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Prognostic value of static cytometry in transitional cell carcinoma of the bladder: Recurrence rate and survival in a group of patients at 10 years follow-up

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Abstract. Many studies have indicated that nuclear DNA content evaluation can be used to predict biological behavior of transitional cell carcinoma (TCC) of the bladder. Some authors also indicated that static cytometry is more useful in DNA content analysis than flow cytometry. The aim of the present study was to evaluate the prognostic significance of DNA ploidy in TCC of the bladder, performed by using static cytometry with an image analyzer, and monitoring patients at 10 years follow-up. Thirty-one consecutive patients underwent transurethral or open surgery for bladder tumors, and neoplastic tissue samples taken from each patient were imprinted on glass slides and sent for histopathological and DNA content evaluation. DNA ploidy evaluation was performed using a CAS 200 image analyzer. Nuclear DNA content evaluation was compared to patient follow-up on recurrence, progression or survival performed 10 years after surgery. Pathological evaluation demonstrated the presence of superficial TCC in 23 patients, while 8 had an invasive bladder tumor. Twenty-nine tumor samples were adequate for DNA content measurement, with 13 showing diploid DNA content and 16 with aneuploid DNA content. At 10 years follow-up, all patients with aneuploid DNA content demonstrated a lower survival time (p=0.049) and higher recurrence rate (p=0.0346). A log-rank test demonstrated that stage, grade and nuclear DNA content are the most useful prognostic parameters for predicting the biological behavior of TCC of the bladder. These results confirm that static cytometry is a good and reliable method to evaluate DNA tumor content and considered a useful prognostic parameter for predicting recurrence rate, disease progression or survival in patients affected by bladder tumors.

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Key words: DNA ploidy, transitional cell carcinoma, bladder cancer, recurrence, progression

Introduction

Transitional cell carcinoma of the bladder is a tumor with variable biological potential (1); the recurrence rate is 50% to 70% (2) and as many as 19% of pTa and 34% of pT1 tumors will progress to muscle-invasive features (3). The ability to predict true tumor biological potential could facilitate patient treatment selection and improve the patient survival rate and quality of life.

Various pathological and clinical parameters, such as tumor grade, stage, multifocality, size, rate and pattern of recurrence, association with Cis and other dysplastic urothelial lesions have been associated with a poor clinical outcome (4). Histopathological stage and grade are conventional prognostic factors for predicting biological behavior (5).

To increase understanding of the cellular mechanisms underlying the development of bladder carcinoma and its natural history, many groups of potential molecular markers and quantitative morphometric methods have been described over the years (6). DNA ploidy identification has been proposed (since the late 1970s) as a diagnostic (7) and prognostic method to evaluate the malignant potential in several neoplasms, such as ovarian (8), endometrial (9) or colorectal cancers (10). Moreover, other studies demonstrated that nuclear DNA content evaluation can be used to predict the risk of recurrence and progression of bladder carcinoma (11,12).

DNA content analysis can be performed using either flow cytometry or static cytometry with an image analyzer. Koss *et al* have demontrated that the percentage of aneuploid neoplasms seems to be lower when using flow cytometry instead of static cytometry (13). This can be due to the presence of a high number of non-neoplastic diploid cells (such as lymphocytes) in the sample. Moreover, the prognostic significance of DNA static cytometry has not yet been prospectively studied in large groups of patients or with a long follow-up. The aim of this study was to evaluate the prognostic significance of DNA ploidy in transitional bladder cell carcinoma, using static cytometry with an image analyzer, and compare the results with the recurrence rate, progression and patient survival at 10 years follow-up.

