RESEARCH COMMUNICATION

TP53 - Molecular Soldier's Mutations in Bladder Cancer in the Kashmiri Population

Mahoor S Nanda[1,2], Zaffar A Shah[2], A Syed Sameer[2], Nidda Syeed[2], Imtiyaz Murtaza[1], Mushtaq A Siddiqi[2], Arif Ali[1*]

Abstract

Purpose: We made a preliminary attempt to study mutations in exons 5-8 (the DNA binding domain) of the tumor suppressor gene *TP53*, in urinary bladder cancer patients from Kashmir. Further the relation of clinicopathological characteristics with mutation status was asessed. Materials and Methods: The study population consisted of 60 patients diagnosed with transitional cell carcinomas who underwent transurethral resection and /or radical cystectomy. Mutations in 5-8 exons of *TP53* gene were detected by means of single strand conformation polymorphism (SSCP). All samples which showed different migration bands in SSCP were confirmed by DNA sequencing. Results: 19 of 60 (31.6%) bladder cancers had mutations of the *TP53* gene (11 transitions and 8 transversions), three were G→A transitions, two G→T transversions, three A→C transversions, five C→T transitions and six A→T transversions. Predominantly missense mutations (66%) were detected but no deletions or insertions were found. Statistically significant associations (<0.05) were noted with higher tumor stage (T2 or higher), recurrence and large tumor size (>3cm). No correlation was found between smoking and tumor grade and the presence of *TP53* mutations. Conclusions: Mutation of the *TP53* gene is one of the commonest genetic changes in human bladder cancer, also in a high risk ethnic Kashmiri population.

Keywords: *TP53* mutations - SSCP - bladder cancer - Kashmir

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Introduction

Bladder cancer is the fourth most incident cancer in males and ninth most incidents in females. Over 67,000 new cases are diagnosed per year in the United States (Crawford James, 2008) and over 350,000 cases are diagnosed globally (Ferlay et al., 2008). By a rate ratio of at least 3:1, men have a higher risk of bladder cancer than women. Urothelium cancers account for 5.6% of males and 1.8% of female cancers in India with actual crude rate (ACR) incidence of about 1 in 174 and 1 in 561 women (Kamarana et al., 2000).

As per a epidemiological survey by Dhar et al in leading hospital of Kashmir, bladder cancer in Kashmir has an annual incidence of 9.66 (2.46%) ranking 9th in all types of cancers (Dhar et al., 1993). This was a partial presentation of bladder cancer incidence as the survey was done in a single hospital catering the needs of 1/3 of the population.

Approximately 66% of bladder cancers are diagnosed among individuals of age 65 years or older. Transitional cell carcinoma (TCC) is the most common type of bladder cancer, accounting for 90% of all cases (Crawford James, 2008), most classified into superficial (pTa and pT1) and muscle invasive (pT2) (Knowles, 1998).

The incidence of bladder cancer was strongly associated with occupational exposure to aromatic amines used in the dye industry, before their potent carcinogenicity to the bladder was demonstrated (Clavel, 2007) .With reduction of such workplace exposure, active smoking is now the strongest environmental risk for bladder cancer, contributing to more than 50% of cases (Ferlay et al., 2008). Recent modest reductions in the incidence of bladder cancer are attributed to decreasing exposure to tobacco smoking and to occupational carcinogens. Although diet might also influence bladder carcinogenesis, owing to the many potential carcinogens or chemo preventive nutrients therein (Tang et al., 2008), no consistent association between intake of selected nutrients or micronutrients and bladder cancer has emerged.

