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Over expression of P27 protein from CDKN1B gene in patients with invasive bladder transitional cell carcinoma

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Abstract
Background: Urinary bladder cancer is the fifth most common cancer in the western world. P27 is an enzyme inhibitor encoded by the CDKN1B gene in humans that belongs to the Cip /Kip family of cyclin-dependent kinase (Cdk) proteins. This protein prevents activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controlling cell cycle progression at G1.
Objective: To assess the relation of over expression of P27 protein to translational in a group of invasive bladder transitional cell carcinoma as compared to their healthy counterparts**.**
Materials and Methods: Forty-two formalin-fixed, paraffin embedded bladder tissues were enrolled in this study; biopsies from 30 invasive bladder transitional cell carcinomas well as 12 apparently normal bladder autopsies were included as a control groups. The translational protein of the expressed P27 gene was evaluated by immunohistochemistry.
Results: Positive immunohistochemical reactions for P27 protein were observed in 15 cases (50.0%) of invasive bladder transitional cell carcinoma while none of bladder tissues in control group revealed P27 immunostaining reactions. Statistically, the difference between the percentages of positive P27 protein-IHC reactions in bladder cancer group with control group was highly significant (P value = 0.002).
Conclusions: The highly significant translational over expression of P27 in our series of invasive bladder transitional cell carcinoma could importantly point for a role for derangement in that protein levels in the carcinogenesis of a subset of such cancer in our country.

Keywords: P27 protein, Bladder Transitional Cell Carcinoma, Immunohistochemistry.

Introduction
Bladder cancer is the most common malignancy in urinary system and the ninth most common malignancy in the world [1].
Cigarette smoking is recognized as the main cause of bladder cancer (about 50%) in developed countries. Occupational risks have shared5-10% in the etiologies of bladder carcinomas in industrialized countries. Drinking tap water with chlorination by-products or arsenic, exposure to certain drugs, like cyclophosphamide and heavy consumption of phenacetin-containing analgesics, have been shown to cause bladder cancer. Infectious agents have a major influence on bladder cancer risks in Schistosoma haematobium- endemic areas. Other potential risk factors for bladder cancer are other urinary tract infections [2].
The P27 is a member of the universal cyclin –dependent kinase (CDK) inhibitor family. The CDK inhibitor P27Kip-1(kinase- inhibitor protein-1) belongs to the Cip/Kip family of proteins. It plays a crucial role finking extracellular growth regulatory signals to progression to or exit from cell cycle [3].
The P27 Kip-1 protein oscillate during the cell cycle and plays a pivotal role in the regulation of cell cycle proteins & cyclin –CDK complex activity [4, 5]. In S – phase, P27 Kip is phosphorylated at Th-187 by cyclin E-CDK2 and is then recognized and targeted for ubiquitination by SCFKip2(S-phase kinase – associated protein2 [6]. One of the key mechanisms involved in the P27 regulation is ubiquitin-dependent proteolysis [7]. The level of P27 protein expression decreases during tumor development and progression in some epithelial, lymphoid and endocrine tissues [8, 9].
Expression ofP27Kip 1 was down regulated in breast cancer indicating marked S-phase progression and G2 phase due to active AAV2 genome (adeno associated virus 2) [10].
The expression and function of an inhibitory receptors like P16 (ink4a) and P27 (kip-1) decreased during HBV vaccine which subsequently enhanced the expression of cycline-

dependent kinase Z and cycline E (11).
Disruption of the balance between proliferation and differentiation in kerationcytes especially affect the expression of miR-24(microRNA24) and miR- 205(microRNA24).The miR-24 effects in the cellmay be due to targeting of cyclin dependent kinase inhibitor p27 (12).
It has been suggested that P27 is an independent prognostic factor in various human cancers. The measurement of both nuclear P27 and associated caspase 3 levels may be useful in monitoring patients with bladder carcinoma in situ. The prognostic value of P27 protein expression is not completely understood in bladder cancer yet (8).
This research work, and up to our best knowledge, is the first in Iraq, that study the expression of P27 protein from CDKN1B gene in a group of Iraqi patients with invasive bladder transitional cell carcinoma.

