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Association of *p53 codon 248* (*exon7*) with urinary bladder cancer risk in the North Indian population

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Abstract **p53 is the most frequently mutated gene in all forms of human cancer. It responds to diverse stresses including UVR-induced DNA damage and regulates many downstream genes to initiate cell-cycle arrest, DNA repair or apoptosis. p53 gene variants at *codon 11*, *Pro47Ser* and *codon 248* (*exon 7*) were evaluated for bladder cancer (BC) risk in North Indians. In the present study, the above encoding regions in p53 genes were analyzed in a hospital based study in 200 BC and 200 healthy controls age and gender matched and of similar ethnicity. The genotyping was assessed by the polymerase chain reaction restriction fragment length polymorphism technique and statistically evaluated using SPSS software ver. 15.0. A significant association was found with *p53 codon 248* polymorphism and BC risk whereas *p53 codon 11* and *p53 Pro47Ser* polymorphism showed no association with BC risk. The individuals carrying the heterozygous genotype (Arg/Trp-Arg/Gln) in the *p53 codon 248* polymorphism showed high BC risk ($p < 0.001$). Combinations with heterozygous and variant genotypes also showed a high risk for BC ($p < 0.001$). The minor allele (Trp/Gln) carriers of the *p53 codon 248* demonstrated a 1.7-fold risk for BC. Furthermore, haplotype analysis revealed that the Glu-Pro-Trp/Gln haplotype is associated with a 1.9-fold risk for BC. A protective role was observed with tumor stage/grade of BC patients with *p53 codon 248* ($p = 0.003$; OR = 0.32). Thus, it is evident from our study that of all the 3 single nucleotide polymorphisms evaluated, only *p53 codon 248* (*exon7*) gene polymorphism has an implication for risk in BC in the North Indian population.**

Keywords: Bladder cancer, p53, gene polymorphism, BCG, haplotype

1. Introduction

Bladder cancer (BC) is one of the most common forms of urogenital cancer. Globally the prevalence of BC is estimated at over 1 million and is steadily increasing (*1*). Despite its low incidence as compared to western countries, it still continues to be a major problem in India. The occurrence rate of BC is three times more common in men than in women (*2*). More than 90% of all newly diagnosed BC patients are transitional cell carcinomas.

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p53 is the most frequently mutated gene in all forms of human cancer (*3*), it is a gatekeeper or guardian of the cell division and plays a critical role in cell cycle control and apoptosis (*4*). It is located on chromosome 17p13. Several other functions of p53 in tumor suppression have been discovered that are independent of its ability to transactivate gene expression. These include direct effects on survival proteins in the mitochondria (*5,6*). The ability of p53 to eliminate excess, damaged or infected cells by apoptosis is vital for the proper regulation of cell proliferation in multi-cellular organisms. The latter activity is crucial for tumor suppression and therefore it is reasonable to analyze the p53 status in human cancer.

Somatic mutations in p53 are found in more than 50% of human cancers (*7,8*). Alterations in the p53

gene are the most frequently documented somatic alterations in human BC, as well as other tumor types, and detection of this altered gene holds prognostic significance.

Several studies have reported on association of p53 polymorphisms with increased risk for various cancers such as esophageal (*9*), gastric (*10*), lung carcinoma (*11*) and colorectal cancer (*12*), respectively. Although the possibility that polymorphic variants in p53 might contribute to cancer susceptibility has been extensively investigated, the issue remains highly complicated. As the genetic nature of BC is complex, individual polymorphisms are likely to have a modest effect on risk. Mounting evidence indicates that, besides environmental factors, genetic components and gene-gene and gene-environment interactions also play important roles in BC development (*13,14*). Considering the complexity of bladder carcinogenesis, single-factor studies may not have sufficient power to detect small genetic effects on cancer risk (*15*). It is also plausible that examining several polymorphisms within biologically relevant pathways may reveal subgroups of individuals who are at a significantly elevated risk for this disease.

Hence the rationale of our study was to investigate the effect of 3 single nucleotide polymorphisms (SNPs) in loss of heterozygosity (LOH) sites of p53 in *codon 11, Pro47Ser* and *codon 248* (*exon 7*) for BC risk. These mutations affect normal protein functions and *codon 248* was characterized to acquire novel oncogenic gain of function and contribute to tumor malignancy and chemoresistance (*16*). Further, modulation by lifestyle habits such as smoking in a hospital based case-control study in the North Indian population was also determined.

