The Possible Role of C-Myc and P-53 on the Pathogenesis of Transitional Cell Carcinoma of the Bladder.

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Abstract

Objective: Estimation of the in situ hybridization expression of c-Myc mRNA and immunohistochemistry expression of p53 protein in patients with transitional cell carcinoma of the bladder.

Methods: A technique utilizing in situ hybridization was performed to detect c-Myc mRNA and immunohistochemistry technique was performed to detect p53 proteins using paraffin embedded sections of tissue that were obtained from 60 patients; who were divided into two groups: 30 patients with transitional cell carcinoma of the bladder (group 1) and 30 cystitis patients as a control group(group2). Patient's ages ranged between 25-81 years (14 females and 46 males).

Results: The levels of the in situ expression of c-Myc mRNA and immunohistochemistry expression of p53 were found to be highly significantly increased in group 1 as compared with group 2 ($P<0.001$),with a significant positive correlation between these two parameters ($P<0.05$) in group 1 and 2.

Conclusion: The results of this study indicated that c-Myc gene and p53 protein may play an important role in pathogenesis of bladder cancer and the expression of c-Myc was associated with expression of p53 in patients with bladder cancer.

Key word: Bladder cancer, p53, c-Myc

Introduction:

Transition cell carcinoma of the bladder is the second-most common malignancy of the genitourinary tract and the second-most common cause of death from genitourinary tumors [1,2]. Several genetic mutations have been identified in bladder cancer. Mutations of the tumor suppressor gene for p53, found on chromosome 17, are associated with high-grade bladder cancer and CIS. Mutations of the tumor suppressor gene for *p15* and *p16,* found on chromosome 9, are associated with low-grade and superficial tumors. Retinoblastoma (Rb) tumor suppressor gene mutations are also noted. Bladder cancer is associated with increased expression of the epidermal growth factor gene and the *e*rb-b2 oncogene, and mutations of the oncogenes p21 ras, c-myc, and c-jun [3].

Several genes govern the postcarcinogen exposure pathway taken by the normal cell. All of these interact and regulate each other in a complex and often unclear ways. It is probably fair to say, however, that p53, c-Myc and Bcl-2 have been identified as key gene complexes involved in the regulation of repair, proliferation, and apoptosis and in the initial selection of the response option [4; 5].

The c-Myc gene, encoding a basic helix-loop-helix transcriptional factor, was first identified through its involvement in neoplasia in humans and animals in 1986 [6]. The c-Myc gene, localized to 8q24, may play an important role in cell proliferation and differentiation and may induce apoptosis under certain conditions[7].The c-Myc gene amplification seemed to be associated with tumor progression[8;9] and an over expression of the c-Myc gene protein may be related with high grade bladder cancer [10].

On the other hand, the human tumor suppressor gene p53 maps to chromosome 17p13.1, consists of 11 exons spanning over 20 kb of DNA and encodes for a 393 amino acids, 53kDa nuclear protein [11; 12]. The p53 protein has several biological functions such as involvement in cell cycle regulation, programmed cell death, senescence, differentiation and development, transcription, DNA replication, DNA repair and maintenance of genomic stability [13]. Genetic changes in the p53 gene are found in almost every kind of human cancer [14; 13].

In this study we attempted to investigate the role of c-Myc gene detected by in situ hybridization(ISH) and p53 protein detected by immunohistochemistry(IHC) in tissue of patient with bladder cancer and shed more light on the relation between these two markers in transitional cell carcinoma of the bladder.

Materials & Methods:

Patients:

A total of 60 Iraqi patients with bladder cancer who were admitted to AL-Yarmouk and Baghdad Teaching Hospital. Patients' ages ranged between 25-81 years (14 females and 46 males). According to histological examination, the patients were divided into two groups:

Group 1: (30) patients with transitional cell carcinoma of the bladder (TCC group).

Group 2: (30) patients with cystitis (control group).

Samples:

For each patient and control included in these study serial sections from paraffin embedded block were taken from the archive of department of pathology of these two hospitals. Tissue sections cut into 5µm thickness put on Fisher brand positively charched slides.

In situ hybridization (ISH) for detection of c-Myc gene expression in paraffin embedded sections:

The use of Biotin – Labeled DNA probe for **c-Myc** (8 µg/10015 µl ddH2O). Probe size: 371 bp (Maxim Biotech, Inc., U.S.A).

