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Diagnostic utility of CK20, p53 and KI67 to differentiate between Papillomas/Non-Invasive papillary urothelial Neoplasm of low malignant potential/non-invasive papillary urothelial carcinoma, low grade

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Abstract
Background: Urinary Bladder cancer is the 7th most common cancer worldwide, with an estimated 260,000 new cases occurring each year in men and 76,000 in women. It is estimated that approximately 70-80% of patients with newly diagnosed bladder cancer present with non-invasive or early invasive. The problem arise mostly in distinguishing urothelial papilloma from non-invasive PUNLMP & papillary carcinoma especially low grade noninvasive ones. It is very important to distinguish these entities to design the therapeutic and monitoring strategies for these patients and it is difficult on the basis of histological features alone.
Aim & Objectives: To study histomorphological spectrum of urothelial tumors according to WHO/ISUP consensus (2004) and to study pattern of expression of CK20, p53 LI & Ki67 LI to differentiate between Papillomas/PUNLMP/PUCLG.
Material & Method: In this study 300 consecutive cases were taken from March 2016 to May 2017. Cases were histomorphologically classified according to WHO/ISUP (2004) classification & investigated the role of IHC panel of CK20, p53 & Ki67 to evaluate their utility for the diagnosis & to differentiate between Papilloma/PUNLMP/PUCLG.
Results: Out of 300 cases, 48 cases were required IHC (CK20, p53, Ki67) for final diagnosis. Out of 48 cases, 16 were diagnosed as PUCLG, 10 were diagnosed as PUNLMP. Urothelial papilloma and Inverted urothelial papilloma were diagnosed in 13 and 9 cases respectively.
Conclusion: The WHO/ISUP (2004) classification for bladder tumor enables to diagnose urothelial tumors into clinically and prognostically relevant entities. IHC panel of CK20, p53 & Ki67 may be a useful diagnostic marker in Papillary urothelial neoplasms, especially Non-invasive urothelial neoplasias at the lower end of the spectrum & help to differentiate papilloma/PUNLMP/PUCLG with borderline histological features.

Keywords: Papilloma, PUNLMP, PUCLG, Immunohistochemistry – CK20, p53 & Ki67

Introduction
Urinary Bladder cancer is the 7th most common cancer worldwide, with an estimated 260,000 new cases occurring each year in men and 76,000 in women [1]. It accounts for about 3.2% of all cancers worldwide and is considerably more common in males than in females (ratio worldwide is about 3.5:1) [2]. It is estimated that approximately 70-80% of patients with newly diagnosed bladder cancer present with non-invasive or early invasive [3]. The most common presenting symptom of bladder cancer is painless gross hematuria which occurs in 85% of patients [4]. The papillary lesions of the urinary bladder vary from benign lesions to lesions showing dysplasia or malignant changes [5].
The classification & grading of papillary urothelial neoplasm has been a long standing subject of controversy. Previously, numerous grading schemes for bladder tumor, including 1973 WHO classification, existed whereby one of the major limitations was poor inter-observer reproducibility [6], In 1998, the WHO/ISUP consensus classification, noninvasive papillary urothelial tumors were divided into 4 groups, namely, papilloma, Papillary Urothelial Neoplasm of Low Malignant Potential (PUNLMP),

Low grade carcinoma & High grade carcinoma [7]. This classification was revised most recently in 2003 (published in 2004) and now has become the 2004 WHO / ISUP consensus classification of urothelial tumors [8]. The current classification system provides detailed histological criteria for papillary urothelial lesions & allows for designation of a lesion PUNLMP with a negligible risk of progression [6]. This classification recognizes papilloma, PUNLMP & low grade papillary transitional cell carcinoma at the low grade end of the transitional cell neoplasia spectrum [8].

