

# Analysis of p53 Tumor Suppressor Gene Mutations and Human Papillomavirus Infection in Human Bladder Cancers

Kun Hong Kim and Yoon Soo Kim<sup>1</sup>

To determine whether the dysfunction of p53 caused either by mutation of the p53 gene itself or by binding to E6 protein of oncogenic HPVs is involved in the transitional cell carcinomas (TCCs) of the bladder, we analyzed 23 TCCs of the bladder. DNA was extracted from each paraffin embedded tissue of TCCs of bladder and polymerase chain reaction (PCR)/ single strand conformation polymorphism (SSCP) analysis were performed to screen mutations in p53 tumor suppressor gene, then PCR/dot blot hybridization were performed to detect infection of HPVs. We found that p53 gene mutation was found in 3 cases and oncogenic HPV infection was detected in 8 cases and thus, the overall incidence of possible p53 dysfunction was 47.8% on DNA analysis (If the results of immunohistochemistry to detect overexpression of p53 protein were included, the incidence was 60.9 %).

Therefore, we concluded that dysfunction of p53 plays a major role in the development of TCCs of bladder in Korean patients.

**Key Words:** p53 tumor suppressor gene, polymerase chain reaction, single strand conformation polymorphism analysis, human papillomavirus, transitional cell carcinoma of bladder

The p53 gene, located on chromosome 17p13.1, encodes a nuclear phosphoprotein. Originally, p53 was thought to be a dominant oncogene as it was found to transform rodent fibroblasts in cooperation with the *ras* oncogene (Eliyahu *et al.* 1984). However, the further investigation has revealed that p53 is a tumor suppressor gene (Knudson, 1985; Lane and Benchimol, 1990). Generally, in the case of p53, one allele is lost through a chromosomal deletion and the second allele undergoes some

types of mutation within the gene. As have been reported in a variety of human tumors, including colon (Vogelstein *et al.* 1988), breast (Mazars *et al.* 1992), lung (Yokota *et al.* 1987), and mesothelioma (Cote *et al.* 1991), chromosome 17p deletions (Olumi *et al.* 1990; Presti *et al.* 1990) or p53 gene mutations have been frequently associated with high grade invasive bladder carcinoma, suggesting a role for p53 in bladder cancer progression (Sidransky *et al.* 1991).

Intracellular function of wild type p53 protein is to arrest cell growth after DNA damage or cellular stress, thus allowing DNA repair to occur prior to cell division, although p53 is not required for normal development (Kastan *et al.* 1991; Lane 1992). When p53 expression is stimulated, the p53 binds to Cipl (Harper *et al.* 1993)/WAF1 (El-Deiry *et al.* 1993) regulatory elements and transcriptionally activates the expression of Cipl/WAF1. Then,

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Department of Biochemistry and Molecular Biology, Yonsei University College of Medicine and the Institute of Genetic Science, Seoul, Korea

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Address reprint requests to Dr. YS Kim, Department of Biochemistry and Molecular Biology, Yonsei University College of Medicine, C.P.O Box 8044, Seoul 120-752, Korea

the Cip1/WAF1 protein binds to and inhibits cyclin dependent kinase (CDK) activity which has been implicated in the control of G1 to S phase transition in mammals and thus blocking cell cycle progression (El-Deiry *et al.* 1994). Therefore, if the p53 is mutated or if wild type p53 protein is inactivated by binding to the cellular oncogene MDM2 (Oliner *et al.* 1992) or to viral oncoproteins, the cell will continue to replicate.

