

P21 and p27 Immunorexpression in Upper Urinary Tract Urothelial Carcinomas

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Abstract p21 and p27 are members of cyclin-dependent kinase family, which function as tumor suppressors and they are involved in development and progression of several malignancies. We investigated their expression in upper urinary tract urothelial carcinoma (UUTUC). Radical nephroureterectomy materials of 34 patients were assessed by immunohistochemistry to evaluate expression of p21 and p27 in UUTUC. Results were correlated with various clinicopathological variables as age, gender, tumor grade and stage, tumor architecture, multifocality, subsequent bladder carcinoma development and clinical outcome.

p21 and p27 expression was observed in 52.9 % ($n = 18$) and 88.2 % ($n = 30$), respectively. A total of 21 tumors (61.7 %) showed either total loss of p21 expression ($n = 16$, 47 %) or lower expression ($n = 5$, 14.7 %). No correlation was found between p21 expression and clinicopathologic variables. Cases showing total loss or lower p27 expression (11.7 % and <25.6 %, respectively) ($n = 19$, 55.8 %) constituted 67.6 % ($n = 23$) of the cases totally. This loss or lower p27 expression correlated with a shorter overall survival in both univariate and multivariate analysis ($p = 0.039$ and

$p = 0.037$, respectively). None of the noninvasive tumors (papillary and nodular tumors) showed loss of p27 ($p = 0.016$) while 33.3 % of invasive ones showed p27 loss. Noninvasive tumor architecture also correlated with subsequent bladder carcinoma development ($p = 0.032$) while invasive tumor architecture correlated with advanced stage (T3 and T4) ($p = 0.003$). p27 is widely expressed in UUTUC, while p21 expression is observed in half of the cases. Loss of p27 expression correlated with tumor architecture and overall survival in UUTUC. However, further research is needed to assess their role in UUTUC.

Keywords Cylin-dependent kinase inhibitors · p21 · p27 · Prognosis · Upper tract urothelial carcinoma · Urinary tract

Introduction

Urothelial carcinomas arising from upper urinary tract (upper urinary tract urothelial carcinoma; UUTUC) account for 5 % of all urothelial malignancies and approximately 10 % of renal tumors [1]. Current standard treatment option for UUTUC is radical nephroureterectomy with removal of a bladder cuff. It has been reported that metastasis occurs in 20–55 % of patients with UUTUC [2]. Tumor stage, histologic grade, lymph node involvement and type of surgical procedure have been demonstrated to be significant prognostic factors [2–4].

p21waf1 (p21) and p27kip1 (p27) are members of *cyclin-dependent kinase (CDK) interacting protein/kinase inhibitory protein (Cip/Kip)* family, which function as tumor suppressors by preventing the cell proceeding from G1 to S phase. They have been reported to be involved in development and progression of several malignancies. Loss of p27 expression has been shown in several organ tumors, including breast [5],

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prostate [6], colon [7], bladder [8] and has been associated with poor prognosis [9].

p21 expression have been found to affect prognosis in previous studies, however, whether p21 levels may be used as an independent prognostic factor is still not clear [10–12]. Although the role of p27 in carcinogenesis of UUTUC has been investigated in previous studies, results are conflicting [13–16]. Similarly, several studies are available in the literature on the role of p21 in urothelial carcinoma of bladder [8, 10, 11]. But no data is available in the literature on its role in UUTUC.

In this study, we investigated expression of p21 and p27 in UUTUC, and evaluated the association of p21 and p27 expression with age, gender, tumor architecture, multifocality and developed subsequent urinary bladder carcinoma and clinical outcome.

Methods

Patient Selection

Thirty-four cases with UUTUC diagnosed between 1992 and 2004, were retrieved from the archives of Pathology Department of Ege University Medical Faculty. Surgical procedures were performed in one center by various surgeons, using approved standard radical nephroureterectomy principles (extrafascial transabdominal dissection of the kidney and ureter, lymphadenectomy, and combined removal of the bladder cuff [17]). All palpable lymph nodes were removed during surgery. The dissected lymph nodes were located in paracaval, para-aortic areas between renal vessels and inferior mesenteric artery, and renal hilar region. Tumor multifocality was defined as synchronous presence of two or more pathologically confirmed tumors in any location (renal pelvis, ureter or bladder). Patients with the history of urothelial carcinoma of the urinary bladder were excluded. Bladder tumors detected six months after the surgery was considered as new-onset malignancy (i.e., not interpreted as recurrence or disease progression). The lifespan of patients who died from UUTUC was measured in months. Patients who died in the perioperative period (i.e., within 30 days after surgery) were excluded from the study. Survival periods were calculated according to patient demographics.