Papillomas have a low recurrence rate with a low risk for subsequent development of higher grade tumors [9]. PUNLMP are lower grade papillary neoplasm that are not intrinsically malignant but associated with a significant risk for development of new papillary tumors (recurrence) [10] It is intermediary stage between Papilloma and Non-invasive Papillary Urothelial Carcinoma, Low Grade (PUCLG) [11]. PUCLG have higher recurrence rate than for PUNLMP [10]. Therefore distinguishing urothelial papilloma from non invasive PUNLMP & papillary carcinoma especially low grade non invasive ones is very important to design the therapeutic and monitoring strategies for these patients [8].

Despite of histological examination being the mainstay in the diagnosis of the urothelial tumors, Immunohistochemistry (IHC) plays a major role in improving the diagnostic accuracy of some urothelial tumors. Combined use of IHC markers may be helpful, especially when histological features are borderline [12].

CK20, p53, and Ki-67 are related either to neoplastic changes or prognosis in urothelial proliferations. Aberrant CK20 expression in urothelial cells plus over expression of p53 and Ki-67 are indicators of early dysplastic changes in urothelial mucosa [13] Thus, the purpose of present study is to investigate the role of IHC especially panel of CK20, p53 & Ki67 in urothelial tumors to evaluate their utility for the diagnosis of the urothelial tumors & to differentiate between Papillomas (Urothelial Papilloma-UP/ Inverted Papilloma-IP) / PUNLMP / PUCLG.

Material and methods

A total of 314 cases were received during study which was carried out in the Histopathology Department at, Santokba Durlabhji Memorial Hospital cum Medical Research Institute, Jaipur, Rajasthan. This study was conducted over a period 15 months, extending from March 2016 to May 2017. It was Observational Prospective Study which included 300 urothelial tumor specimens.

Cases diagnosed as urothelial neoplasm on light microscopy and cases received for the evaluation by IHC markers for further diagnosis were included and biopsy with inadequate/ suboptimal material for light microscopy were excluded. 300 cases completely satisfied the inclusion criteria of our study. 14 cases were excluded as they had suboptimal material for light microscopy.

Statistical analysis will be performed with the SPSS, Trial version 20 for Windows statistical software package (SPSS inc., Chicago, Il, USA). The Categorical data will be presented as numbers (percent) and will be compared among groups using Chi square test. Groups were compared for quantitative data will be present as mean and standard deviation (Range). Probability P value <0.05 was considered statistically significant.

After receiving the specimen in the histopathology department, a detailed clinical history and type of specimen (endoscopic biopsy / TURBT / resected specimen) of the patients under study were recorded. Written consent was obtained after explaining the nature and purpose of study. H & E stained sections were examined for provisional morphologic diagnosis of urothelial neoplasm. An attempt was made to classify urothelial neoplasm according to WHO/ISUP consensus classification (2004) by two senior consultants of the department. If IHC required then the block best representative of the tumor was selected for immunohistochemical staining.

IHC analysis was performed using Antigen retrieval method: BIO GENEX-EZ-Retriever system V.3 (temperature controlled microwaving). Colon cancer for CK20, Breast cancer for p53 & Lymphoma for Ki67 were taken as positive control. Negative control was performed by replacing primary antibodies with phosphate-buffered saline. On IHC type of staining (Cytoplasmic/Nuclear) & intensity of staining was observed.

The pattern of CK20 expression was evaluated as abnormal when immunoexpression extended to deeper layers as clusters of >3 positively stained cells or diffuse staining of the urothelium [7]. For the assessment of p53 and Ki67 expression, 200 cells from the most immunoreactive regions of each lesion was visually counted. The percentage of cells positive for p53 were recorded as p53 Labeling Index (p53 LI) [14]. p53 was considered positive when 10 % of cells show nuclear positivity [15]. The percentage of cells positive for Ki67 were recorded as Ki67 Labeling Index (Ki67 LI).

Morphological & IHC diagnosis of urothelial neoplasms were attempted using the WHO/ISUP consensus classification (2004) & correlation of morphologic diagnosis & final IHC diagnosis were attempted.