Mutations of the *TP53* gene are the most frequent somatic genetic abnormalities detected in human malignant disease and are more common in urinary bladder cancer (Hainaut and Hollstein, 2000; Dalbagni et al., 2001)

TP53 mutation is one of the most universal genetic abnormalities observed in bladder cancer, which results in increased half-life and accumulation of nonfunctioning *TP53* nuclear protein (Sidransky and Messing, 1992; Thomas et al., 1994). The *TP53* gene and protein statuses

*[1]Department of Biotechnology, Jamia Millia Islamia, Jamia Nagar, New Delhi, [2]Department of Immunology and Molecular Medicine, Sher-I-Kashmir Institute of Medical Sciences, Soura, Srinagar, India *For correspondence : mousvi786@gmail.com*

both play a critical role in the regulation of the normal cell cycle, cell cycle arrest, and apoptotic response (Hartwell and Kastan, 1994; Spruck et al., 1994; Levine, 1997). Alterations in the *TP53* protein, leading to a loss of its tumor suppressor function, have been reported previously (Hollstein et al., 1991; Sarkis et al., 1993; Esrig et al., 1994). It has been revealed that *TP53* mutations are found primarily in high-grade and invasive bladder tumors, contributing about 35–72% of muscle-invasive bladder tumors (Prescott et al., 2001; Hartmann et al., 2002; Lu et al., 2002). Wild-type *TP53* protein has a short half-life; however, the protein encoded by mutated *TP53* remains active for a long period. Mutated *TP53* gene is a common genetic abnormality in transitional cell carcinoma (TCC) of the bladder (Esrig et al., 1994). Earlier studies have depicted that over expression of *TP53* occurs in higher stages and grades of TCC (Esrig et al., 1993; 1994).

Materials and Methods

Patient Specimens

Sixty consecutive patients who underwent TURBT in surgery department at the Sher-i-kashmir institute of Medical Sciences 2007-2009 were entered into this study. All of the patients signed written informed consent. The patient group included 10 women and 50 men with ages ranging from 36 to 80 years. Diagnostic slides were reviewed by a panel of two expert pathologists to confirm diagnosis and ensure uniformity of classification criteria. All the samples resected by TURBT by urological surgeon were confirmed to be histologically bladder cancers. 3-5 ml venous blood from each patient was collected in EDTA to serve as controls for this study. The study protocol was approved by the Research Ethics Committee of Sher-I-Kashmir Institute of Medical Sciences.

DNA isolation

Tumor samples (both tumor and adjacent normal) collected after TURBT were snap-frozen immediately and stored at -70°C. DNA from neoplastic tissue was extracted using DNA extraction kit (Qiagen, USA) according to enclosed protocol.

PCR-SSCP analysis

The exons 5, 6, 7 and 8 of *TP53* coding for DNA binding domain, were amplified using four specific oligonucleotide primers (Table 1). PCR was performed in a 25µl total volume reaction mixture containing 50ng of genomic DNA, 100ng of each primer, 100µM of each dNTP, 1.5mM MgCl2, 1X of Taq buffer and 0.1 unit of Taq DNA polymerase (Biotools, Spain). PCR was performed using the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C, annealing at 52-62°C and extension at 72°C, for 1 min each and final extension at 72°C for 7 min in Biorad icycler. In every instance, negative (DNA was replaced with water) controls were amplified by PCR and included in the experiment. The PCR products were run on 2% agarose gel and analyzed under UV illuminator.

The SSCP analysis of the amplicons of exons 5, 6, 7, and 8 was performed on 6% non-denaturing polyacrylamide gel (PAGE) utilizing either non-radioactive silver staining or radioactive procedures (Orita et al., 1989; Bassam et al., 1991).

Table 1. Primers Used for Screening Different Exons of TP53

Amplicon	Primer	Annealing temperature
Exon 5	F: TGTTCACTTGTGCCCTGACT	55 °C
	R: AGCAATCAGTGAGGAATCAG	
Exon 6	F: TGGTTGCCCAGGGTCCCCAG	62 °C
	R: TGGAGGGCCACTGACAACCA	
Exon 7	F: CTTGCCACAGGTCTCCCCAA	62 °C
	R: AGGGGTCAGCGGCAAGCAGA	
Exon 8	F: TCCTGAGTAGTGGTAATCTA	58 °C
	R: GCTTGCTTACCTCGCTTAGT	

