Bladder Cancer Risk Assessment with Quantitative Fluorescence Image Analysis of Tumor Markers in Exfoliated Bladder Cells

Rebecca B. Bonner, M.S., C.T.(A.S.C.P.), George P. Hemstreet III, M.D., Ph.D.,*†§ Yves Fradet, M.D.,‖ Jian Yu Rao, M.D.,* Kyung Whan Min, M.D.,† and Robert E. Hurst, Ph.D.*‡§*

Background. **The detection of potentially highly curable low-grade bladder cancers by noninvasive techniques remains an unsolved problem. Conventional cytology detects such tumors with 50% sensitivity, and addition of DNA measurements to cytology only improves sensitivity incrementally. Tumor-associated antigens potentially offer an additional diagnostic marker.**

Methods. **In this study, the M344 antibody against a tumor-associated antigen expressed mainly by low-grade tumor cells was tested for its sensitivity and specificity, alone and in combination with DNA ploidy and cytology. Voided urine samples from 69 asymptomatic control subjects, urines and bladder washings from 59 patients with cancer, and 195 symptomatic control patients were collected. Cells were double-labeled with M344 monoclonal antibody and Hoechst. Each case was blinded, and the number of positive cells was scored by two independent observers.**

Results. **High-grade and low-grade transitional cell carcinomas (TCC) were detected with equal efficiency (78%, $P < 0.001$ versus symptomatic control patients). Urine samples proved higher specificity in detecting cancers. Patients being monitored for recurrence, but without current detectable cancer, were intermediates between control subjects and patients with cancer, suggesting that this marker also responds to dysplasia or field disease. Patients with outlet obstruction did not significantly differ from patients with previous TCC ($P = 0.95$). When combined with DNA ploidy measurements and cytology, the sensitivity for low-grade and high-grade tumors was 88% and 95%, respectively.**

Conclusions. **The M344 antibody potentially could improve the specificity and sensitivity of detection of low-grade bladder tumors in symptomatic and asymptomatic patients as well as monitoring for recurrence, therapeutic response, and assessment of individual risk.** ***Cancer*** **1993;72:2461–9.**

Key words: cancer, carcinogenesis, M344, monoclonal antibody, DNA, ploidy, field disease, urology, image analysis.

From the Departments of *Urology, †Pathology, ‡Biochemistry and Molecular Biology, and §Environmental Health Sciences, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma; and the ‖Laval University Cancer Center, Quebec, Canada.

Supported in part by grants from the Oklahoma Center for Advancement of Science, the Presbyterian Health Foundation, and CytoDiagnostics, Inc.; CDC/National Institute of Occupational Safety and Health grant R01-OH02647; and a Postdoctoral Research Fellowship awarded to Dr. Rao by the Graduate College of the University of Oklahoma Health Sciences Center.

The authors thank Drs. Robert A. Badalament, Thomas J. Gomella, Robert L. Karp, Timothy D. Moon, Johnny B. Roy, William A. See, Issam T. Shalhoub, and Bobby G. Smith for providing patient samples and follow-up information.

Address for reprints: George P. Hemstreet III, M.D., Ph.D., Department of Urology, Oklahoma University Health Sciences Center, P.O. Box 26901, Oklahoma City, OK 73190.

Accepted for publication May 25, 1993.

Transitional cell carcinoma (TCC) of the bladder is the sixth most prevalent cancer in the United States, with 52,000 new cases and 10,000 deaths expected per year.[1] Bladder cancer appears to develop along two main tracks: a deeply invasive, high-grade form that rapidly becomes life-threatening, and a much less dangerous low-grade form.[2–4] Low-grade tumors are usually cured readily, by simple resection if detected early or by bacillus Calmette–Guerin therapy in the case of multiple tumors. The detection of low-grade tumors is pressing because approximately 15% of patients with such tumors progress to dangerous disease.[5] There is also a need for noninvasive techniques, such as those based on exfoliated cells found in urine. Conventional cyto-

logic methods have poor sensitivity to low-grade tumors,[3,4] whereas the addition of DNA ploidy by image analysis, which only detects the limited class of low-grade tumors with aberrant ploidy or bladders with field disease, improves the sensitivity 15–20% compared with Pap cytology.[6–8]

An exfoliated cell sample will contain cells from both a tumor, if present, and the bladder field. The normal bladder, or any other solid organ, represents a complex ecosystem of interacting epithelial and stromal cells whose growth is highly regulated, and the progressive subversion of growth and differentiation controls[9–13] leads to emergence of cells with altered phenotypes. Some of these altered phenotypes result from genotypic changes and others from altered differentiation arising from a changed cytokine and stromal environment.[14,15] Bladder cancer seems to typically develop by a process of field disease, frequently involving widespread histopathologic or biochemical changes and carrying increased risk for progression and recurrence.[5,16,17] Because phenotypic markers reflect the process of carcinogenesis and may not detect the same cells used to make a morphologic diagnosis, it is therefore perhaps more appropriate to refer to the use of cytologic markers to detect disease as "risk assessment" rather than "diagnosis."

