Journal of Cellular Biochemistry 76:311–321 (1999)

Assessment of DNA Flow Cytometry as a Surrogate End Point Biomarker in a Bladder Cancer Chemoprevention Trial

Silvia Bruno,[1,2]* Rosalba Torrisi,[3,4] Massimo Costantini,[5] Laura Baglietto,[6] Vincenzo Fontana,[7] Beatrice Gatteschi,[8] Giovanni Melioli,[1] Guido Nicolo,[8] Antonio Curotto,[9] Bruno Malcangi,[10] Gian Paolo Bruttini,[11] Marco Varaldo,[10] Paolo Bruzzi,[5] and Andrea Decensi[3,4]

[1]Cytometry Unit, National Cancer Institute, 16132 Genoa, Italy
[2]Department of Experimental Medicine, Human Anatomy Section, University of Genoa, 16132 Genoa, Italy
[3]Unit of Medical Oncology II, National Cancer Institute, 16132 Genoa, Italy
[4]Chemoprevention Unit, European Institute of Oncology, 20141 Milan, Italy
[5]Unit of Clinical Epidemiology and Trials, National Cancer Institute, 16132 Genoa, Italy
[6]Division of Epidemiology, European Institute of Oncology, 20141 Milan, Italy
[7]Unit of Biostatistics, National Cancer Institute, 16132 Genoa, Italy
[8]Unit of Pathology, National Cancer Institute, 16132 Genoa, Italy
[9]Urologic Clinic, S. Martino Hospital, 16132 Genoa, Italy
[10]Division of Urology, Gallino Hospital, 16164 Pontedecimo, Genoa, Italy
[11]Division of Urology, Nervi Hospital, 16167 Genoa, Italy

Abstract Although conventional cytology represents the most widely performed cytometric analysis of bladder cancer cells, DNA flow cytometry has, over the past decade, been increasingly used to evaluate cell proliferation and DNA ploidy in cells from bladder washings. We have investigated whether DNA flow cytometry and conventional cytology of epithelial cells obtained from bladder washings provide reliable surrogate endpoint biomarkers in clinical chemoprevention trials. We used cytometric and clinical data from a chemoprevention trial of the synthetic retinoid Fenretinide on 99 patients with superficial bladder cancer. A total of 642 bladder washing specimens obtained from the patients at 4 month intervals was analyzed. Intra-individual agreement and correlation of flow cytometric DNA ploidy (diploid vs. aneuploid), DNA Index, Hyper-Diploid-Fraction (proportion of cells with DNA content higher than 2C), and conventional cytologic examination, as assessed by kappa statistics and Spearman's correlation test, were poor from baseline through 24 months. Moreover, no correlation was found between DNA ploidy and cytology at each time point. The same results were obtained when the analyses were stratified by treatment group. In addition, the association between the results of bladder washing (by either DNA flow cytometry or cytology) and concomitant tumor recurrence was significant only for abnormal cytology, while neither biomarker was predictive of tumor recurrence at the subsequent visit. During the time of this study only four patients progressed to muscle-invasive bladder cancer, indicating the "low-risk" features of the patient population. We conclude that DNA flow cytometry and conventional cytology on epithelial cells obtained from bladder washings do not appear to provide suitable surrogate endpoint biomarkers during the early stages of bladder carcinogenesis. J. Cell. Biochem. 76:311–321, 1999.

Key words: bladder cancer; DNA flow cytometry; biomarker

Bladder cancer is the sixth most common cancer in the United States, with approximately 54,000 new cases estimated in 1998 [Landis et al., 1998]. Among newly diagnosed cases, the vast majority initially present with papillary superficial bladder cancer (SBC), either in situ (Ta) or invading the lamina propria (T1). After transurethral resection (TUR) of the tumor(s), and despite the efficacy of prophylac-

Grant sponsor: NIH/NCI; Grant number 5UO1 CA-56457; Grant sponsor: Italian Association for Cancer Research (AIRC).

*Correspondence to: Silvia Bruno, Institute of Human Anatomy, Department of Experimental Medicine, University of Genoa, Via de Toni 14, 16132 Genoa, Italy. E-mail: silviab@anatomiau.unige.it

Received 5 April 1999; Accepted 22 July 1999

tic BCG immunotherapy in prolonging recurrence and progression-free survival, approximately 40–80% of the cases will recur as a superficial tumor, while progression to muscle-invasive cancer within 5 years ranges from 10% to 20% [Landis et al., 1998]. Importantly, tumors may recur anywhere in the urothelial lining, possibly as a result of field cancerization effect, thus providing the rationale for chemopreventive interventions which may counteract the carcinogenic transformation of the entirely exposed urothelial tract.

As chemoprevention trials using the incidence of invasive cancer as endpoint require large numbers of subjects and long periods of follow-up, the use of surrogate biomarkers greatly increases the feasibility of these trials to select active agents. Moreover, these trials could aid in learning more about the carcinogenesis process [Hong and Sporn, 1997]. Since two important components in the process of carcinogenesis are genetic instability coupled with increased cell proliferation, DNA ploidy and proliferation assessed by flow cytometry (FCM) may qualify as suitable intermediate biomarkers for chemopreventive trials.

Numerous studies have investigated in the last two decades the role of DNA flow cytometry (DNA-FCM) as a clinical tool in bladder cancer [e.g., Collste et al., 1980; Wijkström et al., 1984; deVere White et al., 1988]. While some of these studies have suggested a good association between DNA content and disease course, others have reached different conclusions. The results of a DNA Cytometry Consensus Conference [Wheeless et al., 1993], together with more recent studies [Shackney et al., 1995; Sole et al., 1995; Vindeløv et al., 1995; Bittard et al., 1996], seem to support the prognostic and diagnostic usefulness of DNA-FCM analysis in superficial bladder cancer only under limited circumstances.

