Is DNA content alteration a consequence of proliferative and differentiation changes in urothelial bladder tumours?

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Abstract

The loss of differentiation, high cell proliferation rate and aneuploidy are usually related with tumour aggressiveness. The accumulation of this phenotype in bladder cancer was observed in invasive tumours. However in superficial ones it is not clarified the meaning and the time of these events. We studied simultaneously cell proliferation, blood group ABH antigen expression and DNA ploidy in urothelial cell bladder carcinoma in order to determine the relationship between these biomarkers and clinical-pathological features of urothelial cell carcinoma of bladder.

In present series we verified that early tumours (Ta/G1) had high proliferative index, loss of blood group antigen expression and were DNA diploid. Invasive tumours (T2/T4G3), also showed loss of A, B and H antigens expression, but were more frequently DNA aneuploids and showed a decreased in proliferation rate.

These results suggest that aneuploidization, i.e., DNA content alteration, might be a consequence of these previous changes.

Resumo

A perda de diferenciação, um elevado índice proliferativo e a aneuploidia são características biopatológicas associadas a maior agressividade tumoral. Este fenótipo é observado com frequência nos tumores invasores. No entanto, nos carcinomas superficiais da bexiga, o significado destes eventos e o momento em que ocorrem não estão esclarecidos.

Estudámos simultaneamente a proliferação celular, a expressão dos grupos sanguíneos e a ploidia de DNA, numa amostra de carcinomas uroteliais da bexiga, com o objectivo de determinar a relação entre estes marcadores biológicos e as características clínico-patológicas.

No presente estudo, verificámos que os tumores mais precoces (TaG1) tinham um índice proliferativo elevado e perda de expressão dos grupos sanguíneos, sendo, no entanto, diplóides de DNA. Os tumores invasores (T2/T4G3) tinham perda de expressão dos antigénios ABH; eram, na sua maioria, aneuplóides de DNA, e a taxa de proliferação era baixa.

Estes resultados sugerem que o processo de aneuploidização, entendido como alterações do conteúdo de DNA, é o resultado da perda de diferenciação e de uma elevada taxa de proliferação.

Introduction

DNA aneuploidy, which indicates a state with an abnormal DNA and chromosome content, has been found in various human cancers[1]. This parameter is considered to be an important biological and prognostic variable in human cancer[2-3].

In bladder carcinoma some authors have reported an objective assessment of grading by cytophotometry, achieving a good correlation between the histological grade of bladder tumours and the DNA content[4]. This correlation was well reported in the Consensus on the clinical utility of DNA ploidy analysis in patients with transitional cell carcinoma of the bladder[5]. Wheeless et al[5] found that the majority of grade 1 bladder tumours were DNA diploid, being the majority of grade 3 tumours DNA aneuploid. Furthermore, superficial bladder cancer, compared with the invasive ones, had a significantly lower number of aneuploid cells[6].

Image cytometry studies report that it is possible to determine the ploidy level in a given tumour using the parameters obtained from the DNA histogram, assessed by Feulgen-stained nuclei. One of them is the DNA histogram type (DHT), whose parameters contribute with discriminatory information on the clinical progression in superficial transitional cell carcinomas of the bladder[8]. The percentage of aneuploid cells with DNA content above 5c (5cER) is another ploidy related parameter, and is important in assessing bladder cancer aggressiveness, being a reliable biomarker in the risk assessment for this type of tumour[8-9].

The histoblood group ABO involves three carbohydrate antigens - A, B, and H. A and B and AB individuals express glycosyltransferases that add sugar residues to H structure, whereas O individuals lack such activity [10]. The ABH gene is mapped on 9q34.1--34.2 region [11]. The presence of blood group antigens (A, B, H) on the surface of the epithelial cells depends on the individual secretor status. About 85% of the population is secretor and express A, B, and H in their normal urothelium[12]. Alterations of the blood group antigen and loss of ABH gene chromosomal region are frequent in bladder cancer. This antigen loss has been reported in bladder cancer as related to progression and poorer prognosis[13-14]. De Cenzo et al [15] and Newman AJ et al[16] observed, particularly among superficial papillary urothelial carcinomas, progression in those with blood group antigens (A, B, H) loss.

