A study of p53 and Bcl-2 genes expression and their relation to histopathological variables using DNA in situ hybridization in bladder cancer patients

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دراسة التعبير الجيني للجينين p53 و Bcl-2 وعلاقتها مع المتغيرات النسيجية المرضية باستخدام طريقة التهجين الموضعي في مرضى سرطان المثانة

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المستخلص

سرطان المثانة هو احد اكثر انواع السرطانات شيوعآ بالعالم, حيث يقدر بحوالي 6.5% من بين امراض السرطان, مع الحدوث الاعلى بالدول الصناعية.كان التحري عن علاقة التعبير الجيني لكلا الجينين بأستخدام تقنية التهجين الموضعي للمقاطع النسيجية لمرضى سرطان المثانه و مقارنتها مع المتغيرات النسيجية محور دراستنا في هذا البحث. استخدم التعبير الجيني للجينات p53, bcl-2 كمعلمات لسرطان المثانة باستخدام طريقة التهجين الموضعي. تضمنت الدراسة 50 مقطعاً نسيجياً لأفراد مصابين بسرطان المثانة مقارنة مع 12 نسيجا لافراد مصابين بالتهابات المثانة كمجموعة مقارنة. أوضحت النتائج وجود فروق معنوية عالية بين المجموعتين لكلا الجينين (p=0.001). كما أوضحت الدراسة وجود ارتباط معنوي بين الجينين p53, bcl-2 ودرجة المرض (p=0.003, p=0.005) على التوالي ومع غزو الانسجة المجاورة (p= 0.002, p= 0.007) على التوالي. كما لوحظ ان شدة التعبير الجيني لكلا الجينين تزداد معنويا مع زيادة درجة المرض (p=0.005, p=0.04) على التوالي ومع غزوه للانسجة المجاورة (p = 0.01, p=0.05) على التوالي .اوضحت هذه الدراسة ان الجينات bcl-2, p53 تلعب دورا مهما في عملية تسرطن وتنظيم انسجة المثانة .

Abstract

Bladder carcinoma (BC) is one of the most common cancers worldwide, it accounts for 6.5% of all cancers, with highest incidence in industrialized countries. The investigation of p53 and bcl-2 expression by using in situ hybridization technique on the formalin fixed paraffin embedded sections and studying their relationships with the histopathological variables (type cells, grade, stage, and invasiveness) of bladder cancer was our aim of this work. In this study we have investigated p53 and bcl2 genes expression as tumor markers in BC. In situ hybridization technique was used to quantitate these markers in tumor tissues. A total of 50 bladder cancer tissues were

examined compared with twelve cystitis tissue sections as control group. The results revealed significant differences between p53 and bcl2 overexpression (p=0.001) for both markers. A significant positive correlation between p53 and bcl-2 expression with grade(p=0.003, p=005 respectively) and invasiveness were found (p= 0.002, p= 0.007 respectively), while both gene expression were found to be negatively associated with other histological variables (histological type, stage, gender and age group). The intensity of p53 and bcl-2 overexpression revealed correlation with high grade (p=0.005, p=0.04) respectively, and invasive status (p = 0.01, p=0.05) respectively. In conclusion, this study confirms that p53 and bcl-2 play crucial roles in carcinogenesis process and BC management.

Key word: Bladder cancer, p53, Bcl-2, In situ hybridization.

