Image analysis and Ki-67 expression in urothelial dysplasia and carcinoma

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Background/aim
Cancer of the urinary bladder is a worldwide disease in which transitional cell carcinoma is the most common histologic type. The diagnosis of dysplasia is particularly important, as it is the precursor of invasive carcinoma. The present study aimed to investigate the role of image analysis together with Ki-67 immunostaining in bladder dysplasia and invasive urothelial carcinoma.
Materials and methods
This study was carried out in 80 urinary bladder paraffin blocks that were selected from the Department of Pathology of Kasr El-Aini Hospital, Cairo University, Egypt. The studied cases were divided into four groups: six cases of normal bladder mucosa, 12 cases of chronic cystitis, 18 cases of epithelial dysplasia, and 44 cases with transitional cell carcinoma. Morphometric analysis and Ki-67 expression were studied in all cases using an image analysis system.
Results
All morphometric parameters, DNA index, and proliferating cells' percent and Ki-67 index were increasing from normal, chronic cystitis, dysplasia to carcinoma cases. However, nuclear area, length, size, and epithelial stromal ratio showed significant differences between dysplasia and carcinoma cases ($P<0.05$). High-grade carcinoma showed significant enlargement of nuclear area and size, as compared with low-grade carcinoma. DNA index and proliferating cells' % showed a significant difference between dysplasia and carcinoma cases. Both parameters were significantly higher in high-grade carcinoma. Normal bladder and chronic cystitis cases exhibited negative stain for Ki-67. However, all cases of dysplasia and carcinoma exhibited a positive stain for Ki-67. The carcinoma cases showed a significantly higher Ki-67 index (68%) than the dysplastic cases (34%).
Conclusion
The present study revealed the usefulness of image analysis together with Ki-67 expression in discriminating cases of bladder dysplasia and carcinoma.

Keywords:
carcinoma, dysplasia, image analysis, immunohistochemistry, Ki-67, morphometry, urothelium

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Introduction

Transitional cell carcinoma comprises 90% of all primary tumors of the urinary bladder. It is the seventh commonest cancer worldwide in male individuals and the 17th commonest cancer in female individuals. Most of the newly diagnosed cases are carcinoma *in situ* (CIS). Smoking is one of the most common risk factors accounting approximately for half of all cases of the urothelial bladder carcinoma with a sharp correlation between smoking habits and occurrence of nuclear atypia in the transitional epithelium. Other important risk factors include occupational exposure to aniline dyes, aromatic hydrocarbons, and aromatic amines [1].

Transitional carcinoma including papillary tumors and flat urothelial CIS are diagnosed primarily on the basis of cytologic atypia with or without papillary growth pattern. The diagnosis of urothelial dysplasia is particularly important because it is the precursor of invasive transitional carcinoma [2].

Nuclear findings are the most important parameters to classify and diagnose urothelial tumors, and nucleomegaly of urothelial cells is a helpful feature for detecting cytologic atypia [3].

Modern technology using image analysis has afforded to make objective measurements to discriminate high-grade dysplasia or CIS and invasive urothelial carcinoma [1]. Rosenthal *et al.* [4] used other morphometric criteria including nuclear to cytoplasmic ratio (N/C) to differentiate atypical urothelial cells and high-grade

transitional carcinoma and identified atypical urothelial cells with N/C ratio greater than 0.5, which has a high prediction for high-grade carcinoma, whereas an N/C ratio less than 0.5 should be considered as indicative of benign disease.

In contrast, the development of many biomarkers enabling the diagnosis of urothelial carcinoma at an earlier stage remains challenging. The Ki-67 antigen is a classic marker of cell proliferation and an important prognostic indicator of aggressiveness, progression and recurrence of tumors [5]. Ki-67 is a DNA-binding nuclear protein that is preferentially expressed through all phases of the cell cycle wherein it is absent from resting cells in GO phase; thus, it is an excellent biomarker that determines the growth fraction of a cell population [6].

However, there is a still debate with regard to the significance of Ki-67 in urothelial carcinomas, because many reports identified it as a poor prognostic marker [7,8], whereas others disagreed with these studies [9,10].

To obtain reliable and objective criteria for diagnosis and predicting the prognosis of urothelial carcinoma, many quantitative methods were developed including nuclear morphometry, flow cytometry, immunohistochemistry, and genetic studies [11].

The present study is focused on the retrospective study of cases of urothelial dysplasia and invasive urothelial carcinoma using morphometric parameters and Ki-67 expression in order to investigate usefulness in differentiating dysplasia from carcinoma cases.

