THE VALUE OF P53 NUCLEAR PROTIEN EXPRESSION IN PREDICTING RESPONSE TO INTRAVESICAL MITOMYCIN C CHEMOTHERAPY

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Abstract

Background: Alterations of p53 gene are the most common mutations in human cancers. In bladder cancer, p53 mutations have been associated with high tumor grades and advanced stages, as well as progression of superficial disease to muscle invasive disease. Moreover, p53 nuclear over expression appears to be an independent predictor of disease progression and decreased survival after cystectomy.

Objective: The objective of the study is to evaluate the P53 expression percentage in patients with transitional cell carcinoma of the bladder and the relation of this expression to superficial cancer, muscle invasive disease, and carcinoma in situ as far as the tumor grade, clinical stage and response to intravesical mitomycin chemotherapy is concerned.

The expression of P 53 in normal bladder mucosa, taken from patients admitted for ureteral endoscopic procedures, was used as a control group

Method: The expression of p53 protein was studied by immunohistochemical analysis in paraffin embedded specimens from 58 patients with transitional cell carcinoma of the bladder and 20 patients with normal urinary bladder (control group), these patients were admitted for insertion or removal of double J ureteric stents. Patients with superficial tumors (stage T1, Ta) were treated with intravesical chemotherapy with mitomycin C (20-40mg). once weekly for 6 weeks and cystoscopy repeated after 3 months , 6 months and 9 months. Patients with superficial muscle invasive disease were subjected to extended transurethral resection followed by intravesical chemotherapy and cystoscopy repeated at 6 weeks, 3 months and 6 months. No treatment other than MMC was given.

Results: P53 over expression was observed in 29 (50%) out of 58 patients with transitional cell carcinoma of the bladder, with no case of p53 over expression observed in the control group.

A statistically significant relation was noticed between p53 over expression and clinical stage, with p53 over expression was more common in muscle invasive tumors. P53 over expression was also more common in high grade tumors; however, no statistically significant relation between p53 expression and tumor grades and response to intravesical chemotherapy with mitomycin C was noticed.

Conclusions: P53 over expression was noticed in half of the patients with transitional cell carcinomas of the bladder, it was much more common in muscle invasive tumors and more frequent in high grade tumors, however, it seems that p53 status did not predict response to intravesical mitomycin C chemotherapy.

Keywords: Bladder Tumor, p53 over expression, response to Mitomycin C

Introduction

The P53 gene is tumor suppressor gene that acts as a guardian of the genome[1] .

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It's located on chromosome 17p 13.1 and encodes a 53 kilo Dalton nuclear phosphoprotein with DNA binding properties[2]. The P53 protein was first discovered in 1979, and it was classified as tumor antigen at that time[3].however, the realization that P53 is a tumor suppressor gene came in 1989[4]. Wild type P53 protein normally

has short half life and lasts only very briefly in the cell nucleus , where as the mutated form often accumulate for longer times and hence are more easily detected by immunohistochemistry (IHC)[5].

Given the pivotal role that P35 plays in DNA repair, cell cycle arrest and apoptosis, it's not surprising that it is the most commonly mutated gene in human tumors, including genitourinary malignancies, close to 50% of all tumors has P53 mutation [6].

Activation of P53 gene:

The main upstream events that activate P53 include: 1. DNA damage. 2. Hypoxia. 3. Low ribonucleoside triphosphate level.

The exact mechanism by which P53 detects DNA damage and subsequent signal transduction pathways involved are not well defined. However, it appears that DNA strand breaks and DNA repair intermediates activate P53 [8].

Functions of P53 gene:

The functions of P53 are diverse and complex. The main cellular responses following activation of P53 include: 1.Cell cycle regulation.2.DNA repair. 3. Apoptosis [1].

P53 in Urologic Malignancies

Mutational inactivation of P53 is a particularly frequent event in bladder carcinoma and has been associated with high-grade muscle-invasive disease [9]. P53 accumulation in the transitional cell carcinoma cell nuclei, as detected by immunohistochemistry, predicts risk of recurrence and death independent of stage, grade, and lymph node status [10].P53 status in primary tumors may predict not only likelihood of recurrence, but also whether patients will respond to chemotherapy [2]. Mutations in prostate specimens range from 10% to 35% in untreated primary tumors and from 40%

to 50% in hormone refractory metastatic disease [11].Mutations in P53 occur in renal cell carcinoma, particularly metastatic lesions. Patients with abnormal expression in primary tumors may have an increased risk of developing metastasis [12]. Penile carcinoma has not been conclusively linked to P53 mutations, even in advanced lesions [13].