These controversial findings, along with encouraging results of a previous feasibility study that we conducted on superficial bladder cancer patients [Bruno et al., unpublished results], prompted us to assess the validity of DNA-FCM in providing surrogate endpoint biomarkers of bladder cancer in "low-risk" patients. For this we utilized the serial bladder washings of patients with superficial bladder cancer participating in a randomized clinical trial of the synthetic retinoid Fenretinide (N-4-hydroxyphenyl-retinamide, 4-HPR). The DNA ploidy (diploid vs. aneuploid), the DNA Index (ratio of aneuploid DNA content to diploid DNA content), and the Hyper–Diploid-Fraction (HDF, which is proportional to the proliferation of diploid and aneuploid cells, as well as to the relative amount of the latter) have been found to be the most reliable and sensitive among DNA-FCM parameters [Wheeless et al., 1991a, 1993]. In clinical chemoprevention studies these parameters should fulfill the necessary, albeit not sufficient, requirement for reliable surrogate endpoint biomarkers, namely the ability to show the tendency of each individual to have similar values over time, thus providing an overall consistency of measurements. To this end, we investigated the intra-individual agreement and correlation over time of DNA-FCM and conventional cytology, as well as the association between the two. Moreover, we analyzed the diagnostic sensitivity and specificity of DNA-FCM and cytology, as well as their ability to predict tumor recurrence.

MATERIALS AND METHODS

Study Design and Patients

Ninty-nine patients with primary or recurrent superficial bladder cancer (stage Ta or T1, any Grade) within the previous three years, were randomized to oral 4-HPR 200 mg/d, or no treatment, for 2 years. A third year of follow-up was planned in both arms. Randomization was performed by phoning to a Central Coordinating Center. The study had received Institutional Review Board approval and all subjects signed an informed consent before randomization. Patients were aged < 80, and had performance status $= 0$ (WHO score), normal liver, renal, cardiac and metabolic functions and no controindications to retinoid use. Moreover, they had to have no cystoscopic evidence of disease at the time of randomization and had to have terminated intravesical treatment within at least 2 months before randomization. Patient and tumor characteristics were evenly distributed between the two arms (data not shown). All subjects underwent physical examination and cystoscopy with bladder washings for conventional cytology and DNA-FCM at baseline and every 4 months. Bladder washing was performed using the technique previously described [Decensi et al., 1992]. Briefly, a double irrigation with five to six vigorous pulses of 100 ml normal saline was performed through a urethral catheter or a resectoscope sheath.

During the trial, tumor recurrence, defined as the presence of a papillary tumor or an infiltrating cancer at cystoscopy, was treated either with diathermy (if the tumor maximum diameter was < 5 mm) or TUR in all remaining cases. All patients undergoing TUR subsequently received weekly intravesical BCG instillations (Pasteur strain) 75 mg for 6 weeks or mitomycin C 40 mg for 8 weeks. Five patients proved to be ineligible after randomization for different reasons; however, these 5 patients were nonetheless included in the present analysis. A total of 82 subjects completed the three year study period. The reasons for the 17 dropouts were the following: progression to muscle-invasive disease ($n = 4$), other neoplasms ($n = 3$), cardiovascular disease ($n = 3$), other diseases ($n = 2$), and loss to follow-up ($n = 5$).

Cytology

The collected fluid specimen was immediately mixed and divided for cytology and flow cytometry, which were performed in two different laboratories. In order to preserve study blinding, each collected fluid specimen was labeled only by a progressive identification number, so that neither the subject's identification nor the treatment allocation could be disclosed during cytologic and DNA-FCM examinations. Specimens for cytologic examination were stained with the Papanicolaou technique and classified in four categories, according to the slightly modified criteria of Murphy [1990]: normal cells; benign changes related to reactive cells (e.g., after intravesical treatment or due to mild dysplasia); low-grade neoplasia (atypical cells of uncertain significance, dysplastic cells, or suspicious abnormal cells); high-grade malignant cells.

Flow Cytometry

Staining for flow cytometry and DNA measurements were performed as previously described [Decensi et al., 1994, 1996]. Briefly, cells contained in 100 ml of fluid specimen were centrifuged, resuspended in 0.5 ml of 0.5% paraformaldehyde for 15 min at 0°C, washed in PBS, and permeabilized with 0.5 ml of 0.1% Triton X-100 for 5 min at 0°C, washed again with PBS, and finally resuspended in the DNA staining solution (30 μg/ml propidium iodide (PI) and 0.5 mg/ml RNase). After 1–2 h at room temperature, flow cytometric measurements were performed on an EPICS Elite Coulter instrument (Coulter Corporation, Miami, FL), equipped with suitable filters for PI excitation and emission fluorescence. For optimal cleaning from debris, the discriminator (trigger) was set on the photomultiplier for PI fluorescence. For each event, the forward scatter, side scatter, and PI fluorescence signals (both Area and Peak, for cell aggregates correction) were acquired and stored in List Mode. A minimum of 10,000 cells for each specimen was analyzed. The PI fluorescence mean channel of the lymphocyte population, which, although to variable proportions, could easily be identified in the vast majority of bladder washings on the log FS-log SS bivariate plot, was utilized as internal reference channel of diploidy. In aneuploid histograms, i.e., histograms with at least one discrete cell population with a DNA content other than diploid, the DNA Index was evaluated as the ratio of the DNA content of G0-G1 aneuploid cells to the DNA content of diploid G0–G1 cells, which are conventionally given a DNA Index of 1.00. Histograms with multiple (n) aneuploid peaks were characterized by n DNA indices and n fractions of aneuploid cells. A histogram was considered aneuploid with DI = 2.00 when the tetraploid peak fraction exceeded by two standard deviations the mean value of the tetraploid cell fractions measured in 15 bladder washings from normal nonsmoking donors, i.e., 15% or higher. For a finer DI evaluation of aneuploid peaks that were very close to, and often partly overlapping, the diploid G0–G1 peak, we utilized the bivariate log SS-lin PI fluorescence plot for easier identification. We operationally defined as being aneuploid those populations with a DI $\geq$ 1.10. However, plots showing a slightly asymmetrical diploid population shifting to higher PI fluorescence (PI fluorescence increase $<$10%), were considered diploid. For all histograms, both diploid and aneuploid, the Hyper-Diploid-Fraction (HDF) was calculated as the percentage of cells above the upper bound of the diploid G0–G1 population.

