Biological characterisation of superficial bladder cancer by bivariate cytokeratin 7/DNA analysis, flow cytometric assessment of MIB-1, and an immunohistochemical study

Elena Leonardi [a,*], Paolo Dalla Palma [a], Alessandro Reich [b], Orazio Caffo [c] and Lucio Luciani [b]

[a] *Department of Pathology, St. Chiara Hospital, Trento, Italy*
[b] *Department of Urology, St. Chiara Hospital, Trento, Italy*
[c] *Department of Oncology, St. Chiara Hospital, Trento, Italy*

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A total of 238 cases of bladder carcinoma stages Ta, Tis, T1 were submitted prospectively to multiparameter flow cytometry and immunohistochemical study in order to determine the biological aggressiveness of the tumour. DNA index (DI), S-phase fraction (SPF) obtained by bivariate cytokeratin 7/DNA analyses, and the immunohistochemical evaluation of p53 and MIB-1 were studied in relation to the traditional prognostic factors in bladder cancer (stage and grade). The variance analysis results showed that DNA aneuploidy was significantly associated with high stage ($p = 0.0001$), high grade ($p = 0.0001$), high SPF value $\geqslant$5.5% ($p = 0.0001$), MIB-1 positivity $\geqslant$31% ($p = 0.0001$) and high expression of p53 (staining involving >50% of cells, $p = 0.0001$). Even if there was no statistical significance the hypotetraploid class ($1.70 < \text{DI} < 1.89$) showed poor prognostic biomarkers more frequently than the other aneuploid classes. Out of 238 cases, 101 were also submitted to flow cytometric measurement of MIB-1 (fMIB-1) to study the correlation between cell proliferation and DNA content. Data obtained from fresh, 3 : 1 methanol/acetone fixed samples were compared with values obtained from both cell cycle analysis methods and routine application of the MIB-1 immunostaining in histological sections. fMIB-1 values were positively correlated with SPF values ($r = 0.801$, $p < 0.01$) and S+G2M fraction (percentage of cells in S and in G2M phases) ($r = 0.763$, $p < 0.01$) but no correlation with paraffin sections was found. A fMIB-1 value >7% was strongly associated with aneuploidy ($p = 0.0001$). The determination of DNA content coupled with the study of the epithelial (cytokeratin 7) and proliferative (MIB-1) markers could be useful in providing important information on the biological behaviour of superficial bladder tumours.

Keywords: DNA content, bladder cancer, p53, cell proliferation, MIB-1

1. Introduction

Superficial transitional cell carcinoma of the bladder represents a disease entity characterised by a wide variation in its natural course and prognosis. Alongside relatively benign non invasive tumours some neoplasms show a highly aggressive attitude because of their high biological potential to progress and metastatize. Often an unpredictable evolution occurs although the parameters in general use today such as T-classification and histological grade, support a low malignant potential [11,37].

In order to improve the prognostic value of the traditional indicators and a better monitoring of patients after therapy, flow cytometric procedures have been introduced as an objective and measurable adjunct to clinical morphology. Flow cytometric analyses give

*Corresponding author: Elena Leonardi, Department of Pathology, Ospedale S. Chiara, via Crosina Sartori 6, Trento, 38100, Italy. Tel.: +39 461 903303; Fax: +39 461 903389; E-mail: leonardi@tn.aziendasanitaria.trentino.it.

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additional information with regard to cellular heterogeneity, state of tumour proliferation and predictive response to therapy. In particular DNA status [2,16, 19,33,39–42] and proliferative activity estimation [3, 6,14,22,36] are reliable prognostic indicators in bladder cancer. A bivariate approach based on the staining of the epithelial component of the tumour with low molecular weight cytokeratin is more accurate in determining DNA content particularly in cases where the inflammatory component is most relevant [18,43]. Immunohistochemical determination of mutational inactivation of the p53 tumour-suppressor gene can also provide useful information about bladder cancer behaviour. Previous studies on p53 protein demonstrated its fundamental role in determining the aggressive clinical outcome of the superficial disease [12,20,21,26,28, 30,31]. Quantification of the "tumour growth fraction", the percentage of cycling cells, represents another of the major objectives in tumour prognosis and diagnosis [1,4,15,25,29,38,45]. Among the several methods in use today [7] tumour cell proliferation is currently estimated either from S-phase values obtained by applying mathematical models to DNA histograms or by quantifying, on tumour sections, the proliferation associated molecules such as Ki67, MIB-1, PCNA with immunohistochemical procedures. In this study we performed a flow cytometric MIB-1 analysis on fresh tumour samples to assess the expression of this antigen usually considered as a valuable marker in superficial bladder cancer [3,22,23]. In order to verify the feasibility and usefulness of this approach, flow cytometric MIB-1 data were compared with data obtained from both cell cycle analysis methods and routine application of the immunostaining in histological sections.

The aim of our prospective study was to investigate the behaviour of superficial bladder cancer by characterising biologically tumour tissue with a number of markers which include DNA content, S-phase fraction obtained by flow cytometric analysis of DNA content with cytokeratin 7, an antigen expressed in urothelial cells, flow cytometric measurement of MIB-1 in fresh tumour tissue and the immunohistochemical expression of p53 and MIB-1 in routine parallel sections of the neoplasms. We also attempted to study the relation among distinct ploidy classes, obtained by subgrouping the DNA Index (DI) values, with the strongest expression of unfavourable markers (high MIB-1 and p53 values).

Data obtained from flow cytometric and immunohistochemical approaches were compared with established prognostic factors (stage and grade) in order to assess their potential usefulness as prognostic markers in superficial bladder cancer.

2. Materials and methods

2.1. Patients

The study population consisted of 238 patients with histologically proven superficial transitional cell carcinoma of the bladder (stage Ta Tis T1) collected prospectively in the Pathology Department of S. Chiara Hospital (Trento, Italy) during the period 1995–1997 (Table 1). The diagnostic samples were obtained from cold transurethral resections biopsies and examined fresh in order to perform the flow cytometric procedures.