Proliferation rate assessed by Ki-67 labelling index (LI) was recently accepted as a prognostic marker in superficial papillary urothelial carcinomas of the bladder[17]. Tumours with high LI show a significant low recurrence-free and progression-free survival[17]. Bush et al [18] observed that invasive bladder tumours had a significantly higher Ki-67 LI than the non-invasive ones. Also, bladder tumours with lymph node involvement had higher LI than those without lymph node metastasis, appearing that the high frequency of S-phase cells within the tumour tissue to indicate a great potential for malignancy and a poor prognosis[19].

The prognostic value of blood group ABH antigen status and DNA ploidy combination, assessed by flow cytometry, was related to the disease outcome [20]. On the other hand, more than 10% of cells with 5cER of DNA content were related to the increase of the cell proliferation rate[21]. However, the relationship between grade and stage and the combination of these three biological markers (cell proliferation, blood group ABH antigen expression and DNA ploidy) has not been performed simultaneously, until now. Therefore, the aim of this study was to analyse the

usefulness of image DNA ploidy and other ploidy--related parameters, blood group antigens (ABH) status and Ki-67 labelling index in predicting prognosis and for therapeutic purposes.

Patients and Methods

This study comprised 94 unselected patients diagnosed between 1990 and 1996 at the Instituto Português de Oncologia, Porto, Portugal; 53 with primary superficial (56.4%) and 41 invasive (43.6%) urothelial bladder carcinoma. The patients' age ranged from 28 to 83 years with a median age 73 years. The patients were treated either by transurethral resection or cystectomy; no previous radiation or chemotherapeutic treatments were performed. All specimens were graded using the World Health Organization (WHO) classification[22] and staged according to the AJCC system[23]. In the series, 68 (72.3%) tumours were papillary, 19 (20.2%) were non-papillary and the 7 (7.5%) remaining were mixed. Ten (10.6%) cases were G1, 50 (53.2%) were G2 and the last 34 (36.2%) cases were G3. The stage of tumours was Ta in 9 (9.6%), T1 in 44 (6.8%) and T2-T4 in 41 (43.6%).

Image Analysis

The nuclear DNA content of cancer cells was measured with CAS 200 image analysis system (Cell Analysis Systems, Inc., Elmhurst, Ill.). Sections from paraffin-embedded blocks were cut at 6 μm and were deparaffinized and rehydrated. The slides were stained by standard Feulgen method with the DNA staining kit (Cell Analysis System, Inc.), according to the manufacturer's instructions. Within each staining batch was also stained one CAS slide with control rat hepatocytes. The image system was first calibrated using the controls, which contained a known quantity of DNA. Twenty to 30 lymphocytes and a minimum of 100 intact non-overlapping tumour nuclei were measured and analysed for each case, using the Quantitative DNA Analysis software program (Cell Analysis System, Inc.). The integrated optical density (OD) of each Feulgen-stained nuclei was considered proportional to the amount of DNA present in the nuclei. The OD of lymphocyte nuclei from each section served as internal control (diploid reference). The resultant DNA histograms were analysed by previously used methods [24]. For each tumour G0/G1 peak visually identified, mean, standard deviation (SD) and coefficient of variation (CV) values were calculated. The control CV provides an indicator of overall precision of the imaging technique. The DNA index (DI) was evaluated as the ratio of tumour G0/G1 peak mean value divided by the internal control lymphocytes G0/G1 peak mean value. Peaks having a DI greater than 3SD from the internal control lymphocytes were considered aneuploid. The 5cER was also evaluated being defined as the percentage of tumour nuclei with DNA values above 5n.

DHT – DNA Histogram Type

To obtain a reproducible DNA histogram classification, the DHT were classified into five categories (Table 1).