Introduction

Bladder cancer (BC) represents the seventh most common cause of cancer in men and the twelfth most common in women worldwide, with a much lower incidence in women that reflects an approximate 3:1 male-to-female ratio (1,2) and the second most common carcinoma in males , and the ninth in females in Iraq (3). More than 90% of BC instances represent urothelial carcinomas, which can be subdivided by grade, stage, and subtype (conventional urothelial carcinoma vs a variant morphology) (4). Bladder cancer is a predominant disease of the elderly male, and although most patients are older than 60 yrs of age, BC may affect younger patients. Like most sporadic human tumors, the bladder cancer incidence increases at late age, supporting the idea of a mounting process of accumulating errors at different age levels. Furthermore, there is evidence that older patients have a less favorable prognosis than younger patients (5, 6). Bladder carcinoma is recorded as the commonest malignancy of the urinary tract (7). The neoplastic changes in the urothelium of bladder are a multistep phenomenon; the exact genetic events leading to urothelial transformation involve the activation of oncogenes, inactivation or loss of tumor suppressor genes, and alterations in the apoptotic gene products (8). Cytogenetic and molecular studies have shown the existence of a strong relationship between urothelial carcinoma and alterations involving specific chromosomes 1, 3, 5, 7, 9 and 17 (9). It has been determined that chromosomal loss and inactivation of tumor suppressor genes play a significant role in the development and progression of tumors (10). The tumor suppressor p53 plays a key role in regulating cell cycle progression, apoptosis, or senescence in response to various stress signals, and inactivation of the p53 pathway accounts for the most common molecular defects in human cancers (11).

Alterations of the p53 tumor suppressor gene or changes in its pathway leading to loss of protein function have also been reported to be frequent events in urothelial cancer (12). Bcl-2, discovered in human B-cell lymphoma, is a proto oncogene intrinsically involved in the apoptosis cascade (13) .The most common genetic alteration of BC associated with high-grade is mutations in p53, as being characteristic of the

carcinogenesis pathway for high-grade invasive of BC (14, 15, 16). The relationship and interaction between Bcl-2 and the tumor suppressor gene p53, whose role in bladder cancer behavior is well established, has also been studied, with controversial results. There is some suggestion that tumors may express Bcl-2 and p53 reciprocally (17), and that their interaction carries prognostic significance depending on their respective expressions (18).

Materials and Methods

Patients and tissue samples

The study involved 50 patients attended to AL-Karamaa Teaching Hospital in Wasit Province in Iraq, from 2010 to 2012. This study was carried out in the unit of molecular biology in AL-Karama Teaching Hospital. Full clinical data were obtained including full medical history and complete clinical examination. The patients with bladder carcinoma included 34 males and 16 females with an age ranged from 32 to 80 years. These cases were collected from the laboratory of histopathology in confirmatory histopathological re-evaluation of each obtained tissue blocks was done by a specialist pathologist. Twelve cystitis tissues (as control group) were collected from the hospital as well. Formalin-fixed, paraffin embedded blocks tissue were sectioned (5μm) thickness, from each tissue block. To simplify the statistical analysis, the patients were divided into five age groups, other histopathological variables, including age, sex, staging, grading and histological types were taken in this study.

In Situ Hybridization

Serial tissue sections were cut 5μm thick and were positioned on positive charged slides. The slides were placed in 60˚C oven over night. The tissue sections were deparaffinized; then the slides were dehydrated by graded ethanol concentration (100%, 95%, and 70%) and distilled water. The slides were treated with proteinase K solution and dehydrated after that treated with citric buffer for 15 minute. We then added one drop of the biotinylated long cDNA probe for human p53 and Bcl.2 (Maxim Biotech, USA). The used Hybridization/ detection kit was purchased from Maxim Biotech/USA Cat. Number IH-6001(IHD-0050) was placed on the tissue section in an oven at 98˚C for 8-10 minutes. After that the slides were placed in a humid chamber and incubated over night at 37°C to allow hybridization of the probe with the target nucleic acid. The slides were soaked in 1X detergent wash (protein block) at 37˚C until the cover slips fall, and then treated with streptavidin-AP-conjugate. Then washed by buffer solution. One to two drops of substrate (5-bromo-4-chloro-3-indolyl phosphartel/nitro blue tetrazolium substrate-chromogen solution (BCIP/NBT) conjugate were placed on tissue section at room temperature for about 30 minutes; the latter was monitored by viewing the slides under the microscope. A blue colored precipitate will form at the site of the probe in positive cells. Slides were then counterstained using nuclear fast red (Santa cruz, USA) and sections were mounted with a permanent-mounting medium (DPX, Sakora, Japan). Finally the

examination and scoring were done under light microscope at power 40X according to the scoring system of (19).

