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DNA cytometry and kinetics of rat urothelial lesions during chemical carcinogenesis

C. PALMEIRA[1,2], P.A. OLIVEIRA[3], R. ARANTES-RODRIGUES[3], A. COLAÇO[3], P.L.F. DE LA CRUZ[4], C. LOPES[5,6] and L. SANTOS[2,7]

[1]Department of Immunology, Portuguese Institute of Oncology, Rua Dr António Bernardino de Almeida, 4200-072 Porto; [2]Health Faculty, Fernando Pessoa University, Porto; [3]Department of Veterinary Sciences, CECAV, University of Trás-os-Montes and Alto Douro, 5001-911 Vila Real, Portugal; [4]Department of Physiology, Veterinary Faculty, University of Santiago de Compostela, Lugo, Spain; [5]Department of Molecular Biology and Immunology, ICBAS, Porto University; Departments of [6]Pathology and [7]Surgical Oncology, Portuguese Institute of Oncology, Porto, Portugal

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Abstract. The aims of this study were to evaluate the DNA content of chemically-induced rat urothelial lesions and their relationship to the proliferation index and histological patterns. Sixty female Fisher 344 rats were divided randomly into six groups, four groups were exposed to N-butyl-N-(4-hydroxybutyl) nitrosamine for a period of 10 and 20 weeks, and two groups of ten rats were used as control animals. Paraffin sections were Feulgen stained and analyzed using DNA image cytometry analysis; histograms were classified as either diploid or aneuploid. Ki-67 immunoreactivity was determined by means of the streptavidin-biotin-complex immunoperoxidase method. All normal urothelium from the control groups were found to have diploid DNA content. The same histogram pattern was found in the simple hyperplasia group. As regards the other histological lesions, the frequency of the aneuploidy varied depending on the lesion type: 20% of aneuploidy were nodular hyperplasia, 32% of aneuploidy were dysplasias, 25% of aneuploidy were papilloma, 44% of aneuploidy were papillary neoplasm of low malignant potential, 22% of aneuploidy were low-grade papillary carcinoma, 100% of aneuploidy were high-grade papillary carcinoma and 100% of the aneuploidy were invasive carcinoma. Our results revealed the existence of a statistically significant relationship between DNA ploidy and histological pattern lesions (r=0.3, p<0.023). The Ki-67 proliferation index was significantly higher in aneuploid lesions than in diploid (r=0.56, p=0.01). There was also a statistically significant difference in the Ki-67 proliferation index in relation to the histopathological pattern (r=0.751, p<0.01). DNA content was associated with the Ki-67 proliferation index and histopathological grade. DNA content and proliferation index have critical roles to play during urothelial carcinogenesis.

Correspondence to: Dr Paula A. Oliveira, Department of Veterinary Sciences, CECAV, University of Trás-os-Montes and Alto Douro, 5001-801 Vila Real, Portugal
E-mail: pamo@utad.pt

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Introduction

Animal models for studying bladder carcinogenesis have reliably demonstrated that the urothelium is a good choice for studying the early alterations that occur during the initial steps of the cancerization process. The main morphological stages have been identified, progressing from simple hyperplasia to invasive carcinoma, and are similar to those observed in man (1-3). For this reason, these models are a valuable tool for understanding the genetic, epigenetic and environmental factors that influence cancer progression and behaviour. Since bladder tumours exhibit heterogeneous behaviour patterns, the study of this disease cannot be based only on anatomopathological criteria. DNA ploidy provide valuable information on the biological behaviour of preneoplastic and neoplasic lesions (4,5). DNA image cytometry is a technique that enables the densitometric analysis of nuclear DNA of cells and therefore the individual evaluation of DNA ploidy. As a complementary method for this kind of individual molecular markers, gross genomic damage measurements in the form of aberrant DNA content could prove to be a valuable method in the identification of premalignant events and markers to evaluate the different prognosis observed from malignant lesions (4,6,7).

A tumour's proliferative rate has been considered to help predict its subsequent evolution (8,9). Ki-67 is a proliferation associated nuclear antigen that is expressed in replicating cells throughout all phases of the cell cycle (G1, S, G2, M) but is not expressed in quiescent cells (G0) (10). The immunohistochemical expression of Ki-67 has been used in tumour proliferation assessment for many malignancies such as gallbladder, lung, prostate, bladder and breast (11-14). It is also considered to be an acceptable proliferative marker in

rodent urothelial carcinogenesis (15,16). The relationship between DNA content and Ki-67 expression in chemically-induced urothelial lesions remains to be clarified. To the best of our knowledge, there are as yet no reports published on the relationship between DNA content and proliferation index during rat urothelial chemical carcinogenesis. This study uses image cytometry analysis to evaluate the DNA content of rat urothelial lesions that are chemically induced by N-butyl-N-(4-hydroxybutyl) nitrosamine. It also aims to assess the kinetic features of the same lesions by determining their proliferation index and correlating these values with histological patterns.

