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Role of Human Papillomavirus and p53 Tumor Suppressor Gene in Cervical Carcinogenesis

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To determine whether the dysfunction of p53 protein, caused either by the mutation of p53 gene itself or by the human papillomaviruses (HPVs), is involved in the development of cervical cancers and to find out the status of p53 tumor suppressor gene in HPV positive or negative cancers, we analyzed 64 cases of primary cervix cancers. First, polymerase chain reaction (PCR) was performed with E6 consensus primer pairs to detect the infection of HPVs in cervix cancer tissues. Second, to screen the p53 gene mutation, PCR of p53 exon 5 through 9 and single strand conformation polymorphism (SSCP) analysis were performed with p53 exon specific primers. Finally, overexpression of p53 protein was checked by immunohistochemical staining with anti p53 antibody. We found HPVs in 43 cases out of 64 cases (67.2%). HPV type 16 was positive in 26 cases, type 18 was positive in 7 cases, and 6 cases were positive for both HPV type 16 and 18. HPV type 31 or 33 was not detected in our experiments, and in 4 cases, type of HPVs were unknown. The SSCP analysis showed mobility shifts in 8 cases (6 in HPV positive cases; 2 in HPV negative cases) and the sequence analysis confirmed the results of SSCP analysis. In addition, the overexpression suggesting the mutation of p53 gene was detected in 27 cases (42.2%, 21 in HPV positive cases; 6 cases in HPV negative cases). Our results support the previous reports that the dysfunction of p53 plays an important role in the development of cervix cancers but contrary to the results obtained from cervix cancer cell lines, there is no inverse correlation between HPV infections and p53 mutations in primary cervix cancers.

Key Words: Cervix cancer, human papillomavirus, p53 tumor suppressor gene, polymerase chain reaction, single strand conformation polymorphism analysis, immunohistochemical staining

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, one allele of the p53 gene is lost through a chromosomal deletion and the second allele undergoes some types of mutations within the gene. Chromosome 17p deletions or p53 gene mutations 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), mesothelioma (Cote *et al.* 1991), and bladder (Olumi *et al.* 1990; Presti *et al.* 1990) cancers.

The normal intracellular function of p53

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protein is to arrest cell growth after DNA damage, thus allowing DNA repair to occur prior to cell division (Kastan *et al.* 1991; Lane, 1992). Actually, p53 is not required for normal development, but in certain cellular environments (such as DNA damage, cellular stress), p53 expression is stimulated. In turn, p53 binds to the regulatory elements of Cip1 (Harper *et al.* 1993)/ WAF1 (El-Deiry *et al.* 1993) and transcriptionally activates its expression. Then, the Cip1/WAF1 protein binds to and inhibits cyclin dependent kinase activity which has been implicated in the control of G1 to S phase transition in mammals and thus preventing phosphorylation of critical cyclin dependent kinase substrates and 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 (hsp 70) that results in a metabolically stable protein with a half life of many hours (Finlay *et al.* 1988), but 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. Of the 60 different types of HPVs which have been isolated thus far from a variety of squamous epithelial lesions, eighteen types have been shown to be associated with anogenital tract lesions (Schwarz *et al.* 1985). Some of these, such as HPV 6 and 11, are associated with benign proliferative tumors (e.g., condyloma acuminatum) which have a low risk for malignant progression, whereas others, such as HPV 16, 18, 31, 33, and HPV 35 are associated with potentially precancerous genital tract lesions, and with a high percentage of anogenital cancers (zur Hausen and Schneider, 1987). In cervical carcinomas and in cell lines derived from cervical carcinomas, the E6 and E7 open reading frames (ORFs) of the high risk HPVs are found to be intact and actively transcribed, implicating the involvement of E6 and E7 genes in the malignant phenotype (Phelps *et al.* 1988). 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, 1987), 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 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 E6 gene product of HPV type 16 or 18 possess wild type p53 gene (Scheffner *et al.* 1991; Yaginuma and Westphal, 1991; Crook *et al.* 1992; Kaelbling *et al.* 1992). In contrast, mutant p53 genes have been detected in cell lines derived from cervical carcinomas bearing no HPV (Srivastava *et al.* 1992). Therefore, it has been reported that the dysfunction of p53, caused either by mutation itself or by HPV, may be closely associated with development of cervix cancers and that the inverse correlation existed between infection of HPVs and mutations of p53 gene.

In this study, we used PCR/SSCP analysis, PCR/Southern blot hybridization and immunohistochemical staining to determine whether the dysfunction of p53 protein, caused either by mutation of p53 gene itself or by HPVs, is involved in the development of cervical cancers, and to find out the status of p53 tumor suppressor gene in HPV positive or HPV negative primary cervix cancers.