As most bladder washing specimens normally contain a variable population of lymphocytes, monocytes/macrophages, granulocytes, and squamous cells in addition to the transitional epithelial cells, adjustment for the nontransitional cell component of HDF values was performed after computer-assisted image analysis (CAS 200, Becton Dickinson, San Diego, CA) on the cytologic smears. The percentage of non-

urothelial cells was calculated over 200 cells representative of the whole sample. This was done under the assumption that non-urothelial cells are mainly quiescent and therefore enrich only the G0–G1 cell fraction.

DNA content histograms were considered non evaluable when the measurement resolution was high, i.e., when the Coefficient of Variation (CV) of diploid G0–G1 cells was higher than 6%, when the non-transitional fraction of cells was higher than 60%, or when the proportion of cell debris was excessive (>60%).

Statistical Analyses

A prerequisite for a reliable biomarker is its ability to trace the expected tendency of each individual to show similar values over time. In addition, the consistency of measurements, indicated by the intra-individual agreement, should decrease with increasing time between two measurements. To this purpose, we evaluated the indices of agreement within the same subject among samples at different times. For binary variables, such as DNA ploidy and cytology, kappa statistics [Fleiss, 1981] was used. Kappa statistics estimates the agreement between two (or more) measurements over and above the agreement expected by chance. The kappa coefficient ranges between –1 and 1, with higher values reflecting higher agreement. It has been suggested that kappa values less than 0.40 reflect a poor agreement [Fleiss, 1981]. For ordinal variables, such as DNA index and HDF, the Spearman correlation coefficient was used [Siegel, 1956]. Spearman correlation is a non-parametric method to quantify the degree of linear association between two variables. It ranges between –1 and +1, with higher values reflecting higher correlation. In our analysis the set of samples taken at each time were contrasted with the set of samples taken at each other time, and the intraindividual agreement or correlation was estimated. No test of statistical significance is presented since the null hypothesis of no agreement or no correlation is devoid of any interest.

In order to evaluate the association between bladder washing measurements (by either flow cytometry or conventional cytology) and the occurrence of tumor recurrences, two analyses were performed: a cross sectional analysis, where the presence of a first recurrence was contrasted with the results of measurements of bladder washing, and a prospective analysis aimed at evaluating the ability of bladder washing measurements to predict the detection of tumor recurrence 4 months later. In both instances a multivariate logistic model was used [Halperin et al., 1971], with presence of recurrence as the dependent variable, and bladder washing measurement as covariate. Both analyses were stratified by time, in that each patient was re-entered in the model several times, until he developed a recurrence, or was either lost to follow-up, or completed without recurrence the 24 months of follow-up.

RESULTS

Quality Standards

In the present study 642 bladder washings were analyzed. The specimens were obtained from 99 patients every 4 months for 2 years. Six hundred and thirty-seven were considered evaluable for cytological measurement and 609 for flow cytometry. Thirty-three bladder washings were rejected for excessive debris (n = 10) or predominant non-transitional cell component (n = 23), while most of the 609 evaluable bladder washings showed minimal debris and less than 10% of non-urothelial cells. No bladder washing was rejected for low resolution of the DNA-FCM measurement, i.e., for a CV > 6% (see Materials and Methods). Moreover, the mean CV of the lymphocyte population present in the bladder irrigation specimens was 2.3% ± 0.4%, providing additional evidence for a good quality standard of the DNA measurements. Eighteen bladder washings showed a slight asymmetry to the right of the diploid peak, but were considered diploid (see Materials and Methods).

Pattern of Distribution of DNA Index, HDF, and Cytology

The DNA Index frequency distributions of the bladder washings collected at baseline, 1 and 2 years after enrollment, are shown in Figure 1. The large majority of bladder washings belong to the diploid and "near-diploid" (DNA Index ≤ 1.25) categories. A trend towards an increase in the proportion of diploid histograms over time was observed. The same pattern of DNA Index distributions was observed on all bladder washings collected at intermediate time intervals (i.e., 4, 8, 12, 16, and 20 months), as well as when the control and the 4-HPR groups were evaluated separately (data not shown).