In this article we examined the use of a tumor-related antigen to assess bladder cancer risk from exfoliated cells. Tumor cells frequently synthesize one or more of several classes of proteins, referred to as tumor-related antigens,[18] which are not usually made by the normally differentiated cells found within the normal tissue. The M344 monoclonal antibody prepared against low-grade tumor cells detects a 300 kD mucin-like glycoprotein[19] (p300 marker) that apparently is preferentially expressed by diploid bladder cancer cells.[19] This marker, therefore, seems ideally suited to detect bladder tumors in combination with aberrant DNA ploidy, as measured by the abundance of cells with more than 5C DNA, which detects aneuploid and genetically unstable tumors.[20] We describe its measurement by fluorescence and the derivation of optimal interpretive thresholds from the context of risk assessment by investigating the distribution of the marker in several different control populations. The combination of this marker with DNA ploidy and DNA cytology was also examined. Included were patients expressing symptoms but in whom cancer was not found, including specifically the category of patients with outlet obstruction, as well as patients with a history of cancer but currently without clinically detectable cancer. This graded risk approach is a powerful technique for rapidly investigating marker use.[21]

Methods

Patient and Control Populations

Patients with cancer were enrolled in the study through several institutions including Oklahoma Medical Center, Oklahoma City, Oklahoma; Laval University, Quebec, Canada; Tulane Medical Center and Charity Hospital, New Orleans, Louisiana; Thomas Jefferson University Hospital, Philadelphia, Pennsylvania; and Veterans Administration Medical Centers, New Orleans, Louisiana, Iowa City, Iowa, and Muskogee and Oklahoma City, Oklahoma. A total of 36 voided urines and 49 bladder washings were obtained from patients with histologic diagnosis of Grade 1 or 2 TCC, and 23 voided urines and 16 bladder washings were obtained from patients with histologic diagnosis of Grade 3 TCC. The Grade 1 and 2 tumors were grouped together as low-grade tumors and the Grade 3 tumors were characterized as high-grade tumors. A total of 195 control voided urine specimens were collected from randomly selected urologic patients primarily from the VA Medical Center Urology Clinic. A total of 124 bladder wash specimens were collected from patients undergoing cystoscopy for a variety of conditions, including outlet obstruction, hematuria, follow-up from bladder cancer, or recurrent cystitis. An additional 69 voided urines were collected from asymptomatic individuals age-matched and sex-matched to the population with bladder cancer.

Collection and Processing of Specimens

Voided urines or bladder washings were collected in the usual manner and immediately fixed in an equal volume of 50% buffered ethanol as described previously.[6] Samples were stored at 4°C until processed. The sample was split, and one aliquot was prepared for immunofluorescence and the other for DNA ploidy and cytology.

Slides were prepared for immunofluorescence by collecting the cells on a 5 or 8 μm polycarbonate 25 mm filter and fixing for two minutes with 2% polyethylene glycol 1450 in 50% buffered ethanol. Cells were transferred by imprinting the filter onto poly-L-lysine coated Probe-On microslides (Fisher, Pittsburgh, PA). The slide was then quickly sprayed with Carbofix-E (Stat-Lab Medical Products, Dallas, TX) and allowed to air-dry. Slides were stored at room temperature until analysis.

Slides were labeled for immunofluorescence using the computer controlled Code-On Immunostainer (Instrumentation Laboratories, Lexington, MA) that sequentially and reproducibly carries out the sequence of

labeling, incubation, and washing steps. The M344 antibody was prepared as previously described[19] and was used as a 1:10 dilution of the ascites fluid in "Automation buffer," a proprietary solution containing buffer and surfactant (Brij) to assist with wetting. Sodium azide (0.02%) and 0.1% bovine serum albumin were added to the solution. The slides were incubated for 30 minutes in diluted M344 and washed three times followed by 30 minutes in biotinylated goat anti-mouse immunoglobulin G (IgG) diluted in the above buffer, washed three times and incubated for 30 minutes streptavidin conjugated Texas Red diluted 1:500 in the above buffer, and washed five times. Optimal concentrations of these reagents were determined by titration as described previously.[22] The slides were finally incubated with aqueous 8.7 μM Hoechst 33258[23] in 0.1 M NaCl, 0.05 M MOPSO buffer, 5 mM EDTA, with five changes of the solution to ensure saturation with a total incubation time of six minutes. Slides were mounted in 0.09 M n-propylgallate in glycerol. Control slides were included in each batch consisting of a positive control for M344 antibody (MGH-U3 cell line), a negative control cell line (HL-60), and a negative control from each sample treated with 1:2000 mouse IgG instead of the primary M344 antibody.