Materials and Methods
It has recruited 42 selected formalin fixed, paraffin embedded bladder tissue blocks which were belonging to the period group from 2013-2015; among them,
(30) tissue biopsies from invasive bladder transitional cell carcinoma with different grades were collected from the archives of different private laboratories as well as (12) apparently normal bladder tissue autopsies which were collected from the archives of Forensic Medicine Institute / Baghdad and used as bladder healthy control The study was designed as a retrospective one. Following trimming process of these tissue blocks, one section was mounted on ordinary glass slide and stained with hematoxyline and eosin, while another slide was mounted on charged slide to be used for IHC for detection of P27 protein.
The diagnosis of these tissue blocks were primarily based on their accompanied histopathological records. However, histopathological re-examination was done by a consultant pathologist to further confirm the diagnosis of these tissue blocks.
Immunohistochemistry / Detection system (Abcam. England) was used to demonstrate the P27 tumor suppressor genes. This technique is based on the detection of the product of gene expression (protein) in malignant and normal cells using specific monoclonal antibodies.
Rehydration process was done at room temperature which includes slides immersion in two changes of absolute ethanol for one minute each, then immersion in ethanol (95%) for one minute each, after that immersed in ethanol (70%) for one minute each, finally immersion in distilled water for 5 minutes to remove residual alcohol. After that, slides were allowed to dry completely by incubating them at 37 °C for 5 minutes.
Primary antibody for specific epitope (usually mouse antihuman monoclonal antibody), which binds to nuclear targeted protein.
The bound primary antibody is then detected by secondary antibody (usually rabbit or goat anti mouse), which contains specific label (in this context we used peroxidase labeled polymer conjugated to goat anti mouse immunoglobulin).
The substrate is DAB in chromogen solution; positive reaction will result in a brown- color precipitate at the antigen site in tested tissues. After that sections were dehydrated by ethyl alcohol, (95%, once for one minute then, 100% twice times for 2 minutes each); cleared by Xylene, then mounted with permanent mounting medium (DPX). Then the slides have been incubated at 37 °C for 30 minutes or until color development was developed completed. Final evaluation was achieved by light microscope where color development has monitored by viewing these slides under the light microscope.
Technical analysis was done according to the specification of the kit, proper use of this ISH detection system gives an intense brown signal at specific sites of the antigenic sites in positive test tissues (by using light microscope).
The signal was evaluated under light microscopy using × 100 lens for counting the positive cells. The IHC results were given intensity and percentage scores based on intensity of positive signals and number of cells that gave these signals, respectively.
Positive cells were counted in 10 different fields of 100 cells for each sample and the average percentage of positive cells within the 10 fields was determined. A scale of 0-3 was used for relative intensity with 0 corresponding to no detectable IHC reaction, and 1, 2, 3 equivalents to weak, moderate, and strong intensity of reaction, respectively. Cases were assigned to one of the following percentage score categories: 1%–25% (score 1; low), 26%–50% (score 2; moderate) or > 50% (score 3; high) (13).
All the statistical analysis was done by SPSS program (Version– 17). Chi – square test was used to detect the significance between variables of our study and P value was considered significant when $p < 0.05$.

Results
The results of the present study (Table 1) have revealed that the mean age of bladder cancer patients was (60.83±12.389), while the mean age of those enrolled as control group was (67.833±8.642). The statistical analysis has showed no significant difference between the mean age of bladder cancer patients and their control group (P=0.082).

Table 1: The Mean Age of Patients with Bladder Cancers and Control Groups

Type	N	Mean	Std. Deviation	Std. Error Mean
Age Patient	30	60.8333	12.39322	2.26268
Control	12	67.8333	8.64274	2.49494

Table (2) shows the mean age of patients according the grades of their bladder cancers. The mean age of (20) patients with grade 1 bladder cancer was (58±13.439) years, while (4) patients with grade 2 bladder cancer have mean age of (66.25±10.43) years and (6) patients with grade3 bladder cancer have mean age of (65.83±7.67) years. The statistical analysis has showed no significant difference between the mean age of bladder cancer patients and their control group (P=0.280).

Table 2: Distribution of Patients with Bladder Cancers According To Their Ages

Grade	N	Mean of Age (Years)	Std. Deviation	Std. Error
1	20	58.2500	13.43944	3.00515
2	4	66.2500	10.43631	5.21816
3	6	65.8333	7.67898	3.13493
Total	30	60.8333	12.39322	2.26268

Table (3) reveals the study groups of the present study according to their gender. There were 21(70.0%) males and 9 (30.0%) females in the patients group, while there was 7(58.3%) males and 5(41.7%) females which consist the control group. The statistical analysis has showed no significant difference between the gender of bladder cancer patients and their control group (P value greater than 0.05).

Table 3: Gender Distribution of Patients with Bladder Cancer and Their Control Counterpart Individuals.

			Group	
			Bladder Cancer Patients	The Controls
Gender	Male	Count	21	7
		%	70.0%	58.3%
	Female	Count	9	5
		%	30.0%	41.7%
Total		Count	30	12
		%	100,0%	100.0%

Table (4) shows that 50% of bladder cancer tissues have p27- gene scion (Figure 1) while none of bladder tissues in the control group have p27- gene expression. The statistical analysis has showed significant between p27- gene expression and bladder cancer (P= 0.002).