Mutated p53 protein complexes with a heat shock protein (hsc 70) that results in a metabolically stable protein with a half life of many hours (Finlay *et al.* 1988) but the wild type p53 has a half life of 6 to 30 minutes (Reich and Levine, 1984). Wild type p53 proteins bind to oncoproteins encoded by some DNA viruses such as simian virus (SV) 40 large T antigen, adenovirus (Ad) E1B protein, and human papillomavirus (HPV) E6 protein. Although the binding of SV 40 large T antigen and Ad E1B protein stabilizes p53, the binding of E6 proteins encoded by HPV type 16 and 18 which are closely associated with anogenital cancers (zur Hausen and Schneider, 1988), results in rapid degradation of p53 via the ubiquitin-directed pathway (Scheffner *et al.* 1990). Thus, it is clear that the role of the E6 oncoprotein is to eliminate or inactivate p53 as a tumor suppressor, although the functional consequences of p53 binding and degradation by E6 in HPV induced cancer remain unclear. The role of E6 protein is supported by the observation that cell lines derived from cervical carcinomas bearing the HPV type 16 or 18 E6 gene product also possess wild type p53 gene (Scheffner *et al.* 1991). In fact, no mutant p53 gene or protein has been detected in tumors containing the HPV E6 gene, such as cervical carcinomas (Scheffner *et al.* 1991; Crook *et al.* 1992) and esophageal carcinomas (Furihata *et al.* 1993a; Furihata *et al.* 1993b). In contrast, p53 gene mutations have been detected in cell lines derived from cervical carcinomas bearing no HPV (Srivastava *et al.* 1992). Therefore, dysfunction of p53 either by mutation itself or by HPV may be closely associated with development of cervix cancers.

In this study, we investigated the frequencies of mutations in p53 gene and HPV infec-

tion in human TCCs of bladder samples isolated from Korean patients by using PCR/SSCP analysis and PCR/dot blot hybridization.

## MATERIALS AND METHODS

### Preparation of DNA from paraffin-embedded bladder cancer tissues

Twenty-three tissue specimens of TCCs of bladder were obtained from paraffin-embedded tissue block at the Yonsei Medical Center. Two 5  $\mu$ m sections from each paraffin embedded tissue block were deparaffinized by extracting with xylene. Then, the sections were incubated with 400  $\mu$ L of a solution (100 mM NaCl, 10mM Tris-HCl, pH 8.4, 25 mM EDTA, 0.5% SDS, 200  $\mu$ g/mL proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA)) at 50°C water bath for 1 hour and further incubated at 37°C for 72 hours (Innis *et al.* 1989). Finally, the incubated solution was extracted with phenol/chloroform. After extraction, DNA was precipitated with ethanol.

### Primers and probes

Primers and probes were synthesized by the phosphoramidite method with DNA synthesizer (Model 381A, Applied Biosystem Inc., Foster city, CA, USA). Primer sequences were as follows;

p53 each exon (Gaidano *et al.* 1991);

p5-5 5'-TTCCTCTTCTGCAGTACTC-3'  
p5-3 5'-ACCCTGGGCAACCAGCCCTGT-3'  
p6-5 5'-ACAGGGCTGGTTGCCAGGGT-3'  
p6-3 5'-AGTTGCAAACCAGCCTCAG-3'  
p7-5 5'-GTGTTGTCTCCTAGGTTGGC-3'  
p7-3 5'-GTCAGAGGCAAGCAGAGGCT-3'  
p8-5 5'-TATCCTGAGTAGTGGTAATC-3'  
p8-3 5'-AAGTGAATCTGAGGCATAAC-3'  
p9-5 5'-GCAGTTATGCCTCAGATTAC-3'  
p9-3 5'-AAGACTTAGTACCTGAAGGGT-3'

E6 consensus primer sequences for detecting HPV (Resnick *et al.* 1990);

E6 Consensus Positive Strand Primers

WD72 19-mer 5'-CGGTCGGGACCGAAAACGG-3'  
WD76 18-mer 5'-CGGTTSA ACCGAAAMCGG-3'

**E6 Consensus Negative Strand Primers**

WD 66 20-mer 5'-AGCATGCGGTATACTGTCTC-3'  
 WD 67 20-mer 5'-WGCAWATGGAWGCGYGTCTC-3'  
 WD 154 20-mer 5'-TCCGTGTGGTGTGTCTGTC-3'

W=A/T, S=G/C, R=A/G, Y=C/T, M=A/C

**HPV type specific probes (Resnick *et al.* 1990)**

HPV type 16 5'-CAACAGTTACTGCGACG-3'  
 HPV type 18 5'-GCAAGACATAGAAATAA-3'  
 HPV type 31 5'-AAATCCTGCAGAAAGACCTC-3'  
 HPV type 33 5'-GTACTGCACGACTATGT-3'

