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Original Article

The cell cycle regulator p27^{Kip1} is associated with urothelial bladder cancer invasion

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Abstract: Background: The expression of p27^{Kip1} has been shown to be associated with the development of various malignancies. The aim of this study was to analyze the clinical significance of expression in Saudi bladder cancer patients. Methods: Analysis of p27 expression was performed by immunohistochemistry using tissue microarray of bladder cancer specimens. Correlation analysis between p27 expression and patients' clinical parameters was undertaken. Results: 50% of patients showed positive nuclear expression of p27 protein. Interestingly, loss of p27 expression correlated significantly with increased tumor grade (P = 0.001) and muscle invasion (P = 0.014). Patients with reduced p27 expression had trend toward poorer survival ($P = 0.07$, log-rank test). Conclusion: This is the first study describing the expression of p27 in bladder cancer patients of Arab origin and indicating that p27 plays an important role in bladder carcinogenesis. p27 might help in the stratification and the management of bladder cancer patients.

Keywords: Bladder cancer, IHC, p27, prognosis, recurrence, tissue microarray

Introduction

Globally, urothelial carcinoma of the bladder is a leading cause of cancer death with an estimated 429,800 new cases and 165,100 deaths of bladder cancer in the year 2012 [1]. The disease is characterised by high recurrence rates and a greater risk for progression to invasive form which necessitates regular surveillance to monitor disease recurrence [2]. Unfortunately, the tumor grading and staging system is inappropriate to accurately predict for bladder cancer progression and recurrence which necessitate the urgent need for a reliable non-invasive biomarker that could be used in clinical setting.

Tumorigenesis is a complex multistep process characterized by uncontrolled cell growth and associated with genetic and epigenetic alterations in genes that control cell proliferation [3]. Recent advances in molecular biology have enhanced our understanding to the molecular signature of the disease and have unveil a number of candidate genes such as FGFR3, Her2/neu, p53 and PI-3-kinase/mTOR, that are associated with cancer development and metastasis [4]. Expression analysis of these genes could have prognostic and therapeutic significances. The cell cycle regulator, p27 is a members of the Cip/Kip family of cyclin kinase inhibitors (CKI) that includes p21 and p57 as well. Members of this family can restrain cell growth during development, differentiation and in response to cellular stresses [5]. The biological functions of p27 depend enormously on its cellular localisation in the cytoplasm or nucleus [6]. Great line of evidence support the tumor suppressor activity of p27 through interaction with cyclin E/CDK2 complex leading to inhibition of G1 to S phase transition during cell cycle [7-9]. Along with other proteins, p27 have been suggested to act as a prognostic biomarker in bladder cancer [10]. Low levels of nuclear p27 protein were associated with the invasive phenotype of bladder cancer and poor survival [11, 12] suggesting that monitoring the p27 level at

Table 1. Clinicopathological characteristics of the urothelial bladder cancer cases included in the study

Parameters		Percentages (%)	
Sex	Male	108/131	82.443%
	Female	023/131	17.557%
Grade	High Grade	065/131	49.618%
	Low Grade	052/131	39.695%
	Unknown	014/131	10.687%
Age	< 60 Years	053/131	40.458%
	≥ 60 Years	076/131	58.015%
	Unknown	002/131	01.527%
Blood group	A^+	022/131	16.794%
	A^-	001/131	00.763%
	B^+	019/131	14.504%
	AB^+	003/131	02.290%
	O^+	038/131	29.008%
	O^-	003/131	02.290%
	Unknown	045/131	34.351%
Type of cancer	MIBC	058/131	44.275%
	Superficial	049/131	37.405%
	Undecided	024/131	18.321%
Subtypes	Transitional	103/131	78.626%
	Transitional/Squamous	019/131	14.504%
	Squamous	006/131	04.580%
	Adenocarcinoma	001/131	00.763%
	Unknown	003/131	02.290%
Smoking	YES	041/131	31.298%
	NO	026/131	19.847%
	Unknown	064/131	48.855%
Survival	Died of Disease	038/131	29.008%
	Alive	091/131	69.466%
	Unknown	002/131	01.527%
Recurrence		051/131	38.931%

early stage of bladder cancer could help identifying patients with increased risk for invasive bladder cancer [13].

Unlike other members of CIP/KIP family, the significance of p27 in bladder cancer is not clearly understood. The current study was undertaken to determine the expression of p27 and analyze the association between p27 expression and patients' clinicopathological parameters in a cohort of 131 patients with urothelial bladder cancer.

Methods

Patients and sample collection

Paraffin-embedded tissue samples were obtained from histologically confirmed bladder cancer patients who undergone bladder resection at King Abdulaziz University Hospital, Jeddah (KSA) between 2005 and 2010. Cancer samples were collected from 131 patients arranged in duplicate on TMA. The study was approved by the local ethics committee (No. 149-14). The clinical and pathological data including age, sex, tumor grade, TNM stage were also collected to carry out correlation analysis.