There were 199 men (84%) with a mean age of 65.1 years (range 27–89 years; STD = 11.4) and 39 women (16%) with a mean age of 66.7 years (range 20–90 years, STD = 13.3). Criteria for inclusion into the study were: patients with a diagnosis of bladder cancer, data available on T-classification and histological grade. Clinical characteristics of the study population are summarised in Table 1.

2.2. Sample preparation for flow cytometry

The fresh surgical specimens were immediately mechanically disaggregated with a scalpel in PBS (Cell dissociation solution non enzymatic, cat. n° C-1544, Sigma St. Louis, MO, USA). Cell suspensions were filtered through a 50 μm nylon mesh, centrifuged at 600 rpm and stored at -80°C in a propylen tube containing 1 ml of cryopreservative solution composed of fetal bovine serum (cat. n° F-3018, Sigma, St. Louis, MO, USA), 10% and dimethylsulfoxide (cat. n° D-5879, Sigma, St. Louis, MO, USA) 10% in RPMI 1640 Medium (cat. n° R-8758, Sigma, St. Louis, MO, USA).

Table 1
Clinico-pathologic characteristics of the study population

Total number of patients	238
Clinico-pathologic stage	
Ta	209 (88%)
Tis	9 (4%)
T1*	20 (8%)
Grading	
G1	80 (34%)
G2	119 (50%)
G3	39 (16%)

*Out of 20 T1 tumours 6 cases showed a carcinoma *in situ* in the same or adjacent biopsies.
Stage was assigned according to the TNM classification; grading was assigned according to Bloom and Richardson.

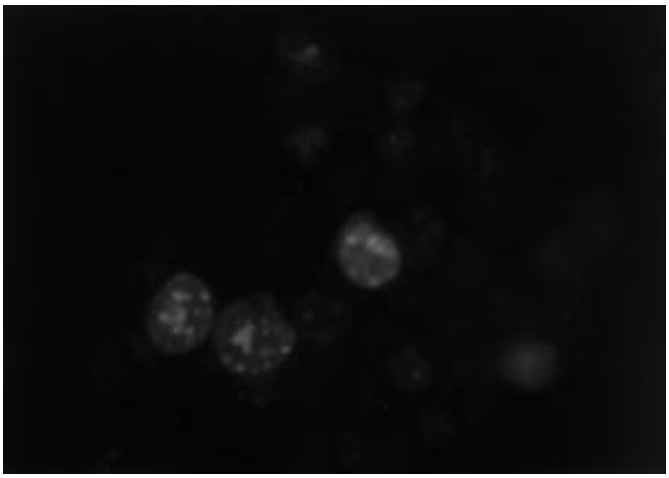

Fig. 1. Dot-like intranuclear staining of MIB-1 in a single-cell suspension prepared from a superficial transitional cell carcinoma of the bladder, grade 2 (original magnification $\times 1000$). Cells were fixed with methanol/acetone and stained with an indirect method of immunofluorescence.

At the time of analysis, tubes were rapidly thawed at 37°C and centrifuged at 600 rpm for 10 min. An indirect immunofluorescent staining was applied on pellets previously fixed for nuclear antigen (MIB-1) and cytoplasmatic antigen (cytokeratin 7).

2.2.1. MIB-1 immunostaining

Pellets were fixed for 20 minutes with 1 ml of a solution composed of absolute methanol and acetone (3 parts of methanol and 1 part of acetone) previously refrigerated at −20°C. After a brief centrifugation the pellets were resuspended within the washing solution (normal goat serum and phosphate buffer 1 : 10) and centrifuged. The pellets were divided in two aliquots and respectively incubated with MIB-1 (MIB-1, Mouse IgG1, cat. n° M-520, Medac Diagnostika, Valter Occhiena) and mouse isotypic IgG (Mouse IgG1 Kappa, MOPC-21, cat. n° M-5284 Sigma, St. Louis, MO) dilutions 1 : 10 for 1 h at room temperature. After washing the pellets were incubated with goat anti-mouse IgG Fab specific FITC conjugate (anti-mouse IgG cat. n° F-2653 Sigma, St. Louis, MO) dilution 1 : 100, for 30 minutes at room temperature in the dark.

At the end of this step the washed pellets were stained with 5 gamma propidium iodide with RNase and Nonidet –P40 overnight at 4°C.

Before flow cytometric analysis aliquots of stained cell suspensions were smeared and examined with fluorescent microscope to verify the MIB-1 labelled nuclei. The presence of a dot-like (Fig. 1) or an homogeneous diffuse intranuclear staining was accounted to positive expression of MIB-1.

2.2.2. Cytokeratin 7 immunostaining

Pellets were fixed with cold 70% ethanol for 30 minutes at 4°C. After centrifugation pellets were washed twice in a washing solution composed of 5% fetal bovine serum, 0.1% albumin bovine (cat. n° A-2153, Sigma, St. Louis, MO) in PBS.

The first aliquot was incubated 1 h at 4°C with a monoclonal antibody against cytokeratin 7 (KER 7, cat. n° M-7018 DAKO) diluted 1 : 20 in PBS. The pellet for negative control was incubated for 1 hour at 4°C with a non specific isotypic mouse IgG1 (Mouse IgG1 Kappa, MOPC-21, cat. n° M-5284 Sigma, St. Louis, MO) in the same experimental conditions as the test sample. At the end of the incubation time, cell suspensions were washed in 1 ml of washing solution that had been added to each tube and mixed.

Tubes were immediately centrifuged at 600 rpm for 10 minutes. Cell pellets were incubated with rabbit anti-mouse IgG FITC (cat. n° F0313, DAKO, Glostrup, Denmark) diluted 1 : 20 for 30 minutes and finally stained with 5 gamma of propidium iodide (PI) solution overnight.

In order to evaluate the presence of cytokeratin-positive cells an aliquot of cell suspension was smeared and examined by fluorescent microscope.

