ARTICLES

Biomarker Risk Assessment and Bladder Cancer Detection in a Cohort Exposed to Benzidine

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Background: **Cancer screening with highly sensitive, specific biomarkers that reflect molecular phenotypic alterations is an attractive strategy for cancer control. We examined whether biomarker profiles could be used for risk assessment and cancer detection in a cohort of Chinese workers occupationally exposed to benzidine and at risk for bladder cancer.** *Methods:* **The cohort consisted of 1788 exposed and 373 nonexposed workers, followed from 1991 through 1997. We assayed urothelial cells from voided urine samples for DNA ploidy (expressed as the 5C-exceeding rate [DNA 5CER]), the bladder tumor-associated antigen p300, and a cytoskeletal protein (G-actin). Workers were stratified into different risk groups (high, moderate, and low risk) at each examination based on a predefined biomarker profile. For workers who developed bladder cancer, tumor risk assessment was analyzed from samples collected 6–12 months before the cancer diagnosis. The associations between risk group and subsequent development of bladder cancer were analyzed by Cox proportional hazards regression analysis and logistic analysis, after adjustment. All statistical tests were two-sided.** *Results:* **Twenty-eight bladder cancers were diagnosed in exposed workers and two in nonexposed workers. For risk assessment, DNA 5CER had 87.5% sensitivity, 86.5% specificity, an odds ratio (OR) of 46.2 (95% confidence interval [CI] = 8.1 to 867.0), and a risk ratio (RR) of 16.2 (95% CI = 7.1 to 37.0); p300 had 50.0% sensitivity, 97.9% specificity, an OR of 40.0 (95% CI = 9.0 to 177.8), and an RR of 37.9 (95% CI = 16.8 to 85.3). The risk of developing bladder cancer was 19.6 (95% CI = 8.0 to 47.9) times higher in workers positive for either the DNA 5CER or p300 biomarkers than in workers negative for both biomarkers and 81.4 (95% CI = 33.3 to 199.3) times higher in workers positive for both biomarkers. G-actin was a poor marker of individual risk.** *Conclusions:* **Occupationally exposed workers at risk for bladder cancer can be individually stratified, screened, monitored, and diagnosed based on predefined molecular biomarker profiles. [J Natl Cancer Inst 2001;93: 427–436]**

People occupationally exposed to carcinogens provide the opportunity to evaluate biomarkers for individual cancer risk assessment, for cancer detection, and as molecular surrogate markers for monitoring the efficacy of cancer prevention agents. Cancer control in people occupationally exposed to carcinogens that cause bladder cancer has focused primarily on early cancer detection *(1,2)* by use of screening methods that include Pap cytology, hematuria testing, and cystoscopy *(3,4)*. However,

these screening methods have been only partially effective in reducing bladder cancer mortality, and better methods of identifying premalignant disease are clearly needed *(5,6)*.

Approximately 260 000 new cases of bladder cancer are diagnosed each year, and bladder cancer accounts for approximately 115 000 cancer deaths per year worldwide *(7,8)*. Nearly 50% of bladder cancers are caused by aryl amine exposure from cigarette smoke, and approximately 20%–25% are related to occupational exposure to chemicals, such as benz[*a*]pyrene, benzidine, and β-napthylamine *(2,9–11)*. Because of the high risk of bladder cancer in some occupationally exposed people, the National Institute for Occupational Safety and Health has recommended bladder cancer control screening and notification programs for such people. A 1989 consensus conference reinforced the appropriateness of screening programs already ongoing worldwide *(12)*. The incidence of bladder cancer in occupationally exposed people is up to 50 times that of the nonexposed population; thus, people involved in ongoing screening for bladder cancer also provide an opportunity to evaluate biomarkers for bladder cancer risk *(2,9,10)*. Currently, however, few occupationally exposed cohorts have been identified worldwide in which to investigate surrogate intermediate end point markers for improved risk assessment and detection of bladder cancer. Bi et al. *(11)* reported one such cohort in a case–control study involving 1972 workers exposed to benzidine in three Chinese cities. The standardized incidence rate of bladder cancer was 25.0, based on 30 cases in the exposed group *(11)*. Conventional screening (Pap cytology and hematuria testing) failed to substantially reduce mortality, indicating the need for new tech-

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niques and biomarkers for risk assessment and detection of bladder cancer.

Previously, we *(13,14)* and other investigators *(15–17)* suggested that biochemical phenotypic alterations in voided urinary urothelial cells precede the cellular morphologic changes associated with malignancy and hypothesized that these biochemical and morphologic alterations could serve as a basis for risk stratification. To test this hypothesis, it would be important to identify biomarkers that have a high sensitivity for detecting bladder cancer and then monitor them over time to identify any changes that occur early during cancer development. However, selecting biomarkers that result from the interaction between environmental factors and inherited genetic risk factors and using them in a clinical setting involve a number of complex issues, such as marker stability, sensitivity, specificity, and reliable automated assay methods for biomarker analysis *(18,19)*. Potential molecular and phenotypic biomarkers include changes in DNA ploidy, changes in gene or protein expression, or changes in cell architecture *(18,19)*.

To test the hypothesis that biomarkers could serve as a basis for risk stratification, we carried out a longitudinal study of individuals in the asymptomatic high-risk occupational Chinese cohort identified by Bi et al. *(20)* by use of a quantitative biochemical phenotypic biomarker panel consisting of DNA ploidy (expressed as the 5C-exceeding rate or DNA 5CER), a bladder cancer-associated antigen (p300), and a cytoskeletal marker associated with differentiation (G-actin). DNA ploidy and p300 were defined as highly sensitive and specific biomarkers in a pilot study *(20)*. In this article, we report on a 6-year prospective longitudinal study of this cohort, many of whose members were occupationally exposed to benzidine. We attempted to determine whether quantitative measures of carcinogenesis-related alterations in the biochemical phenotype of urothelial cells shed into urine could be used to stratify individuals for their risk of bladder cancer. The same markers were also analyzed for their ability to detect bladder cancer at the time of pathologic diagnosis of bladder cancer.

