

# DNA *in Situ* Sensitivity to Denaturation in Bladder Cancer and Its Correlation with Tumor Stage<sup>1</sup>

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## ABSTRACT

DNA content and sensitivity of DNA *in situ* to denaturation by acid were analyzed by flow cytometry of cell nuclei freshly isolated from the bladder tumors of 32 patients and were compared with normal urothelium of 8 subjects. DNA sensitivity to denaturation was assessed in RNase treated cells by acridine orange metachromasia following partial denaturation with hydrochloric acid; the extent of denatured DNA is given as an index ( $\alpha t$ ), representing the ratio of single stranded to total DNA per nucleus.

Of the low stage tumors (papillomas, Ta, Tis, T1) 11 of 18 (61%) were aneuploid. Of the high stage tumors (T2 and T3a) 11 of 14 (79%) were aneuploid. DNA in nuclei of normal transitional epithelium was very sensitive to denaturation, as was papilloma, characterized by nuclear  $\alpha t$  indices of  $0.73 \pm 0.01$  (SD) and  $0.73 \pm 0.04$ , respectively. Nuclear DNA of noninvasive carcinomas (Ta, Tis) was significantly more resistant to denaturation ( $\alpha t = 0.69$ ), and DNA of invasive carcinomas was most resistant, ranging from  $\alpha t = 0.61$  (T<sub>1</sub> tumors) to  $\alpha t = 0.59$  (T<sub>2</sub> tumors) to  $\alpha t = 0.57$  (T<sub>3</sub> tumors).

High stage tumors as a group (T<sub>2</sub>, T<sub>3</sub>) had significantly different (lower)  $\alpha t$  values than low stage tumors (Ta, Tis, T1). In model cell culture systems it is known that a decrease in  $\alpha t$  index, *i.e.*, greater resistance to denaturability, occurs as cells transit from resting phase into the cell cycle. Whether the  $\alpha t$  index can be used to estimate resting *versus* cycling cells of human tumors is still speculative; changes in DNA denaturability also are known to occur with changes in chromatin structure during cell differentiation and in transformation. However, the empirical relationship between  $\alpha t$  index and tumor stage, of itself, may prove clinically useful in identifying more advanced and perhaps more aggressive tumors.

## INTRODUCTION

Prognosis of bladder tumors is generally related to histological grade and pathological stage (1). Although DNA aneuploidy is also known to be of prognostic value (2, 3), there is still a paucity of markers for the biological behavior of these tumors. In model cell systems we have shown that cell proliferation, quiescence, and differentiation are accompanied by changes in resistance to denaturation of DNA *in situ*, presumably due to differences in chromatin structure (4-6). The more condensed, transcriptionally inactive chromatin is generally more sensitive to denaturation. We have also shown, in a small preliminary study, a relationship between tumor stage and DNA denaturability of human colon carcinoma, suggesting that chromatin structural changes progress with progressively advancing cancer (7).

The flow cytometry method used by us to quantify DNA denaturation was originally developed for intact, whole cells. It has been applied to cells in suspension culture (8, 18) and to clinical specimens of leukemias (9) and lymphomas (10). Technical problems encountered with dissociation of individual cells

from solid tumors initially limited wider application of this methodology. However, in the modification used here we examined nuclei freshly isolated from solid tumors, rather than whole cells, and have successfully examined carcinomas of breast (11) as well as colon (7). In the present study we measure the DNA denaturability of 32 transitional cell tumors of the bladder and 8 specimens of normal transitional epithelium. The data suggest that resistance to denaturation of DNA *in situ* may be a marker discriminating tumors of different stage and perhaps of different aggressiveness.

## MATERIALS AND METHODS

**Specimens.** Biopsy specimens of 32 urothelial tumors were obtained by transurethral resection from 32 patients seen at Memorial Sloan-Kettering Cancer Center between September 1988 and February 1989. Normal urothelium was obtained from the renal pelvis of 8 kidneys removed for renal cortical carcinoma. The urothelial tumors were classified according to the American Joint Committee on Cancer staging system (12) and divided into low stage (papilloma, Ta, Tis, T1) and high stage (T2, T3a) categories for statistical analysis. There were 18 low stage tumors; 4 papillomas, 3 Ta, 8 Tis, and 3 T1; and 14 high stage tumors: 12 T2 and 2 T3a.

