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Prognostic significance of p16 immunohistochemical expression in urothelial carcinoma

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Abstract

Background: p16 is the protein product of most commonly involved gene in bladder carcinogenesis. Therefore, we performed an immunohistochemical study to evaluate association of p16 overexpression with prognostic parameters in bladder cancer.

Methods: p16 immunohistochemistry was performed on 121 cases of bladder cancer and association with tumor grade, lamina propria invasion, muscularis propria invasion and survival status was noted.

Results: Low expression of p16 was noted in 86% (104 cases), whereas 14% (17 cases) revealed high p16 expression. We found significant association of p16 expression with tumor grade ($p = 0.000$), muscularis propria invasion ($p = 0.001$), lamina propria invasion ($p = 0.001$) and survival status ($p = 0.020$). Univariate binary logistics showed that low grade tumors were less likely to express high p16 expression as compared to high grade tumors. Similarly, patients with lamina propria and muscularis propria invasion were more likely to exhibit high p16 expression. Significant association of high p16 expression was noted with worse long term survival ($p = 0.020$), while univariate logistic regression showed that patients with low p16 expression were at low risk (HR = 0.194) to die of disease as compared to patients with high p16 expression.

Conclusion: p16 is an important biomarker in bladder cancer as it can be used for prognostic stratification of patients with bladder cancer. Moreover, we suggest that molecular studies should be performed in our population in order to correlate abnormal p16 expression with underlying gene mutations.

Keywords: Bladder cancer, p16, Urothelial carcinoma, Muscularis propria invasion

Introduction

Urothelial carcinoma is the most common morphologic type of bladder cancer. Chromosome 9p21 is the region which is most commonly altered in the onco-pathogenesis of bladder cancer. p16 is the most important gene which is either deleted or mutated in this process. p16 is a tumor suppressor gene; therefore loss of its function results in abnormal cell proliferation leading to cancer development (Cairns et al., 1995; Williamson et al., 1995). Prognostic parameters of bladder cancer include histologic differentiation, grade, lamina propria invasion and deep muscle invasion. Various

prognostic biomarkers have been studied in bladder cancer to help in prognostic stratification of patients (Hashmi et al., 2018a; Hashmi et al., 2018b). These biomarkers include epidermal growth factor receptor (EGFR), p53 and retinoblastoma (Rb) (Sarkis et al., 1993; Cordon-Cardo et al., 1992; Mumtaz et al., 2014). p16 can be lost or abnormally expressed in bladder cancer as a result of deletion or mutations respectively; however the prognostic significance of this biomarker is still unclear. Moreover, p16 mutations or protein expression patterns have not been studied in our population in bladder cancer; therefore in the present study, we aimed to investigate the expression pattern of p16 in bladder cancer and to evaluate the prognostic significance of this biomarker in our setup.

