

Association of ki67 Proliferative Marker Expression with COX2 and iNOS Immunoreactivity in Bladder Cancer

Part -4 (Medical Science)

Chapter-I

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Abstract:

Objective: The objective of this study was to find out (if any) association between ki67 proliferative marker expression with COX2 and iNOS immunoreactivity in bladder cancer.

Methodology: The present study investigated the relationship between immunohistochemical expression of ki67, 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: Significant difference in the expression of all selected markers between carcinomas and cystitis was observed (p.value = 0.000). Ki67 immunoreactivity associated significantly with both COX2 and iNOS intensity, denoting P values of (0.000) with both markers among bladder carcinomas.

Conclusion: Strong link between proliferative markers (Ki67), COX2 and iNOS were detected among bladder carcinomas. Thus the three investigated markers (ki67, COX2 and iNOS) can be used as pre-warning factors of bladder cancers among patients at risk of bladder cancer.

keywords: ki67, COX2, iNOS, Bladder cancer.

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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].

It is now well established that the expression level of some nucleolar proteins is a useful indicator of cell proliferation. Such nucleolar proteins are marker's of proliferation because they are detected only in cycling cells as ki-67 antigens [5, 6]. Thus, the expression of nucleolar proteins is one criteria used for cell proliferation assessment with a prognostic value for human cancer.

Cyclooxygenase-2 (COX-2) is regarded as induced inflammatory mediator involved in the development of tumors. It is an inducible enzyme (also called prostaglandin synthetase) responsible for conversion of arachidonic acid to prostaglandins and other inflammatory mediators [7]. It is not detectable in most normal tissues; however, it is induced at sites of inflammation by cytokines, growth factors and tumor promoters [8]. 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 [9, 10].

Nitric oxide synthase (NOS) is the key enzyme for the conversion of L-arginine to L-citrulline and nitric oxide (NO) [11, 12]. The NOS family consists of endothelial, neuronal, and inducible nitric oxide synthase (eNOS, nNOS, and iNOS, respectively) [12]. iNOS genes located on the human chromosome 17 can be induced by lipopolysaccharide, cytokines in macrophages, or tumor-related immune reactions [13,14] 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 few published data explored the association between ki67 and immunohistochemical expression of COX2 and iNOS in bladder cancer.

Therefore it is interesting to hypothesize that, the ki67 expression 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 immunohistochemical expression of ki67, 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.

Sample processing:

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

Immunohistochemistry staining procedure:

An immunohistochemical assay for Ki67, COX-2 and iNOS was performed on paraffin wax sections (3–5 mm thick) of each tissue were mounted on APES (3-aminopropyltriethoxysilane, Sigma) coated slides. Sections were processed for immunostaining as follows: The sections were deparaffinized with xylene and then 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:

For Ki67 (MIB-1), a primary mouse monoclonal antibody to MIB-1 was used (DAKO Ltd, UK, M7240) at 1: 50, for COX-2 a primary goat polyclonal anti-human COX-2 antibody was used (sc-1745; Santa Cruz Biotechnology, USA) at a dilution of 1:50; polyclonal rabbit anti-iNOS antibody (Ab-1, Lab.Vision, Neo Markers, USA), Using antibody dilution at 1:50, washed in phosphate-buffered saline (PBS) and incubated for 30 minutes with diluted 1 : 200 secondary biotinylated antibody. 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 all 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 evaluation by the following Strategies; for all three markers, when less than 5% of cells were stained positive classified as negative, less than 50% considered low intensity, more than 50% positive for immunostaining classified as high intensity.

Statistical analysis:

SPSS version 17 statistical software was used for statistical analysis. Following the descriptive statistics 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

Results:

The expression of the investigated markers among the different selected bladder samples was summarized in Table (1), ki67 was expressed among 100% of both bladder tumors (SCC and TCC), and among 31(79.5%) of benign bladder cystitis. Significant difference between carcinomas and cystitis was observed (p.value = 0.000).