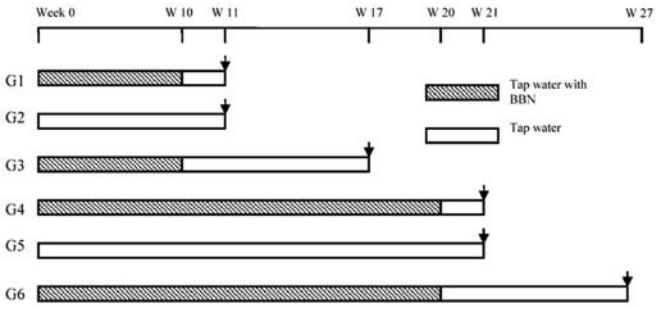

Figure 1. Experimental design (arrow indicates time of death; W-week).

Materials and methods

The Portuguese General Veterinary Direction in accordance with the current regulations and standards currently in place in the European Community, approved the facilities where the animals were kept and the experimental procedures. Sixty, five week-old, female Fisher 344 rats were obtained from Harlan (Amsterdam, The Netherlands). The rats were randomly housed in plastic cages, with hard wood chips used for bedding (cages were cleaned twice a week). They were kept under controlled temperature conditions (23±2˚C), light-dark periods of 12 h and with free access to water and a commercially available diet (Harlan Teklad, Glogal Diet). After a one-week acclimatization period, four groups of ten animals were given drinking water containing 0.05% BBN (Tokyo Kasei Kogyo Co., Tokyo Japan) for a period of 10 (groups 1 and 3) and 20 weeks (groups 4 and 6). Twenty animals were used as negative controls; they drank water without BBN for 10 (group 2) and 20 (group 5) weeks. Rats were sacrificed and necropsies were carried out as detailed in Fig. 1. During the course of the experiment, a daily health check was performed and body weights were measured once per week.

For histological examination purposes, the bladders were filled with 10% buffered formalin, the urethra was ligated with silk thread and the whole bladder was fixed and 12 h later cut sagittaly. The urinary bladders were embedded in paraffin. Sections of 2 μm were cut and stained with haematoxylin and eosin. All sections were reviewed by two researchers and the urothelial lesions classified according to the World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder (17). Urothelial lesions were categorized as either: simple hyperplasia, nodular hyperplasia, dysplasia, papilloma, papillary neoplasm of low malignant potential, low-grade papillary carcinoma, high-grade papillary carcinoma, invasive carcinoma, spinocellular carcinoma or squamous metaplasia.

The nuclear DNA content of preneoplastic and neoplastic lesions was measured using the CAS 200 Image Analysis System (Cell Analysis Systems, Elmhurst, III, Becton-Dickinson®). Urinary bladder sections from paraffin-embedded blocks were cut at 6 μm before being deparaffinized and rehydrated. The slides were then Feulgen-stained with the CAS DNA staining kit (Cell Analysis Systems, Elmhurst, III) as previously described (18). The Feulgen reaction produced a blue staining of nuclear DNA which reflects the stain's stoichiometric binding to the DNA. At least 100 complete, non-overlapping, and focused nuclei in every case and 20-30 normal lymphocytes (used as internal reference diploid cells) were measured. The resultant DNA histograms were analysed and classified as diploid and aneuploid using a previously described methods (19). The 5c exceeding rate (5cER) defined as the percentage of tumour nuclei with DNA content above 5n, was also evaluated.

Immunohistochemistry was performed using the streptavidin-biotin-complex immunoperoxidase method as

Table I. Incidence of urothelial lesions in female Fisher 344 rats exposed to BBN and control groups. No. of animals (%).

