ASSOCIATION BETWEEN AgNORs AND IMMUNOHISTOCHEMICAL EXPRESSION Of COX2 and iNOS IN BLADDER CANCER

Hassan Elsiddig Hassan FARAGALLA[1,2] , Hussain Gadelkarim AHMED[3], Ahmed Abdel Badie MOHAMED[4], Amel Omer BAKHIET[5], Faris Mohammednour ALTAF[6,7], Abdella Emam Abdella BARAGOB [8], and Ashraf Mohamed Elsayed Ali SAKRAN[9]

[1] Department of Public Health, Health Sciences College at Al.leith, Umm Al-Qura University, Saudi Arabia.
[2] Department of Histopathology and Cytology, Faculty of Medical Laboratory Science, Sudan University of Science and Technology, Khartoum, Sudan.
[3] Department of Histopathology and Cytology, Faculty of Medical Laboratory Science, University of Khartoum, Khartoum, Sudan.
[4] Department of Pathology, Faculty of Medicine, Elribat National University. Khartoum, Sudan.
[5] Department of Pathology, Faculty of Vet. Medicine, Sudan University of Science and Technology. Khartoum, Sudan.
[6] Department of anatomy, Faculty of Medicine, Umm Al-Qura University, Makkah, Saudi Arabia.
[7] Health Sciences College at Al.leith, Umm Al-Qura University, Saudi Arabia.
[8] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Umm Al Qura University, Makkah, Saudi Arabia.
[9] Department of anatomy, Faculty of Medicine, Umm Al-Qura University, Makkah, Saudi Arabia.

OBJECTIVE: The objective of this study was to find out if there is correlation between AgNORs (Argyrophilic Nucleolar Organizer Region-associated proteins), COX2 and iNOS expression in bladder cancer
METHODOLOGY: The present study investigated the relationship between means AgNORs count and immunohistochemical expression of COX2 and iNOS in bladder carcinomas in serial paraffin sections from 155 bladder carcinomas. Thirty nine control cases of benign bladder cystitis lesions were included.
RESULTS: Mean AgNOR counts correlated significantly with COX2 and iNOS, denoting P values of 0.000 for all three markers. Mean AgNOR count was significantly correlated with tumor type; the mean was 5.94 for SCC, 4.94 for TCC and 3.72 for benign bladder cystitis.
CONCLUSION: Strong link between cell proliferation and cellular proliferative activity. Thus the three investigated markers (AgNORs, COX2 and iNOS) can be used as pre-warning factors of bladder cancers among patients at risk of bladder cancer.

Keywords: Ag NORs, COX2. iNOS, Bladder cancer.

INTRODUCTION:

Bladder cancer is significant health problem, as it is the one of the most common cancers [1]. Worldwide, bladder cancer is diagnosed in approximately 275,000 people each year, and about 108,000 die of this disease [2].

There are several genetic and environmental factors that contribute to the development of bladder cancer, recently, the role of inflammation in the development and progression of bladder cancer has been increasingly recognized [3]. Inflammation can be directly or indirectly caused by smoking, chronic irritation, or infection [4].

Nucleolar organizer regions (NORs) are loops of DNA that are responsible for ribosomal RNA (rRNA) transcription. They are located in the nucleoli of cells and in the chromosomes 13–15 and 21, 22 in association with proteins [5]. As rRNA molecules are the main sites of protein synthesis, it follows that the number of NORs in each cell nucleus reflects cellular activity [6]. The number and size of Ag NORs can be assessed following staining of tissues or cells with silver stains [7]. Ag NORs correlate with the proliferative activity of neoplasm. Increased Ag NOR counts may reflect increased proliferative activity of cells [8, 9]. Ag NOR increasingly applied in histopathology research following suggestions that there may be a possible association between high Ag NOR counts and malignant transformation [10, 11].

AgNORs is correlated with the proliferative activity of neoplasms. Increased AgNOR counts may reflect increased proliferative activity of cells [12, 13]. AgNOR counts have been studied in bladder carcinoma, but the results have been conflicting.