Tissue microarray

Tissue microarray (TMA) is one of the most revolutionary technologies introduced into research during the past decade. For the purpose of TMA construction, H&E staining was done to obtain representative tumor cores. Two cores from each patient were integrated into the TMA. We successfully transferred 131 blocks of bladder cancer to construct TMA slides making the total of 262 tissue cores in the TMA. Evaluating the expression pattern of p27 was performed using Immunohistochemistry (IHC) staining.

Immunohistochemistry

Immunohistochemical staining was performed using the Bench-Mark XT automated system (Ventana Medical Systems, Inc., Tucson, AZ, USA) as previously described [14]. Briefly, after de-waxing and antigen retrieval using CC1 cell conditioning buffer, slides were incubated with the primary human anti-p27 antibody (code M7203, Dako, Denmark) at a dilution of 1:50 for 32 min. After, the slides were treated with DAB chromogen and counterstained hematoxylin and bluing reagent for 4 min. The sections were then dehydrated with ethanol and xylene and permanently mounted.

Scoring and data analysis

Scoring of the p27 immuno-staining was performed in a blind fashion to the patients' clinicopathological data using x40 objective. The information collected during the scoring process include the sub-cellular localisation, the intensity of the staining on scale 0 to 3 (0 = negative; 1 = weak; 2 = moderate; and 3 = strong) and the extent of staining (percentage of tumor cells showing positive immuno-reac-