Statistical analysis

For all statistical analyses, the SPSS system for personal computer was used, and p values of 0.05 or less were regarded as statistically significant. Sensitivity and specificity of the tests (with 95% exact confidence intervals) were determined in 2 studied groups. Comparison between groups was carried out using Chi-square test. Correlation and Fisher's exact test, Binary Logistics Regression analysis were also performed whenever appropriate.

Results

Results of histopathology

The histopathological features of the studied cases were shown in table (1). Fifty formalin-fixed, paraffin embedded blocks were collected from bladder carcinoma patients. Each carcinoma was distributed according to histologic cell type as transitional cell carcinoma 76% (n=38), and squamous cell carcinoma 24% (n=12). The specimens were graded according to World Health Organization classification (20). As follows: Grade I: well differentiated cell carcinoma of the bladder 14 %(n=7), Grade II: moderately differentiated cell carcinoma of the bladder 40 %(n=20) and Grade III: poorly differentiated cell carcinoma of the bladder 46 %(n=23). And classified according to stage as follows: stage1 28 (n=14) stage2 68 %(n=34), stage3 4% (n=2).68% (n=34) of patients were males while 32% (n=16) of them were females. As well as bladder carcinoma cases were also distributed according to age group, as follows: group1; 4% (n=2), group2; 18 %(n=9), group3; 18 %(n=9), group4; 30 %(n=15), group5; 10 %(n=5). Moreover, muscle invasion was seen in 84% (n=42) cases while non-invasion was seen in 16% 8 cases.

In all sections of control bladder tissues (cystitis), did not express neither p53 nor bcl-2 (Fig 1A, 2A), whereas tumor tissues did demonstrate that the positive expression of both genes in all cases (Fig 1B, 2B), Table (2). The p53 overexpression was reported in 62% (n= 31) out of 50 cases of bladder carcinoma and Bcl-2 overexpression was reported in 28(56%) out of 50 cases of bladder. A Fisher's exact test indicated a highly significant difference between p53 and bcl-2 genes expression in bladder tumors and control bladder tissues (p=0. 0001, p=0.0001) respectively. Table(3) summarized the correlation between p53 and bcl-2 expression with histopathological variables of bladder cancer patients, The χ 2 test was performed for comparing binary responses to see if there was any correlation between p53 and bcl-2.When we Compared results of both p53 and bcl-2 in bladder cancer cases, we found a significant positive correlation among both of p53 and bcl-2 (p= 0.01).This correlation was due to that BC cases displayed both p53 and bcl-2 overexpression compared with control group. Among of overexpression of p53 was obvious, were including 31 cases of TCC, 8 SCC; while Bcl-2 overexpression was obvious in 28, including 20 TCC, 8SCC. There was no statistically significant difference between