Pathologic Evaluation

All surgical specimens were processed according to standard pathological procedures and histologically confirmed to be urothelial carcinoma. No additional histological features were found (e.g. squamous cell carcinoma or adenocarcinoma). Tumors were staged according to the TNM classification

[18] and later dichotomized into two groups for statistical purposes, as T0 ($n = 4$), T1 ($n = 8$), and T2 ($n = 1$) tumors that were classified as early stage, and T3 ($n = 9$) and T4 ($n = 12$) tumors, classified as advanced stage. Tumor grading was performed according to the 2004 World Health Organization–International Society of Urologic Pathology Consensus Classification [19]. Tumor architecture was defined as papillary, nodular or invasive based on the predominant feature of the index lesion.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue specimens of all patients were obtained for immunohistochemical (IHC) staining. A demonstrative block containing normal kidney tissue adjacent to the tumor was selected for IHC evaluation. 4–5 μm thick sections were prepared from paraffin embedded tissues and were placed on electrostatic-charged slides (*X-traTM*, Surgipath Medical Industries, Richmond, Illinois, USA). Sections were deparaffinized and dehydrated through a graded ethanol series using routine protocols. Immunohistochemical study was performed by using mouse monoclonal antibodies anti-p21 (*Neomarkers*, MS 230-P, 200 mg/L, 1/40 dilution) and anti-p27 (*Neomarkers*, MS 256-P, 200 mg/L, 1/50 dilution). Briefly, sections were incubated at 50 °C overnight, deparaffinized in xylol and rehydrated through graded alcohol rinses. To enhance antigen retrieval, slides prepared for p21 staining were heated in 0.1 mmol/L EDTA buffer (pH 8) in a microwave oven (650 W for 5 min plus 450 W for 20 min) and those for p27 were immersed in citrate buffer (10 mmol/L, pH 6) and heated in a vacuum-pan for 2.5 min and cooled for 20 min. Endogenous peroxidase activity was blocked with 30 mL/L H₂O₂ for 5 min. The incubation time with monoclonal antibodies p27 and p21 was optimized as 30 min. Immunostaining of p27 and p21 was performed with the streptavidin-peroxidase method (Labvision, TM-125-HL), and alkaline phosphate-based visualization system. 3,3'-diaminobenzidine (Dako, TA-125 HD) was used as a chromogen. Carcinomatous breast and colon tissue served as a positive control, whereas primary mAb was omitted for negative control sections. Stained lymphocytes were used as an internal control.

Evaluation of Immunohistochemistry Results

Scoring was performed separately by two pathologists who were blinded to the patient characteristics as well as each other's scores. In each case, a total of 1000 cells were counted in two randomly selected representative high power fields (HPFs). Percentage of positive nuclear staining either weak or strong was noted. Mean scores counted by each observer was accepted as expression score.

Statistical Analysis

Statistical analysis was carried on a PC based analysis program SPSS (version 18.0). After initial frequency analysis the cases were categorized into three groups as “total loss of p21 or p27” when there was no immunexpression. The cases with expression scores below the median of all cases’ scores were regarded as ‘low p21 or low p27 expression’ while the scores above the median of all cases’ scores were coded as ‘high p21 or high p27 expression’. Pearson Chi-square or Fisher’s exact tests were used to evaluate the association between categorical variables. Differences in continuous variables were analyzed using the Mann-Whitney U test or Kruskal-Wallis test. Kaplan-Meier analyses with the log-rank test were used for survival analysis. Prognostic variables were analyzed for association with overall survival using the Cox regression model in both a univariate and multivariate analysis. A *p* value of <.05 was accepted as significant.

Results

Patient and Tumor Characteristics

Mean age was 62.15 ± 10.03 (range 46–79) years, and 85.3 % of the patients were male. Patients’ characteristics are shown in Table 1. Histologically, 6 out of 34 tumors (17.6 %) had nodular pattern, 16 tumors (47.1 %) had papillary pattern and 12 tumors (35.3 %) had invasive pattern (Fig. 1/A). Three cases (8.8 %) had low grade tumors, whereas 31 cases (91.2 %) had high grade tumors.