Type 1 - a single distinct G0/G1 peak with a DI within 3SD from the internal control lymphocyte (diploid region) containing at least 70 % of its cell nuclei populations[8], usually with less than 1 % of 5cER

Type 2 - a single G0/G1 peak in the near-diploid region or two G0/G1 peaks, one in the diploid region and the second peak in the near-diploid region

Type 3 - show either a distinct modal value in the tetraploid or near tetraploid region or have two well-defined peaks around the 2n and 4n regions

Type 4 - have a single DNA aneuploid peak outside the tetraploid or near-tetraploid region

Type 5 - contain more than two distinct G0/G1 peaks showing a very pronounced and irregular aneuploidy, with DNA amounts ranging from 2n up to values exceeding 6n or even 9n

Table 1 – Definition of DHTs identified in bladder carcinomas analysed by Image Cytometry

DHT Type	DI Range Value of Tumour G0/G1 DNA Peak
1	$0.83 < DI \leq 1.17$
2	$1.17 < DI \leq 1.20$
3	$1.90 < DI \leq 2.20$
4	$1.20 < DI \leq 1.90$ and $DI > 2.20$
5	—

Table 2 – Studied biomarkers in function of stage and grade

	n	*5cER*		*DHT*					*DNA ploidy*		*Ki-67*			*Blood group ABH antigen*			
		without	**with**	**1**	**2**	**3**	**4**	**5**	**diploid**	**aneuploid**	**neg**	**1-18%**	**≥18%**	**loss**	**partial loss**	**expression**	**NS**
TaG1	5	5	0	5	0	0	0	0	5	0	0	2	3	3	2	0	0
T1G1	5	3	2	2	1	0	2	0	2	3	2	2	1	2	0	2	1
TaG2	4	2	2	2	0	0	0	2	2	2	1	1	2	1	2	0	1
T1G2	33	13	20	6	5	0	9	13	6	27	11	11	11	14	9	8	2
T2-T4G2	13	3	10	3	0	1	2	7	3	10	5	2	6	5	4	1	3
G3	34	1	33	3	1	3	4	23	3	31	25	5	4	27	4	3	0
Total	**94**	**27**	**67**	**21**	**7**	**4**	**17**	**45**	**21**	**73**	**44**	**23**	**27**	**52**	**21**	**14**	**7**

5cER - 5c exceeding rate. DHT - DNA histogram type. NS - non secretor. Neg - negative

Immunohistochemistry

Sections of 4μm were cut from formalin-fixed, paraffin-embedded primary bladder cancers. A microwave oven was used for antigen extraction; endogenous peroxidases were blocked by incubation with 0.3% hydrogen peroxide. The slides were incubated with normal horse serum (VectaStain ABC kit, Vector Laboratories®) for 20 min at room temperature. Tissue sections were then incubated with primary antibodies. Monoclonal antibodies anti-blood group antigen A (1/100 Dako®), antigen B (1/15 Dako®) and antigen H (1/15 Dako®) were incubated for 12 minutes at room temperature. Mib-1 (1/50 Novocastra®) was applied overnight at 4ºC. These sections were sequentially incubated with the secondary biotinylated antibody (VectaStain ABC kit, Vector Laboratories®) and avidin-biotin peroxidase complexes (VectaStain ABC kit, Vector Laboratories®) for 30 min. Reaction products were revealed with diaminobenzidine (DAB) as the chromogen. The sections were counterstained with Harris's hematoxylin.

Paraffin-embedded tissues known to express nuclear Ki-67 in more than 18% of the cells (lymphoma) were used for titration and positive controls. Negative controls were performed by replacing the primary antibody for 2.5% bovine serum albumine (BSA) in phosphate-buffered saline (PBS). For A, B and H immunoreactivity, tissues expressing the appropriate blood group antigen were used for titration of reagents as well as for the positive and negative controls. In addition, stained endothelial and red blood cells present in the tissue sections served as internal positive controls for the secretor phenotype[25].

Immunohistochemical scoring

Positive staining was defined as the presence of ≥ 18% antibody-stained nuclei for Ki-67, according to the cut-off values of the National Cancer Institute Bladder Tumour Marker Network[26]. Secretor tumours were classified according to the semiquantification of A, B and H loss of expression in membrane and/or cytoplasm, being the three categories: total loss of expression, partial loss of expression and with expression. The entire section was screened to find the region with maximum fraction of positive Ki-67 cells and the region without cells stained for ABH blood group antigens. The percentage of positive stained nuclei was scored in this region using the 40x objective. At least 200 malignant cells were counted in each case. Immunohistochemical evaluation was done by two independent observers (T.A. and L.S.).

