

Prognostic Effect of DNA Aneuploidy from Bladder Washings in Superficial Bladder Cancer

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Abstract

Background: Superficial (papillary) bladder cancer is associated with progression and death from muscle-invasive bladder cancer, but no reliable predictors of the outcomes have been identified.

Methods: We analyzed the long-term prognostic effect of DNA flow cytometry in bladder washings from 93 subjects with previously resected T_a and T₁ bladder tumors who participated in a chemoprevention trial of the synthetic retinoid fenretinide. Kaplan-Meier analysis and Cox regression were used to determine the prognostic effect of DNA aneuploidy on cancer progression and mortality in conjunction with conventional clinical factors after a median of 11.5 years (interquartile range, 9.5-11.7 years).

Results: Overall, 58 of 93 (62%) specimens were DNA aneuploid at baseline. Progression-free survival was significantly shorter in subjects with stage T₁ [hazard ratio (HR), 31.6; 95% confidence interval (95% CI), 2.6-386.1; *P* < 0.001] and in subjects with baseline DNA aneuploid washing (HR, 10.5; 95% CI, 1.1-126.1; *P* = 0.03). The risk of death was also greater for stage T₁ tumors (HR, 2.6; 95% CI, 1.04-6.7; *P* = 0.04). DNA aneuploidy was a significant prognostic factor also for overall survival (HR, 2.8; 95% CI, 1.0-9.0; *P* = 0.05). Fenretinide treatment had no significant effect on cancer progression and death.

Conclusions: DNA aneuploidy in washings from endoscopically normal bladder is a significant predictor of progression and death in addition to tumor stage. This biomarker may help to identify and monitor a high-risk group who may benefit from a chemoprevention intervention. (Cancer Epidemiol Biomarkers Prev 2007;16(5):979-83)

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Introduction

Bladder cancer is the fourth most common tumor in men and the 12th most common in women in the United States (1), with ~61,500 new cases being expected in 2006 (2).

Superficial bladder cancer (stage T_a, T₁, T_{is}) represents the great majority (70-80%) of all bladder neoplasms at first presentation (3, 4). Invasion of the lamina propria (T₁) and a high grade of malignancy (G3) are associated with a worse outcome (5, 6). Whereas low-grade noninvasive papillary tumors (stage T_a) carry a small risk of tumor progression (7), high-grade T₁ tumors have a 30% to 50% risk of progression within 10 years (8). In addition, the presence of carcinoma *in situ*, a flat and noninvasive lesion of the surface mucosa that can occur alone or in association with papillary tumors, may develop into a muscle-invasive disease in 20% to 80% of cases (8).

Superficial bladder cancer comprises a spectrum of pre-malignant or early malignant disorders which have recently been incorporated into the concept of dysplasia or intraepithelial neoplasia, which virtually precedes all epithelial cancers (9). Importantly, prevention and treatment of intraepithelial

neoplasia, including superficial bladder cancer, is now considered an important target for the accelerated development of new agents designed to reduce cancer incidence (9).

Despite the efficacy of prophylactic Bacillus Calmette-Guérin intravesical immunotherapy in prolonging recurrence and progression-free survival, a substantial proportion of cases are destined to recur anywhere in the urothelial lining as a result of the field cancerization effect. Moreover, ~15% to 20% of these cases will progress to muscle-invasive cancer (8). However, no reliable quantitative biomarkers that predict progression and death from the field cancerization-affected target organ have been identified in bladder cancer (10, 11).

Considerable effort has recently been devoted to the search for surrogate end point biomarkers which are measurable and repeatable to allow selection of high-risk individuals and efficient drug testing in chemoprevention clinical trials (12-14). Given the promising use of serial flow cytometric DNA content measurements in bladder washings to monitor intravesical treatment outcome (15), we had selected this biomarker as the primary outcome measure in a chemoprevention trial of the synthetic retinoid fenretinide. The results of the trial at 12 and 36 months have previously been published (16). Importantly, recent studies have shown that DNA aneuploidy in papillary tumor tissue predicts the subsequent risk of invasive cancer (17-20).

