

# Expression of p53 and p21 in bladder carcinoma of Iraqi patients

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## Abstract

Bladder cancer is an abnormal cellular multiplication without control arising from the urinary bladder urothelium. Cells cycle is a strictly controlled process regulated by protein complexes composed of cyclins and cyclin-dependent kinases and also by several tumor suppressor gene protein products acting at the Go/G1 checkpoint of the cell-cycle. This study was designed to evaluate the frequency of p53 and p21 expression as biomarkers to monitor the disease and compare the results with those of others. Seventy-three tumors with different stages and grades were collected from patients with bladder carcinoma and ten normal bladder biopsies from forensic autopsy. Sections were immunohistochemically stained for p53 and p21. Results revealed that p53 positive immune reactivity was observed in (57.5%) which were exclusively nuclear and no immune reactivity detectable in (42.5%) of tumors, while all normal tissues expressed negative patterns. Positive immune staining of p21 was recorded in (69.5%) and negative p21 immune staining was recorded in 22 (31.1%), as well as ten normal urothelium. Statistically p53 and p21 over-expression was significantly related to stage and grade of tumors ( $P < 0.05$ ).

## Introduction

Bladder cancer is the most common cancer in the upper urinary tracts, which has the highest rate of any malignancy. In Iraq, the incidence of bladder cancer is the fourth common type of cancer in men and eighth one in women. More than 90% of bladder cancers start from the urothelium known as urothelial or transitional cell carcinoma (TCC) while squamous cell carcinoma and adenocarcinoma are rare types. Cells of bladder carcinoma can spread through the blood and lymph vessels, attach to other tissues then grow to form new tumors, thus characterized by rapid progression from noninvasive to invasive stage. The incidence and mortality rates vary among the countries because of the differences of the risk factors and the availability of treatments.<sup>1-3</sup>

p53 is a tumor suppressor protein. It regulates the cell cycle, apoptosis and is involved in many cellular activities such as metabolism, antioxidant response, and DNA repair. The tumor suppressor gene has been located on the chromosome 17p13, which encodes wild type p53 protein that consists of 393 amino acids, which described as *the guardian of the genome*, because of its role in the conserving stability by preventing genome mutation and tumor development. Tumor's defective p53 could allow abnormal cells to proliferate, resulting in cancer. As many as 50% of all human tumors contain p53 mutants.<sup>4,5</sup> Under usual conditions, p53 is expressed at an extremely low level, tightly suppressed in the normal cells by MDM2 (mouse double minute 2 homolog also known as E3 ubiquitin-protein ligase MDM2). Upon DNA damage, p53 is induced to accumulate in the cell nucleus through post-translational modifications such as phosphorylation and acetylation. These modifications convert p53 to an active form by dissociation of MDM2. Effective p53 turns activate an appropriate set of its target genes to induce cell cycle arrest and/or apoptosis, which is dependent on the magnitude and sites of DNA damage.<sup>6,7</sup>

p53 protein enhances another gene to produce a protein called p21 that interacts with cell division stimulating protein cyclin-dependent kinase 2 (CDK2), when p21 complexes with cdk2, the cell unable to pass through to the next stage of cell division.<sup>8-10</sup> p21 is recognized as the cyclin dependent kinase inhibitor, encoded by the CDKN1A gene located on chromosome 6p21.2 in humans. It facilitates cell cycle arrest by interaction with different stimuli and transition factors such as p53, which acts as a regulator of cell cycle progression by interacting with proliferating cell nuclear antigen, DNA polymerase accessory factor and plays a regulatory role in S phase DNA replication, DNA damage repair.<sup>11,12</sup> Various stress stimuli and p53 protein responsible for activation of the apoptotic protein p21 and cause alteration in the checkpoints which led to arrest the cell cycle at G1 and G2 phases by binding and inhibiting the complexes of CDK and cyclin.<sup>13,14</sup> p21 is sometime expressed without being induced by p53. Kind of induction plays a big role in p53 independent differentiation. Expression of p21 is mainly

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dependent on the stimulus provided and the type of the cell.<sup>15</sup> p21 was expressed in a variety of human malignancies and correlated with tumor progression and a poor prognosis in various carcinomas. Expression of the mutant gene of p21 has been reported to be associated with progression of bladder carcinoma.<sup>16,17</sup> This study was aimed to investigate the pathological significance and prognostic value of p53 and p21 in bladder cancer.

