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Original Research Article.

Papillary Urothelial Carcinoma of Urinary Bladder: A Comparative Study of The Immunohistochemical Staining Pattern of (Cytokeratin 20 and P16) And Its Relation to Tumor Grade and Other Factors

Abdel Wahab AR. Alshaikhly[1], Noora M. Kareem[2*], Zaidoon A. Musa[3]

[1]Head of Pharmacy Department, Alrasheed University College, Baghdad, Iraq.
[2*]Department of Pathology & Forensic Medicine, College of Medicine, Al-Nahrain University, Baghdad, Iraq.
[3]Pathology Unit, Al-Emamain Al-Kadhimain Medical City, Baghdad, Iraq.

ABSTRACT

Background: Bladder carcinoma is the 9th most common malignancy. Papillary urothelial carcinoma represents about 45% of all primary bladder tumors.

Objectives: To compare between the immunohistochemical staining pattern of (CK20 and p16) of (non-neoplastic urinary bladder urothelium, low and high grade papillary urothelial carcinoma) and its relation to tumor grade and invasiveness.

Methods: It was a retrospective study. Formalin fixed paraffin tissue blocks of papillary urothelial carcinoma for 48 patients, and an equal number of non-neoplastic cases were collected. Immunohistochemical marker study of two markers (P16 and CK20) staining pattern was performed.

Results: CK20 stain was positive in (68.8%) of cases, while only (10.4%) of the controls was positive, with a p value of (0.002). p16 stain was positive in (70.8%) of cases, whereas its positivity in the controls was only (20.8%), and the p value was (0.003). CK20 was positive in 5 invasive cases but negative in 12 invasive cases. On the other hand CK20 was positive in 28 noninvasive cases and negative in only 3 noninvasive cases with a p value of (0.000). Significant difference (in favor for the non-invasive cases) in p16 expression was found between invasive and non-invasive urothelial carcinoma (p value 0.018).

Conclusion: Both markers (CK20 and P16) are adequate and useful in assessing bladder biopsies and both can be used in routine practice to confirm the diagnosis of both low and high grade papillary urothelial carcinoma and P16 positivity may indicate a good prognosis in papillary urothelial carcinoma patient.

Keywords: Papillary Urothelial Carcinoma, CK20, P16, Immunohistochemistry.

***Correspondence to:**

Dr. Noora M Kareem,
Department of Pathology and Forensic Medicine,
College of Medicine, Al-Nahrain University, Baghdad, Iraq.

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INTRODUCTION

Bladder carcinoma is the 9th most common malignant neoplasm among all cancers and the most common cancer of the urinary tract.[1] It accounts for about 3.2% of all cancers worldwide and is considerably more common in males than in females (ratio is 3.5:1).[2] The most common type of bladder tumor in the Western world is a non-invasive, papillary tumor, which accounts for approximately 45% of all primary bladder tumors.[3]

In 1998, the WHO/ISUP (International Society of Urologic Pathology) consensus classification was published in an effort to reach a universally acceptable system for the classification and grading of urothelial tumors. The consensus was that on the basis of eight histological features of architecture and cytology, non-invasive papillary tumors should be divided into four categories: papillomas with a benign behavior, papillary neoplasms of low malignant potential (PNLMP) with a low risk of recurrence and progression, low grade papillary urothelial carcinoma (LGPUC), and high grade papillary urothelial carcinoma (HGPUC).[4] The cytokeratin family consists of a broad spectrum of intermediate filaments that are expressed by many epithelial and mesothelial cells. These cytokeratins are expressed by different cell layers within the normal urothelium.[5,6] Specifically, CK20 may be normally expressed in superficial cells (umbrella cells layer), in the papillary neoplastic counterpart, this normal expression pattern may be maintained, lost, or altered as the tumor grade or stage increases, which could be indicative of not only the level of epithelial maturation but also the patient outcome.[7,8]

In human papillary urothelial cell bladder carcinoma, loss of one or both alleles of the p16 gene - also referred to as CDKN2 or INK4a gene - has been proposed to play a major role in early carcinogenesis.[9]

CDKN2A encodes two proteins, p16 and p14ARF. The importance of inactivation of CDKN2A in cancer development stems from the fact that its products are involved in the Rb and p53 tumor suppressor pathways[10] and it has a role in tumor cell invasion.[11]
Among several studies investigating immunohistochemical expression of p16 in urothelial cell bladder carcinoma, only two[12,13] reported a significant prognostic impact of p16 immunoreactivity when regarded as a single parameter.