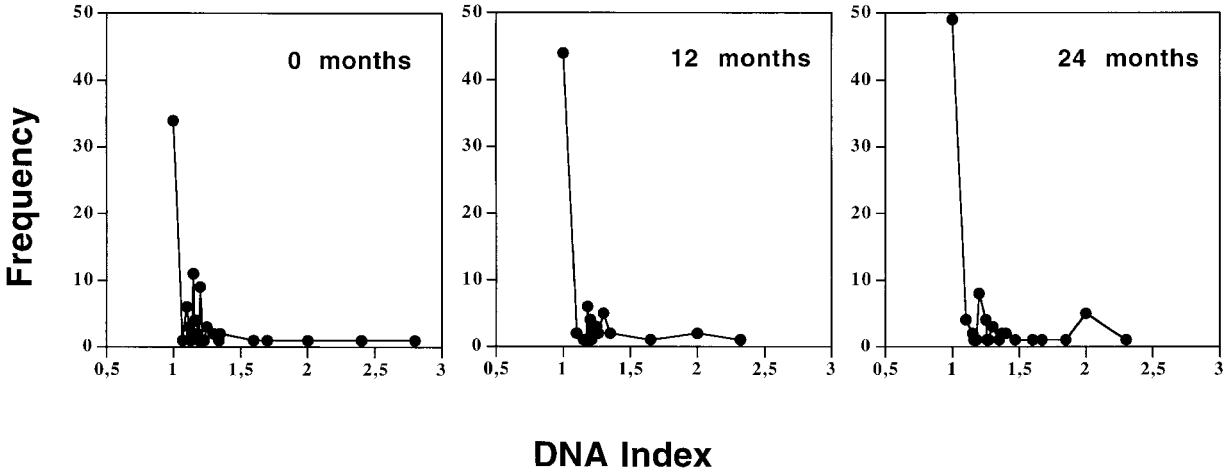

Fig. 1. Frequency distribution of the DNA Index evaluated by DNA flow cytometry on bladder washings of all patients at baseline, 12, and 24 months ater enrollment.

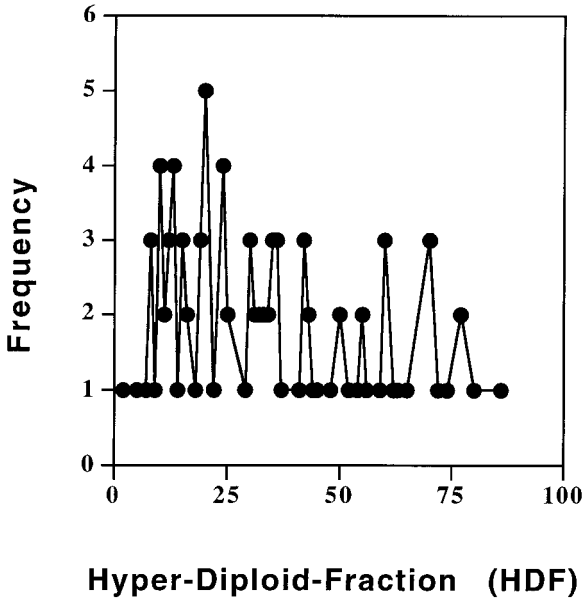

Fig. 2. Frequency distribution of the Hyper-Diploid-Fraction (HDF) evaluated at baseline by DNA flow cytometry for the total patient population.

The values of the HDF at baseline are reported in Figure 2. A scattered distribution can be observed. A similar pattern is observed at all other time points, as well as when the two treatment groups are considered separately (not shown).

Table I shows the distribution of the cytologic examinations at baseline, 1 and 2 years after enrollment. Consistent with the results of DNA-FCM, an increase in the proportion of negative examinations was observed over time.

TABLE I. Distribution of Cytologic Examinations at Baseline, 1, and 2 Years After Enrollment

	Baseline (n = 99)	1 year (n = 94)	2 years (n = 89)
Negative	36	36	58
Benign alterations	52	36	16
Low grade malignant cells	8	18	12
High grade malignant cells	2	3	3
Not evaluable	1	1	0

Intra-Individual Agreement and Correlation

As a good consistency of the measurements is an essential prerequisite for a biological marker to be considered a suitable surrogate endpoint, we investigated the intra-individual agreement of DNA-FCM parameters and cytology of bladder washings collected at 4 month intervals over a period of 2 years. The agreement was first investigated irrespective of treatment arm. Table II reports the k values for DNA ploidy (diploid vs. aneuploid), indicating a poor intra-individual agreement. Similar conclusions emerge from separate analysis by treatment group, indicating no confounding effect by 4-HPR (not shown). Tables III and IV report the intra-individual correlation of DNA Index and HDF, respectively. Again, the Spearman's correlation coefficients show a poor correlation (Tables III and IV), even when stratified by treatment arm (not shown).

As shown in Table V, the k values obtained with the cytology evaluations, which were cat-

TABLE II. Intraindividual Agreement in DNA Ploidy (Diploid vs. Aneuploid) Between Pairs of Bladder Washings Measured by Flow Cytometry Every 4 Months[a]

	4th month	8th month	12th month	16th month	20th month	24th month
Baseline	0.10 (n = 84)	0.18 (n = 83)	0.20 (n = 85)	0.08 (n = 79)	0.22 (n = 75)	0.19 (n = 76)
4th month	—	0.17 (n = 82)	0.21 (n = 82)	−0.10 (n = 78)	0.16 (n = 73)	0.13 (n = 74)
8th month	—	—	0.17 (n = 84)	0.06 (n = 80)	0.23 (n = 76)	−0.01 (n = 77)
12th month	—	—	—	0.26 (n = 82)	0.26 (n = 78)	0.22 (n = 79)
16th month	—	—	—	—	0.46 (n = 77)	0.11 (n = 79)
20th month	—	—	—	—	—	0.42 (n = 77)

[a]Intraindividual agreement was estimated by means of the k values.