In the present study, we analyzed the long-term prognostic value of DNA aneuploidy in washings from endoscopically normal bladder of patients with resected stage T_a and T₁ papillary tumors after a median follow-up time of 11.5 years (interquartile range, 9.5-11.7 years).

Materials and Methods

Subjects, Treatment, and Study Procedures. We used clinical and DNA flow cytometric data from 99 subjects with a median age of 63 years (range, 35-80 years) with resected

Received 7/6/06; revised 1/9/07; accepted 2/27/07.

Grant support: U.S. NIH/National Cancer Institute grant 5U01 CA-56457, the Italian Association for Cancer Research (AIRC), the Italian Foundation for Cancer Research (FIRC), the Italian League against Cancer (LILT), and Gruppo Bancario Credito Valtellinese.

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Note: Presented in part at the Fourth Annual AACR International Conference on Frontiers in Cancer Prevention Research, Oct 30-Nov 2, 2005, Baltimore, Maryland (Abstract #A15) and at the Fifth Annual AACR International Conference on Frontiers in Cancer Prevention Research, Nov 12-15, 2006, Boston, Massachusetts (Abstract #A146).

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doi:10.1158/1055-9965.EPI-06-0538

Table 1. Main subject characteristics at baseline by DNA index (n = 93)

	Diploid (n = 35)	Aneuploid (n = 58)	P*
Age (median), y			
≤63	23 (65.7) [†]	27 (46.6)	0.09
>63	12 (34.3)	31 (53.4)	
Sex			
Male	27 (77.1)	48 (82.8)	0.6
Female	8 (22.9)	10 (17.2)	
Smoking habit			
Nonsmoker	9 (25.7)	13 (22.4)	0.5
Former smoker	10 (28.6)	24 (41.4)	
Current smoker	16 (45.7)	21 (36.2)	
Tumor stage			
pT _a	22 (62.9)	32 (55.2)	0.5
pT ₁	13 (37.1)	26 (44.8)	
Tumor grade			
G1	19 (54.3)	19 (32.8)	0.1
G2	13 (37.1)	33 (56.9)	
G3	3 (8.6)	6 (10.3)	
Tumor history			
First event	19 (54.3)	16 (27.6)	0.02
Recurrent tumor	16 (45.7)	42 (72.4)	
Treatment			
Fenretinide (4-HPR)	15 (42.9)	33 (56.9)	0.2
Control	20 (57.1)	25 (43.1)	
Previous intravesical treatment			
None	7 (20.0)	1 (1.7)	0.01
Bacillus Calmette-Guèrin	15 (42.9)	35 (60.3)	
Chemotherapy	13 (37.1)	22 (37.9)	

*Pearson χ^2 or Fisher exact test.[†]Numbers in parentheses are percentages.

superficial bladder cancer who participated in a randomized chemoprevention trial of the synthetic retinoid fenretinide, administered at the daily oral dose of 200 mg for 2 years. The presence of T_{is} by bladder mapping was not assessed. A detailed description of the trial has been published elsewhere (16). Briefly, the aim of the trial was to evaluate the ability of fenretinide to modulate the flow cytometric DNA content in epithelial cells obtained from serial bladder washings taken over a period of 36 months. The primary outcome measure was the flow cytometric DNA content in epithelial cells obtained after 12 months.

Subjects underwent a complete medical examination and a cystoscopy with a bladder washing, using the technique previously described (21). Irrigation was done with five to six vigorous pulses of 100 mL of normal saline through a urethral catheter or a resectoscope sheath. This procedure was repeated twice to increase cell availability. The design and performance of the study procedures were described in previous reports (12, 16, 22).