Statistical analysis

A descriptive study was performed for all variables included in the study. Chi-square and Fisher's exact tests were used. All statistical analyses were performed with SPSS 8.0 software (SPSS Inc.). A $p<0.05$ was accepted as statistically significant.

Results

From the 94 urothelial bladder carcinomas studied, 73 (77.7%) were DNA aneuploid and 21 (23.3%) were diploid tumours. Type III DNA histogram was the most prevalent, having 71% of the tumours 5cER cells (Table 2). The A, B and H expression was lost in 52

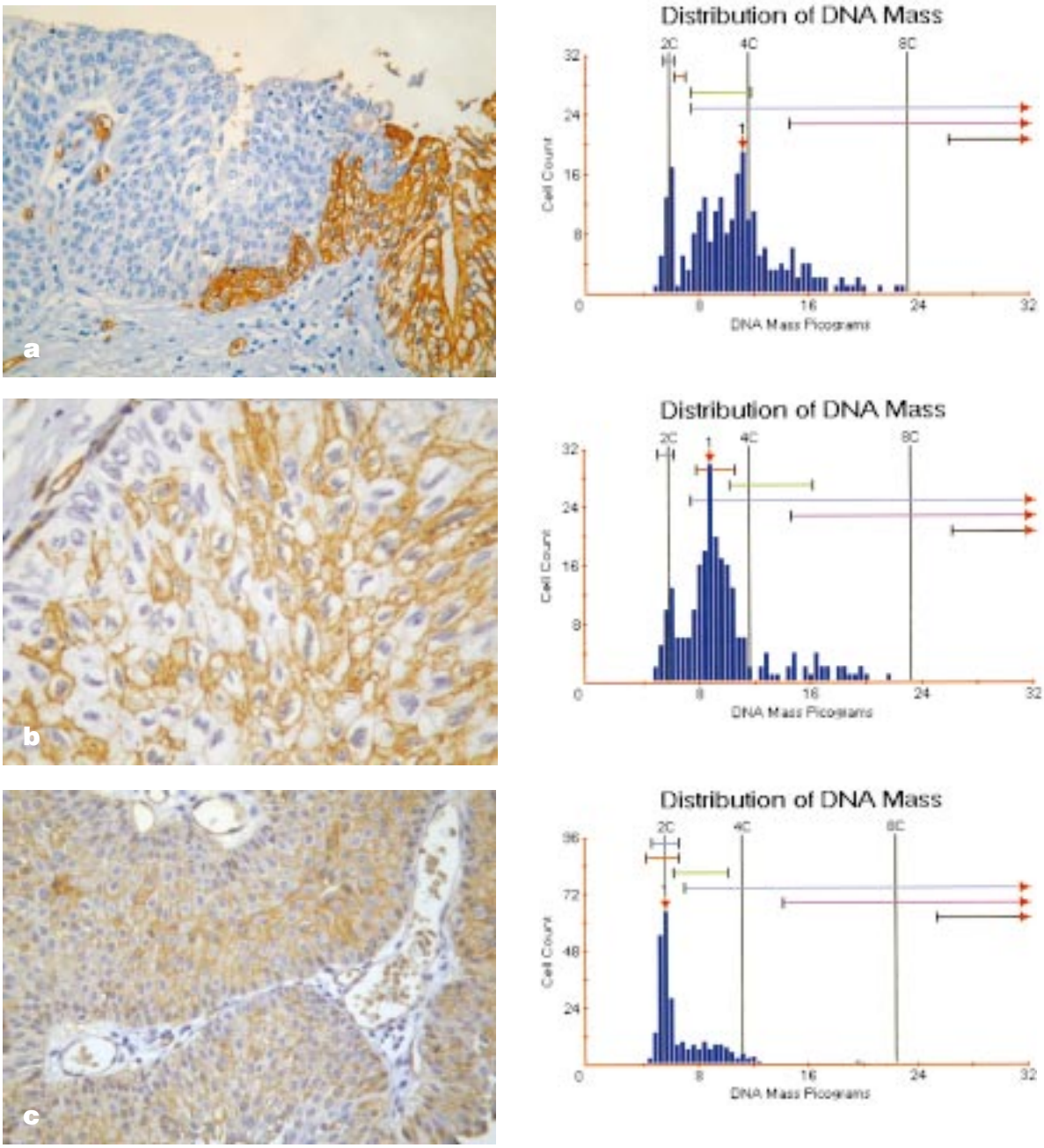

Figure 1

a) Total loss of blood group antigen (ABH) in superficial urothelial bladder cancer and corresponding DNA aneuploid histogram type V (transitional area between tumour and normal mucosa), Ki-67 LI - 0%.

b) partial loss of blood group antigen (ABH) in superficial urothelial bladder cancer and corresponding DNA aneuploid histogram type IV, Ki-67 - 5%.

c) Expression of blood group antigen (ABH) in superficial urothelial bladder cancer and corresponding DNA aneuploid histogram type I, Ki-67 LI - 17%.

(55.3%) tumours, partially lost in 21 (22.3%) and was present in 14 (14.9% - Figures 1 a, b and c, respectively); 7 (7.4%) were non-secretor cases. The immunohistochemical status of Ki-67 protein was positive (>18% of cells) in 27 (28.7%) cases, partially positive (1-18% of positive cells) in 23 (24.5%) and negative in 44 (46.8%) cases. Table 3 shows the relationship between DNA content parameters, Ki-67 protein expression and blood group antigen loss according to tumour morphology, grade and stage. Diploidy

Table 3 – Discriminatory distribution of biomarkers studied according to morphology, grade and stage

	Morphology				Grade				Stage			
	Pap	NP	M	p	G1	G2	G3	p	Ta	T1	T2-T4	p
DNA Ploidy												
Diploid	19	2	0	0.09	7	11	3	0.0001	7	8	6	0.0001
Aneuploid	49	17	7		3	39	31		2	36	35	