Concerning the COX2; 82 out of 87 (94.3%) of bladder SCC were stained positive for COX2, the marker expressed in 41(60.3%) and 18(46.2%) of TCC and bladder cystitis respectively.

74(85.1%) of SCC were stained positive for iNOS, the marker stained positive among 22(32.4%), 17 (43.6%) of bladder TCC and cystitis respectively. For both markers (COX2 and iNOS) significant differences between the different bladder pathology was observed (p. value was 0.000).

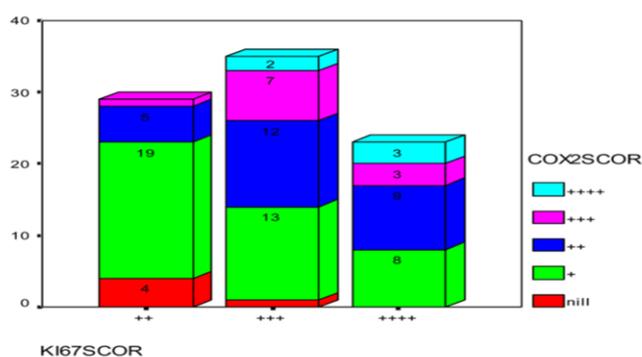
The association between cell proliferative marker intensity (Ki67), and both COX2 and iNOS among the different bladder pathology were expressed in figures (1, 2,3,4,5 and 6); among the SCC group; ki67 reactivity was significantly associated with both COX2 and iNOS scores denoting p.value of 0.018 and 0.001 respectively (figure 1 and 2).

Similar findings were observed between Ki67 and both COX2 and iNOS intensity among TCC group as shown in figure (3 and 4), p.value = 0.032 with COX2, and 0.017 with iNOS.

While for cystitis group as shown in figure 5 and 6; insignificant association between ki67 and COX2 immuonoreactivity was observed, p.value of 0.548 (figure.5), also insignificant association of ki67 with iNOS was detected with p.value of 0.548 (figure.6)

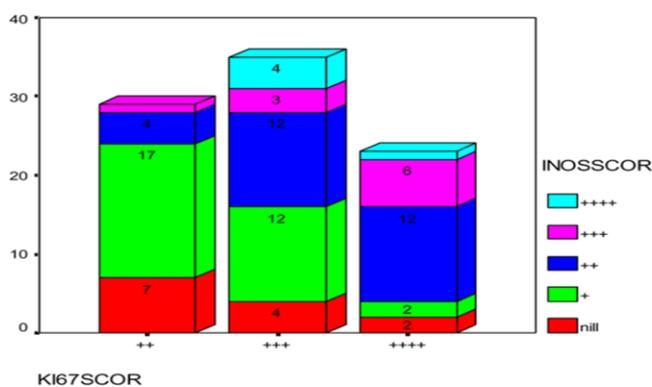
Table (1): The expression of the demonstrated makers among different bladder pathology

Diagnosis	Markers expression						p.value
	Ki67		COX2		iNOS		
	Positive	Negative	Positive	Negative	Positive	Negative	
Bladder SCC(n=87)	87 (100%)	0 0.0%	82 94.5%	5 5.7%	74 85.1%	13 14.9%	0.000
Bladder TCC(n=68)	68 100%	0 0.0%	41 60.3%	27 39.7%	22 32.4%	46 67.6%	0.000
Bladder cystitis(n=39)	31 79.5%	8 20.5%	18 46.2%	21 53.8%	17 43.6%	22 56.4%	