SUBJECTS AND METHODS

Cohort

This study was conducted in The People's Republic of China using a previously defined cohort of workers occupationally exposed to benzidine and nonexposed workers *(11,20)*. The study was approved by the Review Board of the Chinese Academy of Preventive Medicine, a World Health Organization (WHO) Center in Beijing, China, and written informed consent was obtained from participants. The initially defined cohort included 1972 male and female workers from industries in Tinjing, Shanghai, and Jilin who were exposed to benzidine through its production and/or use for at least 1 year between 1972 and 1977. The nonexposed group was selected from industries located in the same city districts and comprised 1974 workers with no known exposure to benzidine, 1-naphthylamine, 2-naphthylamine, 4-aminobiphenyl, or other occupational carcinogens. The nonexposed group was similar to the exposed group for age, smoking status, and employment. A retrospective analysis of the cohort determined that the incidence of bladder cancer for the period of January 1, 1972, through December 31, 1981, was 30 cases in the exposed group and one in the nonexposed group *(11)*.

For this study, we analyzed 1788 male workers from the exposed group and 373 (20% randomly selected, age-matched) male workers from the nonexposed group. We used a small nonexposed group to validate assay drift and to reduce the cost of the study. Both groups were screened sequentially in five cities between September 1991 and May 1992. All workers were screened a second time 3 years after the initial screen. In addition, the high-risk group (*defined*

below) was recommended for screening every 6 months during the intervening 3 years with biomarkers and cystoscopy. The moderate-risk group was screened with biomarkers only on a yearly basis, and the low-risk group was not rescreened during the intervening 3 years. A total of 209 exposed and nonexposed workers entered the program at different time intervals after the initial screen.

The screening involved acquiring informed consent from participants, administering a questionnaire, performing a limited urologic examination, and collecting samples of urine and blood. The questionnaire had been field tested previously and validated for ease of administration and compliance in the pilot study *(20)*. The questionnaire was administered to all workers who were aware of potentially being exposed to benzidine. Trained interviewers collected detailed information on demographics, smoking, occupation (including occupational exposure to benzidine), and individual and family medical history (including urologic diseases and cancer) *(20)*.

Sample Collection and Preparation

A 150–200-mL urine sample was collected from each subject at the initial and follow-up visits. The sample was split immediately into two aliquots. Approximately 50–100 mL of urine was prepared on site for Pap cytology and routine urinalysis in a local laboratory within 2 hours of sample collection. All samples were blind coded before any laboratory analysis. After the first screen, bar coding was incorporated into sample labeling and tracking to reduce the possibility of sample mislabeling. The Pap cytology and urinalysis were performed by use of standard techniques *(22)*. The urine cytology results reported here were based on the results of the Chinese Pathologist (Z. Ma). Hematuria was measured by use of a dipstick test (Chemstrip; Boehringer Mannheim Biochemicals, Indianapolis, IN) and by microscopic analysis.

The other 100 mL of urine was fixed with 5 mL of 10% paraformaldehyde (final concentration, 0.5% paraformaldehyde) for 15 minutes and then mixed with an equal volume (105 mL) of QFIA Fixit *(23)*. The fixed samples were stored at 4 °C until they were shipped on ice in Styrofoam containers to Oklahoma City, OK, for analysis. The total time from collection to receipt in Oklahoma City was 2–3 weeks. On arrival, sample information (date of collection, subject identification number, sample identification number, identification number of individual processing the sample, date of specimen processing, total number of cells in the sample, volume of specimen received, and any special comments) was immediately entered into a database. The samples were centrifuged for 15 minutes at 600*g* at 4 °C and 40 000 cells per aliquot or a maximum of four vials with equal distribution of the sediment cells were frozen at −80 °C. For some subjects, we obtained bladder washings and catheterized urine during cystoscopy, which were processed in a similar manner as the voided urine samples.

In addition, subjects in the high-risk group with or without visual evidence of tumor on cystoscopy or a positive biopsy specimen were recommended for intravenous pyelogram to exclude a neoplasm in the upper urinary tract. Sixty-nine percent of the high-risk group had one or more cystoscopies during the follow-up period, and 92% of the members of the high-risk group were followed longitudinally for a mean of 36 months with repeat biomarker analysis every 6 months throughout the study. Any suspicious findings had a biopsy performed at the time of cystoscopy, or the patient was referred for cystoscopy and biopsy at their regional hospital with subsequent pathologic examination.

All biopsy specimens were reviewed by a primary pathologist in China (Z. Ma) and categorized for WHO grade and tumor–node–metastases stage according to the American Joint Commission on Cancer *(24)*. The results were confirmed by a second pathologist (B. Bane) in the United States.

Biomarker Measurements

All biomarkers were measured by quantitative fluorescence image analysis of urine sediment cells. Samples were prepared for fluorescence as described previously *(21)*. In brief, the frozen urine sediment cells were thawed, collected on 8-μm-pore-size, 25-mm polycarbonate filters, and fixed for 2 minutes with 2% polyethylene glycol-1450 in 50% ethanol buffered with dipotassium–EDTA at 4 °C. Cells were transferred to slides by imprinting the filter onto poly-L-lysine- or silane-coated capillary gap microslides (Probe-On or ChemMate; Fisher Scientific Co., Houston, TX). The slides were then sprayed quickly with Carbofix-E (StatLabs, Inc., Lewisville, TX), allowed to air dry, and stored at −20 °C until analysis.

The fixed samples were sequentially labeled for p300 antigen, G-actin, and DNA by use of a computer-controlled Code-On Immunostainer (Fisher Scientific Co.) to maximize consistency in the staining procedures *(25)*. All labeling

was performed at room temperature. First, the slides were hydrated sequentially in 95% ethanol for 15 minutes, in 70% ethanol for 1 minute, and in 50% ethanol for 1 minute, with at least 2 minutes in Automation buffer (Biomeda Corp., Foster City, CA), a rinsing reagent between each ethanol step. Next, the slides were blocked with 0.45% Tween 20 and 0.35% bovine serum albumin (BSA) in Automation buffer for 15 minutes and incubated for 30 minutes with the M344 primary antibody, which labels the p300 antigen (1 : 25 dilution of ascites fluid in Automation buffer with 0.004% BSA) (25). After three washes in Automation buffer, the slides were incubated for 30 minutes with a 1 : 100 dilution of biotinylated goat anti-mouse immunoglobulin G (IgG) (Life Technologies, Inc. [GIBCO BRL], Rockville, MD) in Automation buffer. After three washes in Automation buffer, the slides were incubated for 30 minutes with a 1 : 500 dilution of streptavidin-conjugated Bodipy-FL (Molecular Probes, Inc., Eugene, OR) in Automation buffer. Next, after three washes in Automation buffer, the slides were incubated in 120 µg/mL deoxyribonuclease Texas Red Conjugate (Molecular Probes, Inc.) for 30 minutes to label G-actin. To stain DNA, the slides were then incubated with 8.7 μM Hoechst 33258 (Polysciences, Inc., Warrington, PA) in 0.1 M NaCl, 0.05 M MOPSO, 5 mM EDTA, with five changes of the solution to ensure saturation, with a total incubation time of 6 minutes. Finally, slides were mounted in 0.09 M N-propyl gallate (Sigma Chemical Co., St. Louis, MO) in glycerol (Fisher Scientific Co.). Control slides of the MGHU3 bladder carcinoma cell line (provided by Y. Fradet, Laval University, Quebec, ON, Canada) were included as a positive control for the M344 immunofluoresence, and slides of the 5637 transitional cell carcinoma cell line (American Type Culture Collection, Manassas, VA) were included for a negative control for M344 immunofluoresence and for a positive control for G-actin (24). An additional slide from each specimen was treated with a 1 : 2000 dilution of mouse IgG (Pierce Chemical Co., Rockford, IL) instead of the primary antibody to serve as a negative control for the M344 immunostaining.