F, Sense primer; R, Antisense primer

Sequencing

Purified PCR products of the samples showing mobility shift on SSCP analysis and randomly chosen normal samples were used for direct DNA sequencing using Automated DNA sequencer ABI prism 310. To minimize the sequencing artifacts by PCR, products from at least two different PCRs were sequenced using forward and reverse primers with Big Dye terminator cycle sequencing ready reaction mix (Applied Biosystems) based on fluorescence labeled dideoxy nucleotides as chain terminators. Purified single-stranded extension products were then resolved on ABI Prism 310, DNA sequencer (see Figures 1 to 3).

Statistical Analysis

All statistical analysis was performed using SPSS

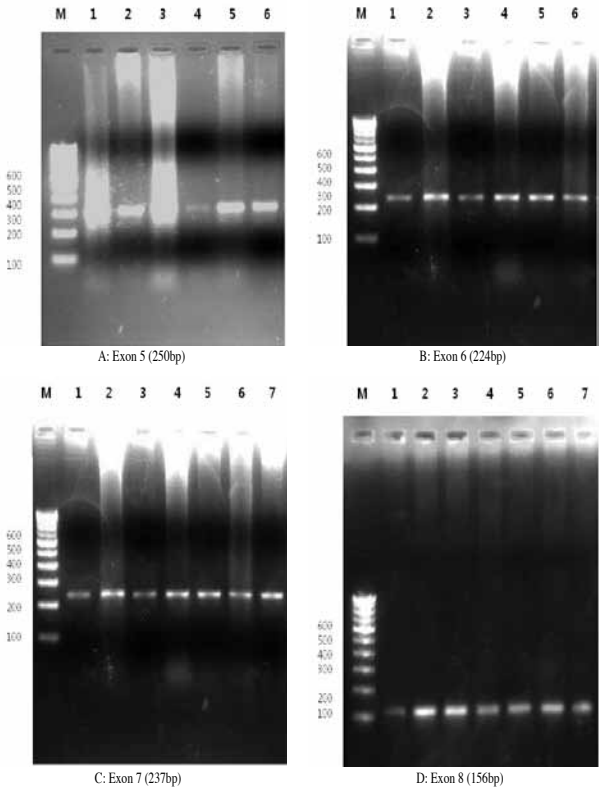

Figure 1. PCR Amplification of Different Exons of the TP53 Gene

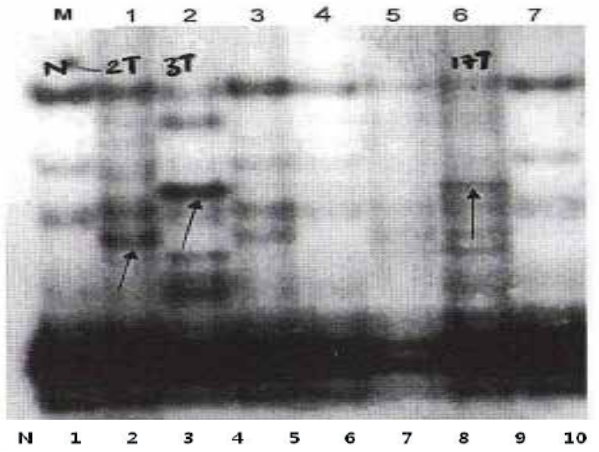

Figure 2. SSCP (Radio-active) Analysis of Exon 5 of p53 Showing Mobility Shift Compared to the Controls

software, version 12 (SPSS, Chicago, IL). Pearson's test for equal distribution was used to determine associations of the presence of *TP53* with various clinico-pathologic parameters. Statistical significance was considered when $p<0.05$.