Table 4: The P27 - gene expression in patients with bladder cancers

			P27- IHC Reaction		
			Positive	Negative	Total
Group	Patient Count s % within Type		15	15	30
			50.0%	50.0%	100.0%
	Control	Count	0	12	12
% within Type			0%	100.0%	100.0%
Total Count			15	27	42
% within Type			35.7%	64.3%	100.0%

Table (5) shows the scoring of IHC- reactions that were used for protein detection of P27 - gene expression in bladder cancer tissues. There were an equal percentages (33.3%) of each scoring grades of IHC- reactions for P27 - protein detection in the examined bladder cancer tissues.

Table 5: Scoring of IHC- reactions for P27 – protein expression in bladder cancer patients group.

	Frequency	**Valid Percent**	**Cumulative Percent**
Valid	5	33.3	33.3
Low	5	33.3	66.7
Moderate	5	33.3	100.0
High Total	15	100.0	

Table(6)shows the intensity scoring of positive- IHC- reactions for detection of P27 - gene expression in bladder cancer tissues, where 33.3% of P27 - IHC- reactions revealed moderate intensity scoring while 66.7% of these IHC-reactions have strong intensity scores.

Table 6: Intensity Scoring of IHC-reactions for P27 – protein expression in bladder cancer tissues.

	Frequency	Valid Percent	Cumulative Percent
Valid Moderate	5	33.3	33.3
	10	66.7	100.0
Strong Total	15	100.0	

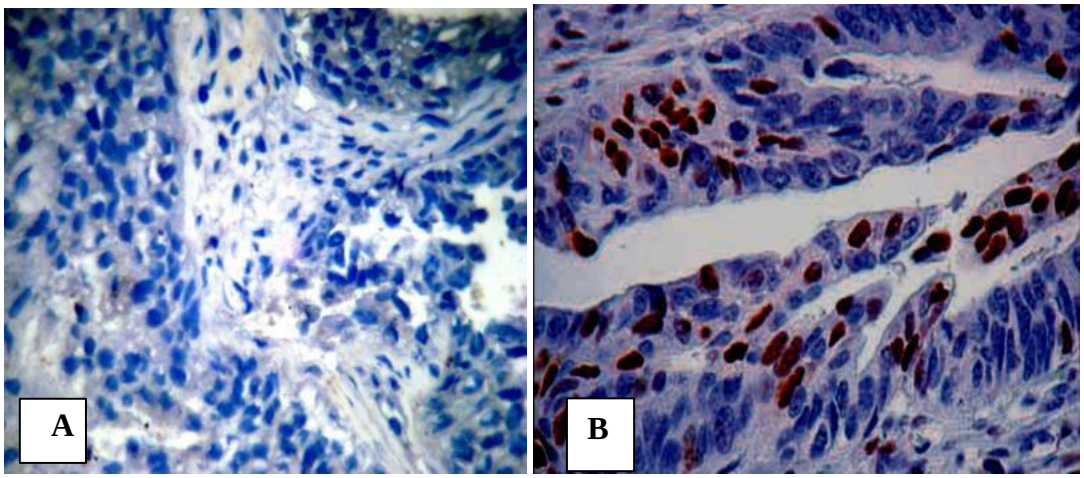

Fig 1: Immunohistochemical results for P27 expression detection in bladder cancers; stained with DAB chromogen (brown) and counter stained by Mayer's hematoxyline (blue);
A. Bladder cancer with negative P27-IHC reaction (20X).
B. Bladder cancer tissue with positive P27- IHC reaction (20X).

Table (7) shows 15 patients have p27- gene expression in their bladder cancer tissues and their mean age was (63.±11.85) years, while there was another 15 patients whom their bladder cancer tissues did not have p27 protein expression and their mean age was(58.6±12.9) years. The statistical analysis has showed no significant difference between the p27- gene expression and the age of bladder cancer patients (P value greater than 0.05).

Table 7: Distribution of P27- gene expression in bladder cancer tissues according to the age of these patients.

P27	N	Mean	Std. Deviation	Std. Error Mean
Age Positive	15	63.0667	11.85909	3.06200
Negative	15	58.6000	12.91621	3.33495

Table (8) shows p27- gene expression distribution according to the gender of patients with bladder cancer. There were 47.6% (10)males have positive p27- gene expression, while there were 55.6%(6)females had tissues with such positive- p27 gene expressions. The statistical analysis has showed no significant difference between the p27- gene expression and the gender of bladder cancer patients (P value greater than 0.05).