To quantify the fluorescence staining, the slides were scanned on a Zeiss Axitron microscope (Zeiss, Thornwood, NY) at a final magnification of ×32 by use of a scanning stage and the IBAS image analysis system (Zeiss). The software developed by our research group for the IBAS image analysis system automatically captures each field, segments each scene, and extracts morphometric features of individual cells (23). Continuous fields were examined until approximately 100 abnormal nucleated epithelial cells had been measured, 1000 epithelial cells had been found, or 100 fields had been examined for DNA and G-actin determinations. The total number of epithelial cells on the slide was counted for p300 determinations.

The details of how the three biomarkers used in this study were quantified has been described in detail (25). Data for DNA 5CER analysis was obtained from 100 to 200 cells per slide, and the total number of image-selected epithelial cells on the slide was counted. The Hoechst 33528 dye incorporation was measured by use of a narrow band quantitative filter set (excitation at 390 nm and measured through a 410-nm emission filter). To determine DNA content, images of the stained cells were captured with the use of a UV filter, and the integrated intensity of the nucleus was determined by use of the IBAS image analysis software. The DNA content in urine sediment cells from a laboratory worker was used as a control. We generated a histogram from the integrated measurements and adjusted the histogram so that the tallest peak fell at 2C. A nonproliferating, diploid cell has a 2C DNA content and a dividing cell has a 4C DNA content. The images of all nuclei with a ploidy greater than 4.5C were reviewed by a cytotechnologist to confirm the validity of the measurements. To determine the 5CER, the number of cells with greater than or equal to 5.0C DNA was divided by the total number of epithelial cells present within the scanned area and expressed as a percentage. A positive DNA biomarker test was one with a 5CER greater than or equal to 0.8% or 2.0% for an extremely positive test to optimize the sensitivity and specificity of the biomarker.

To determine the G-actin concentration, we used the Texas Red narrow band pass quantitative filter set (exciter 560 DF 40, dichroic 595 dichroic long pass, emission 630 DF 23 630 nm [Omega Opical, Brattleboro, VT], with an SIT [Hammamatsu, Bridgewater, NJ]) to capture the images, which were corrected for autofluorescence. The mean gray level of each cell was determined as the mean gray level of the pixels on a scale of 0 (black saturation level) to 255 (white maximum fluorescence saturation level). The assay was standardized against an arbitrary standard cell line, the 5637 transitional carcinoma cell line, as described previously (25). The G-actin content was expressed as the G-actin units compared with the reference cell line. A positive biomarker test had greater than or equal to 90.0 G-actin units, and an extremely positive test was greater than or equal to 140 G-actin units.

For p300 immunoreactivity, the number of M344-positive cells was normalized to the number of cells on the slide by dividing the number of positive cells (visually counted) stained with a Bodipy label by the total number of epithelial cells (machine-counted epithelial DNA nuclei) on the slide and multiplying by 10 000 (reported as number positive/10K). The cells were scored for fluorescence by two independent observers, who were each unaware of each other's findings. On the basis of previously established thresholds (21), a positive biomarker test was 2/10K positive cells or extremely positive 20/10K for a voided urine sample and 8/10K positive cells for a bladder wash specimen.

Risk Assessment

Each individual in the cohort was classified into one of three groups based on the results of the biomarker analysis and the conventional cytologic examination of the urine samples. The criteria for the biomarker thresholds were initially defined before the study and were based on previous clinical studies (6,21). Three groups were defined (Fig. 1): high-risk group, positive for two or more biomarkers or extremely positive for any one of the biomarkers and/or positive for Pap cytology and/or hematuria; moderate-risk group, positive for any one biomarker; and low-risk group, negative for all biomarkers and negative for Pap cytology and hematuria. Some workers in both the exposed (n = 258) and the nonexposed (n = 56) groups could not be classified at some interval during the course of the study because of unsatisfactory urine biomarker tests (Fig. 1).

We had originally defined the thresholds for the biomarker profile on the basis of the results from symptomatic patients with bladder cancer (26). To confirm that the thresholds were appropriate for screening the cohort of asymptomatic workers, we generated cumulative frequency graphs for each of the three biomarkers after the first screen in two of the five cities (Shanghai and Hunan), plotted for exposed workers (smokers and nonsmokers separately) compared with nonexposed workers (smokers and nonsmokers). Because exposure to benzidine and smoking shifted the curves to the right, it was apparent from this analysis that too many individuals in the nonexposed group would be positive for DNA ploidy by use of the previously established DNA cut point of 0.4% 5CER (21). The cut point was adjusted upward to 0.8% based on the plot from the initial screen to avoid too many false positives.
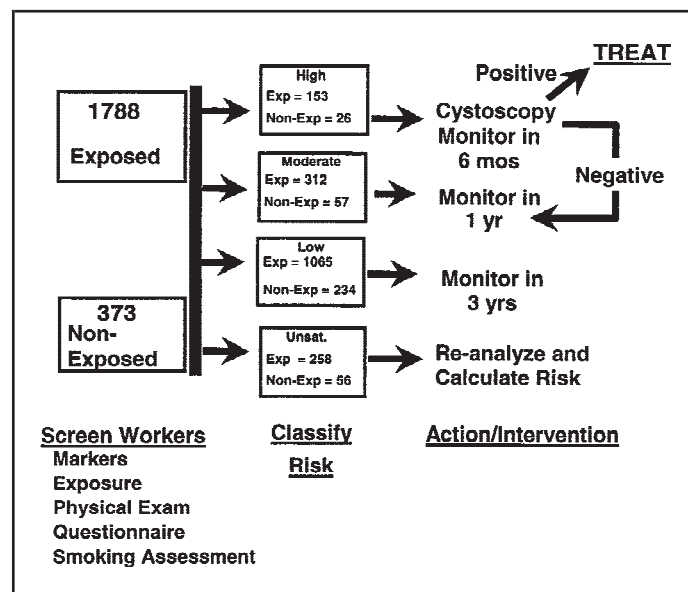