**Specimen Preparation.** Tumor tissue was collected by transurethral resection and placed immediately in Hanks' balanced salt solution without calcium and magnesium. Small representative portions of tumor were selected by one of us (M. R. M.) and stored in the balanced salt solution at 4°C for up to 24 h prior to preparation and measurements; histological sections were prepared from the remaining portions of each tumor fragment sampled and examined for confirmation of tumor type, grade, and stage.

Two types of nuclear isolation buffers were used: (a) for measurement of DNA content, nuclei were isolated in 1.0% citric acid (Sigma Chemical Co., St. Louis, MO) and 0.1% Nonidet P-40 detergent (Accurate Scientific and Chemical Co., Hicksville, NY); (b) for measurement of DNA denaturation, nuclei were isolated in a buffer of 10 mM acetic acid, 1 mM MgCl<sub>2</sub>, and 0.1% Nonidet P-40, pH 1.5.

The bladder tissue was carefully reexamined, nonviable appearing tissue was trimmed and the remainder was divided and placed in two 15-ml conical tubes to which 1 ml of the appropriate nuclear isolation solution was added. This was vortexed for approximately 1 min, and the supernatant was decanted and filtered through gauze to strip remaining cytoplasmic tags from the free nuclei and to remove particles of debris. The procedure was repeated and to the filtered supernatant an equal volume of 50 mM Piperazine-N,N'-bis(2-ethanesulfonic acid) buffer (Sigma) with 5 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub>, pH 7.4, was added.

**DNA Determination.** The DNA content of the citric acid isolated nuclei from malignant and normal urothelium was determined by the two step Acridine Orange (AO)<sup>2</sup> technique of Traganos and Darzynkiewicz *et al.* (13, 14). Briefly, a 0.2-ml aliquot of the nuclear suspension ( $2 \times 10^5$  nuclei) was incubated with 0.4 ml of acid detergent (0.1% v/v Triton X-100, 0.08 N HCl, and 0.15 M NaCl) for 30 s at room temperature. To this was added 1.2 ml of an AO solution [containing 100 ml of 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, 0.1 M citric acid (pH 6.0), 1 mM sodium EDTA, 0.15 N NaCl, and 6 µg/ml of chromatographically purified AO; Polysciences, Warrington, PA]. Measurements were immediately carried out on an Ortho FC-200 flow cytometer (Ortho Diagnostics,

<sup>2</sup> The abbreviation used is AO, acridine orange.

Received 11/6/89; accepted 9/17/90.

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<sup>1</sup> Supported in part by National Cancer Institute Grants R01-CA 14134 and UO-CA 41021.

Westwood, MA) equipped with a Lixel laser (Palo Alto, CA) operating at a wavelength of 488 nm (blue). AO binds to double helical DNA by intercalation and fluoresces green (530 nm) in blue light; the intensity of green fluorescence is proportional to the nuclear DNA content. A total of 5000 nuclei per sample was measured and the data were stored in a Compaq Deskpro 386 microcomputer interfaced to the flow cytometer. Peripheral blood lymphocytes from healthy donors prepared by Ficoll-Hypaque separation (15) were used as a control to establish the 2C (normal) diploid content of DNA. Diploid tumors were those in which the DNA content of the G<sub>1</sub> cells (peak channel) of the tumor was equal to the G<sub>1</sub> peak of the lymphocytes, within the error of measurement [i.e.,  $1 \pm 0.05$ ]. Aneuploid tumors had a G<sub>1</sub> population with increased DNA content (DNA index > 1.05) compared to the normal lymphocyte control.

**Acid-induced DNA Denaturation.** The DNA denaturability of chromatin by acid was studied in acetic acid isolated nuclei as described elsewhere (7, 10, 11). Briefly, a nuclear suspension of 1 million cells was incubated with 2000 units of RNase (Calbiochem-Behring Diagnostics, La Jolla, CA) at room temperature. A 0.2-ml aliquot of nuclear suspension was mixed with 0.5 ml of buffer containing 0.1 M HCl at pH 1.4 and after 40 s 1.5 ml AO solution were added. Acridine orange was used at a concentration of 4  $\mu$ g/ml in buffer containing 0.1 M citric acid and 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, at pH 2.6. Acid denaturation and staining was performed at room temperature and all solutions were warmed to room temperature before use. The fluorescence of individual nuclei was measured in an FC-200 flow cytometer, as above, interfaced to a Compaq Deskpro computer. The red (>600 nm) and green (~530 nm) fluorescence from each nucleus were separated optically and the integrated values of the pulses were quantitated by separate photomultipliers.

