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Original Research Article

EXPRESSION OF KI-67 IN PAPILLARY UROTHELIAL NEOPLASMS OF LOW MALIGNANT POTENTIAL AND NON-INVASIVE PAPILLARY UROTHELIAL CARCINOMA- A RETROSPECTIVE OBSERVATIONAL STUDY FROM SOUTH INDIA

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Abstract

Background: Urothelial carcinoma is the second most common malignancy of the genitourinary tract following prostatic carcinoma. Non-invasive urothelial neoplasms can be flat or papillary. Papillary neoplasms range from benign papillomas to invasive carcinomas. The objective of this study was to evaluate the immunohistochemical expression of Ki-67 in papillary urothelial neoplasms of low malignant potential (PUNLMP) and noninvasive urothelial carcinomas as well as to determine its utility in differentiating these tumors, as they vary in recurrence and progression rates. **Materials and Methods:** A total of 43 cases including PUNLMP(n=7), noninvasive low grade (LGPUC) (n=35) and high grade (HGPUC) (n=1) papillary urothelial carcinomas, diagnosed based on histomorphological criteria as defined by WHO/ISUP 2016 classification, were evaluated for Ki-67 immunohistochemical expression. Chi square test was used to analyse the utility of Ki-67 in differentiating PUNLMP and LGPUC. **Result:** All seven cases of PUNLMP showed less than 10% Ki-67 expression. Out of 35 cases of LGPUC 37% showed less than 10% and 63% showed more than 10% Ki-67 expression (Range= 1-50). One case of HGPUC showed a Ki-67 staining of 70%. Data analysis proved an increasing Ki-67 expression with increasing grade of tumors. **Conclusion:** Ki-67 expression increases with tumor grade. It serves as a useful adjunct to morphology, in differentiating PUNLMP from LGPUC. Small number of HGPUC cases for analysis was a limitation in this study.

INTRODUCTION

Urothelial carcinoma (UC) is one of the commonest cancers of the genitourinary tract with a worldwide incidence of 9.6/100000 in men and 2.4/100000 in women.[1,2] The incidence of UC in India in 2018 was 18921, with an incidence rate of 2.4/100000 in men and 0.7/100000 in women.[3] Men are three times more affected than women.[4,5] Risk factors include cigarette smoking, exposure to arylamines, Schistosoma hematobium infection, chronic analgesic usage, long term exposure to cyclophosphamide and irradiation of the urinary bladder. Genetic alterations of chromosome 9 have also been implicated in the pathogenesis. [5-7]

According to The World Health Organization (WHO) classification of urinary bladder tumors 2016, non-invasive lesions/tumors of the urinary bladder include papilloma, dysplasia, urothelial proliferation of uncertain malignant potential, PUNLMP, LGPUC, HGPUC and carcinoma in situ (CIS).[5]

Despite the defined histomorphological criteria for diagnosis of superficial papillary lesions, inter-observer variation occurs due to architectural and cytological overlap especially between PUNLMP and LGPUC. As lesions differ in prognosis and treatment, they need to be accurately diagnosed.

The recurrence and progression rates for PUNLMP are 36% and 4%, for LGPUC are 50% and 10% and HGPUC are 60% and 25%, respectively.[3]

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Small, low grade papillary neoplasms are resected via the transurethral route and followed up with repeated cystoscopies and urine cytology. Following resection of non-invasive high-grade lesions (HGPUC, CIS), topical intravesical instillation of Bacillus Calmette-Guerin (BCG) is recommended. It elicits an inflammatory response that destroys residual tumor cells. Radical cystectomy is reserved for cases with muscularis propria invasion, CIS or HGPUC unresponsive to BCG and CIS involving the prostatic urethra.[7]

Ancillary tests have been described to objectively diagnose superficial non-invasive neoplasms like immunohistochemistry (IHC) (Ki-67, p53, CK20, E-cadherin) and molecular studies. [9-13]

Ki-67 is a proliferation marker that is absent in the resting phase but excellent for detecting multiplying cells in a given cell population. Ki-67 expression is associated with histological grade, stage, recurrence and progression to a higher grade in bladder cancer. [8,14-22] This study aims to determine the immunohistochemical pattern of expression in PUNLMP and non-invasive papillary urothelial carcinoma and to evaluate its role in differentiating PUNLMP from LGPUC.