AIMS

To compare between the immunohistochemical staining pattern of (CK20 and p16) of (non-neoplastic urinary bladder urothelium, low grade papillary urothelial carcinoma, and high grade papillary urothelial carcinoma). Thus, to investigate if there is any significant difference between the staining pattern of the above three categories, and if there is any relation of these staining patterns to factors like tumor grade and tumor invasiveness.

MATERIALS AND METHODS

The study was designed on a retrospective model. Formalin fixed paraffin tissue blocks of papillary urothelial carcinoma (PUC) for forty eight patients were collected for the period from January 2015 to January 2017. All biopsies were of TURT-bladder mass (Trans-urethral resection bladder mass) type, and all have been already studied by routine H&E histopathological examination for grading purpose. A control category (C) of forty eight formalin fixed paraffin tissue blocks were included in the study, all control cases were non-neoplastic cases with normal urothelial (transitional) bladder lining.
Evaluation of all cases, i.e; (PUC) and (C) cases, was performed by an immunohistochemical tumor markers study for cytokeratin 20 and p16 staining pattern.
Correlation was made also with other parameters including biographic data and histological factors.

Histopathological Categorization

All forty eight (PUC) included in this study were either of (low grade PUC) or (high grade PUC), these two grading categories of cases included were based on the criteria of World Health Organization and the International Society of Urological Pathology (WHO/ISUP) grading system of papillary urothelial neoplasms. Although this (WHO/ISUP) grading system includes other categories like (urothelial papilloma and papillary urothelial neoplasm of low malignant potential) however the latter two groups are not included in this study.

Immunohistochemical Assessment

Cases were stained for (cytokeratin 20 and p16) using the VENTANA (ROCHE) BenchMark-XT fully automated system, using the *ultra*View Universal DAB Detection Kit. Four µm thickness tissue sections were used.
The antibodies used for both markers were:
1. Cytokeratin 20 rabbit monoclonal antibody (clone SP33).
2. p16 mouse monoclonal antibody (clone E6H4).

The *ultra*View Universal DAB Detection Kit detects specific mouse and rabbit primary antibodies bound to an antigen in paraffin-embedded tissue sections. The specific antibody is located by a cocktail of enzyme labeled secondary antibodies (HRP Multimer. The complex is then visualized with hydrogen peroxide substrate and 3, 3'-diaminobenzidine tetrahydrochloride (DAB) chromogen, which produces a brown precipitate that is readily observed by light microscopy. The principal steps of the procedure are illustrated in figure (1).
The staining protocols for both markers were in accordance with standard staining protocols of VENTANA (ROCHE) BenchMark-XT system for each antibody. The immunohistochemistry slides were independently reviewed by two histopathologists, followed by a common open review for discussion and final agreement.

Cytokeratin 20 (CK20)

The cases were categorized according to CK20 staining pattern to (positive pattern) or (negative pattern). A positive pattern is defined as: any collection of cells (group of more than five cells) in the intermediate and/or the deep layers of urothelium stained with moderate or strong cytoplasmic stain reaction. Faint weak cytoplasmic stain pattern was rejected. Needless to mention, cases with diffuse strong cytoplasmic reaction were considered as positive as well.
A negative pattern is defined as: total absence of cytoplasmic stain reaction, or weak-moderate cytoplasmic stain reaction limited only to occasional cells in the very superficial layer (umbrella cells layer).

p16

For p16 the examination was focused on the nuclear staining pattern of the urothelial cells. Again, the cases were classified either as (positive pattern) or (negative pattern), a positive pattern was given to an unequivocal strong nuclear stain pattern in more than 25% of urothelial cells in the intermediate and deep layers of urothelium. While cases with (absence of nuclear staining or with a nuclear stain pattern in less than 25% of urothelial cells in the intermediate and deep layers) were considered negative.