Results

A total of 314 cases were initially considered for study, out of which 14 cases were excluded (based on exclusion criteria). The study thus includes 300 cases of Urothelial tumors for final evaluation. In present study 75 % cases were seen in males. 25% cases were seen in females. Male Female ratio being 3:1.The mean age of the study group was 60.65 year. Minimum 27 years and maximum 88 years age was found in our study. Maximum no. of cases were diagnosed (33 %) in 61-70 years of age group.

Out of 300 cases of urothelial tumors, 84% (n=252) cases had classical histomorphological features and did not required marker studies for final diagnosis. In 16% (n=48) cases IHC (CK20, p53, Ki67) were required for final diagnosis.

4 cases were diagnosed as UP / PUNLMP on histomorphology. CK20 expression was normal in all the 4 cases, p53 LI was 0 % in three cases and 02 % in the fourth case and Ki67 LI was 1 to 2 % in three cases and 10 % in the fourth case. The final diagnosis was UP in three cases and PUNLMP in the fourth case.16 cases were diagnosed as IP / PUCLG on histomorphology. In 9 cases CK20 expression was normal, p53 LI was 0-2 % and Ki67 LI was 1-6% and were diagnosed as IP. In 07 cases, CK20 expression was abnormal, p53 LI was >10% (Range 10-18 %) except one case which showed p53 LI 8% and Ki67 LI was 20-40 %. 15 cases were diagnosed as UP / PUCLG on histomorphology. In 10 cases, CK20 expression was normal, p53 LI was 0-3% and Ki67 LI was 1-6%. In 05 cases, CK20

expression was abnormal, p53 LI was >10% (Range 12-20 %) and Ki67 LI was 15-40%. 13 cases were diagnosed as PUNLMP / PUCLG on histomorphology. In 9 cases, CK20 expression was normal, p53 LI was 0-3 % and Ki67 LI was 2-7 %. In 4 cases, CK20 expression was abnormal, p53 LI was>10 % (Range 14-20 %) and Ki67 LI was 28-38%.

Table 1: Expression of CK20 and percentage of p53 and Ki67 positive cells in cases with differential diagnosis on histomorphology and Distribution of Urothelial tumors diagnosed on the basis of IHC markers (CK20, p53 & Ki67) (n=48) WHO/ISUP consensus classification (2004)

S. No	Diagnosis based on Histomorphology (N=48)	CK20 expression	p53 LI (%)	Ki67 LI (%)	Final Diagnosis based on IHC (N=48)
1.	UP / PUNLMP (n=4)	Normal	0	1-2	UP (n=3)
			2	10	PUNLMP (n=1)
2.	IP / PUCLG (n=16)	Normal	0-2	1-6	IP (n=9)
		Abnormal	>10 (10-18)*	20-40	PUCLG (n=7)
3.	UP / PUCLG (n=15)	Normal	0-3	1-6	UP (n=10)
		Abnormal	>10 (12-20)	15-40	PUCLG (n=5)
4.	PUNLMP / PUCLG (n=13)	Normal	0-3	2-7	PUNLMP (n=9)
		Abnormal	>10 (14-20)	28-38	PUCLG (n=4)
***** p53 LI was >10% (10-18 %) except one case which showed p53 LI 8%					

Out of 48 cases, 16 were diagnosed as Non-invasive papillary urothelial carcinoma low grade (PUCLG), 10 were diagnosed as Non-invasive papillary urothelial neoplasm of low malignant potential (PUNLMP). Urothelial papilloma and Inverted urothelial papilloma were diagnosed in 13 and 9 cases respectively.