P53 relevance in the management of bladder cancer.

Loss of P53 function confers genomic instability, impaired apoptosis, and diminished cell cycle restraint. Alteration of P53 is most common mutation in human cancer, roughly half of all human malignancies, including many urological cancers, exhibit P53 mutations. In bladder cancer, P53 mutation has been associated with higher tumor grade, and advanced stage, as well as progression of superficial disease to muscle invasive. Moreover, P53 nuclear over expression appears to be an independent predictor of disease progression and decreased survival after cystectomy [1].

1. *Superficial disease:* In general, P53 mutations and resultant protein over expression seen on immunostaining in superficial bladder cancer. are much less common than in muscle invasive disease, however, when present, P53 mutations in superficial disease has been correlated with increased grade, recurrence, progression and decreased survival[1].

2. *Muscle invasive disease:* The vast majority of the studies have been demonstrated that P53 mutations and nuclear over expression to be more common among patients with muscle invasive bladder cancer compared to those with superficial disease [1].

P53 over expression was associated

with an increased risk of recurrence and decreased overall survival 20.In some study, P53 status was the only independent predictor of survival in case of organ confined disease at cystectomy [14].

3. *Carcinoma in situ:* A significant proportion of patients with carcinoma in situ have P53 mutations and nuclear over expression on IHC. Moreover, P53 mutations antedated the development of stage T2 or T3 disease by a mean of 8 months [1].

4. P53 status and response to BCG: Definitive conclusions for P53 status predicting response to BCG are not currently available.[1] still; it seems that P53 nuclear over expression after BCG therapy is an ominous harbinger of disease progression.15. P53 status shows promise in the selection of patients after BCG failure for early cystectomy versus repeats intravesical therapy [16].

5. *P53 status and response to radiation:* Theoretically P53 could modify tumor response to radiation through regulation of cell cycle kinetics and apoptosis. Loss of P53 function may increase radiosensitivity via impaired P53 dependent DNA repair processes, whereas over expression by up-regulation has been suggested to confer radio resistance. Overall, P53 status does not seem to predict response to radiation reliably [1].

6. *P53 status and response to chemotherapy:* P53 function may also influence tumor response to chemotherapy via regulation of the cell cycle and apoptosis, however, data regarding P53 status as a predictor of response of bladder cancer to chemotherapy is contradictory, and appears to depend on the specific mechanism of action of the chemotherapeutic agent as well as tumor type.

Initially P53 was thought to confer chemoresistance in bladder cancer via impaired apoptosis as seen in breast, colon, and hematological malignancies. In contrast, studies by other investigators support the concept that P53 mutations may confer a chemosenstive phenotype [1].

Detection of P53 Mutations:

P53 mutations can be detected by: 1.Single strand conformation polymorphism. 2. Direct DNA sequencing. 3. IHC [6].

P53 genetic mutations most commonly result in nuclear over expression of the P53 protein and positive immunostaining.

The P53 protein normally has a short half-life within the cell due to degradation via ubiquitin mediated pathways. However, the half-life is markedly prolonged after missense mutation (by far the most common genetic change of P53), although deletions can cause the same effect. Mutations in the DNA binding region (missense) account for 80% of point mutations and subsequently lead to the greatest decrease in P53 function. Missense mutations of P53 lead to accumulation of protein and nuclear over expression as demonstrated by various IHC techniques. It is crucial to understand that "positive" immunostaining implies missense mutation of the P53 gene with prolonged half-life and nuclear accumulation of the mutant P53 protein.

It is also important to appreciate that up to 20% of P53 mutations are deletions or none sense mutations that result in negative immunostaining despite complete absence of the P53 protein. They are also specific instances in which P53 is over expressed in the absence of mutation, resulting in

"positive" immunostaining without genetic alterations.

Nonetheless, P53 genetic mutations most commonly result in nuclear over expression of the P53 protein and positive immunostaining [17].

IHC analysis has many inherent limitations: 1. Variations in technique. 2. Use of diverse antibodies. 3. Tumor heterogeneity. 4. None standardization of values used to define positive staining. All these factors contribute to the unreliability of results based purely on immunostaining. Nevertheless, a strong correlation exists between P53 mutations and positive IHC for the P53 nuclear protein [1].