## Materials and Methods

Seventy-three biopsies have been collected from urinary bladder cancer (UBC) patients after cystoscopy surgery, 60 male and 13 female with an average age 65.2 years and a range of (43 to 85) years, 32 patients had UBC and 30 healthy control. Subjects are collected through the period from March 2014 to the November 2014. They attended to the Urology Unit at Al-Yarmook and Al-Jabchi Hospitals. The tumors were graded as low or high on the basis of World Health Organization (WHO) classification criteria. While ten normal bladder biopsies have been collected from forensic autopsy.

### Immunohistochemical staining

Immunohistochemically staining was performed by using labeled streptavidin biotin immune staining method. Briefly, 5-mm thick sections were deparaffinized and rehydrated. Most formalin fixed tissue requires an antigen retrieval step before immune staining. Antigen retrieval was performed by using the Tris EDTA buffer pH=9 for p53 and Sodium Citrate buffer pH=6 for p21, then placed in microwave histoprocesser at 850 w for 20min. After cooling for 15min, slides were removed and washed by the washing buffer for 10 min. The hydrogen peroxidase block was added for 10 min, protein block was applied and incubated for 10 min room temperature, then washed 3 times in the PBS buffer. Diluted mouse monoclonal primary antibody at a ratio (1/50 for p53 and 1/200 for p21) was added to tissue sections and incubated 20 min primary antibody then washed 4 times in PBS buffer. Biotinylated goat anti-mouse was applied and incubated for 15 min in humidity chamber at room temperature after washing 4 times in PBS buffer; Streptavidin peroxi-

dase was applied and incubated for 10 min at room temperature then rinsed 4 times in the PBS buffer. Two hundred mL of 3,3'-Diaminobenzidine solution was and incubated 10 min at room temperature. Sections were minimum rinsed 4 times in the PBS buffer, then counter stain was added for 2 min at room temperature then washed in the tap water. The slides were dehydrate and mounted with DPX and coverslip, then slides were examined by light microscope 10×, 20× and 40×. Results were compared with the positive control, which determined according to leaflet of the kit. Positivity was assessed semi quantitatively by the intensity and percentage of staining. The score was determined according to a scale when the nucleus and membrane of the cell has been stained with brown color for p53 and p21 respectively.<sup>18-20</sup>

### Statistical analysis

The relationship between p53 over-expression and the clinic pathologic variables were analyzed by the Statistical Analysis System program and Chi-square test was used to significantly compare between the percentage of observed and expected frequencies. The results were considered statistically significant if the  $P < 0.05$ .<sup>21</sup>

## Results

Seventy-three bladder tumors were collected from patients. Stage and grade of each tumor were diagnosed by a consultant of histopathology according to WHO. All tumors were classified as TCC. Fourty (54.8%) tumors were characterized by high grade and 33 (45.2%) low grade while according to stage of tumors 12 (16.4%) Ta, 28 (38.4%) T1 invaded the subepithelial connective tissue, 22 (30.1%) T2 invaded the muscle, 7 (9.6%) T3 invaded the perivesical wall of the bladder and 4 (5.5%) T4 invaded other organs as shown as in Table 1.