Fig. 1. Schematic of the screening study design indicating that subjects were stratified on the basis of biomarker results into three monitoring groups with their associated course of action. Subjects were grouped as high risk (High) if one marker extremely positive or two markers were positive, as moderate risk (Moderate) if G-actin or DNA ploidy as determined by DNA 5CER was positive or M344 was positive, and as low risk (Low) if all biomarkers were negative. Subjects were grouped as unsatisfactory (Unsat.) if the samples were mislabeled, there were too few cells, or there was a bad batch analysis. Exp = exposed; Non-exp = nonexposed. **Numbers** refer to the number of subjects in each group.

Statistical Analysis

The associations between the presence of each biomarker or biomarker combination and bladder cancer were assessed by means of the odds ratios (ORs) to determine the power of the biomarkers to detect bladder cancer in this cohort at the time of bladder cancer diagnosis and before the diagnosis for risk assessment. For those workers who developed cancer during the follow-up period, biomarker measurements made 6–12 months before the cancer was diagnosed were used for risk-assessment analysis, and biomarker measurements made 0–6 months before the cancer was diagnosed were used for detection analyses. For nonexposed workers screened initially and after 3 years, the biomarker measurements in years 1 and 3 were used for both risk assessment and detection analysis. If any worker developed a tumor, the biomarker results from the specimen collected at the time of tumor diagnosis were included in the analysis.

The bladder cancer incidence rate was reported as the number of cases per 100 000 person-years. The sensitivity, specificity, and predictive value of positive tests were derived on the basis of two-by-two tables (marker positive/negative by cancer/noncancer) for all biomarkers and their combinations. The age-adjusted ORs were estimated with logistic regression models. The 95% confidence intervals (CIs) of the adjusted ORs were derived on the basis of the likelihood function for the models. Exposure status was not used in adjusting the ORs because there were no cancers in the nonexposed group for risk-assessment analysis and only two cancers in the nonexposed group for detection analysis. The individual contributions and interactions of the biomarkers were assessed by the relative risk (RR) ratio (relative hazards ratio) from Cox proportional hazards regression models with time-dependent covariates. In the Cox proportional hazards regression models, all biomarker observations before the diagnosis of bladder cancer for cancer cases, all biomarker observations until the end of the study for noncancer cases, and the respective age and exposure duration at the time of each of these biomarker measurements were treated as time-dependent covariates (e.g., *see* PHREG Procedure in SAS, SAS software, version 6.12; SAS Institute, Cary, NC). A stepwise selection procedure was used to select the optimal model among all biomarkers and combinations of biomarkers. All statistical tests were two-sided.

RESULTS

To determine whether one or a combination of the three biomarkers can be used to identify individuals who are at high risk for developing bladder cancer and to detect bladder cancer, we screened a Chinese cohort of workers who were exposed occupationally to benzidine *(11)* for several biomarkers. The mean age of the nonexposed workers was 57.7 ± 10.8 years, whereas that of the exposed workers was 55.4 ± 10.5 years. Table 1 shows the incidence rates of bladder cancer in the nonexposed and exposed workers. Two cancers were identified in the nonexposed workers during the screening program, for an incidence rate of 87.2 per 100 000 person-years. The initial screen identified three cases of bladder cancer in exposed workers; an additional 25 cases of bladder cancer were identified in exposed workers during the screening program, for a total of 28 cases and an incidence rate of 263.4 per 100 000 person-years.

All exposed and nonexposed workers were initially screened for all biomarkers and then classified into one of three risk groups as detailed in the "Subjects and Methods" section (Fig. 1). On the basis of the anticipated risk profile, each group was subject to a predefined intervention protocol. Low-risk individuals were screened for the biomarkers every 3 years, moderate-risk individuals were screened every year, and high-risk individuals were screened every 6 months. Individuals in the high-risk group were also recommended for cystoscopy every 6 months. During the course of the study, 69% of the high-risk group had at least one cystoscopy. Individuals who were in the high-risk group and refused cystoscopy or who were in the moderate-risk group and were negative for all markers on two repeat screens were reclassified into the low-risk group. Ninety-two percent of the members of the high-risk group were screened sequentially (at least twice) for Pap cytology, urinalysis, and biomarker analysis and reported regularly to their occupational health unit.

The biomarker analysis results, biomarker risk stratification categories, clinical stages at diagnosis, and pathologic grades are listed in Table 2. Of the 30 total bladder cancer cases in the cohort, 28 were classified as transitional cell carcinomas, one was classified as squamous cell carcinoma, and one was not classified. Twenty cancers were classified as low or intermediate grade (grade 1 or 2), eight were classified as high grade (grade 3), and one was classified as a high-grade squamous cell carcinoma. At the time of diagnosis, four cancers had invaded the surrounding muscle, but none had clinically metastasized. Twenty-three of the 30 bladder cancers occurred in men from the high-risk group, three occurred in men from the moderate-risk group, three occurred in men from the low-risk group, and one occurred in a man who could not be classified because there was no urine sample available at the time of diagnosis. Five men who did not provide voided urine or who had an unsatisfactory urine result were determined to be in the high-risk group by either a catheterized urine or bladder wash specimen. Because the majority of the cancers were detected in the high-risk group, these results confirm the validity of the risk stratification scheme. In fact, the primary objective of this study was to identify individuals at risk in an occupationally exposed cohort on the basis of a pre-established biomarker profile derived from several different studies in symptomatic patients. Of the total individuals screened, 229 (10.6%) who had a positive biomarker profile on sequential screens were in the high-risk group and 218 (10.1%) were in the moderate-risk group. On the basis of these data and the combined specificity of the high- and moderate-risk profiles (Table 3) only 20%–30% of the exposed and nonexposed workers required continuous longitudinal surveillance.