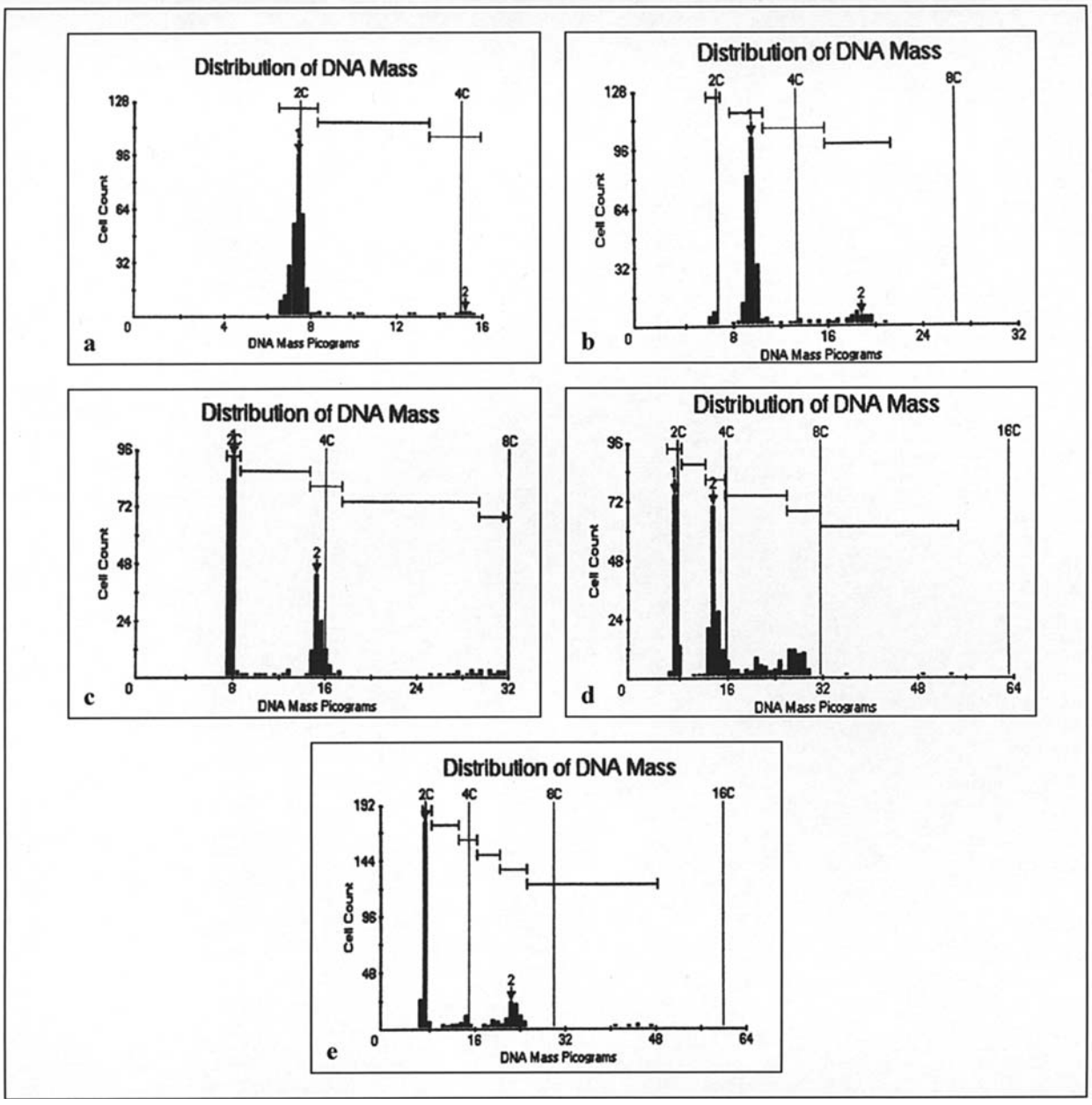

Figure 1. Examples of DNA histograms obtained using CAS 200 image analyzer (Becton-Dickinson) in transitional cell carcinoma of the bladder. (a) Diploid, (b) aneuploid, (c) tetraploid, (d) triploid and (e) hypertetraploid histograms.

Materials and methods

Eligibility criteria. Inclusion criteria were the presence of cystoscopically demonstrated bladder tumors and informed consent by the patients. Exclusion criteria were the presence of other neoplastic diseases, upper urinary tract tumors and lower urinary tract diseases, such as urinary tract infection or ematuria.

Patient characteristics. In January 1994, 31 patients [27 men and 4 women, with a male:female ratio of 6.75:1 with an age range of 44 to 77 years (mean 63.9)], who had undergone transurethral resection (TUR) or open surgery for single or multiple bladder tumors, were consecutively selected for the study. Twenty patients were at the first recurrence, while 11 were at the second or more recurrence. Twelve patients had a single lesion, while 12 had two lesions, 3 had four lesions and 4 had five lesions. Some 23 patients had a lesion diameter ≤3.0 cm, while 8 patients had a lesion diameter >3.0 cm (mean tumor diameter 2.8±0.5 cm). Four patients had Cis associated with the tumor. Of 23 patients with a superficial TCC, 2 were classified as low risk, 15 as intermediate risk and 6 as high risk, according to the EAU Working Group on Oncological Urology classification (14).