2. Materials and Methods

2.1. *Study subjects*

BC patients for the study were acquired from an on-going case-control study of BC, which started patient recruitment in 2008. All patients (200) (mean age 58.5 years; 175 men and 25 women) were incident cases of histologically confirmed invasive or superficial BC recruited from the Urology Department at Sanjay Gandhi Postgraduate Institute of Medical Sciences, from May 2008 to June 2010. BC patients with a previous history of other cancer, cancer metastasized to the bladder from another origin, or previous radiotherapy treatments were excluded. Healthy and genetically unrelated individuals visiting the hospital for a routine checkup or health awareness camps and hospital employees were recruited as controls (n = 200). All the controls were age and sex matched (56.8 years, and M/F ratio 179/21) with similar ethnicity and had no evidence of malignancy or chronic disease.

2.2. *Epidemiology data collection*

An epidemiologic questionnaire was designed for study participants to collect data on demographic characteristics such as smoking, occupation, and other lifestyle factors involved. Informed consent was obtained from all subjects when interviewing for demographic details and a blood sample collection. The Ethical Review Board of the Institute approved the study. The response rate from the interview was 95% for the subjects. Individuals who smoked once a day for more than 5 years were defined as smokers. The individuals who had never smoked in their lifetime were regarded as non-smokers. At the conclusion of the interview, a 5 mL blood sample was drawn into coded vials.

2.3. *Clinical data collection*

The demographic and clinical characteristics of the patients are presented in Table 1. The clinical information about tumor size, number, stage and tumor grade, intravesical therapy and dates of recurrence, chemotherapy, radical cystectomy and pathological findings at cystectomy were provided by the uro-oncologist in our department. The classification of tumor stages were from the American Joint Committee on Cancer's TNM staging system (*17*). Of the 200 total

Table 1. Demographical details of BC patients and healthy controls

Variable	Cases (n = 200) n (%)	Controls (n = 200) n (%)	p value*
Sex			
Female	25 (12.5)	21 (10.5)	0.531
Male	175 (87.5)	179 (89.5)	
Age (years)			
Mean age ± S.D.	58.5 ± 12.4	56. 8 ± 10.8	0.117
Smoking**			
Non-smokers	47 (30.1)	155 (77.5)	< 0.001
Smokers	109 (69.9)	45 (22.5)	
Tumor number**			
Single	115 (60.8)	–	–
Multiple	74 (39.2)	–	
Tumor size (cm)**			
< 1	35 (24.3)	–	–
1-3	73 (50.7)	–	
> 3	36 (25.0)	–	
Stage			
Ta	64 (32.0)	–	–
T1	85 (42.5)	–	
T2	51 (25.5)	–	
Grade			
G1	67 (33.5)	–	–
G2	43 (21.5)	–	
G3	90 (45.0)	–	
Intravesical therapy			
Non-treated	71 (47.7)	–	–
BCG Induction (BCG i + m)	78 (52.3)	–	
Event			
Recurrence	65 (43.9)	–	–
Non-recurrence	84 (56.1)	–	

* Student's t-test was used to determine the p value; ** The sum could not add up to the total due to some missing values.

patients enrolled in the study, 149/200 (74.5%) patients had non-muscle invasive BC (NMIBC) while the rest 51/200 (25.5%) had muscle invasive BC (MIBC). Patients with NMIBC at high risk (high grade, multiple, and large tumors) were treated with intravesical *Bacillus* Calmette-Guerin (BCG) (n = 78). The patients with NMI cancer of low risk (low grade and single small tumor) were kept on cystoscopic surveillance and considered as non-BCG patients. Subsequently, all the patients were examined by cystoscopy every 3 months in the first and second years and later at six monthly intervals as long as there was no tumor recurrence. BCG treatment consisted of 6 weekly instillation induction BCG (n = 78). Since the number of patients receiving maintenance BCG was too low, we did not categorize the patients according to BCG regime for statistical analysis. The end point of the study included tumor recurrence, defined as a newly found bladder tumor following a previous negative follow-up cystoscopy, or end of the study time. Patients with invasive BC (n = 51) were treated with radical cystectomy with or without adjuvant chemotherapy, which included cisplatin, and gemcitabine followed by periodical cystoscopy. A blood sample was collected in EDTA from all subjects for genotyping at the time of enrollment and stored at −70ºC.

2.4. *Genotyping*

Genomic DNA was extracted from peripheral blood lymphocytes by the salting out method (*18*). Polymorphisms in p53 (*codon 11, Pro47Ser* and *codon 248*) genes were analyzed using the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method. Details of the primers and PCR conditions for *p53 codon 11* and *codon 248* have been previously described (*19*) and for *p53 Pro47Ser* (*20*). Genotyping was done on 10% polyacrylamide gel using molecular weight markers and visualized after staining with ethidium bromide. Positive and negative controls were used in each genotyping assay, and 10% of the samples were randomly selected and run in duplicate with 100% agreement. The results were reproducible with no discrepancies in genotyping.

