Expression of *p21* and *p27* in gallbladder cancer

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ABSTRACT

Objectives: To investigate the expression of *p21* and *p27* factors in gallbladder cancer (GBC), and to correlate their expression with clinicopathological parameters: age, gender, stage, invasion, and grade.

Methods: Thirty-two surgically resected specimens were collected between 1994-2001 from different health centers in north Jordan. Tissues belong to 25 females and 7 males were examined immunohistochemically. The study took place in the Pathology Department, Jordan University of Science and Technology, Jordan.

Results: Levels of *p21* were found in 75% and *p27* in 25%. Furthermore, *p21* was expressed in 50% of the specimens which are belong to patients with ages ≤64 years, whereas all specimens for ages >64 years have *p21*WAF1/CIP1 expression (p=0.001). The expression of *p21* between advanced stages (stages III and IV) was 89.5% and early stages (stages I and II) was 53.8 % (p=0.031).

Conclusion: The *p27* expression was markedly decreased in GBC cases (25%) and there were no significant correlation between *p27*KIP1 expression and all clinicopathological parameters including gender, World Health Organization grades, stages, and invasion, whereas the expression of *p21* was 75% and there was a significant correlation between *p21* and the clinicopathological parameters including gender, stages, and invasion.

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The gallbladder is a small muscular organ that lies in the undersurface of the liver. It stores bile that is used mainly to emulsify fats.[1] Gallbladder, histologically, includes lining of simple columnar epithelium, lamina propria that is a thin layer of smooth muscle, serosa of loose fibrous tissue and mucosa.[2] Gallbladder carcinoma (GBC) is the most common malignancy of the biliary tract. Despite advances in diagnostic and surgical techniques, the prognosis of GBC remains one of the worst among all cancer types. Understanding and identifying biomarkers correlated with the prognosis of GBC are important for improving the clinical outcome.[3] *p21* and *p27* are members of the Cip/Kip families of cyclin dependant kinase inhibitors which preferentially inhibit cdk2 containing complexes (cyclin A/e-cdk2). Cyclin dependant kinases (Cdk) inhibitors negatively regulate the cell cycle complexes (cyclin/cyclin dependant kinase) which phosphorelate the retinoblastoma protein; where hypophosphorelated active retinoblastoma protein bounds to transcription factors E2F family leading to transcription of genes responsible for S phase progression. Any loss of their function could lead to malignancy transformation.[4,5] *p27* plays a significant role in controlling the G1 to S phase transition.[5,6] Evidences suggest that *p27Kip1* function as a tumor suppressor.[7-9] Through the binding to and inhibition of Cyclin/Cyclin dependant kinases.[10,11] Loss or reduced expression of *p27* has been found in a variety of human cancers and associated with more aggressive tumor behavior.[12] *p21* plays an important role in repairing cell cycle arrest during DNA damage, if this mechanism fails it triggers apoptosis.[13,14] Its also binds the proliferating cell nuclear antigen (PCNA) in order to inhibit replicative DNA synthesis.[15] Expression of these cell cycle molecules may be predictive markers for the efficiency of the chemotherapy drugs.[16] Down regulation of expression of these molecules was proved to be a late event in gallbladder carcinogenesis.[17-22] In Jordan, reports on GBC are scarce. According to the cancer incidence registry for the year 2000 gallbladder carcinoma constituted 1% of all cancers.[23] On the other hand, report

on the epidemiological, clinical, and pathological features of gallbladder cancer, which depended on the histopathological reports and the hospital records for all cases found that the prevalence was 33/4502 cholecystectomies performed between 1994-2004. In the Middle East and North Africa only scattered reports are available. Of 7352 cholecystectomies performed, 89 cases of gallbladder carcinoma were found in Libya over a period of 16 years.[24] Several risk factors for gallbladder carcinoma have been studied. Some of these risks are attributed to genetic factors.[25] These genetic factors had not been widely studied because of the rarity of the tumor and difficulties arise due to late development and location of gallbladder. However, several studies showed deregulation and overexpression of some proteins in GBC such as mamoglobin B, K-ras, growth factors, p53, *MDR1*, and cyclin D1.[26-31] The objective of our study was to evaluate the expression of *p21*and *p27* in GBC. On the other hand, we aimed to correlate the expression of the previous markers with clinicopathological parameters of the patients.

Methods. Paraffin embedded tissues from surgical specimens of 32 GBC patients were retrieved from the Pathology Department, Jordan University of Science and Technology, Jordan. Specimens were collected between 1994-2001 from different health centers in north Jordan. Tissues belong to 25 females and 7 males.