MATERIALS AND METHODS

This is a retrospective observational study carried out in a tertiary health care center in Mangalore, Karnataka, over a period of 5 years (2013-2017). Ethical committee clearance was obtained before the commencement of the study

The study sample comprised 43 cases diagnosed as PUNLMP, and non- invasive LGPUC and HGPUC, according to the morphological criteria defined by WHO 2016 classification of urinary bladder tumors on bladder biopsies or transurethral resection specimens. Cases with prior radiotherapy and chemotherapy were not included in this study to prevent diagnostic and interpretative challenges with therapy induced histological changes. Cases with inadequate epithelial tissue for assessment as well as extensive cautery, crush and fixation artefacts were excluded from the study.

In slides retrieved from the archives, the histomorphological parameters and IHC were evaluated by a pathologist (blinded study).

Both cytological and architectural features studied at low and high-power magnifications were taken into consideration while grading the neoplasms. Abnormalities in nuclear size, shape, and quality of chromatin were criteria for cytological disorder; whereas abnormalities in the cell polarity with respect to each other and to the basement membrane were criteria for architectural disorder.[3]

Papillary lesions with more than 7 layers of urothelium, well-preserved polarity, uniform nuclei and homogenous chromatin were diagnosed as PUNLMP.[2,3] Focal nucleomegaly and crowding was acceptable.[3]

Lesions with delicate branching papillae, orderly arrangement of cells under low power, loss of polarity and mild nuclear pleomorphism under higher power were diagnosed as LGPUC. Mitotic figures in these lesions are typical and seen towards the superficial layers of the urothelium.[3]

Lesions were diagnosed as HGPUC when the papillae were fused and solid, and the cells showed marked anisonucleosis, pleomorphism and loss of polarity under low power itself. Brisk mitoses, including atypical forms, is another feature of this lesion.[3]

Post histopathological study, paraffin blocks of selected slides with representative material were retrieved from the archives, 5-micron thick sections were cut, taken onto poly-l-lysine slides, kept at 37^0 C overnight, deparaffinized with xylene and rehydrated with graded alcohols. IHC was done according to the immunoperoxidase technique. Antigen retrieval was carried out by microwave technique. Power block was applied followed by primary (Ki-67) (DAKO company) and secondary antibodies. Di-amino-benzidine (DAB) and hematoxylin were used as chromogen and counterstain respectively. The same procedure was performed on a positive control slide of carcinoma colon, simultaneously.

Ki-67 staining was assessed by a quantitative score i.e., the number of cells with nuclear positivity (moderate to strong) per 1000 epithelial cells, in areas with maximum staining under high power magnification.[7] A minimum of 5 high power fields (field diameter of 40x lens 0.65mm, area corresponding to 1.66mm2), each with at least 800 epithelial cells, were studied. Two observers (pathologist, principal investigator) independently analyzed the slides and the arithmetic mean of their observations was used for statistical analysis.

Statistical package for social sciences- IBM SPSS statistics for windows, version 25.0 Armonk NY: IBM corp was used for analysis. The data was expressed in terms of percentages, mean, median and range using appropriate tables and figures. For comparison across groups, Chi square test was used and a p value of less than 0.05 was considered statistically significant.

RESULTS

Forty-three cases that included PUNLMP (n=7), LGPUC (n=35) and HGPUC (n=1) were studied. The overall mean age at presentation was 60.62 years (range 37 to 84 years). The mean age of cases with PUNLMP and papillary noninvasive urothelial neoplasms was 52.43 years and 62.22 years respectively. The male: female (M: F) ratio was 6:1.