To determine which biomarker profile is predictive of risk, we determined the overall sensitivity and specificity for each of the biomarkers and conventional markers alone and in various combinations at different time intervals (Table 3). Tumor risk assessment was analyzed from samples collected 6–12 months before the cancer diagnosis. When the biomarkers were considered individually, DNA ploidy, determined by DNA 5CER, was the strongest single marker for risk assessment. The sensitivity of DNA ploidy was 87.5%, its specificity was 86.5%, and the OR for bladder cancer risk in men positive for this marker was 46.2 (95% CI = 8.1 to 867.0).

The tumor-associated antigen p300, measured by M344 immunostaining, was the next most sensitive biomarker for risk assessment, with a sensitivity of 50.0%, a specificity of 97.9%, and an OR for bladder cancer risk of 40.0% (95% CI = 9.0 to

Table 1. Incidence rate (per 100 000 person-years) of bladder cancer in a cohort of Chinese workers occupationally exposed and nonexposed to benzidine (1991 through 1997)*

Group	No. of persons followed	Age, y, mean ± SD	No. of bladder cancer cases	Incidence rate
Nonexposed	373	57.7 ± 10.8	2	87.2
Exposed	1788	55.4 ± 10.5	28	263.3
Total	2161	55.8 ± 10.5	30	232.1

*SD = standard deviation. Notation-incidence rate was calculated on the basis of computer analysis and reflects actual person-years of follow-up.

Table 2. New cases of bladder cancer in the cohort of Chinese workers occupationally exposed and nonexposed to benzidine (1991 through 1997)*

Case No.	WHO grade	TNM stage	Biomarkers at the time of tumor diagnosis, voided urine						Remarks
			G-actin	p300	DNA	Pap	Hematuria	QFIA risk	
1	2	T2N0M0	−	+	+	N/A	N/A	High	
2	2	Ta	−	−	+	Positive	−	High	
3	2	Ta/T1†	−	+	+	Positive	+	High	
4	2	Ta	−	+	+	Atypical	−	High	
5	SqCa	Ta/T1†	+	−	+	Suspicious	N/A	High	
6	2	T1	−	−	+	Suspicious	−	High	
7	2	Ta	−	+	+	Negative	−	High	
8	3	T1	−	+	+	Positive	+	High	
9	1	Ta	−	+	−	Negative	+	High	
10	3	T1	−	+	+	Positive	+	High	
11	2	T2N0M0	Unsatisfactory	+	+	Positive	−	High	
12	3	Ta/T1†	−	+	+	Suspicious	N/A	High	
13	2	Ta	−	+	−	Negative	N/A	High	
14	3	Ta	−	+	+	Positive	−	High	
15	3	Ta/T1†	+	+	+	Atypical	−	High	
16	1	Ta	−	+	+	Positive	−	High	
17	N/C	Ta/T1†	Unsatisfactory	+	−	Positive	−	High	
18	2	Ta	−	−	−	Atypical	−	Low‡	CU,BW = high
19	1	Ta	−	−	+	Positive	N/A	Low‡	CU,BW = high
20	3	Ta/T1†	N/A	N/A	N/A	N/A	N/A	N/A‡	CU,BW = high
21	2	Ta	−	+	+	N/A	N/A	N/A‡	BW = high
22	2	Ta	−	−	+	Atypical	−	Moderate	
23	2	T2	−	−	+	Negative	−	Moderate	
24	2	T1	−	−	+	Negative	−	Moderate	
25	2	T	−	−	−	Negative	−	Low	
26	1	Ta	−	−	−	Positive	N/A	Low	
27	3	T2N0M0	−	−	−	Negative	−	Low	
28	3	T1	N/A	N/A	N/A	N/A	N/A	N/A	Sample error
29‖	2	Tx	+	+	−	Suspicious	−	High	Cancer of ureter
30‖	1	Ta	−	−	−	Negative	−	Low‡	BW = high

*WHO = World Health Organization tumor grading system *(23);* TNM (tumor–node–metastasis) = International Tumor Staging Classification *(23);* G-actin = cytoskeletal protein; p300 = bladder cancer antigen detected by M344 immunostaining; DNA = percentage of cells with DNA content exceeding 5C; Pap = Pap cytology; QFIA = quantitative fluorescence image analysis; N/A = no sample available; high = two markers positive or one marker extremely positive; N/C = not classified; SqCa = squamous cell carcinoma; low = all markers negative; CU = catheterized urine; BW = bladder wash; moderate = G-actin or DNA positive or M344 positive; and sample error = sample was labeled incorrectly and could not confirm which patient the sample came from.

†Pathologic stage could not be determined because of lack of muscle tissue in the transurethral specimen. Patients are assumed to have Ta/T1 disease because of lack of clinical evidence of invasive disease during 2 years of follow-up.

‡Quantitative fluorescence image analysis risk profile was measured from sediment cells in voided urine as low risk and N/A but from a bladder washing and catheterized urine as high risk. N/A means that there was no voided urine sample collected at the time of diagnosis.

§The original tissue samples did not include muscle tissue; pathologic stage could not be assessed.

‖Cases from nonexposed group.

Table 3. Sensitivity, specificity, odds ratios, and predictive values of positive biomarkers and conventional markers for detection and risk for bladder cancer in the cohort of Chinese workers occupationally exposed and nonexposed to benzidine*

Marker	Sensitivity†		Specificity†		Odds ratio (95% CI)†		Predictive value of positive test, %†	
	Detection	Risk	Detection	Risk	Detection	Risk	Detection	Risk
DNA 5CER‡	68.2	87.5	86.5	86.5	13.4 (5.8 to 36.6)	46.2 (8.1 to 867.0)	5.5	2.7
G-actin‡	5.0	25.0	80.7	80.7	0.2 (0.01 to 1.1)	1.6 (0.2 to 7.1)	0.3	0.6
p300‡	56.5	50.0	97.9	97.9	58.7 (24.4 to 145.6)	40.0 (9.0 to 177.8)	24.5	9.1
Hematuria§	23.5	20.0	98.8	98.8	23.7 (6.3 to 72.8)	17.5 (0.9 to 125.7)	13.3	3.7
Pap§	59.1	14.3	99.3	99.3	219.0 (80.6 to 636.1)	18.6 (0.9 to 126.0)	48.2	6.7
H‖	69.6	62.5	90.9	90.9	23.1 (9.7 to 60.8)	17.2 (4.2 to 85.0)	8.8	2.9
M or H‖	78.3	87.5	71.0	71.0	9.1 (3.6 to 27.6)	19.2 (3.4 to 360.5)	3.3	1.3
H or M or P¶‖	87.0	87.5	70.0	70.0	15.8 (5.4 to 67.2)	17.8 (3.1 to 334.6)	3.5	1.3

*Odds ratios are adjusted for age. CI = confidence interval.