The additional marker of DNA cytology was added to the study protocol while still in progress. Both markers were analyzed on all bladder washings and voided urines from the following groups: 69 asymptomatic, 56 symptomatic without previous TCC (4 had insufficient sample for all tests), 12 with outlet obstruction, 15 with a history of TCC, 30 low-grade tumors, and 20 high-grade tumors. Slides were prepared for DNA cytology as previously described[6,23] after splitting a second time. Cells were collected on filters as described above, one aliquot being stained with 8.7 μM Hoechst 33258 in 25% ethanol and the other with 15 μM acridine orange on the filter and wet-mounted under a coverslip. Cells were evaluated under fluorescence using criteria developed more than 30 years ago.[24–27]

Immunofluorescence and DNA Ploidy Analysis

Preliminary studies showed that positive cells were present as rare events in many samples. The number of cells that were positive with M344 antibody was then scored under fluorescence by two independent observers who were each unaware of the other's findings or of clinical outcome. Cells were observed under fluorescence using green excitation and a barrier filter to isolate red emission. Cells were scored positive on the basis of observing intense cherry-red granules within the cytoplasm of transitional cells. Nonspecific binding was characterized by orange-red granular staining in the cytoplasm which was also seen under ultraviolet (UV) excitation. The positive M344 binding was not excited by the UV. The number of cells per slide was counted on the TAS-Plus (E. Leitz, Inc., Rockleigh, NJ) in which the computer automatically controls the focus, scanning stage, and image acquisition using the fluorescence of the aqueous Hoechst 33258 to locate nuclei. The number of positive cells was normalized to the number of cells on the slide by number of M344 positive cells/total number of cells on the slide $\times$ 10,000 and was reported as "positive cells/10 K." M344 test result thresholds were determined by analysis of receiver operating characteristics (ROC) plots (see Results) for each type of sample.

DNA ploidy and DNA cytology were performed using the QFIA methods previously described.[20,6] Two different scoring systems were used. One considered only DNA ploidy and was scored as positive if more than 0.04% of cells examined contained more than 5C DNA. The other, "risk analysis" scoring, included visual assessments of the cytology from the separate acridine orange-stained and Hoechst 33258-stained slides as previously described.[20] This scoring system combines a visual cytology with the quantitative marker of cells with more than 5C DNA as described in Table 1 and ranks patients according to their risk for bladder cancer in general and of high-grade, invasive lesions. The numerical system previously described[20] was reversed, so that Group 1 is healthy and Group 5 is the highest-risk group. Group 1 indicates the lack of any abnormal findings. Group 2 is generally negative, but may contain some entirely diploid, genetically stable tumors, as well as other abnormalities leading to the presence of atypical cells (inflammation). Group 3 is a borderline group that is usually considered negative, but may be considered positive if maximal sensitivity is desired. Many smokers fall into this category. Groups 4 and 5 are both positive, with Group 4 being indicative of low-grade tumors, and hence of lower risk, whereas Group 5 is indicative of high-grade processes. Such a risk categorization provides a base model onto which other markers can be added to improve sensitivity, specificity, or risk categorization.

Results

Quantitative Labeling of Cells Viewed Under Fluorescence

Figure 1 shows the appearance of urothelial cells obtained from a patient with a Grade 2 TCC doubly labeled for both DNA and p300 with the M344 antibody. Cells that are positive for the p300 marker show a char-

Table 1. Risk Categories for Fluorescence Cytology and DNA Ploidy*

Risk category	Visual cytologic findings	No. of cells with > 5 C *dsNA*	No. of cells with > 5 C *DNA*
1	Negative or viral changes	0/500	0/500
2	Atypical	< 1/500	0/500
3	Atypical	< 2/ 500	1–2/500
4	Atypical	≥ 2/500	≥ 2/500
5	Suspicious or positive	≥ 2/500	≥ 2/500

* Cells were labeled quantitatively with acridine orange (15 μM) and Hoechst 33258 (8.7 μM), and cells exceeding 5 C dsNA (acridine orange) and 5 C DNA were identified in separate scans. The cytologic features were assessed under fluorescence with both dyes. The finding of any positive cells was sufficient for risk group 5, regardless of DNA ploidy findings, because there are associated with aggressive, high-grade lesions.

acteristic red fluorescence in the cytoplasm, and cells that are negative are devoid of this marker. The identification of positive cells is usually straightforward.

Determination of Thresholds for p300 in Voided Urines and Bladder Wash Specimens

The subjects were grouped into the following three groups: patients with bladder cancer, symptomatic control subjects, and asymptomatic control subjects. Symptomatic control patients were further categorized into three subgroups: patients with no history of TCC, patients with current bladder outlet obstruction, and patients being followed for recurrence of TCC. The samples showed three patterns when the number of positive cells per 10,000 cells were examined. One group consisted of asymptomatic subjects: more than 90% of such urines contained no positive cells, and only a small number contained more than a single positive cell. Among symptomatic patients, those without history of TCC or outlet obstruction were indistinguishable from asymptomatic control subjects. In contrast, the distribution of patients with current bladder outlet obstruction closely resembled patients with previous TCC, with several patients having p300/M344-positive cells in the 5–25/10 K range. These patients comprised a second group. Patients with TCC comprised the third group and expressed a wide range of positive rates, with approximately 50% expressing more than 50/10 K. Comparison of the distribution of the control sample histograms showed that more p300-positive cells were present in bladder washings than in voided urines, but the results with bladder washings were otherwise similar.