Tissue sampling, slide preparation and histopathological examination. All neoplastic tissue samples taken from each patient were imprinted on glass slides at least twice and sent for conventional histopathology. Imprinted tissues were dried, fixed for 10 min in 10% buffered neutral formalin (BNF) at pH 7.2 and briefly washed. The glass slides were sent for DNA content analysis. Histopathological evaluation was performed using the WHO classification (1998) and TNM classification of malignant tumors defined by the

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ummary of clinical and pathological patient data.

a. Patient characteristics

No. of patients	31
Gender	
Male	27
Female	4
Age (years)	
Mean	63.9
Range	44-77

b. Clinical data

Presentation	
First recurrence	20 (64.5%)
Second or more recurrence	11 (35.5%)
No. of lesions	
1	12 (38.7%)
2	12 (38.7%)
4	3 (21.0%)
≥5	5 (1.6%)
Diameter of lesion (if multiple, diameter of the largest)	
≤3 cm	23 (74.2%)
>3 cm	8 (25.8%)
EAU risk classification (sTCC)	
Low risk	2 (8.7%)
Intermediate risk	15 (65.3%)
High risk	6 (26%)
Treatment	
TUR	22 (71.0%)
Radical cystectomy	9 (29.0%)

c. Pathological data

	Stage				
	pTa	pT1	pT3	pT4	Total
Grade					
G1	10	2	-	-	12
G2	4	5	-	-	9
G3	-	2	7	1	10
Total	14	9	7	1	31
Cis	4				

sTCC, superficial transitional cell carcinoma; Cis, associated carcinoma *in situ*; TUR, transurethral resection.

International Union Against Cancer (1997). The anatomopathological analysis of all specimens was performed by the same oncopathologist.

Cell preparation. In accordance with the manufacturer's procedures, the slides were fixed again for 30 min in 10% BNF at pH 7.2 and briefly washed. They were then stained using the CAS-Feulgen-Thionine quantitative DNA staining kit (Becton-Dickinson, San Jose, CA, USA). Commercially available slides of rat hepatocytes, which were stained along with each batch of specimen slides, were used for system calibration.

DNA measurement. Following the steps described by Bacus *et al* (15), DNA cytometry analysis was performed using a CAS 200 image analyzer (Becton-Dickinson) with the following components: 1) a conventional optical microscope optimized for resolution and corrected for shading and stray light. The images were captured using a x40 dry objective, then stored in the computer; 2) a solid state charged image sensor coupled with a digital video-camera (CCD) having a spectral range of 380 to 1100 nm. The potential digital image size was 384 pixels horizontally and 491 pixels vertically. Only 256 x 256 center pixels were used for measurements. 3) measurement processing by means of a PC compatible, Intel 486 processor; and 4) two color display monitors, with one for cell image display and the other for DNA histograms.

The cytometer was calibrated with rat hepatocytes according to the manufacturer's procedures, with granulocytes, lymphocytes and normal urothelial cells always measured as a diploid internal control in each sample.

DNA analysis and test reading and interpretation. For each slide, the cells were identified using an image analyzer, and more than 300 urothelial cell nuclei were selected and analyzed at a wavelength of 560 nm to integrate optical density, which is related to DNA content. Of 31 cases provided, 29 were suitable for measurement. Two cases were inadequate owing to the lack of cells on the slides. The DNA index (DI) and percentages of the G0/G1 cell peak and S and G2/M phases, and coefficient of variation (CV) of the representative peaks were assessed for each case. The diploid region limits were established on the basis of the control cell CV (≤5%). As the CV is equal to the ratio between standard deviation (SD) and the mean value, considering a double SD, the diploid range was between 0.90 and 1.10 and, consequently, the tetraploid range was between 1.80 and 2.20 (16). The limits of the G0/G1 and G2 peaks of the tumor cells were obtained as in the control cells (±10% the modal value of the G0/G1 peak, calculated by means of the software utilized). In this way, it was possible to find the mean value of the DI and the percentage of cells included in the diploid, tetraploid, S-phase and hypertetraploid regions (16).