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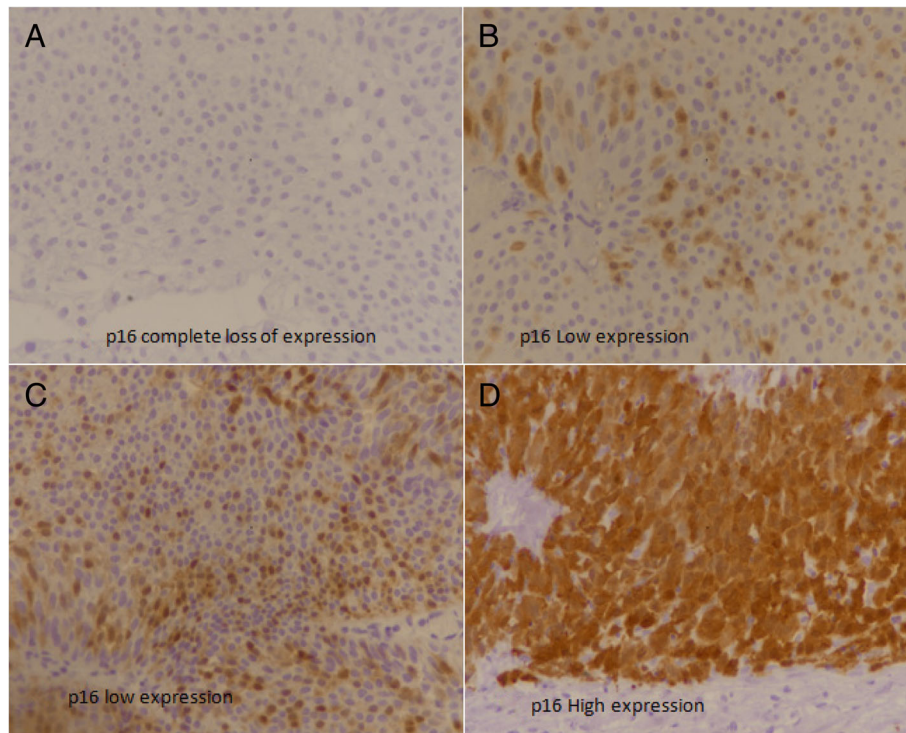
Fig. 1 p16 expression in Urothelial carcinoma. **a** Complete loss of p16 expression, H-score 0; (**b**) Low p16 expression, weak expression (1+) in 10% of tumor cells, H-score 10 (1 × 10), (**c**) Low p16 expression, Intermediate expression (2+) in 25% of tumor cells, H-score 50 (2 × 25); (**d**) High expression p16 expression, strong expression (3+) in 95% of tumor cells, H-score 285 (3 × 95). Magnification = 400×

Table 1 Association of p16 expression with Clinicopathologic features of Urothelial carcinoma

		n (%)			P-Value
		Low Expression (n = 104)	High Expression (n = 17)	Total (n = 121)	
Gender	Male	77 (74)	11 (64.7)	88 (72.7)	0.557
	Female	27 (26)	6 (35.3)	33 (27.3)	
Age group	≤25 years	1 (1)	0 (0)	1 (0.8)	0.346
	26–50 years	27 (26)	2 (11.8)	29 (24)	
	> 50 years	76 (73.1)	15 (88.2)	91 (75.2)	
Tumor grade[a]	Low grade	62 (59.6)	1 (5.9)	63 (52.1)	0.000
	High grade	42 (40.4)	16 (94.1)	58 (47.9)	
Muscularis propria Invasion	Can't assessed	39 (37.5)	4 (23.5)	43 (35.5)	0.001
	Present	12 (11.5)	9 (52.9)	21 (17.4)	
	Absent	53 (51)	4 (23.5)	57 (47.1)	
Lamina propria Invasion	Present	22 (21.2)	11 (64.7)	33 (27.3)	0.001
	Absent	82 (78.8)	6 (35.3)	88 (72.7)	
Recurrence (n = 54)	Yes	18 (39.1)	5 (62.5)	23 (42.6)	0.264
	No	28 (60.9)	3 (37.5)	31 (57.4)	
Survival Status (n = 54)	Alive	41 (89.1)	4 (50)	45 (83.3)	0.020
	Died of disease	5 (10.9)	4 (50)	9 (16.7)	

Fisher Exact test was applied
[a]Chi-square test was applied
P-value≤0.05 considered as significant