Statistical Analysis

The SPSS (Statistical Packages for Social Sciences) program, version 18 was used in statistical analysis. Descriptive data were expressed as means and standard deviations for continuous measurements and as frequencies and percentages for categorical measurements. Differences of ck20 and p16 between cases and controls were compared using chi square test. $P<0.05$ was set as statistically significant.

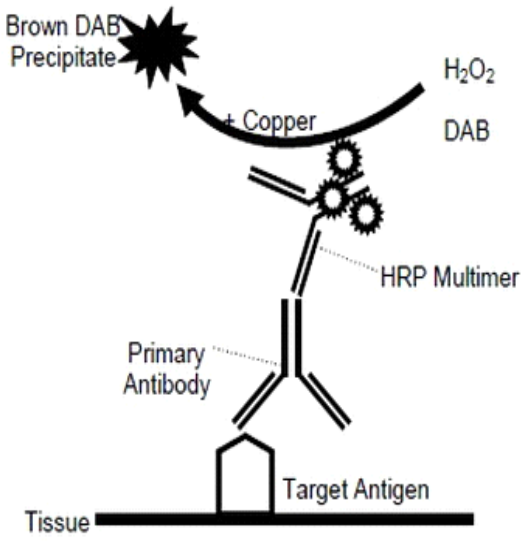

Figure 1: ultraView Universal DAB Detection Kit Reaction, Roche/Ventana. (Accessed on 10/12/2016)
[Availableat: productlibrary.ventana.com/ventana_portal/OpenOverlayServlet?launchIndex=1&objectId=760-50021090EN]

RESULTS

Forty eight cases of papillary urothelial carcinoma and 48 control cases were examined. The mean age of urothelial carcinoma patients was (60.9), 70.8% of them were male and 29.2% were female. The mean age of the control group was (59.02), 66.7% were male and 33.3% were female. Among the 48 cases of the papillary urothelial carcinoma: 24 cases (50%) were high grade, 24 cases (50%) were low grade. 31 cases (64.6%) were not invading the muscularis layer (T1 stage), whereas 17 cases (35.4%) were invading the muscle layer (T2 stage) (table1).

CK20 stain was positive in 33 of cases (68.8%) (Fig 2B, 2C), while CK20 positivity in the control group was only in (10.4%), with a p value of (0.002) which is highly significant.

p16 stain was positive in 34 cases (70.8%) (Fig 3B, 3C), whereas its positivity in the control group was only in (20.8%), and the p value was (0.003) which is significant (table 2).

Table 1: General characteristic of cases and controls

Variable		Cases		Control	
		Number	%	number	%
Age (years)	Mean±SD (Range)	60.9±12.55	(25-85)	59.02±11.53	(28-80)
Sex	Male	34	70.8	32	66.7
	Female	14	29.2	16	33.3
Grading	High	24	50		
	Low	24	50		
Invasion	Positive	17	35.4		
	Negative	31	64.6		

Table 2: Expression of CK20 and P16

Variable		Cases		Control		Chi square	P value
		Number	%	number	%		
CK20	Positive	33	68.8	5	10.4	12.27	**0.002**
	Negative	15	31.2	43	89.6		
P 16	Positive	34	70.8	10	20.8	10.19	**0.003**
	Negative	14	29.2	38	79.2		