p53and bcl2 expressions among different histological types of the tumors ($p > 0.05$).Analysis of overexpression p53 in relation to grade of tumor revealed that positive p53 was reported in 2(6.5%)cases out of grade I, 9(29%) grade II, and 20(64.5%) grade III, with a highly positive correlation between the overexpression of p53 and grade of tumor, i.e., as tumor grade increases more p53 overexpression was noticed ($P < 0.01$).While positive Bcl-2 overexpression was reported in1(3.6%) cases out of grade I, 9(32.1%) grade II, and 18(64.3%) grade III, with a highly positive correlation between the overexpression of Bcl2 and grade of tumor ($p < 0.05$). There was a highly significant difference between overexpression of p53, Bcl2 and muscle invasive ($p < 0.01$). p53 positive overexpression was reported, in 27(96.4%) cases of invasive and1 (3.6%) cases of noninvasive while Bcl-2 positive overexpression was reported in in 27(96.4%) cases of invasive and1 (3.6%) cases of noninvasive. There was no a significant difference between the stage of bladder carcinoma in relation to p53 or Bcl-2 expression ($p > 0.05$). In p53 positive overexpression was reported , in 7(22.6%) cases out of stage I, 22(71%) stage II, and2(6.5%) stage III, with also no correlation between the overexpression of Bcl2 and stage of tumor($p > 0.05$).While Bcl-2 positive overexpression was reported , in 5(17.9%) cases out of stage I, 21(75%) stage II, and 2(7.1%) stage III. Also, there was no significant difference between p53 or Bcl-2 expression with age groups ($P > 0.05$), In p53 positive overexpression was reported , in 2(4%) cases out of group I, 9(18%)cases of group2, 19(38%) cases of group3 , 15(30%)cases of group4 and 5(10%) cases of group5. When we take the effect of all histopathological altogether, our findings showed that a statistically significant relation was found between p53 gene mutation and grade ($p=0.01$) as well as the muscle invasive of the tumor ($p=0.02$) ($p=0.02$), while expression of bcl-2 showed correlation only with tumor grade ($p=0.02$) table 4, no effect of the other variables. We further investigated the relationship of p53 and bcl-2 gene score expression (Intensity) with histopathological features of bladder tumor (Table 5), our results found a significant correlation between p53 score expression and grade ($p=0.005$) and invasive ($p=0.01$), no significant relationship of p53 score expression with increasing pathological stage ($p = 0.962$) nor age ($p = 0.557$), and gender ($p=0.271$) was observed. Regarding bcl-2 score expression, the findings referred to a significant correlation between bcl-2 score overexpression and histological type ($P=0.05$), grade ($p=0.04$) and invasive. ($p=0.05$), There was no relation, however between p53 and bcl-2 and other histopathological variables studied (stage, age and gender) table 5.

Table (1): Histopathological characteristics of the studied 50 patients with bladder cancer

Histopathological Features	Frequency	Percent 100%
Histological type		
SCC	12	24%
TCC	38	76%
Tumor grade		
Grade 1	7	14%
Grade 2	20	40%
Grade 3	23	46%
Tumor stage		
Stage 1	14	28%
Stage 2	34	68%
Stage 3	2	4%
Gender		
Male	34	68%
Female	16	32%
Age group		
31-40	2	4%
41-50	9	18%
51-60	19	38%
61-70	15	30%
71-80	5	10%
Invasiveness		
Invasive	42	84%
Noninvasive	8	16%
Total	50	100%

Table (2): Expression of p53 and bcl-2 in 50 patients of bladder cancer and 12 control group

Type of tissue	P53 expression		Total	Bcl-2 expression		Total
	Positive	Negative		positive	negative	
Control	0(0%)	12(100%)	12(100%)	0(0%)	12(100%)	12(100%)
Patients	31(62%)	19(38%)	50(100%)	28(56%)	22(44%)	50(100%)
P value	0.0001**			0.0001**		

****highly significant p value at 0.0001**

Table (3): Correlation between histopathological variables of the patients and related to p53 and bcl-2 genes expression