MATERIALS AND METHODS

Preparation of DNA from paraffin-embedded cervix cancer tissues

Sixty four tissue specimens of cervical cancer patients were obtained from paraffin-embedded tissue block from the Department of Pathology of the Yonsei University College of Medicine, Seoul, Korea. Two 5 μm sections

from each paraffin embedded tissue block were deparaffinized by extracting with xylene. Then, the sections were incubated with 400 μL of a solution (100 mM NaCl, 10 mM Tris-HCl, pH 8.4, 25 mM EDTA, 0.5% SDS, 200 μg/mL proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA)) at 50℃ water bath for 1 hour and further incubated at 37℃ 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 using 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′-TTCCTCTTCCTGCAGTACTC-3′
p5-3 5′-ACCCTGGGCAACCAGCCCTGT-3′
p6-5 5′-ACAGGGCTGGTTGCCCAGGGT-3′
p6-3 5′-AGTTGCAAACCAGACCTCAG-3′
p7-5 5′-GTGTTGTCTCCTAGGTTGGC-3′
p7-3 5′-GTCAGAGGCAAGCAGAGGCT-3′
p8-5 5′-TATCCTGAGTAGTGGTAATC-3′
p8-3 5′-AAGTGAATCTGAGGCATAAC-3′
p9-5 5′-GCAGTTATGCCTCAGATTCAC-3′
p9-3 5′-AAGACTTAGTACCTGAAGGGT-3′

E6 consensus positive strand primers (Resnick *et al.* 1990)

WD72 19-mer 5′-CGGTCGGGACCGAAAACGG-3′
WD76 18-mer 5′-CGGTTSAACCGAAAMCGG-3′

E6 consensus negative strand primers (Resnick *et al.* 1990)

WD66 20-mer 5′-AGCATGCGGTATACTGTCTC-3′
WD67 20-mer 5′-WGCAWATGGAWWGCYGTCTC-3′
WD154 20-mer 5′-TCCGTGTGGTGTGTCGTCCC-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, Norwlak, CT, USA), 1.0 units; 10x PCR buffer (100mM Tris-HCl, pH 8.3, 500 mM KCl, 20 mM $MgCl_2$, 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℃, 90 seconds of annealing at 63℃ (for p53 exon 5, 6, 7, and 9) or 58℃ (for p53 exon 8, and HPV), and 90 seconds of extension at 72℃. The cycle was repeated 35 times and final extension step was performed for 10 minutes. For HPV detection, 4.0 mM of $MgCl_2$ was used and cycle was repeated 40 times.

SSCP analysis

For SSCP analysis, PCR reactions were carried out as described above under the presence of α-^{32}P-dCTP. Samples for electrophoresis were prepared 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℃ 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℃ 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, Piscataway, NJ, USA) according to the manufacturer's instruction. Some cases were sequenced by Sequenase PCR product sequencing kit (Amersham LIFE SCIENCE Inc., Arlington Heights, Illinois, USA).

Southern blot hybridization of PCR products for HPV typing

After agarose gel (2%) electrophoresis, amplified HPV DNA was blotted on to Zeta-probe GT blotting membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA) using the method of Sambrook *et al.* (1989). The membranes were UV-cross linked with UV-Stratalinker 2400 (Stratagene Inc., La Jolla, CA, USA). Each blotted membrane was hybridized with ^{32}P-end labeled HPV type specific probes.

Immunohistochemical staining

To detect overexpression of p53, the paraffin embedded cervix cancer tissues were stained with mouse anti p53 monoclonal Ab (p53(DO-1), Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). First, tissues were boiled in the microwave oven and were incubated with mouse anti p53 monoclonal Ab for

A.

2	3	4	5	6	7	12	13	14	M	15	16	17	18	20	21	22	23	24	25
26	27	28	29	30	32	33	34	35	M	37	42	43	44	62	61	59	58	57	55
M	51	48	65	67	70		β-actin												

B.

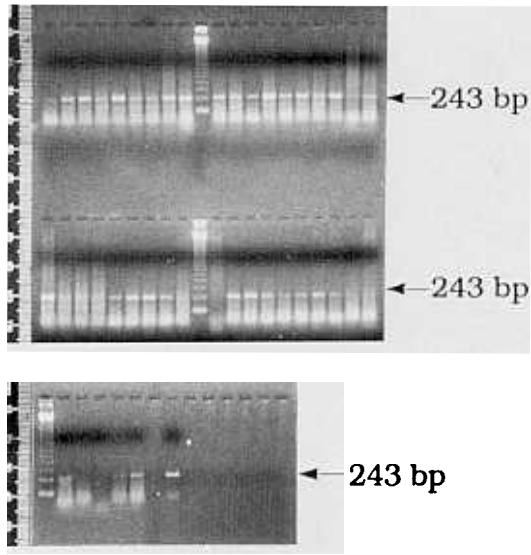

Fig. 1. *Detection of HPVs by PCR using E6 consensus primers.*
To detect the infection of HPVs in each cervix cancer tissue, chromosomal DNA was extracted from each tumor tissue section, and PCR was performed to amplify the HPV E6 consensus region by using E6 consensus primer pairs. A: table for case No. assignment; B: result of agarose gel (2%) electrophoresis. The arrow indicates amplified HPV DNA band. The 123 bp DNA ladder was used as a size marker (Lane M).

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.