**PCR amplification**

Typical PCR conditions were: template DNA, 200 ng; primers, 10 pmoles each; Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA), 1.0 units; 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 500mM KCl, 20mM MgCl<sub>2</sub>, 0.1% (w/v) gelatin), 2 μL; each 50 μM dNTP in a total volume of 20 μL. Each cycle of PCR consisted of 60 seconds of template denaturation at 94°C, 90 seconds of annealing at 63°C (for p53 exon 5, 6, 7, and 9) or 58°C (for p53 exon 8, and HPV), and 90 seconds of extension at 72°C. The cycle was repeated 35 times and final extension step was performed for 10 minutes. In case of PCR for HPV detection, 4.0mM of MgCl<sub>2</sub> was used and cycle was repeated 40 times.

**SSCP analysis**

For SSCP analysis, PCR primers were end labeled with  $\gamma$ -<sup>32</sup>P-ATP (Sambrook *et al.* 1989) and PCR reactions were carried out as described above. Samples were prepared for electrophoresis as described (Orita *et al.* 1989). Electrophoresis with the amplified DNA fragments was performed at 10 watts for 5 to 8 hours in 6% non-denaturing polyacrylamide gel containing 5% (v/v) glycerol at 20°C using LKB 2010 MacroPhor sequencing system (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). After electrophoresis, gels were dried and exposed to X-ray film at -70°C for overnight.

**DNA sequencing**

PCR products which showed mobility shifts

on SSCP analysis were subcloned into TA cloning vector (Invitrogen Corporation, San Diego, CA, USA) and double strand sequencing was performed with T7 sequencing kit (Pharmacia LKB Biotechnology) according to the manufacturer's instruction.

**Dot blot hybridization of PCR products for HPV typing**

Five microliters of each PCR products were denatured in 100 μL of a solution (0.4N NaOH and 25mM EDTA) and were applied to Zeta-probe GT blotting membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA) using Bio-Dot apparatus (Bio-Rad Laboratories Inc.). The membranes were UV-cross linked with UV-Stratalinkler 2400 (Stratagene Inc., La Jolla, CA, USA). Each blotted membrane was hybridized with <sup>32</sup>P-end labeled HPV type specific probes.

**Immunohistochemical staining**

To detect overexpression of p53 in the formalin fixed paraffin embedded bladder cancer tissues, the tissues were stained with mouse anti p53 monoclonal Ab (p53(DO-1), Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). First, the tissues were boiled in the microwave oven and were incubated with mouse anti p53 monoclonal Ab for 1 hour. Second, the tissues were incubated with biotinylated anti-mouse IgG Ab and then incubated with peroxidase conjugated streptavidin. Finally, 3, 4-diaminobenzidine (DAB) was added and then counterstained with hematoxylin.

**RESULTS****Histopathology of the tumor**

Among the 23 TCCs of bladder, 6 cases were superficial type including 1 pT1a, 5 pT1, and 14 cases were invasive type, including 2 pT2, 6 pT3, 6 pT4, and the stage of remaining 3 cases were uncertain. All cases were graded on a scale of 1 to 3 according to the WHO classification. All the superficial cancers showed low grade (grade 1 to 2). Among the

invasive cancers, 7 cases showed low grade (grade 2), 7 cases showed high grade (grade 3).

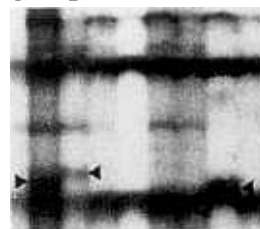
### PCR-SSCP analysis

To screen the p53 gene mutations in each tumor samples, PCR of each exon of p53 (from 5 to 9) was done first and then, non denaturing (6%) polyacrylamide gel electrophoresis was performed. The summary of PCR-SSCP analysis of exons 5 to 9 of p53 tumor suppressor gene was given in Table 1. Autoradiographies from SSCP analysis showed that there were 3 mutant cases among the samples screened and that all the mutations found were in exon 7 (Fig. 1).