Accurate histopathological typing, grading and staging of tumors are of proven value in the clinical management of cancer. In many cases, however, histopathological assessment does not correlate accurately with clinical outcome and may not reveal all possible markers of prognostic importance. These problems have motivated the development of new techniques to augment routine methods and to improve the accuracy and reproducibility of prognostication [14,15]. One of the most accurate conventional for the assessment of neoplastic is staining of the nucleolar organizing regions by silver compound (AgNORs), this method has become popular for its simplicity, ease of use, low cost and its good correlation with other proliferative markers, as their frequency within nucleus are significantly higher in malignant cells than in normal, reactive or benign neoplastic cells [16].

Cycloxgenase-2 (COX-2) is regarded as induced inflammatory mediator involved in the development of tumors. It is an inducible enzyme (also called prostaglandin syntheses) responsible for conversion of arachidonic acid to prostaglandins and other inflammatory mediators [17]. It is not detectable in most normal tissues; however, it is induced at sites of inflammation by cytokines, growth factors and tumor promoters [18]. Also, prominent COX-2 expression has been described in bladder cancers including transitional cell and squamous cell carcinomas and this expression correlates with tumor grade and invasion [19, 20].

Nitric oxide synthase (NOS) is the key enzyme for the conversion of L-arginine to L-citrulline and nitric oxide (NO) [21, 22]. The NOS family consists of endothelial, neuronal, and inducible nitric oxide synthase (eNOS, nNOS, and iNOS, respectively) [22] . iNOS genes located on the human chromosome 17 can be induced by lipopolysaccharide, cytokines in macrophages, or tumor- related immune reactions [23,24] they reported that iNOS was detected in human bladder cancer tissues but not in normal bladder tissues, and that it was found in macrophages and neutrophils of bladder cancer tissues and some tumor cells.

To our knowledge no published data explored the association between Ag NORs and immunohistochemical expression of COX2 and iNOS in bladder cancer

Therefore it is interesting to hypothesize that, the numbers, and the distribution of AgNORs within the nucleus, COX2 and iNOS expression might be of potential use in predicting behavior in bladder carcinomas. In this study, we investigated the association between AgNORs and immunohistochemical expression of COX2 and iNOS in bladder lesions.

MATERIALS AND METHODS:

One hundred and ninety four formalin-fixed, paraffin embedded tissue block samples from the bladder lesions were investigated. These included 87 cases of bladder squamous cell carcinoma, 68 bladder transitional cell carcinomas and 39 cases of benign bladder cystitis lesions. Data related to the studied subjects were retrieved from NHL and Soba teaching hospital- Khartoum. The biopsies were obtained from Sudanese patients with bladder lesions.

Sample processing: Serial sections on poly-L-lysine–coated slides for (immunohistochemistry and AgNORs) and one section on a regular slide for Hematoxylin and Eosin (H&E) procedure were prepared from each case. The immunohistochemistry staining was performed as followed.

Immunohistochemistry staining procedure: Sections were processed for immunostaining as follows:

The sections were de paraffinized with xylene and then both sections were hydrated through 100%, 90%, 70% and 50% ethanol. The sections then were treated for antigen retrieved by microwave treatment for 30 minutes in citrate buffer (pH 6.0). The slides were allowed cooling for 20 minutes in the citrate buffer before further treatment. After a quick rinse in phosphate buffered saline.

Endogenous peroxidase was blocked by immersing slides in methanol with 0.3% hydrogen peroxide for 30 minutes (Dako k0411 kit).

The specimens were incubated in 5% goat serum for 10 min to block non-specific binding. Primary antibodies were incubated for 1 hour in a humidity chamber using the following dilutions: COX-2 at a dilution of 1:50; polyclonal rabbit anti-iNOS antibody (Ab-1,Lab.Vision, Neo Markers,USA), Using antibody dilution at 10-20μg mL-1 and incubated for 1 hour in a humid chamber, washed in phosphate-buffered saline (PBS) incubated for 30 minutes with the secondary biotinylated antibody followed by avidin peroxidase complex for another 30 minutes according to the manufacturer's instructions (Universal Detection Kit, Dako, Denmark). A brown color was developed with 3, 3-diaminobenzidine tetra hydrochloride (DAB, Dako k0411 kit) for 5 minutes, washed in distilled water, and counterstained with Mayer's haematoxylin for 1 minute. The entire procedures were performing at room temperature. In addition, a negative control for both markers in which the primary antibody was omitted and replaced by phosphate buffered saline was used. Positive control sections were added to process with the bladder tissue sections in the same run for precision and standardization of the elaborated IHC results.

