ORIGINAL ARTICLE

p53 and cytokeratin 20 immune marker expression as dual marker in papillary lesions of urinary bladder

Anita P. Javalgi[1], Vidisha S. Athanikar[1], Priyanka P[1], Anushree S[1]*

[1]Department of Pathology, Shri Dharmasthala Manjunatheswara College of Medical Science and Hospital, Shri Dharmasthala Manjunatheswara University, Dharwad-580009 (Karnataka) India

Abstract

Background: The classification and grading of papillary urothelial neoplasm has been a long-standing subject of controversy and various immunohistochemistry markers have been studied, out of which p53 and cytokeratin 20 (CK20) are emerging as useful indicators for neoplastic changes and prognosis in urothelial proliferations. *Aim and Objectives*: To determine the expression status of p53 and CK20 immune markers in papillary lesions of urinary bladder. *Material and Methods*: This was a prospective study of 2 years. All lesions favouring papillary lesions were included. Metastatic lesions and recurrent papillary lesions were excluded from the study. For statistical analysis, IBM SPSS-21.1 version software was used and the levels of significance were calculated using Chi-square test and Fisher's exact test. *Results*: A total of 59 papillary lesions of urinary bladder were studied. Males were affected more than females. The commonest lesion noted was high grade non-invasive papillary carcinoma. The combined usage of CK20 and p53 as a dual immune markers showed statistical significance ($p = 0.046$) in differentiating papillary lesions. *Conclusions*: The present study concluded that differentiating papillary lesions of urothelium, depending on morphology is challenging and thus staining for CK20 and p53 may be helpful in differentiating the lesions.

Keywords: Invasive Papillary Carcinoma, Immunohistochemistry, Bladder Biopsy

Introduction

Urinary bladder lesions especially carcinoma is common in men following prostate, lung and colorectal carcinoma [1-2]. Various risk factors like cigarette smoking, arylamines, aniline dyes, auramines, phenacetin, and cyclophosphamide exposure are associated with development of bladder carcinomas. Schistosoma haematobium infestation and radiation exposure especially for the treatment of prostate cancer also plays a role in bladder tumours [3]. It is estimated that approximately 70-80% of patients with newly diagnosed bladder cancer present with non-invasive or early invasive carcinoma. The most common presenting symptom of bladder cancer is painless gross haematuria which occurs in 85% of patients. The papillary lesions of the urinary bladder vary from benign lesions, dysplastic changes to malignant ones [4]. The classification and grading of papillary urothelial neoplasm has been a long standing subject of controversy. Previously, numerous grading schemes for bladder tumor, including 1973 World Health Organization (WHO) classification, existed whereby one of the major limitations was poor interobserver reproducibility. In 1998 the WHO/International Society of Urological Pathology (ISUP) consensus proposed and classified non-invasive papillary urothelial tumours into 4 groups which included papilloma, Papillary Urothelial Neoplasm of Low Malignant Potential (PUNLMP), Low Grade Urothelial

Carcinoma (LGUC) and High-Grade Urothelial Carcinoma (HGUC). This classification is revised regularly considering progress and prognosis of disease. The current classification system provides detailed histological criteria for papillary urothelial lesions and allows for designation of a lesion as PUNLMP with a negligible risk of progression [5-7]. Immunohistochemical markers like p53 and cytokeratin 20 (CK20) have been investigated in several international studies for their use as diagnostic and prognostic aids in urothelial tumours [8]. In the search for reliable markers in literature; p53 and CK20 are emerging as useful indicators of neoplastic change and prognosis in urothelial proliferations [9]. CK20, p53, and Ki-67 are related either to neoplastic changes or prognosis in urothelial proliferative lesions. Aberrant CK20 expression in urothelial cells plus over expression of p53 and Ki-67 are indicators of early dysplastic changes in urothelial mucosa [10]. This study aimed to determine the immune expression of p53 and CK20 as dual markers in papillary lesions of urinary bladder.

Material and Methods

The present study was a prospective study of 2 years. Ethical clearance was obtained (SDMCDS IEC NO. 2021/Medical/Pathology/S/02) from Institutional Ethics Committee. Considering the prevalence of bladder lesions in the last 2 years at our hospital, the sample size was calculated as approximately 50 cases.

Inclusion criteria: All the bladder tissue samples (Formalin fixed) with morphology favouring papillary lesions were included in the study.