5cER												
Without	27	0	0	0.001	8	18	1	0,0001	7	16	4	0,0001
With	41	19	7		2	32	33		2	28	37	
DHT												
I	19	2	0	0.01	7	11	3	0.001	7	8	6	0.0001
II	7	0	0		1	5	1		0	6	1	
III	1	3	0		0	1	3		0	0	4	
IV	14	1	2		2	11	4		0	11	6	
V	26	13	5		0	21	23		1	19	24	
Ki-67												
0%	25	14	5	0.007	2	17	25	0.003	1	19	24	0.09
1-18%	18	5	0		4	14	5		3	13	7	
>18%	25	0	2		4	19	4		5	12	10	
ABH												
Expression	11	0	3	0.01	2	9	3	0.02	0	10	4	0.31
Partial loss	18	2	1		2	15	4		4	9	8	
Total loss	31	16	3		5	19	26		4	21	25	
No secretor	7	0	0		1	6	0		1	3	3	

5cER - 5c exceeding rate. ABH - blood group antigens. NS - non secretor, Pap - papillary, NP - non papillary, M - mixed. DHT – DNA histogram type.

was correlated significantly (p=0.0001) with TaG1 tumours. The percentage of cases having cells with more than 5c (5cER) was significantly correlated with G2/G3, T1 and T2-4 tumours (p=0.0001). Aneuploid histograms type (II-V) were more frequent in G2/G3 and T1, T2-4 tumours. Low proliferative LI was observed in 73.5% of G3 tumours. The majority of G2/G3 tumours lost partial or totally the ABH immunoreactivity. As can be seen in table 4, about 70% of the tumours without proliferation (Ki-67 negative cases) and 5cER cells lost totally or partially the A, B, H immunoreactivity (p=0.008).

Discussion

Molecular and cytogenetic analyses have shown that multiple genetic alterations are involved in the genesis and progression of urothelial cell carcinoma of the bladder[27]. Among these alterations, deletions of chromosome 9p (9p21 - p14, p15 and p16 proteins) or 9q (9q13-31, 9q32-33 and 9q34) are the most frequent, suggesting that the loss of tumour suppressor genes lead to cell uncontrolled proliferation and increasing in the DNA amount[28]. On the other hand, these 9q altered regions are close to the 9q34.1-34.2, the mapped area for the ABH genes[11].