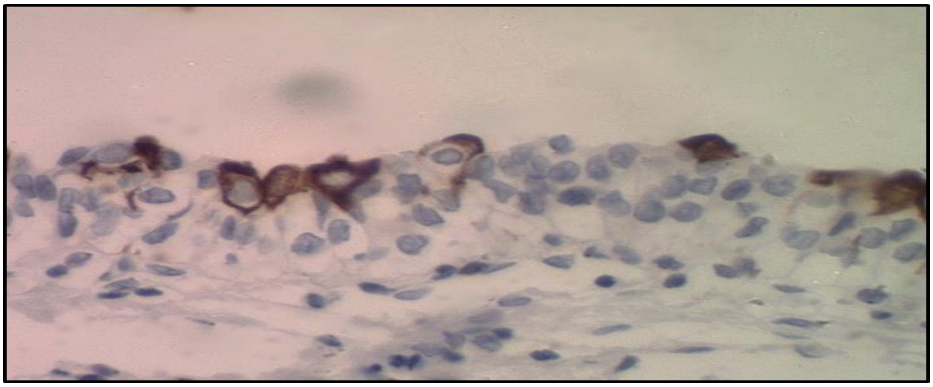

Fig 2A: Cytokeratin 20: Normal (non-neoplastic) transitional epithelium of bladder (urothelium), moderate cytoplasmic stain reaction limited to only few cells in the very superficial layer (umbrella cells layer) of urothelium, the intermediate and deep layers are totally negative to stain, the overall assessment is negative staining pattern, power x40.

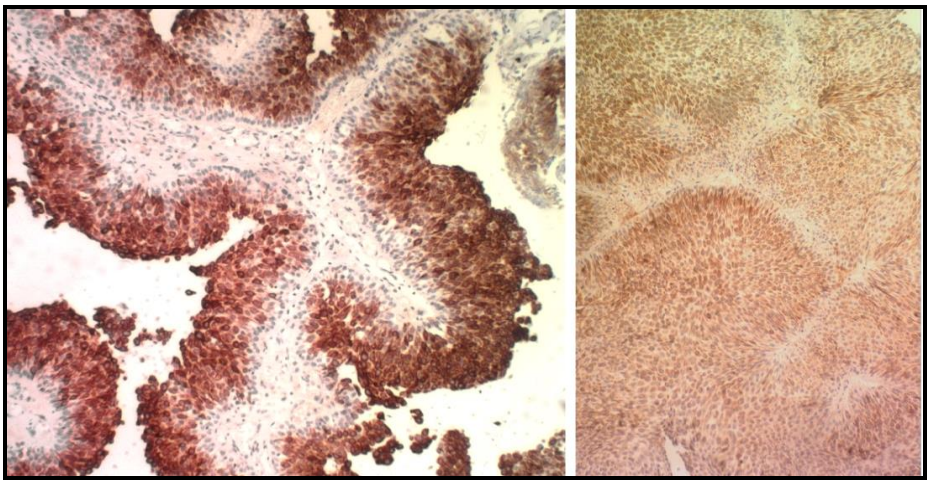

Fig 2B:Cytokeratin 20: low grade papillary urothelial carcinoma of bladder with a (strong: left, & moderate: right) diffuse cytoplasmic stain reaction of almost all the layers of the malignant urothelium including the intermediate and deep layers, the overall assessment is positive staining pattern, power x10.

