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The prognostic value of proliferating cell nuclear antigen, Ki-67 and nucleolar organizer region in transitional cell carcinoma of the bladder

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Abstract. *Objectives*: To investigate the value of proliferating cell nuclear antigen (PCNA), Ki-67 antigen labelling indices and nucleolar organizer region (NOR) score in relation to histological grade, stage, recurrence and progression of the bladder tumor. *Materials and methods*: Tissue specimens from 77 bladder cancer patients (43 superficial, 34 invasive) were immunostained with PCNA and Ki-67 and stained with AgNOR. Thirteen specimens of normal bladder mucosa served as controls. *Results*: In comparison to normal bladder mucosa the values of the three indicators were significantly greater ($p < 0.001$). There was a significant relationship between PCNA, Ki-67 indices, AgNOR scores and grade and stage of the tumor ($p < 0.001$). All indicators also correlated with each other ($p < 0.001$). The Kaplan-Meier curves for recurrence-progression free survival revealed that patients with a PCNA labelling index >36.22%, Ki-67 labelling index >29.68% and AgNOR score > 3.34 had a worse prognosis than those with <36.22%, <29.68% and <3.34, respectively. *Conclusions*: PCNA, Ki-67 indices and AgNOR scores correlated with each other and with tumor grade and stage. These proliferation markers may give objective and accurate information about the biological behavior of bladder transitional cell carcinoma.

Key words: Bladder, Ki-67, Proliferating cell nuclear antigen, Nucleolar organizer region, Transitional cell carcinoma

Introduction

Although the current system of classifying transitional cell carcinoma (TCC) of the bladder by stage and histological grade is useful, they are not reliable enough to predict the biological behavior of the tumor [1, 2]. Recently, several antibodies have been developed for estimating, immunohistochemically, the proliferative activity of TCC [3–5]. In addition, to estimate this activity morphometrical and cytochemical indicators have also been studied [6–10]. Information about the cellular proliferation rate on paraffin sections of the same tissue can be obtained by immunohistochemical detection of the proliferating cell nuclear antigen (PCNA) and Ki-67 and the histochemical assessment of nucleolar organizer region (NOR) number using a colloid silver nitrate staining technique. These three methods are relatively simple, rapid and can be applied on archival material. Although, different markers of proliferation have been studied separately in many studies [8, 11], to the best of our knowledge, in only one study these three parameters were studied simultaneously on paraffin sections of the same tissue of TCC of the bladder [4]. In that study, Krüger and Müller showed that these three parameters could be correlated with tumor grade, but not with stage of transitional bladder carcinoma. They reported that especially for the heterogenous group of grade 2 carcinomas, the Ki-67 index was better than the other indicators of proliferation and allowed significant differentiation between invasive and noninvasive carcinomas. On the other hand, some

other studies showed that significant statistical correlation existed between the PCNA index and grade or stage of the tumor [1, 5, 12–14]. The aim of this study was to investigate the correlation of different proliferation markers with grade and stage of bladder TCC and to determine whether the PCNA, Ki-67 indices or AgNOR score predict recurrence and progression.

Materials and methods

A total of 77 patients (60 men and 17 women) with newly-diagnosed TCC of the bladder, who underwent transurethral resection or partial or radical cystectomy between February 1993 and September 1998, formed the basis of this study. The mean age of the patients was 58.6 years (range 26–80 years). Thirteen biopsies of bladder were taken from patients who underwent transvesical prostatectomy for benign prostatic hyperplasia (BPH) served as controls. The histological assessment of grading was performed according to the grading system, proposed by Ash [15]. In this grading system, benign-appearing papillary tumors were classified as grade 1 TCC and tumors were divided into four grades. Tumors were staged according to the UICC system [16]. Follow-up investigations were carried out regularly. The patients were followed for a period of 6–72 months (median, 45 months).

Tissues were fixed in 10% formalin and embedded in paraffin wax. Sections were cut at 5 μm and stained with haematoxylin and eosin.