In situ hybridization (ISH) is a technique that makes use of the high specificity of complementary nucleic acid binding to detect specific DNA or RNA sequence in the cell. For detection of these markers , the biotinylated DNA probe hybridize to the target sequence (c-myc mRNA sequence) then a streptavidin-AP (streptavidin-alkaline phosphatase) Conjugate is applied followed by addition of the substrate promo-chloro – indolyl – phosphatel / nitro-blue tetrazolium (BCIP/NBT) which yield an intense blue – black signal which appears at the directly specific site of the hybridized probe. This strepteividin – AP conjugate like the biotinylated probe provides a rapid and highly sensitive detection method. Hybridization /Detection System will give an intense blue –black color at the specific sites of the hybridization probe in positive test tissues. Evaluation of the in situ staining was done with the assistance of a histopathologist.

A scoring system that includes evaluation of the staining percentage of stained cells was employed for the expression of c-Myc gene. Counting the number of the positive cells in the bladder tissue which gave a blue-black nuclear staining under the light microscope.

The extent of the ISH signaling the cells of the examined tissue was determined in 10 fields under high power microscope (100X). The total staining score was divided by the number of whole cells per field in 10 fields, so the percentage of positively stained cells in the 10 fields was calculated for each case by taking the mean of the percentage of the positively stained cell in the 10 fields. Tissues were regarded as c-Myc positive when their ISH signaling scores were ≥ 5% [15, 16].

Immunohistochemical analysis (IHC) for detection of p53 protein expression in paraffin embedded sections:

The use of universal DakoCytomation streptavidin- biotin system purchased from DakoCytomation (USA) Immunohistochemistry detection kit. The mouse anti-human monoclonal antibodies p53 protein (code No. /M7203) (Denmark).

Mucosal biopsies were immunoassaying with polyclonal antibodies to gastrin and somatostatin by the avidin-biotin complex (DakocCrop, Denmark). The primary antibody reacts with antigen in the tissue, and then a biotin labeled secondary antibody (link antibody) binds to the primary antibody. When the conjugate is added, the biotinylated secondary anti-body will form a complex with the peroxidase-conjugated streptavidin and by adding the substrate, which contains 3, 3 □-diaminobenzidine (DAB) in a chromogen solution, a brown-colored precipitate will form at the antigen site. In the peroxidase secondary detection system, the presence of a brown reaction product at the site of the target antigen is indicative of positive reactivity. Counter stain will be pale to dark blue coloration of the cell nuclei. Evaluation of the immunostaining was done with the assistance of a histopathologist. The observer was blinded to the clinical diagnosis of the tissues at the time of assessment, and tissues were independently assessed by two observers positive or negative cases, positive immunostaining gave nuclear and/or cytoplasmic dark brown granules. Counting the number of positive cells which gave brown cytoplasmic staining system under light microscope. The extent of the IHC signal was determined in 10 fields (X100magnification). In each field the total number of cells was counted and the extent of cytoplasmic staining cells was determined as a percent. The total staining score was divided by the number of whole cells per field in 10 fields, so the percentage of positively stained cells in the 10 fields was calculated for each case by taking the mean of the percentage of the positively stained cell in the 10 fields .Tissue were regarded as p53 positive when their immunoreactivity scores were ≥ 5% [17].

Statistical analysis:

Student test (t-test) was used for the quantitative data. The relationship between the indicators was measured qualitatively by using the correlation coefficient(r). The lowest level of significance was when the probability ($p<0.05$) and the highly significance was ($p<0.01$) [18].

Results:

The c-Myc mRNA in bladder cancer patient (group 1) and cystitis patient (group 2) as control samples were measured by ISH. A statistically significant difference in c-Myc mRNA expression was noted between the patient and control groups **(Table 1**). Higher c-Myc mRNA expressions were found in bladder cancer patients than in controls (the mean percentages, 17.1 versus 4.5; $P < 0.01$). In addition, p53 protein expression were measured by IHC were higher in patients than in controls (the mean percentages, 28.4versus 2.2; $P < 0.01$**) (Table2**).

Table (1): Mean percent of the expression of c-Myc (ISH assay) among studied groups.

Studied groups	No.	Mean± SEM	Comparison of significant *P*-value
Group1	**30**	**17.1 ±1.4**	**(*P*<0.01)***
Group2	**30**	**4.5±0.6**	
Total	**60**		

*= highly significant difference (p<0.01)

Table (2): Mean percent of the expression of p53 protein (IHC assay) among studied groups.

Studied groups	No.	Mean± SEM	*P*-value
Group1	**30**	**28.4±1.5**	**(*P*<0.01)***
Group2	**30**	**2.2±0.8**	
Total	**60**		

*= highly significant difference (p<0.01)

In addition **(table 3)** show the frequency of c-Myc mRNA and p53 protein in patients with transitional cell carcinoma (TCC).