Fig. 2 p16 expression among studied population
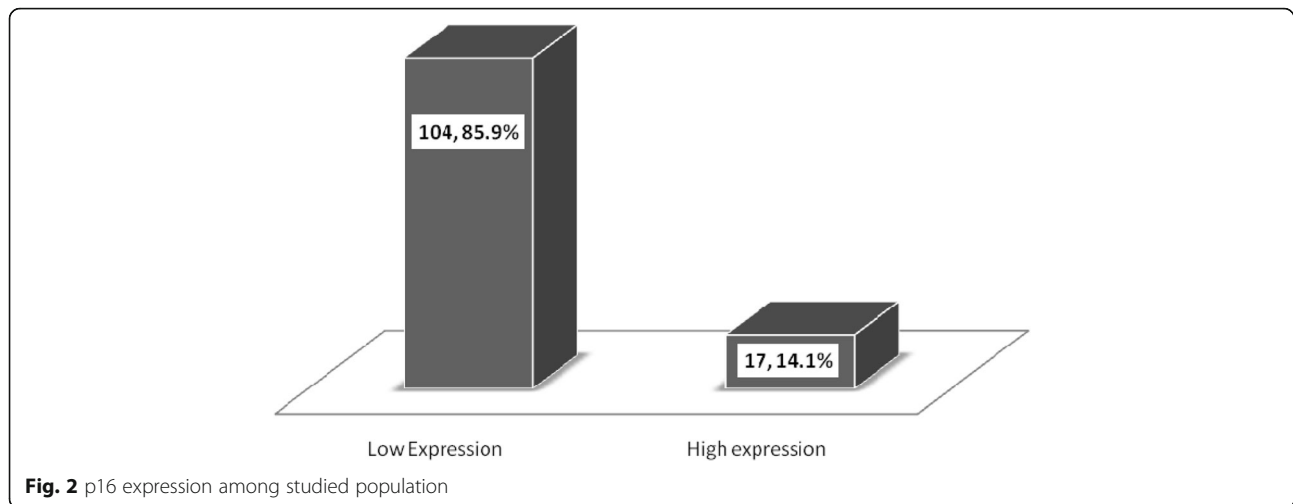
Methods

We retrospectively evaluated 121 diagnosed cases of urothelial carcinomas from January 2010 till December 2014 over a period of 5 years from records of pathology department archives. All patients had undergone elective surgeries at Liaquat National hospital, Karachi. The study was approved by research and ethical review committee of Liaquat National Hospital. Informed written consent was taken from all patients antecedent to surgery. Specimens included 108 (89.3%) transuretheral resections (TUR) and 13 (10.7%) radical cystectomies specimens. Slides of all cases were retrieved and reviewed by senior two histopathologists to evaluate pathologic characteristics. Clinical records of 54 patients were available and were thus evaluated from institutional records to determine recurrence and survival status of the patients. Moreover, representative tissue blocks were selected for p16 immunohistochemistry. For TUR specimens, if the specimen is entirely submitted in 2–3 cassettes then, IHC was applied on all tissue blocks, whereas if the tissue is submitted in more than 3 cassettes then 3 representative tissue blocks were selected for IHC. For radical cystectomy specimens, IHC was applied on 3 representative tissue blocks.

p16 antibody was purchased from Roche Ventana and IHC was performed using antibody CINtec R p16[INK4a], clone E6H4™ according to manufacturers protocol. Tonsils and carcinoma cervix was used as positive controls. Both nuclear and cytoplasmic staining was evaluated. Intensity of staining was scored into no staining (0), weak (1+), intermediate (2+), strong (3+) while percentage of positively stained cells were scored from 0 to 100. Intensity and percentage scores were multiplied to calculate a total H-score. An H-score of less than 200 was considered as low p16 expression while an H-score of more than 200 was taken as high p16 expression i.e., moderate staining (2+) in 100% tumor cells ($2 \times 100 = 200$) or strong staining (3+) in more than > 67% tumor cells ($3 \times 67 = 201$) (Fig. 1).

Recurrence status and follow-up were determined by evaluating hospital records. Overall survival was defined as time from surgical excision till death or last follow-up.

Statistical package for social sciences (SPSS 21) was used for data compilation and analysis. Mean and standard deviation were calculated for quantitative variables. Frequency and percentage were calculated for qualitative variables. Independent t-test and ANOVA were used to compare mean difference. Chi-square and fisher

Table 2 Odds ratio for patients with high expression of p16 expression

		odds ratio (95% CI)	*P*-Value	Adjusted Odds Ratio	*P*-Value
Tumor grade	Low grade	0.042 (0.005–0.331)	0.003	0.144 (0.011–1.854)	0.137
	High grade[a]	1			
Muscularis propria	Can't assessed	1.359 (0.320–5.772)	0.678	4.851 (0.413–56.969)	0.209
	Present	9.937 (2.618–37.728)	0.001	16.119 (0.53–488.4)	0.110
	Absent[a]	1			
Lamina Propria Invasion	Present	6.833 (2.274–20.537)	0.001	0.374 (0.024–5.797)	0.482
	Absent[a]	1			
Survival Status	Alive	0.122 (0.023–0.647)	0.013	0.169 (0.025–1.154)	0.070
	Died of disease[a]	1			