The commonest presenting complaints were painless intermittent hematuria (n=16, 61.5%), urine voiding symptoms (n=3, 11.5%) and lower back pain (n=3, 11.5%). Cystoscopy records of 23 cases showed the following location of tumors; multiple sites (n=6,

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26.1%), lateral wall of bladder (n=12, 52.2%), vesico-ureteric junction (VUJ) (n=4, 17.4%) and dome of bladder (n=1, 4.3%). Unifocal lesions were seen in 14 cases (58%) and multifocal lesions in 10 cases (42%).

All cases of PUNLMP showed a Ki-67 staining of less than 10% (Figure IA, IB). Majority (63%) of LGPUC cases showed imuunoexpression of above 10% with a range between 1% and 50 % (Figure IC)[Table 1]. Strong Ki-67 staining of 70% was observed in the single HGPUC case studied. This was the highest percentage of staining seen among the cases studied.

The association of Ki-67 staining was measured with above parameters using Chi-square test. The cases were divided into two categories based on Ki-67 staining (less than10% and greater than 10%).[6] [Table 2]

The difference in expression of Ki-67 between PUNLMP and LGPUC was statistically significant with a p value of 0.006.

Table 1: Mean, median and range of Ki-67 proliferation index in PUNLMP and LGPUC.

Parameter measured	Ki-67 staining in PUNLMP	Ki-67 staining in LGPUC
Mean	4.86	14.78
Median	5	10
Range	1-8	1-50

Table 2: Correlation of Ki-67 staining with clinical features and histological grade.

Clinical/histological parameter		Ki-67<10%	Ki-67>10%	Total	p Value
Gender	Male	16	21	37	0.286
	Female	4	2	6	
Age	<61 years	13	9	22	0.091
	>61 years	7	14	21	
Focality	Unifocal	6	7	13	0.874
	Multifocal	4	6	10	
Site	Lateral wall	5	7	12	0.448
	VUJ	3	1	4	
	Dome	0	1	1	
	Multiple sites	2	4	6	
Symptoms	Painless hematuria	8	8	16	0.881
	Voiding difficulty	2	1	3	
	Hematuria and Voiding difficulty	2	2	4	
	Lower back pain	1	2	3	
Histological grade	PUNLMP	7	0	7	0.006
	LGPUC	13	22	35	
	HGPUC	0	1	1	

Table 3: Comparison of Ki-67 staining in different studies. [26-28]

	Present study		Cina et al,[26]		Shim et al,[27]		Gajjar et al,[28]	
	Mean	Median	Range	Mean	Median	Range	Mean	Range
PUNLMP	4.86	5	1-8	2.5	1	0.5-15	8.29	2-10
LGPUC	14.78	10	1-50	7.3	3.7	0.5-38.5	38.74	15-40
HGPUC	70	NA	NA	15.7	11	1-65	58.32	NA

NA-Not applicable

DISCUSSION

The most commonly encountered lesions on bladder biopsies are superficial urothelial neoplasms (80%).[1] The neoplastic non-invasive papillary lesions range from benign papillomas to high grade carcinomas. These lesions have diverse biological behavior and differ considerably in terms of prognosis, treatment and surveillance. Despite defined histomorphological criteria,[3] differentiating the close mimics, PUNLMP and LGPUC, still pose a dilemma to the pathologist. Identification of molecular signatures of these lesions improves diagnostic accuracy. However, these modalities are expensive and not easily available. Hence, our study explores the utility of Ki-67 in grading and as an aid to conventional morphological studies as a differentiating marker.