The immunostaining was evaluated by the following Strategies; for both markers, when less than5% of cells were stained positive classified as negative , less than 50% considered low intensity, more than 50% positive for immunostaining classified as high intensity.

AgNOR Staining Method: The sections were stained according to the AgNOR method. Working solution was freshly prepared by mixing one volume of 2% gelatin in 1% formic acid solution and two volumes of 50% aqueous silver nitrate solution. All sections were incubated with this silver solution for 30 minutes at room temperature in a dark medium, and they were protected in the dark until each slide was analyzed. Two investigators, blind to the study groups, analyzed the silver-stained cells under light microscope (Olympus BX-51, Japan) at 1000x magnification. All sections were screened horizontally from left to right, and AgNORs were counted in the nuclei of the first 50 nucleated cells. Cells with pyknotic nuclei were not counted.

The AgNOR count was made adopting the method described by [25].

AgNORs, which were visible as black-dark brown dots located within the nuclei of the cells, were counted; overlapped black dots were counted as one structure.

Statistical analysis: SPSS version 17 statistical software was used for statistical analysis. The numeric results (AgNOR counts and tumor markers scores) were expressed as mean $\pm$ SD, and the 95% confidence intervals (CIs) of the means were calculated.

The X2 test was used to compare the differences in categorical variables between the groups. Relationships between variables were analyzed using Pearson's correlation analysis. A $P < 0.05$ was considered statistically significant.

Table 1 - Association between mean AgNOR counts and different variables

Histopathological type	Tumor marker	Result	NO (%)	Ag NORs			p.value
				Minimum	Maximum	Mean±STD	
Bladder SCC	COX2	+ve	82 94.3%	2.00	6.00	3.4878 ±1.3988	.044
		-ve	05 5.7%	2.00	3.00	2.200 ± 0.4472	
	iNOS	+ve	74 85.1%	2.00	6.00	3.6351±1.39052	.000
		-ve	13 14.9%	2.00	3.00	2.1538±0.37553	
Bladder TCC	COX2	+ve	39 57.4%	2.00	5.00	2.4359±.94018	.027
		-ve	29 42.6%	2.00	3.00	2.0345±.18570	
	iNOS	+ve	18 29.4%	2.00	5.00	2.9444±1.21133	
		-ve	50 70.6%	2.00	3.00	2.0200±.14142	.000
Bladder cystitis	COX2	+ve	18 46.2%	1.00	3.00	1.9444±.80237	.001
		-ve	21 53.8%	1.00	2.00	1.2381±.43644	
	iNOS	+ve	17 43.6%	1.00	3.00	2.2353±.56230	
		- ve	22 56,4%	1,00	2,00	1,0455±,21320	.000

Ethical consent: The study was submitted and approved by the Faculty Research Board of Sudan University for Science and Technology.

RESULTS:

AgNORs dots were clearly visible on light microscopical examination as black silver binding dots within nuclei stained orange. AgNOR silver stain and immunohistochemistry for COX2 and iNOS, was applied in all benign and malignant lesions.

Details of mean AgNORs counting and association with other variables were summarized in **Table 1.** In malignant SCC group, AgNORs counts were significantly different with both COX2 and iNOS results (COX2 +ve =3.4878 versus COX2 –ve = 2.200 with P.value of .044 and iNOS +ve 3.635 versus iNOS –ve = 2.1538 with p.value of .000).

Similar finding detected in TCC group; (COX2 +ve =2.4359 versus COX2 –ve = 2.0345 with P.value of .027 and iNOS +ve 2.9444 versus iNOS –ve = 2.0200 with p.value of .000).

Also AgNORs counting show significant differences with both COX2 and iNOS markers results in benign Cystitis group (COX2 +ve =1.9444 versus COX2 –ve = 1.2381 with P.value of .001 and iNOS +ve 2.2353 versus iNOS –ve = 1.0455 with p.value of .000).

