and Hoehst 33258 (DNA) were quantified by QFIA, and individual cells were scored visually as positive or negative for M344 labeling.

M344	No. of cells	DNA C value	Actin content in phosphor particle units	
			Average intensity	Integrated intensity[a]
Negative	164	0.20 ± 0.06	0.30 ± 0.22	1.34 ± 0.57
Positive	52	0.21 ± 0.10	0.13 ± 0.07[b]	0.89 ± 0.35[b]

[a] Integrated intensity = $\frac{\text{average intensity}}{\text{cell area (mm}^2\text{)}} \times 1000$.

[b] $P < 0.001$ comparing with negative cells by Student's t test.

not completed their terminal differentiation programs, and the presence of such cells may well be an early marker for field disease rather than frank cancer and probably precedes the development of aneuploidy. The phenomenon of finding biochemically abnormal cells that are definitely associated with carcinogenic activation, but which are in tissue that is not histologically abnormal, is now well accepted (28).

This study compared F-actin content in a group of patients who had been stratified by bladder cancer risk using biopsy results and a combination of DNA analysis, from either QFIA cytology or flow cytometry, a history of bladder cancer, and hematuria. The risk stratification approach, while not eliminating the need for long-term follow-up studies, provides early information on the association between a given marker and disease development as well as the marker's association with other markers and risk factors (29). QFIA cytology was used instead of conventional Papanicolaou cytology because of its enhanced sensitivity to lower grade cancers due to incorporation of a DNA marker (12, 13). Among the patients with biopsy-proven cancer, 94% showed abnormal F-actin results. The prevalence of abnormal F-actin results decreased in step monotonically with decreasing risk as defined with QFIA cytology and almost as cleanly with risk defined by flow cytometry. The clear separation of the hematuria patients into 2 groups (C and D as well as c and d) by history accurately reflects the differential risk of these groups in that over 50% of patients with a bladder tumor will develop a second tumor within 5 years, but only 20% of patients with unexplained hematuria will develop a tumor within 8 years (6). The finding that 36% of Group D and 34% of Group d showed abnormal F-actin results probably reflects this significant potential for cancer development and suggests that significant abnormalities in F-actin precede significantly altered DNA ploidy. The broader distribution of F-actin among groups A–D (Fig. 2), as compared with the controls, suggests that these groups are more heterogeneous and may consist of subgroups of high and low risk, respectively. Thus, F-actin may provide a means to assess such risk.

There were some interesting differences in the results when stratifying using QFIA cytology and flow cytometry. False-positive and false-negative results seem to have clouded the stratification using flow cytometry. Some of the reasons for this can be seen in Table 6, which shows the correlation between DNA ploidy and flow cytometry and risk stratification by QFIA cytology. Although 80% of the highest risk group showed an abnormal proliferation index, that parameter failed to distinguish a difference between the cytology-negative patients with hematuria when stratified by history (Groups c and d). Inflammatory processes unrelated to carcinogenesis probably induce increased proliferation in such a symptomatic population and, hence, proliferation index may be of limited use in distinguishing patients with low-grade cancer or precancer from those with other problems. Using aneuploidy as the sole marker improved correlation on the low-risk end of the scale, but at the cost of a significant decrease in sensitivity among the highest risk group of cytologically positive patients. The results indicate that most of the enhanced "sensitivity" of proliferation index actually results from false-positive results. It is possible that at least some of these false-positive results could result from cell doublets that are not gated out. However, even with these false-positive results, the correlation between risk and F-actin is strong and confirms the findings with QFIA cytology.

Taken together, the results of this study suggest that the presence of cells with low F-actin probably reflects the presence of cells with altered differentiation programs, but that they divide at a normal rate and are not necessarily aneuploid. F-actin and other markers including epidermal growth factor receptors, blood group substances, and a large number of tumor-associated antigens, may well be useful for indicating elevated risk. If F-actin indeed represents a marker for increased risk by identifying patients with an altered urothelium, then it could well provide a very sensitive monitor for therapeutic efficacy and follow-up, as well as a tool to evaluate hematuria patients by potentially identifying low- and high-risk subgroups among

Table 6 *Proliferation index and DNA ploidy determined by flow cytometry in 204 urological patients stratified by risk*

Cells were collected, fixed, and analyzed according to Table 2. Proliferation index and DNA aneuploidy were obtained from DNA histogram by flow cytometry. The proliferation index was the percentage of cells in G2+M and S phases and was considered "normal" if proliferation index was <10% and "abnormal" if proliferation was ≥10%. The DNA histogram was scored as "aneuploidy" if a distinct nondiploid peak was discernible.

Group	Proliferation index[a]		DNA aneuploidy[b]	
	Normal (%)	Abnormal (%)	No (%)	Yes (%)
A	10 (20)	41 (80)	23 (45)	28 (55)
B	14 (58)	10 (42)	15 (63)	9 (37)
C	27 (53)	25 (47)	38 (74)	14 (26)
D	22 (61)	14 (39)	28 (78)	8 (22)
E	26 (63)	15 (37)	39 (95)	2 (5)
Total	99	105	143	61

[a] $\chi^2 = 17$, $P < 0.0001$.
[b] $\chi^2 = 27$, $P < 0.0001$.

those individuals with other risk factors. F-actin measurements could also drastically shorten the time needed to evaluate chemopreventive agents, and could be very useful in identifying groups at higher and lower risk among chemically exposed workers or others at high epidemiologically defined risk. It will be of interest to determine whether therapies such as BCG (bacillus Calmette-Guerin) produce normal F-actin patterns in some patients and whether patients with normal F-actin patterns have reduced risk of recurrence.

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