Exclusion criteria: Metastatic lesions and recurrent papillary lesions, autolyzed or inadequate biopsies and non-representative biopsy found in microscopy with respect to cystoscopy features were excluded.

Clinical details were simultaneously obtained from hospital information system.

The tissue samples received were fixed in 10% buffered formalin for 24 hours. After detailed gross examination and extensive sampling, the tissue was processed in automatic tissue processor. Processed tissue was paraffin embedded. Sections were cut at 4-5 μm thickness and stained with Hematoxylin and Eosin (H&E) stain and then thoroughly examined. Slides with deparaffinized sections were dipped in 100% ethanol for 2 minutes. Then they were dipped in 70% ethanol for 2 minutes and washed in water for 2 minutes. Slides were then kept in H&E stain for 10-15 minutes and washed in water for 2 minutes, then dipped in 1% acid alcohol and washed again in water for 2 minutes. The slides were stained with eosin for 2-3 minutes and washed in water for 2 minutes and then one dip in 100% alcohol and then xylene. Finally, the slides were mounted with Dibutylphthalate Polystyrene Xylene (DPX).

Immunohistochemistry procedure

Tissue of 4 μm thick sections were taken on positively charged slides. After overnight incubation at 37º C and deparaffinization with repeated washes of Xylene (10 min each), rehydration of tissues with graded alcohol was done. The slides were then washed with running water (10 min) and distilled water (5 min). Antigen retrieval with citrate or EDTA buffer at 95º C was done and cooled at room temperature. Peroxide block was carried out for 10-15 min and washed in Phosphate Buffer Solution (PBS) for 5 min and incubated with primary antibody p53 and CK20 rabbit monoclonal antibody for 45 min. Subsequently,

tissues were incubated with Poly Excel Target Binder for 15 to 20 min and washed with PBS for 5 min. Again, incubation was done with Poly Excel Poly Horse Radish Peroxidase for 15 to 20 min, washed with PBS for 5 min and developed with Diaminobenzidine (DAB) chromogen for 5-8 min. Sections were counterstained with hematoxylin after washing with running water. Each set was run with positive and negative controls. Primary antibody was not used instead Tris buffer was used. Positive controls used for p53 and CK20 were lymph node and colonic mucosa, respectively. Interpretation of CK20 and p53 was done as follows:

CK20 expression was considered as negative (when stained normal) in figure 1 and positive (when stained abnormal) in figure 2. Negative expression was defined as CK20 staining restricted to superficial cells of the urothelium or less than three cells in intermediate cells of the urothelium. Positive expression was called when immunoexpression was seen in deeper layers of urothelium as clusters of more than three positively stained cells or diffuse staining of urothelium [1]. Expression of p53 was calculated as a percentage of labelled nuclei per 500 cells counted in most immunoreactive region of the tumour and categorized into negative and positive. Negative (normal) expression of p53 was reported when < 5% of the cells, counted from the most immunoreactive regions of the section showed nuclear staining for p53 (Figure 3). Positive (abnormal) expression of p53 was considered when > 10% of the cells counted from most immunoreactive region of the section showed nuclear staining for p53 (Figure 4) [1].

Statistical analysis

After data collection, the results were tabulated and analysed with the help of tables, proportions and percentages. IBM SPSS-21.1 version software was used for statistical analysis, and the results were compared using Fischer's exact test. The value of $p < 0.05$ was considered as statistically significant.

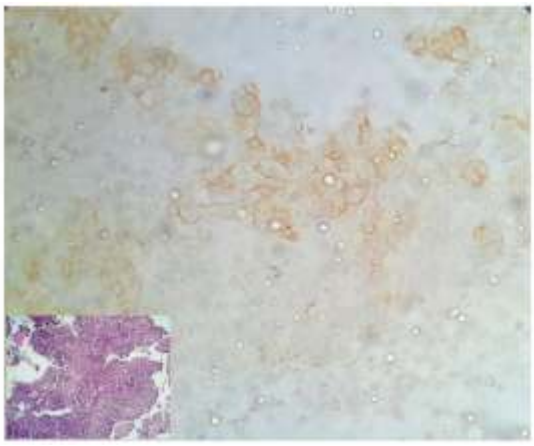

Figure 1: IHC 400× CK20 shows normal expression. Inset shows H&E stained section 400× Case of invasive papillary urothelial carcinoma.