Table 2: Association of IHC markers p53, Ki67and CK20 with type of urothelial neoplasia

	P53		Ki67		CK20	
	<10%	>10%	0-10 %	15-40 %	Normal	Abnormal
IP (n=9)	9	0	9	0	9	0
UP (n=13)	13	0	13	0	13	0
PUNLMP (n=10)	10	0	10	0	10	0
PUCLG (n=16)	1	15	0	16	0	16
Total (n=48)	33	15	32	16	32	16

Out of 48 cases all the cases of Urothelial papilloma, Inverted urothelial papilloma and PUNLMP showed normal CK20 expression (100%) and all the cases of PUCLG showed abnormal CK20 expression (100 %).

There was significant association between p53 LI with type of urothelial neoplasia. p53 LI (<10%) was seen in 100% cases of IP, PUNLMP and UP. p53 LI (>10%) was seen in 93.75% (n=15) cases of PUCLG and it was <10% in only 6.25% (n=1) of cases. Chi-square = 39.529 with 3 degrees of freedom; $P< 0.001$ (for p53)

There was significant association between Ki67 LI with type of urothelial neoplasia. Ki67 LI (0-10%) was seen in 100% cases of IP, PUNLMP and UP. Ki67 LI (15-40%) was seen in 100% cases of PUCLG. Chi-square = 48.00 with 3 degrees of freedom; $P<0.001$ (for ki67)

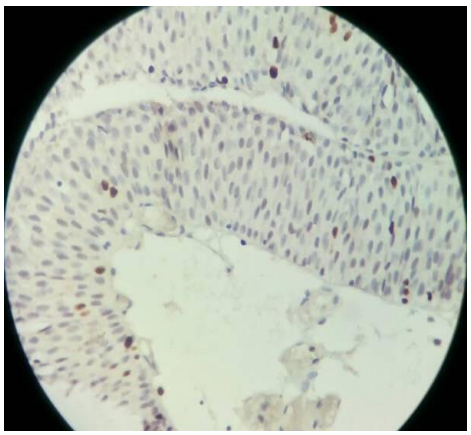

Fig 2: Inverted urothelial papilloma, Ki67 LI 2-3% (IHC; 40X)

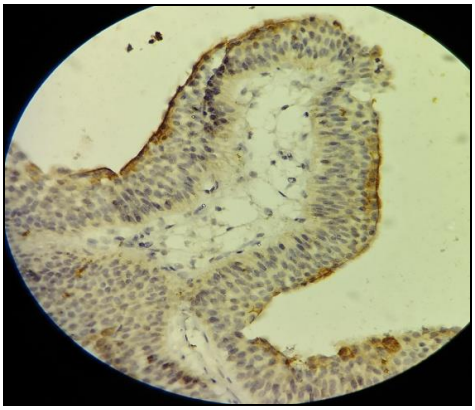

Fig 1: Inverted urothelial papilloma showing normal CK20 expression in superficial umbrella cells (IHC; 40X)

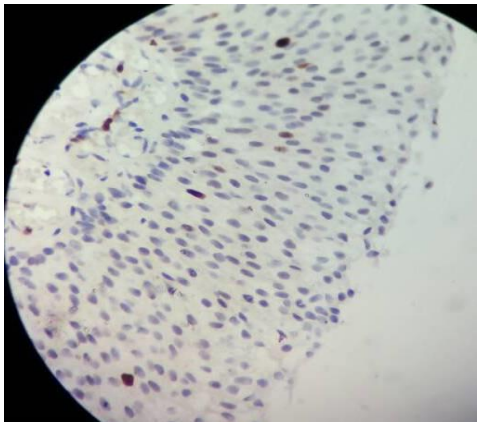

Fig 3: Inverted urothelial papilloma, p53 LI 1% (IHC; 40X)

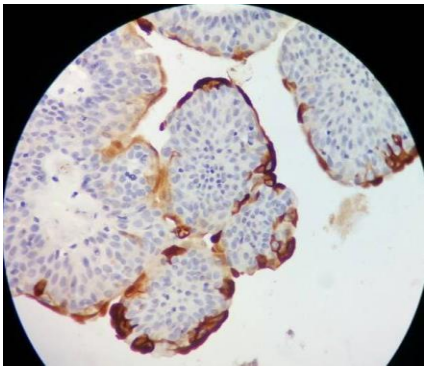

Fig 4: Papillary urothelial neoplasm of low malignant potential (PUNLMP) normal CK20 expression (IHC; 100X)