Immunohistochemical and histochemical assays

Immunostaining with PCNA (PC 10/Zymed) and Ki-67 (MIB-1/Zymed) antibodies was performed by streptavidin-biotin-peroxidase method using the Zymed histostain kit, with microwave pre-heating method for antigen retrieval. Briefly, sections of 5 μm thick were mounted on poly-L-lysin (Sigma) coated glasses. Sections were deparaffinized and then placed in plastic jars containing 10 mM Na-citrate buffer (pH 6). Jars were heated in a microwave oven at 700W for four 5 minutes cycles with an interval of 1 minute between cycles. Jars were then removed from the oven and allowed to cool for 20 minutes at room temperatures. Sections were washed with phosphate buffered solution (PBS) for 5 minutes and then incubated with 3% H_2O_2 for 5 minutes to block endogeneous peroxidase activity. Slides were incubated with protein blocking solution to block nonspecific background staining and then sections were covered with monoclonal primary antibodies (anti-PCNA with a dilution of 1/50 and anti-Ki-67 with a dilution of 1/50) for 2 hours. The sections were treated with biotin-conjugated secondary antibody for 15 minutes in room temperature and then with streptavidin-peroxidase complex for 15 minutes. The sections were incubated with amino-ethyl-carbozole for 5 minutes and counter staining with Mayer's hematoxylin was performed. A normal human tonsil was served as positive control for both PCNA and Ki-67 immunoreactivity. The negative controls were performed by substituting primary antibody for PBS.

About 1000 tumor cells were counted from well-labeled areas to determine the PCNA and Ki-67 indices. Counting was performed at $\times$400 magnification with a light microscope. PCNA and Ki-67 indices were expressed as the percentage ratio of total labeled cells to the total number of cells counted.

AgNOR staining

For the demonstration of NORs, we employed the colloid silver nitrate technique. Briefly, the sections were deparaffinized and incubated with AgNOR solution in a dark room for 45 minutes. After washing with distilled water, sections were incubated in alcohol for 4 minutes, in xylene for 3 minutes and then mounted with mounting medium. The number of nuclear dots (AgNORs) from 100 cells was counted using $\times$1000 immersion lens. The mean score of AgNORs per cell was expressed as AgNOR score.

Statistical analysis

The mean value was expressed as the mean $\pm$ standard deviation. Differences between the means were tested by Kruskal-Wallis analysis of variance (multiple groups), Student's test, and Mann-Whitney U test (2 groups). Survival analysis were constructed using method of Kaplan-Meier with statistical significance determined by the Log-rank test. Relationship between two variables were assessed by Spearman rank correlation coefficient.

Table 1. The distribution of patients into clinical stage groups and grades

Histological grade	Clinical stage			
	Ta	T1	T2 or higher	Total
1	11	2	2	15
2	14	11	14	39
3	0	4	11	15
4	0	1	7	8
Total	25	18	34	77

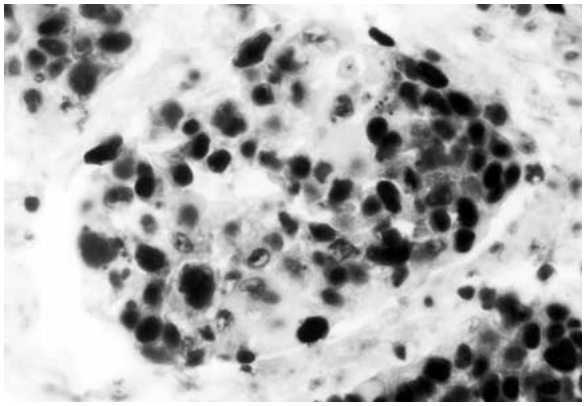

Figure 1. Tumor cells expressing PCNA in a high grade TCC (×100).