### Sequence analysis

To get the sequence data from the mutant

Case No. C 12 20 22



**Fig. 1.** PCR/SSCP analysis of p53 exon 7.

Nondenaturing PAGE(6%) of  $^{32}$ P-labeled PCR products of p53 exon 7 showed mobility shifts in 3 cases. Numbers written at the top of the figure indicate the case No. which showed mobility shift on SSCP analysis and each arrow head indicates shifted band. Human WBC DNA was used for control(Lane C).

**Table 1.** p53 gene mutations and HPV infection in TCCs of bladder

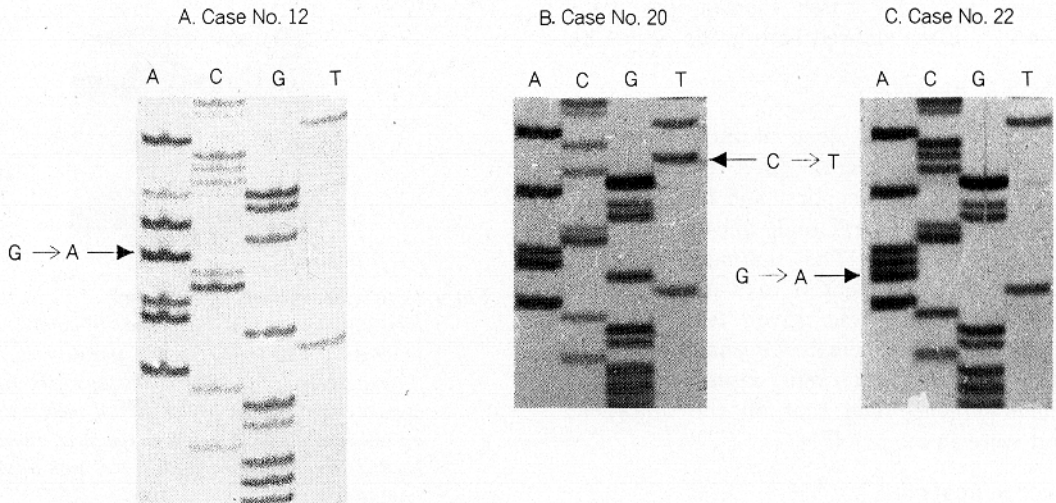
Case No.	Grade	Stage	HPV(Type)	Mutation of p53	Codone	Overexpression of p53 <sup>c</sup>	Aminoacid substitution
		pT1aN0M0		ND <sup>a</sup>		NT	
		pT1N0N0	+(18)	ND			
		pT1N0M0	+(18)	ND			
		pT1N0M0		ND		NT	
		pT1N0M0	+(16, 18)	ND		NT	
		pT1N0M0		ND		NT	
		pT2N0M0	(16,18)	ND			
8		pT2N0M0		ND		NT	
9		pT3N0M0		ND			
10		pT3N0M0	+(16, 18)	ND			
11		pT3N0M0	+(18)	ND			
12		pT3N1M1		exon 7	248		Arg→Gln(CGG→CAG)
13		pT3bN1M0		ND		NT	
14		pT3bN0M0		ND			
15		pT4N1M0		ND		NT	
16		pT4N1M1		ND		NT	
17		pT4N1M1		ND		NT	
18		pT4N1M1		ND		NT	
19		pT4N1M1	+(18)	ND		NT	
20		pT4N1M1		exon 7	250	NT	Pro→Leu(CCC→CTC)
21		NF <sup>a</sup>	(16,18)	ND		NT	
22		NF		exon 7	246	NT	Met→Ile(ATG→ATA)
23		NF		ND		NT	

<sup>a</sup>Not found

<sup>b</sup>Not detected

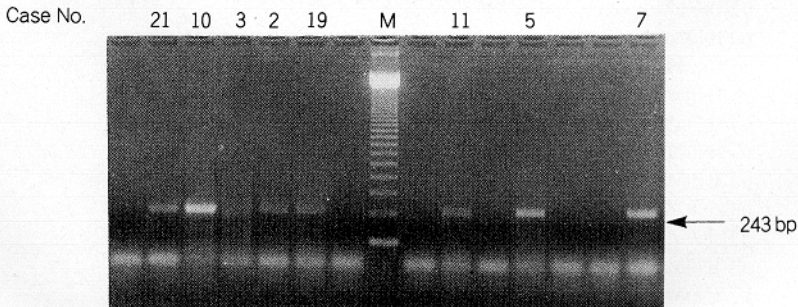
<sup>c</sup>Detected by immunohistochemical staining