The amount of DNA denatured was expressed as a ratio of the red fluorescence (denatured DNA) to total fluorescence (7, 11). The L1210 cell line was used as an external standard for calibration of the flow cytometer. The photomultiplier sensitivities for red and green fluorescence detection were adjusted so that the mean  $\alpha t$  value of the G<sub>1</sub> L1210 cell population was always the same, and between 0.4 and 0.5. During the course of this study the mean value was  $0.44 \pm 0.07$ .

**Statistical Analysis.** The cumulated frequencies (i.e., empirical distributions) of the  $\alpha t$  index of various subsets of cases have been plotted and the differences are analyzed statistically. Two tests have been used: Kolmogorov-Smirnov (16) and Wilcoxon (17) (Mann-Whitney). The more conservative result has been reported.

## RESULTS

There were 32 transitional cell tumors of the bladder and 8 samples of normal transitional epithelium examined. All samples of normal urothelium were diploid. Eleven tumors (34%) were diploid and 21 (66%) were aneuploid; papilloma: 4 of 5

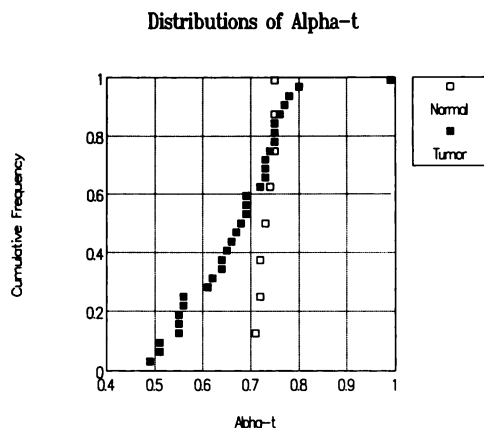


Fig. 1. Cumulative frequency of distribution of  $\alpha t$  of normal urothelium ( $\square$ ) versus tumor ( $\blacksquare$ ).

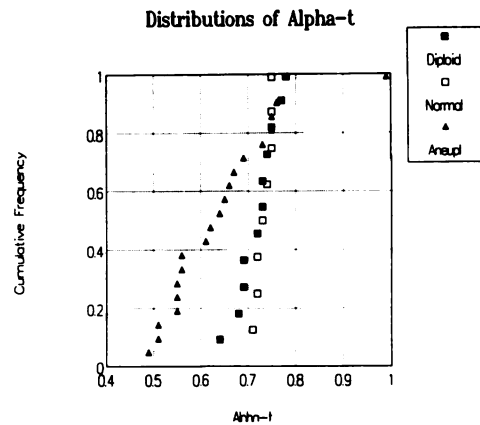


Fig. 2. Cumulative frequency of distribution of  $\alpha t$  of normal urothelium ( $\square$ ) versus diploid tumors ( $\blacksquare$ ) versus aneuploid tumors ( $\blacktriangle$ ).

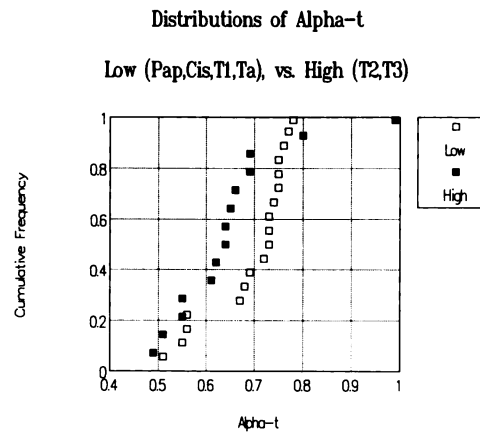


Fig. 3. Cumulative frequency of distribution of  $\alpha t$  of low (Pap, Cis, T1, Ta) stage ( $\square$ ) versus high (T2, T3) stage tumors ( $\blacksquare$ ).

Table 1 Mean  $\alpha t$  values of bladder tumors by stage

	Mean $\alpha t$
Normal urothelium	$0.73 \pm 0.015^a$
Papilloma	$0.73 \pm 0.047$
Noninvasive carcinoma	
Ta	$0.69 \pm 0.021$
Tis	$0.69 \pm 0.07$
Invasive carcinoma	
T1	$0.61 \pm 0.081$
T2	$0.59 \pm 0.094$
T3a	$0.57 \pm 0.075$

<sup>a</sup> Mean  $\pm$  SD.

diploid, 1 of 5 aneuploid; Ta: 1 of 3 diploid, 2 of 3 aneuploid; Tis: 1 of 7 diploid, 6 of 7 aneuploid; T1: 1 of 3 diploid, 2 of 3 aneuploid; T2: 3 of 12 diploid, 9 of 12 aneuploid; T3a: 2 of 2 aneuploid. When separated into low stage and high stage tumors, low stage were 7 of 18 (39%) diploid versus 11 of 18 (61%) aneuploid; high stage were 3 of 14 (21%) diploid versus 11 of 14 (79%) aneuploid. There was no difference in DNA index of aneuploid low and high stage tumors.