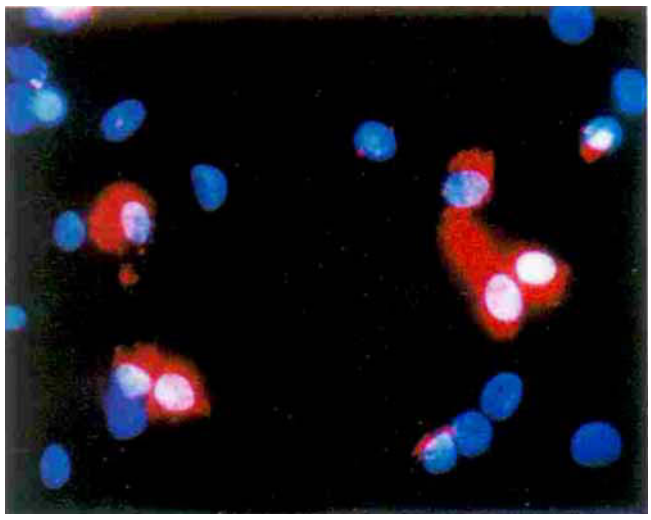

Figure 1. Photomicrograph demonstrating the typical binding pattern of the M344 antibody to urothelial cells in urine from a patient with Grade 2 TCC. The photograph was prepared by double exposure with exposure to ultraviolet wavelengths, which stimulate the blue fluorescence of the Hoechst 33258 and green, which stimulates the Texas Red fluorochrome attached to the M344 antibody. The photographs were obtained on a Leitz Orthoplan microscope at ×312.5 magnification.

To determine diagnostic threshold values of the p300/M344 positive rate and to demonstrate the effect of different choices of threshold on specificity and sensitivity, the percent of positive samples with a given threshold was plotted against the threshold value by group. These ROC plots are shown in Figures 2 and 3 for voided urines and bladder washings, respectively, using the symptomatic patients without history of bladder cancer or current outlet obstruction as the comparison group for determining specificity. Examination of these plots showed that approximately 40% of the tumors in the voided urines and 60% of the bladder washings showed high p300-positive rates. The major differences between the findings with bladder wash cells as opposed to voided urines are that the marker is more prevalent in bladder wash cells from individuals without cancer and the difference between low-grade and high-grade tumors is more pronounced. Approximately 60% of the urologic control bladder wash samples contained at least one p300-positive cell per slide, whereas only 15% of matching urine specimens from the same population contained at least one positive cell

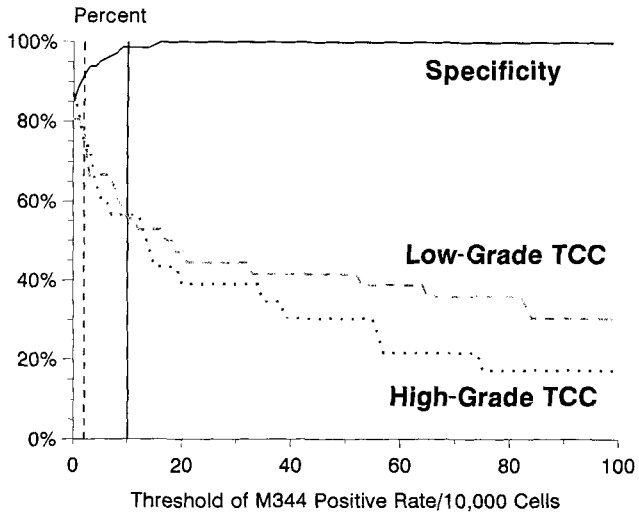

Figure 2. ROC plots for p300-positive cells in urine by grade of tumor. Specificity was established from the distribution of symptomatic control patients without a history of bladder cancer. The two vertical lines represent two threshold choices, the dotted line (2/10 K) represents optimal sensitivity, and the solid line (10/10 K represents optimal specificity.

per slide. Examination of the control urines suggested two thresholds would be more useful in interpreting such data than would a single value. Thresholds of greater than or equal to 10/10 K in urines and greater than 21/10 K in bladder washings yielded optimal specificity while sacrificing some sensitivity. In contrast, threshold values of less than 2/10 K and less than 8/10 K for urines and bladder washings, respectively, achieved a high sensitivity at the expense of some loss in specificity. An intermediate category consisted of those patients with results falling between the two thresholds. In some urine samples, a single positive cell was occasionally found in samples that had fewer than 5000 total cells. These samples (n = 20) were not considered in calculations listed in the tables or figures.

The significance of differences between groups was tested using the lower threshold (maximized sensitivity) with the χ^2 test. The P values in Tables 2 and 3 indicate that patients with bladder outlet obstruction and those with previous tumor are not significantly different from one another but are significantly different from symptomatic patients with no history of tumor ($P = 0.005$). Those patients with previous tumor versus no previous tumor are significant at $P = 0.05$ in bladder washings but less significant in voided urine samples ($P = 0.10$). Voided urines from age-matched and sex-matched control subjects were not significantly different from symptomatic patients with no history of tumor ($P = 0.90$).