<sup>d</sup>Not tested



**Fig. 2.** Sequence analysis of the 3 cases that showed mobility shift on SSCP analysis.

PCR products of 3 cases that showed mobility shift on SSCP analysis were cloned into TA cloning vector, and sequenced by the method described in the text. A: Case No. 12 (codon 248 mutation); B: Case no. 20 (codon 250 mutation); C: Case No. 22 (codon 246 mutation). Each arrow indicates the position of mutated base.



**Fig. 3.** Agarose gel(2%) electrophoresis of PCR products amplified with HPV E6 consensus primers.

To detect the infection of HPVs in TCCs of bladder, chromosomal DNA was extracted from each tumor tissue, and PCR was performed to amplify the HPV E6 consensus region by using E6 consensus primer pairs. Numbers written at the top of the figure indicate the case No. which showed amplified HPV DNA band(indicated by arrow). The 123 bp DNA ladder was used as a size marker(Land M).

cases, double strand sequencing was done. We found that case No. 12 contained CGG→CAG transition mutation resulting in arginine to glutamine substitution at codon 248 (Fig. 2A), case No. 20 contained CCC→CTC transition mutation resulting in proline to leucine substitution at codon 250 (Fig. 2B), and case No. 22

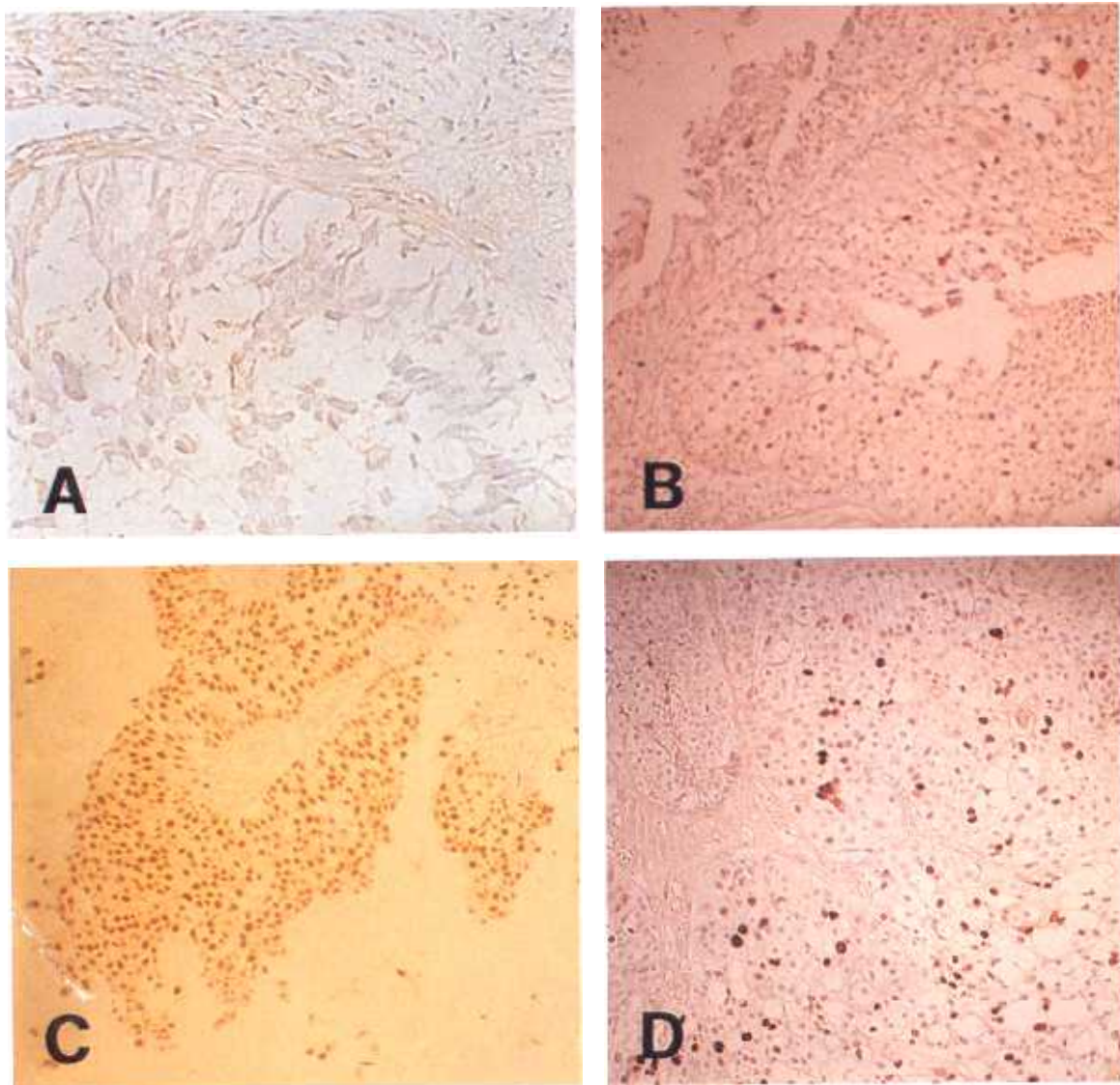
contained ATG→ATA transition mutation resulting in methionine to isoleucine substitution at codon 246 (Fig. 2C). The sequence data showed that there were no codon preference in the mutations of p53 gene, indicating that tumor samples screened were heterogeneous.