Table 1. HPV infection and p53 overexpression according to pathologic diagnosis

	CIN(11)	CIS(23)	Invasive Cancer(30)
HPV positive	3/11(27.3%)	19/23(82.6%)	21/30(70%)
HPV negative	8/11(72.7%)	4/23(17.4%)	9/30(30%)
p53 overexpression	1/11(9.1%)	6/23(26.1%)	20/30(66.6%)

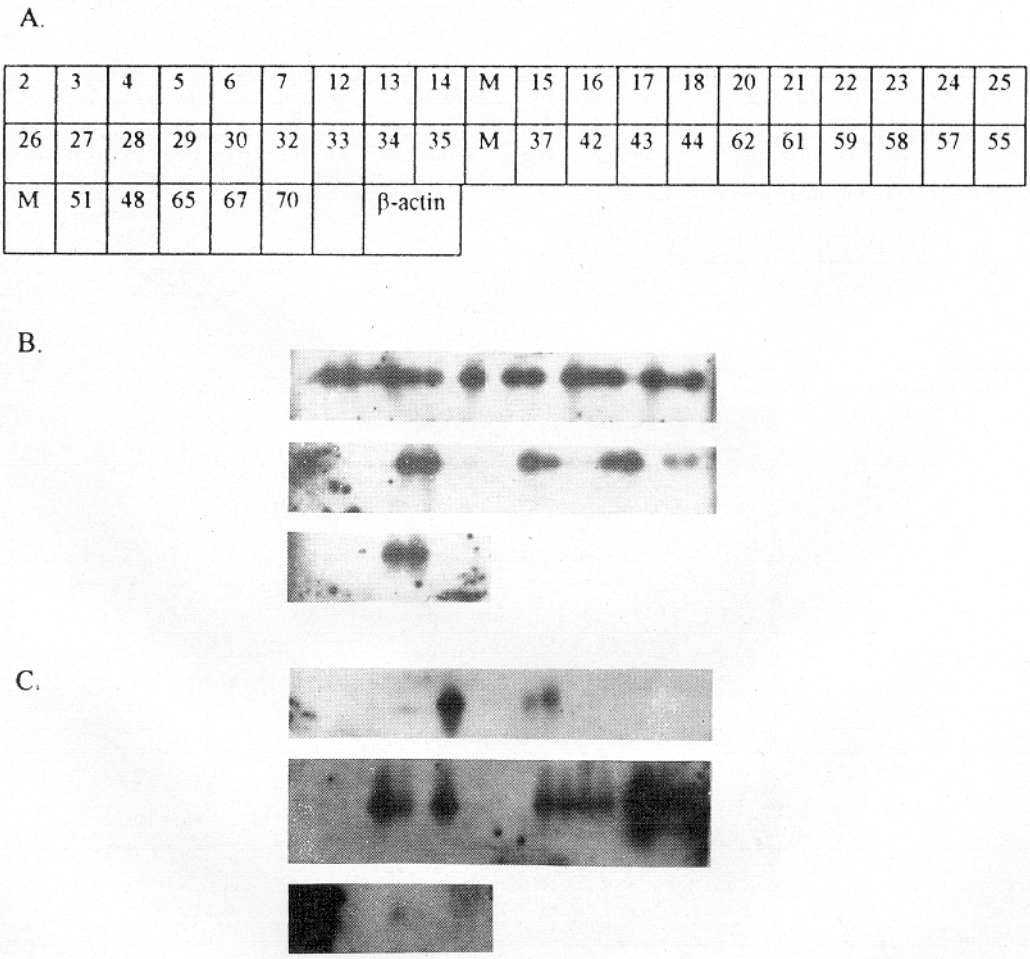

Fig. 2. *HPV type determination by Southern blot hybridization probed with HPV type specific probes.*
After agarose gel (2%) electrophoresis, amplified HPV DNA was blotted onto nylon membrane. Each membrane was hybridized with HPV type 16, 18, 31, or 33 specfic probe. A: table for case No. assignment, B: blot probed with HPV type 16 specific probe, C: blot probed with HPV type 18 specific probe. The amplified β-actin is used as a negative control.

Table 2. Pathology, HPV type detected, mobility shift and overexpression of p53

Case No.	HPV(Type)	Pathology	Mobility shift	p53 overexpression	Mutation (nucleotide)
48	+(ND[1])	CIN	−	−	
58	+(18)	CIN	−	−	
33	+(16)	CIN	−	−	
21	+(16)	CIS	−	−	
23	+(16)	CIS	exon 5	+	CAC→CAT
24	+(16)	CIS	−	−	
25	+(16)	CIS	−	−	
26	+(16)	CIS	−	−	
27	+(16)	CIS	−	−	
37	+(ND)	CIS	−	+	
42	+(16)	CIS	exon 8	+	CAC→CCC
43	+(16, 18)	CIS	−	−	
62	+(16, 18)	CIS	−	+	
22	+(16)	CIS	−	+	
28	+(16)	CIS	−	−	
29	+(16)	CIS	−	−	
30	+(18)	CIS	−	−	
35	+(16)	CIS	−	−	
44	+(18)	CIS	−	−	
51	+(18)	CIS	−	−	
55(60)	+(16, 18)	CIS	−	−	
57	+(16, 18)	CIS	−	+	
3	+(16)	Invasive cancer	−	+	
4	+(16)	Invasive cancer	−	+	
6	+(16)	Invasive cancer	−	++	
14	+(16)	Invasive cancer	−	−	
18(19)	+(16)	Invasive cancer	−	−	
20	+(16)	Invasive cancer	−	+	
32	+(16, 18)	Invasive cancer	−	−	
13	+(18)	Invasive cancer	−	+	
17	+(18)	Invasive cancer	exon 5	++	GGC→AGC
34	+(18)	Invasive cancer	−	−	
2	+(ND)	Invasive cancer	−	+	
5	+(16)	Invasive cancer	exon 5	+	CAC→CAT
7(8)	+(16)	Invasive cancer	−	+	
11(12)	+(16)	Invasive cancer	exon 5	++	GAT→GAC
15	+(16)	Invasive cancer	exon 5	+	ACG→CCG
16	+(16)	Invasive cancer	−	−	
59	+(16)	Invasive cancer	−	+	
61	+(16, 18)	Invasive cancer	−	++	
65	+(ND)	Invasive cancer	−	++	
67	+(16)	Invasive cancer	−	+	
70	+(16)	Invasive cancer	−	−	