DNA histograms were classified into two categories: diploid and aneuploid. The aneuploid tumor population was classified into four categories: triploid, multiploid, tetraploid and hypertetraploid. Diploid tumors included those with a G0/G1 peak, which fell within DI values of 1.00±10.0% (Fig. 1a). Aneuploid were considered those presenting a G0/G1 peak with DI values greater than the diploid range (Fig. 1b). Tetraploid tumors were considered a subset of DNA aneuploid tumors, where DI was 1.8-2.2 (Fig. 1c). Triploid were considered those presenting a DI intermediate between diploid and tetraploid limits (Fig. 1d). Hypertetraploid were considered those presenting a DI >2.2 (Fig. 1e). Multiploid tumors presented a histogram with two consistent (>10%) aneuploid peaks or having consistent diploid and tetraploid peaks.

Table II. Summary of DNA content tumor characteristics in 31 patients with bladder tumors, and the association of DNA ploidy with stage and grade.

		P1		P2		P3		P4			
Stage	Grade	C	mDI	C	mDI	C	mDI	C	mDI	DI	Ploidy
pTa	G1	341 (91.7)	1.01	5 (1.34)	1.20	26 (6.99)	2.04			1.01	Diploid
pTa	G1	25 (7.4)	0.98	291 (86.60)	1.90	2 (0.60)	2.46			1.90	Tetraploid
pTa	G1	332 (97.1)	1.01	8 (2.34)	1.27	2 (0.58)	1.89			1.01	Diploid
pTa	G1	286 (89.4)	1.00	7 (2.19)	1.47	27 (8.40)	2.01			1.00	Diploid
pTa	G1	303 (97.8)	1.03	3 (0.98)	1.36	4 (1.29)	2.06			1.03	Diploid
pTa	G1	299 (94.3)	1.00	15 (4.70)	1.22	3 (0.95)	1.97			1.00	Diploid
pTa	G1	299 (96.7)	0.98	3 (0.90)	1.14	7 (2.30)	1.96			0.98	Diploid
pTa	G1	-	-	-	-	-	-	-	-	-	NA
pTa	G1	298 (96.8)	0.98	7 (2.20)	1.98					0.98	Diploid
pTa	G1	224 (63.5)	1.00	38 (10.80)	1.25	46 (13.00)	2.04	16 (4.50)	2.46	1.00+2.04	Multiploid
pTa	G2	304 (96.0)	1.00	3 (0.95)	1.61	10 (3.10)	1.92			1.00	Diploid
pTa	G2	273 (84.2)	1.00	28 (8.60)	1.31	23 (7.10)	2.05			1.00	Diploid
pTa	G2	180 (57.3)	0.98	11 (3.50)	1.44	99 (31.53)	1.92	13 (4.10)	3.82	0.98+1.92	Multiploid
pTa	G2	211 (74.3)	1.03	12 (3.90)	3.13					1.03	Diploid
pT1	G1	242 (76.4)	0.99	9 (2.80)	1.30	35 (11.00)	1.82	19 (5.60)	1.97	0.99+1.82	Multiploid
pT1	G1	302 (95.8)	1.01	6 (2.00)	2.06					1.01	Diploid
pT1	G2	286 (94.1)	1.00	8 (2.60)	1.36	10 (3.30)	2.01			1.00	Diploid
pT1	G2	12 (3.9)	0.95	238 (78.80)	1.46	15 (5.00)	1.93			1.46	Triploid
pT1	G2	273 (86.9)	0.99	13 (4.10)	1.18	23 (7.30)	2.05	5 (1.60)	3.01	0.99	Diploid
pT1	G2	-	-	-	-	-	-	-	-	-	NA
pT1	G2	10 (3.8)	0.99	240 (78.90)	1.44	7 (1.90)	1.92			1.44	Triploid
pT1	G3	60 (17.9)	1.01	6 (1.80)	1.31	224 (67.10)	1.97			1.97	Tetraploid
pT1	G3	118 (38.2)	0.97	3 (0.97)	1.35	100 (32.36)	1.81	17 (4.80)	2.40	0.97+1.81	Multiploid
pT3	G3	43 (14.1)	0.99	46 (15.10)	1.55	181 (59.50)	1.94			1.94	Tetraploid
pT3	G3	90 (28.2)	0.98	3 (0.94)	1.48	128 (40.30)	1.78			1.78	Triploid
pT3	G3	24 (7.5)	1.00	4 (1.25)	1.36	237 (74.00)	1.61			1.61	Triploid
pT3	G3	20 (6.0)	0.99	109 (33.00)	2.05	139 (42.10)	2.45			2.45	Hypertetraploid
pT3	G3	208 (65.2)	1.06	21 (6.60)	1.28	63 (19.80)	1.93			1.06+1.93	Multiploid
pT3	G3	202 (65.6)	0.99	10 (3.30)	1.44	15 (4.90)	1.95	63 (20.45)	3.04	3.04	Hypertetraploid
pT3	G3	237 (76.5)	0.99	21 (4.20)	2.00	12 (4.20)	1.98	42 (12.30)	3.02	3.02	Hypertetraploid
pT4	G3	312 (67.4)	1.00	23 (4.50)	1.37	35 (7.60)	2.00	69 (14.90)	2.81	2.81	Hypertetraploid

