FREQUENCY OF IMMUNOHISTOCHEMICAL EXPRESSION OF Ki67 IN UROTHELIAL CARCINOMA BLADDER

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ABSTRACT

Objective: Bladder carcinoma is a prevalent urinary malignancy causing morbidity and mortality worldwide. The main aim of this study was to determine the frequency of immunohistochemical expression of Ki67 in urothelial carcinoma on TURBT bladder biopsies at our centre.

Material and Methods: A descriptive cross-sectional study was conducted in the department of Histopathology, Combined Military Hospital Multan from 13 June 2017 to 12 December 2017 on 79 bladder biopsies from the patients of urothelial carcinoma. Age, gender, grade of the tumor and results of immunomarker Ki67 were recorded. Computer software program SPSS version 21 was used for data analysis. Mean and SD calculation was done for numerical variables like age. Effect modifiers like age, gender and tumor grade were controlled through stratification. Post stratification Chi square test was applied. Percentages and frequency calculations were performed for gender, age, immunohistochemical expression of Ki67 as well as tumor grade. p-value <0.05 was considered as statistically significant.

Results: Out of 79 patients of urothelial carcinoma, 47 (59.49 %) were males and 32 (40.51 %) were females with a male to female ratio of 1.5:1. Patients age varied between 40 and 90 years with an average age of 66.7 years and SD of ±12.49. Among 79 biopsies, 51 (64.56%) were low grade tumors and 28 cases (35.44%) were high grade tumors. Out of 79 cases,23 cases (29.11%) were muscle invasive and 56 cases (70.81%) were noninvasive. 59 (74.68%) urothelial carcinoma cases were Ki67 positive whereas 20 cases (25.32%) did not exhibit Ki67 positivity. No significant statistical association was observed among age, tumour grade, gender and Ki67 IHC results.

Conclusion: The high immunoexpression of Ki67 in urothelial carcinoma bladder is associated with a poor prognosis. Ki67 ASOs (Antisense deoxyoligonucleotides) markedly inhibit tumor growth and can be a promising target for molecular therapies. Ki67 immunoexpression can identify the subpopulation of patients which are more likely to respond to a given therapy. The precision of prognosis can be improved which can be useful in pinpointing the patients with greater risk of disease progression, thereby furnishing a valuable indicator for the clinical management of these patients, which might get advantage from adjuvant treatments.

Key Words: Ki67, Urothelial carcinoma, Immunohistochemical expression.

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INTRODUCTION

Urinary bladder cancer is one of the most common cancers globally, being the fourth most common malignancy in males and eighth amid the females [1]. Urothelial carcinoma is the leading cause of cancer deaths amongst the urinary system malignancies [2]. Almost 95% bladder malignancies are of epithelial derivation, and transitional cell carcinoma accounts for 90% of all of them [3]. In United States, bladder carcinoma is the fourth most frequent tumor [4]. In Pakistan, the actual figures regarding the incidence and urinary bladder related cancer deaths are not known [5]. However, the incidence is high which makes it one of the top ten malignancies in men [6]. Cigarette smoking, tea and coffee intake and exposure to organic dyes are major risk factors in this region of the world [7].

Ki67 is a nuclear and nucleolar non-histone DNA binding protein which has a brief life. This cell proliferation marker is encoded by the MKI67 gene in humans. Many reports in literature prove a correlation between Ki67 and well-known prognostic factors such as grade and stage. High Ki67 proliferation indices in high grade bladder carcinomas whether invasive or noninvasive have been documented in literature. Current studies have determined it to be an autonomous forecaster for recurrence, progression and response to treatment in invasive urothelial cancer [1]. The parameters that regulate the prognosis of bladder carcinoma are` tumor grade, stage, age of the patient and lymph node status [8].

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New studies have validated that blocking of Ki67 by microinjection of antibodies or through using antisense oligodeoxynucleotides (ASOs) causes arrest of cell multiplication. In animal models, Ki67 ASOs markedly inhibit tumor growth and it can be a promising target for molecular therapies [9].

71.1% cases showed Ki67 positivity in a study carried out in Romania in 2012 [10]. Another study conducted in Brazil demonstrated Ki67 positive expression in 58.1% cases [11]. Chen *et al* [12] verified in 2012 that Ki67 was an independent predictor of recurrence of tumor and progression in a 72 cases study of non-muscle invasive bladder cancer. Tanabe *et al* [13] in 2015 demonstrated increased Ki67 expression status might assist in the choice of chemoradiotherapy based multimodal methods in regards to disease prognosis and quality of life. However, no native study is existing currently in this context.