Table 2. Continued

Case No.	HPV(Type)	Pathology	Mobility shift	p53 overexpression	Mutation (nucleotide)
31	−	CIN	−	−	
40	−	CIN	−	−	
47	−	CIN	−	−	
50	−	CIN	−	+	
41	−	CIN	−	−	
45	−	CIN	−	−	
54	−	CIN	−	−	
64	−	CIN	−	−	
36	−	CIS	−	−	
38	−	CIS	−	−	
39	−	CIS	−	−	
49	−	CIS	−	−	
1	−	Invasive cancer	−	+	
9	−	Invasive cancer	−	++	
10	−	Invasive cancer	−	−	
53	−	Invasive cancer	−	−	
56	−	Invasive cancer	−	+	
63	−	Invasive cancer	−	−	
66	−	Invasive cancer	exon 8	+	GAG→AAG
68	−	Invasive cancer	exon 8	+	NT[2]
69	−	Invasive cancer	−	−	

[1]: not determined
[2]: not tested

RESULTS

PCR to detect HPV in human cervical cancer tissues

To detect the infection of HPVs in the primary cervix cancers, PCR using E6 consensus primers which could amplify the consensus E6 region of HPV type 16, 18, 31, and 33 was performed. Then, Southern blot hybridizations were performed using type 16, 18, 31 or 33 specific probes to identify the type of HPVs. We found that HPVs were positive in 43 cases out of 64 cases (67.2%, Fig. 1 and Table 2) and that the type of HPVs were either type 16 (in 26 cases, Fig. 2B) or type 18 (in 7 cases, Fig. 2C) but there were no HPV type 31 and 33(data not shown). In 6 cases, double infection by HPV type 16 and 18 was observed (Fig. 2). The incidence of HPVs according to the pathologic diagnosis was 27.3% (3 of 11 cases) in cervical intraepithelial neoplasia (CIN), 82.6% (19 out of 23 cases) in carcinoma in situ (CIS), and 70% (21 out of 30 cases) in invasive cervix cancer, respectively (Table 1). These results indicated that high risk HPVs were involved in the development of cervix cancer. In the following experiments, the cases were divided into 2 groups; HPV positive and negative group.

PCR /SSCP analysis of p53 exons

To screen the p53 gene mutations in each tumor sample, PCR of each exon of p53 (from exon 5 to 9) was performed first and then

non denaturing polyacrylamide gel (6%) electrophoresis was performed. The summary of PCR-SSCP analysis of exons 5 to 9 of p53 tumor suppressor gene was given in Table 2. Autoradiographies from SSCP analysis showed that there were 8 cases that showed mobility shifts (Fig. 3). Among them, 6 cases were HPV positive and 2 cases were HPV negative. These results suggested that contrary to the results obtained from the cell lines derived from cervix cancers, p53 mutation might occur in HPV positive primary tumors.

Sequence analysis

To confirm the result of SSCP analysis, sequence analysis was performed. To get the sequence data from the cases that showed mobility shift in SSCP analysis, double strand sequencing or PCR/direct sequencing was done. We found that case No. 5 contained CAC→CAT transition mutation resulting in silent mutation (histidine to histidine) at codon 168 (data not shown), case No. 12 contained GAT→GAC transition mutation resulting in also silent mutation (aspartic acid to aspartic acid) at codon 184 (Fig. 4A), case No. 15 contained ACG→CCG transversion mutation resulting in threonine to proline substitution at codon 170 (Fig. 4B), case No. 17 contained GGC→AGC transition mutation resulting in glycine to ser-