[a]Reference Category
P-value≤0.05 considered as significant
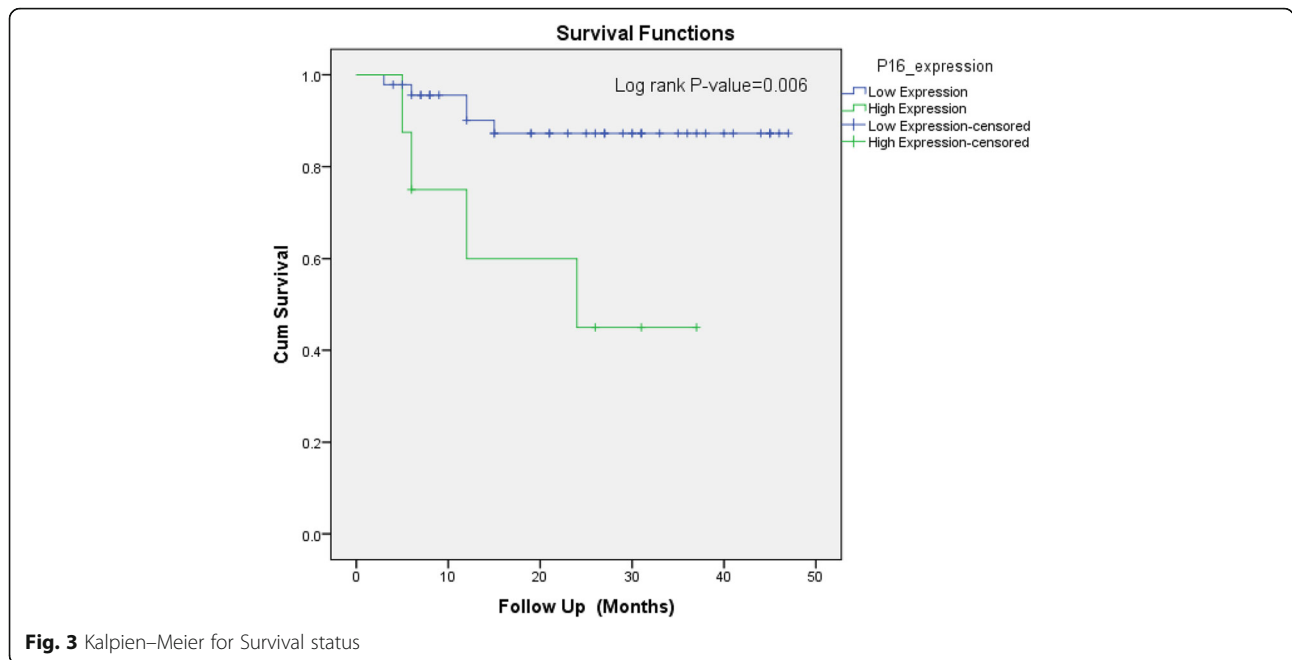
Fig. 3 Kalpien–Meier for Survival status

exact were applied to determine association. Significant variables were included in univariate and multivariate binary logistic regression analysis. Survival curves were plotted using Kaplan-Meier method and the significance of difference between survival curves were determined using log-rank test. *P*-value of ≤0.05 was taken as significant.