Variables	P53 expression		Total	P value	Bcl-2 expression		Total	P value
	Positive	negative			Positive	negative		
Histological type								
TCC	23(74.2)							
SCC	15(78.9)		38(76)		20(71.4)	18(81.8)	38(76)	0.393
	8(25.8)	4(21.1)	12(24)	0.702	8(28.6)	4(18.2)	12(24)	
Tumor grade								
Grade 1	2(6.5)	5(26.3)	7(14)	0.003**	1(3.6)	6(27.3)	7(14)	
Grade 2	9(29)	11(57.9)	20(40)		9(32.1)	11(50)	20(40)	0.005**
Grade 3	20(64.5)	3(15.8)	23(46)		18(64.3)	5(22.7)	23(46)	
Tumor stage								
Stage 1	7(22.6)		14(28)		5(17.9)	9(40.9)	14(28)	
Stage 2	7(36.8)		34(68)	0.335	13(49.1)	34(68)	21(75)	0.113
Stage 3	22(71)	12(63.2)	2(4)		2(7.1)	0(0)	2(4)	
	2(6.5)	0(0)						
Gender								
Males	19(61.3)	15(78.9)	34(68)		16(57.1)	18(81.8)	34(68)	
Females	12(38.7)	4(21.1)	16(32)	0.194	12(42.9)	4(18.2)	16(32)	0.063
Age group								
31-40	2(6.5)	0(0)	2(4)		2(7.1)	0(0)	2(4)	
41-50	5(16.1)	4(21.1)	9(18)		6(21.4)	3(13.6)	9(18)	0.236
51-60	10(32.3)	9(47.4)	19(38)	0.572	7(25)	12(54.5)	19(38)	
61-70	10(32.3)	(26.3)	15(30)		10(35.7)	5(22.7)	15(30)	
71-80	4(12.9)	1(5.3)	5(10)		3(10.7)	2(9.1)	5(10)	
Invasiveness								
Invasive	30(96.8)		42(84)	0.002**	27(96.4)	15(18.5)	42(84)	0.007*
Noninvasive	12(63.2)		8(16)		1(3.6)	7(31.8)	8(16)	
	1(3.2)	7(36.8)						