TABLE III. Intraindividual Correlation in DNA Index Between Pairs of Bladder Washings Measured by Flow Cytometry Every 4 Months[a]

	4th month	8th month	12th month	16th month	20th month	24th month
Baseline	0.13 (n = 84)	0.25 (n = 83)	0.22 (n = 85)	0.17 (n = 79)	0.24 (n = 75)	0.05 (n = 76)
4th month	—	0.12 (n = 82)	0.34 (n = 82)	0.03 (n = 78)	0.16 (n = 73)	0.11 (n = 74)
8th month	—	—	0.11 (n = 84)	0.05 (n = 80)	0.12 (n = 76)	−0.02 (n = 77)
12th month	—	—	—	0.37 (n = 82)	0.27 (n = 78)	0.19 (n = 79)
16th month	—	—	—	—	0.48 (n = 77)	0.14 (n = 79)
20th month	—	—	—	—	—	0.40 (n = 77)

[a]Intraindividual correlation was estimated by means of the Spearman's correlation coefficients (r).

egorized as negative (including benign alterations) or positive (low and high grade malignant cells), again demonstrate that the intra-individual agreement is poor. The same conclusions emerge from separate analysis by treatment group (not shown).

The correlation between the flow cytometric and the cytologic results was also investigated. The k values reported in Table VI indicate that the intra-individual agreement between DNA ploidy (diploid vs. aneuploid) and cytology (negative vs. positive) is low through the 24 months. A similar conclusion was found when the analysis was repeated in each treatment arm (not shown).

Detection and Prediction of Tumor Recurrence

We finally investigated the role of DNA-FCM and conventional cytology in detecting and predicting tumor recurrence. The association between tumor recurrence and the results of DNA ploidy, HDF, or cytology in the concomitant or 4 months earlier bladder washing are reported in Table VII. While a positive cytology was suggestive of a concomitant tumor recurrence, an association between an aneuploid histogram and concomitant recurrence was not found. Paradoxically, a diploid histogram was predictive of tumor recurrence 4 months later, while cytology had no predictive effect. The HDF, arbitrarily categorized in three levels, as suggested by the distribution shown in Figure 2, was not able to predict or to detect tumor recurrence.

DISCUSSION

In the present study we investigated whether DNA-FCM and conventional cytology of urothe-

TABLE IV. Intraindividual Correlation in HDF Between Pairs of Bladder Washings Measured by Flow Cytometry Every 4 Months[a]

	4th month	8th month	12th month	16th month	20th month	24th month
Baseline	0.18 (n = 84)	0.06 (n = 83)	0.21 (n = 85)	0.21 (n = 79)	0.32 (n = 75)	0.10 (n = 76)
4th month	—	−0.01 (n = 82)	0.25 (n = 82)	0.06 (n = 78)	0.04 (n = 73)	−0.02 (n = 74)
8th month	—	—	−0.05 (n = 84)	−0.09 (n = 80)	0.01 (n = 76)	0.06 (n = 77)
12th month	—	—	—	0.37 (n = 82)	0.17 (n = 78)	0.06 (n = 79)
16th month	—	—	—	—	0.55 (n = 77)	−0.01 (n = 79)
20th month	—	—	—	—	—	0.32 (n = 77)

[a]Intraindividual correlation was estimated by means of the Spearman's correlation coefficients (r).

TABLE V. Intraindividual Agreement in Cytologic Examination (Negative[a] vs. Positive[b]) Between Pairs of Bladder Washings Evaluated Every 4 Months[c]

	4th month	8th month	12th month	16th month	20th month	24th month
Baseline	0.28 (n = 93)	0.08 (n = 91)	0.06 (n = 94)	0.09 (n = 87)	0.32 (n = 83)	0.12 (n = 89)
4th month	—	0.29 (n = 89)	0.19 (n = 90)	0.31 (n = 84)	0.35 (n = 80)	0.34 (n = 85)
8th month	—	—	0.24 (n = 90)	0.33 (n = 84)	0.27 (n = 80)	0.23 (n = 85)
12th month	—	—	—	0.12 (n = 87)	0.16 (n = 83)	0.40 (n = 89)
16th month	—	—	—	—	0.27 (n = 80)	0.38 (n = 85)
20th month	—	—	—	—	—	0.34 (n = 83)

[a]Negative also includes benign alterations.
[b]Positive: low and high grade neoplasm.
[c]Intraindividual agreement was estimated by means of the k values.

TABLE VI. Intraindividual Agreement (k Values) Between DNA-FCM Ploidy (Diploid vs. Aneuploid) and Cytologic Examination (Negative[a] vs. Positive[b])

Baseline	−0.06 (n = 92)
4th month	0.01 (n = 88)
8th month	−0.01 (n = 88)
12th month	0.16 (n = 90)
16th month	−0.02 (n = 85)
20th month	0.20 (n = 81)
24th month	0.16 (n = 82)

[a]Negative also includes cells with benign alterations.
[b]Positive: low and high grade malignant cells.

lial cells obtained from bladder washings could provide suitable surrogate endpoint biomarkers in a randomized chemoprevention trial of bladder cancer. The results obtained with 642 bladder washings show poor intra-individual agreements and correlations for both techniques over the 24 month study period. A low correlation between the two methods was also observed. Moreover, DNA ploidy and HDF were unable to detect concomitant tumor recurrence, and cytology was not predictive of subsequent tumor recurrence. Overall, these results seem to discount the use of these biomarkers on bladder washing specimens as reliable surrogate endpoints for clinical trials of superficial bladder cancer.