2.5. *Statistical analysis*

The study power was calculated using Quanto software, version 1.0 (available from: *http://hydra.usc.edu/gxe*) with input of the following variables: case-control study design, significance level (alpha) > 0.05 (Two sided), model of inheritance was log additive, allele frequency was 0.28, and the genetic effect for odds ratio (OR) was 1.5 or greater. The present study achieved 80% of the statistical power. The goodness-of-fit chi square test was used to analyze any deviation from the Hardy-Weinberg equilibrium in controls. A binary logistic regression model was used to estimate the risk as the OR at the 95% confidence interval (CI). Haplotypes of each individual consisting of SNP in p53 was constructed, and the maximal likelihood haplotype frequencies were estimated using the expectation-maximization algorithm using the Arlequin program, version 2000. Bonferroni's correction was applied in the case of multiple comparisons using the formula $P_c = p \times n$ (P_c represents corrected value where n is the number of comparisons performed). The statistical analysis was done using the Statistical Package for Social Sciences software, version 15.0 (SPSS, Chicago, IL), and $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Characteristics of subject*

No significant age difference between the cases (58.5 ± 12.4 years) and the controls (56.8 ± 10.8 years; p = 0.117) was observed. The cases had a significantly higher percentage of smokers (69.9%) than the controls (22.5%) (p = 0.001) (Table 1).

3.2. *Genotypic frequency of p53 gene polymorphisms and BC risk*

The genotype and allele frequencies of p53 gene polymorphism in healthy individuals (controls) and BC patients are presented in Table 2. The genotype frequencies of controls were in Hardy Weinberg Equilibrium (HWE) except for *p53 codon 11*. We found a significant association between the *p53 codon 248* with BC risk, whereas *p53 codon 11* and *p53Pro47Ser* showed no association with BC. In *p53 codon 248* the heterozygous genotype was at higher risk of BC ($p < 0.001$; OR = 2.69; 95% CI, 1.58-4.59). While combining the heterozygous and variant genotypes a high risk for BC ($p < 0.001$; OR = 2.23; 95% CI, 1.42-3.49) was observed. This effect was also evident in the case of Trp/Gln allele carrier (p = 0.004; OR = 1.78; 95% CI, 1.20-2.62).

3.3. *Association of p53 genotypes with tumor stage/grade*

The patients with a similar stage but with different grades respond to treatment differently. Hence, we stratified the patients into three sub-groups according to stage/grade [TaG1 (low risk NMIBC), $TaG_{2,3}$+$T1G_{1-3}$ (high risk NMIBC) and T2+ (muscle invasive)]. TaG_1 was taken as a reference. Significant association was observed in the case of *p53 codon 248* combining with heterozygous and variant (Arg/Trp, Arg/Gln) + (Trp/Trp, Gln/Gln) genotypes (p = 0.003; OR = 0.32; 95% CI, 0.15-0.69) when TaG1 and $TaG_{2,3}$ + $T1G_{1-3}$ tumor stage/grade was considered for calculation. The association between group TaG1 and T2+ was not significant in any of the three polymorphic sites of

Table 2. *p53 codon 11*, *p53 Pro 47 Ser* and *p53 codon 248* gene polymorphisms in BC polymorphisms and susceptibility to BC

Genotype	Controls (n = 200) n (%)	Cases (n = 200) n (%)	p value	Age-gender-smoking adjusted OR (95% CI)[a]
p53 codon 11				
Glu/Glu	193 (96.5)	198 (99.0)	–[b]	
Glu /Gln, Glu/Lys,	7 (3.5)	2 (1.00)	0.281	0.31 (0.04-2.61)
Gln/Gln, Lys /Lys	0 (0.0)	0 (0.0)	NC[c]	NC
Glu	393 (98.3)	398 (99.5)	–	
Gln/Lys	7 (1.8)	2 (0.5)	0.116	0.28 (0.06-1.37)
p53 Pro 47 Ser				
Pro/Pro	176 (88.0)	181 (90.5)	–	
Pro/Ser	7 (3.5)	12 (6.0)	0.355	1.66 (0.57-4.86)
Ser/Ser	17 (8.5)	7 (3.5)	0.247	0.55 (0.20-1.51)
Pro/Ser + Ser/Ser	24 (12.0)	19 (9.5)	0.421	0.77 (0.41-1.46)
Pro allele	359 (89.8)	374 (93.5)	–	
Ser allele	41 (10.3)	26 (6.5)	0.058	0.61 (0.37-1.02)
p53 codon 248				
Arg/Arg	159 (79.5)	127 (63.5)	–	
Arg/Trp, Arg/Gln	34 (17.0)	68 (34.0)	< 0.001	2.69 (1.58-4.59)
Trp/Trp, Gln/Gln	7 (3.5)	5 (2.5)	0.929	1.06 (0.30-3.7)
(Arg/Trp, Arg/Gln) + (Trp/Trp, Gln/Gln)	41 (20.5)	73 (36.5)	< 0.001	2.23 (1.42-3.49)
Arg	352 (88.0)	322 (80.5)	–	
Trp/Gln	48 (12.0)	78 (19.5)	0.004	1.78 (1.20-2.62)