### HPV detection and typing

To detect the infection of HPVs in TCCs of bladder, PCR using consensus primers which could amplify the consensus E6 region of

HPV type 6, 11, 16, 18, and 33 was performed and to identify the type of HPVs, dot blot hybridizations were performed using type 16, 18, 31 or 33 specific probes. We found that HPVs were positive in 8 cases (34.7%, Fig. 3)



**Fig. 4.** Detection of p53 overexpression by immunohistochemical staining.

To detect overexpression of p53, the tissues were stained with mouse anti p53 monoclonal Ab. First, tissues were boiled in the microwave oven and were incubated with mouse anti p53 monoclonal Ab for 1 hour. Second, the tissues were incubated with biotinylated anti-mouse IgG Ab, and then incubated with peroxidase conjugated streptavidin. Finally, 3,4-diaminobenzidine(DAB) was added and then counterstained with hematoxylin. A: case No. 2, B: case No. 7, C: case No. 12, D: case No. 8. In case of negative control, primary Ab was omitted(data not shown).

and that the type of HPVs were either type 16 (in 4 cases) or type 18 (in 8 cases) but there were no HPV type 31 and 33. In 4 cases, double infection by HPV type 16 and 18 was observed (data not shown). These results suggested that oncogenic HPVs might be involved in the development of TCCs of bladder.

### Immunohistochemical staining

To support the results of p53 gene mutations and HPV infection in terms of p53 protein expression in the tumor tissues, immunohistochemical staining was done using mouse anti p53 monoclonal Ab. Cases were grouped into three according to the presence (+) or absence (-) of HPV infection and mutation in p53 gene on DNA analysis: first group, HPV (+) and p53 (-); second group, HPV (-) and p53 (+); third group, HPV (-) and p53 (-). Because tissue sections of all the cases were not available for immunohistochemistry, only some cases from each group could be included. Although, as we expected, 2 cases (case No. 2 and 3) of first group showed no p53 on immunostaining (Fig. 4A), case No. 7 showed unexpected overexpression of p53 (Fig. 4B), suggesting that, not in cell lines but in tumor samples, both HPV infection and mutation in p53 suggested by overexpression may exist in the same tumor. Case No. 12, one of second group, showed marked overexpression of p53 (Fig. 4C). The overexpression of p53 was observed in nearly all the transitional cells. In addition, 2 cases (case No. 8 and 9) of third group showed overexpression of p53 (Fig. 4D), suggesting that the mutations may be present in other exons which are not included in our experiment and that SSCP analysis may not be sensitive enough for detecting mutations if the cell population which contained mutation of p53 gene is small.