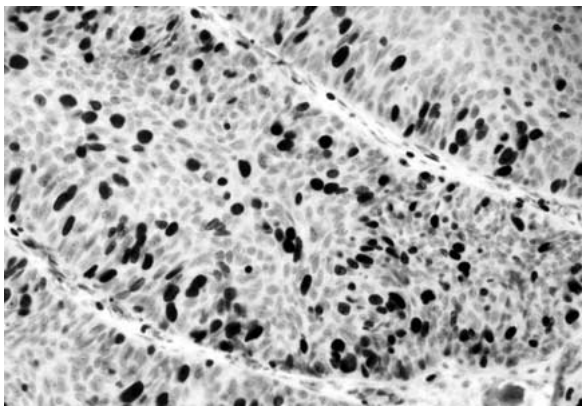

Figure 2. Ki-77 immunostaining of tumor cells in a low grade papillary TCC (×50).

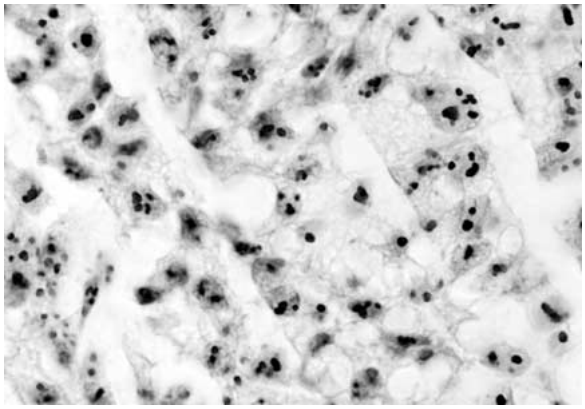

Figure 3. NORs stained with silver in tumor cell nuclei (×1000, immersion).

Results

The clinical stage and histological grade of 77 patients are summarized in Table 1. Majority of superficial TCCs of the bladder were grade 1 and 2. In contrast, most invasive TCCs were grade 3 or 4. There was a significant correlation between grade and stage (r: 0.59, $p < 0.001$). With PCNA and Ki-67 monoclonal antibodies, red-brown granular nuclear staining is observed in tumor cells. PCNA and Ki-67 positive tumor cells were mostly located in basal parts of the papillary formations (Figure 1, 2). It was noticed that nuclear NORs were small and dispersed in high grade tumors, in contrast, NORs were grouped in low grade tumors (Figure 3).

The mean (±SD) values of PCNA, Ki-67 and AgNOR scores of normal bladder mucosa and TCCs (grade 1–4) are given in Table 2. There was a significant relationship between PCNA, Ki-67 indices, AgNOR scores and grade ($p < 0.001$). One may notice that the higher the tumor grade, the higher the PCNA, Ki-67 and AgNOR scores. Compared to normal mucosa the values for all tumors were significantly greater ($p < 0.001$). All indicators also correlated significantly with each other ($p < 0.001$) (Table 3).

The mean (± SD) values of PCNA, Ki-67 and AgNOR scores of different stages are shown in Table 4. It is apparent that the more advanced the stage, the higher the PCNA, Ki-67 and AgNOR scores. On the other hand, all invasive carcinomas (T2 or higher) showed significantly greater proliferative activity for all indicators, than had superficial carcinomas ($p < 0.001$) (Table 5).

The average values of PCNA and Ki-67 indices, and AgNOR score of all the patients were 36.22 ± 14.07%, 29.68 ± 12.70% and 3.34 ± 0.85 (mean ± standard deviation), respectively. In each group,

Table 2. Relationship of normal bladder mucosa and histologic grade with PCNA, Ki-67 and AgNOR scores (*: $p < 0.001$ vs. Normal mucosa)

Parameters	Normal mucosa (n = 13)	Carcinoma grade 1 (n = 15)	Carcinoma grade 2 (n = 39)	Carcinoma grade 3 (n = 15)	Carcinoma grade 4 (n = 8)
PCNA score, % (mean ± SD)	2.24 ± 0.43	21.21 ± 6.49*	31.35 ± 6.11*	51.36 ± 7.82*	59.65 ± 2.50*
Ki-67 score, % (mean ± SD)	1.69 ± 0.47	16.06 ± 6.41*	25.23 ± 5.29*	44.65 ± 6.47*	49.72 ± 4.15*
AgNOR score (mean ± SD)	1.67 ± 0.28	2.60 ± 0.45*	3.11 ± 0.61*	4.01 ± 0.79*	4.53 ± 0.34*