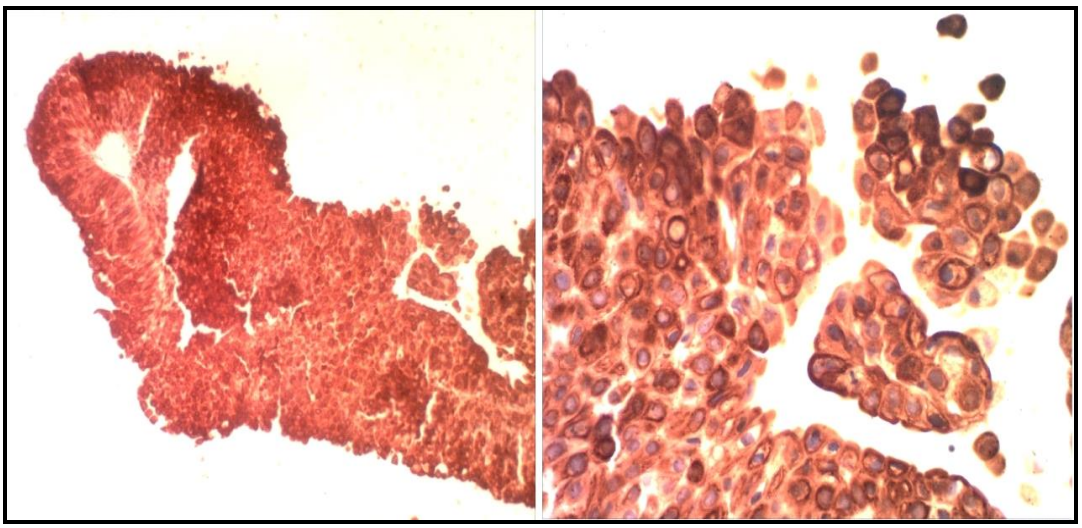

Fig 2C: Cytokeratin 20: high grade papillary urothelial carcinoma of bladder with a strong diffuse cytoplasmic stain reaction of almost all the layers of the malignant papillary urothelium, the overall assessment is positive staining pattern, left: power x10, right: power x40.

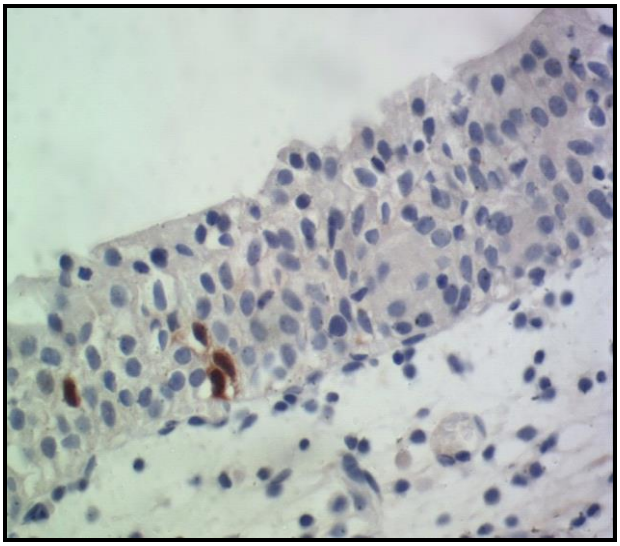

Fig 3A: p16: normal (non-neoplastic) transitional epithelium of bladder (urothelium), moderate to strong nuclear stain reaction limited to less than 25% (in this case about 4%) of the intermediate and deep layers cells of urothelium, the overall assessment is negative staining pattern, power x40.

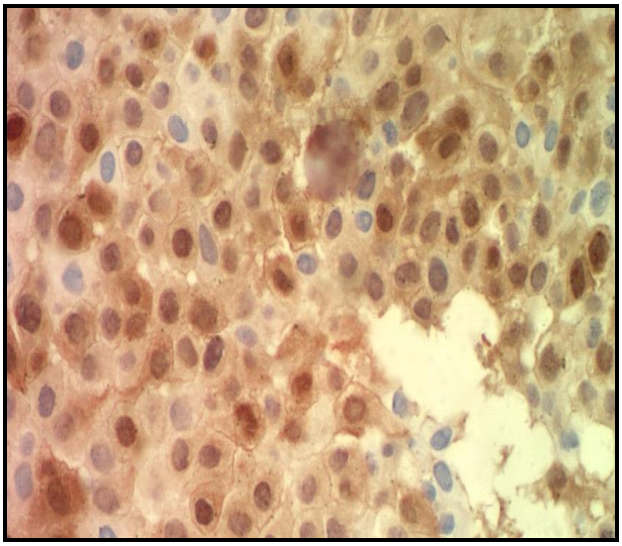

Fig 3B: p16: low grade papillary urothelial carcinoma of bladder with a moderate to strong nuclear stain reaction of more than 25% (in this case about 70%) of cells of the malignant papillary urothelium, the overall assessment is positive staining pattern, power x40.