The mean age of presentation (60.62 years), male gender predilection, unifocality, lesions in the lateral wall of the bladder and complaints of painless hematuria correlate with previously published literature. [4,5,7,8,24]

This study essentially evaluated the expression of Ki-67 in PUNLMP, LGPUC and HGPUC. Significant differences in expression (p value=0.006) of Ki-67 were found between the three categories. The Ki-67 staining was directly proportional to increasing grade of the tumour, as seen in several other studies [Table 3]. [6,20,25-26]

In literature, Ki-67 staining in LGPUC spans over 0.5% to 40%; It went up to 50% in our study. This range could reflect varied experimental conditions pertaining to gender, race, age, diverse technical platforms, varying antibody concentrations, different monoclonal antibodies used for IHC, etc. [6] Ki-67 staining also depends on the duration of fixation in

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formalin. Fixation for over 50 hours lowers the expression of Ki-67.[27] A high normal value of LGPUC may reflect transformation to a higher grade or an invasive carcinoma adjacent or elsewhere in the bladder.[24] An overlap exists between PUNLMP and LGPUC in the category of less than 10% Ki-67 staining which emphasizes the need for combined use of morphology and IHC in differentiating these lesions.

Selection bias of having only one case of HGPUC was probably the reason for discrepant mean values of Ki-67 staining in contrast to other studies.

In 2 cases, ambiguous histomorphological features made diagnosis difficult. Each case showed a papillary lesion with predominantly low-grade features with focal areas showing increased cytological pleomorphism. WHO criteria require presence of a higher-grade focus in excess of 5% of the entire tumour to upgrade the neoplasm. [3] Ki-67 index was instrumental in assessing this parameter more effectively than morphology alone as described below.

The first case showed a papillary neoplasm with increased thickness of urothelium, the cells showed mild distortion in architecture, loss of polarity, atypia and anisokaryosis (Figure ID). However, one focus showed increased pleomorphism and hyperchromasia with a high nuclear cytoplasmic(N:C) ratio (Figure IE). Ki-67 expression was higher in this area but it comprised less than 5% of the tumour. Therefore, this case was classified as LGPUC and not upgraded to HGPUC

The other case showed morphological features intermediate between PUNLMP and LGPUC with only a small focus showing mild pleomorphism, nucleomegaly and loss of polarity. Ki-67 in this area showed a Ki-67 expression of 18% in more than 5% of the tumour, confirming the diagnosis of LGPUC.

Interpretation of Ki-67 in areas of regeneration following ulceration and degenerative changes in neoplasms is another quandary. Few biopsies showed areas of ulceration with granulation tissue and regenerative epithelium. These foci showed reactive nuclear features and increased Ki-67 staining representing the proliferative ability of the epithelium as a part of wound healing (Figure IF). Caution in the interpretation of such areas is needed to prevent upgradation of the tumour grade.

Matoso et al reviewed 16 consult cases of LGPUC with degenerative features based on morphology and Ki-67 immunostaining. The cases showed large atypical cells with nuclei five times the size of stromal lymphocytes having smudgy chromatin, intranuclear vacuoles and multinucleation. However, a combination of preserved polarity, few mitoses, Ki-67 less than 5% and negativity in the large smudged cells confirmed a diagnosis of LGPUC.[29]

The limitations of our study include a small sample size with only one HGPUC case. The numbers of LGPUC were much higher than the cases of PUNLMP. A larger sample needs to be studied to minimize random errors. Recurrent cases were not studied to eliminate morphological and interpretative difficulties secondary to BCG and chemotherapy induced pathological changes. Long term follow up studies are recommended as the prognostic utility of Ki-67 cannot be understated. Studies using Ki-67 in conjunction with other markers such as p53 and CK20 have proved that these markers have significant correlation with grade, stage, recurrence and prognosis. [6,24,25]

CONCLUSION

Non-invasive papillary neoplasms like PUNLMP and LGPUC are close mimics that need to be differentiated based on morphology and IHC, as they differ in biological behaviour. Ki-67 index is a useful diagnostic tool to aid the pathologist in grading on a routine basis. There is a direct relationship between Ki-67 expression and the grade of the lesion. This will help in identifying patients with increased risk of recurrence and progression. The cut off value for significant Ki-67 expression varies across studies and standardization will improve quality of future studies and practical applications. In our study, in addition to a majority in literature a 10% cut-off value was found to be most reliable.

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