Table 4 shows the sensitivity of detecting bladder cancer in urines and bladder washings, respectively, using two thresholds together with the corresponding specificities for the control groups. A high degree of specificity was achieved with voided urines among age-matched and sex-matched control subjects and symptomatic patients with no history of tumor (94.2% and 91.7%, respectively) using a threshold of 2/10 K. With the higher thresholds, the corresponding specificities are 97.1% and 98.6%, respectively. Sensitivity under these criteria was near 80% with the lower threshold and approximately 55% at the higher threshold, regardless of tumor grade, and there was no significant difference in the detection rates between high-grade and low-grade tumors in urines. Specificity was lower with patients having outlet obstruction or a history of TCC. Among the patients with bladder outlet obstruction, the specificity was improved to 94% at the higher threshold, but increasing the threshold from 2/10 K to 10/10 K offered little improvement in specificity for patients with a history of TCC. Also shown are the corresponding results for bladder wash samples. Higher thresholds were necessitated with bladder washes because of the higher prevalence of positive cells in bladder wash samples, including patients with no history of TCC. Except for the significantly decreased sensitivity to high-grade tumors, the results with bladder washes were comparable to those with urines.

Combined p300/M344 and Ploidy/Cytology

Combining the p300 marker as detected by M344 antibody with DNA ploidy combined with DNA cytology

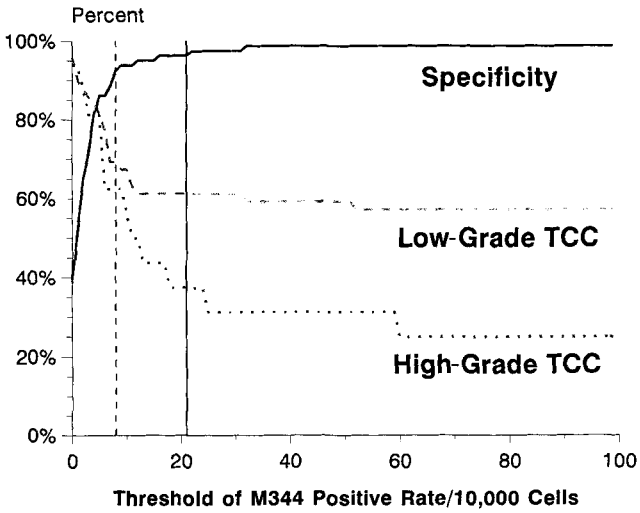

Figure 3. ROC plots for p300-positive cells in bladder washes by grade of tumor. Specificity was established from the distribution of symptomatic control patients without a history of bladder cancer. The two vertical lines represent two threshold choices, the dotted line (8/10 K) represents optimal sensitivity, and the solid line (21/10 K) represents optimal specificity.

Table 2. Chi-Square Test for the Significance of the p300 Marker in Voided Urine*

		No history of TCC (n = 145)		Previous history of TCC (n = 32)	
Group	*No.*	*Chi-square*	*P value*	*Chi-square*	*P value*
Asymptomatic age-matched and sex-matched control subjects	69	0.4	0.90	7.7	0.01
Patients with outlet obstruction	18	6.5	0.025	0.05	0.95
Patients with low-grade TCC	36	80.8	< 0.001	28.7	< 0.001
Patients with high-grade TCC	23	66.2	< 0.001	15.2	< 0.001
Patients with no history of TCC	145			7.3	0.01

TCC: transitional cell carcinoma.

* Samples were scored as positive or negative according to the criteria listed in the text. Chi-square values were calculated for the groups shown in the first column against the results of symptomatic subjects with no history and with a previous history, respectively.

to improve the detection of bladder cancers was also tested with those samples having both analyses (Table 5). The complementarity of p300/M344 and ploidy/cytology are illustrated by the low sensitivity and high specificity when "and" logic was used, that is, p300 is expressed preferentially in tumors that are not highly aberrant by cytology. Consequently, adding the ploidy/cytology tests to p300/M344-negative samples ("or" logic) improves the sensitivity, particularly for high-grade tumors, but also for low-grade tumors (88% versus 78%). With low-grade tumors, cytology makes the largest contribution (the sensitivity is not improved by the cells more than 5C DNA marker), but aberrant ploidy makes the largest contribution for high-grade tumors. The specificity, however, decreased by inclusion of the second test. With the age-matched and sex-matched control subjects, the specificity decreased by only 4%, but the decrease was larger among the symptomatic patients. As expected, the decrease was larger for the group with a history of TCC than for the group without such a history. Similar trends (not shown) were observed for bladder wash samples.

Discussion

The process of carcinogenesis results from progressive subversion of the normal controls of cellular growth and differentiation resulting from an interaction between genetic and epigenetic mechanisms.[9-13] The evidence suggests that at least two different pathways of genetic alterations lead to bladder cancer,[28] but the possibility of multiple genotypes leading to the malignant phenotype complicates the use of genetic markers to assess the process of carcinogenesis. A small number of phenotypic markers may yield information concerning the status of a carcinogenic process with multiple genotypes. In this article we investigated a tumor-related antigen, the p300 glycoprotein detected by the M-344 antibody,[19] as a marker for bladder cancer, testing it alone and in combination with DNA ploidy and fluorescence cytology to determine if the detection of bladder cancers and individuals at high risk for bladder cancer development can be improved with these tests alone or in combination.