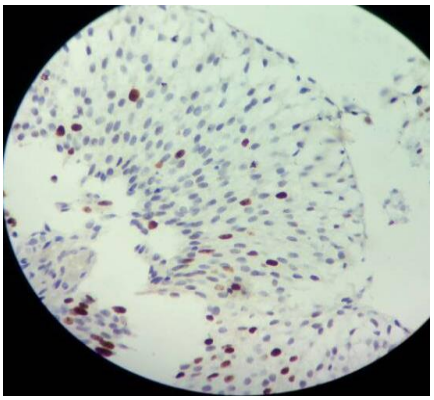

Fig 5: Papillary urothelial neoplasm of low malignant potential (PUNLMP) Ki67 LI 5% (IHC; 40X)

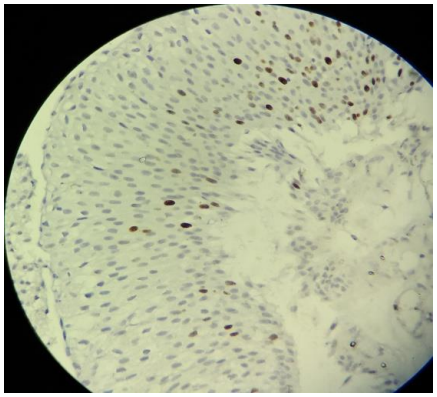

Fig 6: Papillary urothelial neoplasm of low malignant potential (PUNLMP) p53 LI 3-5% (IHC; 40X)

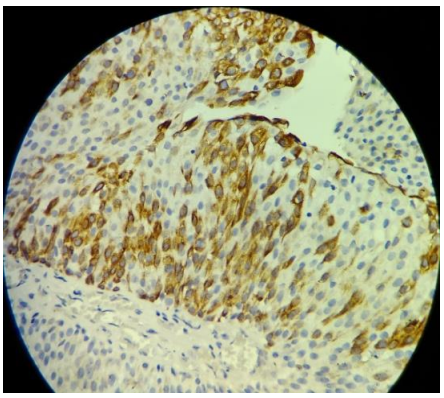

Fig 7: Papillary urothelial carcinoma, low grade (PUCLG), abnormal CK20 expression (diffuse & strong immunoreactivity) (IHC; 40X)

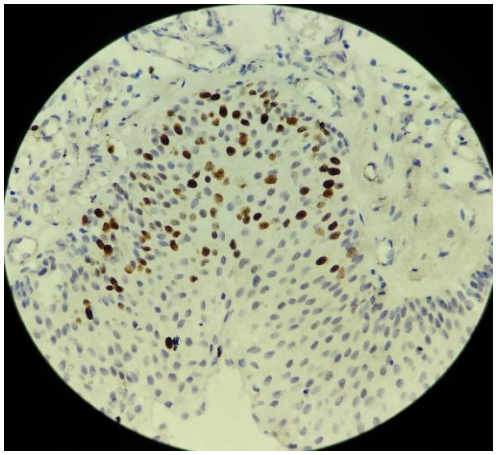

Fig 8: Papillary urothelial carcinoma, low grade (PUCLG) Ki67 LI 30% (IHC; 40X)

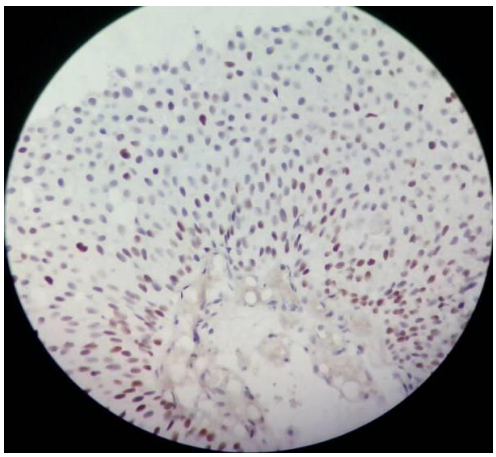

Fig 9: Papillary urothelial carcinoma, low grade (PUCLG) p53 LI 18-20%, (IHC; 40X)