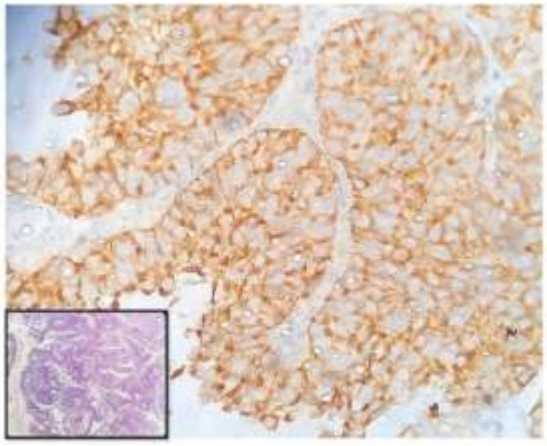

Figure 2: IHC 400× CK20 shows abnormal expression. Inset shows H&E stained section 400× Case of high grade papillary urothelial lesion.

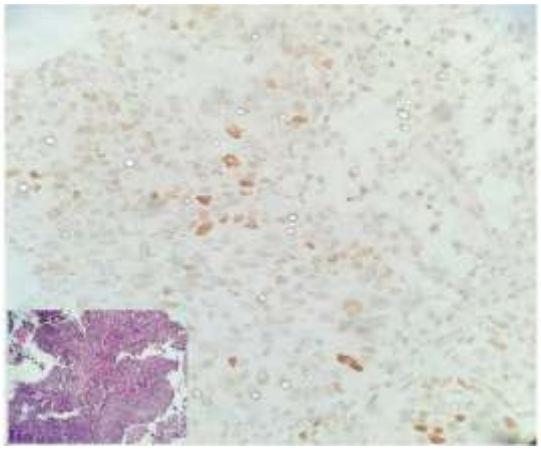

Figure 3: IHC 400×p53 shows normal expression. Inset shows H&E stained section 400× Case of invasive papillary urothelial carcinoma.

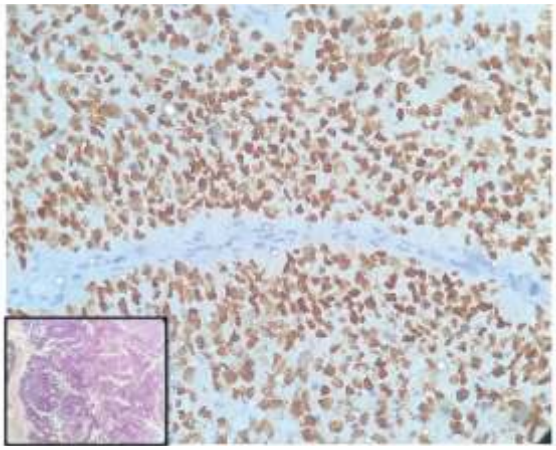

Figure 4: IHC 400×p53 shows abnormal expression. Inset shows H&E stained section 400× Case of high grade papillary urothelial lesion

Results

A total of 59 bladder biopsies were studied in study period which showed varied papillary lesions on morphology. Males were affected more than females i.e., 54 (90%) cases were seen in males and 5 (10%) cases in females. Average age affected was 63.2 years with youngest being 38 years old and oldest being 88 years old. The commonest clinical presentation was abdominal pain followed by dysuria and hematuria. These features were more obvious in malignant lesions than benign lesions. On cystoscopy the growth pattern varied from flat to polypoidal growth as shown in Table 1. Commonest was papillary growth on cystoscopy.

The morphological spectrum noted was from papillary urothelial hyperplasia to invasive papillary carcinoma as shown in Table 2. The commonest lesion noted was high grade non-invasive papillary carcinoma which accounted for 33.9% cases. Only 1 case of papillary urothelial hyperplasia was encountered in our study period. Immunohistochemistry of CK20 and p53 was performed on these 59 cases and Table 3 shows the expression pattern of these IHC markers. It was observed that CK20 as a single marker was not statistically significant in differentiating papillary lesions; how-ever it was observed that there was abnormal staining pattern in non-invasive papillary carcinoma (low grade and high grade) and invasive urothelial carcinoma. p53 immunostaining showed statistical significance ($p = 0.032$) in differentiating urothelial papilloma and PUNLMP from both non-invasive low grade and high-grade papillary carcinoma. p53 was abnormally expressed in low grade and high-grade lesions compared to hyperplasia, papilloma and PUNLMP, which showed normal to weak pattern of staining. CK20 and p53 were abnormally expressed in low grade, high grade and invasive papillary carcinoma compared to normal expression in papillary hyperplasia, inverted papilloma and PUNLMP. Statistical significance for CK20 and p53 as dual markers was significant ($p = 0.046$).