Table 1 Patient characteristics

Patient characteristics (n = 34)	
Gender	
Male	29 (85.3)
Female	5 (14.7)
Age at diagnosis (yrs.)	62.15 ± 10.03
Tumor Grade	
Low	3 (8.8)
High	31 (91.2)
Stage	
Ta-T1-T2 (Early stage)	13 (38.2)
T3-T4 (Advanced stage)	21 (61.7)
Multifocality	9 (26.5)
Metastasis	4 [11]
Outcome	
Alive	7 (20.6)
Dead	16 (47.1)
Unknown	11 (32.4)

p21 and p27 Expression

The median p21 and p27 expression scores were 6.97 % ± 10.03 (range 0–36) and 25.62 % ± 25.92 (range 0–84), respectively. The results of p21 and p27 nuclear immunoreactivity are shown in Fig. 1. p21 and p27 expression was observed in 52.9 % (*n* = 18) and 88.2 % (*n* = 30) of the cases respectively. A total of 21 tumors (61.7 %) showed either total loss of p21 expression (*n* = 16, 47 %) or showed lower p21 expression (*n* = 5, 14.7 %). But no correlation was found between p21 expression and the tested clinicopathological variables. However, cases showing loss of p27 expression (*n* = 4, 11.7 %) and cases with lower p27 expression (*n* = 19, 55.8 %) constituted 67.6 % (*n* = 23) of the cases totally (Table 2).

Associations between Variables

The immunostaining in relation to the tumor characteristics of the patients are summarized in Table 2. There was no significant association between p21 expression and tumor grade, stage, multifocality, architecture, development of subsequent bladder tumor and p27 expression (Table 2). On the other hand, presence of p27 expression correlated with tumor architecture, none of the noninvasive tumors (papillary and nodular tumors) showed total loss of p27 (*p* = 0.016, Table 2). However, grade, stage, multifocality and development of subsequent bladder tumor did not correlate with p27 expression either. Six of the 34 (17.6 %) patients treated for UUTUC had subsequent bladder carcinoma development, at a mean interval of 66 (range 24–120 months) months. Of these, 16,7 % cases had low-grade and 83.3 % had high-grade tumors. Noninvasive (papillary and nodular) tumor architecture of the UUTUC correlated with subsequent bladder cancer development (*p* = 0.032) and invasive tumor architecture correlated with late stage (*p* = 0.003).

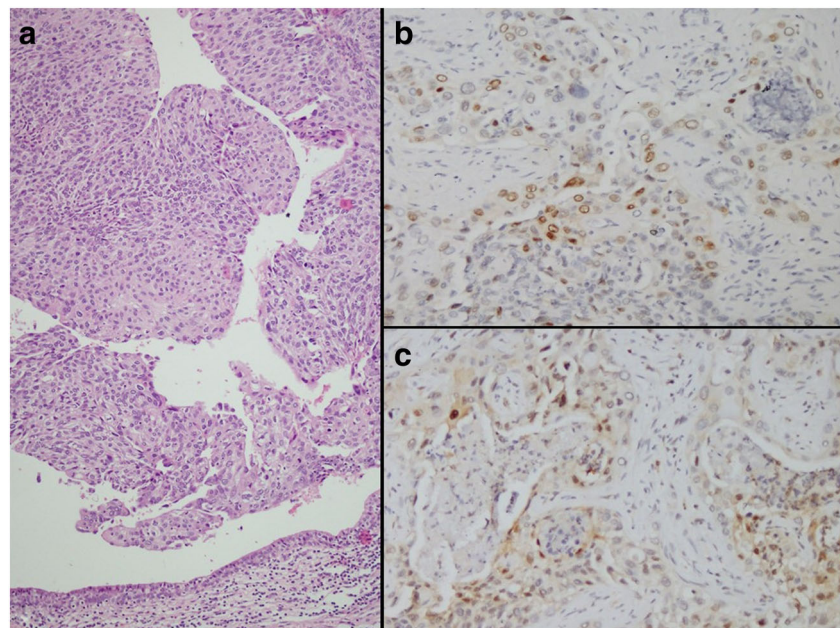
Outcome

The follow-up period comprised the time between the diagnosis and the time of death or last control. The mean follow-up period in this series was 59.13 ± 52.0 (range, 2–180) months. The mean survival period was 87.35 ± 14.55 months (95%CI of 59–116 months). In the overall group, 1, 5 and 10 year-survival rates were 86 %, 49 % and 29.4 %, respectively.