A significant advantage of IHC over DNA sequencing is that IHC is commonly used for the assessment of other antigens as tumor markers in many pathology laboratories. Furthermore, identification of P53 nuclear accumulation in the tumor cells in the absence of gene mutation has been noted, indicating alternative disruption of this pathway [18].

P53 autoantibodies: Tumor specific P53 autoantibodies have been found in the serum of patients with a variety of malignancies on immunoprecipitation, immunofluorescence, western blot test and enzyme-linked immunosorbent assay, however, Even among patients with known P53 mutations only 20% to 40% will have development of P53 autoantibodies. Although further investigation is clearly warranted, it is suggested that P53 autoantibodies may have a predictive role in bladder cancer survival [1].

Patients, Materials, and Method:

This study is a case-control study conducted at AL-KADIMYA teaching hospital from September 2003 to December 2004 (16 months). 58 patients with transitional cell carcinoma (TCC) of the bladder were included, the mean age of the patients was 58 years (range, 23_ 79 years), 43 patients are male and 15 are females (male / female = 2.8 / 1).

Patients:

Patients with bladder tumors were subjected to: 1.Cystoscopy. 2. Transurethral resection of any visible growth. 3. Bimanual examination under anesthesia before and after tumor resection. Tumor specimens obtained at time of resection were divided into two halves, one half subjected to histopathological examinations and the other half spared for IHC studies. In every case, experienced pathologist examined the tumor.

Patients involved in the study met the following eligibility criteria:
1. The primary tumors were histologically confirmed TCC.
2. Tumor specimens were large enough for histopathological examination and IHC studies.
3. Patients with history of any form of previous treatment, were excluded from the study

The patients were properly staged according to the TNM staging system developed by the American joint committee on cancer, 1997.

Patients with superficial tumors (stage T1, Ta) were treated with intravesical chemotherapy with mitomycin C (20-40mg). once weekly for 6 weeks and cystoscopy repeated after 3 months , 6 months and 9 months. Patients with superficial muscle invasive disease were subjected to extended transurethral resection followed by intravesical chemotherapy and cystoscopy repeated at 6 weeks, 3 months and 6 months.

Control

In the current study, P53 alterations had been studied also in 20 healthy

young adults admitted to cystoscopy for ureteric stent removal or insertion. All these patients were healthy young adults with no history of malignancy, chemotherapy or radiotherapy. The procedure had been explained to these patients and a proper informed consent obtained. Multiple biopsies had been taken from healthy looking bladder mucosa from the posterior wall of the bladder.

Out of 58 patients, 34 had superficial tumors and 24 had muscle invasive tumors. In the former group, the male/female ratio was 27/7, while in the later group it was 16/8. The histological grade showed G1.G2 and G3. 11patients showed G1, 14 patient's shows G2 and10patients showed G3 in the superficial group, while in the muscle invasive group it was 1, 10 and 12 respectively. The clinical staging showed 10 cases with Ta and 24 cases inT2 in the superficial tumor group, while in the muscle invasive group it showed 14 cases with T2,6 cases with T3 and 4 cases withT4 respectively. Twenty six patients received chemotherapy, 8 patients (31%) had recurrence and 18(69%) showed response to chemotherapy.

Table 1: Patients Characteristics.

	Superficial Tumors	Muscle invasive Tumors	Total
No.	34	24	58
Male/ female	27/ 7	16/8	43/15
Histological grade			
G1	11	1	12
G2	14	10	24
G3	10	12	22
Clinical stage			
	Ta 10	T2 14	-
	T1 24	T3 6	-
		T4 4	-
Patients receiving chemotherapy	26	–	26
Recurrence after chemotherapy	8(31%)	–	–
Response to chemotherapy	18(69%)	–	–

Materials:

Antigens in the sections are detected by two stages process, the binding of the primary antibody to a specific antigen, and the subsequent detection of this binding by a colorimetric reaction. The section incubated with: 1.Primary antibody: anti P53 (JC70A mouse IgG1, Kappa, Dako, Denmark). 2. Biotinylated secondary antibody. 3. Diaminobenzidine (DAB) chromogen. 4. Streptovidin horse redishes peroxidase.
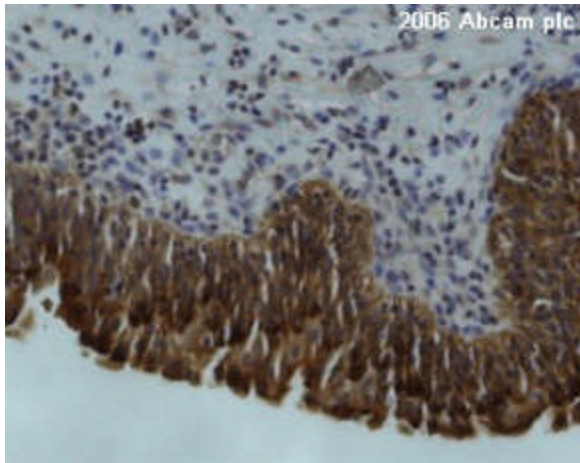
Method:

Immunohistochemical analysis:

  A standard labeled streptavidin-biotin technique was used for IHC staining.

 Four um sections were cut and deparaffinized. Antigenicity were retrieved by microwave treatment in citrate buffer ph = 6.0. The sections were incubated overnight at 4c with 1/100 dilution of anti p53 monoclonal antibody. The incubation was followed by sequential 10-min. incubation with biotinylated-linked antibody and peroxidase-labeled streptovidin. The final incubation used diaminobenzidine (DAB) as the chromogen. Both positive and negative control was includ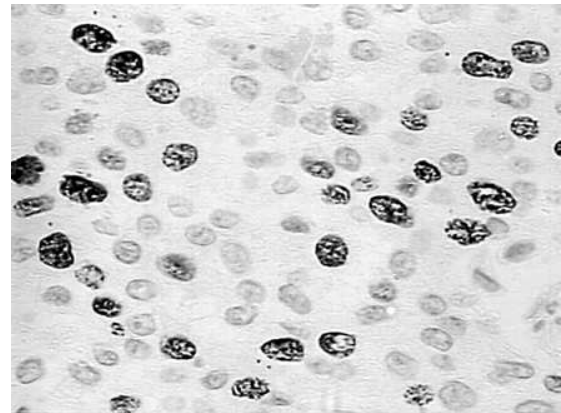ed for each run of IHC: a-The negative control was obtained by replacing the primary antibody with a buffer, phosphate buffered saline (PBS). b-A known positive section was used as a positive control.

Evaluation of immunostaining:

  The staining results were evaluated in a blinded manner without knowledge of the clinical data by two independent researchers. The cutoff point for P53 positivity was established at > 5% cells with nuclear staining. The nuclear intensity of P53 was classified as negative and positive, positive results included weak, moderate and strong staining.

Figure1: P53 Immunohistochemical staining x 400

Figure 2: Staining of human urothelium (bladder cancer) tissue sections by IHC (Formalin-fixed paraffin-embedded sections).

Statistical analysis

Statistical analyses were performed using Chi-square test (X2). All P-values less than 0.05 reflected statistically significant differences. Statistics could not be applied in table 8 because one of the values was zero.

Results

The immunohistochemical expression of P53 was observed in 29 (50%) out of 58 patients with TCC of the bladder, however, no case of P53 over expression observed in the IHC of the normal looking bladder mucosa (control group).

A statistically significant difference was noticed between P53 expression and clinical stage, $P < 0.025$ (Table 2), P53 positivity was particularly observed in stage T2-4 invasive TCC of the bladder.

Table 2: P53 Immunoreactivity in Relation to Tumor Types.

Types of Tumor	No. Patients	No.+ve P53 Cases	Percentage
Superficial Tumors	34	11	32.3%
Muscle Invasive Tumors	24	18	75%

$X^2 = 8.6$, $P < 0.005$

P53 over expression was more frequent in grade 3 (68%) than in grade 2 (41%) and in grade 1 (25%), however, no statistically significant difference was noticed between P53 over expression and tumor grades, $P > 0.05$ (Table 3).

Table 3: P53 Immunoreactivity in Relation to Tumor Grades.

Grade	No. Patients	No.P53 + ve	Percentage
I	12	4	25%
II	24	10	41%
III	22	15	68%
Total No.	58	29	50%

$X^2 = 4.91$, $P > 0.05$.