Results

This study comprised 60 patients and equal number of controls, with a distribution of 50 males and 10 females. The mean age of the patients was 50 years (range, 36 to 80 years), and 63.3% of them were older than 50 years (Table 2). Fifty (83.4 %) patients were men and 45 (75%) among them were smokers. Almost all the patients had attended the hospital with a clinical presentation of haematuria. There were 21 grade-2, 21 grade-3, 18 grade-4. In total 39 (65%) were pTa/pT1, 21 (35%) were pT2. There were 35 (58.3%) superficial and 25 (41.7%) muscle invasive. We screened 60 confirmed transitional cell carcinoma samples for mutations in *TP53* by single-strand conformation polymorphism (SSCP) analysis to screen for mutations in exons 5-8 of followed by direct sequencing of the samples showing mobility shift in SSCP. The overall mutations of our study in exon 5-8 of *TP53* identified in bladder cancer were 19 0f 60 cases (31.6%).

Table 2. Clinico-epidemiological Variables of Bladder Cancer Patients Versus the Mutant Phenotypes of the TP53 Gene

Variable	Total N =60 (%)	Mutants M (n = 18*) (%)	P value
Sex	Males:50 (83.4)	14/50 (28.0)	>0.05
	Females:10 (16.6)	4/10 (40.0)	
Age	≤50: 22 (36.7)	7/22 (31.8)	>0.05
	>50: 38 (63.3)	11/38 (28.9)	
Dwelling	Rural: 48 (80)	14/48 (29.1)	>0.05
	Urban: 12 (20)	4/12 (33.3)	
Smoking status	Smokers: 45 (75)	14/45 (31.1)	>0.05
	Nonsmokers: 15 (25)	4/15 (26.7)	
Differentiation grade	II: 21 (35)	4/21 (19.0)	>0.05
	III: 21 (35)	7/21 (33.3)	
	IV:18 (30)	7/18 (38.8)	
Histological type	S:35 (58.3)	4/35 (11.4)	<0.01
	MI:25 (41.7)	14/25 (56.0)	
Site[e]	RPL:22 (36.6)	7/22 (31.8)	>0.05
	LRL:24 (40)	5/24 (20.8)	
	BN:7 (11.7)	3/7 (42.8)	
	O:7 (11.7)	3/7 (42.8)	
Size	≤ 3cm: 26 (43.3)	4/26 (15.4)	<0.05
	> 3cm: 34 (56.7)	14/34 (41.2)	
Lymph node status	NO:55 (91.7)	15/55 (27.3)	>0.05
	YES:5 (8.3)	3/5 (60.0)	
Status	NR:47 (78.3)	11/47 (23.4)	<0.05
	R:13 (21.7)	7/13 (53.8)	
Stage	PTa/PT1:39 (65)	6/39 (15.4)	<0.01
	PT2:21 (35)	12/21 (47.6)	

*one patient had double mutations; MI, muscle invasive; S, superficial; LRL, left posterior lateral; RPL, right posterior lateral; O, orifide; BN, bladder neck; NR, non-recurrent; R, recurrent

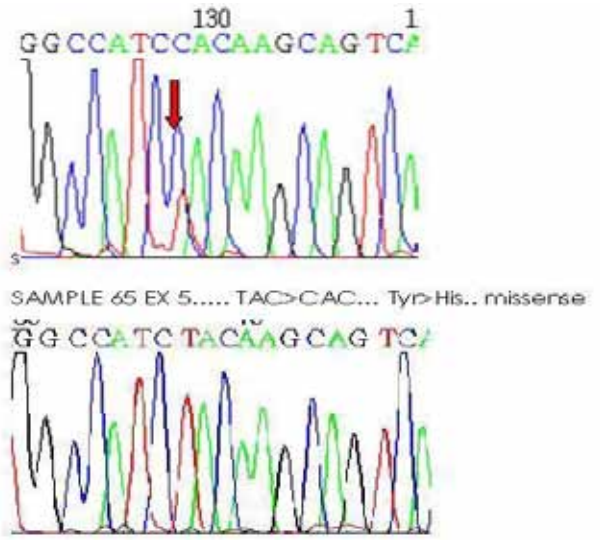

Figure 3. Partial Electropherograms Representing the Normal (Lower Panel) and Mutant (Upper Panel) of Exon 5

Analysis of the mutation spectrum of *TP53* included 19 mutations and significant amount of mutations were found in exon 5(16.6%), exon 6 (27.7%), exon 7 (16.6%) and exon 8 (38.9%) respectively. One sample had a double mutation in exon 8 in codon 283 and 284.