p.value = 0.018

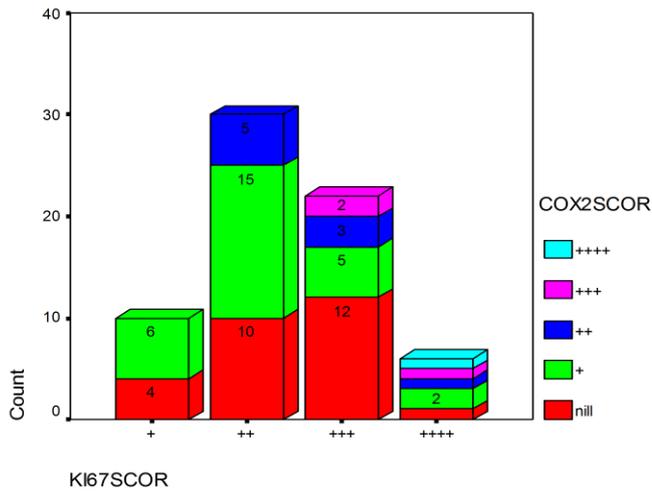
Figure (1): The association between Ki67 and COX2 scores among SCC group



p.value = .001

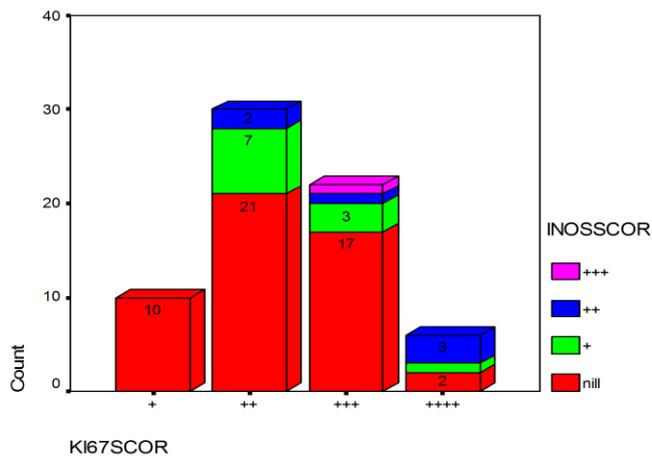
Figure (2): The association between Ki67 and iNOS scores among SCC group

Figure (2): The association between Ki67 and iNOS scores among SCC group



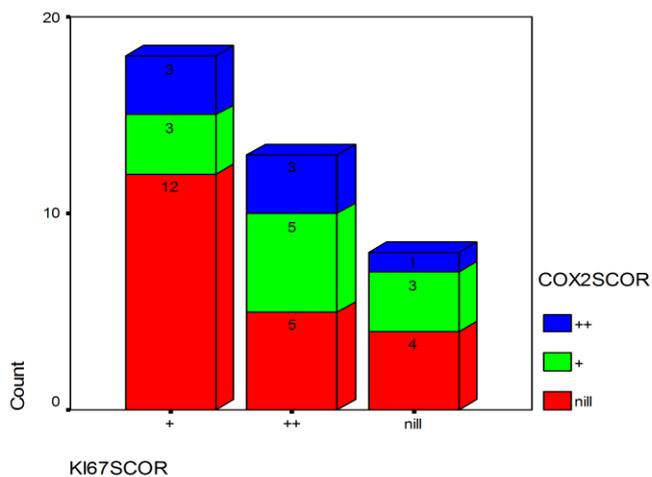
p.value =.032

Figure. 3: The association between Ki67 and COX2 scores among TCC group



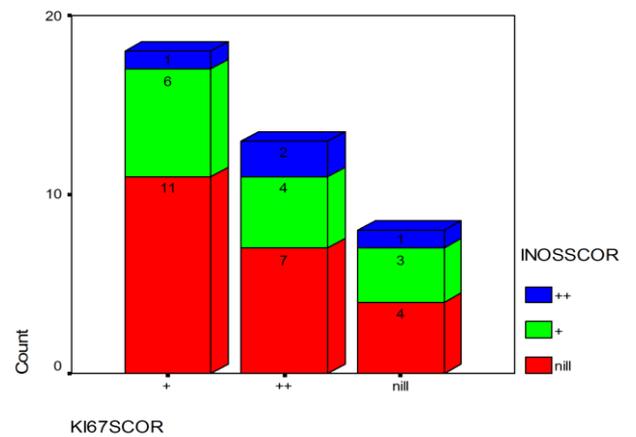
p.value =.017

Figure. 4: The association between Ki67 and iNOS scores among TCC group



p.value =.548

Figure.5: The association between Ki67 and COX2 scores among Cystitis group



p.value =.548

Figure.6: The association between Ki67 and iNOS scores among Cystitis group

Discussion:

The Ki-67 protein is a nuclear and nucleolar protein that is strictly associated with cell proliferation. It has been suggested to play a role in the control of the higher order chromatin structure [15]. Because the protein is produced only in dividing cells, the anti Ki-67 antibody MIB-1 has been widely used in histopathological procedures to estimate the growth fraction of human neoplastic tissue samples in situ. In the present study and compared to benign bladder cystitis, Ki67 reactivity was significantly greater (P = 0.000) in both SCC and TCC than cystitis. Ki67 marker was expressed among 100% of both malignant groups (SSC and TCC). In benign cystitis group, the marker was expressed among 79.5% of the samples; this indicates that the inflammation process activates the proliferation of the cells which may lead to carcinogenesis. Our findings agree with study by [16]

The contribution of COX-2 to carcinogenesis and the malignant phenotype of tumor cells have been thought to be related to its abilities: (1) increase production of prostaglandins [17]; (2) convert procarcinogens to carcinogens [18]; (3) inhibit apoptosis [19]; (4) promote angiogenesis [20]; (5) modulate inflammation and immune function [21, 22]; (6) increase tumor cell invasiveness [23, 24]. Like COX-2, iNOS is also involved in the process of carcinogenesis. Sustained induction of iNOS in chronic inflammation may be mutagenic through NO-mediated DNA damage or hindrance to DNA repair, and thus potentially carcinogenic. In addition, NO can favor tumor growth and development by stimulating angiogenesis [25, 26] and causing immunosuppression [27]

One of the major findings in this study was that COX-2 was markedly expressed in the cytoplasm of most of bladder squamous cell carcinomas (94.5%). In contrast to squamous cell carcinoma, transitional cell carcinoma of the urinary bladder expressed COX-2 less frequency (60.3%),

statistically significant different between these groups was detected ($p = 0.000$), these different expression patterns in the two malignancies appear to be logical because they have different etiologies. The development of squamous cell carcinoma closely correlates with chronic urinary tract infection; therefore, COX-2 plays an important role in inflammation-induced carcinogenesis. These findings agree with the study by [28 and 29], both studies found that COX-2 is expressed in squamous cell carcinomas of the urinary bladder and in the precursor lesions.

Also the present findings agree with study by [30] in that COX-2 was slightly expressed in chronic cystitis but disagree with the present findings in that they found that the marker reactivity was higher in transitional cell carcinoma (TCC) than in squamous cell carcinoma (SCC) ($P < .01$).

Similar to COX2, iNOS was expressed in 85.1% of bladder SCC, 32.4% of TCC and 43.6% of bladder cystitis group, significant different between groups was detected denoting p.value of 0.000.

The present study was aimed to verify the association if any between Ki67, COX2 and iNOS Immunohistochemical expression in bladder lesions.

The present study revealed significant association between ki67 intensity and the immunoreactivity of COX2 in both malignant types (SCC and TCC) but not in benign cystitis groups (p.value = 0.018, 0.032, and 0.548) respectively. Similarly ki67 immunoreactivity associated with iNOS intensity in malignant groups (SCC and TCC) denoting p.value of = 0.001, 0.017 respectively, but not with cystitis group p.value = 0.548. 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 [14].

The present study revealed significant positive association between ki67 immunostaining intensity with both COX2 and iNOS score in the selected groups 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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