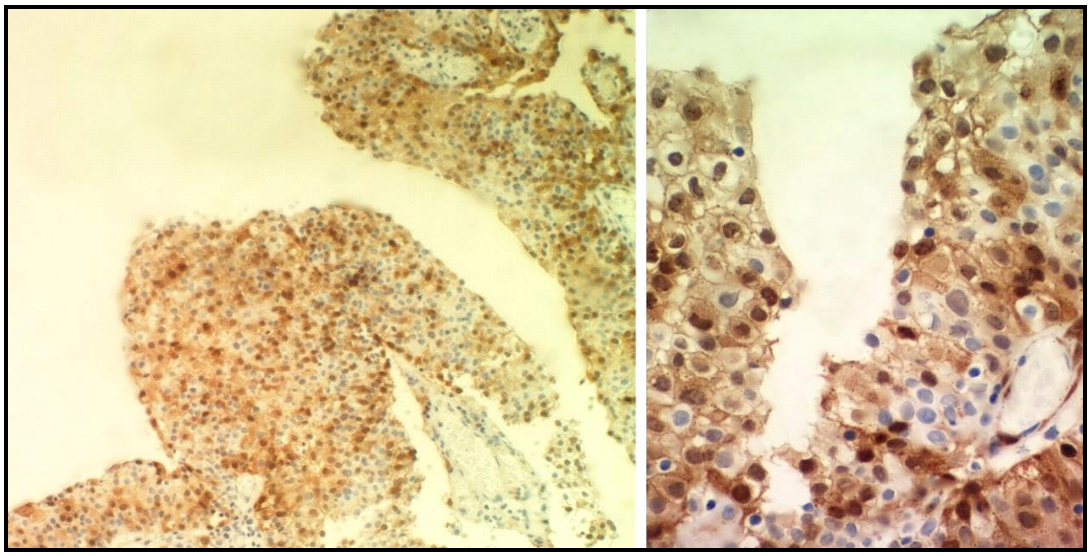

Fig 3C: p16: High grade papillary urothelial carcinoma of bladder with a moderate to strong nuclear stain reaction of more than 25% (in this case about 80%) of cells of the malignant papillary urothelium, the overall assessment is positive staining pattern, left: power x10, right: power x40.

Table 3: Relation of CK20 with the grade and invasion

Variable		CK 20		Chi square	P value
		Positive	Negative		
Grading	High	16	8	(0.097)	0.5
	Low	17	7		
Invasion	Present	5	12	(18.96)	0.000
	Absent	28	3		

Table 4: Relation of P16 with the grade and invasion

Variable		P 16		Chi square	P value
		Positive	Negative		
Grading	High	20	4	(3.630)	0.055
	Low	14	10		
Invasion	Present	8	9	(7.202)	0.018
	Absent	26	5		

Non-significant difference in CK20 expression was found between low and high grade urothelial carcinoma (p value 0.5). Positive expression of CK20 was seen in 16 high grade cases while 17 low grade tumor cases showed such positivity. Regarding the relation between CK20 and invasion, CK20 was positive in 5 invasive cases but negative in 12 invasive cases. On the other hand CK20 was positive in 28 noninvasive cases and negative in only 3 noninvasive cases with a p value of (0.000) which is highly significant in favor for the non-invasive cases (table 3).

Regarding P16, it was positive in 20 high grade cases and 14 low grade cases, negative in 4 high grade cases, and 10 low grade cases with a p value of (0.055) which were not significant.

Significant difference (in favor for the non-invasive cases) in p16 expression was found between invasive and non-invasive urothelial carcinoma (p value 0.018). Positive expression of p16 was seen in 8 invasive cases while 26 non-invasive cases showed such positivity (table 4).

DISCUSSION

In the present study, 48 paraffin blocks of papillary transitional carcinoma of the bladder and another 48 paraffin blocks that did not showed any histopathological features of malignancy were selected and reviewed.

The cytokeratin are intermediate filament proteins characteristic of epithelial cells. About 20 different isotypes of cytokeratin have been identified. Epithelial cells express between two and ten cytokeratin isotypes and the consequent profile which reflects both epithelial type and differentiation status may be useful in tumor diagnosis. The transitional epithelium or urothelium of the urinary tract shows alterations in the expression and configuration of cytokeratin isotypes related to stratification and differentiation. The most important recent finding is the demonstration that a normal CK20 expression pattern is predictive of tumor non-recurrence and can be used to make an objective differential diagnosis between non-neoplastic urothelium and papillary urothelial carcinoma. Its expression beyond superficial layers of the urothelium is considered positive.[5,14]

Different studies have previously evaluated various markers for the diagnosis and follow up of urothelial carcinoma.

p16 is a protein that belong to CDKI family; its function is to restrict the activity of kinases. This protein is able to stop the cellular cycle and to restrict mitotic activity to assist in the process of DNA repair. The gene encoding p16(CDKN2 or INK4a gene) is mutated or downregulated in several cancer cells like breast[15] and head and neck malignancy, and also in aggressive subtypes of bladder cancers.[9,16] This goes with our findings that showed a significant difference (p value 0.003) between the collected cases and the control group.