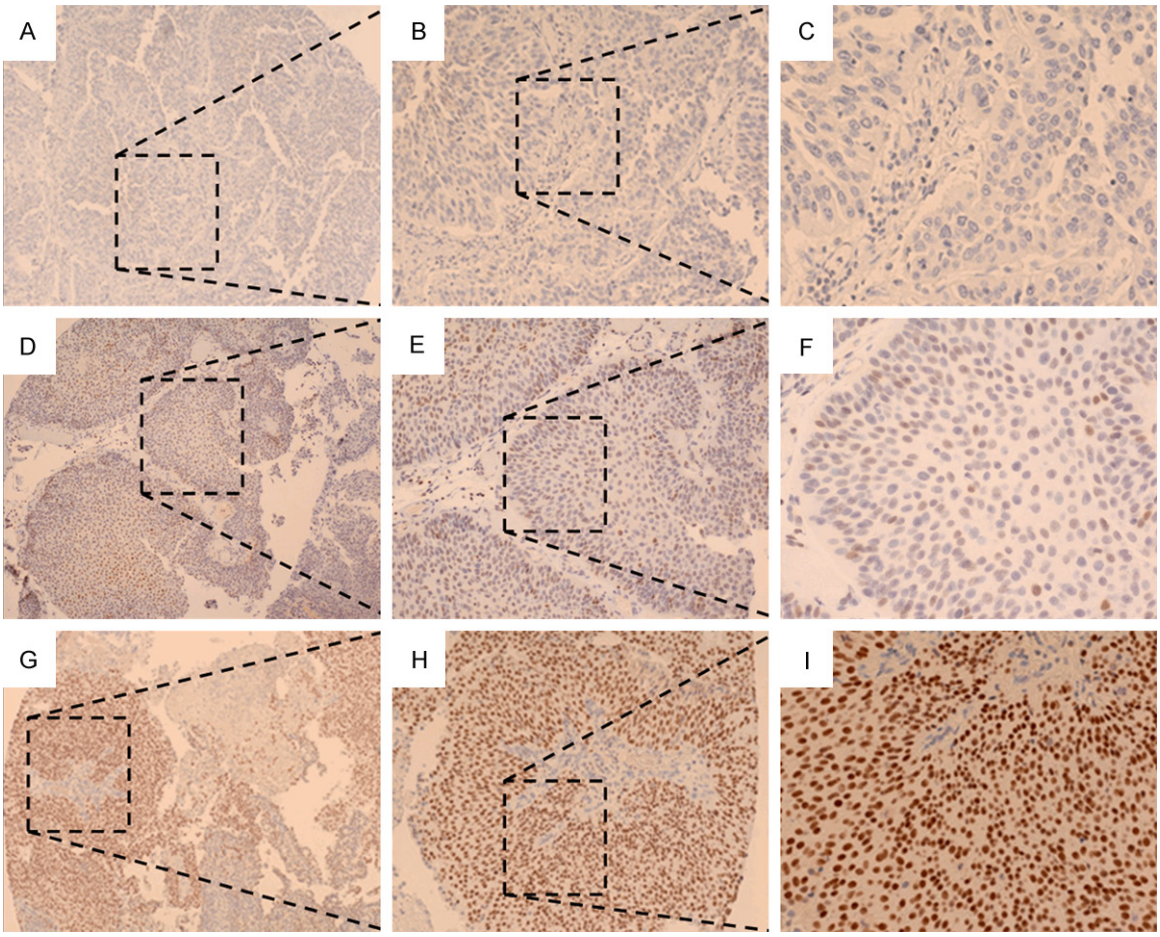

Figure 1. Expression of p27 in bladder cancer. Immunohistochemical staining of bladder cancer tissue microarray using p27 antibody. A-C. No p27 expression. D-F. Moderate p27 expression. G-I. Strong p27 expression. Images were taken using different objectives (x10, x20, x40).

tivity: 0-100% of cells). A staining index was calculated by multiplying the staining intensity by the percentage of positive tumor. The staining index varies from 0-300.

For association analysis between the level of p27 expression and patients' clinical and pathological parameters Fisher's two-sided exact tests was applied. Correlation between SHh levels and cancer-specific survival, Kaplan-Meier curve was used and *P* value was calculated using the log-rank test. Statistical analysis of data was processed using SPSS (version 21) and data were considered significant for values of $P < 0.05$.

Results

Clinical data

In an attempt to examine the expression pattern of the cell cycle regulator p27 and evaluate its value as a marker of good/bad prognosis, which may help in clinical setting, we collected tissue samples from patients affected with bladder cancer. Formalin-fixed paraffin-embedded samples from 131 patients, some of whom had cancer recurrence, were collected and arranged in a tissue microarray making the total number of cores 262 on 3 TMA slides. To perform correlation analysis, clinical and pathological data related to cancer patients were collected retrospectively (**Table 1**).

p27 expression and correlation analysis

We first examined the pattern of p27 expression in human bladder tumor tissues, using immunohistochemistry. As shown in **Figure 1**, p27 immuno-reactivity varied among BC tissue sections. Evaluation of the staining pattern revealed that p27 is solely expressed in the nuclei of cancer tissues. A total of 63 out of

Table 2. Association between p27 protein expression and clinicopathological parameters in urothelial bladder cancer

Clinicopathological parameters	Number	P27 cytoplasmic expression		*P* value
		Low (%)	High (%)	
Age group (years)	128			0.513
< 60		26 (50.0%)	26 (50.0%)	
≥ 60		39 (51.3%)	37 (48.7%)	
Marital status	114			0.088
Single		1 (16.7%)	5 (83.3%)	
Married		58 (53.7%)	50 (46.3%)	
Gender	128			0.36
Male		52 (49.1%)	54 (50.0%)	
Female		14 (60.9%)	9 (39.1%)	
Smoking	66			0.3
Yes		19 (46.3%)	22 (53.7%)	
No		14 (56%)	11 (44%)	
Type of cancer	111			0.014
MIBC		38 (64.4%)	21 (35.6%)	
NMIBC		21 (40.4%)	31 (59.6%)	
Histological grade	114			0.001
Low grade		15 (30.0%)	35 (70.0%)	
High grade		42 (65.6%)	22 (34.4%)	
Family history	66			0.21
Yes		28 (47.5%)	31 (52.5%)	
No		5 (71.4%)	2 (28.6%)	
Lymph node status	84			0.48
Negative		40 (55.6%)	32 (44.4%)	
Positive		6 (50.0%)	6 (50.0%)	
Metastasis	81			0.61
Positive		38 (56.7%)	29 (43.3%)	
Negative		8 (57.1%)	6 (42.9%)	

128 (49.2%) cancer specimens showed positive nuclear staining for p27 while 51.8% of the analyzed cancer samples were negative. There was no expression of p27 in the cytoplasm in all bladder cancer samples. We next analyzed the relationship between p27 protein expression and patients' clinicopathological parameters. The cut-off value for discriminating between positive and negative staining was determined using the median score value for the immunostaining. A significant correlation was reported between low expression of p27 and muscle invasive bladder cancer ($P = 0.014$), high tumor grade ($P = 0.001$). We found no significant association between the nuclear p27 levels and patients' age, gender, family history, marital status, smoking, lymph node metastasis and vascular invasion (**Table 2**).

The relationship between p27 expression and cumulative survival rate

Patient survival was evaluated for a follow-up period ranging from 0-152 months. In uni-variate analysis, Kaplan Meier survival curve demonstrated a trend towards an association between *p27* expression and survival (log-rank test, $P < 0.07$). Reduced p27 could predict for poor survival in patients with bladder cancer (**Figure 2**).