In all there were nineteen mutations (11 transitions and 8 transversions), three were G→A transitions, two G→T transversions, , three A→C transversions, five C→T transitions and six A→T transversions. There were also a significantly high percentage of missense mutations (57.8%), nonsense mutation (10.52%) and silent mutations (31.6%). (16.6%) of the *TP53* mutation were detected in patients at hotspot codon 245 but no mutations were detected at other hotspot codon 175,273,248,196 and 282. A non sense mutation at codon 280 in bladder cancer (Arg > Stop) was found in two samples. The patients when compared for the presence of *TP53* mutation with studied histological type showed a statistically significant association ($p<0.01$) with the incidence of *TP53* mutations in muscle invasive rather than superficial cancer. A significantly higher frequency of *TP53* mutations was seen in higher stage in bladder cancers ($p<0.01$). A significant association ($p<0.05$) was seen in the incidence of *TP53* mutations in bigger size tumors >3cm (as compared to <3cm) and recurrences of the tumor. The investigation did not show significant association with age, sex, dwelling,

Table 3. Clinicoepidemiological Variables of Bladder Cancer Patients Versus the Mutant Phenotypes of the TP53 Gene

Age/ Sex	Rural/ Urbn	Smoking Status	Grade	Stage	Lymph node status	Histopatho-logical type	Site	Status	Size (cm)	Exon	Codon number	Base change	Amino acid change	Effect
81/F	R	NS	II	PTa/PT1	No	S	RPL	NR	<3	8	292	AAA>AAT	Lys>Asn	MS
60/M	U	S	III	PT2	No	MI	O	R	>3	5	163	TAC>CAC	Tyr>His	MS
45/M	R	S	IV	PT2	Yes	MI	RPL	NR	>3	6	199	GGA>GGC	Gly>Gly	S
38/M	R	S	III	PT2	No	MI	LRL	NR	>3	8	283	CGC>CGT	Arg>Arg	S
											284	ACA>ACT	Thr>Thr	S
57/F	R	S	IV	PT2	No	MI	BN	R	>3	8	280	AGA>TGA	Arg>Stop	NS
69/M	R	S	III	PT2	No	MI	LRL	R	>3	7	245	GGC>AGC	Gly>Ser	MS
68/F	R	NS	IV	PT2	Yes	MI	RPL	NR	>3	6	202	CGT>CTT	Arg>leu	MS
51/M	R	S	IV	PT2	No	MI	RPL	R	>3	8	292	AAA>AAT	Lys>Asn	MS
57/M	U	S	IV	PT2	No	MI	O	NR	>3	7	245	GGC>AGC	Gly>Ser	MS
71/M	R	S	III	PT2	No	MI	LRL	NR	>3	8	292	AAA>AAT	Lys>Asn	MS
45/M	U	S	III	PT2	No	MI	BN	R	>3	6	199	GGA>GGC	Gly>Gly	S
46/M	R	S	II	PTa/PT1	No	S	LRL	NR	<3	5	163	TAC>CAC	Tyr>His	MS
41/M	R	NS	II	PTa/PT1	No	S	BN	NR	<3	6	199	GGA>GGC	Gly>Gly	S
48/M	R	S	IV	PT2	No	MI	RPL	NR	<3	7	245	GGC>AGC	Gly>Ser	MS
52/M	R	S	III	PTa/PT1	No	MI	O	NR	>3	5	163	TAC>CAC	Tyr>His	MS
38/M	U	NS	II	PTa/PT1	No	S	RPL	R	>3	6	202	CGT>CTT	Arg>Leu	MS
55/F	R	S	III	PTa/PT1	No	MI	RPL	NR	>3	8	283	CGC>CGT	Arg>Arg	S
62/M	R	S	IV	PT2	No	MI	LRL	R	>3	8	280	AGA>TGA	Arg>Stop	NS

MI, muscle invasive; S, superficial; LRL, left posterior lateral; RPL, right posterior lateral; O, orifide; BN, bladder neck; NR, non-recurrent; R, recurrent

differentiation grade, smoking status and lymph node status (see Table 3).