Table 3. Correlation between different parameters of proliferation in bladder carciomas (n = 77) (Spearman rank correlation coefficient) ($p < 0.001$)

	Stage	Grade	PCNA	Ki-67	AgNOR
Stage	–				
Grade	0.61	–	–	–	–
PCNA	0.64	0.88	–	–	–
Ki-67	0.63	0.87	0.95	–	–
AgNOR	0.52	0.70	0.74	0.71	–

Table 4. Mean (± SD) values of PCNA, Ki-67 and AgNOR scores of our cases according to the stage

	Stage		
	Ta	T1	T2 or higher
PCNA score,% (mean ± SD)	23.26 ± 5.86	38.23 ± 11.41	44.66 ± 12.68
Ki-67 score, % (mean ± SD)	18.02 ± 6.03	31.56 ± 10.76	37.24 ± 11.01
AgNOR score (mean ± SD)	2.72 ± 0.53	3.47 ± 0.50	3.72 ± 0.93

patients were divided into two subgroups, depending on the amount of PCNA, Ki-67 and AgNOR stainings. The Kaplan-Meier curves for recurrence-progression free survival of these two subgroups are shown in Figures 4, 5, and 6. Patients with a PCNA labelling index >36.22%, Ki-67 labelling index >29.68%, and AgNOR score >3.34 had a worse prognosis than those with <36.22%, <29.68%, and <3.34, respectively. The difference between each subgroups was statistically significant ($p < 0.001$).

Table 5. Mean (± SD) values of PCNA, Ki-67 and AgNOR scores in superficial and invasive bladder carciomas

	Superficial carcinoma (n = 43)	Invasive carcinoma (n = 34)	*P*-value
PCNA score, % (mean ± SD)	29.53 ± 11.32	44.66 ± 12.68	<0.001
Ki-67 score, % (mean ± SD)	23.69 ± 10.64	37.24 ± 11.01	<0.001
AgNOR score (mean ± SD)	3.03 ± 0.63	3.72 ± 0.93	<0.001

Discussion

It is well known that histological characteristics of TCC of the bladder are not reliable enough to predict their biological behavior [2, 17, 18]. To get more objective and accurate information about the biologic behavior of the tumors, several immunohistochemical antibodies have been developed for estimating the proliferative activity of TCC. The ideal marker for cellular proliferation should be one that is simple to measure, applicable to different fixation processes and is easy to interpret results with high reproducibility and reliability. PCNA, Ki-67 and AgNOR are the markers of proliferation which have been widely used to determine the proliferation activity of tumors [1, 4, 11].

PCNA, a 36-KD nuclear acidic protein, is necessary for cellular proliferation and its expression is elevated in the nucleus during the late G1 phase with a maximum expression during the S-phase and a decline during G2 and M-phases [5, 12]. Several

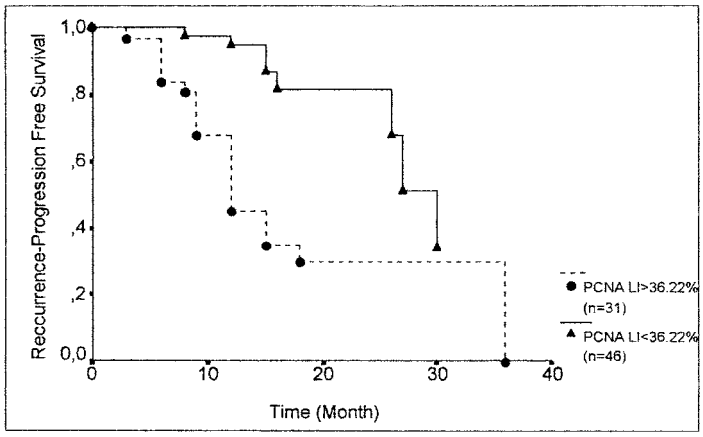

Figure 4. Kaplan-Meier curves for recurrence-progression free survival according to the PCNA labelling index (Log-Rank test, $p < 0.001$).