†Detection = biomarker profile measured 0–6 months before cancer diagnosis. Risk = biomarker profile measured 6–12 months before cancer diagnosis.

‡Biomarker: DNA 5CER = measures DNA ploidy as a function of the percentage of cells with DNA content exceeding 5C; G-actin = cytoskeletal protein; p300 = bladder cancer antigen detected by M344 immunostaining.

§Conventional marker.

‖Risk profile based on biomarker results. H = high-risk (one marker extremely positive or two markers positive); M = moderate risk (G-actin or DNA positive or M344 positive).

¶Risk profile based on biomarker results or Pap positive.

177.8). G-actin was the weakest single biomarker for risk assessment, with a sensitivity of 25.0%, a specificity of 80.7%, and an OR of 1.6 (95% CI = 0.2 to 7.1) (Table 3). The conventional markers of hematuria and Pap cytology were both poor markers for risk assessment, with sensitivities of 20.0% and 14.3%, respectively; specificities of 98.8% and 99.3%, respectively; and ORs of 17.5 (95% CI = 0.9 to 125.7) and 18.6 (95% CI = 0.9 to 126.0), respectively.

We next determined the sensitivity and specificity of various biomarker combinations for risk assessment. When considering the high- or moderate-risk biomarker profiles, the sensitivity was 87.5%, the specificity was 71.0%, and the OR was 19.2 (95% CI = 3.4 to 360.5). When DNA ploidy and p300 were combined as "or" markers, the sensitivity and specificity were both 85% (data not shown), comparable with three markers (hematuria, Pap cytology, and p300) combined as "or" markers (Table 3). Because of the high specificity of hematuria and Pap cytology, these two conventional markers may be combined with other biomarkers with little loss of specificity. Thus, when the combinations of markers were analyzed for sensitivity, specificity, and ORs, the combination of a high- or moderate-risk biomarker profile and Pap cytology yielded a sensitivity of 87.5%, a specificity of 70.0%, and an OR of 17.8 (95% CI = 3.1 to 334.6).

We next determined the sensitivity and specificity of each biomarker for the accuracy of tumor detection from the samples obtained at the time of cystoscopy (diagnosis) or within the previous 6 months. Of the three individual biomarkers, DNA ploidy was again the most sensitive for cancer detection (68.2%), p300 was of intermediate sensitivity (56.5%), and G-actin was least sensitive (5.0%) (Table 3). The high- or moderate-risk biomarker profile combinations had a sensitivity of 78.3%. By contrast with the risk-assessment results, p300 was the strongest individual biomarker for cancer detection, with an overall specificity of 97.9%, an OR of 58.7 (95% CI = 24.4 to 145.6), and a positive predictive value of 24.5. DNA ploidy had an overall sensitivity of 68.2%, a specificity of 86.5%, an OR of 13.4 (95% CI = 5.8 to 36.6), and a positive predictive value of 5.5. Pap cytology had a sensitivity of 59.1%, a specificity of 99.3%, an OR of 219.0 (95% CI = 80.6 to 636.1), and a positive predictive value of 48.2.

We performed a Cox proportional hazards regression model with time-dependent covariates after applying a stepwise selection procedure to assess interactions among the biomarkers and their relative contributions to risk assessment and detection. First, adjusting only for age and duration of exposure, the relative RR was strongest for p300 alone (RR = 37.9 [95% CI = 16.8 to 85.3]), followed by the high-risk biomarker profile (RR = 25.5 [95% CI = 11.0 to 59.1]) and then by DNA ploidy alone (RR = 16.2 [95% CI = 7.1 to 37.0]) (Table 4). G-actin alone was the weakest predictor of bladder cancer risk, with an RR of 3.2 (95% CI = 1.2 to 8.4) (Table 4). Next, we applied the stepwise selection procedure and considered all variables and combinations of variables (Table 5). When the model was selected from all variables, only the variables p300 (RR = 15.4 [95% CI = 6.3 to 37.7]) and DNA ploidy or hematuria (RR = 8.3 [95% CI = 3.5 to 19.9]) were statistically significant (model 1). When the model was selected only from age, duration of exposure, the three individual biomarkers, and the biomarker combinations, only the variables DNA ploidy and p300 (RR = 14.7 [95% CI = 5.4 to 39.6]) and DNA ploidy or p300 (RR = 11.6 [95% CI = 4.3 to 31.2]) were statistically significant (model 2).

Table 4. Relative risk of bladder cancer in individuals with different positive biomarkers and conventional marker combinations as determined by Cox proportional hazards regression model with time-dependent covariates*

Biomarker	Risk ratio†	95% confidence interval
DNA 5CER	16.2	7.1 to 37.0
G-actin	3.2	1.2 to 8.4
p300	37.9	16.8 to 85.3
Pap cytology	14.7	6.2 to 34.6
Hematuria	5.1	1.2 to 22.1
High or moderate biomarker risk profile‡	13.5	5.3 to 34.7
High biomarker risk profile	25.5	11.0 to 59.1

*DNA 5CER = measures DNA ploidy as a function of the percentage of cells with DNA content exceeding 5C; G-actin = cytoskeletal protein; p300 = bladder cancer antigen detected by M344 immunostaining.
†Risk ratios were adjusted by age and total months of exposure.
‡High risk (one marker extremely positive or two markers positive); moderate risk (G-actin or DNA positive, or M344 positive).