Patients with superficial tumors who received intravesical mitomycin C chemotherapy, 31% showed recurrence within 9 months, while 69% showed no recurrence (Table 1).
Among those who were P53 positive, 44% developed recurrence, compared to 24% in those with p53 negative expression (Table 4), and no statistically significant relation between intravesical mitomycin C chemotherapy and P53 expression was noticed, $P > 0.05$ (Table 4).

Table 4: Patients Outcome Following Intravesical Chemotherapy

Clinical Stage	P53 + ve (No. = 9)		P53 – ve (No. = 17)		Total
	Response	Recurrence	Response	Recurrence	
Ta	1	1	3	1	6
T1	4	3	10	3	20
Total	5	4	13	4	26

$X^2 = 0.923$, $P > 0.05$

In the current study, eight Patients with T2a disease had been treated with extended transurethral resection, intravesical mitomycin C chemotherapy, and close follow up. Among p53 negative patients, two of three respond to this mode of therapy, while all patients (five) with p53 positive expression failed treatment and showed recurrence.(Table 5),and two of them progressed to stage T2b.

Table 5. Outcome of Intravesical Chemotherapy in Patients with T2a Disease

Stage	P53 + ve (No. = 5)		P53 – ve (No. = 3)	
	Response	Recurrence	Response	Recurrence
T2a (no. = 8)	_	5	2	1

Statistics cannot be applied.

Discussion

Different studies have indicated that alterations in the cell cycle regulation are a key event in determining the biological behavior of bladder cancer.[3] an extensive body of literature regarding P53 had accumulated during the last two decades [1].

Incidence of P53 over expression in TCC of the bladder:

In the current study, 50% of patients with TCC showed P53 over expression. Although the sample size is small, similar results had been observed in other studies, Toyoaki et al, 2001, retrospectively studied 119 patients with TCC and showed that P53 was over expressed in 61 % .[19]In Iraq, Al-Qaysi, 2002, showed that P53 over expressed in 23(57.5%) out of 40 bladder cancer patients[20] .

P53 over expression and clinical stage:

In the current study, P53 over expression showed strong statistical relation with tumor stage, P53 over

expression was seen in 25% of superficial tumors (T1, Ta) and 75% of muscle invasive tumors (T2-T4). (Table 2, 6).

Toyoaki et al, 2001, showed statistically significant difference between P53 over expression and tumor stage (P=0.0209), P53 positivity was particularly observed in stage T2-T4 invasive type TCC of the bladder (P=0.0089). [19] Investigators from the international study initiative on bladder cancer analyzed data sets from 25 different centers in an attempt to examine more clearly and definitely the association of P53 mutations in superficial versus muscle invasive disease. Their preliminary report on 1706 patients used a cut off value of 23%, only 25% of patients with superficial tumors had positive P53

immunostaining compared to 48% of those with muscle invasive tumors [21].

It seems that the incidence of p53 over expression in muscle invasive tumors was higher in this study compared to other studies; this difference may be due to 1. Different cut off values used for P53 over expression, for example, in Toyoaki et al study, the cut off value was 10%, while in the current study, it was 5%.2. Variation in intensity scoring, for example, in our study tumors specimens showing weak intensity staining were considered positive while in Toyoaki et al study was considered negative.3.Non uniform methodology for p53 staining, ranging from use of different antibodies to application of dissimilar technique for enhancing epitope expression.

Table 6: P53 Immunoreactivity in Relation to Clinical Stage.

Stage	No. Patients	No. P53+ve	Percentage
Ta	10	2	20%
T1	24	9	37.5%
T2	14	10	71.4%
T3	6	5	83.3%
T4	4	3	75%
Total No.	58	29	50%

$X^2 = 11.34$, p < 0.025

P53 status and tumor grade:

In the current study, P53 over expression was more frequent in high grade tumors, being 68% in grade 3, and only 25% in grade 1 tumors and 41% in grade 2 tumors. However, no statistically significant relation between P53 over expression and tumor grade was obtained (Table 3).

These results were in agreement with Toyoaki et al study which showed no statistically significant relation between grades and P53 over expression,

with more frequent expression of P53 in grade 3 tumors (72%) compared to 54% in grade 2 tumors and 58% in grade 1 tumors [19]. However AL-Qaysi, 2002, showed significant correlation (p<0.05) between P53 over expression and tumor grade [20].