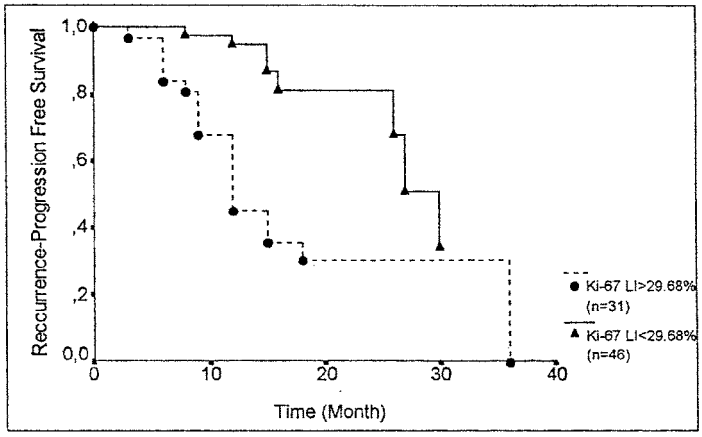

Figure 5. Kaplan-Meier curves for recurrence-progression free survival according to the Ki-67 labelling index (Log-Rank test, $p < 0.001$).

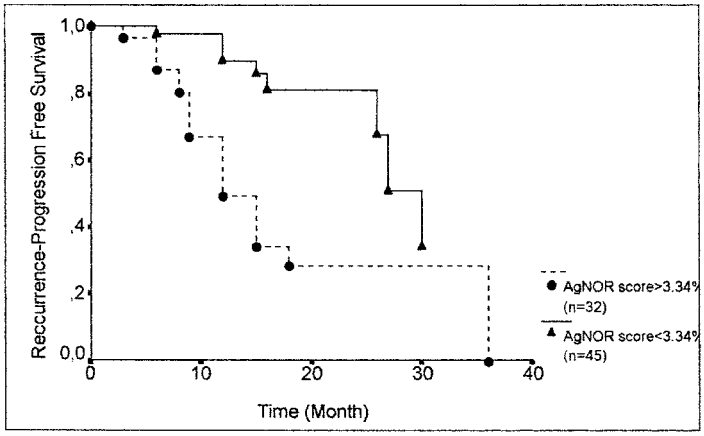

Figure 6. Kaplan-Meier curves for recurrence-progression free survival according to the AgNOR score (Log-Rank test, $p < 0.001$).

studies revealed that in TCC of the bladder, expression of PCNA immunostaining gradually increased as the grade and stage became higher [1, 5, 13, 19]. However Krüger and Müller reported that PCNA index although correlated with tumor grade, it did not correlate with stage of the tumor (4). In our study, we like many others, found that PCNA index correlated with tumor grade and stage.

The relationship between PCNA labelling index and survival was investigated by Hattori et al. [5] in 56 bladder cancer patients (39 superficial, 17 invasive). Using anti-PCNA monoclonal antibody (Novo Casta, Newcastle, UK) they reported that patients with an index of <12% had a good prognosis than those with an index of >12%. In our study survival analysis revealed that, those patients with a PCNA labelling index >36.22% had a worse prognosis than those with an index <36.22%. As we had more patients with invasive carcinoma in our study group than Hattori et al.'s study group, this difference might be explained by this factor. Additionally, histopathological heterogeneity and sampling might also effect the PCNA labelling index values. Finally, we used a different PCNA antibody for immunostaining (PC 10/ Zymed) than Hattori et al's. Other studies, like our study, reported high mean PCNA index scores [1, 4, 14].