The main rationale and foundation of this study is to estimate the immunohistochemical expression of Ki67 in urothelial carcinoma bladder in our population. Increased Ki67 expression is intensely related with poor prognosis and it is a potential therapeutic target. It will not only provide the local statistics of the problem but will also be a useful addition in existing literature. The results will benefit the physicians to plan routine practice guidelines for appropriate management of these high-risk patients in order to reduce the mortality and morbidity of community as expression of Ki67 recognizes population of bladder cancer patients who have more chances to respond to a given treatment.

MATERIAL AND METHODS

After approval from ethical committee, a descriptive cross-sectional study was conducted at Histopathology department of Combined Military Hospital, Multan in six months. (13th June 2017 to 12th December 2017). 79 cases of urothelial carcinoma were comprised in the study using non-probability, consecutive sampling technique between 40 to 90 years of age. Sample size was calculated using WHO sample size calculator according to following parameters:

Confidence Level (1 – α) = 95%
Anticipated Population Proportion (P)=71.1% [10]
Absolute Precision Required (d) = 10%
Minimum Sample Size (n) = 79

Poorly preserved, autolyzed and inadequate bladder biopsies were omitted from the study. The specimens were fixed in 10% buffered formalin. After 24 hours of processing, they were grossed and stained with hematoxylin and eosin to examine the morphological features. Data collection performa of the patients was filled. Cases were selected as per inclusion criteria. Immunohistochemical assays for Ki67 were done by using DAKO kit as per the manufacturer's guidelines as follows: The FFPE tissue sections were cut at 3 µm thickness and placed on clean glass slide with pre-attached adhesive on its surface. They were incubated at 58 degrees Celsius for 4 hours. The sections were deparaffinized with xylene 1 and 2, for 3 minutes each. They were rehydrated in decreasing concentrations of alcohol, 90%, 80% and 70% for 3 minutes each, followed by running tap water for 5 seconds. The slides were placed in coplin jar with 0.01 M Tris-EDTA buffer at pH of 9.0. 750 W domestic microwave was used to treat the slides for 20-30 minutes for heat mediated antigen retrieval. Slides were washed with distilled water for 20-40 minutes. After cooling down the sections, they were brought to phosphate buffered saline (PBS) at pH 7.3 for 5 minutes. PBS was washed and excess was wiped off the sections. Endogenous peroxidase activity was blocked by incubating in 0.5% hydrogen peroxide in methanol for 5 minutes. The slides were washed in three series of PBS, 2 minutes each. 100 uL of primary antibody of Ki67 was instilled on the sections and incubated for 60 minutes. The slides were again washed in three series of PBS, for 60 minutes. The slides were then incubated in avidin-biotin complex for 10 minutes. They were rinsed with distilled water and then incubated in DAB (diaminobenzidine) substrate solution for 5 minutes. Then the slides were washed with water and counter stained with haematoxylin for 40 seconds. The slides were dehydrated by placing them in increasing concentrations of alcohol, 70%, 80%, 90% and 100% alcohol for 3 minutes each. Clearing was done by placing slides in xylene for 3 minutes. The slides were mounted with Canada balsam.

The results were elucidated by two experienced histopathologists having more than 15 years of experience to minimize bias. In a predesigned performa, all data was entered. Normal bladder without the primary antibody and urothelial carcinoma paraffin embedded sections functioned as negative and positive controls, respectively. To explain expression level of Ki67 staining, it was defined as: <10% of tumour cells stained (Score 1-Negative), 10-50% of tumour cells stained (Score 2-Positive), >50% of tumour cells stained (Score 3 -Positive) [10].

Gender, age, Ki-67 immunohistochemical expression and grade of tumor was recorded. Computer software program SPSS version 21 was employed for statistical analysis. Calculation of SD and mean was done for numerical variables like age. Gender, age, tumor grade and immunohistochemical expression of Ki67 were expressed in frequencies and percentages. Through stratification, effect modifiers including age, tumor grade and gender were controlled. Post stratification, Chi-square test was applied by taking p value of less than 0.05 as significant.

RESULTS

This study was carried out over a period of six months.79 patients of urothelial carcinoma were included. In Table-I, case distribution in keeping with different age groups, gender, tumour grade and immunohistochemical expression of Ki67 is summed up.