Results

Mean age of patients was 63.43 + 14.88 years with male to female ratio of 2.66:1. 52.1% (63 cases) were of high grade morphology, whereas 47.9% (58 cases) showed low grade histology. All cases were those of papillary urothelial carcinoma. Out of total 121 cases, 33 (27.3%) cases were those of invasive urothelial carcinoma, while 88

Table 3 Association of Clinicopathologic features with survival status of the patients

		n (%)			P-Value
		Alive (*n* = 45)	Died of disease (*n* = 9)	Total (*n* = 54)	
Gender	Male	35 (77.8)	7 (77.8)	42 (77.8)	1.000
	Female	10 (22.2)	2 (22.2)	12 (22.2)	
Age group	≤25 years	0 (0)	0 (0)	0 (0)	0.178
	26–50 years	11 (24.4)	0 (0)	11 (20.4)	
	> 50 years	34 (75.6)	9 (100)	43 (79.6)	
Tumor grade	Low grade	27 (60)	2 (22.2)	29 (53.7)	0.065
	High grade	18 (40)	7 (77.8)	25 (46.3)	
Muscularis propria Invasion	Can't assessed	18 (40)	4 (44.4)	22 (40.7)	0.541
	Present	5 (11.1)	2 (22.2)	7 (13)	
	Absent	22 (48.9)	3 (33.3)	25 (46.3)	
Lamina propria Invasion	Present	11 (24.4)	4 (44.4)	15 (27.8)	0.244
	Absent	34 (75.6)	5 (55.6)	39 (72.2)	
Recurrence	Yes	14 (31.1)	9 (100)	23 (42.6)	0.000
	No	31 (68.9)	0 (0)	31 (57.4)	
P16 Expression	Low expression	4 (8.9)	4 (44.4)	8 (14.8)	0.020
	High expression	41 (91.1)	5 (55.6)	46 (85.2)	

Fisher Exact test was applied
P-value≤0.05 considered as significant

Table 4 Hazard Ratio for patients died of disease

		Hazard ratio (95% CI)	P-Value
Recurrence	Yes	328.01 (0.384–280,517.73)	0.093
	No[a]	1	
p16 Expression	Low	0.194 (0.052–0.722)	0.015
	High[a]	1	

[a]Reference Category
P-value≤0.05 considered as significant

(72.7%) cases were of non-invasive urothelial carcinoma. There was no case of isolated carcinoma insitu (CIN). Lamina propria invasion was seen in 27.3% (33 cases), while muscularis propria invasion was noted in 17.4% (21 cases). Mean follow up of patients involved in the study was 23.11 + 13.574 months and recurrence was seen in 42.6% (23 cases) as shown in Table 1. Among 54 cases in which follow-up were available, 16.7% (9 cases) died of disease.

Low expression of p16 was noted in 86% (104 cases), whereas 14% (17 cases) revealed high p16 expression as shown in Fig. 2. Significant association of p16 expression with clinicopathologic parameters was noted. We found significant association of p16 expression with tumor grade ($p = 0.000$), muscularis propria invasion ($p = 0.001$), lamina propria invasion ($p = 0.001$) and survival status ($p = 0.020$). Univariate binary logistics showed that low grade tumors were less likely to express high p16 expression as compared to high grade tumors. Similarly, patients with lamina propria and muscularis propria invasion were more likely to exhibit high p16 expression. Detailed results of univariate and multivariate binary

Table 5 Mean p16 expression (mean H-score) and complete loss of p16 expression in urothelial carcinoma

Clinicopathologic features	Mean ± SD	P-Value	Complete Loss of p16 expression n (%)
Overall	64.61 ± 93.13		33 (27.3)
Gender			
Male	64.23 ± 92.46	0.942	23 (19)
Female	65.63 ± 96.34		10 (8.26)
Age group			
26–50 years	59.34 ± 73.78	0.707	5 (4.13)
> 50 years	66.87 ± 99.13		28 (23.14)
Tumor grade			
Low grade	26.93 ± 38.96	0.000	18 (14.87)
High grade	105.55 ± 115.48		15 (12.39)
Muscularis propria Invasion[a]			
Can't assessed	46.97 ± 77.86	0.000	14 (11.57)
Present	156.80 ± 124.37		4 (3.30)
Absent	43.96 ± 68.23		15 (12.39)
Lamina propria Invasion			
Present	131.30 ± 120.79	0.000	8 (6.61)
Absent	39.61 ± 65.40		25 (20.66)
Recurrence (n = 54)			
Yes	85.21 ± 107.69	0.287	4 (3.30)
No	56.29 ± 89.59		8 (6.61)
Survival Status (n = 54)			
Alive	51.33 ± 85.37	0.003	12 (9.91)
Died of disease	155.00 ± 114.94		0 (0)