Compared with flow cytometry, image cytometry has limitations in distinguishing cell populations with small alterations in DNA content, like near-diploid populations, however, it requires a lower number of cells allowing the study of small tumour sample[29]. Furthermore, microscopic selection of tumour cells allows the rejection of artefacts as well as the detection of aneuploid cells that may represent a small proportion of the whole tumour cell population. In the present study the DNA content was assessed by ima-

Table 4 – Discriminatory distribution of Ki-67 and A.B.H. blood group antigen expression for 5cER

Ki67			5cER Without	5cER With	Total	p
>18%	**A.B.H**	Expression	2	1	3	
		Partial Loss	2	6	8	
		Total Loss	4	8	12	0.282
		NS		4	4	
	Total		8	19	27	
1-18%	**A.B.H.**	Expression	2	3	5	
		Partial Loss	4	2	6	
		Total Loss	5	6	11	0.590
		NS	1		1	
	Total		12	11	23	
negative	**A.B.H.**	Expression		6	6	
		Partial Loss	1	6	7	
		Total loss	4	25	29	0.008
		NS	2		2	
	Total		7	37	44	

ge cytometry. Regarding all studied DNA parameters (DNA ploidy, DNA histogram type and percentage of 5cER cells) similar trends were observed when compared with grade and stage. All TaG1 urothelial tumours were DNA diploid, being the incidence of the aneuploid pattern (ploidy and DHTs) and the 5cER value increased with grade and/or stage. Among these DNA content related variables, 5cER was shown to be the variable that represents the less time consuming procedure if we previously set-up the morphometric image filter to select only these aneuploid cell nuclei. This parameter has been showed to be a useful tumour marker in bladder cancer [4-9].

Previous studies have demonstrated an association between cell proliferation and tumour grade, stage, recurrence and progression in bladder carcinoma[30-33]. In our series, the majority of G3 tumours (T1 – 6 cases and T2/T4 – 28 cases) showed no proliferative cells, however, these cases were aneuploid and lost the ABH antigens. Similar results where observed by Baithun et al [34], who found a distinct genetic and kinetic profile (slower cell turnover) in musclenvasive bladder cancer. Blanes et al[35], studying kinetic parameters like mitotic figures counting, Ki-67 LI and proliferative rate assessed by image cytometry in same bladder carcinomas, observed a lower proliferation rate in deep compartment of tumours. This lower rate could be a consequence of the absence of tumour suppressor gene alterations, as referred by Diaz--Cano et al [36].

Loss of blood group antigen was an early event in our series of secretor superficial low-grade tumours of urinary bladder. Juhl et al[37] also observed a small number of superficial bladder tumours exhibiting a normal blood group antigen expression. In their work, Ornotof et al[38] concluded that in normal and malignant urothelium ABH glycosylation is regulated at the mRNA level. This mechanism is associated with cell proliferation that may trigger down the regulation of ABH mRNA[38]. In our series we did not show a significant association between high Ki-67 labelling index and loss of ABH expression. Therefore, alteration of glycosyltranferase encoding genes may occur, as suggested by Orlow et al[39].

In this series we verified that early tumours (Ta/G1) had high proliferative index and blood group antigens loss of expression. Nevertheless, these tumours remained diploid. Therefore, we can say that, indeed, chromosome 9 loss (partial or entire) is an early event and is accompanied by cell proliferation. When we analysed more invasive tumours, the proliferation rate is diminished, the ABH antigens expression loss remains, but they are no longer diploid. According to

our results, aneuploidy i.e., DNA content alteration is not an early event. During the progression of urothelial cell carcinoma of the bladder there seems to be a selection of those cells with gains of genetic material, conferring the DNA aneuploid status. The alteration in the parameters studied were usually related with worse prognosis and consequently determining more aggressive therapy[5, 7, 14-20]. The loss of ABH antigen immunoreactivity is a surrogate marker of urothelial cancer cells aberrant glycosylation. This suggests that adjuvant immunization-based strategies can be used to mobilise the immune system against criptic carbohydrate antigens, displayed on the surface of those cancer cells.

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