Paraffin embedded GBC tissues were cut into 3-µm thick sections and stained by Mouse polyclonal antibodies against specifically to *p21*WAF1/CIP1 (clone 187) and *p27KIP1* (clone F-8) which were purchased from Santa Cruz Biotechnology Incorporation, USA. Sections were floated on vectabond treated frosted glass slides (Vector Laboratories, USA) and heated in oven at 70°C for 5 mins. Tissue sections were cleared in xylene for 2 mins and rehydrated in a descending series of ethanol (70-100%). Thereafter, sections were cooked under pressure in the reveal solution (Biocare Medical, (location)) 10x for 2-4 mins in the Decolaking chamber (Biocare Medical, (location)). Sections were left in a cooled room temperature then washed and incubated with phosphate buffer solution (PBS) for 15 mins. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 minutes. Thereafter, sections were incubated for one hour at room temperature. This was followed by incubation with biotinylated secondary antibody for 10-20 min, then immunoperoxid- ase staining was carried out using streptavidin biotin reagent (Biocare Medical LSAB kit) for 10-20 mins at room temperature. However, blocking of endogenous peroxidase activity incubation with secondary antibody and immunoperoxidase staining must be followed by PBS washing. Diaminobenzidine (DAB) chromogen was used to develop staining (7 mins incubation) and sections were counterstained with hematoxylin (Gain Land Chemical Company/UK) for 20-40 seconds. Dehydration, clearing, and mounting were carried out then slides were examined under light microscope. Positive controls for *p27KIP1* and *p21WAF1/CIP1* were prepared from breast cancer tissue known to express these markers. *p27KIP1* has positive control from normal breast tissue. Negative controls were performed by incubation of sections with PBS buffer only instead of the primary antibodies. Expression of investigated markers was evaluated by intensity of staining and incidence of positively stained cells. The intensity was graded as absent (0), mild (1), moderate (2), and intense (3). The incidence was scored for *p21WAF1/CIP1* as absent (0), <10% (1), 10-50% (2), and >50% (3). For *p27KIP1* as absent (0), <5% (1), 5-30% (2) and >30% (3). Intensity and incidence of positively stained cells were added to derive a staining score range from 0-6. A score equal to or greater than 3 was considered as overexpression.[32] Frequencies of *p21* and *p27* over expression were compared with clinical characteristics and pathological parameters. Correlation between markers expression and clinicopathologic factors was determined by using Pearson's chi-square test. Statistical analysis was performed using the statistical software SPSS version 10.0, p values ≤0.05 were considered statistically significant.

Results. During this study, 2 molecular markers (*p21* and *p27*) were investigated in 32 cases of GBC using immunohistochemistry. *p21WAF1/CIP1* expression in association with the age, stages (according to WHO), grading, and invasion was studied. Twenty four specimens (75%) showed positive staining for *p21WAF1/CIP1* (**Figure 1**). Approximately 50% of the specimens which belong to patients with ages ≤64 years were positive (*p21WAF1/CIP1* expression), whereas all specimens for ages >64 years have *p21WAF1/CIP1* expression, which indicates statistical differences on age groups (p=0.001). In this study, we were unable to demonstrate differences in *p21* expression according to patient's gender. Approximately 71.4% of males and 76% of females were positive, and similar results were obtained when the grade of the disease was considered (grades 1 and 2 were 70% and grades 3 and 4 were 83.3%). Although the expression was higher in moderate and well differentiated cases (grades 3 and 4) 10 out of 12 cases (**Table 1**) in comparison with the poorly and undifferentiated ones (grades 1 and 2). No statistical differences between the 2 groups were demonstrated (p=0.344). In contrast to the grade, there was a significant difference (p=0.031) in the expression of *p21*

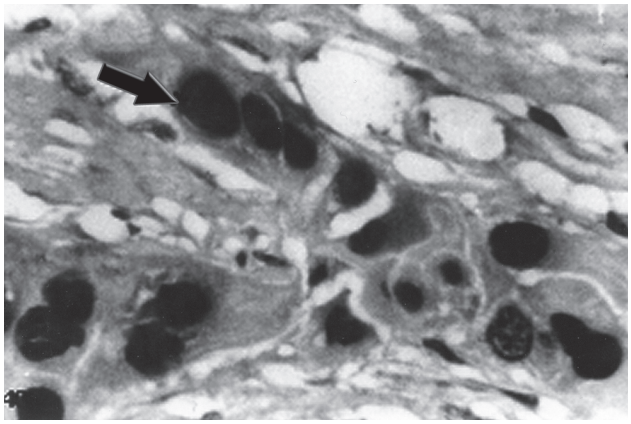

Figure 1 - Strong nuclear and cytoplasmic expression of *p21* in gallbladder cancer (black arrow).