2.3. Flow cytometric analysis

FCM analysis was performed on a FACS can flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA), equipped with a 15 mW argon laser (488 nm) and doublet discrimination module. Cell FIT Software Package was used for instru-

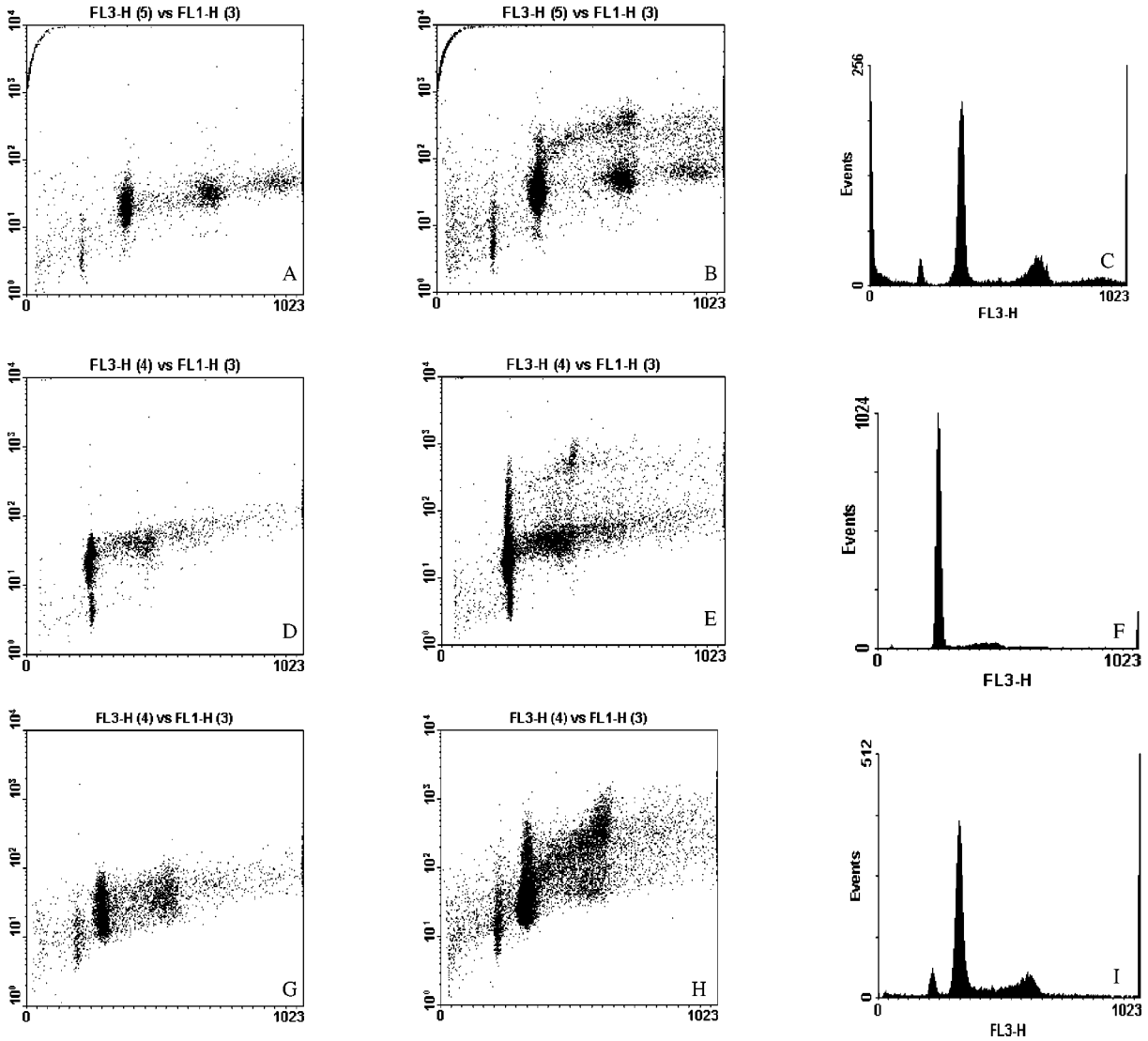

Fig. 2. Examples of bivariate MIB-1/DNA FCM analyses. Dot-plots relate to MIB-1 expression (FL1-H) versus DNA content (FL3-H). Figures A, B, C refer to isotypic controls; D, E, F to test sample with positivity for MIB-1 of 11, 8 and 28%, respectively; G, H, I to DNA histograms with DIs = 1.77; 1; 1.51, respectively.

ment set-up, data acquisition and histogram deconvolution. Instrument set-up and calibration were performed using a propidium iodide stained diploid normal tissue. For MIB-1/DNA samples almost 15,000 events were recorded. For cytokeratin 7/DNA samples at least 20,000 events were recorded in list mode data using 1024 channels.

2.3.1. Gating methods

To assess the MIB-1 antigen expression a threshold level of fluorescence for the negative control was empirically fixed allowing not more than 1% of positive cells in the control sample. All the events falling above this cutoff are considered positive for the expression of MIB-1 (Fig. 2).

In order to calculate the percentage of the positive events WinMdi software was used (Win MDI Version 2.1.4).

A population with positive staining for cytokeratin 7 was identified by comparing FL3-H and FL1-H dot plots of the tested sample to the negative control. Samples were considered adequately labelled only if a distinct visually separation in green fluorescence intensity (FL1-H histogram) was present.

2.3.2. Histogram interpretation

Cell cycle analysis was calculated by rectangular curve fitting (RFIT, Becton Dickinson, CellFIT Software) for diploid tumour and by polynomial model (POLY Model) when the sample contained two cell populations with different DNA contents. Histograms including a single G0/G1 peak were considered DNA diploid (DI = 1.00).When more than one G0/G1 peak was present, histograms were classified as follows: DNA hypodiploid (DI $<$ 1.00), DNA near-diploid ($1.00 < \text{DI} < 1.39$), DNA near-triploid ($1.40 < \text{DI} < 1.69$), DNA hypotetraploid ($1.70 < \text{DI} < 1.89$), DNA tetraploid ($1.90 < \text{DI} \leqslant 2.10$), DNA hypertetraploid ($\text{DI} > 2.10$), DNA multiploid when more than one aneuploid population was found.