Table 1: Distribution of growth pattern on cystoscopy

Growth patterns	Malignant	Benign
Papillary	25 (49.02%)	2 (25%)
Solid and Papillary	7 (13.73%)	0
Solid	10 (19.61%)	3 (50%)
Sessile	0	1 (25%)
Polypoidal	5 (9.80%)	0
Solid and Sessile	1 (1.96%)	0
Proliferative	3 (5.88%)	2
Total	51 cases	8 cases

Table 2: Spectrum of papillary lesions of Urinary Bladder

Type of lesion	Number of lesions (Percentage)
PUH	1 (1.6%)
IUP	4 (6.8 %)
PUNLMP	8 (13.6%)
LGPUC	12 (20.4%)
HGPUC	20 (33.9%)
IPUC	14 (23.7%)
Total	59 cases

PUI: Papillary urothelial hyperplasia, IUP: Inverted urothelial papilloma, PUNLMP: Papillary urothelial neoplasm of low malignant potential, LGPUC: Low grade papillary urothelial carcinoma, HGPUC: High grade papillary urothelial carcinoma, IPUC: Invasive papillary urothelial carcinoma

Table 3: CK20 and p53 staining pattern in papillary lesions of UB

Type of lesion	Number of lesions	CK20		p53	
		Abnormal	Normal	Abnormal	Normal
PUH	1	-	1	-	1
IUP	4	-	4	1	3
PUNLMP	8	2	6	6	2
LGPUC	12	6	6	8	4
HGPUC	20	9	11	17	3
IPUC	14	2	12	13	1
Total	59	19	40	46	13

PUI: Papillary urothelial hyperplasia, IUP: Inverted urothelial papilloma, PUNLMP: Papillary urothelial neoplasm of low malignant potential, LGPUC: Low grade papillary urothelial carcinoma, HGPUC: High grade papillary urothelial carcinoma, IPUC: Invasive papillary urothelial carcinoma

Table 4: Comparison of various studied in literature with present study

Lesions	IHC markers	Gajjar *et al*., (2019) [26]		Ahadi *et al*., (2021) [29]		Raheem *et al*., (2014) [30]		Present study	
		Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
PUH	**CK20**	9	-					1	-
	p53	-	-					1	-
IUP	**CK20**	9	-					4	-
	p53	-	-					3	1
PUNLMP	**CK20**	10	-					6	2
	p53	-	-					2	6
LGPC	**CK20**	4	16	11	14	6	12	6	6
	p53			12	23	8	10	4	8
HGPC	**CK20**			2	23	1	6	11	9
	p53			5	20	0	7	3	17
IPC	**CK20**					45	117	12	2
	p53					55	107	1	13

IHC: Immunohistochemical

Discussion

Since decades the three-tiered classification for the morphological grading of papillary neoplasms is maintained, aside from the rare urothelial papillomas. The papillary lesion and its grading were first proposed in 1998 and since then the WHO has been revising the grading system in consequent series. The morphological findings considered for grading are increased cellularity, nucleus accretion, nuclei pleomorphism, chromatin pattern, abnormal mitosis, loss of cell polarity, cell differentiation from the basement membrane to the cell surface, cell size differences and giant cell [11-13]. In 1998 the WHO/ISUP divided non-invasive papillary urothelial tumours into 4 groups namely, papilloma, PUNLMP, low grade carcinoma and high- grade papillary carcinoma [14].

The various papillary lesions, its histological classification and grading is one of the most important determinants for prognosis. This classification system closely reflects the two major molecular pathways of evolution of urothelial neoplasms and is based on the level of architectural and cytological disorder, which has been proved to be clinically relevant [13, 15-16]. Previously, the precursor lesions of low-grade non-invasive papillary carcinoma were designated as "papillary urothelial hyperplasia" or "urothelial proliferation with undetermined malignant potential". Such lesions have a tented architectural appearance, with short, non-branching papillae covered by mildly atypical urothelium that has cytological features similar to those of low-grade non-invasive papillary carcinoma. In this fifth-edition of WHO classification, such lesions are no longer recognized as a unique entity; rather, they are considered early low-grade non-invasive papillary carcinoma or an extension of such tumours. This classification of papillary tumours and its grading system is accepted by pathologists, urologists, and oncologists worldwide [17-19].