Analysis of 73 bladder tumors and 10 biopsies of normal bladder tissues revealed that 42 (57.5%) gave positive nuclear immune staining of p53 and 31 (42.5%) gave a negative staining while all normal transitional epithelium biopsies were reflected negative immune staining as shown as in Table 2. P21 (cyclin-dependent kinases Inhibitor) suppresses tumors by promoting the cell cycle arrest in

**Table 1. Classification of urinary bladder cancer patients into stage and grade.**

Grade	Stages					Total
	Ta	T1	T2	T3	T4	
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Low	12 (16.4)	21 (28.8)	0	0	0	33 (45.2)
High	0	7 (9.6)	22 (30.1)	7 (9.6)	4 (5.5)	40 (54.8)
Total	12 (16.4)	28 (38.4)	22 (30.1)	7 (9.6)	4 (5.5)	73 (100)

**Table 2. p53 and p21 expression in urinary bladder cancer and control.**

Group	p53 expression		P-value	p21 expression		P-value
	Positive N (%)	Negative N (%)		Positive N (%)	Negative N (%)	
UBC	42 (57.5)	31 (42.5)	0.0004*	51 (69.9)	22 (30.1)	0.0026*
Control	-	10 (100)		-	10 (100)	

UBC, urinary bladder cancer. \*Significant.

response to cellular and environmental signals to promote tumor suppressor activities. The results showed that 51 (69.9%) out of 73 bladder tumors gave a positive staining of p21 with different scores, 22 (30.1%) gave negative immune staining and all ten normal urothelium consistently demonstrated no p21 immune reactivity, providing a negative staining pattern as shown in Table 2.

The bladder tumors gave positive expression of p53 and p21 with different scores, summarized in Table 3. Positive staining of p53 with score +3 reflected a high frequency 17 (23.3%), 15 (20.5) for score +4 then 9 (12.3%) score +2 and 1 (1.4%) for score +1 while score +2 represented the highest frequency 17 (23.3%) of p21, score +3 represented 16 (22 %) and score +4 represented 12 (16.4 %) while score +1 gave the lowest percentage 6 (8.2%) with significant differences.

In addition, the results in Figure 1 and Table 4 showed a higher significant positive expression of p53 according to tumor stage. Results observed that invasive stages reflected strong positive immune expression of p53 and p21 when compared with non-invasive stages with significant difference. Score +3 represented the most prevalent one which expressed by tumors had invasive stages (20.6%) followed by score +4 (17.8%) then score +2 (4.1%), no one expressed score +1 of positive expression and only 2 (2.7%) gave negative expression of p53. Most of tumors had non-invasive stages 29 (39.8%) gave negative immune staining. The results demonstrated in Figure 2 and Table 4 showed that the highest positive expression of p21 with score +3 and score +2 was observed among tumors had invasive stages. Moreover, the highest negative p21 expression was recorded among tumors had non-invasive stages.

Table 5 revealed that the highest significant positive expression of p53 according to tumor grade was recorded in high grade 36 (85.7%) out of 42 and 6 (14.3%) low grade. The highest negative expression in the low grade was 27 out of 31 (87.1%) and 4 (12.9%) in high grade as shown as in Figure 1.

The expression of the cyclin dependent kinase inhibitor p21 provides important prognostic information in patients with UCC of the bladder. Patients with tumors that maintained p21 expression demonstrated a statistically significant decreased rate of tumor recurrence and a statistically significant increased overall survival in comparison with those whose tumors had lost expression of p21 and statistically significant association between p21 expression and tumor progression was observed at all pathologic stages. The

results in Table 5 revealed that a higher positive expression was recorded in high grade 29 (56.9%) out of 51 and 22 (43.1%) out of 51 in low grade with significant differences while the negative expression in low grade was 11 (50%) out of 22 and 11(50%) in high grade Figure 2.