[a] Adjusted for Age, gender and smoking habits; [b] Reference; [c] Not calculated (NC).

Table 3. Influence of *p53 codon 11*, *p53 Pro 47 Ser* and *p53 codon 248* gene polymorphisms on the Tumor-stage/grade in BC patients

Genotypes	TaG1 (a) n (%)	TaG_{2-3} + $T1G_{1-3}$ (b) n (%)	T2+ (c)[a] n (%)	p value (a - b)	OR (95% CI)[b]
p53 codon 11					
Glu/Glu	36 (97.3)	111 (99.1)	51 (0.0)	–[c]	
Glu/Gln,Glu/Lys	1 (2.7)	1 (0.9)	0 (0.0)	0.430	0.32 (0.20-5.32)
Gln/Gln, Lys /Lys	0 (0.0)	0 (0.0)	0 (0.0)	NC[d]	NC[d]
p53 Pro47Ser					
Pro/Pro	35 (94.6)	104 (92.9)	42 (82.4)	–	
Pro/Ser	2 (5.4)	3 (2.7)	7 (13.7)	0.464	0.51 (0.08-3.15)
Ser/Ser	0 (0.0)	5 (4.5)	2 (3.9)	NC	NC
Pro/Ser + Ser/Ser	2 (5.4)	8 (7.2)	9 (17.6)	0.160	0.45 (0.15-1.37)
p53 codon 248					
Arg/Arg	25 (67.6)	71 (3.4)	31 (60.8)	–	
Arg/Trp, Arg/Gln	12 (32.4)	37 (33.0)	19 (37.3)	0.839	1.09 (0.49-2.40)
Trp/Trp, Gln/Gln	0 (0.0)	4 (3.6)	1 (2.0)	NC	NC
Arg/Trp, Arg/Gln) + (Trp/Trp, Gln/Gln)	12 (32.4)	41 (36.6)	20 (39.3)	0.003	0.32 (0.15-0.69)

[a] The association between groups a and c was not significant (data not shown); [b] Adjusted for Age, Gender and Smoking habits; [c] Reference; [d] Not calculated (NC).

the codon (data not shown). However, the other two polymorphic sites of *p53 codon 11* and *p53 Pro47Ser* showed no significant association with tumor stage/grade (Table 3).

3.4. *Association of p53 genotypes with smoking*

We evaluated the gene smoking interaction to study the modulation of BC risk with respect to p53 gene polymorphisms. The patients were grouped as non-smokers and smokers. However, no significant association was observed for any of the three polymorphic sites of p53 with BC risk (data not shown).

3.5. *Association of p53 haplotypes with BC risk*

The ability of haplotypes to further substantiate the detection of association over the single locus analysis incited us to analyze haplotypes and their association with BC susceptibility in p53. Seven haplotype combinations were possible from haplotype analysis of p53. In the case of p53, Glu-Pro-Arg was taken as reference. The haplotype results demonstrated that Glu-Pro-Trp/Gln was associated with a two fold (OR = 1.91; 95% CI, 1.27-2.87; p = 0.002) increased risk in BC patients. After applying the Bonferroni correction the p value remained

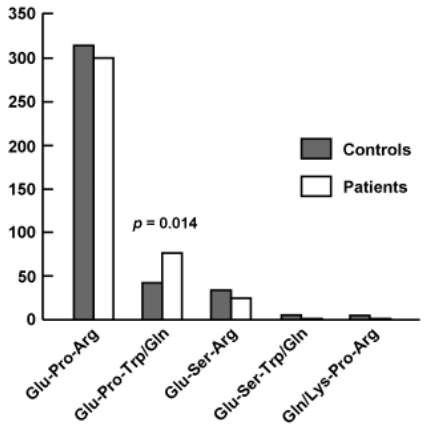

Figure 1. Haplotype analysis of p53 gene polymorphisms and BC risk.

significant ($p = 0.014$) for BC risk (Figure 1).