Furthermore, a significant positive correlation (p<0.05) between c-Myc and p53 in group 1 and 2; was observed **(table 4)**.

Table (3): The In situ expression of c-Myc mRNA and immunoexpression p53 protein in patients with transitional cell carcinoma (TCC).

Markers	Marker expression in patients (N=30)			
	Negative		Positive	
	No	%	No	%
c-Myc	2	6.7	28	93.3
P53	6	20	24	80

Table (4): Correlation (r) between c-Myc and p53 in the studied groups.

Variable	Studied groups	Correlation Coefficient r =	*P*-Value
C-Myc and p53	**Group1**	0.386	**< 0.05**
	Group2	0.291	**<0.05**

P<0.05 = a significant difference

The expression of c-Myc mRNA was heterogeneous blue-black nuclear staining in the tissue, as shown in **(figure 1),** whereas the expression of p53 proteins was heterogeneous dark brown nuclear staining in the tissue, as shown in **(figure 2).**

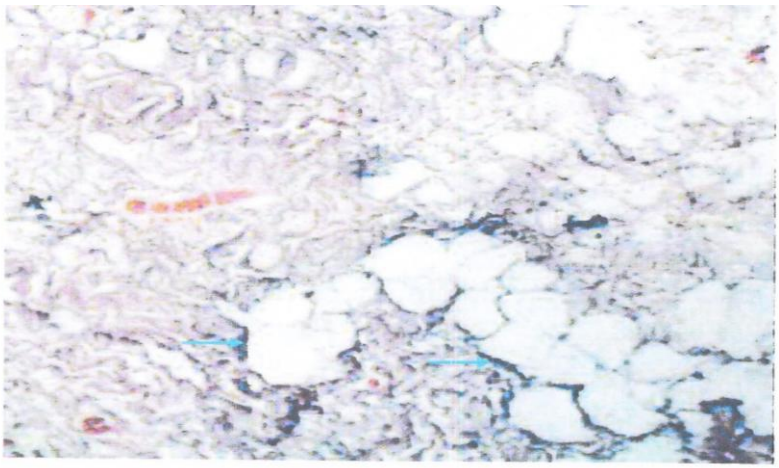

Figure (1): Detection of c-myc in studied groups by in situ hybridization (ISH). Staining of c-myc mRNA by BCIP/NBT (blue-black) counterstained with nuclear fast red. Tissue from bladder cancer patients shows positive c-myc hybridization signals (X400).

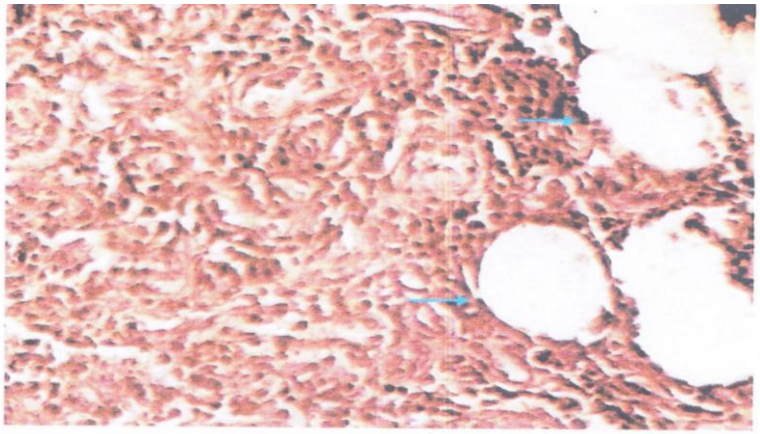

Figure (2):Immunohistochemical staining(IHC) of p53 proteins in studied groups. Staining by DAB chromogen (dark brown) counterstained with nuclear fast red . Tissue from bladder cancer patients shows positive p53 immunostaining (X400).

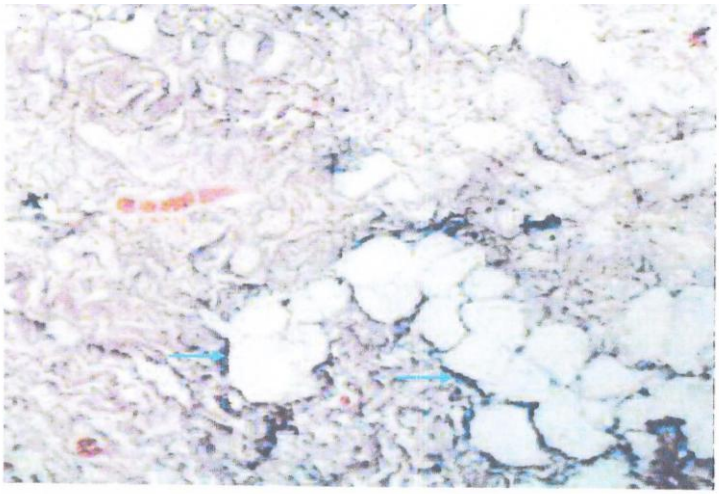

Figure (1): Detection of c-myc in studied groups by in situ hybridization (ISH). Staining of c-myc mRNA by BCIP/NBT (blue-black) counterstained with nuclear fast red. Tissue from bladder cancer patients shows positive c-myc hybridization signals (X400).