Tumours were classified as tetraploid only when a corresponding G2/M peak in the 8C region was identifiable. Histograms were considered reliable when CV (coefficient of variation) of the G0/G1 diploid peak was lower than 3.5%. SPF values obtained by biparametric procedure were divided in tertiles: low ($\text{S} \leqslant 1.90\%$), medium ($1.91\% < \text{S} < 5.5\%$), high ($\text{S} \geqslant 5.5\%$).

In order to identify the ploidy class in which a higher frequency of cases with high cellular proliferation (MIB-1 $\geqslant$ 31%, SPF $\geqslant$ 5.5%) and high expression of p53 (score 2) occured, a subdivision of the cases in six ploidy classes was performed as follows: DNA diploid and peridiploid ($0.90 < \text{DI} < 1.39$), DNA near-triploid ($1.4 < \text{DI} < 1.69$), DNA hypotetraploid ($1.70 < \text{DI} < 1.89$), DNA tetraploid ($1.90 < \text{DI} < 2.10$), DNA hypertetraploid ($\text{DI} > 2.10$), multiclonal (two or more clones).

2.4. Statistical analysis

The differences between diploid and aneuploid cases in terms of stage, grading, MIB-1 value, p53 expression and SPF values were assessed using the one way analysis of variance (ANOVA test). Similarly, after stratifying the DNA index into six classes, the differences in terms of MIB-1 value, p53 expression and SPF values were analysed by ANOVA test; a post hoc multiple comparison test was based on both Duncan's multiple range test and the Sidak's test. These tests allowed us to compare each pairs of means stratified by ploidy classes. The statistical analysis was performed by SPSS software package version 8.0 for Windows.

3. Immunohistochemistry

Formalin fixed, paraffin embedded tissues were cut in sections 5 μm thick. Antigen retrieval was performed using an 800 W microwave oven at full power in citrate buffer pH 6.0 for two cycles 10 minutes each [35]. Slides were incubated overnight at 4°C with the following antibodies: MIB-1 (MIB-1, Mouse IgG1, cat. n° M-520, Medac Diagnostika, Valter Occhiena), p53 (clone DO7, DBA, Milano, Italy) dilution 1 : 1000. Biotinylated anti-mouse IgG and streptavidin–biotin enhanced immunoperoxidase technique (StrepABComplex/HRP, Duet, Mouse/Rabbit, DAKO, Glostrup, Denmark) were applied in sequence. The immunostaining for MIB-1 was evaluated on the basis of the percentage of stained nuclei counting at least 500 cells in more than 10 high power (1000×) representative fields. In cases where intratumoral heterogeneity of staining was seen, examined fields included those with the highest and the lowest percentage of stained cells (Fig. 3). Percentile expression of MIB-1 in neoplastic cells was divided in tertiles: low ($\leqslant$10%), medium (11–30%), high ($\geqslant$31%).

Quantification of nuclear p53 immunostaining was estimated analysing 10 consecutive 1000× microscopic fields (100× objective and 10× oculars) containing malignant cells.

A bladder cancer biopsy specimen known to contain a mutational inactivation of p53, as detected by DNA sequence analysis, and showing an intense positivity for p53 was used as positive control. As negative control each tumour was incubated with nonimmune mouse immunoglobulin instead of the primary antibody. All slides were reviewed independently in a blinded fashion by two of us (E.L. and P.D.P). The immunohistochemical positivity for p53 was scored by using an arbitrary system as follows: score 0, p53 negative carcinomas;

score 1: focal darkly staining areas or moderate staining of $<$50% of cells; score 2: dark nuclear staining that involves $>$50% of cells (Fig. 4).

P53 was evaluated in this way following the suggestion of a previous report [26] in which positive immunohistochemical staining for p53 represented a good indication of mutation in the p53 gene if the designation of positive immunostaining was limited to the cases where more than one-half tumour cells were stained instead of occuring as isolated single cells or in small clusters.

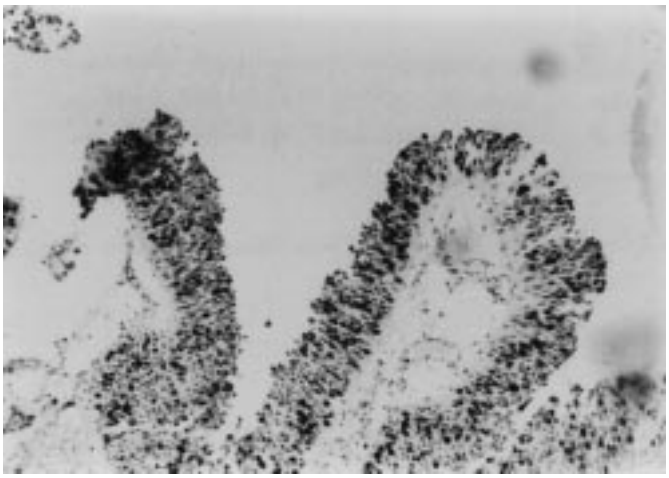

Fig. 3. Immunoreactivity for MIB-1 in transitional cell carcinoma of the bladder, Ta grade 2 (original magnification ×250).