P1, first cell peak; P2, second cell peak; P3, third cell peak; P4, fourth cell peak. C, number and percentage of cells within each cell peak. DI, mean DNA Index. With two cell peaks the first (P1) is considered G0/G1 phase, the second (P2) S-G2 phase. With more than two cell peaks, P1 and P2 are considered G0/G1 phase, while P3 and P4 are considered S-G2 phase. NA, not analyzable.

Patient follow-up. For superficial bladder tumors, urine cytology, cystoscopy and complete blood tests were conducted 1 month after the conclusion of the treatment. In tumor-free cases, cystoscopy and urinary cytology were repeated at 3-monthly intervals for the first 2 years, at 6-monthly intervals for the next 3 years and annually thereafter. An ultrasound tomography of the urinary tract was required every 6 months. Recurrence was defined as a positive finding in cystoscopy or bladder biopsy, while progression was defined as an increase in grade or stage of the tumor. The time to first recurrence was defined as the time from surgery until recurrence was detected during cystoscopy or primary cytology, as confirmed by multiple random bladder mucosa biopsies.

For invasive tumors, complete blood tests and urine analysis were performed 1 month after treatment. Serum creatinine and urea, blood gas analysis, sonography of the kidney, liver and retroperitoneum, and a chest-X-ray were repeated at 3-monthly intervals. A CT scan and bone scintigraphy were performed annually.

The follow-up was performed at 10 years (9-11 years). All patients were contacted by telephone, and tumor recurrence, progression and survival were evaluated.

Statistical analysis. Fisher's exact test and the Chi-square test were used to assess the significance of all parameters, with $P<0.05$ accepted as significant. A univariate analysis of

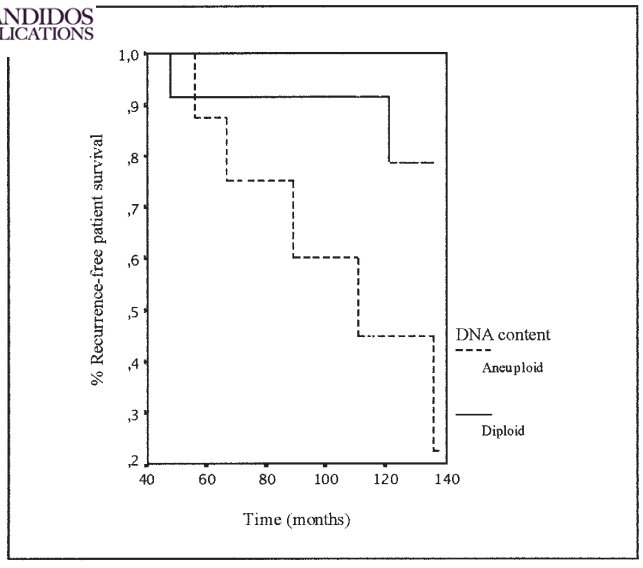

Figure 2. Kaplan-Meier survival curves in sTCC patients (p=0.049). Relationship between recurrence-free patient survival (%) and cause-specific survival in accordance with DNA content.

recurrence/progression was performed using the life-table method (log-rank analysis). Pearson's coefficient was adopted to evaluate the correlation between DNA content and all prognostic parameters in both superficial and invasive bladder cancer patients. Kaplan-Meier survival curves were also used to evaluate survival in superficial bladder cancers. The 95% CIs were calculated for the probability of survival for the Kaplan-Meier estimates. The Mann-Whitney test was also used to compare different parameter mean values. All statistical analyses were performed using SPSS 11.0 for Apple-Macintosh (SPSS, Inc. Chicago, IL, USA).