Discussion

The focus of our study was to determine the incidence of mutations in the *TP53* gene in patients with bladder TCC analyzing the characteristics of the mutations, their locations and importance with respect to various clinicopathological characteristics in Kashmir population. Our aim was also to look for the existence of aggregated mutations in certain regions of *TP53* gene with respect to the TCC.

Previous studies of *TP53* gene mutations in bladder cancers have revealed mutation frequencies in the range from 15 to 60% (Sidransky et al., 1991; Berggren et al., 2001). The diverse frequencies are to some extent determined by variations in the tumor stage and high grade but much higher number of mutations is found in tumors of high stage and grade (Fujimoto et al., 1992; Spruck et al., 1994). The results for mutation frequency in the present group (31.6%) are similar to those described for high-grade, invasive urinary bladder cancers (Sidransky et al., 1991).

The *TP53* gene is frequently altered in UCC, 270 different mutations have been registered in the IARC database26 up to October 2006, of which 262 (97%) are in exons 4 to 9. The most common mutations are missence (72.5%), 12.2% and 5.55% being nonsense and silent, respectively. The main hotspots are codons 285, 248, 280, 175 and 213. There were also a significantly high percentage of missense mutations (61.1%), nonsense mutation (11.1%) and silent mutations (22.2%). (16.6%) of the *TP53* mutation were detected in patients at hotspot codons 245, but no mutations were detected at other hotspot codons.

All of *TP53* gene mutations in our study were point mutations and most caused amino acid substitutions in the *TP53* although silent and nonsense mutations were also observed. These findings are consistent with those of other studies in which point mutations accounted for 95% of the mutation detected (Xu et al., 1997), and were the type most frequently found in bladder cancer (Levine, 1997). Our investigation could not trace any deletion or insertion in the *TP53* gene in all TCC samples .

Mutations in the *TP53* gene are usually located in functionally important regions that have been highly conserved over the evolution of the species (Van der Poel et al., 1998; Gen et al., 2001). These regions are located in exon 5-8 (codon 126-306).In our series exon 8 showed the largest number of mutations, a finding consistent with other articles (Abdel-Fattah et al., 1998; Lorenzo-Romero et al., 2003; 2004). In our study out of 100% of mutations in exon 5-8, 16.6% were found in mutational hot points.

Significant amount of mutations were found in exon 5 (16.6%), exon 6 (27.7%), exon 7 (16.6%) and exon 8 (38.9%) respectively. (16.6%) of the *TP53* mutations were detected at hotspot codons 245 , but no mutations were detected at other hotspot codon 196,175, 248, 273, and 282. A non sense mutation at codon 280 in bladder cancer (Arg > Stop) was found in one sample. The most commonly mutated codons in our study were 280, 283, 163 and 245: these findings support the notion that codon 280 is a hotspot in bladder cancer because it is mutated in 4% of bladder tumors versus 1% of tumors from all sites (Spruck et al., 1993; Sigal and Rotter, 2000).We also got a double silent mutations, one sample with two mutations in exon 8 in codon 283 and 284.