Table 5. Risk ratios of single and multiple biomarker combinations by Cox proportional hazards regression model with time-dependent covariates using a stepwise selection method

Model*	Biomarker	Risk ratio	95% confidence interval
1†	p300 (+)	15.4	6.3 to 37.7
	DNA 5CER (+) or hematuria (+)	8.3	3.5 to 19.9
2‡	DNA 5CER (+) and p300 (+)	14.7	5.4 to 39.6
	DNA 5CER (+) or p300 (+)	11.6	4.3 to 31.2
3§	DNA 5CER (+)	9.2	3.8 to 22.5
	p300 (+)	18.4	7.5 to 44.9
4‖	DNA 5CER (+) and p300 (+)	81.4	33.3 to 199.3
5¶	DNA 5CER (+) or p300 (+)	19.6	8.0 to 47.9

*All models selected among age and total exposure time. p300 = bladder cancer antigen detected by M344 immunostaining; DNA 5CER = measures DNA ploidy as a function of the percentage of cells with DNA content exceeding 5C.
†Model 1 selected among all variables.
‡Model 2 selected among all biomarker combinations.
§Model 3 selected among individual biomarkers.
‖ Model 4 selected among DNA 4CER and p300 biomarkers.
¶Model 5 selected among DNA 5CER or p300 biomarkers.

When the model was selected only from age, duration of exposure, and the three individual biomarkers, again, only the variables p300 (RR = 18.4 [95% CI = 7.5 to 44.9]) and DNA ploidy (RR = 9.2 [95% CI = 3.8 to 22.5]) were statistically significant (model 3). Selecting from age, duration of exposure, and the variable DNA ploidy or p300, the regression model predicted that exposed workers have 19.6 times (95% CI = 8.0 to 47.9) higher risk of developing bladder cancer than nonexposed workers (model 5). Changing the variable to DNA ploidy and p300, the model predicted that the risk for exposed workers increases to 81.4 times (95% CI = 33.3 to 199.3) that for nonexposed workers (model 4).

The lead time from when a patient developed a positive marker profile (moderate or high risk) compared with conventional screening tests (Pap or hematuria) until tumor detection provided a useful clinical observation. On average, for individuals in the moderate-risk group who developed bladder cancers, the cancers were predicted 33 months before diagnosis. For individuals in the high-risk group who developed bladder cancers,

92% of the cases were predicted a mean of 15 months before diagnosis. For individuals with positive Pap cytology or positive hematuria, cancers were predicted approximately 8 or 3 months before diagnosis, respectively. Of interest, of the patients with bladder cancer who were originally classified in the moderate-risk group, all but two had a positive high-risk biomarker profile some time before tumor detection. The results confirm the usefulness of the risk stratification scheme and the previous observation *(4,11,27)* that Pap cytology and hematuria have poor sensitivity as primary screening methods for bladder cancer detection.

DISCUSSION

Molecular epidemiologic research is aimed at identifying the sequence of phenotypic and genotypic changes in carcinogenesis and at improving cancer control through individual risk assessment using these changes *(12,14,18,20)*. Conventional epidemiologic methods have been useful for defining groups at risk due to exposure, but advances in biomarker research have facilitated individual risk assessment through the detection of premalignant disease. For example, biomarkers can be used to identify high-risk groups from exposure-defined cohorts *(20)*. The high incidence of bladder cancer in the Chinese cohort of workers occupationally exposed to benzidine presented an opportunity to evaluate the usefulness of biomarkers for primary disease risk assessment and disease detection in a relatively small group compared with screening a general population *(11,20)*.

To identify individuals at high risk for bladder cancer, we had first defined the biomarkers used in this study in a pilot study with symptomatic patients, at which time the cut points for optimal sensitivity and specificity for each biomarker were determined *(6,21,26,28)*. Herein, we report a confirmatory study of these biomarkers evaluated for prospective risk assessment and for the detection of bladder cancer. Biomarker profiles would be especially beneficial for the detection of cancers with low incidence, such as bladder cancer. In China, bladder cancer is the 12th most common cancer, with 25 500 new cases diagnosed in 1985 *(7)*. The incidence of bladder cancer in the benzidine-exposed workers was 263 per 100 000 person-years during the time frame of the study and thus represents a major health problem for these workers. The incidence of bladder cancer in the nonexposed workers was 87 per 100 000 person-years, substantially higher than the anticipated incidence rate of 3.3 per 100 000 person-years projected in China in the general population for 1999 *(29)*. The difference in incidence between the nonexposed workers and the general population is due, in part, to the two cases in the control group. However, the incidence in the nonexposed workers is also higher than reported in the previous cohort study *(11)* and is probably biased by the small sample size. Moreover, the quantitative fluorescence image analysis screening for specific biomarkers in the current study may have contributed to the increased detection of bladder cancers that would otherwise have gone undetected in the absence of an intensive screening program. Similarly, Messing et al. *(30),* in a 14-day dipstick bladder cancer screening program of smokers more than 50 years of age, noted an incidence of 1300 per 100 000 person-years compared with an expected incidence of 28 per 100 000 person-years in males in the United States.

The prospective risk stratification scheme allowed us to iden-

tify the subset of occupationally exposed workers most likely to develop bladder cancer. The high percentage of cancers detected may reflect the fact that this cohort study was conducted over a 6-year longitudinal interval *(31)*. However, some selection biases may also have occurred because the study design specified more frequent cystoscopy of exposed workers with positive biomarker results *(32)*. Although compliance for biannual cystoscopy was low, at only 69%, this weakness was offset by a mean longitudinal follow-up of 36 months in 92% of the workers. In addition, all exposed workers were required to report any symptomatic disease to their respective occupational health units. Biased cystoscopy follow-up is unlikely because follow-up in the exposed and nonexposed groups was equal.

The prospective risk stratification scheme (high, moderate, and low risk) was used to stratify workers by risk for more or less intensive follow-up. Knowing the mean time interval from the time an individual is positive for a given marker or marker profile to the time of diagnosis is clinically useful. For instance, there was an impressive difference between the time an individual was classified into the moderate-risk group (33 months) or the high-risk group (15 months) and the time an individual was positive for hematuria (3 months) before a clinically detectable tumor. Therefore, knowing the mean number of months from the time an individual was biomarker positive until the development of disease not only is clinically important but also confirms the validity of the classification schema. These results also provide an explanation for the ineffectiveness of conventional biomarkers for early bladder cancer detection, which are weakened by the intermittent shedding of cancer cells and red blood cells into the urine. It is rational to assume that the premalignant changes that occur in the majority of normal cells are likely to be stronger markers. Other studies *(6,33,34)* in bladder, breast, and colon cancers also support this hypothesis.

Although the sensitivity results of the biomarker panel were consistent with a previous study *(20),* the sensitivity of each of the biomarkers used in the profile varied. DNA ploidy was the most sensitive individual biomarker, confirming its usefulness, along with conventional Pap cytology, for the detection of bladder cancer *(21)*. Although image analysis for DNA 5CER measurements can detect rare cells *(35,36)* and provide the additional advantage of easily testing voided urine samples (and thus avoiding invasive screening practices), the thresholds for a positive test need to be carefully determined for each population. Furthermore, the biomarker thresholds for a given population need to be assigned with an awareness of confounding factors, such as smoking, premalignant lesions, or sample processing.