Mean age was of 66.77 ± 12.49 years with age group ranging from 40 to 90 years (table-I). 39 cases (49.35%) belong to 40 -65 age group and 40 cases (50.63%) belong to 66-90 age group (figure-I). Out of these 79 patients, 47 (59.49%) were males and 32 (40.51%) were females with a male to female ratio of 1.5:1. Out of 79 cases ,51 cases (64.56%) were low grade and 28 cases (35.44%) were high grade (figure-ii). 59 cases (74.68%) of urothelial carcinoma bladder showed positivity for Ki67 (Score 2+ or 3+) while 20 cases (25.32%) were negative (Score 1) (figure-III). Out of total 79 cases, 23 cases (29.11%) were muscle invasive and 56 cases (70.81%) were noninvasive. Figure-IV shows high grade urothelial carcinoma bladder showing a predominantly disordered pattern and loss of polarity. Figure-V is the same case at high power(40x) showing marked cytological atypia, nuclear pleomorphism and frequent mitotic figures. In figure-VI, Ki67 nuclear expression is seen in urothelial carcinoma. In figure-VII, there is no immunohisto-chemical expression of Ki67.

Table-I: Stratification of Ki67 expression according to Age groups, Gender, Grade of tumor and Muscle Invasion.

Clinical and pathological variables	Cases (n)	Percentage (%)	Ki67 expression intensity		*P* Value
			Positive cases	Negative cases	
Age Groups in years (n=79)					0.137
40-65	39	49.35	32 (82.05%)	7 (17.95%)	
66-90	40	50.63	27 (67.50%)	13 (32.50%)	
Gender (n=79)					0.562
Male	47	59.49	34 (72.34%)	13 (27.66%)	
Female	32	40.51	25 (78.13%)	7 (21.87%)	
Tumour Grade (n=79)					0.622
Low Grade	51	64.56	39 (76.46%)	12 (23.53%)	
High Grade	28	35.44	20 (71.43%)	8 (28.57%)	
Muscle Invasion					0.503
Invasive	23	29.11	16 (69.55%)	7 (30.43%)	
Non-Invasive	56	70.81	43 (76.79%)	13 (23.21%)	

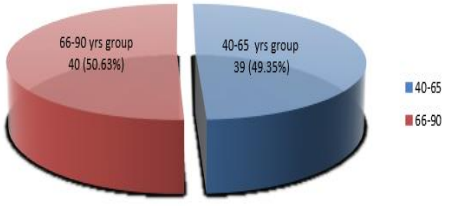

Figure-I: Cases distribution in accordance with different age groups (n=79) Mean Age 66.77+-12.49.

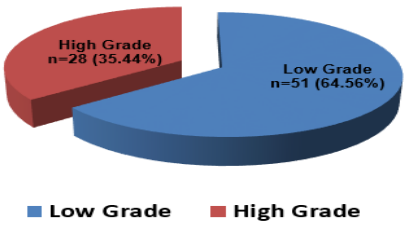

Figure-II: Distribution of patients according to Grade of tumor.

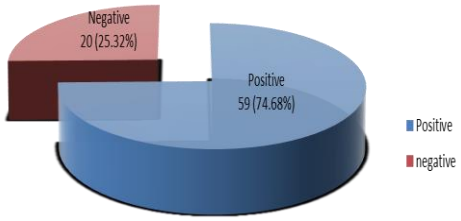

Figure-III: Frequency of immunohistochemical expression of Ki67 in urothelial carcinoma bladder (n=79).

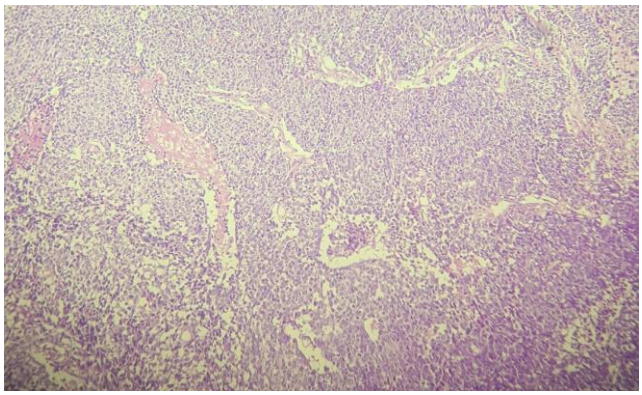

Figure-IV: Photomicrograph of Urothelial carcinoma bladder (10 x).