Independent t test was applied
[a]ANOVA was applied
P-Value≤0.05, considered as significant
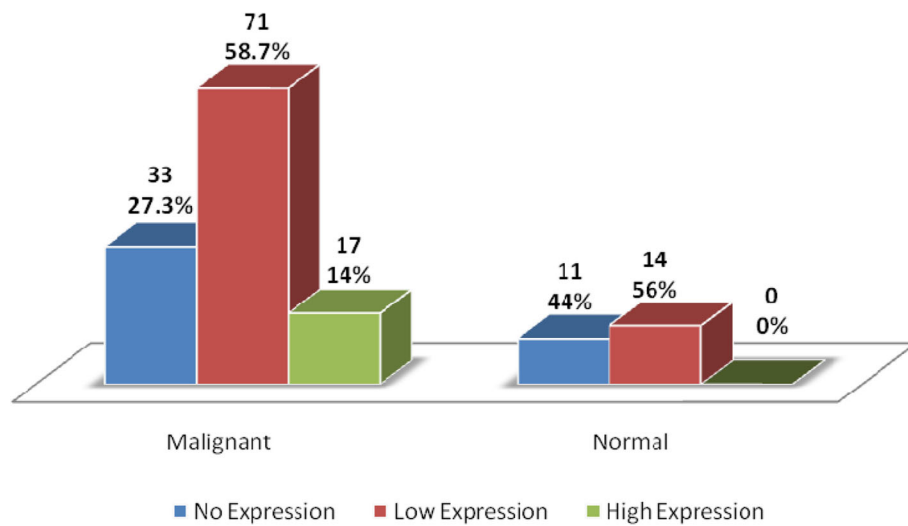
Fig. 4 Comparison of p16 expression between normal and malignant specimens

logistic regression are presented in Table 2. Significant association of high p16 expression was noted with worse long term survival (Table 1 and Fig. 3). In our study, most of cases were of conventional histology, there were only two cases of micropapillary variant, one case showing microcystic pattern, while there was one case each with squamoid and glandular differentiation. Among these cases, only one case with micropapillary and one case with squamoid differentiation showed positive p16 expression. Cases with variant histology were not sufficient enough to get evaluated for statistical correlation.

We found significant association between survival status of patients and p16 expression ($p = 0.020$) as shown in Table 3 while univariate logistic regression showed that patients with low p16 expression were at low risk (HR = 0.194) to die of disease as compared to patients with high p16 expression (Table 4).

Table 5 shows mean H-scores and cases with complete loss of p16 expression and its comparison with clinico-pathologic features. Overall 33 cases (27.3%) showed complete loss of p16 expression and mean H-score was 64.61 ± 93.13.

We also compared p16 expression in urothelial carcinomas with 25 cases of normal urothelium. 44% of normal urothelium showed complete loss of p16 expression while 56% showed low p16 expression. None of the cases revealed high p16 expression as shown in Figs. 4 and 5.