Flow Cytometry. Staining for flow cytometry and DNA measurements were done as previously described (22, 23). Briefly, cells contained in 100 mL of fluid specimen were centrifuged, suspended in 0.5 mL of 0.5% paraformaldehyde for 15 min at 0°C, washed in PBS, permeabilized with 0.5 mL of 0.1% Triton X-100 for 5 min at 0°C, washed again with PBS, and finally suspended in the DNA staining solution (30 μ g/mL propidium iodide and 0.5 mg/mL RNase). After 1 to 2 h at room temperature, flow cytometric measurements were obtained. A minimum of 10,000 cells were analyzed for each specimen. The propidium iodide fluorescence mean channel of the lymphocyte population was used as the internal reference channel of diploidy. Aneuploid histograms were defined as those with at least one discrete cell population with a DNA content other than diploid. The DNA index was evaluated as the ratio of the DNA content of G₀-G₁ aneuploid cells to the DNA content of diploid G₀-G₁ cells, which are conventionally given a DNA index of 1.00. For a finer DNA index evaluation of aneuploid peaks that were very close to and often partially

overlapping the diploid G₀-G₁ peak, we used the bivariate log Side Scatter-lin propidium iodide fluorescence plot for easier identification. Because SS-propidium iodide plots of bladder washings from 10 healthy volunteers displayed in a few cases a slightly asymmetrical diploid population with propidium iodide fluorescence increased by <10% (i.e., 1 \leq DNA index < 1.1), we operationally defined as being aneuploid those populations with a DNA index \geq 1.1.

Because most bladder washing specimens contain a variable population of lymphocytes, monocytes/macrophages, granulocytes, and squamous cells in addition to transitional epithelial cells, adjustment for the nontransitional cell component was done by computer-assisted image analysis of the cytologic smears. The percentage of nonurothelial cells was calculated over 200 cells that were representative of the whole sample (CAS 200; Becton Dickinson). DNA content histograms were not considered evaluable when the resolution of the measurement was poor [i.e., when the coefficient of variation of the diploid G₀-G₁ peak was higher than 6%, when the nontransitional fraction of cells was higher than 60%, or when the proportion of cell debris was excessive (>60%)].

Cohort Follow-up. Information on patients' health status, cancer progression, and deaths was ascertained from the Urology Units who participated in the trial or, when the patient was lost to follow-up, through computer linkage with the local Tumor/Mortality Registry or by phone.

Table 2. Subjects' vital status according to DNA index (n = 93)

	All subjects, N	Diploid, n (%)	Aneuploid, n (%)
Alive	66	30 (45)	36 (55)
Deaths	27	5	22
Non-cancer-related deaths	10	3 (30)	7 (70)
Cancer-related death (other than bladder cancer)	9	2 (22)	7 (78)
Bladder cancer-related death	8	—	8 (100)

Progression was defined as the onset of a muscle-invasive bladder cancer (stage pT₂ or higher) or metastatic disease (N₊ or M₁) as documented by review of the hospital records. Causes of death were ascertained through hospital records or by death certificates or by review of the mortality registry.

Statistical Analysis. Pearson χ^2 and Fisher exact test were used to compare all baseline factors with DNA index value (<1.1 versus ≥ 1.1). Kaplan-Meier estimates (24) of the cumulative probability of progression, defined as the time from randomization to the onset of a muscle-invasive bladder cancer (T₂ or higher), and mortality from any cause were obtained for all baseline factors including allocated treatment (fenretinide versus control), age, sex, smoking habit, tumor stage, tumor grade, recurrence history, previous intravesical treatment, and DNA index (<1.1 versus ≥ 1.1). In the analyses of progression, follow-up times of patients dying before diagnosis of progression were censored at the time of death. A multivariate Cox proportional hazard model (25) was used to assess the independent prognostic effect of all factors under investigation. All calculations were done using SPSS version 11.5 and STATA version 9 software.

Results

DNA index analyses were done on 93 (94%) subjects with assessable baseline bladder washing specimens. Specimens with a DNA index ≥ 1.1 were classified as DNA aneuploid. Six subjects were not assessable because the coefficient of variation of the diploid G₀-G₁ peak was >6% ($n = 3$), the nontransitional fraction of cells was >60% ($n = 1$), or the proportion of cell debris was >60% ($n = 2$).

The distribution of DNA aneuploid washings according to established clinical factors is summarized in Table 1. Overall, 58 of 93 (62%) specimens were DNA aneuploid at baseline. Among the eight subjects who did not receive any previous intravesical treatment, only one had an aneuploid washing, whereas aneuploidy was more frequent in Bacillus Calmette-Guèrin-treated versus chemotherapy-treated subjects ($P = 0.01$). Moreover, recurrent tumors were more frequent among DNA aneuploid washings (72%; $P = 0.02$, Fisher exact test). All other factors including smoking habit were evenly distributed between diploid and aneuploid washings.