In human urinary bladder cancers, no specific bp substitution pattern for the p53 gene has hitherto been described, and there has been no pointer to any specific mutagen (Greenblatt et al., 1994; Xu et al., 1997;

Hainaut et al., 1998). In this study 4 mutations (26.3%) were G: C > A: T transitions which could more possibly be due to endogenous formation of urinary N-nitroso compounds that leads to O6-alkylguanine formation and G: C > A:T transitions (Warren et al., 1995). The prevalence of exogenous mutations is not surprising as cigarette smoking and occupational exposure to aryl amines are thought to account for more than half of all cases of bladder cancer, with smoking being the more important risk factor. It is also suggested that there is a certain pattern of mutation in smoking patients (Spruck et al., 1993; Bernardini et al., 2001). But on contrary our findings were statistically insignificant (>0.05) in relation to smoking where 31% mutations were seen in smokers as compared to non smokers harboring mutations in 27% subjects. Interestingly in our study we found in only one case G >T transversions at codon 202 suggesting that a tobacco carcinogen may be responsible for this mutation (Denissenko et al., 1996).

Mutation of *TP53* is found in mostly muscle invasive bladder cancers (Sidransky et al., 1991; Fujimoto et al., 1992; Habuchi et al., 1993; Spruck et al., 1993 ;Williamson et al., 1994). As many mutations increase the half-life of the protein, detection of high levels of protein by immunohistochemistry provides a useful surrogate marker for mutation (Esrig et al., 1993) and has been used extensively to measure *TP53* alteration. p53 accumulation has been associated with adverse prognosis in all types of TCC (Sarkis et al., 1993; Esrig et al., 1994; Sarkis et al., 1993; 1994).Our study has shown a significant association (P<0.001) of *TP53* mutation incidences(56.6%) in patients with muscle invasion as compared to superficial bladder cancer (11.4%) depicting the *TP53* as a worst prognostic factor in concordance with the above data. Tumor stage and grade are considered to be significant factors in disease progression to muscle invasive bladder cancer (Heney et al., 1983; Kaubisch et al., 1991). Of superficial carcinomas, Ta, tumors (confined to the bladder epithelium) have a progression rate of 3%, and T1 tumors (invading the lamina propria) have a rate of 25% during the 2 years after diagnosis. About 50% of muscle invasive tumors (T2-T4) progress and metastasize (Anderstrom et al., 1980; Cutler et al., 1982). In previous studies, mutations of p53 have been linked with high-stage and high-grade bladder tumors, whereas the lowest frequencies were observed in noninvasive, low-grade tumors (Kaubisch et al., 1991; Sidransky et al., 1991; Esrig et al., 1993; Fujimoto et al., 1992). In our study, the p53 mutations appeared to be equally common in low-grade and high grade bladder cancers, but the prevalence of p53 mutations was greatest in stage T2 or higher stage tumors with a mutation rate of 47% as compared to15% in lower stages (pTa/pT1). These data are consistent with many recent immunohistochemical and molecular analyses, which suggest that there is no clear association between p53 nuclear over expression and either stage or grade of the primary tumor (SchmitzDrager et al., 1994; Harney et al., 1995; Serth et al., 1995). Low incidences of p53 mutations in Ta/T1 possibly suggests that p53 mutations in superficial cancer are related to a more aggressive phenotype and a high risk of recurrence.

Mutations in our study were randomly detected throughout the various grades presenting with around 19%, 33%, and 38% *TP53* mutations in grade-I, grade-II and grade-III respectively and thus had no association with a particular grading.

There is evidence that mutations often correlate with the incidence of recurrent neoplasm (Sachs et al., 2000; Friedrich et al., 2001; Shigyo et al., 2001). In our series a significant association with recurrence of tumor was found with *TP53* mutations in accordance with the published data. Moreover recurrent tumors in our series presented with bigger size lesions and majority of tumors had size >3cm which showed a significant association (<0.05) with the incidence of *TP53* mutations. This implicates the aggressive nature of tumors harboring *TP53* mutations

In conclusion, in conclusion the frequency of *TP53* gene mutations in patients with urinary bladder carcinoma from the Kashmir is comparatively same as that shown in reports from other countries. Mutations of the *TP53* gene in this study are detected mainly in the advanced stages of the histopathological and clinical development of the disease. The high frequency of *TP53* gene mutations implicates *TP53* as a predominant factor for bladder cancer in high risk ethnic Kashmiri population.

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