Discussion

In the current study, we found that high expression of p16 to be associated with higher grade and poor prognostic factors including muscularis propria invasion and poor long term survival.
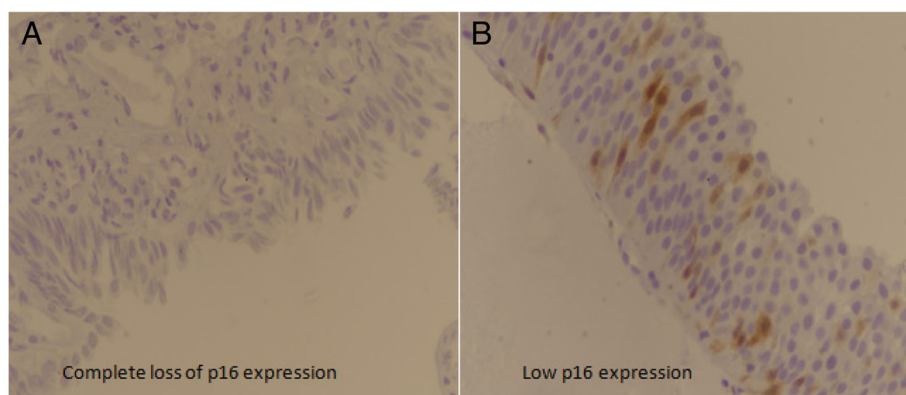
Fig. 5 p16 expression in normal urothelium, (**a**) Complete loss of p16 expression, H-score 0; (**b**) Low p16 expression in normal urothelium, Intermediate supression (2+) in 10% of tumor cells, H-score 20 (2 × 10)

p16 expression in bladder cancer has been studied previously (Kruger et al., 2005; Bartoletti et al., 2007; Yang et al., 2002); however, its prognostic significance is still unclear. In a meta-analysis of 37 studies including 2246 patients revealed that downregulated p16 expression was linked to poor prognosis in bladder cancer (Gan et al., 2016). In most of the studies analyzed, IHC markers were used to evaluate p16 expression and marked heterogeneity exists in the definition of abnormal IHC expression of p16. p16 is a tumor suppressor gene, which is most commonly involved in bladder carcinogenesis. Mutations involving p16 gene can be in the form of deletions of chromosome 9, in that case there would be complete loss of IHC expression of p16 protein. Conversely, inactivated or mutated gene product is generally not easily digestible leading to abnormally high expression as it is noted in the case of p53 (another tumor suppressor gene product) which is known to be overexpressed (immunohistochemically) in endometrial and ovarian serous carcinoma. Many studies evaluating p16 IHC expression didn't take into account this important fact and therefore didn't categorize p16 expression on the basis of intensity and percentage. Significant association of high p16 expression by IHC with poor prognostic parameters of bladder cancer in our study may be due to aberrant protein expression as a result of underlying gene mutations.

Yin M et al., found that strong p16 expression was seen in 100% of cases of urothelial carcinoma insitu (CIS) that can be used in differentiating CIS from adjacent normal epithelium showing normal or focal loss of p16 expression (Yin et al., 2008). Similarly, Raspollini MR et al., found a statistically significant association of p16 expression with disease stage (Raspollini et al., 2006). They found strong p16 expression in 28.2% cases of urothelial carcinoma. Moreover, they reported a statistically significant association of p16 expression with disease stage, however no significant association was noted with tumor grade or disease progression. On the contrary, we found a significant association of p16 expression with recurrence status and tumor grade. p16 expression is considered as a surrogate marker of HPV infection in oral and cervical squamous cell carcinoma, however studies revealed that no such association was found in case of bladder cancer (Alexander et al., 2012).

One of the major limitations of our study was that molecular studies were not performed to evaluate patterns of mutations in p16 gene; therefore, we suggest that correlation of abnormal p16 expression with underlying gene mutations should be performed in our setup for better understanding of disease pathogenesis in our population.

Conclusion
p16 is an important biomarker in bladder cancer as it can be used for prognostic stratification of patients with bladder cancer. Moreover, we suggest that molecular studies should be performed in our population in order to correlate abnormal p16 expression with underlying gene mutations.

Availability of data and materials
Please contact author, Atif Ali Hashmi (doc_atif2005@yahoo.com) for data requests.

Authors' contributions
AAH and ZFH: main author of manuscript, have made substantial contributions to conception and design of study. MI, SKH, HA and NF: have been involved in requisition of data. MI, SKH, HA and NF have been involved in analysis of the data and revision of the manuscript. All authors read, revise and gave approval of the manuscript.

Ethics approval and consent to participate
Ethics committee of Liaquat National Hospital, Karachi, Pakistan approved the study. Written informed consent was obtained from the patients for the participation.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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