Our study showed increased P16 expression in the non-invasive papillary urothelial carcinoma cases (26 positive p16 cases) (table 4) which was consistent with Ching-Hsiu Yang a et al[17] that showed increased P16 expression in non-invasive urothelial carcinoma and considered P16 as a good prognostic factor. They also found increased P16 expression in low grade urothelial carcinoma while we did not find any significance in P16 expression in relation with the grading of the cases (p value 0.055).

Similar to our results, a study done by M. Yin et al[9] had shown no significant difference in p16 expression between the low grade and the high grade urothelial carcinoma. S. Kruger et al[18] found significant correlation between loss of p16 expression and tumor progression in patients with minimally-invasive bladder cancer and confirms the results of our study. Other authors[12,13] reporting an association between decreased p16 immunoreactivity and worse prognosis of urothelial carcinoma patients. Many studies found that high grade urothelial carcinoma have shown diffuse and strong expression of CK20 and P16[19,20] which is consistent with our study that showed 66.7% of high grade cases were positive for CK20and 83.3% of them were positive for P16.

AW Hitchings et al[13] studied the assessment of the prognostic value of immunohistochemical evaluation of the cell cycle markers p53, p16 and pRb and they found that immunohistochemical evaluation of p53 and p16 may identify a subset of patients at high risk for progression to more aggressive disease. This may help in selecting patients for early aggressive therapy.

Our study shows increase CK20 expression in non-invasive cases (28 case) with a p value of (0.000) but no significant difference between its expression and the grade of the cases (p value 0.5) while Sangeeta Desai et al. who performed CK20 on 42 low grade and 62 high grade urothelial carcinoma.[6] They found cytokeratin 20 positivity was associated with increasing tumor grade and stage. Also Mumtaz et al. 2014[20], who studied dual

immunohistochemical staining of CK20 and P53 on 95 cases of bladder carcinoma, found significant difference in CK 20 expression between low and high grade urothelial carcinomas.

Abdul Raheem et al 2014[21] studied immunohistochemical panel of CK20, P53 AND P63 on 213 bladder lesions (hyperplasia, carcinoma in situ, dysplasia, non-invasive and invasive urothelial carcinoma) and found that CK20 was expressed in 76% of non-invasive cases and in 84% of invasive urothelial carcinoma. Similar findings were reported by Moll et al., 1992 and Peiguo Chu et al., 2000[22,23] who found the expression of CK20 in different human cancers and found that the majority of cases of urothelial carcinoma showed positive CK20 expression. These studies validate our finding that CK20 is highly expressed in papillary urothelial carcinoma (68.8% positive cases in compared to only 10.4% of the control group).

Yildiz et al[24] investigated the utility and advantages of p53+CK20 dual immunohistochemistry as a tool for detecting the synchronous expression of both markers in bladder biopsies and for objectively distinguishing the cases with CIS and dysplastic urothelial changes from reactive non-neoplastic atypia and found an abnormal expression of CK 20 in (90%) of dysplasia cases, (89%) of CIS and (71%) of invasive carcinoma cases whereas the rest of the cases lacked abnormal CK20 expression.

CONCLUSION

Our study concludes that our proposed immunohistochemical panels of CK20 and P16 for studying papillary urothelial carcinoma is both adequate and useful in assessment of bladder biopsies, the two markers, in our opinion, could be used in routine practice, together with careful clinical and morphological correlation in confirming the diagnosis of both low and high grade papillary urothelial carcinoma and P16 positivity may indicate a good prognosis in papillary urothelial carcinoma patients.

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