By contrast with DNA 5CER, the tumor-associated antigen p300 was the single most specific biomarker (97.9%). However, the sensitivity for p300 was 56.5%, which is lower than the approximately 70% reported by Fradet et al. *(37)* in an immunohistochemical analysis of bladder tumor tissue. One explanation for the lower sensitivity in our study may be that the M344 antibody is sensitive for detecting low-grade (grade 1) tumors, whereas our Chinese cohort developed primarily grade 2 or 3 cancers. Indeed, the sensitivity was approximately 78% in a population of symptomatic patients with a mixture of high- and low-grade tumors *(21)*. It is unlikely that the decreased sensitivity in our study is a result of decreased or lost antigen expression incurred during sample transit because p300 expression is generally stable, at least compared with that of the bladder cancer antigen DD23 *(38).* We hope to increase the sensitivity of

the assay by automating the counting of the M344-positive cells. The addition of neural networks to the image analysis system *(39)* may further improve the test by detecting subtleties of cellular staining patterns.

The third biomarker incorporated into the risk-assessment profile was G-actin. G-actin was included because this monomeric component of the cytoskeletal protein F-actin is important in cellular differentiation *in vitro,* is often used as a measure of transformation *(40),* is often dysregulated early in carcinogenesis *(25),* and has been shown not to polymerize to F-actin in the dedifferentiation of multiple tumors, including those of the bladder, prostate, and breast *(33,40,41).* However, the sensitivity of G-actin in the biomarker profile was considerably less than that reported previously *(6,25),* possibly because of an increased tendency of G-actin to degenerate during sample shipping or because of a high variability in the deoxyribonuclease-binding assay compared with the antibody-based systems to detect DD23 or p300 *(37,38).* Because G-actin is an early marker for dedifferentiation, it is unlikely that the poor sensitivity of this biomarker for cancer detection in our cohort is related to a lack of progression through this premalignant phenotype. A Will Rogers effect (i.e., a selection bias based on biomarkers analyzed longitudinally) cannot be excluded as a reason for the poor performance of G-actin in this study *(42).* Likewise, assay drift also cannot be excluded on the basis of the consistency of the G-actin results in the nonexposed control group. The inclusion of a longitudinally monitored, nonexposed control group strengthened the design and facilitated the analysis of biomarker assay drift *(42).*

The biomarker and biomarker combinations tested in this study were also analyzed by ORs for bladder cancer to gauge their usefulness in a population at high risk for bladder cancer. The ORs were high for DNA ploidy (OR = 46.2), p300 (OR = 40.0), and Pap cytology (OR = 240). Although Pap cytology has a high OR, the reduced sensitivity of this conventional marker for risk assessment and tumor detection is the reason additional markers are needed and were evaluated in the cohort of benzidine-exposed and nonexposed workers. In addition to assaying all of the markers, the longitudinal monitoring of the cohort resulted in enhanced tumor detection. The targeting of a select subset of individuals at risk for more invasive diagnostic tests is important when the next step in a clinical evaluation is costly and invasive. Moreover, the concept of molecular fingerprints in morphologically normal-appearing cells from the premalignant field has broad application to individuals at high risk of other diseases, such as smokers with a positive computerized axial tomographic scan for lung cancer, who may require bronchoscopy, biopsy, or thoracotomy as the next diagnostic step.

Additional biomarker studies evaluating genetic changes, such as mutations or deletions, or microsatellite DNA associated with chromosomes 9 and 17 may improve the risk-assessment profile *(43)* because genetic changes on these chromosomes are associated with bladder cancer *(44,45).* Incorporation of chromosomal markers may also be associated with a substantial loss of specificity because many of the genetic changes are associated with genetic instability, and premalignant changes may not progress to the malignant phenotype.

Alternative to chromosomal markers, Pham et al. *(46)* have defined a urine biomarker that may be particularly useful for detecting invasive bladder cancer. Moreover, since we initiated the pilot study in 1989, other biomarkers expressed late in car-

cinogenesis have been defined, including the bladder tumor antigen DD23, telomerase, and cytokeratin 20 *(38,47–49).* Quantifying these additional biomarkers at the single-cell level should reduce the number of false positives associated with known confounding variables, such as umbrella cells and white blood cells *(23).* Quantitative fluorescence image analysis of single cells also avoids some of the problems with dipstick or gene microarray analyses, neither of which distinguishes tumor cells from nontumor cells or high levels of antigen expression from low levels of antigen expression. Ultimately, effective screening requires establishing when (i.e., early versus late) a biomarker is altered during carcinogenesis and identifying functional pathways that may be modulated by various chemopreventive agents *(19,50,51).* Improved sensitivity and specificity may be possible by altering the cut points of the biomarkers in this study and are goals for subsequent analysis. Further improvement in biomarkers for individual risk assessment and early bladder cancer detection is important because, in the past, 50% of the patients in occupationally exposed groups monitored on a yearly basis with Pap cytology died of their disease *(10).* To date, only two patients detected to have bladder cancer in this prospective biomarker-based screening program have died of bladder cancer, but we cannot exclude the possibility that lead-time bias may account for the decreased number of deaths to date. However, although the overall impact on mortality cannot be discerned from this study, early detection and improved bladder cancer control with intravesical vaccines such as bacille Calmette-Guérin are likely to contribute to reduced bladder cancer mortality in this current cohort *(52).* Early detection of breast, colon, and cervical cancers has reduced cancer mortality, and this may prove to be true for bladder cancer *(53).*

Previously, we predicted that biochemical changes would precede the usual morphologic and cytologic alterations associated with malignant transformation *(14).* The detection of positive biomarkers before the cystoscopic or cytologic diagnosis of a primary tumor suggests that the premalignant changes, represented by the biomarkers, occur in an organ at risk and are not a consequence of the molecular changes that occur secondary to the cancer *(50).* This study demonstrates the importance of studying the premalignant field to elucidate the mechanisms of carcinogenesis and clearly points to the value of biomarkers for individual risk assessment. Simple screening tests that can be performed on site or in a doctor's office and/or the incorporation of other biomarkers should further improve individual risk assessment and detection of bladder and other cancers *(54–56).*

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