Patients with lower levels of p27 showed a shorter overall survival period (*p* = 0.023) as 63 months (95 % CI 30–95). For overall survival, p27 was found to be a significant prognostic marker in univariate and multivariate analysis (*p* = 0.039 and *p* = 0.037, respectively, Table 3). On the other hand, p21 expression did not correlate with survival (*p* > 0.05) and the mean survival of cases with low level p21 expression was 71 months (95 % CI 46–96).

Stage was also found to be significantly associated with prognosis in both univariate and multivariate analysis

Fig. 1 Papillary pattern and high grade tumor (A: Hematoxylin-eosin $\times 10$) Immunohistochemical staining in tumor cells (B: p21 immunorexpression, $\times 20$; C: p27 immunorexpression ($\times 20$))



($p = 0.014$ and $p = 0.022$, respectively, Table 3). However, only invasive architecture was found to be significant in univariate analysis ($p = 0.047$, Table 3).

Discussion

Different factors determine the prognosis and clinical outcome in patients with UUTUC. Tumor grade, stage, lymph node

involvement and type of surgical procedure are widely considered as significant prognostic factors [2–4]. In the present study, stage was found to be an independent predictor of overall survival.

The role of p21 and p27 in the pathogenesis of different malignancies have been investigated in several previous studies [5–16]. We showed that higher p21 and lower p27 expression was correlated with adverse prognostic factors in prostate carcinoma in a previously published study [21]. Similarly,

Table 2 Comparison of p21 and p27 staining with clinicopathological features

		Total number of cases	p21 total loss n (%)	p	Low p21 n (%)	High p21 n (%)	p	p27 total loss n (%)	p	Low p27 n (%)	High p27 n (%)	p
Total number of cases		34	16 (47)		5 (14.7)	13 (38.2)		4 (11.7)		19 (55.8)	11 (32.3)	
Architecture				0.14			0.38		0.016*			0.101
	Nodular	6	5 (83.3)		0	1 (16.6)		0		2 (33.3)	4 (66.6)	
	Papillary	16	6 (37.5)		4 [20]	6 (37.5)		0		11 (68.7)	5 (31.2)	
	Invasive	12	5 (41.6)		1 (8.3)	6 (50)		4 (33.3)		6 (50)	2 (16.6)	
Multifocality				0.55			0.72		0.25			0.44
	Present	9	5 (55.5)		1 (11.1)	3 (33.3)		2 (22.2)		5 (55.5)	2 (22.2)	
	Absent	25	11 (44)		4 [16]	10 (40)		2 [8]		14 (56)	9 (36)	
Stage				0.53			0.71		0.12			0.54
	Early	13	7 (53.8)		2 (15.3)	4 (30.7)		0		8 (61.5)	5 (38.4)	
	Advanced	21	9 (42.8)		3 (14.2)	9 (42.8)		4 [19]		11 (52.3)	6 (28.5)	
Grade				0.47			0.27		0.50			1.00
	Low	3	2 (66.6)		1 (33.3)	0		0		2 (66.6)	1 (33.3)	
	High	31	14 (45.1)		4 (12.9)	13 (41.9)		4 (12.9)		17 (54.8)	10 (32.2)	
Subsequent bladder carcinoma development				0.28			0.78		1.00			0.07
	Present	6	4 (66.6)		0	2 (33.3)		0		2 (33.3)	4 (66.6)	
	Absent	28	12 (42.8)		5 (17.8)	11 (39.2)		4 (14.2)		17 (60.7)	7 [20]	

Table 3 Cox regression analysis for various potential prognostic factors in overall survival

Overall survival	Unfavourable/ favourable characteristic	No. of patients	Relative risk (95 % CI)	p
Univariate				
Grade	High/low	31/3	0.246–14.406	0.542
Stage	Late/early	21/13	0.057–0.724	0.014*
p21	Low/high	21/13	0.292–2.457	0.760
p27	Low/high	23/11	1.071–13.491	0.039*
Pattern	Invasive/nodular/papillary	12/6/16		0.079
	Papillary	16	Referent	-
	Nodular	6	0.139–1.185	0.099
	Invasive	12	0.040–0.982	0.047*
Multivariate				
Grade	High/low	31/3	0.027–36.313	0.431
Stage	Late/early	21/13	0.018–0.729	0.022*
p21	Low/high	21/13	0.224–2.289	0.573
p27	Low/high	23/11	0.104–25.421	0.037*
Pattern	Invasive/nodular/papillary	12/6/16		0.961
	Papillary	16	Referent	-
	Nodular	6	0.323–3.948	0.849
	Invasive	12	0.106–7.732	0.929

* $p < 0.05$

Romics et al. have reported that grade progression in prostate carcinoma was accompanied with down-regulation of p27kip1 protein [22]. Down-regulation of p21 was found to be associated with HPV-18 positive cervical carcinomas, suggesting that loss of p21 molecule may contribute to the malignant transformation of HPV-18 positive carcinomas [23].