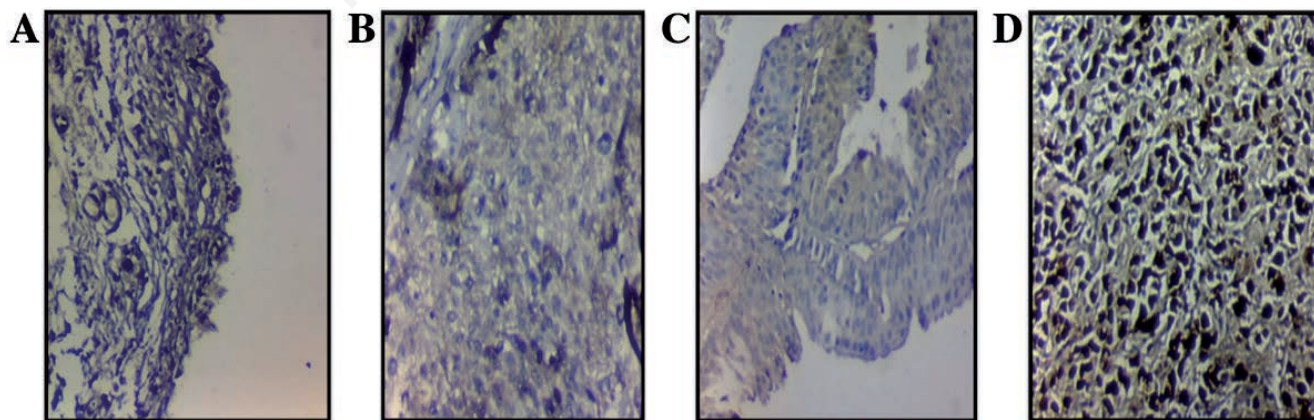
## Discussion

TCC is one of the most common cancers in developed countries which accounts for the 90-95% of bladder carcinoma. The tumor suppressor protein p53 plays an essential role in human cancer, which prevents the neoplastic transformation with induction of senescence or cell death by apoptosis. Inactivation of tumor suppressor genes can occur either primarily through mutations, or without any change in the structure of the given genes. Thus, tumor suppressor function should be analyzed at the level of the genes as well as at the level of proteins, and in the context of the pathways in which these genes are involved.<sup>22-24</sup>

In this study p53 over-expression was mainly found in advanced stage. Statistically, the over-expression of p53 in relation to stage was significant confirming the observation by, some other studies. Puzio-Kuter *et al.*<sup>25</sup> reported that inactivation of p53 promotes tumorigenesis in human bladder and p53 wild-type (undetectable) in non-invasive papillary tumors while p53 alteration,

**Table 3. Frequency of p53 and p21 scores in urinary bladder cancer.**

Score	p53 N (%)	p21 N (%)
Score 0	31 (42.5)	22 (30.1)
Score +1	1 (1.4)	6 (8.2)
Score +2	9 (12.3)	17 (23.3)
Score +3	17 (23.3)	16 (22)
Score +4	15 (20.5)	12 (16.4)
Total	73 (100)	73 (100)



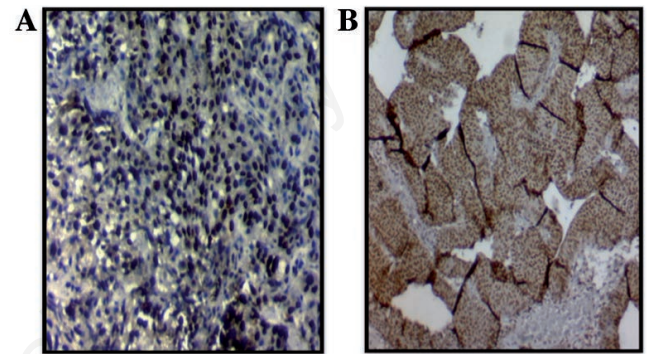
**Figure 1. Immunohistochemical staining of p53. (A) Negative normal urothelium (10x). (B) Negative non-invasive transitional cell carcinoma (TCC), stage T1, low grade. (C) Negative invasive TCC, stage T3, high grade (10x). (D) Positive invasive TCC, stage T4 section under 10x.**