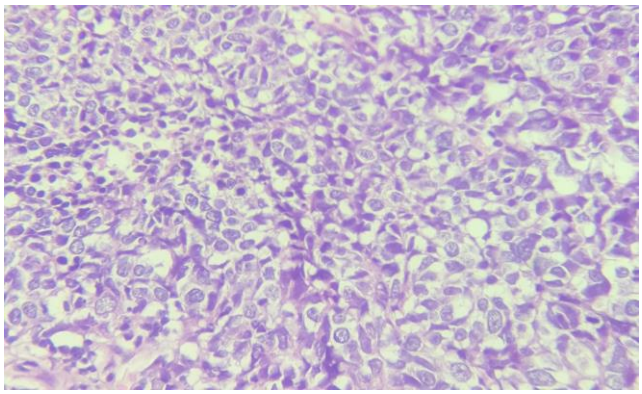

Figure-V: Photomicrograph of Urothelial carcinoma bladder (40 x).

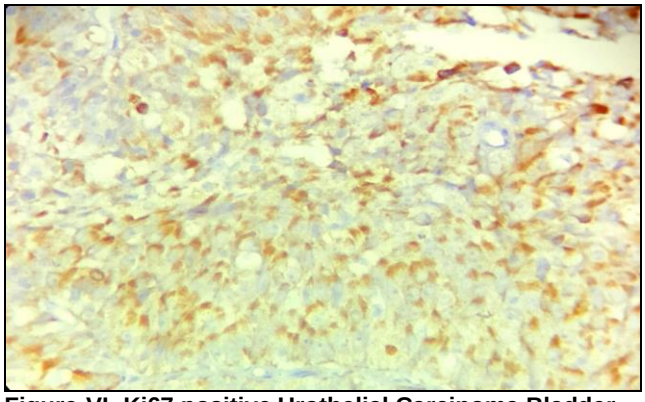

Figure-VI: Ki67 positive Urothelial Carcinoma Bladder (40X).

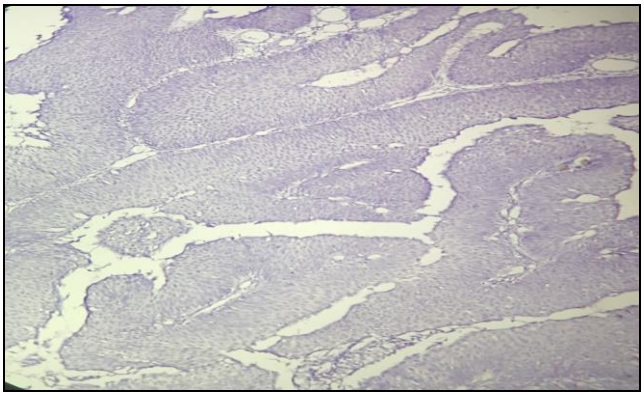

Figure-VII: Ki67 Negative Urothelial Carcinoma Bladder (10X).

DISCUSSION

Urothelial cancer is one of the most common tumors all over the world having a poor prognosis, frequent chances of recurrence and metastasis and is accountable for substantial percentage of cancer morbidity and mortality [14]. Genetically and morphologically, urothelial carcinomas are diverse tumors, with various malignant cell lines and precise alterations [15]. Known prognostic factors for bladder cancer survival are tumor stage and tumor grade [16]. TP53, Ki67 and TP63 are common markers employed worldwide and a combination of these biomarkers may aid in foreseeing the prognosis of bladder cancer and postoperative recurrence in cases of non-muscle invasive bladder cancer (NMIBC) [17].

Ki67 is widely used in routine pathological testing and research as a proliferation marker as its expression is intensely related with tumor cell multiplication and growth [18]. For the assessment of biopsies from patients with tumors, Ki67 nuclear protein is a recognized predictive and prognostic indicator. Correlation of pKi67 with metastasis and the clinical tumor stage has been demonstrated [19]. Furthermore, in comparison with normal tissue, Ki67 staining intensity is found to be remarkably stronger in malignant tumors with poorly differentiated tumor cells [20]. Keeping in view its prognostic value, pKi67 expression ascertains subsets of patient population who are more probable to respond to a particular treatment regimen [21]. In a study carried out in India in 2017 by Thakur B *et al* [14], low Ki67 was noted in 56 cases and high expression in 54 patients. Low grade tumors showed mainly low expression (48 cases) and high-grade tumors exhibited high expression (53 cases).