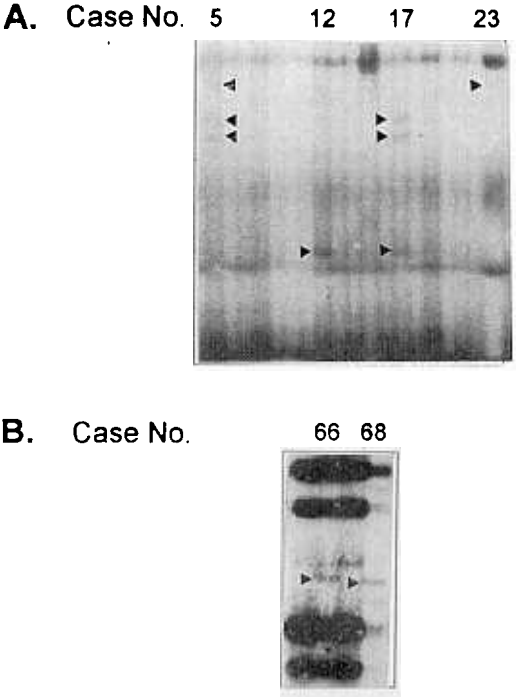

Fig. 3. PCR/SSCP analysis of p53 exon 5 and 8. Nondenaturing PAGE (6%) of ^{32}P-labeled PCR products showed mobility shifts in p53 exon 5 and 8. Numbers written at the top of the figure indicate the case No. Each arrow head indicates shifted bands. A: p53 exon 5, B: p53 exon 8. Human WBC DNA was used for control (data not shown).

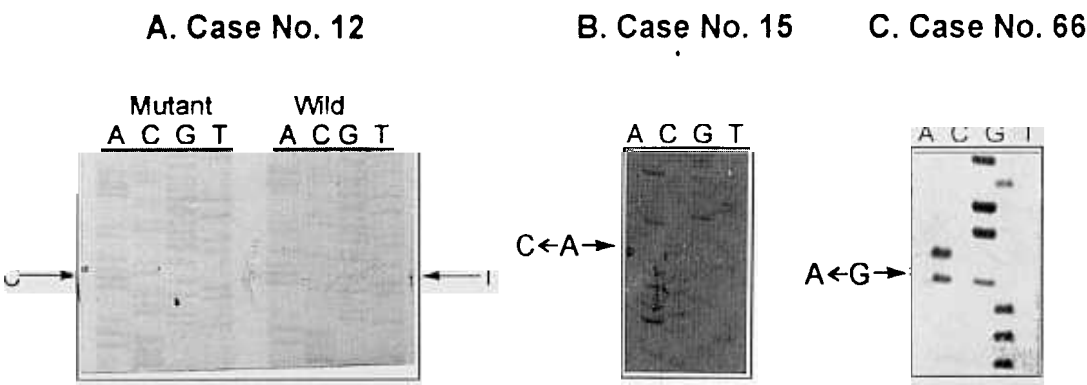

Fig. 4. Examples of sequence analysis of the cases that showed mobility shift on SSCP analysis. PCR products that showed mobility shift on SSCP analysis were sequenced. A: Case No. 12 (codon 184 mutation, GAT→GAC), B: Case No. 15 (codon 170 mutation, ACG→CCG), C: Case No. 66 (codon 271 mutation, GAG→AAG). Each arrow indicates the position of mutated bases.

ine substitution at codon 154 (data not shown), case No. 23 contained CAC→CAT transition mutation resulting in silent mutation (histidine to histidine) at codon 168 (data not shown). In addition, case No. 42 contained CAC→CCC transversion mutation resulting in histidine to proline substitution at codon 297 (data not shown), and case No. 66 contained GAG→