p53 nuclear overexpression and/or gene mutations were frequently observed in invasive bladder tumors. On the contrary, over-expression of p53 in relation to grade was significant statistically as shown by Tawfeeq and Al-Talib<sup>26</sup> who reported significant correlation with higher grades while over-expression of p53 in association with grade was not significant statistically as shown by others.<sup>27-29</sup> These contradictory results might be due to sample size and the presence of inter- and intra-individual variations in evaluation of tumor grades in patients with bladder tumors and differences in staining procedure. Mutated p53 is dysfunctional and has a prolonged half-life resulting in the nuclear accumulation of the abnormal protein. Mutations in p53 gene are not the only reason for accumulation of this protein in cancer cells. Kobayashi and Tsukamoto established that p53 could be up regulated by genes, such as jun, myc and other cascade proteins in signal transduction. Studies demonstrated other tumors with non-mutational patterns of TP53 overexpression might be due to mutations in the TP53 that occurred outside exons 5-8, or to the overexpression of TP53 caused not only by TP53 mutations but also by other factors (such as MDM2) which bind to TP53 protein, thus increasing its half-life and allowing it to accumulate in the nucleus. This observation may reflect alterations in the TP53 pathway rather than in the TP53 gene itself.<sup>30,31</sup> Results revealed that p21 over-expression of p53 in relation to stage and grade was significant. On other hand, noted by comparison that 76% of the tumors exhibited abnormal p21 expression, some of which were stages Ta and T1, but the majority were stage T2 or higher. This results came in accordance with Shahrokh *et al.*,<sup>32</sup> who reported that altered expression of p21 is associated with an increased risk of bladder cancer progression and death after adjusting for the effects of pathologic stage, grade, invasion, and lymph node metastases. Alteration of p21 occurred in more than half of pT1 bladder tumors and remained equally frequent throughout all stages. It may be attributed to that progressive cancers accumulate p21 due to impairment of the p21-induced

inhibitory pathway or mutations in the gene, resulting in the overproduction of abnormal genes. Patients with tumors that maintained p21 expression demonstrated a statistically significant decreased rate of tumor recurrence and overall survival in comparison to those whose tumors had lost expression of p21.<sup>33</sup>

## Conclusions

p53 and p21 served as useful biomarker of disease outcome for urological cancers and may help identify patients who might benefit from additional treatments and closer surveillance after cystectomy.



**Figure 2. Immunohistochemical staining of p21. (A) Positive invasive transitional cell carcinoma (TCC), stage T2, high grade, score 4+, section under 10x. (B) Positive non-invasive TCC, stage T1, low grade, score 2+, section under 10x.**

**Table 4. Association between p53 and p21 expression with urinary bladder cancer stages.**

Stages	p53 expression score					p21 expression score				
	Negative N (%)	+1 N (%)	+2 N (%)	+3 N (%)	+4 N (%)	Negative N (%)	+1 N (%)	+2 N (%)	+3 N (%)	+4 N (%)
Non invasive (Ta-T1)	29 (39.8)	2 (2.7)	6 (8.2)	2 (2.7)	1 (1.4)	13 (17.9)	6 (8.2)	7 (9.6)	9 (12.3)	5 (6.8)
Invasive (T2-T3-T4)	2 (2.7)	-	3 (4.1)	15 (20.6)	13 (17.8)	9 (12.3)	-	9 (12.3)	9 (12.3)	6 (8.2)
Total	31 (42.5)	2 (2.7)	9 (12.3)	17 (23.3)	14 (19.2)	22 (30.2)	6 (8.2)	16 (21.9)	18 (24.6)	11 (15.1)
P-value			0.00537*					0.0013*		

\*Significant.

**Table 5. Association between p53 and p21 expression with urinary bladder cancer grade.**

Grade	p53 expression score					p21 expression score				
	Negative N (%)	+1 N (%)	+2 N (%)	+3 N (%)	+4 N (%)	Negative N (%)	+1 N (%)	+2 N (%)	+3 N (%)	+4 N (%)
Low	27 (37)	2 (2.7)	3 (4.1)	1 (1.4)	-	11 (15.1)	6 (8.2)	5 (6.8)	7 (9.5)	4 (5.5)
High	4 (5.5)	-	6 (8.2)	16 (21.9)	14 (19.2)	11 (15.1)	-	11 (15.1)	11 (15.1)	7 (9.6)
Total	31 (42.5)	2 (2.7)	9 (12.3)	17 (23.3)	14 (19.2)	22 (30.2)	6 (8.2)	16 (21.9)	18 (24.6)	11 (15.1)
P-value			0.00627*					0.043*		

\*Significant.

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