Results

Twenty-two patients underwent TUR, while 9 patients had a radical cystectomy with non-continent urinary diversion. Histopathological examination demonstrated a superficial TCC in 23 patients (10 pTaG1, 4 pTaG2, 2 pT1G1, 5 pT1G2, and 2 pT1G3), while 8 had an invasive TCC (7 pT3G3 and 1 pT4G3). Follow-up ranged from 110-136 months (mean ± SD, 127.20±4.92) (Table I).

The mean of slides analyzed was 2.77 (range, 1 to 6) for each patient. In 29 out of 31 patients, it was technically possible to perform adequate DNA content measurements, while in 2/31 (pTaG1-pT1G2) there was no tumor DNA analysis due to technical problems or an insufficient number of tumor cells on the slides. All patients had a complete clinical follow-up and were available for the study.

The mean DNA content (DNA index) of all tumor samples ranged from 0.98 to 3.04 (mean 1.50±0.63) when compared to granulocytes, lymphocytes and normal urothelial cells. Thirteen out of 29 patients (44.8%) showed normal DNA content (ranging from 0.98 to 1.03, mean 1.00±0.01) and were classified as diploid. Sixteen patients (55.2%) showed DNA content ranging from 1.40 to 3.04 (mean 1.91±0.58) and were classified as aneuploid.

The aneuploid tumor population showed 4 triploid tumors (mean 1.57±0.15), 3 tetraploid tumors (mean 1.93±0.03), 6 multiploid tumors (multiple stemline DNA distribution) (first peak mean of 1.00±0.03 and second peak mean of 1.90±0.09), and 3 hypertetraploid tumors (mean 2.83±0.27) (Table II). Stratification according to grade showed that 8 out of 11 G1 presented normal DNA content, while 2 presented multiploid DNA content and 1 tetraploid with DNA content. Of 8 G2 tumors, 5 were classified as diploid, 2 as triploid and 1 as multiploid. Among G3 tumors, no case presented normal DNA content; 2 were multiploid, 2 tetraploid, 2 triploid and 4 hypertetraploid.

A statistically significant correlation was reported between grade and DNA content ($r=0.658$, $p\leq0.0001$). Stratification according to stage showed that 10 out of 13 pTa presented normal DNA content, while 2 presented multiploid and 1 presented tetraploid DNA content. Of 8 pT1 cases, 3 were classified as diploid, 2 as multiploid, 2 as triploid and 1 as tetraploid. No pT3 presented normal DNA content; 2 were triploid, 1 multiploid, 1 tetraploid and 3 hypertetraploid. The pT4 presented hypertetraploid DNA content.

A strong correlation was reported between stage and DNA content ($r=0.767$, $p\leq0.0001$). No significant correlation between DNA content and other prognostic parameters (such as multiplicity, size or associated Cis) was reported.

The follow-up showed that 5 out of 13 patients with diploid DNA content were alive with no evidence of disease (NED) at a mean time of 131.8 months (range, 118 to 138 months) (4 pTaG1 and 1 pTaG2), 4 out of 13 (3 pTaG1 and 1pTaG2) were alive with NED and a previous single or multiple recurrence(s) invariably treated by TUR (with no progression in stage and grade) at a mean time of 116.5 months (range, 110 to 124 months), 2 out of 13 died due to unrelated causes and 2 out of 13 died of disease progression (1 pT1G1 and 1 pT1G2) at 121 and 48 months. Of 16 patients with aneuploid DNA content, 13 died of disease progression at a mean time of 54 months (range, 18 to 136 months).

One out of 16 patients died due to unrelated causes at 72 months, while 2 out of 16 were alive with NED and a previous single or multiple recurrence(s) invariably treated by TUR (with no progression in stage and grade) at 115 and 138 months (pT1G1 and pTaG1). DNA content analysis in the aneuploid tumor population showed that all of those with triploid tumors died at a mean time of 70.25 months (range, 36 to 111 months) (2pT1G2 and 2pT3G3), 2 out of 3 tetraploid tumors died of disease progression at 18 and 89 months (pT1G3 and pT3G3), all hypertetraploid patients died of disease progression at a mean time of 24.75 months (range, 21 to 31 months) (3pT3G3 and pT4G3) and 3 out of 5 multiploid tumor patients died of disease progression at 23, 56 and 136 months respectively (pTaG2, pT1G3, and pT3G3).