The  $\alpha t$  values of normal urothelium (mean,  $0.73 \pm 0.015$ ) were not significantly different from those of the bladder tumors as a whole, but  $\alpha t$  values of normal urothelium varied little whereas  $\alpha t$  of tumors varied widely according to tumor type and stage (Fig. 1). Thus, diploid tumors (mean,  $0.72 \pm 0.014$ ) did not differ from normal, whereas  $\alpha t$  values of aneuploid tumors ( $0.62 \pm 0.02$ ) were significantly different ( $P < 0.005$ )

from those of normal urothelium, and also different ( $P < 0.05$ ) from those of diploid tumors (Fig. 2). Correlations with stage revealed a significant difference ( $P < 0.05$ ) in mean  $\alpha t$  values of low versus high stage tumors (Fig. 3), given in detail in Table 1.

## DISCUSSION

Earlier studies from this laboratory demonstrated that sensitivity to denaturation of DNA *in situ* was greater in quiescent and mitotic cells than in interphase cells progressing through the cell cycle (5). The increased DNA sensitivity to denaturation in quiescent and mitotic cells was explained as due to the higher degree of chromatin condensation in these cells (6). Treatment of nuclei with heat or acid causes dissociation of histones and perhaps other nuclear proteins from DNA prior to DNA denaturation; such free DNA attached to nuclear matrix is less stable in condensed chromatin compared to euchromatin. Thus, the present method discriminates between cells with varying degrees of chromatin condensation, which in turn may be correlated with quiescence versus progression through the cell cycle. Differences in DNA denaturability also have been demonstrated in association with nuclear chromatin changes during differentiation (18) and in transformed (leukemic) versus corresponding benign cells (19) and may be related to the overall transcriptional activity of the cell nucleus, which is known to correlate inversely with the degree of chromatin condensation (hetero-/euchromatin ratio) (20).

Kunicka *et al.* (7) adapted this methodology to human solid tumors by measuring DNA stability in freshly isolated nuclei rather than in whole cells; isolation of nuclei from solid tissues is often readily accomplished even when dissociation of intact cells is not possible. In their first report, these authors described significant differences in DNA stability of normal colonic mucosa and adenomas compared to colon carcinoma. They noted that the DNA of adenomas and superficially invasive (Dukes A) carcinoma was more sensitive to denaturation than deeply invasive (Dukes B, C/D) carcinomas. In a later study of breast tumors, DNA was again found to be more sensitive to denaturation in adenomas and normal tissue compared with cancer, and DNA denaturability also correlated with the presence of progesterone receptors (11). However, there have been no long-term studies to evaluate the possible prognostic value of these observed differences.

Gustafson *et al.* (21) reported that in a group of 229 patients with low stage (Ta or T1) bladder cancer 175 (76%) tumors were diploid and 54 (24%) were aneuploid. In our patients with low stage bladder cancer 7 of 18 (39%) were diploid and 11 of 18 (61%) were aneuploid. The greater proportion of aneuploid tumors in our series may be due to the presence of 6 aneuploid carcinomas *in situ* (T1s), or simply to the small sample number. Interestingly, nearly all cases of T1s were aneuploid but the mean  $\alpha t$  value of this group ( $0.69 \pm 0.07$ ) was not significantly different from other low stage tumors (Table 1). Thus,  $\alpha t$  does not correlate with aneuploidy. Whether it signifies the proportion of cycling versus noncycling cells or will yield information on tumor behavior unrelated to DNA content is still speculative. Sufrin *et al.* (22) found that thymidine labeling of flat carcinoma

*in situ* is the same as papillary and sessile carcinoma, consistent with our finding that the  $\alpha t$  index is the same for these tumor types. Although it is still too early to assess the clinical value of the  $\alpha t$  index, we did find a significant difference in the mean  $\alpha t$  values of low versus high stage tumors ( $P < 0.05$ ), and this difference was seen even though there was no significant difference in DNA content between low and high stage tumors. These data suggest that subtle differences in chromatin structure which are independent of DNA content may be a sensitive marker of tumor behavior.

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*Cancer Res* 1990;50:7912-7914.

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