Discussion:

Cancer may progress with the accumulation of various genetic abnormalities in a multistep process of carcinogenesis [19; 20]. A number of abnormalities in genes such as K-ras, p53, RB, and c-myc, have been reported [21]**;** in this study we attempt to analyze the possible role of p53 and c-Myc in patients with transitional cell carcinoma of the bladder (TCC).

The results of the present study demonstrated that the in situ expression of c-Myc was significantly higher ($p<0.001$) in bladder cancer patients than in cystitis patients. Evidence supporting this suggestion include the fact that c-Myc oncogene over expression has been shown to be associated with bladder cancer [22; 10]. The proto-oncogene c-Myc encodes a transcription factor that regulates cell proliferation, growth and apoptosis. Dysregulated expression or function of c-Myc is one of the most common abnormalities in human malignancy [23].

Furthermore, the c-Myc oncogene deregulates both cell growth and death checkpoint, and thereby can rapidly accelerate oncogenesis [24; 25].

It is well known, the c-Myc gene family encodes for nuclear phosphoproteins containing DNA-binding activity [24]. The c- Myc oncogene has been shown to be over expressed in several human tumors including bladder cancer [25]**.** Deregulation of the c-Myc gene family occurs with chromosomal translocation and gene amplification**,** [26] **.**Several mechanisms, including gene amplification, point mutations, and chromosomal rearrangements, have been found to activate c- Myc in human tumors. Myc mediates tumor genesis through the activation of genes involved in cell metabolism, proliferation, and apoptosis and through the repression of genes that may promote cellular differentiation and cell cycle arrest [22; 27]**.**

Another study considering that Ras and c-Myc are significantly associated with tumor progression in many types of malignant cells,

nucleophosmin/B23 together with Ras and c-Myc could then be potential molecular targets for therapeutic intervention of cancer development [28]

On the other hand, in our study, we found a highly significant increased expression of p53 protein (p<0.001) in patients with bladder cancer compared with cystitis patients. Based on the previous study that had shown the highly expression of p53 was associated with bladder cancer [29; 10]. Under physiologic conditions in normal cells, p53 protein is expressed at low levels and has a short half-life due to rapid turnover mediated by ubiquitination and proteolysis [30]. The p53 protein becomes stabilized and activated in response to a number of stimuli, including exposure of cells to DNA-damaging agents and oncogene activation. The activation of p53 allows it to carry out its function as a tumor suppressor through cell cycle arrest, apoptosis, DNA repair, differentiation, and antiangiogenesis [31; 32; 33].

In addition, the current study revealed that 93.3% of patients showed positive expression of c-Myc and 80% was positive expression of p53. Previous study by Kadhim and colleagues found that the positive immunostaining of c-Myc and p53 was observed in 56.7% and 53.3 % of patients with bladder cancers, respectively [34].

According, to the current study it a significant positive correlation was found between the expression of c- Myc mRNA and p53 protein in all studied groups. This might indicate that expression of c- Myc mRNA was an association with expression of p53 in patients, previous findings done in agreement with study that showed that c- Myc and p53 genes may be linked to progression of bladder cancer and both polysomy 8 and c- Myc copy gain were significantly correlated with p53 deletions and DNA ploidy [35].

Additionally, the c- Myc and p53 have been reported to act synergistically in the induction of apoptosis [36]. Both c-Myc and p53 serve as critical regulators of the cell cycle and of apoptotic mechanisms in normal and malignant cells [37; 38].

Similarly another report confirmed that abnormal or ectopic over expression of c- Myc in primary cells activates a protective pathway through the induction of p19/p14 and p53-depend cell death pathway .Hence the normal cells that over expression of c- Myc that are eliminated from the host organism through apoptosis thereby protecting the organism from lethal neoplastic change [39].

In conclusion, our data suggest that c- Myc and p53 may play an important role in pathogenesis of bladder cancer and found the expression of c-Myc was associated with expression of p53 in patients with bladder cancer. Therefore, further studies are needed to understand the exact mechanisms of c-Myc action, at the p53 expression in different stages of bladder cancer.

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