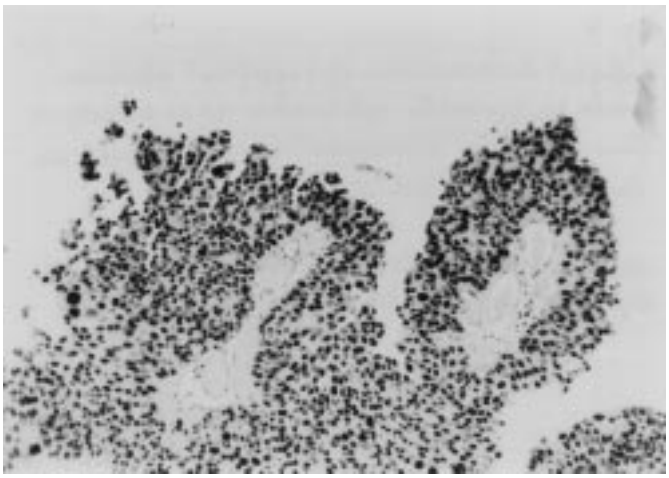

Fig. 4. Expression of p53 in a transitional cell carcinoma of the bladder, Tis, grade 3 (original magnification ×250). Immunoreactivity is present in most of the neoplastic cells.

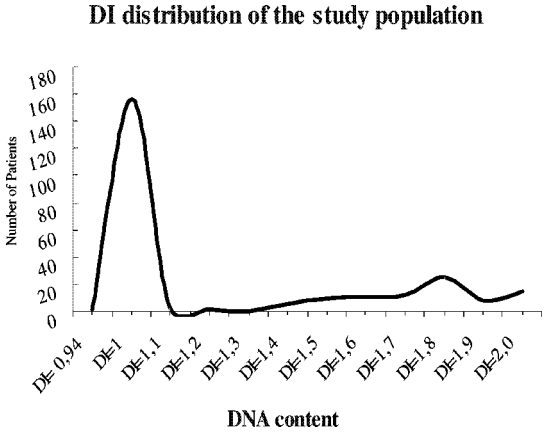

Fig. 5. Distribution of DIs of the study population.

4. Results

Out of 238 cases of superficial transitional cell carcinoma of the bladder diploidy was present in 156 cases (66%) and aneuploidy in 82 cases (34%).

Distribution of DIs is shown in Fig. 5. Application of bivariate cytokeratin 7/DNA analysis was useful to determine more accurately DNA abnormalities and SPF values without the presence of a contaminanting population such as inflammatory cells and debris which increase the risk of underestimating the S-phase value. In particular the bivariate approach was most useful in cases where a possible misinterpretation of DNA histograms may occur as in hypodiploid or multiclonal cases (Fig. 6).

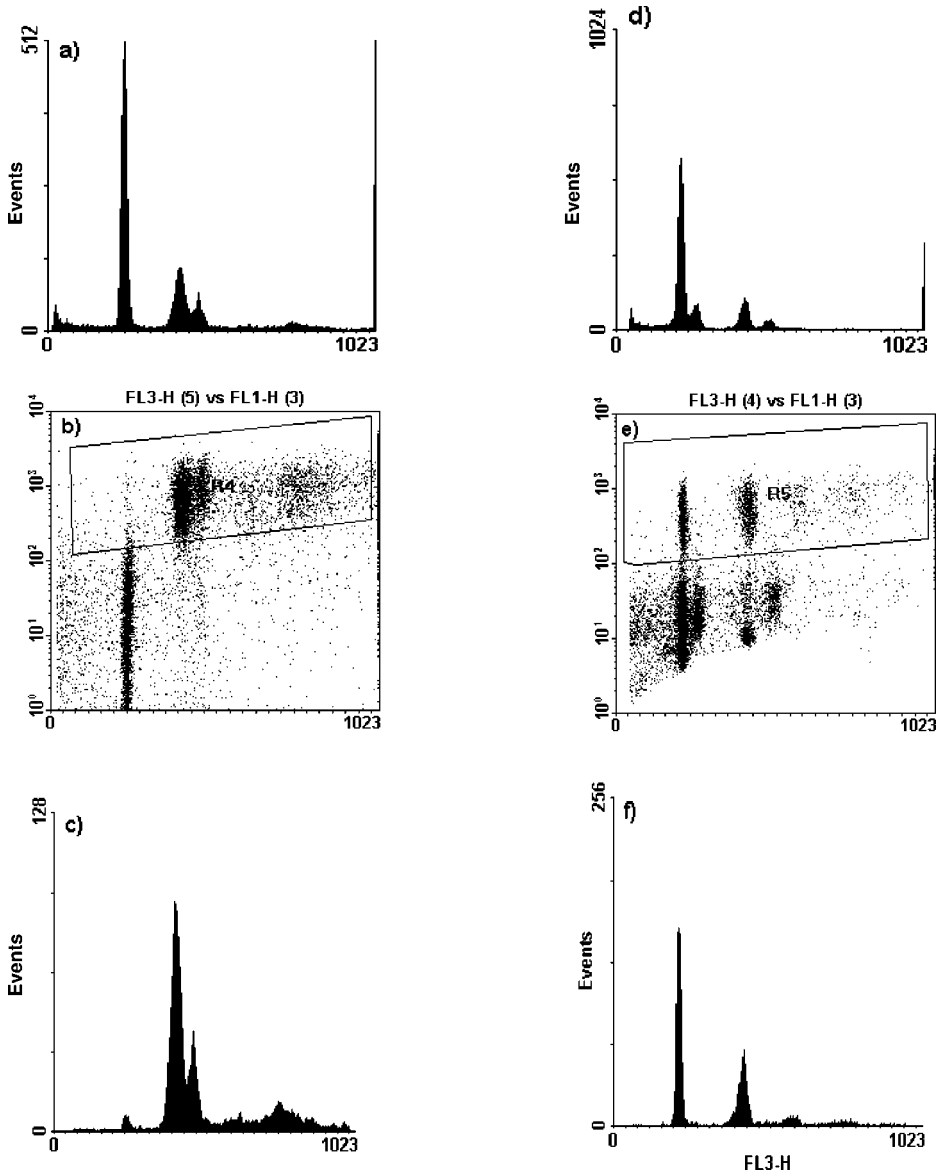

Fig. 6. Examples of bivariate CK7/DNA FCM analyses in superficial transitional cell carcinoma. Ungated DNA histograms (a, d), dot-plots (b, e) and DNA histograms after gating with cytokeratin 7 (c, f); (a) DI = 1.67 + 1.90; (d) DI = 0.88 CK-labelled cells were gated to generate histogram (c) and (f) from the epithelial component only.