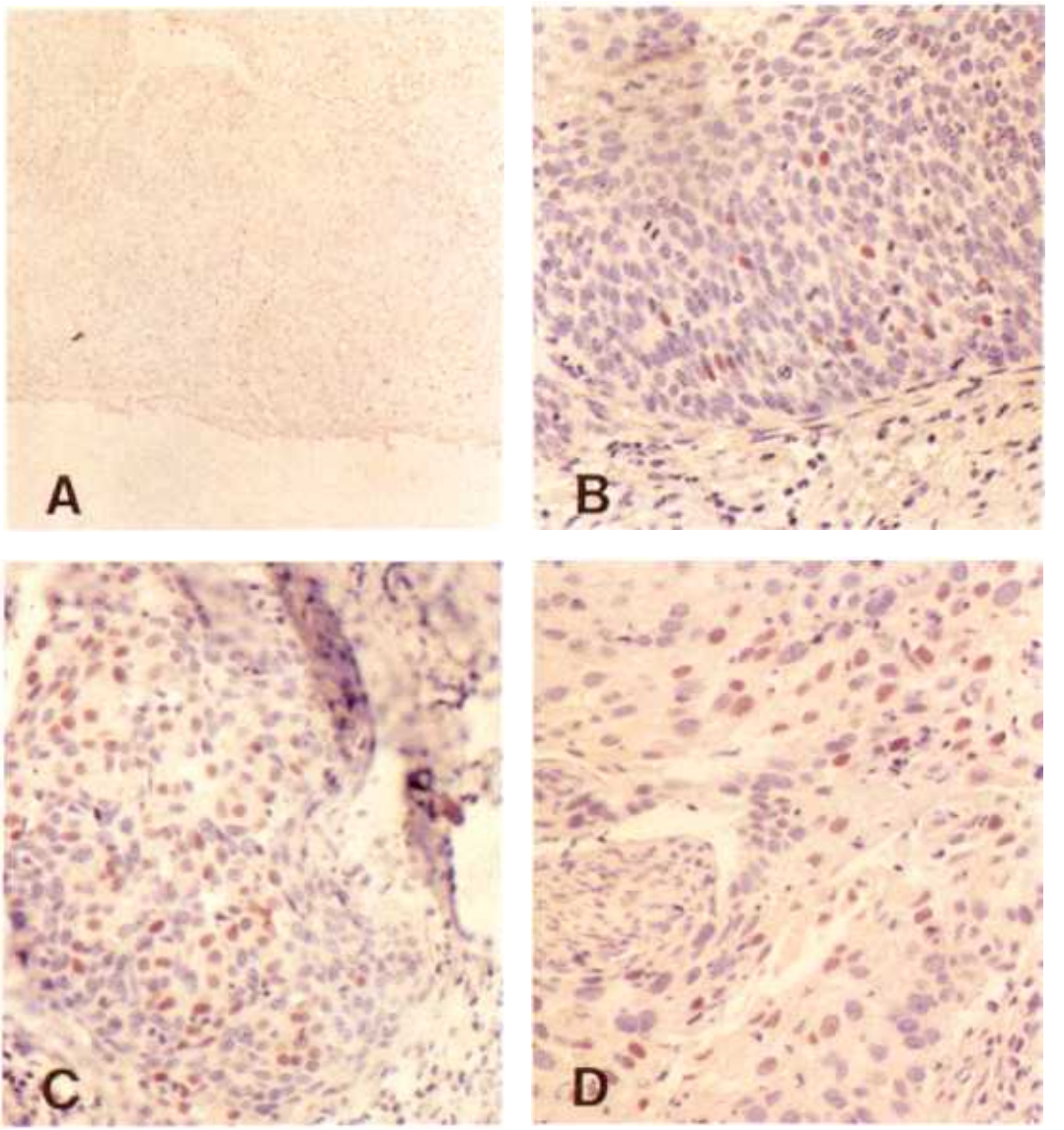

Fig. 5. *Detection of p53 overexpression by immunohistochemical staining.*

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. 14, B: case No. 13, C: case No. 6, D: case No. 9. In case of negative control, primary Ab was omitted (data not shown).

AAG transition mutation resulting in glutamic acid to lysine substitution at codon 271 (Fig. 4C). The sequence data showed that there were no codon preference in the mutations of the p53 gene, indicating that mutagenic events leading to the mutation of p53 gene were heterogeneous.

Immunohistochemical staining

To support the results of the p53 gene mutations in terms of p53 protein expression in the tumor tissues, immunohistochemical staining was done using mouse anti p53 monoclonal Ab.

We found that p53 overexpression was detected in 27 cases out of 64 cases (42.2%). Twenty one cases out of 27 cases belonged to HPV positive group and remaining 6 cases belonged to HPV negative group (Table 3). Six cases out of 27 cases showed strong nuclear

Table 3. p53 overexpression and mobility shifts according to the presence or absence of HPV infection

	HPV positive		HPV negative	
	No. Tested	No. Positive	No. Tested	No. of Positive
HPV detected	64	43(67.2%)	64	21(33.8%)
p53 overexpression	43	21(48.8%)	21	6(28.6%)
Mobility shift	43	6(13.9%)	21	2(9.5%)

Table 4. Site of p53 mutations in current and other studies

Codon	Mutation	HPV status	Reference
133	ATG→CTG	positive	Fujita *et al.*, 1992
143	GTG→ATG	negative	Paquette *et al.*, 1993
154	GGC→AGC	positive	current study
168	CAC→CAT	positive	current study
170	ACG→CCG	positive	current study
173	GTG→GTA	positive	Borrensen *et al.*, 1992
173	GTG→TTG	positive	Crook and Vousden, 1992
175	CGC→CCC	posirive	Crook and Vousden, 1992
181	CGC→CYC	negative	Crook and Vousden, 1992
184	GAT→GAC	positive	current study
240	AGT→ATT	negative	Crook *et al.*, 1992
245[1]	GGC→GTC	negative	Crook *et al.*, 1991
248	CGG→CAG	positive	Pauquette *et al.*, 1992
248	CGG→TGG	positive	Fujita *et al.*, 1992
249	AGG→AGT	negative	Crook *et al.*, 1992
265	CTG→TG[2]	positive	Borrensen *et al.*, 1992
271	GAG→AAG	negative	current study
273[1]	CGT→TGT	negative	Crook *et al.*, 1991
273	CGT→TGT	negative	Crook *et al.*, 1992
273	CGT→CAT	negative	Park *et al.*, 1994
297	CAC→CCC	positive	current study

[1]: Cervical cancer cell lines, [2]: Frame shift

signals in the major population of tumor cells (Fig. 5 C, designated as ++ in Table 2) but in the remaining 21 cases, staining was focal in distribution, with a minority of positive cells (Fig. 5B, designated as + in Table 2). These results suggested that first, contrary to the reports on the status of the p53 gene in HPV positive or negative cervical cancer cell lines, mutation of the p53 gene might occur in HPV positive tumors; second, immunohistochemical staining might be more sensitive method to detect the mutations of p53 than SSCP (such as case No. 9 which showed no mobility shift on SSCP analysis, Fig. 5 D); third, p53 overexpression detected in some cases might not represent the mutation of the p53 gene.