Discussion

The conventional H and E staining is useful for histomorphological diagnosis in majority of the cases but the problem arises in mainly papillomas (UP/IP), non-invasive PUNLMP and non-invasive PUCLG. The present study includes 300 cases presenting as urothelial tumors and attempt has been made to study histomorphological spectrum of urothelial tumors according to WHO/ISUP classification 2004 and to evaluate the role of Immuno-histochemical expressions of CK20, p53 and Ki67 to differentiate between Papillomas (UP/IP)/PUNLMP/ PUCLG.

In the present study 84 % cases could achieve a histological diagnosis on basis of histomorphology alone, and in 16 % cases IHC was required for final diagnosis, mainly to differentiate between Papillomas / Non-invasive PUNLMP/Non-invasive PUCLG.

Many studies done by Alrashidy *et al.* [15] Eble *et al.* [8] Mallofre *et al.* [13] Helpap *et al.* [16] have also reported that distinguishing urothelial papillomas/ PUNLMP and papillary carcinomas especially non-invasive low grade one is sometimes difficult on the basis of histomorphological features alone and IHC markers may be used as an adjuvant to histology to arrive at a final diagnosis in these cases.

IHC markers (CK20, p53 and Ki67) were applied on cases with differential diagnosis of UP / PUNLMP, IP/ PUCLG, UP/ PUCLG and PUNLMP/ PUCLG.

16 cases were diagnosed as Non-invasive PUCLG which showed abnormal CK20 expression, p53 LI = 8-20 % and Ki67 LI = 15-40 %.The immunoreactivity of all these cases of PUCLG were in accordance with studies performed by Cina *et al*. [14] Das *et al*. [17] and Shim *et al*. [11] One case diagnosed as PUCLG showed p53 LI = 8 % (<10%) whereas CK20 expression was abnormal and Ki67 LI = 40 %. Though the p53 LI was <10% but due to abnormal CK20 expression and high Ki67 LI, the case was diagnosed as PUCLG. This was in concordance with the study performed by Y. Leong *et al*. [7] in which p53 LI <5% in 79% of cases with abnormal CK20 expression and high Ki67 LI (15-40%). Since the exact number of mitosis are difficult to identify on H and E stain, Ki67 can be used to assess the proliferative activity of the tumor and proliferative activity being quite high (Ki67 LI 40%) in this case, we diagnosed it as PUCLG.

10 cases were diagnosed as PUNLMP which showed normal CK20 expression, p53 LI = 0-3 % and Ki67 LI = 2-10 %. The immunoreactivity of all these cases were in concordance with the studies performed by Alsheikh *et al*. [18] Y. Leong *et al*. [7] Cina *et al*. [14] Das *et al*. [17] However, study performed by Shim *et al*. [11] showed Ki67 LI = 6.15-10.43 %, abnormal CK20 expression in 45.5% cases of PUNLMP and p53 LI = >15% in 31.8% cases.Ki67 LI correlated well with our study but the p53 LI and CK20 expression was not in accordance with our study. The possible explanation for higher p53 LI may be indicative of the presence of abnormal molecular events with the potential for neoplastic progression and/ or may serve as a marker for the concurrent presence of dysplasia or carcinoma elsewhere in the bladder [14]. Regarding abnormal CK20 expression in their study could be that expression of cytokeratins are determined by cell type, stage of development, differentiation and may represent those PUNLMPs which are prone to recurrence.