Group (n)	Normal urothelium	Simple hyperplasia	Nodular hyperplasia	Dysplasia	Papilloma	PNLMP	Low-grade papillary carcinoma	High-grade papillary carcinoma	Invasive carcinoma	Spinocellular carcinoma	Squamous metaplasia
1 (10)	0	10 (100)	4 (40)	10 (100)	3 (30)	4 (33.3)	1 (10)	0	0	0	4 (40)
2 (10)	10 (100)	0	0	0	0	0	0	0	0	0	0
3 (10)	0	8 (80)	9 (90)	10 (100)	5 (50)	5 (50)	6 (60)	1 (10)	0	0	3 (30)
4 (10)	0	3 (30)	9 (90)	10 (100)	5 (50)	5 (50)	7 (70)	2 (20)	2 (20)	1 (10)	6 (60)
5 (10)	10 (100)	0	0	0	0	0	0	0	0	0	0
6 (10)	0	0	9 (90)	10 (100)	3 (30)	6 (60)	10 (100)	9 (90)	1 (10)	0	5 (50)

PNLMP, papillary neoplasm of low malignant potential.

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y described (20). Immunohistochemical staining (1:20, M7248, Dako®) were performed to study proliferation index. For each lesion, cells showing positive staining within several microscopic fields with the highest immunoreactivity were counted at a higher magnification (x400), using a 10x10 grid. The Ki-67 labelling index (LI) was calculated as the percentage of positive nuclei divided by the total number of cells examined. At least 500 cells per lesion were examined. DNA content and proliferation analysis were performed on the same areas.

Statistical analysis of the data was carried out using the Statistical Package for Social Sciences for Windows (SPSS, Inc., Chicago, IL). Data were expressed as the mean ± standard deviation. The numeric parameters were tested by ANOVA or Kruskal Wallis tests. Histological grade was evaluated by χ^2 or Fisher tests (two-sided). The Spearman correlation was used to evaluate the association of ploidy with histological pattern. P-values <0.05 were considered statistically significant.

Results

There were no significant differences in the body weight gained during the course of the experiment between the BBN-treated and control rats. No deaths attributable to the BBN treatment were observed. No macroscopic or microscopic changes were seen in the liver, lungs, kidneys and gastrointestinal tract. Macroscopically, the urinary bladder was normal in all rats belonging to the control group. Urinary bladders from animals treated for 10 weeks with BBN were apparently normal. Greyish-white urinary bladder masses varying in size between 0.1-7 mm (size at largest diameter) were observed in those groups exposed to BBN for 20 weeks. The surfaces of the lesions were irregular with necrotic and, haemorrhagic areas as well as focal ulcerations. The lesions were distributed randomly throughout the entire urinary bladder. The majority of those lesions were pedunculate. Stone formation in the urinary bladder was not observed in any rat. No histopathological changes in urothelial cells were observed in the control group. The incidence of histopathological lesions in each group is shown in Table I. Simple and nodular hyperplasia, dysplasia, papilloma, papillary neoplasm of low malignant potential, low-grade papillary carcinomas were observed in all the bladders of all the BBN-treated rats. High-grade papillary carcinomas and invasive carcinomas were only observed in animals euthanized after the 7th week.

Adequate DNA histograms were obtained for all the urothelial lesions examined by image cytometry analysis. ICM DNA analysis was successfully performed on 92 lesions and 20 normal urothelium. All the DNA histograms of the normal urothelium from control groups were DNA-diploid. DNA-aneuploid histograms were found only in urothelial lesions. The DNA content results are summarized in Table II.

Despite the period of observation and the BBN treatment, the frequency of the DNA aneuploid pattern increased with the degree of tissue transformation and this variation was statistically significant ($p<0.05$).

Urothelium from the control group were negative for Ki-67 expression, and in the urothelial lesions Ki-67 immuno-

Table II. DNA content results according to the histological pattern and animal group.

Histological pattern	n	DNA content	
		Diploid n (%)	Aneuploid n (%)
Group 1			
Simple hyperplasia	5	5 (100)	0
Nodular hyperplasia	1	1 (100)	0
Dysplasia	7	3(42.8)	4 (57.2)
Papillary neoplasm of low malignant potential	2	1 (50)	1 (50)
Squamous metaplasia	1	1 (100)	0
Group 2			
Normal urothelium	10	10 (100)	0
Group 3			
Simple hyperplasia	3	3 (100)	0
Dysplasia	6	5(83.3)	1 (16.7)
Papillary neoplasm of low malignant potential	5	4 (80)	1 (10)
Low-grade papillary carcinoma	4	3 (75)	1 (25)
High-grade papillary carcinoma	1	0	1 (100)
Group 4			
Nodular hyperplasia	3	2(66.7)	1 (33.3)
Dysplasia	7	5(71.4)	2 (28.6)
Papilloma	4	3 (75)	1 (25)
Papillary neoplasm of low malignant potential	5	2 (40)	3 (60)
Low-grade papillary carcinoma	4	3 (75)	1 (25)
High-grade papillary carcinoma	2	0	2 (100)
Invasive carcinoma	2	0	2 (100)
Squamous cell carcinoma	1	1 (100)	0
Squamous metaplasia	1	0	1 (100)
Group 5			
Normal urothelium	10	10 (100)	0
Group 6			
Nodular hyperplasia	1	1 (100)	0
Dysplasia	5	4 (80)	1 (20)
Papillary neoplasm of low malignant potential	4	2 (50)	2 (50)
Low-grade papillary carcinoma	10	8 (80)	2 (20)
High-grade papillary carcinoma	5	0	5 (100)
Invasive carcinoma	1	0	1 (100)
Squamous metaplasia	2	2 (100)	0