DISCUSSION

Our study showed that HPV infection and mutation of p53 gene plays an important role in the development of cervix cancers as have been reported. However, contrary to the reports on the status of p53 gene in cervix cancer derived cell lines, mutations of the p53 gene might occur in HPV positive primary tumors. The evidences were that 43 out of 64 cases (67.2%) were HPV positive and the types of HPVs detected were oncogenic HPV type 16 and 18 (HPV type 31 or 33 was not detected and type of HPVs were unknown in 4 cases in our experiment) and that the overexpression of p53 suggesting the mutation was detected in 27 cases out of 64 cases (42.2%, 21 in HPV positive cases; 6 in HPV negative cases) and on SSCP analysis, mobility shifts were observed in 8 cases (6 in HPV positive cases; 2 in HPV negative cases).

Inverse correlation between infection of oncogenic HPVs and mutation of p53 gene (Crook *et al.* 1991; Yaginuma and Westphal, 1991; Srivastava *et al.* 1992) is not always true in case of primary cervix tumors. In this study, we found that the mutation of p53 was detected in 8 cases on SSCP analysis and among them, 6 cases were HPV positive and 2 cases were HPV negative and that the p53 overexpression was detected in 27 cases and among them, 21 cases out of 27 cases belonged to HPV positive group and remaining 6 cases belonged to HPV negative group. These results suggested that contrary to the results obtained from the cell lines derived from cervix cancer, mutations of the p53 gene might occur in HPV positive tumors and that some of cervix cancers could develop without involvement of either HPV infection or p53 gene mutation.

Cervix cancer may develop without either infection of oncogenic HPVs or mutation of p53. Although substantial epidemiologic and laboratory evidences links development of cervical carcinoma with HPV infection (Howley, 1991; zur Hausen, 1991) or mutation of p53 (Crook *et al.* 1991; Yaginuma and Westphal, 1991; Srivastava *et al.* 1992), minor proportions of cervix cancer could develop without any evidences of HPV infection or mutation of p53 (Kessis *et al.* 1993; Park *et al.* 1994). Thus, Park *et al.* (1994) screened the amplification of MDM2 as a alternative mechanism for cervix carcinogenesis. In this study, we screened 21 HPV negative cases for the presence of p53 gene mutation and among 21 cases, mutation of p53 gene was detected in 2 cases on SSCP analysis and the overexpression of p53 protein was detected in 6 cases on immunohistochemical staining out of 21 cases. However, all the p53 overexpression might not represent the mutation of p53 gene itself because the staining was focal in distribution with only a minority of positive cells, and these overexpression might not be the cause of these HPV negative cervix cancers.

p53 overexpression may not represent the mutation of p53 gene itself because the half life of wild type p53 is very short and thus, immunohistochemical detection can not detect the wild type p53 but, mutated p53 can be easily detected because mutated p53 could complex with heat shock protein and become metabolically stabilized. However, Lane (1992) reported that rather than indicating the mutation in the p53, the presence of p53 may reflect increased p53 expressions in cells undergoing DNA repair. In this study, we found the overexpression of p53 in 27 cases but in 21 cases among them, the staining was focal in

distribution suggesting that these tumors might not be caused by p53 gene mutation.

However, immunohistochemical detection of p53 might be a more sensitive method than PCR/SSCP to detect p53 gene mutations. Although PCR/SSCP is a very sensitive method to detect the mutations (Gaidano *et al.* 1991; Hayashi, 1991), it might produce false negative results. 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 the experiment.

The p53 gene mutation or p53 overexpression is more frequently found in invasive cervix cancers than CIN or CIS. As reported (Fujimoto *et al.* 1992), p53 gene mutation might be a rather late event in tumor development and might be involved in the progression of bladder cancer. Therefore, in the course of cervix cancer development, HPVs may play a major role and initiate the tumorigenesis and then p53 mutation may occur subsequently in the advanced stage. Crook and Vousden (1992) reported that acquisition of p53 mutation might play a role in the progression of some HPV associated primary cancers.

The mutations of p53 found in this study might not be caused by a 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 cervix cancer investigated in this study. In addition, mutations of p53 found in this experiment were found at codon 154, 168, 170, 184, 271, and 297. The mutations involving these codons have been reported infrequently (Table 4).

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, plays a major role in the development of cervix cancers, but the inverse correlation between the infection of oncogenic HPVs and mutation of p53 gene is not always true in case of primary cervix cancers. In addition, this experiment implicates that another mechanism(s) irrespective of infection of oncogenic HPVs and mutations of p53 gene may be involved in the carcinogenesis of cervix cancers.