***Significant p value 0.05** ****Significant p value 0.01**

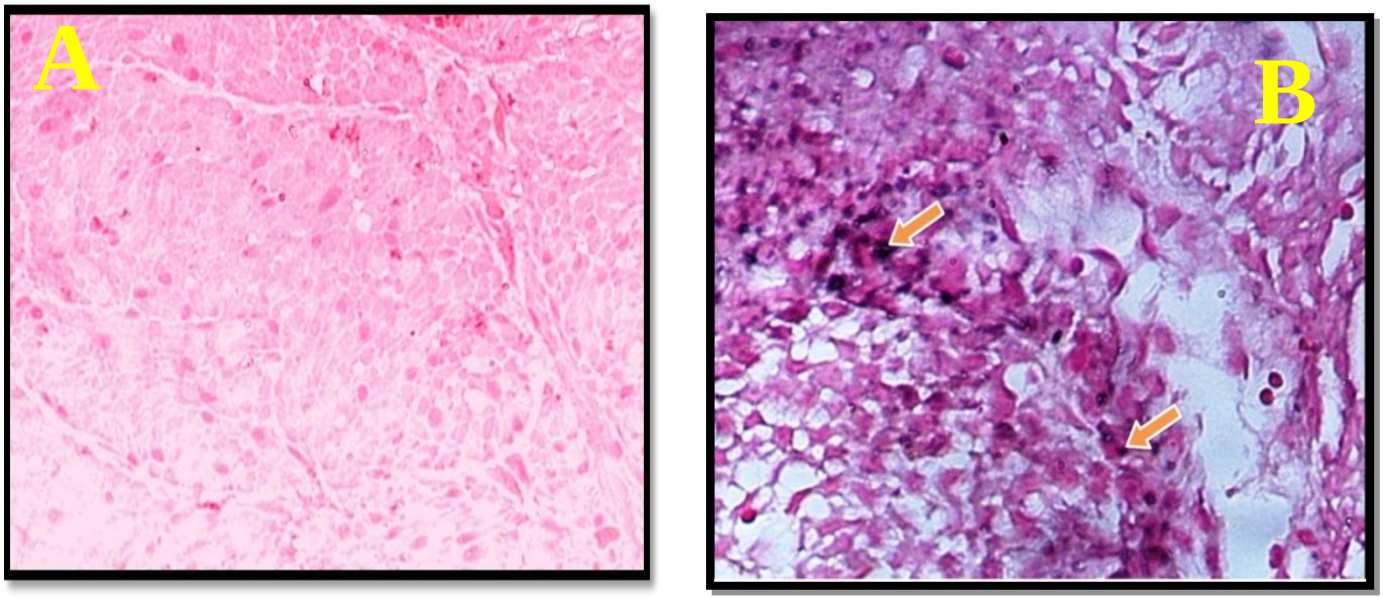

Figure 1: In situ hybridization staining of P53: A- benign section of bladder (cystitis) by nuclear fast red stain 40X. B-Bladder cancer section by nuclear fast red stain 40X

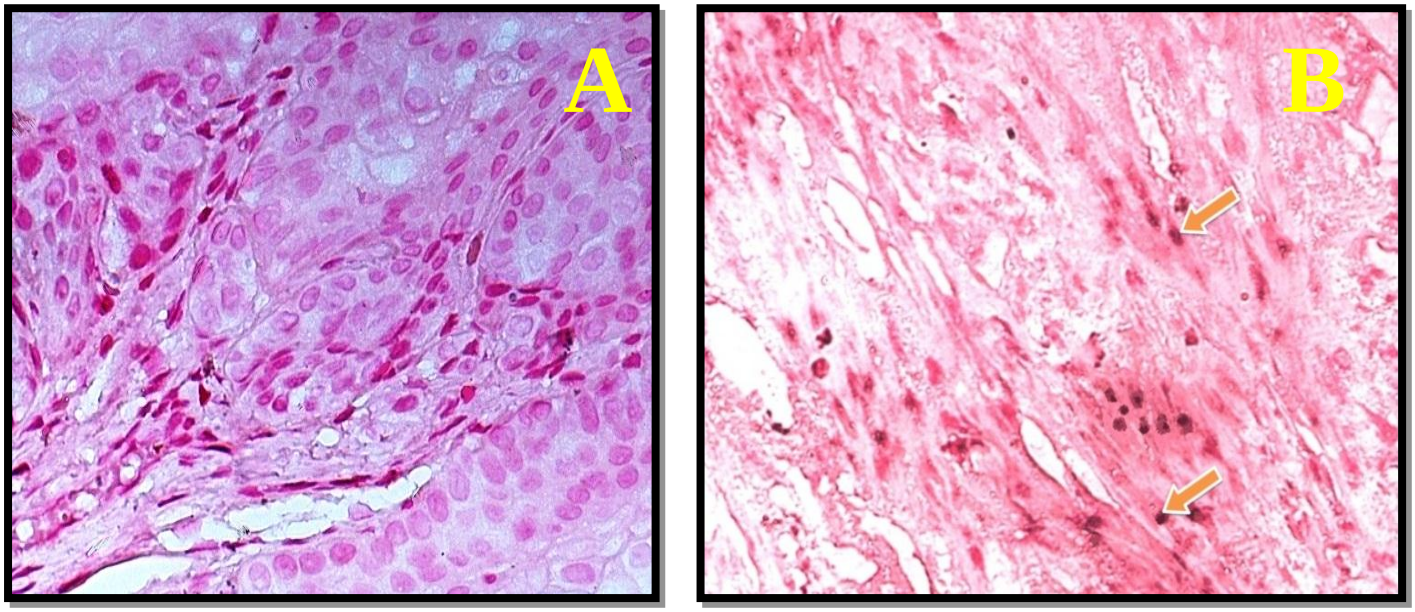

Figure 2: In situ hybridization staining of Bcl-2: A- benign section of bladder (cystitis) by nuclear fast red stain 40X. B-Bladder cancer section by nuclear fast red stain 40X

Table (4):Correlation between p53 and bcl-2 genes expression and histopathological variables of the bladder cancer patients