In the cell cycle, Ki-67 expression is observed in multiplying but not dormant (G0) cells [22]. It is considered to be a strong predictive sign for cancer development, and its correlation with poor prognosis in different types of cancers has been

proven [23]. However, its prognostic role in bladder cancer remains debated from time to time. Makboul and Gontero *et al* [24, 25] established that expression of Ki-67 was only an autonomous forecaster of disease progression and not recurrence in patients of non-muscle invasive bladder cancer. Few studies have discovered Ki-67 having no correlation or to be a sovereign predictor of recurrence, progression, and death due to bladder carcinoma. Similarly, Acikalin *et al* [26] in a study of 68 patients with stage T1 disease who underwent transurethral resection of the tumor, testified that there was no correlation between Ki-67 and tumor recurrence, progression or mortality related to tumor.

Ki 67 is a potentially strong target therapeutically and greater Ki67 expression rates are intensely linked with poor prognosis. Within the South East Asian populations and particularly Pakistani populace, comparatively less is known about Ki67 expression. Furthermore, no local research work is available which has studied the frequency of expression of Ki 67 in urothelial carcinoma.

We studied patients ranging from 40-90 years of age. In the studies carried out by Senturk N *et al* [27] in Denizli, Turkey, and Enanche *et al* [10] in Craiova, Romania, the age ranges were 40 -80 years and 50-70 years respectively which is analogous to our study.

66.77 years was the average age in this study, which is in consonance with a study carried out by Jawad NA *et al* [1] in Baghdad, Iraq (58.72 years) and Senturk N *et al* [27] in Denzili, Turkey (65.15 years). Male to female ratio in our study is 1.5:1 depicting male predominance but it is 4:1 in the studies conducted by Haque S *et al* [28] and 7.46:1 in the study carried out by Thakur B *et al* [14], respectively.

In our study, 64.56% tumours were low grade and 35.44% cases were high grade. However, it is different in the studies conducted by Thakur B *et al* [14] (44.5%, 55.5%) and Elkady N *et al* [16] (48% and 64 %). The expression of Ki67 observed in our study is 74.68% and 25.32% were negative, which is comparable to the study conducted in Romania by Enanche M *et al* [10], which is 71.1%. However, it is 25% in the Turkish study conducted by Senturk N *et al* [27]. The regional variations owing to diverse genetic makeup of the population or dissimilarity in sample sizes might dictate the difference in percentage expression of Ki67.

Among males 72.34% cases are Ki67 positive and 27.66% are Ki67 negative in my study work. Among females, 78.13% cases are Ki67 positive and 21.87% are Ki67 negative. It is in concordance with the study conducted in Beijing, China by Pengjie Wu [29] *et al* in which 27/57 (47.3%) males showed high Ki67 expression and 32/58 (55.17%) females showed high Ki67 expression.

Among the low-grade tumors,71.43% cases were Ki67 positive and 28.57% were Ki67 negative. Among high grade tumors 76.47% cases were Ki67 positive and 23.53% cases were Ki67 negative. It is similar to the study conducted by Jawad NA [1] in which 84% high grade cases showed high Ki67 expression and only 50% low grade cases showed high Ki67 expression. Out of total 79 cases of urothelial carcinoma,23 cases (29.11%) were muscle invasive and 56 cases (70.81%) were non-muscle invasive. Out of invasive cases, 16(69.55%) cases were Ki67 positive and 7(30.43%) cases were Ki67 negative. Among noninvasive cases,43(76.69%) cases were Ki67 positive and 13(23.21%) cases were Ki67 negative. However, in a study by Thakur B *et al* [14] in 2017,29/29(100%) cases of muscle invasive carcinomas revealed high Ki67 expression.

CONCLUSION

High Ki67 immunoexpression rates in urothelial carcinoma bladder can enhance and refine the prognostic accuracy and validity. This in turn will be beneficial substantially in recognizing and pinpointing the bladder cancer patients who are likely to have greater risks of progression of disease. Ultimately, it will offer an important indicator for the appropriate and timely management of these patients. It can serve as a promising therapeutic target as Ki67 ASO are presently under clinical trials and thorough scrutiny.

AUTHOR CONTRIBUTION

Maria Aslam: Concept, planning and design of research work, data collection, analysis, result interpretation and manuscript writing.

Muhammad Tahir Khadim: Design, Analysis, result interpretation and critical revision.

Syed Naeem Raza Hamdani: Result analysis, critical revision

Farah Ahsan: Result interpretation, data analysis

Syed Salman Ali: Statistical analysis, data analysis, Revised manuscript

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