Cells positive for p300 are uncommon, even in cancer urines, but such cells still can function effectively as markers. Positive cells are found rarely in normal urines or bladder washings, and of noncancer conditions investigated, only outlet obstruction was significant in producing p300-positive cells. In only a few cases did the abundance of positive cells exceed 1% of cells (100/10 K). A practical consequence of such rarity is that a method capable of distinguishing such rare

Table 3. Chi-Square Test for the Significance of the p300 Marker in Bladder Washes*

		No history of TCC (n = 145)		Previous history of TCC (n = 32)	
Group	*No.*	*Chi-square*	*P value*	*Chi-square*	*P value*
Patients with outlet obstruction	21	6.5	0.025	0.05	0.95
Patients with low-grade TCC	49	80.8	< 0.001	28.7	< 0.001
Patients with high-grade TCC	16	66.2	< 0.001	15.2	< 0.001
Patients with no history of TCC	80			7.3	0.01

TCC; transitional cell carcinoma.

* Samples were scored as positive or negative according to the criteria listed in the text. Chi-square values were calculated for the groups shown in the first column against the results of symptomatic patients with no history and with a previous history, respectively.

Table 4. Sensitivity and Specificity of the p300/M344 Marker at Two Thresholds in Bladder Cancer Detection With Voided Urine and Bladder Washes*

	Threshold					
	Voided urine			*Bladder wash*		
Subject group	*No.*	*≥ 2/10 K*	*≥ 10/10 K*	*No.*	*≥ 8/10 K*	*≥ 20/10 K*
TCC group sensitivity (%)						
All TCC	59	77.9	55.9	65	67.7	55.4
Low-grade TCC	36	77.8	55.6	49	69.8	61.2
High-grade TCC	23	78.3	56.5	16	48.2	37.5
Control group specificity (%)						
Asymptomatic	69	94.2	97.1	—	—	—
Symptomatic, no previous TCC	145	91.7	98.6	80	92.5	96.2
Outlet obstruction	18	72.2	94.4	21	76.2	86.7
Previous TCC	32	75.0	81.2	23	73.9	83.6

TCC: transitional cell carcinoma.

* The samples were analyzed for p300/M344-positive cells as described and scored with two thresholds. Values listed for transitional cell carcinoma groups represent sensitivity, whereas values listed for control groups represent specificity.

cells must be used and the sample itself must contain sufficient cells to make their detection likely, if present, at abundances of 2/10,000 cells. In this study the human eye was used to find such cells and to distinguish nonspecific fluorescence from that due to the bound antibody.

The M344 antibody was generated from a low-grade diploid bladder cancer, and the p300 protein is generally expressed by diploid tumor cells and much less frequently by high-grade tumor cells.[19] The p300 protein is not normally expressed on superficial cells of the normal bladder as evidenced by the virtual absence of positive cells in the asymptomatic control urines and as previously reported from immunohistochemical studies of tissue sections.[19] In the symptomatic patient group without a history of bladder cancer or outlet obstruction, positive cells were consistently more abundant in bladder washes than in the corresponding urines although the abundance in the urines was not significantly different from that found in asymptomatic control subjects. This finding suggests that normal cells from deeper in the urothelium are more likely to express p300 than are terminally differentiated cells sloughed into urine. Alterations of the differentiation program may be one factor in expression of p300.

This study used a symptomatic control group in addition to an asymptomatic control group to establish the test characteristics in a population more representative of that in which bladder cancer is normally being diagnosed. The symptomatic control group included pa-

Table 5. Multiple Marker Studies in Urine Samples, Combining p300/M344, Abnormal Numbers of Cells With More Than 5C DNA, and DNA Cytologic Features*

Subject group	**No.**	**p300/M344 alone**	**p300/M344 and cells > 5 C**	**p300/M344 and risk category 4 or 5**	**p300/M344 or cells > 5 C**	**p300/M344 or risk category 4 or 5**
Sensitivity						
Low-grade TCC	30	78	10	33	78	88
High-grade TCC	20	78	30	50	95	95
Specificity						
Asymptomatic	68	94	99	100	90	90
Symptomatic, no previous TCC	52	92	100	100	80	75
Outlet obstruction	12	72	100	100	75	75
Previous TCC	15	75	100	100	60	53

TCC: transitional cell carcinoma.

* Samples were split and analyzed separately for p300/M344 and QFIA cytologic features for both the number of cells with more than 5 C DNA and abnormal DNA cytologic findings. The threshold of greater than 2/10 K was used for p300/M344. Samples were scored as positive for DNA ploidy if the fraction of cells with more than 5 C DNA was 0.4% or greater. Risk categorization, which combines ploidy analysis and cytologic findings, is described in Table 1. Samples in risk categories 4 and 5 were positive.

tients with outlet obstruction, patients being followed for recurrence of bladder cancer, and a variety of other urologic problems. This design presents a graded risk spectrum. Patients with no history of bladder cancer are unlikely to significantly manifest the processes of carcinogenesis and serve to identify processes not related to cancer that can produce p300 expression. Outlet obstruction represents one such condition, apparently by producing a hyperplastic response in the bladder with altered urothelial differentiation.[29] The ablation of p300 that was observed after correction of outlet obstruction suggests that removal of the promoting effect allows the phenotype to return to normal, unless other irreversible changes have occurred, in which case p300 can be used to identify patients with outlet obstruction who are at risk for cancer development.