We believe that these negative results are intrinsically related to the distribution of the DNA Index in our "low-risk" patient population. The large majority of DNA aneuploid cell populations lie in the "near-diploid" region, with DNA Indices ranging from 1.00 to 1.25, due to the early-stage bladder cancer history (Ta and T1) of the patients under study. In a previous

TABLE VII. Association Between Tumor Recurrence and Concurrent or Previous Bladder Washing Evaluation by DNA Flow Cytometry and Cytology

	Concurrent bladder washing[a]	Previous bladder washing[b]
	OR (95% CI)[c]	OR (95% CI)[c]
DNA flow cytometry		
Diploid	1.00 (ref.)	1.00 (ref.)
Aneuploid	1.07 (0.54–2.15) ($P = 0.843$)	0.43 (0.22–0.86) ($P = 0.015$)
HDF		
≤25%	1.00 (ref.)	1.00 (ref.)
26%–50%	1.20 (0.58–2.48)	0.55 (0.25–1.19)
>50%	0.93 (0.31–2.79) ($P = 0.847$)	0.73 (0.29–1.89) ($P = 0.293$)
Cytology		
Negative-benign alterations	1.00 (ref.)	1.00 (ref.)
Neoplasms (low and high grade)	8.88 (4.42–17.8) ($P < 0.001$)	1.30 (0.54–3.16) ($P = 0.565$)

[a]DNA flow cytometric or cytologic results concurrent to the tumor recurrence at the follow-up visit.
[b]DNA flow cytometric or cytologic results obtained 4 months prior to the detection of the recurrence (i.e., at the previous follow-up visit).
[c]Odd ratio of recurrence estimated by means of a logistic model.

DNA flow cytometric study on a similar population of bladder cancer patients (n = 197), 41% of bladder washings from patients with Ta and T1 stage tumors were diploid and 92% of the 59% aneuploid bladder washings had a DNA Index ranging from 1.00 to 1.25, again indicating that most Ta and T1 tumors are diploid or near-diploid [Bruno et al., unpublished data]. Similarly, Vindeløv et al. [1995] showed that the large majority of superficial papillary tumors had a DNA Index in the range 1.00–1.25, while a DNA Index >1.25 was strongly correlated to invasiveness. Moreover, Shackney et al. [1995] recently showed that the series of genetic evolutionary changes in early bladder cancer start with the initial development of near-diploid aneuploid stemlines. Therefore, the compression of DNA Index values in the narrow range of the diploid and near-diploid region observed in our "low-risk" patients might increase the possibility of erroneous DNA ploidy estimations, since the experimental error variance, due to measurement variability [Wheeless et al., 1991a,b], may be as large as the slight difference between the diploid and the close near-diploid DNA Index values. In fact, we observed a random stepping up and down of DNA Indices between diploid and near-diploid values over time in a significant proportion of patients. According to the model of evolution of aneuploidy in bladder cancer of Shackney et al. [1995], this is unlikely given to repeated cycles of growth and subsequent apoptosis of near-diploid stem cell populations.

We minimized possible artifacts due to low sensitivity of the DNA flow cytometric measurements (false negative diploid histograms). According to the results of different mathematical approaches [Rabinovitch et al., 1992; Benson et al., 1994], our measurements were carried out under experimental conditions that warranted a high resolution of near-diploid peaks at a DI of approximately 1.10 in most bladder washings. In fact, none of the DNA histograms was rejected for poor resolution, while the low CV of the lymphocyte population present in almost all washing samples assured a good quality of the measurements. In addition, this lymphocyte population provided the best internal reference standard for diploidy [Chabanas et al., 1993; Hijazi et al., 1991]. Another factor that may affect the resolution, and thus the sensitivity of the technique, is the presence of a high proportion of debris and non-urothelial cells. In only 10 out of 642 bladder washings was debris unacceptably high, and only 23 specimens were rejected for an excessive non-urothelial fraction (>60%). The very large majority of the remaining bladder washings showed a urothelial cell component higher than 90%. Nevertheless, the HDF values were always adjusted for the non-urothelial component (as mentioned in Material and Methods).

However, we recognize that histograms classified as "DNA diploid" are "DNA flow diploid" only, as the DNA-FCM technique is insensitive to the gain or loss of small chromosomes or to minor chromosomal deletions or translocation.

While one laboratory which investigated chromosomal aberrations in bladder washing cells by fluorescence in situ hybridization (FISH) has found the same sensitivity of FCM and FISH [Cajulis et al., 1995], other studies have shown that at least half of tumors diploid for FCM are in fact non-diploid [Sauter et al., 1997; Matsuyama et al., 1994; Hopman et al., 1991], thus calling for the inaccuracy of DNA-FCM in detecting early genetic events in bladder tumorigenesis.

We overcame the problem of possible false tetraploid histograms caused by aggregates of diploid G1 cells or multinucleated cells by chemically disaggregating cell clumps and multiple nuclei and by electronically gating out aggregates using the Doublet Discrimination Method included in the flow cytometer software. However, we cannot completely exclude, in some cases, the occurrence of false aneuploid near-diploid histograms. Propidium Iodide was chosen for its high stoichiometric binding compared to other dyes, which minimizes the dependence of DNA accessibility on the chromatin structure. In addition, we minimized this effect by increasing the duration of PI staining to up to 3 h. However, in some urothelial cells with high cytoplasm/nucleus ratio (e.g., umbrella cells of the bladder wall) the possible dim staining of some cytoplasmic structures may provide non-specific contributions to the DNA fluorescence [Wheeless et al., 1991b]. In addition, an inflammatory bladder may also contribute to a false near-diploid DNA histogram, as previously reported [Cajulis et al., 1995; Klein et al., 1988].