Table III. Proliferation index in different urothelial lesions and experimental groups.

Group (n)	Simple hyperplasia	Dysplasia	Papilloma	PNLMP	Low-grade papillary carcinoma	High-grade papillary carcinoma
1	6.6±1.5	15.5±1.3	-	-	-	-
3	19.5±7.7	20±2.8	-	25.5±4.9	29.3±0.6	-
4	-	20.8±4.6	17.6±5.8[a]	24.4±3.1	29±6.8	29±1.41
6	-	17.3±4.2[b]	-	22.3±4.7	24.8±4.5	32±1.58

PNLMP, papillary neoplasm of low malignant potential. [a]Statistically different from low-grade papillary carcinoma (p=0.026). [b]Statistically different from high-grade papillary carcinoma (p=0.003).

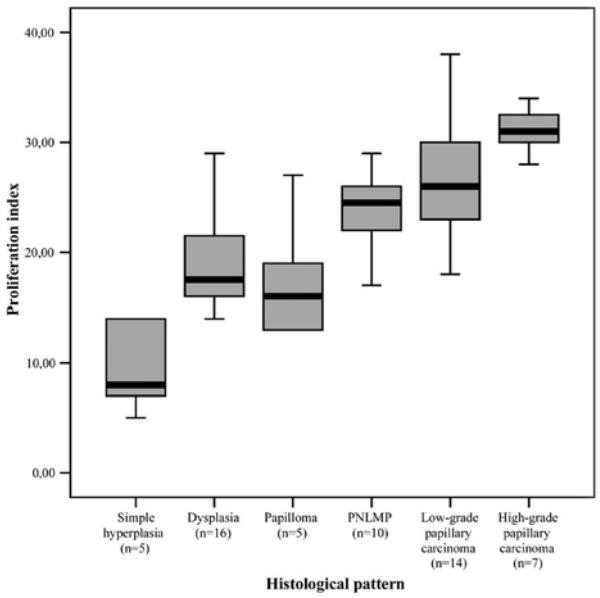

Figure 2. Proliferation index in different histological patterns.

Table IV. Significance of proliferation index between different histological patterns.

	PNLMP	Low-grade papillary carcinoma	High-grade papillary carcinoma
Simple hyperplasia	SD	SD	SD
Dysplasia	-	SD	SD
Papilloma	-	SD	SD

SD, statistically different (p<0.05).

staining was evident as diffuse or dotlike nuclear and nucleolar staining. Ki-67 expression was evaluated in 57 lesions and the mean values of the proliferation index for each lesion in each group evaluated are summarized in Table III.

When the mean values of the proliferation index for the various lesions were analyzed, a tendency to increase Ki-67 expression was observed in lesions with a high grade of malignancy (Fig. 2). Variance analysis using the Bonferroni test showed significant differences (p<0.05) between the mean value of the high-grade malignancy when compared with other lesions (Table IV).

To clarify the biological significance of Ki-67 during urothelial carcinogenesis, a fixed linear model was fitted, which had the histological pattern and ploidy status as the independent variables. Both factors had a statistically significant influence on the proliferation index (p<0.001). A significant correlation was found between DNA ploidy and histological pattern (r=0.3, p<0.02). Correlation between proliferation index and DNA ploidy was r=0.563 and p<0.01. Correlation between proliferation index and histological pattern was r=0.751 and p<0.01 (Fig. 3).