Several lines of evidence suggest the p300 protein is produced by dysplastic cells in addition to low-grade tumors. Positive cells have been reported to be uncommon in tissue sections from high-grade tumors.[19] Yet they were detected with almost the same efficiency as low-grade tumors with the antibody, although p300-positive cells are less abundant in urines and bladder washings from patients with high-grade tumors. The positive cells must have originated in either more differentiated portions of a heterogenous tumor or from premalignant cells within the urothelium. The higher abundance of positive cells observed among patients being monitored for recurrence, but in no other control group other than outlet obstruction patients, strongly supports the concept that the M344 antibody is a marker for field disease and low-grade tumors. Field disease is usually considered to represent morphologically altered cells that can be observed and diagnosed by classical histopathology. The findings in this study suggest that biochemical field disease occurs in cells before expression of characteristic morphologic aberrations. The conventional diagnosis of field disease is primarily weighted toward those lesions most likely to develop into high-grade TCC. The p300 protein is clearly a marker for diploid premalignant cells and, when combined with markers for aneuploid premalignant or malignant cells, can form an effective battery for use in management of bladder cancer.

Analysis suggested that two thresholds would be useful in assessing the clinical significance of p300 expression. This approach is analogous to that used for prostate-specific antigen. The higher threshold offers the highest specificity while sacrificing some sensitivity and is probably equivalent to that achieved with flow cytometry.[30] The ability to visually identify positive cells allows a lower threshold to be set as well. The lower threshold offers the highest sensitivity while sacrificing some specificity for certain patient populations. The intermediate group falling between the two thresholds represents the main diagnostic problem and may require elimination of confounding factors, such as bladder outlet obstruction, before the significance of the findings in terms of cancer diagnosis is clear. Voided urine samples provide the sharpest distinction between these patients with outlet obstruction and those with field disease, further supporting the use of voided urine over bladder washes for this marker. Not only can urines be obtained noninvasively, but the discrimination between abnormal and normal is enhanced with urines.

Combining the two tests, as shown in Table 5, shows that adding DNA and morphologic markers increases the sensitivity to 88% for low-grade tumors and at least 95% for high-grade tumors. In contrast, conventional cytology, even with highly trained personnel, rarely achieves 50% sensitivity for low-grade tumors.[31] Clinical usefulness is facilitated by use of two thresholds, whether the tests are used independently or in combination. A high degree of confidence can be placed in those exceeding the higher threshold and falling below the lowest threshold. Achieving this high sensitivity and specificity also entailed careful adherence to the sample collection, fixation, shipping, and storage protocols. These results will not be duplicated when the procedures usually applied to urines are used. The single high-grade tumor that was missed is illustrative. The cells were found to be degenerated, and the sample had not been handled properly. It is also necessary to ensure that when samples are split, as they are frequently in research studies, that each test receives sufficient cells.

As research into the genetic events involved in bladder carcinogenesis and their effects on protein expression identifies significant phenotypic and genotypic markers and the order in which they develop, the possibility of establishing the individual's risk of cancer development and its biological potential through multiple marker measurements becomes possible. In conventional Papanicolaou diagnostic cytopathology, numerous malignant criteria are used to identify cancer cells and, for a cell to be considered diagnostic, it must display a combination of several of these criteria. There may literally be hundreds of cancer cells on a slide, but few that are diagnostic. The criteria for dysplasia are contained within those for cancer, but the degree of abnormality and combination of criteria are restricted. The approach of using biochemical markers is analogous, except that criteria are quantified based on specific marker substances within cells. As our knowledge of normal and abnormal cell biochemistry increases, biochemical criteria will become more powerful, and the combination of biochemical criteria is likely to achieve high specificity similar to that achieved by us-

ing combinations of morphologic criteria. The main advantage of this biochemical approach is that the criteria are machine-sensible and thus capable of automation, although much research remains before equivalent malignancy criteria will be derived for routine use. This approach may well have sensitivity and specificity adequate enough to use in groups that are asymptomatic but at high risk, an example being to stratify subjects for chemoprevention or other intervention programs to reduce bladder cancer among carcinogen-exposed workers.