Histopathological variables	P53 P value	95%CI		Bcl-2 P value	95%CI	
		Lower	Upper		Lower	Upper
Histological type	0.50	0.10	3.08	0.26	0.07	2.03
Tumor grade	0.01*	1.27	14.72	0.o2*	1.17	12.02
Tumor stage	0.18	0.04	1.87	0.87	0.15	5.15
Gender	0.67	0.14	3.48	0.27	o.o9	1.97
Age group	0.66	0.95	1.09	0.40	0.91	1.4
Invasiveness	0.02*	1.52	588.03	0.21	0.33	144.0

***Significant p value at 0.01**

Table (5): Intensity of p53 and bcl-2 expression among histopathological variables of bladder cancer patients

Histopathological Features	score of p53 overexpression					score of Bcl-2 overexpression				
	0	+1	+2	+3	Total	0	+1	+2	+3	Total
Histological type										
TCC	15	5	13	5	38	18	12	8	0	38
SCC	4	4	2	2	12	4	5	1	2	12
p-value	P=0.366				50	P=0.05				50
Tumor grade										
Grade 1	5	0	2	0	7	6	1	0	0	7
Grade 2	11	5	4	0	20	11	7	2	0	20
Grade 3	3	4	9	7	23	5	9	7	2	23
p-value	P=0.005				50	P=0.04				50
Tumor stage										
Stage 1	9	4	1	0	14	9	4	1	0	14
Stage 2	13	12	7	2	34	13	12	7	2	34
Stage 3	0	1	1	0	2	0	1	1	0	2
p-value	P=0.439					P=0.683				50
Gender										
Male	15	5	11	3	34	18	8	7	1	34
Female	4	4	4	4	16	4	9	2	1	16
	P=0.271					P=0.109				
Age group										
31-40	0	1	1	0	2	0	2	0	0	2
41-50	4	4	1	0	9	3	3	3	0	9
51-60	9	3	5	2	19	12	5	1	1	19
61-70	5	1	5	2	15	5	5	4	1	15
71-80	1	0	3	1	5	2	2	1	0	5
p-value	P=0.242				50	P=0.536				50
Invasiveness										
Invasive	12	8	15	7	42	15	16	9	2	42
Noninvasive	7	1	0	0	8	7	1	0	0	8
p-value	P=0.01				50	P=0.05				

Discussion

The distributions of bladder tumors from Wasit Province by type, grade, stage, gender, age, and invasiveness status are shown in table (1). The proportion of TCC more than SCC ,high grade and stage more than low grade and stage ,middle and orderly effect more than young and males more than females with bladder cancer. These results are concordant with other