The compressed and narrow DNA Index distribution of Ta and T1 tumors also affects the HDF parameter, in that there is a substantial increase in HDF values as the histogram shifts from diploid to near-diploid (due to the addition of the "near-diploid" cell fraction to HDF). This also could partly explain the lack of intra-individual correlation.

As no reliable evaluation of the S-phase fraction is possible for DNA histograms containing aneuploid populations with a DI lower than 1.40, which account for the great majority of our aneuploid histograms (87.5%), we were not able to investigate the S-phase performance in our study. However, we analyzed the S-phase fraction in the diploid subgroup (299 histograms out of 609) and found no intra-individual correlation (data not shown).

Importantly, all results are not affected by the treatment with 4-HPR, since the same conclusions emerged when the untreated control group was analyzed separately.

Progression to muscle invasive disease by far represents the most relevant clinical event in this type of patient population [Kalish et al., 1987]. However, due to the remarkably low number of disease progressions during our study (four out of 99 patients) compared to other series [Kurth et al., 1995], we were unable to assess whether DNA-FCM and/or cytology provide predictive information of this event. In contrast, previous pilot studies in subjects with in situ non-papillary carcinoma at high risk for disease progression showed that DNA-FCM in cells obtained from bladder washings can predict the response to intravesical BCG [Bretton et al., 1989].

We therefore investigated the predictive value of DNA-FCM and cytology with respect to a less severe, yet more frequent, clinical event, namely, superficial tumor recurrence. We found that DNA-FCM not only had no value in predicting recurrence 4 months apart, but could even not correlate with concomitant recurrence. In this study, where patients underwent frequent follow-up visits, the transformed area of the tumor recurrence at the time of the visit might still have been minimal compared to the total lumen of the bladder. Therefore, bladder washings may have contained a low proportion of abnormal cells, thus significantly affecting the sensitivity of the DNA-FCM measurement. While the predictive value of DNA-FCM on papillary tumor recurrence had been suggested in some studies [Giella et al., 1992], other studies, in agreement with our findings, showed no predictive power [Tetu et al., 1996]. Cytology was unable to predict recurrence 4 months apart, but it showed a good association with detection of concomitant tumor recurrence.

The observation that conventional cytology, similarly to DNA-FCM, showed poor intra-individual agreement seems to strengthen the contention that mechanisms other than technical limitations should be advanced to account for our results. Like DNA-FCM, the categorization of cytology may decrease its sensitivity, as a shift between intermediate classes (2 and 3)

over time may be expected. The search for finer, more quantitative, biomarkers is thus recommended.

In conclusion, the data collected from 642 serial bladder washings in a "low-risk" population of 99 patients with previous superficial bladder cancer enrolled in a clinical chemoprevention trial seem to discount the utilization of DNA flow cytometric and cytologic parameters as reliable surrogate end point biomarkers. In contrast to the results of studies in subjects at higher risk for disease progression or with more advanced stages of bladder cancer, the accuracy of these biomarkers during the early stages of bladder carcinogenesis appears to be limited and, thus, of poor clinical utility.

REFERENCES

Benson NA, Braylan RC. 1994. Evaluation of sensitivity in DNA aneuploidy detection using a mathematical model. Cytometry 15:53–58.

Bittard H, Lamy B, Billery C. 1996. Clinical evaluation of cell deoxyribonucleic acid content measured by flow cytometry in bladder cancer. J Urol 155:1887–1891.

Bretton PR, Herr HW, Kimmel M, Fair WR, Whitmore WF Jr, Melamed MR. 1989. Flow cytometry as a predictor of response and progression in patients with superficial bladder cancer treated with bacillus Calmette-Guerin. J Urol 141:1332–1336.

Cajulis RS, Haines GK 3rd, Frias-Hidvegi D, McVary K, Bacus JW. 1995. Cytology, flow cytometry, image analysis, and interphase cytogenetics by fluorescence in situ hybridization in the diagnosis of transitional cell carcinoma in bladder washes: a comparative study. Diagn Cytopathol 13:214–223.

Chabanas A, Rambeaud J-J, Seigneurin D, Fradet Y, Lawrence J-J, Faure G. 1993. Flow and image cytometry for DNA analysis in bladder washings: improved concordance by using internal reference for flow. Cytometry 14:943–950.

Collste LG, Devonec M, Darzynkiewicz Z, Traganos F, Sharpless TK, Whitmore WF Jr, Melamed MR. 1980. Bladder cancer diagnosis by flow cytometry. Cancer 45: 2389–2394.

Decensi A, Bruno S, Giaretti W, Torrisi R, Curotto A, Gatteschi B, Geido E, Polizzi A, Costantini M, Bruzzi P, Nicolò G, Costa A, Boccardo F, Giuliani L, Santi L. 1992. Activity of 4-HPR in superficial bladder cancer using DNA flow cytometry as an intermediate endpoint. J Cell Biochem suppl 16i:139–147.

Decensi A, Bruno S, Costantini M, Torrisi R, Curotto A, Gatteschi B, Nicolò G, Polizzi A, Perloff M, Malone WF, Bruzzi P. 1994. Phase IIa study of fenretinide in superficial bladder cancer, using DNA flow cytometry as an intermediate end point. J Natl Cancer Inst 86:138–140.