There are discrepancies in previous reports regarding the association between low p27 expression and high tumor stage and grade for UUTUC. Although Masuda et al. [13] did not find any association between these parameters, Kamai et al. [14] reported that low p27 expression correlated with higher tumor stage. In the present study, we did not find any association between p27 expression and tumor stage or grade; however, p27 expression was associated with overall survival. This finding is consistent with Kamai et al.'s [14] report suggesting that p27 may play a role in the progression of urothelial carcinoma arising from pelvis renalis and ureter. However, in a more recent study of 62 patients, using tissue microarray technology, Fromont et al. reported that p27 had no prognostic value in UUTUC [15].

Tumor architecture has been demonstrated to predict clinical outcomes in international multicenter studies [20, 24, 25]. Sessile pattern was shown to be significantly associated with metastasis and an independent predictor of cancer specific mortality [24]. In a multi-institutional study of 1363 patients, Remzi et al. found that in 40 % of patients with papillary tumors and in 77 % of those with sessile tumors disease recurred five years after radical nephroureterectomy [20]. Similarly, all invasive tumors were advanced-stage tumors in

our study group and invasive architecture was found to be a significant prognostic factor in univariate analysis.

Previous studies have been conducted on the association between tumor architecture and p21 and p27 expression in bladder tumors. Korkolopoulou et al. reported that no significant relationship was detected between p21 expression and noninvasive papillary carcinoma of bladder [10]. On the contrary, Franke et al. reported that the highest p27 expression was observed in papillary tumors of bladder [26]. However, the prognostic significance of tumor architecture and p21 or p27 expression has not been conclusively investigated in UUTUC. In the present study, we observed p27 negativity in 33.3 % of cases with invasive pattern whereas p27 expression was present in all cases with noninvasive UUTUC, similar to papillary bladder carcinomas. Similar to loss of p27 expression, cases with lower p27 expression was also more frequent among cases with invasive pattern of growth pattern. Of note; in this study "low expression" for both p21 and p27 was defined as expressions lower than median expression ratios. Therefore, the cut-off levels presented herein should be evaluated with caution as there is no pioneering study on their expression in UUTUC and as there is a lack of agreed cut-off levels in the literature for other tumors either.

Few studies to date have focused on the potential risk factors for the development of subsequent bladder tumors after managing their UUTUC. It has been reported that 15–50 % of patients who underwent radical nephroureterectomy for UUTUC have subsequent cancer development in the bladder [27–29]. Tumor multiplicity, stage, grade, size, and ureteric

location, as well as patient gender and surgical method are the reported risk factors [27–29]. In our study, we analyzed whether p21 and p27 expression of the UUTUC is associated with the development subsequent urinary bladder cancer but no significant relationship was found, possibly because of the low number of cases. Our study showed that noninvasive (papillary and nodular) tumor architecture correlate with development of subsequent bladder tumor. This finding may be the result of longer survival in patients with noninvasive tumors.

p21 immunopositivity occurred in 52.9 % of our cases and 61.7 % of those expressed low levels of p21 (Table 2). The frequency of p21 expression in bladder cancer was observed between 30 and 64 % in the literature [10, 30–32]. No significant relationship was detected between p21 expression and clinicopathological features consistent with previous bladder cancer studies. It has been proposed that p21 activation may be an early event in urothelial carcinogenesis [30, 32]. Also, although p21 and p27 are the members of the same protein family, p21 may be less effective on tumor suppression, as suggested by Philipp-Staheli et al. recently [33]. Observation on p21 is more limited and further investigation is needed to explore its role in urothelial tumorigenesis.

We appreciate that our study has limitations, especially related with smaller number of cases due to lower prevalence of UUTUC. However, we believe that the observations presented above can help to enlighten the prognosis of upper tract urothelial carcinoma. Further studies are needed to confirm the role of p21 and p27 in upper tract urothelial carcinoma.

Compliance with Ethical Standards

Conflicts of Interest None declared.

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