studies (21,22,23).On the other hand muscle invasive showed vice versa results, when the proportion of invasive muscle(84%) were more than noninvasive (16%)(24), this may be due to the patients diagnosed at late phase of disease. Most previous reports on the expression of p53 and bcl-2 have carried out by multiple molecular genetic technique, Northern blot, dot blot or PCR-based approaches, FISH and immunohistochemical, while just a few involved in situ hybridization ISH technique, which were primarily performed on paraffin embedded tissue sections. Activation of proto-oncogenes and inactivation of tumor suppressor genes are frequently involved in tumorigenesis. Among other things, proto-oncogenes control normal cell growth pathways and cell cycle regulation, proto-oncogenes undergo mutations, rearrangements, insertions, or amplification, and they can activate genes (oncogenes) and result in uncontrolled cellular proliferation (25).Alterations of the p53 are the most common genetic changes detected in human cancers. In the present study, all sections of control bladder tissues (cystitis), did not express neither p53 nor bcl-2 table (2) other results were found p53 and Bcl-2 expressed in less than 1% of normal urothelial cells (26). P53 overexpression was demonstrated in 62%.This result is compatible to those of Al-Kashwan *et al* (27), El-chennawi *et al* (28) , Comperat *et al* (29), Serdar *et al* (30) who detected nuclear overexpression in 58.6% , 66%, 63.9%,and 64%of their cases, respectively. However, it is slightly higher than that reported by Venyo *et al* (31), Abdul-Hameed *et al* (32) and Turk *et al* (33) who reported positive of tumor nuclei in 54%, 50%, and 47.6% of their cases, respectively. The discrepancy in the results of different studies could be attributed to the difference in the type of p53 marker used (34, 35).or due to differences of the genetic pool of human. Given the main reason of the high p53 positive expression in bladder cancers is the formation of dysfunctional mutated p53, therefore the higher the p53 in bladder cancer, the higher the mutated p53 leading to less functional wild p53. P53-mediated apoptosis can be blocked at multiple death checkpoints, by inhibiting p53 activity directly, by Bcl-2 family members regulating mitochondrial function, by E1B 19K blocking caspase-9 activation, and by caspase inhibitors. The same table showed bcl2 positive overexpression 56% in BC patients. Renouf *et al* (2009) (36) and Gross *et al* (1999)(37), identified three of Bcl-2 members that play important roles in the apoptotic response. The Bcl-2 subfamily (e.g., Bcl-2 and Bcl-xL) functions to inhibit apoptosis. The Bcl-2 proto-oncogene encodes an inner mitochondrial membrane protein that blocks programmed cell death leading to initiation cancer cells. In our human bladder tumor tissue study, a high correlation was found between p53 and bcl-2 ISH upregulating, we found a significant positive correlation between p53 and bcl-2 expression ($p= 0.001$). It is of interest that these findings are similar to those previously reported, in which a similar correlation between p53 and bcl-2 status was observed in TCC patients (38).Among histopathological variables, p53 and bcl-2 overexpression was found to be

positively correlated with grade (p=0.003 ,p=0.005) respectively, and invasiveness (p=0.002, p=0.007) respectively. However, in the present study we could not able to show any correlation between neither p53 nor Bcl-2 expression and histological type, tumor stage, age group, and gender as cleared in table 3.These results indicate, in contrast to other studies, that P53 and bcl-2 expression is not restricted to a certain stage of tumor progression and might play a role in different levels of bladder tumorigenesis. Our results were in agreement with several studies (28, 39).We then investigated the effect of pathological variables together on p53 and bcl-2 score expression in bladder carcinoma using binary logistic test table (4), our results demonstrated that there were a strong correlation between the effect of grade and invasiveness together on p53 expression (p=0.01,p=0.02) respectively .On the other hand, only the grade among all histopathological variables had an effect on bcl-2 expression. Exposure to cellular stress can trigger the p53 tumor suppressor, a sequence-specific transcription factor, to induce cell growth arrest or apoptosis. The choice between these cellular responses is influenced by many factors, including the type of cell and stress, and the action of p53 co-activators. P53 stimulates a wide network of signals that act through two major apoptotic pathways. The extrinsic, death receptor pathway triggers the activation of a caspase cascade, and the intrinsic, mitochondrial pathway shifts the balance in the Bcl-2 family towards the pro-apoptotic members, promoting the formation of the apoptosome, and consequently caspase-mediated apoptosis.(40).As shown in table (5), the intensity of p53 expression revealed correlation with tumor grade (P=0.005) and invasiveness (p=0.01).While bcl-2 intensity revealed correlation with histological type (p=0.05) and tumor grade (p=0.04) and invasiveness (p=0.05). Within tumor tissues p53 and bcl-2 expression were elevated in high grade (n=43/50 for p53 and bcl-2), and invasive lesions (42 /50 for p53 and bcl-2). This suggested that p53 and bcl-2 might play an important role in bladder cancer progression. Indeed, several other studies show that p53 and bcl-2 overexpression correlates with grade and invasive bladder carcinoma (26). However, this study found that the intensity of p53 and bcl-2 overexpression revealed no correlation without significant difference with different histopathological variables.

Ethical consent

The study was submitted and approved by the Faculty of the College of Science, University of Wasit in collaboration with al Karamaa Teaching hospital, Wasit, Iraq.

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