Value of the silver-stained nucleolar organizer regions technique in the differentiation between benign and malignant lesions in urine cytology

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Urinary bladder cancer is a common disease. In Iraq, it represents 12.3% of cancer cases among men, and 4.9% of cancer cases among women.[1] Cytological examination of urinary specimens is a relatively simple diagnostic procedure that can aid in the diagnosis of disease anywhere in the urinary tract; and nowadays, examination of urine cytology is regarded as part of the routine investigation of patients with hematuria, prostatism, and suspected urinary tract neoplasia. It is most useful in detecting high-grade bladder cancers; however, its limitation lies in the diagnosis of low-grade urothelial carcinoma since the cells usually do not exhibit the typical features of malignancy.[2] This problem has motivated the development of new techniques to augment routine methods, and to improve the accuracy, and reproducibility of prognostication. One of these ancillary techniques is the study of proliferative markers. Ncleolar organizer regions [NORs] are loops of ribosomal DNA (rDNA) occurring in the nucleoli of cells. Its evaluation is regarded as an indicator of cell proliferation. So, the aim of this study is to evaluate the application of the argyrophilic nucleolar organizer region (AgNOR) technique on urine cytology in the diagnosis of neoplastic, and non-neoplastic lesions of the bladder, using AgNOR count, size, and dispersion as parameters for evaluation and to study the correlation of these parameters with tumor grade.

The study was performed on 64 urinary specimens. Thirty of these were positive of malignant cells (22 males and 8 females with a male to female ratio of 2.75:1) and the selection criteria included patients complaining of gross hematuria and/or prostatism with radiological investigations suggestive of bladder tumor and confirmed by histopathological study of biopsy specimens taken cystoscopically. The other set represents 34 urine samples that were obtained from patients complaining of other urological diseases (such as vesical stone and urinary tract infection) and they were confirmed to be negative for malignancy both by pylogram and cytological study of urine samples and their stained slides were adequately cellular so they were considered as a control. Urine samples were collected from Iraqi patients attending Medical City and Al-Kadhimiya Teaching Hospitals, Baghdad, Iraq between May 2006 and December 2006. From each patient, 30 ml of mid stream freshly voided urine samples was collected. The samples were kept in an ice box, and processed within 2 hours from collection. Urine samples were centrifuged at 3000 rpm for 15 minutes. The supernatants were decanted, and the sediments were smeared on pre-albumenized slides using the micropipette method for obtaining the sediments. Four slides were prepared for each sample, and stained by routine Hematoxylin and Eosin stain. From these slides, the most representative ones were chosen to be re-stained with silver stain without prior de-staining.

Following the standard procedure adopted by Khan et al,[3] solution A was prepared by dissolving 500 mg gelatin in 25 ml de-ionized water along with 250 µl of formic acid (2% gelatin and 1% formic acid), and freshly prepared solution B was prepared by dissolving silver nitrate in distilled de-ionized water to make a 50% solution. Then, the working solution was prepared just before the staining by mixing one volume of solution A and 2 volumes of solution B. The nuclei were stained light yellow, and AgNORs were visualized as brown black discrete dots of variable size within the nuclei. Using oil immersion (× 1000), all distinguishable black to brown dots within the nucleus were identified, and treated as one AgNOR, and fine focusing was carried out to reduce the effect of dust particles, and deposit debris that interfered with the AgNOR dots. The mean AgNOR (mAgNOR) per nucleus was calculated by counting the AgNOR dots in 50 nuclei, and the average was counted. In addition to the AgNOR counts, variations in AgNOR size, and distribution were also recorded using the criteria of Ahsan et al.[4] The final diagnosis (gold standard) was determined by the histopathological report of subsequent bladder biopsies. There were no cases with confirmed diagnosis of grade 1 urothelial carcinoma (papillary urothelial neoplasms of low malignant potential according to the World Health Organization/International Society of Urological Pathology classification system.)[5] So, the diagnoses were grouped in 3 categories: (I) non-neoplastic lesions; (II) low grade carcinoma; and (III) high grade carcinoma. The AgNOR results of all nuclei were calculated, and expressed as the mean (± standard deviation). Student's t test was used to compare mean

AgNOR counts, and Chi-square test was used for evaluating AgNOR size, and distribution. A *p*-value <0.05 was considered statistically significant. Absolute confidentiality of the patients' vital information was maintained for ethical purposes, and ethical approval was obtained from the institution in which the study was carried out.