After a median follow-up time of 11.5 years, 11 subjects had progressed to muscle-invasive bladder cancer and 27 subjects had died. The distribution of aneuploid washings according to subsequent disease status is summarized in Table 2. There were 10 non-cancer-related deaths and 17 cancer-related deaths, including 8 bladder cancers, 6 lung cancers, 1 leukemia, 1 pancreatic cancer, and 1 biliary tract cancer, all of whom, except for 1 lung and 1 pancreatic cancer, were DNA aneuploid.

Progression-free survival, bladder cancer survival, and overall survival curves according to DNA ploidy in bladder washings are illustrated in Fig. 1. The cumulative probability of progression to muscle-invasive cancer, bladder cancer mortality, and all-cause mortality at 10 years was 0%, 0%, and 11.4% for DNA diploid washings and 17.2%, 15.4%, and 34.5% for DNA aneuploid washings [$P = 0.02$, $P = 0.02$, and $P = 0.008$, respectively (log-rank test)].

Progression-free survival and overall survival curves according to fenretinide treatment are illustrated in Fig. 2. There was no effect of the retinoid on both outcomes, nor was there any interaction between retinoid treatment and smoking status (data not shown).

Table 3 shows Cox multivariate analysis of progression and death adjusted for age, smoking habit, and previous intravesical treatment. Stage T₁ and, to a lesser extent, DNA aneuploidy in bladder washing were associated with a significant increased risk of tumor progression [hazard ratio

(HR), 10.5; 95% confidence interval (95% CI), 1.1-126.1; $P = 0.03$]. Likewise, DNA aneuploidy was, in addition to stage T₁, the only independent predictor of all-cause mortality (HR, 2.8; 95% CI, 1.0-9.0; $P = 0.05$). The Cox model was not applicable to bladder cancer survival given the lack of events in the DNA diploid group.

Discussion

Superficial bladder cancer comprises a spectrum of premalignant or early malignant disorders that are known precursors of muscle-invasive bladder cancer arising from the field