All three indicators AgNORs count, COX2 and iNOS scores were significantly positively correlated with tumor grades in SCC **(Figure 1(A, B, C)**; AgNORs with tumor grade denoting r=.600(**), P= .000, for COX2 r= .582(**), P=.000, and for iNOS r=.528(**) with p.value of .000. Similarly positive correlation between the investi-

gated indicators and tumor grade was detected in TCC group; AgNORs with tumor grade denoting r= .300(*) p= .013, for COX2 r= .385(**), p= .001, and for iNOS r=.450(**) with p.value of .000 (Figure 2(A, B, C).

Among the study samples, AgNORs dots/cell was significantly positively correlated with both tumor markers (COX2 and iNOS) scores, COX2 denoting r= .693(**), p= .000, and for iNOS

r= .807(**), p= .000 **(Figures 3 and 4)**.

DISCUSSION

The increased number of AgNOR dots is, in many cases, considered to be of diagnostic and prognostic significance in tumor pathology, because of its direct relationship to the frequency of cell proliferation and other requirements for ribosome biogenesis [26]. Although the number of AgNORs is increased in malignancy, some workers considered it as not diagnostic due to overlap with benign proliferation [27]. It seems that although the number of AgNORs per cell is not discriminatory enough on its own to determine malignancy, the addition of size or area measurements using image analysis gives improved diagnostic and prognostic specificity [28, 29].

The present study was aimed to verify the usefulness of AgNORs in characterization of different histological bladder lesions and to detect the association if any between AgNORs and Immunohistochemical expression of COX2 and iNOS in bladder lesions.
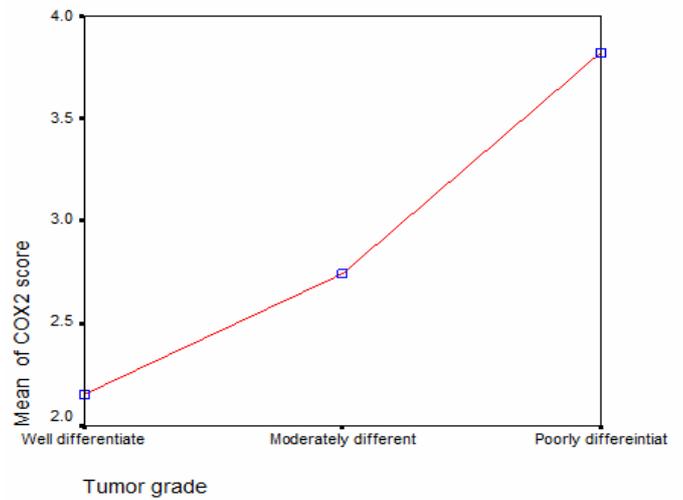
The present study revealed significant association between mean of AgNORs count and the positivity of

Figure 1.(A) - The correlation between tumor grade and mean of AgNORs among SCC group
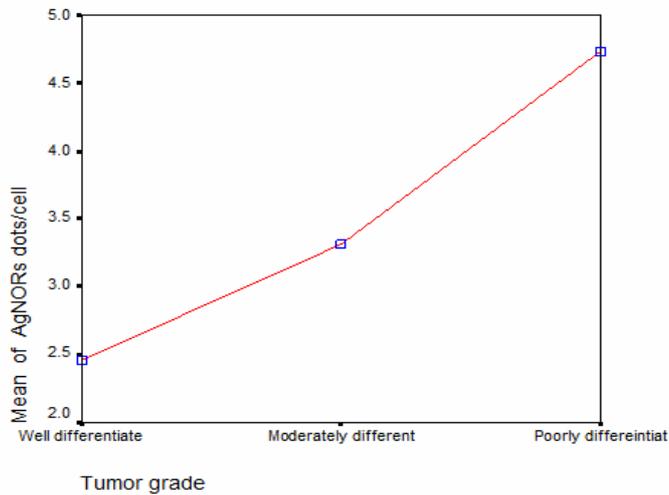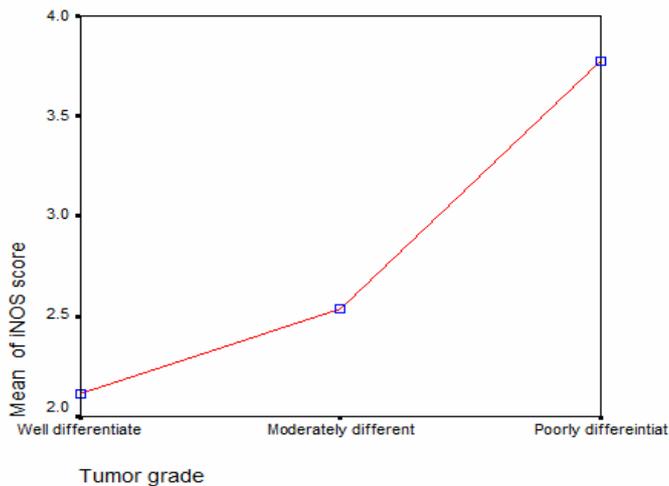