Table 8: Percentage of P27 gene expression results in patients with bladder Cancer according to their gender

			P27- IHC Reaction		Total
			Positive	Negative	
Gender	Male	Count	10	11	21
		% within Gender	47.6%	52.4%	100.0%
	Female	Count	5	4	9
		% within Gender	55.6%	44.4%	100.0%
Total		Count	15	15	30
		% within Gender	50.0%	50.0%	100.0%

Table (9) shows the correlation between the grades of bladder cancers and the expression of P27 gene. Among those tissues with grade 1 bladder cancers, 8 tissues (40%) had showed p27-protein expression, while there were 3 tissues with grade 2 of bladder cancer (75%) had showed p27-protein expression and 4 tissues with grade 3 of bladder cancer (66.7%) had p27 gene expression. The statistical analysis has showed no significant difference between the p27- gene expression and the grade of bladder cancers (P value greater than 0.05).

Table 9: Percentage of P27- gene expression results in bladder cancer tissues according to their grading of differentiation

		P27- IHC Reaction		Total
		Positive	Negative	
Grade	1 Count	8	12	20
	% within Grade	40.0%	60.0%	100.0%
	2 Count	3	1	4
	% within Grade	75.0%	25.0%	100.0%
	3 Count	4	2	6
	% within Grade	66.7%	33.3%	100.0%
Total Count		15	15	30
% within Grade		50.0%	50.0%	100.0%

Discussion

Tumor suppressor proteins are commonly expressed in non-bilharziasis bladder adenocarcinoma. Mutations in genes regulating the expression of these cell cycle regulatory proteins are involved in the development or progression of both conventional urothelial and adenocarcinoma of bladder tumors [14-15].

As a cyclin-dependent kinase inhibitor which inhibit cell entry from G1 to S phase, a decreased expression and / or alterations of p27 have been well associated with the evolution of most invasive bladder cancers in referred to the high stage, poor grade, DNA ploidy, lymphatic metastasis, recurrence and survival in urothelial carcinoma [15].

It was found in the present study that 50% of bladder cancer tissues have p27- gene expression while none of bladder tissues in the control group have shown p27- gene expression. The authers [16] and [17] in their works have found p27 in 71.0% and 83.4%,respectively.These results are in agreement with our results.

Also in this study, and among those studied bladder cancer tissues with grade 1- cancers, 8 tissues out of 20 cases (40%) had showed p27-protein expression, while 3 bladder cancer tissues out of 4 cases with grade 2- cancers (75%) had showed p27-protein expression and 4 tissues out of 6 cases with grade 3- bladder cancers (66.7%) had p27 gene expression.

The absence of p27 gene expression was significantly associated with both high tumor grade and solid tumor growth pattern. Similar association between decreased p27 Kip1 staining and higher tumor grade has been reported [18]. No reports are available regarding the tumor growth pattern and p27 expression level [19, 20].

Although it has reported that a significant association between the low p27 protein level and poor survival, several studies were unable to detect such correlation [3, 21-22]. This discrepancy would be explained by difference in the sample size and detection of protein instead of mRNA.

P27 alterations were associated with high stage and grades well as have showed a trend of lymph nodes metastasis [17, 23]. Low protein levels of p27 were associated with poorly differentiated grade, muscle and lymph node invasion, and unfavorable prognosis. It is likely that p27 affects differentiation pathways and acts as a tumor suppressor gene in different human tumors; and therefore evaluation of p27 protein levels may indicate the biological behavior of human tumors [18].

In addition, up-regulation of P27 in high-grade bladder tumor tissues was associated with over-expression of cyclin E. However, p27 did not suppress the cyclin E kinase activity in those complexes, then resulted in progression of cells through the cell cycle via phosphorylation of cyclin E/Cdk2 downstream substrate targets, In addition, and as a result of activation of receptor tyrosine kinases and the ras signaling pathways, the acceleration of proteolysis as well as cytoplasmic accumulation of p27- protein in the majority of human malignancies can lead to a reduced ability of p27 to bind and inhibit nuclear cyclin E/Cdk2 complexes[24- 27].

Moreover, some studies have showed that high p27 levels are associated with better clinical outcome, while others have denied any prognostic relationship of p27 expression [28-30]. These contradictory studies could related to the fact that p27 could be up-regulated yet it cannot function as a Cdk inhibitor when LMW cyclin E isoforms (which are refractory to p27 inhibition) are over expressed.

Furthermore, over-expressed of LMW isoforms of cyclin E with high p27 levels, especially in an early stage of the disease would thus be a prediction for poor clinical outcome [30] whereas a high p27would be predictive of good clinical outcome in tumors without LMW cyclin E over- expression. The highly significant translational expression of P27 gene in bladder carcinoma in our results could indicate an important role of these molecular factor in the bladder carcinogenesis of subset of bladder malignant tumors.

Conclusions

The highly significant translational over expression of P27 from CDKN1B gene in invasive bladder transitional cell carcinoma in this study could the studied series of point for an important role for P27 protein amongst factors in the carcinogenesis of such subset of invasive bladder cancer.

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