There are many morphological challenges faced in histological examination of urothelial lesions, even though histological examination stays a main stream of diagnostic tool. Now few IHC markers have evolved and in difficult cases, it plays a major role in improving the diagnostic accuracy. Usage of multiple IHC markers may be helpful, especially when histological features are borderline [20]. CK20, p53, p16 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 [21].

p53 and CK20 have been investigated in several international studies for their use as diagnostic and prognostic aids in urothelial tumors [22-23]. p53 is a cell proliferation regulating and pro-apoptotic gene. It is a nuclear phosphoprotein which acts as tumor suppressor and plays a role in apoptosis, genetic stability, and inhibition of angiogenesis. Mutation in p53 can nullify its physiological functions and increase expression of the mutant protein, and this is regarded as a predictor of poor prognosis of urothelial tumors [24]. Wild-type p53 protein has a short half-life; however, the protein encoded by mutated p53 remains active for a long period. Therefore, mutation of p53 gene results in p53 accumulation in cells nuclei. This accumulation is detectable with immunohistochemical methods and correlates with p53 gene mutation, thus, detection of p53 protein in the nuclei of cells by immunohistochemical methods [1].

Studies done by Alrashidy *et al.,* (2016), Eble *et al.*, (2004), Mallofre *et al.*, (2003), mentioned that distinguishing urothelial papillomas/PUNLMP and non-invasive low grade papillary carcinoma is sometimes difficult on the basis of morphological features alone and IHC markers can be useful as an adjuvant investigation along with histopathological findings to arrive at a final diagnosis [7, 10, 25].

Mutations of the p53 gene and immunohistochemical positivity for the p53 protein have been found in 40% to 60% of urothelial carcinomas as recorded in literature. In parallel to these results, 60% (21/35) of the benign/ reactive group cases were found to be p53 negative in study done by Yildiz *et al.,* (2009) [27]. In contrast, p53 was considered positive (abnormal) by Mallofre *et al.,* (2003) in 80% of the CIS cases, with 70% of those cases showing positivity in 50% of the cells [10]. Similarly, studies by Yildiz *et al.,* (2009) found that p53 was positive in 57% of the CIS cases, with all of the cases exhibiting positivity in more than 50% of malignant cells [27].

Present study found p53 expression to be statistically significant in differentiating hyperplasia, papilloma from high grade, low grade and invasive papillary carcinoma. p53 is abnormally expressed in low grade, high grade and invasive urothelial carcinoma.CK20 staining is a useful marker to differentiate dysplasia from hyperplasia and reactive urothelial epithelium. Hence CK20 is a biomarker used for differentiation between urothelial papilloma, PUNLMP and urothelial carcinoma [7, 9]. Alrashidy *et al*., (2016) indicated in their study that CK20 immunomarker can be used to demonstrate the change of urothelial cells in the direction of malignancy [25].

Their study emphasized that CK20 is particularly useful in differentiating between papilloma and PUNLMP. However present study observed no statistical significance but noticed that CK20 is abnormally expressed in low grade, high grade and invasive papillary carcinoma. The statistical insignificance could be due to small sample size. Ceylan discussed extensively about CK20 that it could be significant in differentiating between high grade PUC and low-grade PUC [28].

Table 4 shows various studies' comparison with present study [26, 29]. It was observed in the present study that using both CK20 and p53 as dual markers showed statistical significance in differentiating low grade, high grade and invasive papillary carcinoma from papillary hyperplasia, papilloma and PUNLMP. This was in concordance with study done by Raheem *et al.,* (2014) [30]. Papillary urothelial carcinomas have a high rate of recurrence and progression and these are important factors affecting mortality and morbidity. Patients diagnosed with LGNIPUC/HGNIPUC and LGIPUC/HGIPUC limited to lamina propria have a high recurrence rate and require long-term close clinical follow up and are usually treated conservatively. Treatment options include transurethral surgical resection and intravesical therapy such as Bacille-Calmette-Guerin or mitomycin C [28].

Conclusion

Present study concludes differentiating papillary lesions of urothelium morphologically is challenging and thus CK20 and p53 are very helpful markers to differentiate the lesions.

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Author for Correspondence:
Dr. Anita P Javalgi, Department of Pathology, Shri Dharmasthala Manjunatheswara College of Medical Science and Hospital, Shri Dharmasthala Manjunatheswara University, Dharwad-580009, Karnataka
Email: anitajawalgi@gmail.com Cell: 959016666

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