The SPF percentages were achieved in 222 cases. It was not possible to determine the SPF value in 16 cases due to inadequate fitting of the mathematical models because no distinct G2+M peak for the reference population was visible. A strong correlation between DNA aneuploidy and staging,grading, SPF value $\geqslant$5.5%, MIB-1 $\geqslant$ 31% and high expression of p53 (score 2) was found (Table 2).

The post hoc analysis performed by Duncan's test and Sidak's test failed to demonstrate any statistically significant difference among the ploidy classes: the distribution of cases according to MIB-1 values, SPF values and p53 expression is shown in Fig. 7.

Flow cytometric measurement of MIB-1 (fMIB-1) on fresh tissue was evaluated in 101 cases. Not enough quantity of cellular suspension was available for immunostaining in the remaining cases. Preliminary experiments were performed to determine the method of choice to preserve MIB-1 antigen such as paraformaldehyde, ethanol, etc. but without improve-

Table 2
Correlation of DNA ploidy by biparametric DNA/cytokeratin 7 flow cytometry versus clinico-morphologic and biologic parameters

	Number	DNA diploid	DNA aneuploid	
pT				
pTa	209	153 (73%)	56 (27%)	
pTis	9	0 (0%)	9 (100%)	
pT1	20	3 (15%)	17 (85%)	$p = 0.0001$
	238			
Grade				
G1	80	77 (96%)	3 (4%)	
G2	119	79 (66%)	40 (34%)	
G3	39	0 (0%)	39 (100%)	$p = 0.0001$
	238			
S-Phase fraction(%)*				
S$\leqslant$1.90	75	75 (100%)	0 (0%)	
1.91$\leqslant$S$<$5.5	72	63 (88%)	9 (12%)	
S$\geqslant$5.5	75	13 (17%)	62 (83%)	$p = 0.0001$
	222			
MIB-1(%)				
$\leqslant$10	86	79 (92%)	7 (8%)	
11–30	86	65 (76%)	21 (24%)	
$\geqslant$31	66	12 (18%)	54 (82%)	$p = 0.0001$
	238			
p53				
p53 0	154	138 (90%)	16 (10%)	
p53 1	32	12 (38%)	20 (62%)	
p53 2	52	6 (12%)	46 (88%)	$p = 0.0001$
	238			

* It was not possible to determine S-phase values in 16 cases due to inadequate fitting of mathematical models.
pT: assigned according to the TNM classification; grade: histological differentiation according to Bloom and Richardson; MIB-1: percentile immunohistochemical expression of MIB-1 in neoplastic cells; SPF: fraction of cells in S phase evaluated by flow cytometry; p53: 0,1,2 immunohistochemical reactivity for p53 (score 0: no positive cells; score 1: positivity involves $<$50% of cells; score 2: positivity cells involves $>$50%).

ments. In all cases a good distinction between negative and positive cells was obtained (Fig. 2).

The tertiles of fMIB-1 were divided as follows: low fMIB1: $\leqslant$3%, medium fMIB-1: 4–7%, high fMIB-1: $>$7% The relation between ploidy and tertiles of fMIB-1 is represented in Fig. 8.

Most diploid cases exhibited a low fMIB-1 value, while aneuploid cases showed high fMIB-1 values ($>$7%, $p = 0.0001$).

The distribution of fMIB-1 values was as follows: in the lowest tertile diploid (24 cases) and tetraploid (4 cases); in the medium tertile diploid (15 cases) and hypotetraploid (9 cases), in the higher tertile diploid (4 cases), near-triploid (12 cases), hypotetraploid (16 cases), tetraploid (5 cases), hypertetraploid (2 cases) multiclonal (8 cases), hypodiploid (2 cases). MIB-1 labeling indices were matched with SPF and S+G2M values by correlation method. The flow MIB-1 values were positively correlated with S-phase values ($r = 0.801$, $p < 0.01$) and S+G2M ($r = 0.763$, $p < 0.01$) (Fig. 9). The Pearson's correlation coefficient was significant at 0.01 level. No correlation between flow MIB-1 values and MIB-1 data from paraffin sections were obtained.

5. Discussion

Tumour progression in human solid tumours is currently believed to result from genetic instability and consequent acquisition of new genetic properties in some of the tumour cells. However, not all DNA