The survival analysis, performed with Kaplan-Meier curves, demonstrated a statistically significant difference between patients with normal DNA content (DI 0.90-1.10) and those with aneuploid DNA content (DI >1.10) in superficial TCC ($p=0.049$) (Fig. 2). No significant difference between patients with a normal DNA content and those with aneuploid DNA content was reported in invasive TCC. Four out of 13 diploid patients presented a mean recurrence rate of 5.8 months (range, 1 to 9 months) with a mean time of 7.8 months at first recurrence (range, 5 to 13 months). Two out

Table III. Univariate analysis of factors affecting recurrence-free survival.

Categories (variable)	No. of patients	Recurrence-free patients		p-value
		n	%	
Stage				<0.0001
pTa	14	10	71.42	
pT1	9	1	11.11	
pT3	7	0	0	
pT4	1	0	0	
Grade				<0.0001
G1	12	9	75.00	
G2	9	2	22.22	
G3	10	0	0	
No. of previous recurrences				0.3886
1	20	5	25.00	
≥ 2	11	6	54.54	
Diameter				0.4599
≤3 cm	23	9	39.13	
>3 cm	8	2	25.00	
No. of lesions				0.7893
1	12	1	8.33	
2	12	4	33.33	
4	3	3	100.00	
5	4	3	75.00	
DNA ploidy				0.0006
Diploid	13	9	69.23	
Aneuploid	16	2	12.50	

of 16 aneuploid patients presented a recurrence rate of 5 and 7 months, with the first recurrence at 3 and 5 months. The DNA content status showed a statistically significant correlation with recurrence (p=0.0346).

Univariate analysis, performed with a log-rank test, demonstrated that stage, grade and DNA content are the most useful prognostic parameters for predicting the biological behavior of transitional cell carcinoma of the bladder (Table III).

Discussion

DNA tumor content evaluation is considered a good prognostic factor in transitional cell carcinoma (17,18). However, it is rarely used due to technical difficulties, either in sample preparation and analysis or in choosing the right method to evaluate the samples.

Flow cytometry results are considered to be less reliable compared with static cytometry, due to the presence of non-neoplastic cells (lymphocytes) in the cellular suspension used for tumor DNA evaluation (19). On the other hand, static cytometry allows direct evaluation of tumor cell selection of at least 300 or 400 tumor cell nuclei.

In the present study, we evaluated 29 patients affected by transitional cell carcinoma of the bladder, comparing the mean DNA content of their tumors with recurrence or progression of disease at 10 years follow-up. Although the prognosis of patients with a diploid DNA content was better than those with aneuploid content, we found a strong correlation between grade (r=0.658, p<0.0001) and stage (r=0.767, p<0.0001).

Superficial and/or low grade tumors presented a prevalent diploid DNA content, while the invasive or high grade ones were mainly aneuploid. Nevertheless 2 out 16 aneuploid patients were alive with no evidence of disease. Their tumor stages were T1G1 and TaG1, respectively. In these cases, pathological tumor stage and grade appear to be stronger prognostic factors than tumor DNA content evaluation. These results are comparable with those reported in other studies (20). Joachim-Liossi *et al* demonstrated a strong correlation between recurrence status and DNA ploidy (p<0.001) in 80 patients with superficial transitional cell carcinoma of the bladder (17).

On the other hand, no correlation was found between DNA content and other prognostic parameters, such as multifocality, tumor size or associated carcinoma *in situ*. In 1994, Al-Abadi *et al* demonstrated a statistically significant correlation between tumor ploidy and disease recurrence or progression. They found that tumor DNA content was the strongest predictor of patient survival (21). The patients affected by superficial tumors evaluated in our study presented a significant impact of tumor DNA content both on the survival and recurrence rate at 10 years follow-up (p=0.048 and p=0.0346 respectively). These data were confirmed by univariate analysis.

In conclusion, static cytometry is a good and reliable method to evaluate DNA tumor content in transitional cell carcinoma. It is a useful prognostic parameter for predicting the recurrence rate in superficial tumors and disease progression or survival in patients affected by invasive tumors. However, the method is time-consuming and requires adequate instrumentation and funding.

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