Ki-67 monoclonal antibody is widely used to determine the cellular proliferation rate of malignant tumors [11, 20]. This antigen is expressed in all stages at the cell cycle except for Go. It is also well established that, MIB-1 monoclonal antibody which recognizes the Ki-67 antigen on routinely fixed paraffin-embedded sections, is Ki-67 equivalent on the basis of immunostaining [20]. Several studies showed that Ki-67 labeling index correlated very well with the two prognostic factors for bladder cancer, tumor stage and grade [11, 17, 21]. In accordance with these studies, we demonstrated that Ki-67 labeling index correlated with tumor grade and stage. On the other hand, Krüger and Müller showed that, although Ki-67 index correlated with tumor grade, the correlation of this index was not evident for the stage. However, Ki-67 index still found to be the most suitable proliferation marker for discriminating between invasive and noninvasive carcinomas especially for the heterogeneous group of G2 carcinomas, in that study.

Few studies investigated the relationship between Ki-67 and recurrence of bladder cancer [11, 22, 23]. Stavropoulos et al evaluated recurrence in 26 superficial bladder cancer cases and reported significantly higher Ki-67 labelling index values with recurrence [22]. Asakura et al reported that recurrence rate of superficial bladder cancer was significantly higher in cases with a Ki-67 labelling index of 5.35 or greater than in those with a value less than 5.35 [11]. Gontero et al emphasised that for Ta-T1/G1-G2 tumors, only multifocality and Ki-67 considered either as absolute value (p = 0.03) or with the cut-off value of 20% (p = 0.019) were found to be independent prognostic factors of recurrence. In our study, survival analysis revealed that, those patients with a Ki-67 labelling index >29.68% had a worse prognosis than those with an index <29.68%. As we had both superficial and invasive bladder carcinoma patients in our study group, this might be the reason of a higher Ki-67 labelling index value in our study. Additionally, histopathological heterogeneity might cause difficulty in obtaining a representative sample from the tumor. So, sampling might also effect the Ki-67 labelling index values in different studies.

The number of AgNORs per cell also correlated with cellular activity and possibly with proliferation [24]. They reflect the transcriptional activity of the rRNA gene and play an important role in protein synthesis. Although Skopelitou et al. [14] reported a significant correlation of AgNOR scores with histologic grade and clinical stage in urinary bladder cancer, Krüger and Müller could not manage to correlate AgNOR count with tumor stage [4]. Few studies investigated the relationship between AgNOR scores and recurrence of bladder tumor [25, 26]. Although Tomobe et al shown that proliferating cell NOR had a predictive value for local recurrence in patients with superficial bladder tumor, Korneyev et al reported that AgNOR scores were of no value in distinguishing superficial and invasive tumors or in predicting tumor related survival. In our study AgNOR counts correlated well with the grade and stage of the tumor. In addition, survival analysis revealed that patients with an AgNOR score >3.34 had a worse prognosis than those with <3.34.

Nevertheless, in only one study, different markers of proliferation have been studied simultaneously on paraffin sections of the same tumor and that study revealed that three parameters (PCNA, Ki-67 and AgNOR) correlated with tumor grade, but not with stage of the tumor [4]. However, they reported that for the heterogeneous group of G2 carcinomas Ki-67

index seems to be the most suitable for discriminating between invasive and noninvasive carcinomas. Our study is the second study which used the same three parameters simultaneously on paraffin sections of the same tumor. We found that all three parameters correlated well with the tumor stage and grade. All indicators also correlated significantly with each other.

Using the grading system proposed by Ash [15], we consider the benign appearing papillary tumors as grade 1 carcinomas. We have shown that there is a significant difference between normal bladder mucosa and grade 1 TCC according to the PCNA, Ki-67 indices and AgNOR score. So, there must be an increased proliferative activity in grade 1 TCC (or papilloma in some other grading systems) compared to normal bladder mucosa. That's why we think that, papillomas must be graded as grade 1 TCCs.

In conclusion, the three indicators, PCNA, Ki-67 and AgNOR correlated with each other and with tumor grade and stage. We found no difference between these three indicators in discriminating the invasive tumors from the noninvasive ones. Our results suggest that these proliferative markers may give objective and accurate information about the biological behavior of bladder TCCs.

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