The sensitivity of the conventional urine cytology was 61%, and the specificity was 100% (there were 19 false negative cases, and there were no false positive cases). The mean age for the non-neoplastic group was 47.17 (range 30-65 years), and for the malignant group was 63.5 (range 41-82 years). The largest number of patients in the malignant group was within 50-69 years (19 cases out of 30 with a percentage of 63%). The main clinical presentation for 27 patients with bladder cancer was hematuria (only 2 patients presented with chronic cystitis-like symptoms, and one patient with micturition disorders). Their percentage was 90%. The samples were classified into 3 groups: non-neoplastic lesions, low grade urothelial carcinoma and high grade urothelial carcinoma according to the cytomorphological study of the hematoxylin and eosin stain stained slides. The final diagnosis of each patient was established from the histological reports of bladder biopsies. Eighty-three percent of high grade carcinoma cases showed a heterogenous pattern of distribution of AgNOR dots, while 58% of low grade cases were heterogenous in staining, and distribution) (**Figure 1**). The mean number of AgNORs per nucleus had the highest value in the high grade carcinoma group (**Table 1**). The mean differences were significant (p=0.0001) between urothelial carcinoma, and non-neoplastic cases. Regarding the histological grade; the mean AgNORs count of cancer cells increases from low grade to high-grade tumor, but the differences were statistically non-significant (p>0.05) (**Table 1**).

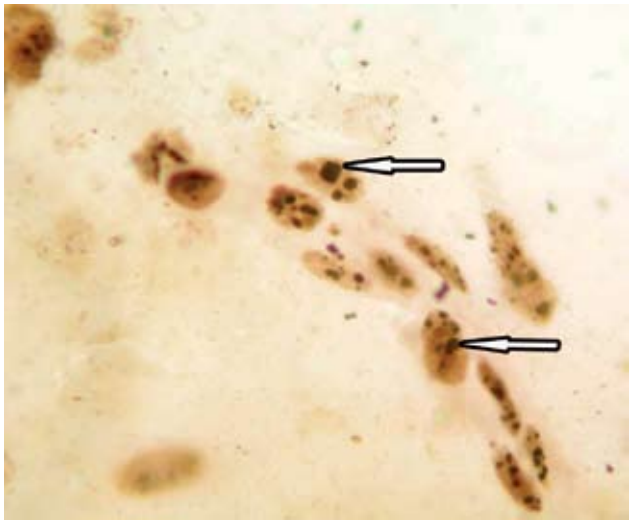

Figure 1 - Urine cytology showing high grade urothelial carcinoma cells. There are more than 2 different sizes of argyrophilic nucleolar organizer region (AgNOR) (arrows) with moderate distribution (size 2^+, distribution 2^+) (AgNOR stain × 1000).

By comparing the variation in AgNOR size, a significant (p=0004) proportion of high-grade urothelial carcinoma cases had an AgNOR size of (2+ to 3+) as compared to the low-grade carcinoma group (**Table 1**). There was no case of high-grade carcinoma with zero grade size; likewise, there was no non-neoplastic case with grade 3^+. At the same time, there was a significant difference p=0001 in AgNORs size between the non-neoplastic cases, and the carcinoma groups (**Table 1**). Results of the AgNORs distribution were similar. The AgNOR distribution was of higher grade in significantly greater proportion of high-grade urothelial carcinoma group (p=0.0003) when compared with the low-grade carcinoma group; and the difference in AgNOR distribution between non-neoplastic, and malignant cases was also significant p=0.0001 (**Table 1**). The AgNOR grade of 3+ distribution was not detected in any of the non-neoplastic cases.

The results of this study show that the "mean number of AgNORs per nucleus" is an important variable for the separation between the non-neoplastic urothelium, and low-grade carcinoma, but, is not very efficient in separating low, and high grade neoplasia. This observation might reflect a slight difference

Table 1 - Comparison of argyrophilic nucleolar organizer region (AgNOR) counts, size and distribution in low grade, high grade urothelial carcinoma cells and non-neoplastic urothelial cells.

Groups	No. of cases	Mean AgNOR counts/cell ± SD	AgNOR variation in size		AgNOR variation in distribution	
			0 to 1^+	2^+ to 3^+	0 to 1^+	2^+ to 3^+
Non-neoplastic cases	34	2.82±0.73	28	6	30	4
Low grade urothelial carcinoma	12	6.32±1.87	5	7	5	7
High grade urothelial carcinoma	18	7.94±3.51	3	15	4	14

in proliferation states between the different grades of urothelial carcinoma, and to some extent might be explained by the subjectiveness of grading as a quantitative method, with considerable intra-observer, and inter-observer variations. The addition of other parameters (namely size, and distribution of AgNORs within the nucleus) allows a better separation between low, and high-grade carcinomas. These results are in agreement with the study conducted by Khan et al[3] who investigated the usefulness of the AgNOR technique in evaluation of different grades of transitional cell carcinoma of urinary bladder, taking into account the differences in size, and distribution of the AgNORs as an additional parameter besides the mean number of the dots, and reported that the use of a combination of various parameters regarding the AgNOR dots might be helpful for differentiating bladder mucosa lesions. Typing of AgNOR size, and distribution was found to be easy, and reproducible in addition to traditional AgNOR counts for differentiating malignant from non-malignant urothelial cells, and low grade from high-grade urothelial carcinoma cells. However, it needs a lot of dedication, standardization, and meticulous bench work to achieve good results.

There are also certain limitations in handling urine sample that should be taken into account including the rapid deterioration of cells in urine (within one hour in hot weather) and the general hypocellularity of urine so pre-staining with Hematoxylin and Eosin stain can help to choose cellular slides to be stained for AgNOR and not to waste the materials on hypocellular slides.

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