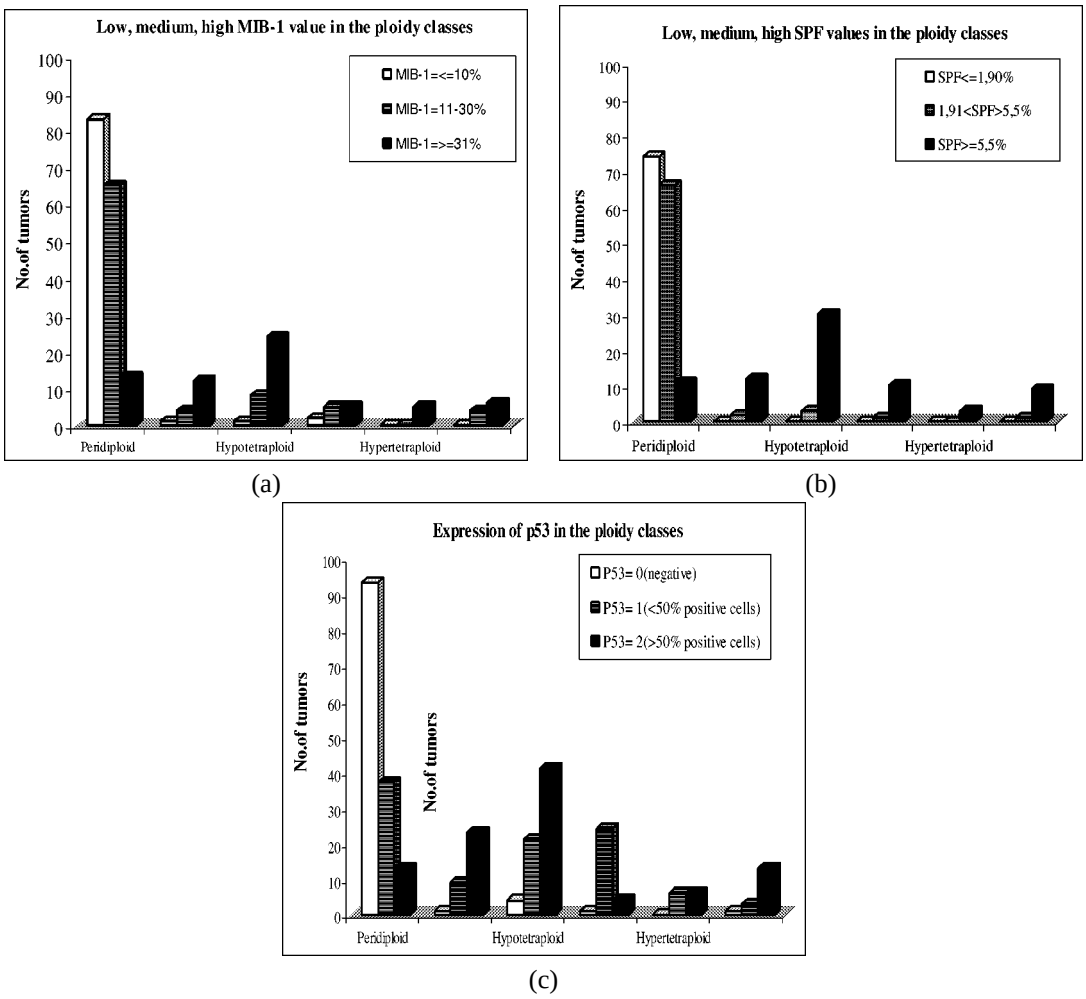

Fig. 7. Bar histograms showing the relation existing between ploidy classes and MIB-1 (a), SPF values (b) and expression of p53 (c). The hypotetraploid class was the one showing the highest percentage of cases with poor prognostic biomarkers.

changes are biologically functional for tumour progression [5]. Accumulation of specific genetic abnormalities in the same tumour cells such as DNA aneuploidy, loss of function of many suppressor genes or oncogenes activation [34] give the tumour a high malignant potential. An increased cellular proliferation may also be seen as a decisive step for tumour progression [27]. Previous studies in bladder cancer showed that aneuploidy and in particular DNA hypertetraploidy and DNA multiploidy represent markers of poor outcome [33]. Moreover, p53 abnormalities [12,20,21,26,28,30,31] and an increase in cellular proliferation [22,23] were reported to play a key role in the transition from pre-invasive to invasive disease. Among the available methods which estimate cellular proliferation those based on the determination of the proliferation-associated markers are the more frequently employed. The antigen known as MIB-1 has been proposed as the indicator of choice for tumour cell proliferation studies. Some reports showed that in bladder cancer immunohistochemical expressions of 15% [23] and 25% [44] of MIB-1 were considered as cut-off values to distinguish between tumours with favourable course and tumours with an aggressive disease in terms of recurrence or progression to invasive cancer. Moreover by using immunohistochemical procedures it is possible to evaluate not only the quantification of this antigen but also its expression pattern (basal *v* diffuse staining); the loss of basal staining represents a worse prognostic marker indicating a poorly differentiated tumour [10].

In our previous preliminary study [17] we have described the usefulness of the bivariate cytokeratin 7/DNA analysis and flow cytometric measurement of

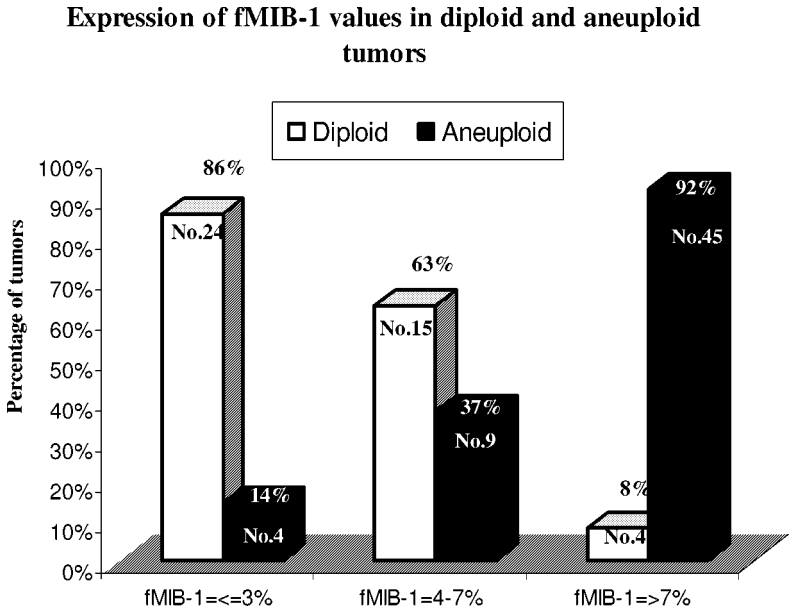

Fig. 8. Expression of flow MIB-1 values in diploid and aneuploid tumors.