Discussion

During the transformation from a normal to malignant state a number of DNA alterations occur. These according to the concept of genetic instability, enable a stepwise evolutionary selection of tumour cell populations with the biological characteristics of tumour progression (21). Few studies have been reported on the correlation between tumour DNA ploidy and proliferation index in urothelial preneoplastic and neoplasic lesions (22,23). As regards rat urothelial lesions, no other study on this subject can yet be found in the available literature. The results of our research revealed that simple hyperplasia were 100% diploid and that a progressive increase in aneuploid frequency was observed as the malignant potential of the lesion increased, until there were 100% of aneuploidy in invasive carcinoma. Our findings are in general agreement with those previously reported by us on chemically-induced preneoplastic lesions in mice (18). Urothelial dysplasia has been considered a putative precursor of carcinoma *in situ* (CIS), and CIS of invasive urothelial carcinoma (17). DNA aneuploidy of dysplasia reflects the precancerous nature of this histological lesion, since the presence of aneuploidy provides unequivocal proof of malignancy. The detection of this neoplastic transformation prior to observing the morphological evidence of the neoplasia, enhances the usefulness of DNA content evaluation in preneoplastic lesions. Evaluating DNA content via image cytometry analysis makes it possible to distinguish between neoplastic cells and void non-tumour cells, as image

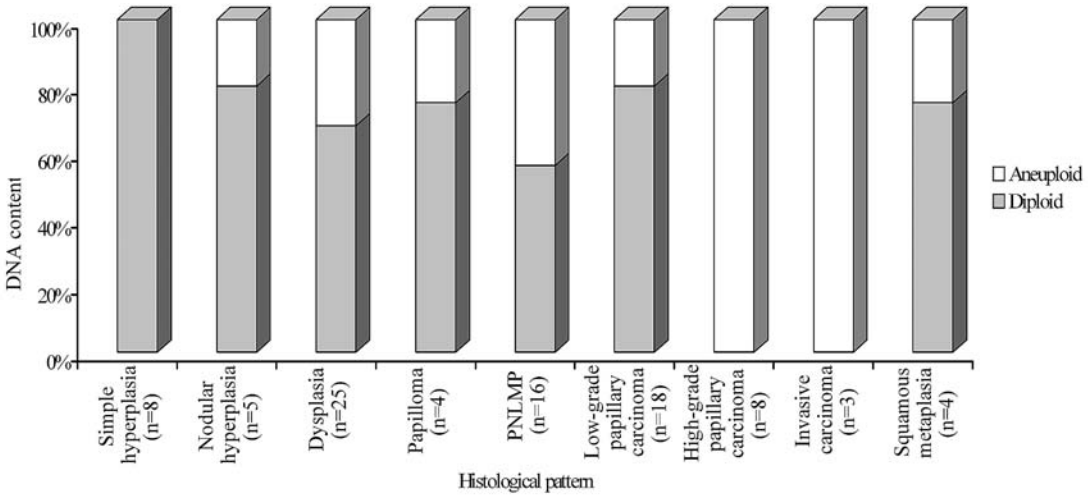

Figure 3. DNA ploidy observed in the different histological patterns analysed.

cytometry analysis enables the simultaneous analysis of different types of histopathological lesions within the same sample (18). In the present study, a strong correlation was established between DNA-ploidy status and histopathological grading. Nevertheless, we found that lesions of the same histological type might have different DNA content depending on their exposure to BBN. These results indicate an increased specificity, of the positive benefits of using DNA cytometry, and confirm this method's suitability for studying urothelial carcinogenesis. DNA content analysis has been reported as diagnostically useless in differentiating benign from malignant conditions (24,25). The data presented here show that the evaluation of cell proliferation using Ki-67 is closely related to the malignancy grades determined by histopathological methods. In common with the results obtained by other researchers (26), the data in this study showed a statistically significant difference between the histopathological grade and the Ki-67 expression in preneoplastic and neoplastic cells. Lesions with a high grade of malignancy showed a greater percentage of Ki-67 positive cells than preneoplastic lesions or low-grade papillary carcinomas. These findings suggest that the evaluation of Ki-67 expression in urothelial lesions can improve our knowledge of bladder cancer development. As regards DNA content, this study demonstrated that DNA content is correlated with tumour proliferation; the Ki-67 proliferation index was significantly higher in aneuploid lesions than in diploid lesions. Other researchers studied the relationship between cellular proliferation and DNA content in adrenocortical proliferative lesions and oesophagus neoplasic conditions and concluded that both factors were related (27,28).

Collectively taken, our data indicate that DNA ploidy and tumour cell proliferative activity, as determined by the expression of Ki-67 nuclear antigen are correlated with the various histological patterns observed during the course of the urothelial malignant transformation process. If the conclusions drawn by our study were to be confirmed by other studies on urothelial carcinoma in man, then DNA ploidy status and Ki-67 taken together may help us to better understand urothelial carcinogenesis.

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