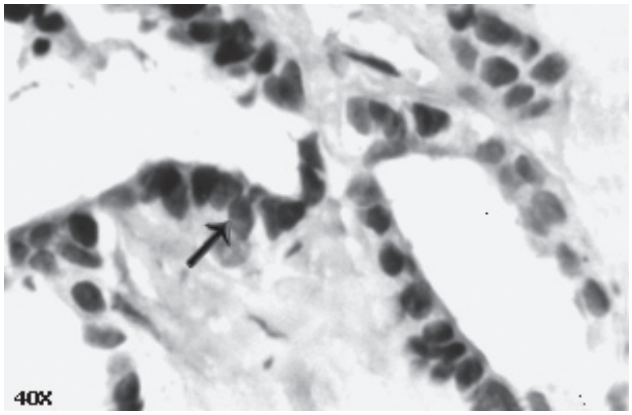

Figure 2 - Positive expression of *p27* in gallbladder cancer (black arrow).

Table 1 - Expression of *p21WAF1/CIP1* according to clinicopathological characteristics (n=32).

Clinicopathological characteristics	*p21WAF1/CIP1*			
	N	No. of positive patients (%)	No. of negative patients (%)	*P* value
Age				
≤64	16	8 (50)	8 (50)	0.001
>64	16	16 (100.0)	0 (0)	
Gender				
Female	25	19 (76)	6 (25)	0.577
Male	7	5 (71.4)	2 (28.6)	
Grades				
1 & 2	20	14 (70)	6 (30)	0.344
3 & 4	12	10 (83.3)	2 (16.7)	
Stages				
I, II	13	7 (53.8)	6 (46.2)	0.031
III, IV	19	17 (89.5)	2 (10.5)	
Invasion				
Presence	21	17 (81)	4 (19)	0.256
Absence	11	7 (63.6)	4 (36.4)	

Table 2 - Expression of *p27KIP1* according to clinicopathological characteristics (n=32).

Clinicopathological characteristics	*p27KIP1*			
	N	No. of positive patients (%)	No. of negative patients (%)	*P* value
Age				
≤64	16	2 (12.5)	14 (87.5)	0.110
>64	16	6 (37.5)	10 (62.5)	
Gender				
Female	25	7 (28)	18 (72)	0.423
Male	7	1 (14.3)	6 (85.7)	
Grades				
1 & 2	20	6 (30)	14 (70)	0.344
3 & 4	12	2 (16.7)	10 (83.3)	
Stages				
I, II	13	2 (15.4)	11 (84.6)	0.271
III, IV	19	6 (31.6)	13 (68.4)	
Invasion				
Presence	21	6 (28.6)	15 (71.4)	0.425
Absence	11	2 (18.2)	9 (81.8)	

between advanced stages (III and IV) and early stages (I and II). On the other hand, there was no significant relationship between invasion and *p21WAF1/CIP1* staining; however, cases with invasion features showed greater staining (81%) than those without invasion features (63.6%). Nuclear immunoreactivity showed a clear decrease in the expression of *p27KIP1* in the tumor tissue. Twenty-four specimens were observed to loose the expression of *p27KIP1*, where as only 8 cases (25%) displayed *p27KIP1* expression (**Figure 2**).

Table 2 shows that there was no significant correlation between *p27KIP1* expression and all clinicopathological parameters including gender, WHO grades, stages, and invasion.

Discussion. The increased level of *p21* in the tumor may be due to a feedback mechanism that halts proliferation.[31,32] Overexpression of *p21* was strongly associated with advanced stages, and age which suggests that *p21* is not an early event of GBC carcinogenesis.[33] Our study showed this clearly where 100% of patients with age over 64 years have *p21*expression, while 50% of patients with age less than 64 years were positive (p=0.001), at the same time 89.5% of the advanced cases showed positive expression of *p21* while only 53.8% in early stages (p=0.031). Low expression of *p27* in GBC cases (25%) was significantly correlated with advanced stages, and invasion of GBC (31.6% and 28.6%) respectively, suggesting that down-regulation of *p27* expression is a late event in GBC.[34] Expression of *p27* in combination with *p21* was not of significance in the present study, similar results were reported in previous studies.[22,34] Overexpression of *p21* was strongly associated with advanced stages, and age related which suggests that *p21* is not an early event of GBC carcinogenesis. Low expression of *p27* in GBC cases (25%) has significantly correlated with advanced stages, and invasion of GBC (31.6% and 28.6%) respectively,

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