Materials and methods

Study samples, design, and ethical approval

The material used in this study consisted of 80 urinary bladder specimen paraffin blocks that were selected from the Department of Pathology of Kasr El-Aini Hospital, Cairo University, Egypt. The studied cases were divided into four groups: six cases of normal bladder mucosa, 12 cases of chronic cystitis, 18 cases of epithelial dysplasia, and 44 cases with transitional cell carcinoma. The samples were obtained as paraffin blocks. Detailed history and clinical data of patients were taken. The study was approved by the Institutional Ethical Committee of National Research Centre, Cairo.

The carcinoma cases were graded according to the criteria of WHO [3]. In this study, the carcinoma cases of grades I and II were considered low grade and those of grade III were considered high grade.

Three sections (4 μm thick) were cut from each block. One section was stained with hematoxylin and eosin for histopathologic evaluation and morphometric analysis. The second section was stained with Feulgen' stain for the measurement of proliferation parameters. The third section was mounted on a positively charged glass slide for immunohistochemical staining using anti-Ki-67 antibody as a proliferation marker.

Morphometric parameters included in this study were nuclear area, nuclear length (long axis), nuclear perimeter, nuclear roundness, nuclear size, and epithelial/stromal ratio. Proliferation parameters included in this study were DNA index (DI), proliferating index, and Ki-67 labeling index.

Image analysis

The image analysis was performed at the Pathology Department, National Research Centre, using the Leica Qwin 500 Image analyzer (LEICA Imaging Systems Ltd, Cambridge, UK), which consisted of a Leica DM-LB microscope with a JVC color video camera attached to a computer system.

Morphometric parameters

The examined slides were placed on the stage of the microscope. The light source was set to the required level. Successful adjustment of illumination was checked for on the monitor. The morphometric analysis was carried out on hematoxylin and eosin-stained slides.

Nuclear parameters were measured at magnification ×400. The selected nuclei were surrounded by a line to be covered automatically by a green mask, which is called a binary image. The parameters of this binary image appeared automatically in the form of a table in micrometers, and, finally, the mean and standard variation of all parameters examined were determined.

Epithelial stromal ratio was measured at magnification ×50. The epithelial tissue to be measured was determined by drawing a line around it. Then, it was covered automatically by a green mask, which is called binary image. The area percentage of the binary image is calculated automatically by the software. The mean percentage of all fields examined is determined.

DNA cytometry parameters

The Feulgen staining reaction is specific for DNA; it gives specific blue staining to the nuclear DNA. Nucleoli and cytoplasm should show no staining. The stained DNA can then be quantified using the cytometry program of the Leica Qwin 500 Image Analyzer.

We place the slide to be examined on the stage of the microscope and focus it at high-power magnification (×400). The light source is set to the required level by the software. DNA cytometry was performed on real-time image from the microscope, which we visualized on the video monitor.

DNA analysis was performed first on the normal control specimens to determine the reference DNA values. Selection of nuclear boundaries is usually performed automatically by the image analysis system; however, some degree of interaction or editing is usually needed for optimal nuclear selection 'Touching' nuclei can be 'cut' from each other, and cellular fragments or extraneous cells can be erased before DNA measurements. Only separate, intact nuclei were measured. Distorted or overlapping nuclei and nuclear fragments were manually eliminated from the measurement. All these facilities were supplied as editing function in the Leica Qwin 500 image analysis systems.

Care was taken to measure various nuclei representative of the examined lesion, so that measurements were not biased toward the bizarre or anaplastic nuclei. Each field was focused before the measurement to exclude cut nuclei and blurred ones. The optical density of the selected nuclei in each microscopic field was then measured and automatically converted by the system into DNA content. We selected many fields until the desired number of nuclei (100–150) had been measured. The percentages of proliferating cells and the DI were calculated and determined automatically by the system. All collected data were stored to be reanalyzed.

Immunohistochemical study

For immunostaining, the sections were deparaffinized and rehydrated through a graded series of alcohol. Endogenous peroxidase activity was blocked by freshly prepared 0.3% hydrogen peroxide in methanol for 20 min. Thereafter, microwave antigen retrieval was used, followed by incubation with the Ki-67 antibody (clone MIB-1, 1 : 50 dilution; DAKO, Glostrup, Denmark). The Ultravision LP polymer system (Labvision, Egypt) and the chromogen diaminobenzidine were used to amplify and visualize the antigen–antibody complex. The expression of Ki-67 was evaluated in the entire section at a magnification of ×400. Ki-67 showed nuclear staining. The Ki-67 labeling index was determined as the percentage of positively stained cells to the total number of cells. The cases were considered positive when the Ki-67 labeling index was greater than 20% [12].