P53 status and response to intravesical mitomycin C chemotherapy:

In the current study, 26 patients with stage T1and Ta disease treated with intravesical mitomycin C chemotherapy,

among P53 positive cases ,4 of 9 patients failed chemotherapy(44%), compared to 4 of 17 patients in p53 negative group(24%),and no statistically significant relation obtained between p53 expression and intravesical mitomycin C chemotherapy ,P > 0.05.(Table 4).

To the best of our knowledge, no study has reported on the P53 over expression and intravesical chemotherapy. Kawasaki et al showed in vitro abrogation of apoptosis induced by mitomycin C or cisplatin in bladder cell lines with P53 mutations, thus P53 mutations may confer resistance to chemotherapy via impaired apoptosis .[2] On the other hand, Kielb et al suggested that paclitaxel may be more effective against cells with P53 mutations as the mechanism of action requires functionally mutated P53 to induce cell death in human bladder cells.[23] So, data regarding P53 status as a predictor of response of bladder cancer to chemotherapy is contradictory [1].

Caliskan et al followed 30 patients with stage Ta and T1 disease subsequently treated with BCG, among P53 positive cases, 5 of 6 failed BCG and progressed to muscle invasion or metastasis, compared to only 6 of 24 that were P53 negative.[24] Pages et al evaluated 43 patients with stage T1 tumors and found that P53 status before treatment with BCG could not define a group of BCG non responders.[25] So definitive conclusions for P53 status predicting response to BCG are not currently available[1] .

P53 status and T2a disease:

In the current study, eight patients with T2a disease had been treated with transurethral resection, intravesical mitomycin C chemotherapy, and close follow up. All p53 positive cases (five cases) failed this mode of therapy and two of these cases progressed to T2b disease, while among p53 negative cases, two out of three patients respond and showed no recurrence.(Table 5).This may suggest that patients with T2a disease and negative p53 expression may respond to bladder sparing approach ,however two points should be taken in considerations 1.The short time of the follow up (only 6 months),so patients who had respond to this modality may develop recurrence in the future.2.The small number of the patients(only eight patients).

Herr et al suggested that patients with stage T2 bladder cancer and negative P53 status may be watched if responsive to methotrexate, vinblastine, doxorubicin and cisplatin (MVAC) therapy, while those with stage T3 disease or T2 tumors with positive P53 immunostaining should proceed directly to cystectomy. In their study, P53 status was the only independent predictor of survival in cases of organ confined disease at cystectomy. Patients with T3b or greater pathological stage carried a poor prognosis regardless of P53 status.[24] In contrast; Tiguert et al were unable to correlate P53 mutations in patients with muscle invasive bladder cancer treated with cystectomy to cancer specific survival. Furthermore, P53 status has not been shown to predict disease free survival in patients with nodes positive disease, [26] Cote and Chatterjee reported that the only patients with bladder cancer to benefit from adjuvant chemotherapy were those with P53 altered tumors [28]. On the other hand, Qureshi et al found that P53 status did not predict response to cisplatin based chemotherapy and failed to predict overall cancer specific survival in their series of patients with

stage T2-4 disease[27]. so data regarding p53 status as a predictor of response of bladder cancer to chemotherapy is contradictory and appears to depend on the specific mechanism of action of the chemotherapeutic agent as well as tumor type. The final conclusion of this work showed that Close to 50% of TCC of the bladder showed P53 over expression which was more common among patients with muscle invasive bladder TCC compared to those with superficial disease, and it was more frequent in high grade tumors (G3), but no statistical relationship between P53 expression and tumor grade had been noticed. P53 status did not predict response to intravesical mitomycin C chemotherapy. Patients with muscle invasive disease and positive p53 immunostianing may need more aggressive intervention than those with negative p53 expression.

Furthers studies with a larger size sample are suggested taking the following points into consideration:
1. Using a uniform methodology for p53 staining, using similar antibodies and similar technique for enhancing antigen expression.
2. Adherence to a generally accepted cut- off value and staining intensity.
3. Study the effect of p53 over expression on response to different chemotherapeutic agents and to other modalities of therapy like radiotherapy and cystectomy.
4. Extending the time of the study for longer period.
5. Study the impact of p53 over expression on bladder sparing procedure in muscle invasive T2 disease.
6. Study the correlation between p53 expression and other types of bladder tumors like seqamous cell carcinomas and adenocarcinomas.

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