r=.600(**), p= .000

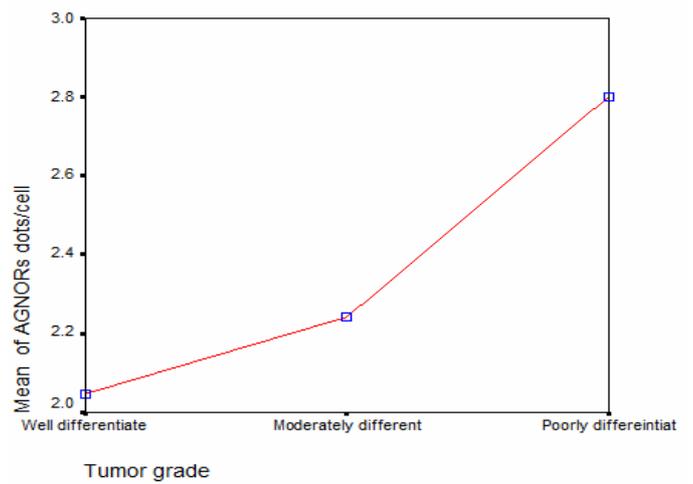***Figure 1.(B) - The correlation between tumor grade and COX2 score among SCC group***

r= .582(**), p=.000

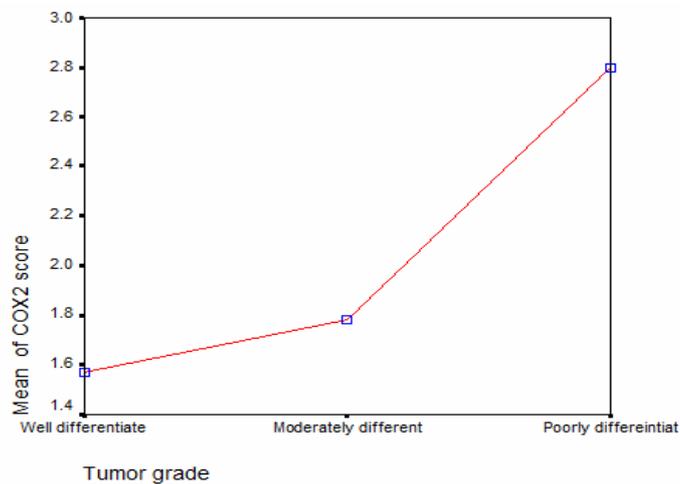***Figure 1 (C) - The correlation between tumor grade and iNOS core among SCC group***

r=.528(**), p=.000

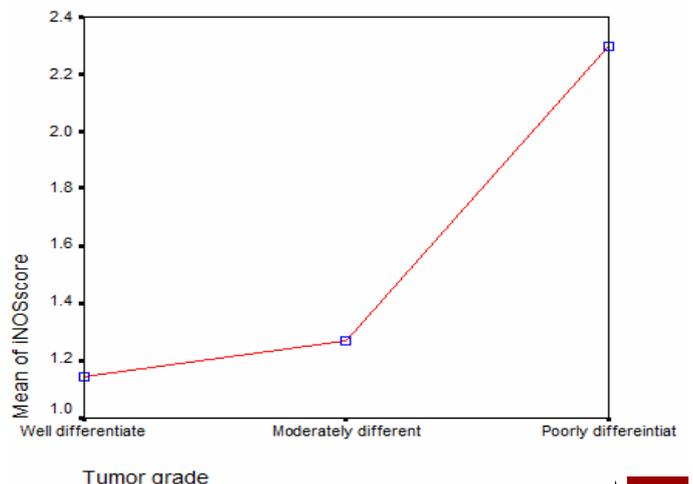***Figure 2.(A) - The correlation between tumor grade and AgNOR among TCC group***