13 cases were diagnosed as Urothelial papilloma and 9 cases were diagnosed as Inverted papilloma which showed normal CK20 expression, p53 LI = 0-3 % and Ki67 LI = 1-6 %. The immunoreactivity of all these cases of Urothelial papilloma / Inverted urothelial papilloma correlated well with the studies performed by Alrashidy *et al*. [15] Desai *et al*. [19] Cina *et al*. [14] and Shim *et al*. [11]

Thus, we infer that IHC panel composed of CK20, p53 and Ki67 can be used as an adjuvant to routine histological section in the problematic cases and to diagnose and clarify the histological entities describe in the new WHO/ISUP consensus classification system (2004).

In our study we faced major difficulty in placing the tumor in the category of non-invasive PUNLMP as the critical point for separating non-invasive PUNLMP from papillomas and non-invasive PUCLG according to the morphological criteria laid down by WHO/ISUP classification system (2004) can be overlapping and thus leading to inter-observer variability. The studies performed by Cheng L. *et al*. [20], Murphy WM *et al*. [21] and Yorukoglu K. *et al*. [22] also observed severe discrepancies in cases of non-invasive PUNLMP by even the most experienced urologic pathologists.

Since distinguishing non-invasive PUNLMP from Papillomas (UP/IP) / Non-invasive PUCLG is very important to design the therapeutic and monitoring strategies for these patients. It is of utmost important to exactly categorize the tumor according to the WHO/ISUP classification (2004).

Thus, the use of these markers (CK20, p53 and Ki67) has been of particular help in the final diagnosis of these tumors (Papillomas/ Non-invasive PUNLMP/ Non-invasive PUCLG).

Limitations of the present study were, due to shorter span of study (March 2016 to May 2017), not a single case of Urothelial carcinoma in situ was received during the study period. Second, not all the histological subtypes of infiltrating urothelial carcinoma could be seen in the study. Thus, the percentage of subtypes may not be the actual representation of the frequency of tumors and third, the follow up, disease progression and recurrence could not be incorporated into the study.

Table 3: Comparison of CK20 expression, p53 LI and Ki67 LI in Papilloma, Non- invasive PUNLMP and Non-invasive PUCLG in different studies.

Final Diagnosis	IHC	Cina *et al*. [14].	Shim *et al*. [11].	Das *et al*. [17].	Y. Leong *et al*. [7].	Alsheikh *et al*. [18].	Alrashidy *et al*. [15].	Desai *et al*. [19].	Present study
Papilloma	CK20	-	Normal	-	-	-	Normal	Normal	Normal
	p53	0 %	0 %	-	-	-	<5 %	-	0-3 %
	Ki67	1-8 %	4.06-11.2%	-	-	-	<5 %	-	1-6 %
PUNLMP	CK20	-	-	Normal	Normal	Normal	-	-	Normal
	p53	0-2 %	-	<10 %	-	-	-	-	0-3 %
	Ki67	0.5-15 %	-	-	0-10 %	-	-	-	2-10 %
PUCLG	CK20	-	Abnormal	Abnormal	-	-	-	-	Abnormal
	p53	0-20.5 %	>15 %	10-50 %	-	-	-	-	10-20 %
	Ki67	0.5-38.5 %	26.49-50.99%	-	-	-	-	-	15-40 %

Conclusion

The WHO/ISUP consensus classification (2004) for urinary bladder tumors enables us to diagnose the urothelial tumors into clinically and prognostically relevant entities. Although histopathological examination is the mainstay for diagnosis of urothelial tumors, some cases can often have overlapping features especially Non-invasive urothelial neoplasias at the lower end of the spectrum (Papilloma / Non-invasive PUNLMP/ Non-invasive PUCLG). IHC markers especially panel of CK20, p53 and Ki67 are useful for accurate diagnosis of the papillary urothelial neoplasms those are histologically "on the fence" with regard to tumor type. Three markers in our opinion could be used in routine practice, together with careful clinical and morphologic correlation.

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