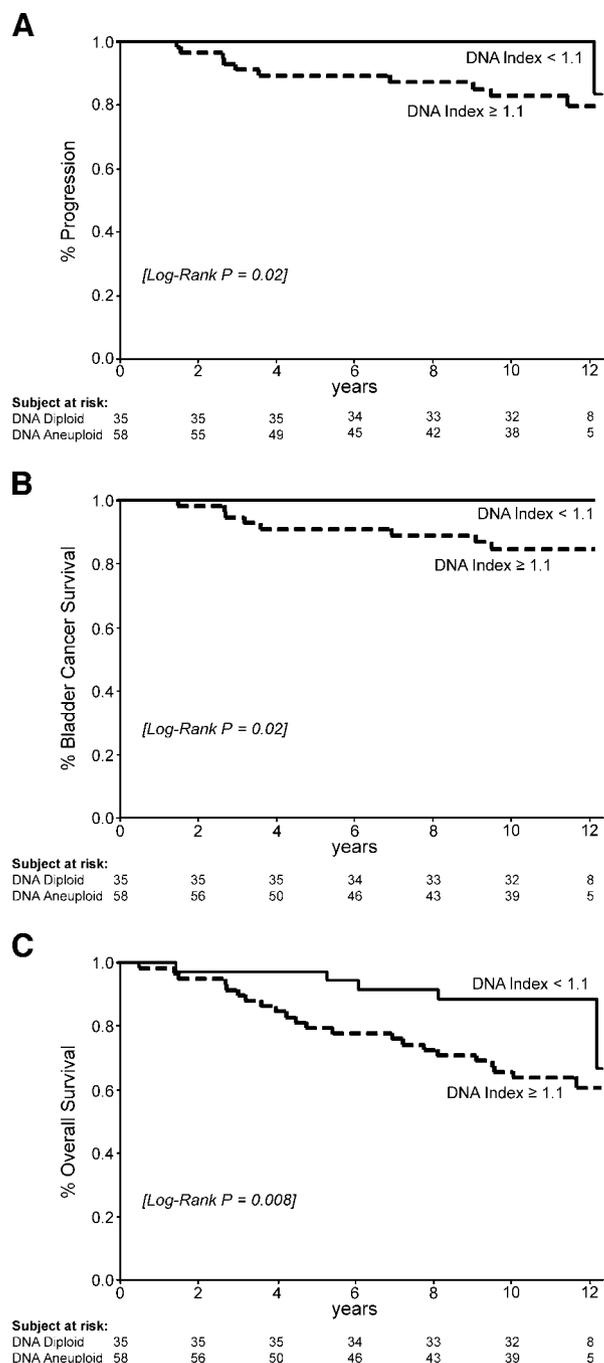


Figure 1. Kaplan-Meier progression-free survival (A), bladder cancer survival (B), and overall survival (C) curves according to DNA index (DNA index < 1.1, diploid; DNA index ≥ 1.1 , aneuploid).

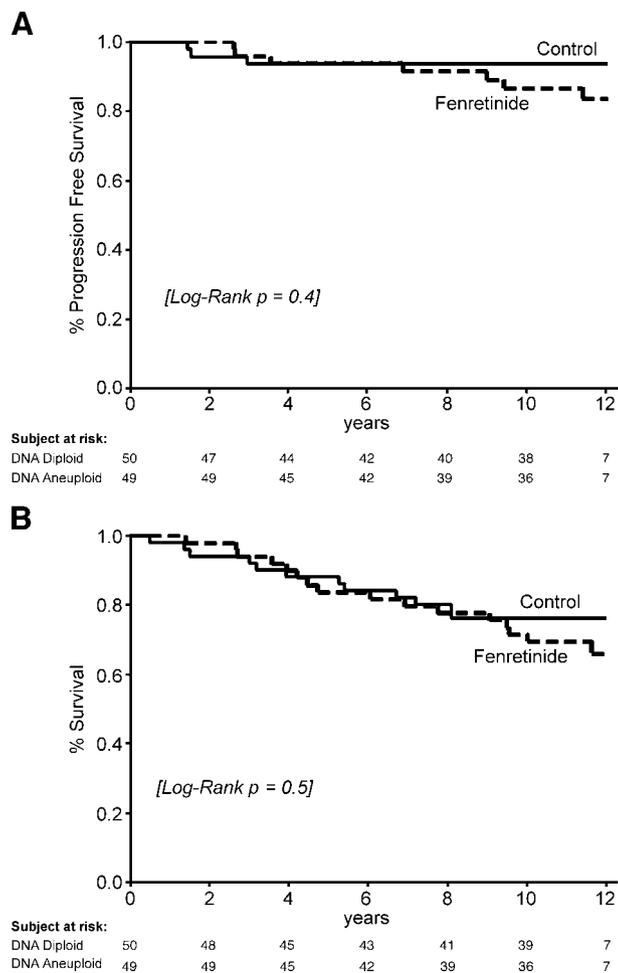


Figure 2. Kaplan-Meier progression-free survival (A) and overall survival (B) curves in the two treatment arms [fenretinide (dotted line); control (continuous line)].

cancerization-affected organ. Although high tumor stage, grade, and recurrence history have been associated with a greater risk of progression (10, 11), no reliable quantitative biomarkers have been identified that will predict progression and death in the entire target organ after tumor resection.

Our results after a median follow-up time of 11.5 years indicate that 12% of subjects with superficial bladder cancer who participated in a chemoprevention trial of fenretinide progressed to muscle-invasive cancer and as many as 29% died. In line with literature findings (3), conventional clinical factors such as tumor stage, grade, and, to a lesser extent, recurrence history were associated with a greater risk of progression and death, thus strengthening the reliability of our results.

Our findings indicate that DNA aneuploidy in bladder washings, which was the primary outcome measure of the trial, was associated with a statistically significant increased risk of progression to muscle-invasive bladder cancer, although the small sample size precludes any firm conclusion, as reflected by the wide confidence interval of the estimate. Interestingly, DNA aneuploidy in bladder washings also had a statistically significant prognostic effect on overall mortality, even when other factors, including age and smoking habit, were adjusted for in multivariate analyses. Finally, DNA aneuploidy was associated with all cancer-related deaths and particularly with bladder cancer-related death.