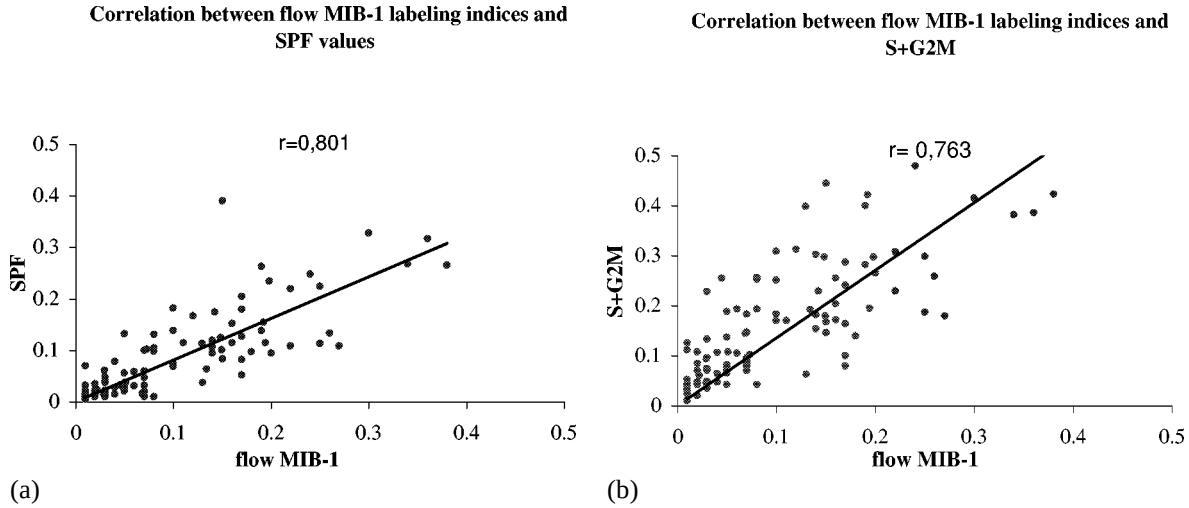

Fig. 9. Linear regression showing the correlation between flow MIB-1 expression and SPF (9a) and S+G2M (9b) values.

MIB-1 in bladder cancer. However we did not investigate the correlation between fMIB-1 values and DNA aneuploidy or with G2+M fraction.

The purpose of our present work was to study the parameters which could be a risk for progression of the disease. We attempted also to verify if tumours with different DNA content were biologically characterised by different associations with unfavourable biomarkers. In order to reach these objectives a multiparameter flow cytometry and an immunohistochemical analysis were performed on tumour tissue. Flow cytometric analyses of DNA/cytokeratin 7 were helpful to obtain more accurate DNA ploidy and SPF values. In particular the bivariate approach avoided some possible mistakes regarding the histogram interpretation as in hypodiploid or in multiclonal cases.

The acquisition of more objective proliferation data in adjunct to cell cycle data analysis constituted a useful tool to understanding the true malignant biological potential of the superficial bladder cancer. Flow cytometric MIB-1 analysis represented a rapid and objective tool in quantifying cellular proliferation.

In our laboratory the best method to fix and permeabilize the cellular suspensions was represented by methanol/acetone for 20 min as previously reported [17]. The method allowed us to examine a large number of cells in a short time. By use of an indirect immunostaining procedure we obtained enhancement of the fluorescent signal and reduced background staining. At the fluorescent microscope MIB-1 expression ranged from small bright intranuclear dots to some large, irregular shaped nucleoli, a staining pattern corresponding to cell cycle related changes of Ki-67 antigen [29]. We found that flow cytometric MIB-1 data $>7\%$ strongly correlated with aneuploidy suggesting that this parameter could be effective to discriminate tumours with high aggressive behaviour. We also observed a strong correlation between fMIB-1 data and flow cytometric proliferative values (SPF) demonstrating that fMIB-1 measurement provides an accurate and useful method to assess cellular proliferation comparable to the cell cycle analysis method. We suppose that the methanol/acetone procedure represents a method to selectively identify the cells in S phase but thiscan only be confirmed by further parallel experiments with bromodeoxyuridine, similar to those conducted on the PCNA molecule [1]. Absence of significant correlations between fMIB-1 and immunohistochemical expression of MIB-1 in the corresponding tissue section could be ascribed to the different fixation protocol (buffered formalin *v* methanol/acetone) or to a partial degradation of the MIB-1 epitope by proteolytic enzymes during thawing and mechanical disaggregation.

The diploid cases fell mostly in the lowest and medium tertile of fMIB-1. Interestingly in these groups the aneuploid cases were represented by tetraploid cases only, suggesting that some tetraploid tumors were characterised by a low cellular proliferation similar to diploid tumors while others showed a very high proliferation similar to hypertetraploid and multiclonal tumors.

Tetraploidy which is generally considered the first step in the process of aneuploidization [8,9,32,34] appears to represent the ploidy class which benefits more from this analysis.

Data arising from our prospective study showed a strong correlation between aneuploidy and staging, grading, SPF value $\geqslant 5.5\%$, MIB-1 positivity $\geqslant 31\%$ and score 2 of p53 expression. In agreement with published findings [13,24,41] we observed that flow cytometry was particularly useful to discriminate a subgroup characterised by a more aggressive potential within grade 2 tumours. By stratifying the cases into six classes according to different DNA content we also attempted to identify a ploidy class with the worst biological profile being characterised by a MIB-1 value $\geqslant 31\%$, SPF $\geqslant 5.5\%$ and p53 score 2 expression. Although statistical analysis failed to demonstrate any significance, probably due to the low number of the patients in the classes it seemed worthwhile to note that the hypotetraploid class showed the highest percentage of cases with poor prognostic biomarkers. In fact 73% of the hypotetraploid tumors had MIB-1 $\geqslant 31\%$, 91% showed SPF values $\geqslant 5.5\%$ and 64% showed p53 score 2. In addition to multiploidy and hypertetraploidy which are considered as highly aggressive ploidy abnormalities [33,39], hypotetraploidy also seems to represent a grave prognostic factor.

In conclusion the present prospective study suggests a strong potential role for flow cytometric and immunohistochemical methods in the biological assessment of bladder cancer behaviour. The determination of DNA content coupled with the study of the epithelial and proliferative components of the tumours could be useful in providing important information on the biological evolution of individual tumours. Biological markers such as DNA hypotetraploidy, high expression of p53, and high cellular proliferation (SPF $\geqslant 5.5\%$, MIB-1 $\geqslant 31\%$, fMIB-1 $> 7\%$) could be critical factors in the acquisition of metastatic potential.

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