Discussion

Bladder cancer continues to pose challenge to healthcare specialists due to high recurrence rate and progression to muscle invasive carcinoma. In an attempt to find a prognostic marker that could be associated with the grading and staging system to better predict for disease recurrence and help clinician their curative decisions, we examined the expression pattern of p27 protein in a cohort of 131 bladder cancer samples. Correlation analysis between the level of p27 protein and clinicopathological parameters indicated that p27 was expressed in 49.2% of the analysed samples. Low p27 expression was associated with high histological grade and with muscle invasive phenotype of the disease. Several attempts have been made in the past to analyze the predictive value of the cell cycle regulators including p27 in bladder cancer [10, 15-17]. Our data is consistent with previous published reports indicating an inverse relationship between low p27 levels and high grade and more aggressive tumors. In an independent study, Kamai et al. [18] used a cohort of 145 Japanese patients affected with bladder cancer and revealed that the expression p27 is associated with progression from superficial into invasive bladder cancer. Pantazis et al. 2011 [19] reported a normal expression of p27 in the majority of malignant specimens analyzed. Contrary to the aforementioned studies, Doganay et al. [20] demonstrated no relationship between p27 and patients' pathological

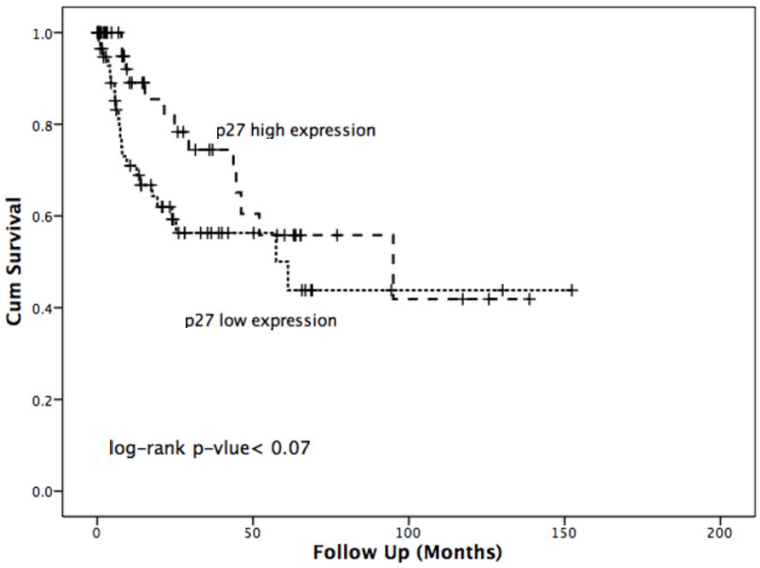

Figure 2. Kaplan Meier survival curve for patients with urothelial bladder cancer. Level of p27 protein was used as a determinant for patients' survival. Test statistics: log-rank test (P = 0.07).

data. The prognostic relevance of p27 was also examined in other malignancies. It has been reported that low expression of p27 was associated with high tumor grade/stage and poor patients' outcome in several cancer types including breast, gastric, colon, prostate and lung cancer suggesting that p27 is an independent prognostic and diagnostic factor [21-25].

p27 is a member of the CIP/KIP sub-family of cyclin-dependent kinase inhibitors, with p21 and p57, plays major roles in the pathophysiology of numerous cancer types. The biological effects of p27 are mainly mediated through binding to and inhibiting cyclin/CDK complex leading to growth arrest and suppression of tumor growth [5]. Nevertheless, alteration of p27 expression and its sub-cellular localisation in cytoplasm can result in contradicting effects in tumor cells [6]. Indeed, the oncogenic effect of p27 is mainly attributed to the fact that localisation of p27 in the cytoplasm would prevent its binding to and inhibiting nuclear cyclin-Cdk targets [26]. Cytoplasmic p27 may also contribute to tumor growth through cooperation with other genes such as Ras oncogene, as described previously in animal model [27].

Moreover, our analysis of p27 expression indicated that p27 is associated with poor survival for bladder cancer patients. The Kaplan-Meier survival curve showed poor survival rates in those patients with low levels of p27 expression (P = 0.07). Sgambato et al. [28] reported an association between low levels of p27 protein and reduced disease-free survival in primary superficial bladder cancers. Similar results were reported in bladder cancer and other cancers suggesting that p27 is a powerful marker for worse prognosis [11, 21, 29].

In summary, the current study has supported the previous findings indicating the role of p27 in tumorigenesis. P27 hold special promise, as a potential marker for cancer progression, to assist in clinical setting and improve outcomes for bladder cancer patients. To our knowledge this is the first study to investigate the prognostic value of p27 expression in bladder cancer patients of Arab origin. Further work is needed to further validate these findings in a larger cohort of patients with bladder cancer.

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Disclosure of conflict of interest

None.

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