To our knowledge, this is the first report indicating an association between DNA aneuploidy in bladder washings from subjects with superficial bladder cancer and progression and death. Whereas recent studies showed an association between DNA aneuploidy in the resected tumor tissue and progression to invasive cancer (17-20), none had addressed the prognostic effect of DNA aneuploidy in cells obtained from bladder washings of an endoscopically normal organ. Conceivably, DNA aneuploidy in bladder washing may reflect the presence of T_{is} in bladder mapping, which was not assessed in our study. Whereas these results need to be confirmed in larger series, the prognostic effect of DNA aneuploidy in bladder washings after papillary tumor resection may be clinically important to stratify subjects at higher risk of progression and death, given the ability of this procedure to noninvasively sample the entire target organ so as to detect field cancerization abnormalities.

A strength of our observation is the long-term follow-up and the source of data derived from an unselected cohort participating in a clinical trial conducted under Good Clinical Practice procedures, where DNA aneuploidy was the primary end point. In addition, our finding is important for future clinical trials because this procedure may be repeated serially without significant discomfort, thus providing adequate cell samples for biomarker modulation assessment by experimental agents. Finally, the association between DNA aneuploidy in

Table 3. Multivariate Cox proportional hazard model of progression-free survival and overall survival (n = 93)

	Progression-free survival		Overall survival	
	HR (95% CI)	P*	HR (95% CI)	P*
Tumor stage				
pT _a	1.0		1.0	
pT ₁	31.6 (2.6-386.1)	<0.001	2.6 (1.0-6.7)	0.04
Tumor grade				
G1	1.0		1.0	
G2/G3	1.7 (0.2-15.8)	0.6	1.4 (0.5-3.9)	0.5
Tumor history				
First event	1.0		1.0	
Recurrent tumor	3.6 (0.7-19.1)	0.1	1.02 (0.4-2.5)	0.9
Treatment				
Control	1.0		1.0	
Fenretinide	0.8 (0.2-3.2)	0.7	0.8 (0.3-1.9)	0.6
DNA index				
DNA index < 1.1 (diploid)	1.0		1.0	
DNA index ≥ 1.1 (aneuploid)	10.5 (1.1-126.1)	0.03	2.8 (1.0-9.0)	0.05

NOTE: The risk estimates are adjusted for age, smoking habit, and previous intravesical treatment.

*Two-sided likelihood ratio test.

bladder epithelial cells and subsequent death from any cause, most of which were tobacco related, suggests that this biomarker may reflect a systemic genetic damage possibly due to smoking, which affects different target organs (26).

Our data show that fenretinide treatment at 200 mg/d for 2 years did not affect disease outcome after 11.5 years of follow-up. This is consistent with our initial report of a lack of modulation of flow cytometric DNA content, conventional cytology, and recurrence-free survival by the retinoid after 3 years (16). Despite evidence of activity in animal studies (27-29) and its ability to induce apoptosis in bladder cancer cell lines at relevant pharmacologic concentrations (30), fenretinide has proved to be ineffective in two independent clinical trials (16, 31). Accumulating evidence suggests that retinoids fail to modulate tobacco-related carcinogenesis (32, 33). Several explanations have been proposed (34, 35), which point to a complex interaction with host or lifestyle characteristics, underlying the importance of appropriate subject selection. Additional explanations for the lack of activity of fenretinide might include an insufficient dose to attain the apoptotic effect through ceramide production and induction of reactive oxygen species (36-39).

In conclusion, our data show that DNA aneuploidy in urothelial cells obtained from washings of clinically normal bladder after tumor resection predicts progression to invasive cancer and death. This biomarker may help to select individuals with bladder intraepithelial neoplasia at high risk for progression to muscle-invasive disease, who may benefit from a chemoprevention intervention. In addition, the feasibility of repeated washings provides an opportunity for monitoring of drug intervention through serial biomarker measurements.

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