Statistical analyses

Comparison of quantitative variables between the study groups was carried out using the one-way analysis of variance test. The correlation was carried out using the Pearson correlation test. *P* value less than or equal to 0.05 was considered statistically significant (S). Statistical calculations were performed using Microsoft Excel version 7 (Microsoft Corp., Redmond, Washington, USA) and SPSS for Windows version 16 (SPSS Inc., Chicago, Illinois, USA) software.

Results

The cases included in this study were classified into six cases of normal bladder tissue, 12 cases of chronic cystitis, 18 cases of urinary bladder dysplasia, and 44 cases of transitional cell carcinoma.

Of the 18 dysplastic lesions, only four cases were of high grade (CIS), and the remaining 14 cases were of low grade.

The carcinoma cases were graded according to WHO. Six cases were grade I, 22 cases were grade II, and 16 cases were grade III. In this study, the cases of grades I and II were considered of low grade (28 cases) and those of grade III were considered of high grade (16 cases).

Image analysis

Morphometric parameters

Nuclear parameters: the mean values of nuclear parameters including area, length, perimeter, roundness, and size in the all studied groups are shown in Table 1.

All parameters were increasing from normal, chronic cystitis, dysplasia to carcinoma cases with nonsignificant differences between normal, cystitis, and dysplasia cases. However, area, length, and size showed significant differences between dysplasia and carcinoma cases (Table 1). However, nuclear morphometry of the four cases of CIS revealed large cell nuclei (nucleomegaly) with nonsignificant differences (area: 56.7, length: 10.2, and size: 4.3) when compared with cases of urothelial carcinoma. Moreover, carcinoma cases showed significantly higher area and size in high-grade cases than in low-grade ones (Table 2).

Epithelial stromal ratio: epithelial stromal ratio in the studied groups was increasing from normal, chronic cystitis, dysplasia to carcinoma cases with nonsignificant differences between normal, cystitis, and dysplasia cases. However, it showed significant differences between dysplasia and carcinoma cases

Table 1 Comparison of morphometric parameters, DNA cytometry, and Ki-67 index in the studied group

	Normal (*n*=6)	Chronic cystitis (*n*=12)	Dysplasia (*n*=18)	Carcinoma (*n*=44)
Image analysis				
Nuclear area	19.95±5.76[a]	22.25±8.96[a]	30.18±9.90[b]	55.16±20.78[c]
Nuclear length	5.12±1.10[a]	6.34±1.33[a]	7.65±1.46[b]	10.17±2.05[c]
Nuclear perimeter	14.39±2.60[a]	18.40±3.45[a]	21.71±3.64[a]	28.71±5.45[a]
Nuclear roundness	1.01±0.08[a]	1.17±0.10[a]	1.18±0.12[a]	1.18±0.09[a]
Nuclear size	2.52±0.89[a]	2.66±0.95[a]	3.10±1.10[b]	4.16±1.26[c]
Epithelial stromal ratio	10.85±1.76[a]	12.95±2.37[a]	14.50±3.21[b]	47.79±14.56[c]
DNA cytometry				
DNA index	1.01±0.30[a]	1.13±0.46[a]	1.54±0.51[b]	2.77±1.13[c]
Proliferating cells (%)	20.78±2.65[a]	25.27±2.97[a]	42.00±4.94[b]	72.29±1.89[c]
Ki-67 index%))	3±2.83[a]	5±3.32[a]	34±7.36 [b]	68±14.44[c]

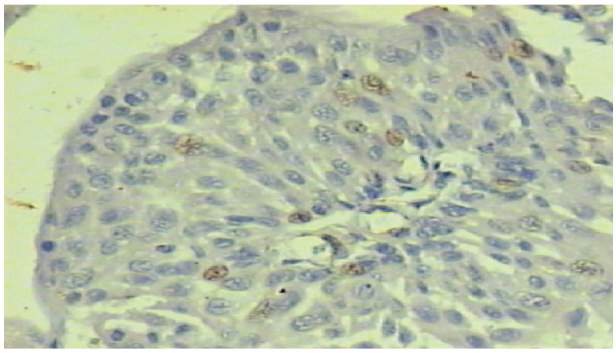
All data with different letters (a, b, c, d) are significant at $P<0.05$, using the analysis of variance test.