3.6. *Modulation of genotype variants and outcome after BCG immunotherapy*

To analyze the association of p53 gene polymorphisms and risk of recurrence in NMIBC patients, further analysis was restricted to NMIBC patients only ($n = 148$). The median follow-up of NMIBC patients was 14 months. We analyzed the association of genotypes and risk of recurrence after BCG immunotherapy. We grouped patients into BCG treated ($n = 78$) and non-treated ($n = 71$) because these patients with low grade tumors did not require BCG immunotherapy. None of the polymorphisms of p53 genes were associated with risk of recurrence free survival (data not shown).

4. Discussion

p53 is a nuclear protein that is essential for cell-cycle control, DNA repair and induction of apoptosis from many stresses. The strongest and undisputed fact about p53 is the high frequency of p53 alterations in human cancer and that mutant p53 proteins constitute a complex family of several hundred proteins with heterogeneous properties. Alterations of the p53 gene are some of the most frequently documented somatic alterations in human BC, as well as other tumor types, and detection of this altered gene holds prognostic significance. Several studies, including our own, have clearly shown that inactivation of the p53 pathway is prevalent in this disease. Several studies (*21,22*) and even including our own, have clearly shown that the p53 pathway plays an important role in this disease.

Most cancer-related mutations of p53 are clustered in the four hot spots (*23*) *codon 175*, *248*, *273*, and *281/282*. To the best of our knowledge, this is the first study reporting an increased risk of BC among individuals with the *p53 codon 248* heterozygous genotype, showing a 2.69-fold risk. The combination of heterozygous and variant genotypes demonstrated a 2.23-fold risk for BC susceptibility. In the case of Trp/Gln allele carrier, we found a significant association with BC predisposition having a 1.78-fold risk. However, there was no significant association found between *p53 codon 248* and endometriosis (*19*). There is no other study reported of this SNP in other cancers, though some mutational studies have been stated as in the case of ovarian cancer (*24*) UL-3A cells that contained two point mutations, in codon *248 of exon 7* of the *p53* gene.

In the case of *p53 codon 11*, we found no significant association with BC predisposition; and this result supports the results showing no-significant association between *p53 codon 11* and endometriosis (*20*). However mutation in *p53 codon 11* in gastric adenocarcinomas has been reported earlier by direct DNA sequencing analysis (*25*). For the *p53 Pro47Ser* SNP, we observed Pro/Ser frequency to be 3.5% in controls as compared to 6.0% in cases. The heterozygous Pro/Ser genotype has been reported for the first time in the Indian population. However, we did not find significant association of BC risk with this p53 gene variant that complemented the observation in colorectal cancer in the Kashmiri population (*20*). A significant association was observed between the mutant Ser/Ser genotype and lung cancer (*26*). The effectiveness of mutant Ser/Ser could be due to a decreased capacity for inducing apoptosis (*27*).

Recent studies have demonstrated that haplotypes analysis may be more affirmative in predicting disease association compared with an analysis of a single polymorphism (*28*). Because an individual polymorphism is likely to confer modest effects to the risk of BC, we examined the effects of multiple p53 polymorphisms by constructing haplotype analyses for all three SNPs. A risk of 1.91-fold was observed in case of haplotypes Glu-Pro-Trp/Gln for BC. Other haplotypes did not show any association in connection with BC risk.

We analyzed the association of p53 polymorphisms with risk of recurrence in NMIBC patients. The NMIBC patients were categorized on the basis of BCG treatment in the BCG group and no BCG group. According to age, gender, and smoking adjusted multivariate Cox regression hazards model, no statistically significant association was observed for any SNP of p53 taken in the present study. Thus, there is no role of BCG immunotherapy in the case of this polymorphism. We also studied the gene smoking interaction for modulation of BC risk but did not find any association with any of three gene variants of the p53 gene SNPs in the present study. A significant association was observed in the case of *p53 codon 248* combining heterozygous and variant genotype with a p value of 0.003 when TaG1 and $TaG_{2,3}$+$T1G_{1-3}$ tumor stage/grade was considered for calculation. However the other two polymorphic sites of *p53 codon 11* and *p53Pro47Ser* showed no significant association with tumor stage/grade. The genetic variations that might be

stabilized among the population are those that cause no pivotal effect under normal conditions. However, under special circumstances, the variations may predispose the carriers to diseases. Therefore, analysis combined with the genetic variations and the other internal and external factors may lead to a better understanding of the role of genetic makeup in cancer pathogenesis.

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