### DISCUSSION

This study shows that dysfunction of p53 caused either by mutation of p53 gene itself or by binding to E6 protein of oncogenic

HPVs is involved in TCCs of the bladder. The evidence is that p53 gene mutation screened by PCR/SSCP was found in 3 cases and HPV infection detected by PCR/dot blot hybridization was found in 8 cases out of 23 TCCs of bladder, respectively, and thus, overall incidence of possible p53 dysfunction was 47.8% (If the results of immunohistochemistry were included, the incidence was 60.9%).

The p53 gene mutation may be more closely associated with invasive TCCs than with superficial TCCs. The evidence is that the stage of all the cases that had p53 mutation (either detected by SSCP analysis or detected by immunohistochemistry) was pT2 or pT3. As reported (Fujimoto *et al.* 1992), p53 gene mutation might be a rather late event in tumor development and might be involved in progression of bladder cancer.

Immunohistochemical detection of p53 might be more sensitive method than PCR/SSCP to detect p53 gene mutations. There are several possible reasons for this as described by Esrig *et al.* (1993). First of all, all of the tumors used for SSCP analysis contained non neoplastic tissue that was intermingled with the tumor cells in various proportions. If the proportion of tumor cells (containing the p53 mutation) was relatively low compared to normal cells (containing wild type p53), the SSCP analysis may not have detected the mutation in the tumor. Second, the mutations may be present in other exons which are not included in experiment. In this study, exon 4 of p53 was excluded in PCR because most p53 mutations in human tumors were found within exons 5 through 9 (Soussi *et al.* 1990; Hollstein *et al.* 1991). However, p53 mutations in exon 4 were found in bladder cancers (Fujimoto *et al.* 1992). Actually, we found 3 cases that showed no evidence of p53 mutation in SSCP but showed p53 overexpression in immunohistochemistry.

The mutations of p53 found in this study might not be caused by specific mutagen. p53 gene mutations investigated from patients with known exposure to cigarette smoke were specifically found in codon 280 (Spruck *et al.* 1993). In this study, however, there were no codon preference, indicating that no specific mutagens were associated with the TCCs of

bladder investigated in this study.

Oncogenic HPVs might play important roles in bladder carcinogenesis. Besides squamous epithelium, other types of epithelia also are susceptible to the papillomavirus infection, such as transitional carcinoma of the urethra (Mevorach *et al.* 1990), and adenocarcinoma of the prostate (McNicol and Dodds, 1990). In bladder cancers, oncogenic HPVs have been demonstrated in 5 to 81 % of the cancer patients (Kitamura *et al.* 1988; Bryant *et al.* 1991; Anwar *et al.* 1992; Furihata *et al.* 1993c) and multiple infections were also reported (Furihata *et al.* 1993c). In this study, HPVs were found in 8 cases (34.7%), and their types were either 16 or 18, but there were no HPV type 31 or 33 and double infections by HPV type 16 and 18 were observed in 4 cases.

Contrary to the reports on the status of p53 in HPV positive or negative cervical cancer cell lines (Crook *et al.* 1992; Yaginuma and Westphal, 1991; Srivastava *et al.* 1992), both p53 gene mutation and HPV infection could exist in same tumors. Furihata *et al.* (1993c) demonstrated that p53 overexpression and HPV infection coexisted in single cancer cell nuclei of bladder TCCs. In addition, Crook and Vousden (1992) reported that acquisition of p53 mutation might play a role in the progression of some HPV associated primary cancers. In this study, we also demonstrated that the both HPV infection detected by PCR and p53 mutation suggested by overexpression in immunohistochemical staining existed in the same case (case No. 7), although there remained possibility that HPV infection and p53 mutation existed in the independent population of tumor cells.

In conclusion, this study supports the previous observations that dysfunction of p53 caused either by mutation of p53 gene itself or by binding to the E6 protein of oncogenic HPVs also plays a major role in the development of TCCs of the bladder in Korean patients as have been reported in cervix cancers.

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