Table 2 Morphometric parameters, DNA cytometry, and Ki-67 index in the histologic grading of urothelial carcinoma

	Low grade (*n*=28)	High grade (*n*=16)
Image analysis		
Nuclear area	41.91±15.64	68.42±27.28*
Nuclear length	9.02±1.47	11.33±1.89
Nuclear perimeter	25.36±4.21	32.07±5.35
Nuclear roundness	1.20±0.09	1.17±0.12
Nuclear size	3.65±1.31	4.66±1.67*
Epithelial stromal ratio	56.85±15.45	67.74±16.34
DNA cytometry		
DNA index	2.11±1.03	3.43±1.79*
Proliferating cells (%)	67.13±1.44	92.19±2.57*
Ki-67 index (%)	52±13.25	84±16.78*

*Significant difference than low-grade carcinoma at $P<0.05$.

Figure 1



Normal urothelium negatively stained for Ki-67 (Ki-67 index <20%) (immunohistochemistry, ×200).

(Table 1). Epithelial stromal ratio was nonsignificantly different between high-grade and low-grade carcinoma cases (Table 2).

DNA cytometry parameters

In this study, DI and proliferating cells' % were increasing from normal, chronic cystitis, dysplasia to carcinoma cases with nonsignificant differences between normal, cystitis, and dysplasia cases. These two parameters showed significant differences between dysplasia and carcinoma cases (Table 1).

Furthermore, in carcinoma cases, high-grade carcinoma cases showed significantly higher DI and proliferating cells' % than low-grade carcinoma (Table 2).

Immunohistochemical study

In this study, the cases were considered positive when the Ki-67 index was greater than 20%. Normal bladder and chronic cystitis cases were negatively stained for Ki-67 wherein the mean of Ki-67 index was less than 20% (3 and 5%, respectively). Cases of dysplasia and carcinoma cases were positively stained for Ki-67, wherein the carcinoma cases showed a significantly higher Ki-67 index (68%) than the dysplastic cases (34%) (Table 1 and Figs 1, 2).

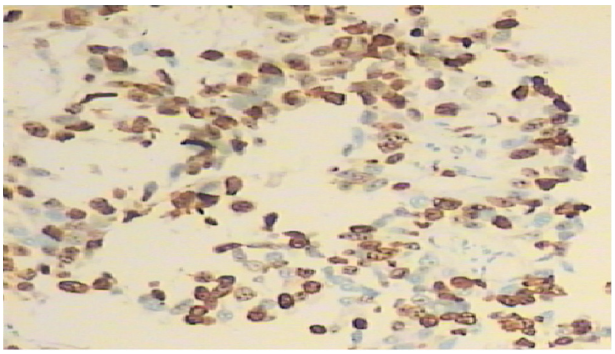
Ki-67 index was significantly higher in high-grade carcinoma cases than in low-grade ones (Table 2).

Discussion

Transitional cell carcinoma of the urinary bladder is one of the important malignant tumors in both sex groups [13]. The major problems in managing patients with transitional cell carcinoma are intravesical recurrence and disease progression. Although radical cystectomy is the standard treatment, unfortunately, many patients with apparently adequate surgical resection subsequently develop recurrence and die of metastases [14].

Much attention has been focused on papillary urothelial tumors that are diagnosed primarily by cytologic atypia. In contrast, less clear morphologic criteria are applied to dysplastic lesions or urothelial CIS, which are particularly important being the precursor lesions to invasive carcinoma [2].

Figure 2



High expression of Ki-67 (Ki-67 index 80%) in transitional cell carcinoma (immunohistochemistry, ×200).

Nuclear findings are important parameters in diagnosing urinary bladder malignancies [3]. Recent advances in computer technology have been utilized for diagnosis and research purposes in many tumors using image analysis [15,16]. In contrast, the development of ideal biomarkers for bladder cancer would enable diagnosis at an earlier stage of disease, and many have predictive value of disease progression [17].

Therefore, the present study aimed to assess the correlation of image morphometry and the proliferative marker Ki-67 in diagnosing cases of bladder dyplasia, and invasive transitional carcinoma.

In this study, digital image analysis of nuclear parameters of urinary bladder lesions was increasing from normal, chronic cystitis, dysplasia to cases of carcinoma with nonsignificant difference between normal, cystitis, and dysplasia cases. In contrast, a significant difference was found in the nuclear area, length, and size between dysplastic lesions and carcinoma.