References

1. Boring C, Squires TS, Tong T. Cancer statistics, 1993. *CA* 1993; 43:7–26.
2. Mostofi FK, Davis CJ, Sesterhenn IA. Current understanding of pathology of bladder cancer and attendant problems. *J Occup Med* 1990; 32:793–6.
3. Farrow GM. Urine cytology in the detection of bladder cancer: a critical approach. *J Occup Med* 1990; 32:817–21.
4. Koss LG. Tumors of the urinary tract and prostate. In: Koss LG, editor. Diagnostic cytology and its histologic bases. 3rd ed. vol. 2. Philadelphia: Lippincott, 1979:749–811.
5. Heney NM, Ahmed S, Flanagan MJ, Frable W, Corder MP, Hafermann MD, et al. Superficial bladder cancer: progression and recurrence. *J Urol* 1983; 130:1083–6.
6. Bass RA, Hemstreet GP, Honker NA, Hurst RE, Doggett RS. DNA cytometry and cytology by quantitative fluorescence image analysis in symptomatic bladder cancer patients. *Int J Cancer* 1987; 40:698–705.
7. Badalament RA, Kimmel M, Gay H, Cibas ES, Whitmore WF, Herr HW, et al. The sensitivity of flow cytometry compared with conventional cytology in the detection of superficial bladder carcinoma. *Cancer* 1987; 59:2078–85.
8. Wheeless LL, Berkan TK, Patten SF, Reeder JE, Robinson RD, Eldidi MM, et al. Multidimensional slit-scan detection of bladder cancer preliminary clinical results. *Cytometry* 1986; 7:212–6.
9. Weinberg RA. Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis. *Cancer Res* 1989; 49:3713–21.
10. Pienta KJ, Partin AW, Coffey DS. Cancer as a disease of DNA organization and dynamic cell structure. *Cancer Res* 1989; 49:2525–32.
11. Heldin CH, Betscholz C, Claesson-Welsh L, Westermark B. Subversion of growth regulatory pathways in malignant transformation. *Biochim Biophys Acta* 1987; 907:219–44.
12. Tzen C-Y, Estervig DN, Minoo P, Filipak M, Maercklein PB, Hoerl BJ, et al. Differentiation, cancer and anticancer activity. *Biochem Cell Biol* 1988; 66:478–89.
13. Couture J, Hansen MF. Recessive oncogenes in tumorigenesis. *Cancer Bull* 1991; 43:41–50.
14. Ruoslahti E, Yamaguchi Y. Proteoglycans as modulators of growth factors. *Cell* 1991; 64:867–9.
15. Nathan C, Sporn M. Cytokines in context. *J Cell Biol* 1991; 113:981–6.
16. Norming U, Nyman CR, Tribukait B. Comparative flow cytometric deoxyribonucleic acid studies on exophytic tumor and random mucosal biopsies in untreated carcinoma of the bladder. *J Urol* 1989; 142:1442–7.
17. Frang D, Somogy L, Jilling A. Current diagnostic and therapeutic methods in the treatment of vesical tumors. *Int Urol Nephrol* 1988; 20:597–609.
18. Herlyn M, Menard A, Koprowski H. Structure, function and clinical significance of human tumor antigens. *J Natl Cancer Inst* 1990; 82:1883–9.
19. Fradet Y, Islam N, Boucher L, Parent-Vaugeois C, Tardiff M. Polymorphic expression of a human superficial bladder tumor antigen defined by mouse monoclonal antibodies. *Proc Natl Acad Sci U S A* 1987; 84:7227–31.
20. Hemstreet GP, Hurst RE, Bass RA, Rao JY. Quantitative fluorescence image analysis in bladder cancer screening. *J Occup Med* 1990; 32:822–8.
21. Rao JY, Hemstreet GP, Hurst RE, Bonner RB, Min KW, Jones PL. Cellular F-actin levels as a marker for cellular transformation: correlation with bladder cancer risk. *Cancer Res* 1991; 51: 2761–7.
22. Jones PL, O'Hare CM, Bass RA, Hemstreet GP, Hurst RE. Quantitative immunofluorescence, anti-ras p21 antibody specificity, and cellular oncoprotein levels. *Biochem Biophys Res Comm* 1990; 167:464–70.
23. McGowan P, Hurst RE, Bass RA, Hemstreet GP, Postier RG. Equilibrium binding of Hoechst 33258 and Hoechst 33342 fluorochromes with rat colorectal cells. *J Histochem Cytochem* 1988; 36:757–62.
24. Weid GL, Manglano JI. A comparative study of the Papanicolaou technic and the acridine orange fluorescence method. *Acta Cytol* 1962; 6:554–68.
25. Dart LH Jr., Turner TR. Fluorescence microscopy in exfoliative cytology. *Lab Invest* 1959; 8:1513–22.
26. Bertalanffy FD. Evaluation of the acridine orange fluorescence microscope method for cytodiagnosis of cancer. *Ann NY Acad Sci* 1962; 93:715–50.
27. Wellman KF, McDermott MA, Gray EH. An evaluation of acridine-orange fluorescence microscopy in cytology. *Acta Cytol* 1963; 7:111–7.
28. Presti JC, Jr, Reuter VE, Galan T, Fair WR, Cordon-Cardo C. Molecular genetic alterations in superficial and locally advanced human bladder cancer. *Cancer Res* 1991; 51:5405–9.
29. Martin BF. Cell replacement and differentiation in transitional epithelium: a histological and autoradiographic study of the guinea-pig bladder and ureter. *J Anat* 1972; 112:433–55.
30. Fradet Y, Gauthier J, Bedard G, Charrois R, Naud A. Monitoring and prognostic determination of bladder tumors by flow cytometry with monoclonal antibodies on bladder irrigations. *J Urol* 1991; 145:250A.
31. Koss LG, editor. Diagnostic cytology and its histopathologic bases. 3rd ed. vol 2. Philadelphia: Lippincott, 1979:767–8.