Decensi A, Fontana V, Bruno S, Gustavino C, Gatteschi B, Costa A. 1996. Effect of Tamoxifen on endometrial proliferation. J Clin Oncol 14:434–440.

DeVere White RW, Deitch AD, West B, Fitzpatrick JM. 1988. The predictive value of flow cytometric information in the clinical management of stage 0 (Ta) bladder cancer. J Urol 139:279–282.

Fleiss JL. 1981. Statistical methods for rates and proportion. 2nd ed. New York: Wiley-Liss.

Giella JG, Ring K, Olsson CA, Karp FS, Benson MC. 1992. The predictive value of flow cytometry and urinary cytology in the followup of patients with transitional cell carcinoma of the bladder. J Urol 148:292–296.

Halperin M, Blackwelder WC, Verter J. 1971. Estimation of the multivariate logistic risk function: a comparison of the discriminant function and maximum likelihood approaches. J Chronic Dis 24:125–131.

Hijazi A, Devonec M, Bouvier R, Bringuier P, Revillard JP. 1991. Use of leukocytes infiltrating bladder tumors as an internal reference for determining the DNA index by flow cytometry. Anal Quant Cytol Histol 13:139–142.

Hong WK, Sporn MB. 1997. Recent advances in chemoprevention of cancer. Science 278:1073–1077.

Hopman AHN, Moesker O, Smeets AWGB, Pauwels RPE, Vooijs GP. 1991. Numerical chromosome 1, 7, 9 and 11 aberrations in bladder cancer detected by in situ hybridization. Cancer Res 51:644–651.

Kalish LA, Garnick MB, Richie JP. 1987. Appropriate endpoints for superficial bladder cancer clinical trials. J Clin Oncol 5:2004–2008.

Klein FA, White FKH. 1988. Flow cytometry deoxyribonucleic acid determinations and cytology of bladder washings: practical experience. J Urol 139:275–278.

Kurth KH, Denis L, Bouffioux C, Sylvester R, Debruyne FM, Pavone-Macaluso M, Oosterlinck W. 1995. Factors affecting recurrence and progression in superficial bladder tumours. Eur J Cancer 31A:1840–1846.

Landis SH, Murray T, Bolden S, Wingo PA. 1998. Cancer Statistics. CA Cancer J Clin 48:6–29.

Matsuyama H, Bergerheim US, Nilsson I, Pan Y, Skoog L, Tribukait B, Ekman P. 1994. Nonrandom numerical aberrations of chromosomes 7, 9, and 10 in DNA-diploid bladder cancer. Cancer Genet Cytogenet 77:118–124.

Murphy WM. 1990. Current status of urinary cytology in the evaluation of the bladder neoplasm. Hum Pathol 21:886–896.

Rabinovitch PS. 1992. Practical considerations for DNA content and cell cycle analysis. In: Bauer KD, Duque RE, Shankey TV, editors. Clinical flow cytometry. Baltimore: Williams and Wilkins. p 117–142.

Sauter G, Gasser TC, Moch H, Richter J, Jiang F, Albrecht R, Novotny H, Wagner U, Bubendorf L, Mihatsch MJ. 1997. DNA aberrations in urinary bladder cancer detected by flow cytometry and FISH. Urol Res 25 suppl 1:S37–43.

Shackney SE, Berg G, Simon SR, Cohen J, Amina S, Pommersheim W, Yakulis R, Wang S, Uhl M, Smith CA, Pollice AA, Hartshock RJ. 1995. Origins and implications of aneuploidy in early bladder cancer. Cytometry 22:307–316.

Siegel S. 1956. Non-parametric statistics for behavioral science. New York: McGraw-Hill.

Sole M, Alos L, Mallofre C, Romero JA, Muntane J, Cardesa A. 1995. Bladder wash flow cytometry in transitional cell carcinoma: useful or misleading? Urol Res 22:361–365.

Tetu B, Allard P, Fradet Y, Roberge N, Bernard P. 1996. Prognostic significance of nuclear DNA content and S-phase fraction by flow cytometry in primary papillary superficial bladder cancer. Hum Pathol 27:922–926.

Vindeløv LL, Christensen IJ, Engelholm SA, Guldhammer BH, Hojgaard K, Sorensen BL, Wolf H. 1995. Prognostic significance of DNA content in bladder cancer based on flow cytometric analysis of 249 transitional cell carcinomas. Cytometry 22:93–102.

Wheeless LL, Badalament RA, de Vere White RW, Fradet Y, Tribukait B. 1993. Consensus review of the clinical utility of DNA cytometry in bladder cancer. Cytometry 14: 478–481.

Wheeless LL, Coon JS, Cox C, Deitch AD, de Vere White RW, Fradet Y, Koss LG, Melamed MR, O'Connell MJ, Reeder JE, Weinstein RS, Wersto RP. 1991a. Precision of DNA flow cytometry in inter-institutional analyses. Cytometry 12:405–412.

Wheeless LL, Reeder JE, O'Connell MJ, Robinson RD, Cosgriff JM, Fradet Y, Frank IN, Cockett ATK. 1991b. DNA Slit-Scan flow cytometry of bladder irrigation specimens and the importance of recognizing urothelial cells. Cytometry 12:140–146.

Wijkström H, Granberg-Öhman I, Tribukait B. 1984. Chromosomal and DNA patterns in transitional cell bladder carcinoma. Cancer 53:1718–1723.