Nuclear morphometry of the four cases of CIS revealed large cell nuclei (nucleomegaly) with nonsignificant differences when compared with cases of urothelial carcinoma. Suggesting being the precursor lesion to invasive carcinoma with high-grade cytologic atypia. However, these results must be confirmed in the future using a large number of cases of CIS. However, measured nuclei of high-grade urothelial carcinoma had the greatest nuclear parameters compared with the other categories. Results of the study by Poropatich *et al.* [2] demonstrated nuclear parameters of CIS, the width, length, and area being larger than low-grade and high-grade urothelial carcinoma.

Poropatich *et al.* [2] studied nuclear size for distinguishing urothelial carcinoma from reactive urothelium on tissue sections, and they showed that nuclear size is highly sensitive and can be used to differentiate the two categories in daily practice.

In the present study, a significant difference in the nuclear area and size was demonstrated between low and high-grade carcinoma. Sangwan *et al.* [18] support our results as they showed that the mean nuclear area for high-grade malignant potential was significantly higher than that of low-grade malignant potential.

Al-Obaidi and Al-Obaidi [13] checked nuclear morphometric features (nuclear area and roundness) using image analysis in different grades (I–III) of transitional cell carcinoma. Their results showed no statistical difference in nuclear roundness between the three grades, whereas there was a statistical difference in the mean nuclear area of grades I and III ($P<0.05$). No such difference was found between grades I and II or grades II and III ($P>0.05$).

On studying epithelial stromal ratio, a nonsignificant difference was demonstrated between normal bladder epithelium, cases of chronic cystitis, and dysplasia. However, a significant difference was found between cases of dysplasia and carcinoma ($P<0.05$) and between low-grade and high-grade carcinoma.

Epithelial stromal ratio was found to have a role in discriminating atypical endometrial hyperplasia and well-differentiated endometrial carcinoma, as the distinction between both lesions continues to be difficult in diagnostic surgical pathology [19].

DI is defined as the DNA content of tumor cells in comparison with that of normal cells, which have a DI of 1 [20]. DI and proliferating cells' percentage are generally evaluated using image cytometry. Discrimination of cells in particular phases of cell cycle and their quantitation based on differences in DNA content is helped by computer analysis [21].

In the present study, both DI and proliferating cells' percentage were increasing from normal, cystitis, dysplasia to cases of carcinoma. However, both parameters showed only a significant difference between dysplasia and carcinoma. Many published literature related to the study of DNA content focused on clinical and prognostic correlations rather than their possible role in the therapeutic response [5,20,22]. These studies were in agreement on one issue, that abnormal DNA content and high proliferating cells' percentage are specific for tumor progression [23].

With the discovery of genetic alterations in oncogenes that accompany bladder tumorogenesis, it was found that they could be used as prognostic markers and targets for clinical therapy [24]. In contrast, cellular proliferation is an excellent prognostic indicator of aggressiveness, progression and recurrence of tumors [6]. Ki-67 is a classic marker of cell proliferation that is strongly associated with clinical outcome after local therapy in many tumors such as soft tissue, cervix, lung, breast, melanoma, prostate and hepatocellular carcinoma [25–28].

While providing important information for prognosis in cases of transitional cell carcinoma, current clinical and pathological variables have a limited ability to predict progression, recurrence or overall survival. In the present study, normal bladder and cases of cystitis were negatively stained for Ki-67. It was overexpressed in cases of dysplasia and infiltrating carcinoma showing indices of 34 and 68%, respectively. In cases of carcinoma, high expression was more common in high-grade cases. Similar results were obtained from other previous studies showing high expression of Ki-67 in high-grade carcinoma [8]. Tian *et al.* [29] showed high Ki-67 expression in association with the high T-stage, recurrence and tumor size. In contrast, Fan *et al.* [30] studied the association between Ki-67 expression and the clinicopathological features of the patients with transitional cell carcinoma. They demonstrated that higher expression of Ki-67 was present in muscle invasive than in noninvasive forms of the tumor, suggesting that its expression may be related to tumor aggressiveness. However, Feng *et al.* [10] found a negative association between expression of Ki-67 and tumor prognosis and recurrence. These conflicting results may be due to different experimental conditions such as sex-specific or age-specific factors; moreover, different techniques and varying concentrations of the antibody used may influence the immunehistochemical results.

In conclusion, the present study revealed the usefulness of image analysis for evaluating nuclear morphometric features together with Ki-67 expression in discriminating cases of bladder dysplasia and carcinoma and helps to identify cases of high-grade carcinoma. Further study with a large number of high-grade dysplasia (CIS) cases is recommended to evaluate morphometric parameters and to distinguish it from other benign and malignant urothelial lesions.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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