r= .300(*), P= .013

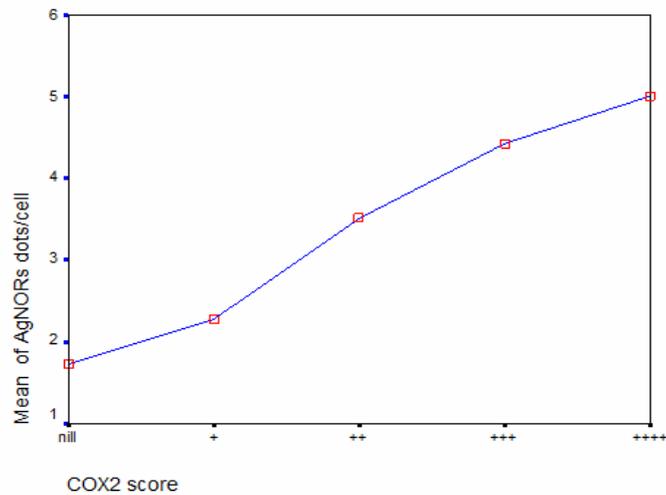***Figure 2.(B) - The correlation between tumor grade and COX2 core among TCC group***

r= .385(**), p= .001

Figure 2.(C) - The correlation between tumor grade and iNOS score among TCC group

r=.450(*), P= .000

Figure 3 - The correlation between COX2 score and Ag-NOR among study samples

r=.693(**), p= .000

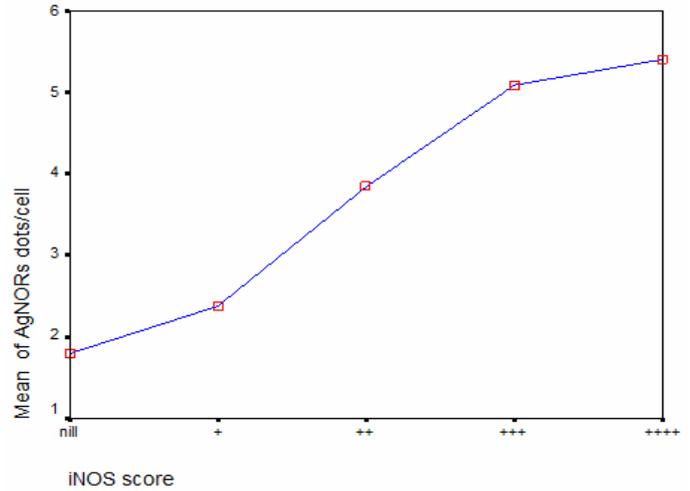**Figure 4 - The correlation between iNOS score and Ag-NOR among study samples**

r=.807(**), p= .000

COX2 in both malignant and benign cystitis groups (SCC, TCC and cystitis groups) (p.value = 0.044, 0.027, and 0.001) respectively .Similarly AgNORs associated with iNOS results in all groups denoting p.value of 0.000. As both COX-2 and iNOS are inflammatory marker they may play a synergistic role in the pathogenesis of bladder cancers; they play- markers of tumor angiogenesis [24]. COX-2 and iNOS could regulate the production of nearly all angiogenetic factors in cancer cells [25].

In the present study, it can be seen that in the group of carcinomas, the expression of COX-2 and iNOS depended on the histological grading, and higher malignant tumors showed more enhanced COX-2 and iNOS expression. A dependence of histological grade with COX2 was also described by [26]; they found that Cyclooxygenase-2 (COX-2) over expression has been associated with the grade,

prognosis and recurrence of transitional cell carcinoma of the bladder.

The present study revealed significant positive correlation between AgNORs count and both COX2 and iNOS score in the study samples which provide the evidence for a strong link between cell proliferation and chronic inflammatory markers in bladder lesions.

CONCLUSION

Overall this study provides evidence for a strong link between cell proliferation and chronic inflammatory markers in bladder lesions. Thus the three investigated markers can be used as pre-warning factors of bladder cancers among patients at risk of bladder cancer.

These biomarkers can also be exploited to develop new anti-inflammatory drugs to prevent and treat bladder cancer.

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