REFERENCES

Borrensen AL, Helland A, Nesland J, Holm R, Trope C, Karen J: Papillomaviruses, p53 and cervical cancer. *Lancet 339: 1350-1351, 1992*

Cote RJ, Jhanwar SC, Novick S, Pellicer A: Genetic alterations of the p53 gene are a feature of malignant mesothelioma. *Cancer Res 51: 5410-5416, 1991*

Crook T, Wrede D, Vousden KH: *p53 point mutation in HPV negative human cervical carcinoma cell lines. Oncogene 6: 873-875, 1991*

Crook T, Vousden KH: Properties of p53 mutations detected in primary and secondary cervical cancers suggest mechanisms of metastasis and involvement of environmental carcinogens. *EMBO J 11: 3935-3940, 1992*

Crook T, Wrede D, Tidy JA, Mason WP, Evans DJ, Vousden KH: Clonal p53 mutation in primary cervical cancer: association with human papillomavirus negative tumors. *Lancet 339: 1070-1073, 1992*

El-Deiry WS, Harper JW, O'Conner PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrell M, Hill DE, Wang Y, Wiman KG, Mercer WE, Kastan MB, Kohn KW, Elledge SJ, Kinzler KW, Vogelstein B: WAF1/CIP1 in induced in p53-mediated G1 arrest and apoptosis. *Cancer Res 54:1169-1174, 1994*

El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B: WAF1, a potential mediator of p53 tumor suppression. *Cell 75:817-825, 1993*

Eliyahu D, Raz A, Gruss P, Givol D, Oren M: Participation of p53 cellular tumor antigen in transformation of normal embryonic cells. *Na-*

ture 312: 646-649, 1984

Esrig D, Spruck III CH, Nichols PW, Chaiwun B, Steven K, Groshen S, Chen SC, Skinner DG, Jones PA, Cote RJ: p53 nuclear protein accumulation correlates with mutations in the p53 gene, tumor grade, and stage in bladder cancer. *Am J Pathol 143: 1389-1397, 1993*

Finlay CA, Hinds PW, Tan TH, Eliyahu D, Oren M, Levine AJ: Activating mutations for transformation by p53 produce a gene product that forms an hsc 70-p53 complex with an altered half life. *Mol Cell Biol 8: 531-539, 1988*

Fujimoto K, Yamada Y, Okajima E, Kakizoe T, Sasaki H, Sugimura T, Terada M: Frequent association of p53 gene mutation in invasive bladder cancer. *Cancer Res 52: 1393-1398, 1992*

Fujita M, Inoue M, Tanizawa O, Iwamoto S, Enamoto T: Alterations of the p53 gene in human primary carcinoma with and without human papillomavirus infection. *Cancer Res 52: 5323-5328, 1992*

Gaidano G, Ballerini P, Gong JZ, Inghirami G, Neri A, Newcomb EW, Magrath IT, Knowels DM, Dalla-Favera R: p53 mutations in human lymphoid malignancies: Association with Burkitt lymphoma and chronic lymphocytic leukemia. *Proc Natl Acad Sci USA 88: 5413-5417, 1991*

Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ: The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinase. *Cell 75: 805-816, 1993*

Hayashi K: PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. *PCR Methods Appl 1:34-38, 1991*

Howley PM: Role of the human papillomaviruses in human cancer. *Cancer Res 51: 5019s-5022s, 1991*

Innis MA, Gelfand DH, Sninsky JJ, White TJ: *PCR protocols: A Guide to Methods and Applications.* California, Academic Press, *1989, 153-158*

Kaelbling M, Burk RD, Atkin NB, Johnson AB, Klinger HP: Loss of heterozygosity on chromosome 17p and mutant p53 in HPV-negative cervical carcinomas. *Lancet 340: 140-142, 1992*

Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW: Participation of p53 protein in the cellular response to DNA damage. *Cancer Res 57: 6304-6311, 1991*

Kessis TD, Slebos RJ, Han SM, Shah Keerti, Bosch XF, Munoz N, Hedrick L, Cho KR: p53 gene mutations and MDM2 amplification are uncommon in primary carcinomas of the uterine cervix. *Am J Pathol 143: 1398-1405, 1993*

Knudson AG: Hereditary cancer, oncogenes and antioncogenes. *Cancer Res 45: 1437-1443, 1985*

Lane DP: p53: guardian of the genome. *Nature 358: 15-16, 1992*

Lane DP, Benchimol S: p53: oncogene or antioncogene? *Genes Dev 4:1-8, 1990*

Mazars R, Spinardi L, Ben Cheikh M, Simony-Lafontaine J, Jeanteur P, Theillet C: p53 mutations occur in aggressive breast cancer. *Cancer Res 52: 3918-3923, 1992*

Oliner JD, Kinzler KW, Meltzer PS, George DL, Vogelstein B: Amplification of a gene encoding a p53 associated protein in human sarcomas. *Nature 358: 80-82, 1992*

Olumi AF, Tsai YC, Nicholas PW, Skinner DG, Cain DR, Bender LI, Jones PA: Allelic loss of chromosomes 17p distinguishes high grade from low grade transitional cell carcinomas of the bladder. *Cancer Res 50: 7081-7083, 1990*

Orita M, Suzuki Y, Sekiya T, Hayashi K: Rapid and sensitive detection of point mutations and DNA polymorphism using the polymerase chain reaction. *Genomics 5: 874-879, 1989*

Paquette RL, Lee YY, Wilczynski SP, Karmaker A, Kizaki M, Miller CW, Koeffer HP: Mutations of p53 and human papillomavirus infection in cervical carcinoma. *Cancer 72: 1272-1280, 1993*

Park DJ, Wilczynski SP, Paquette RL, Miller CW, Koeffler HP: p53 mutations in HPV-negative cervical carcinoma. *Oncogene 9: 205-210, 1994*

Phelps WC, Yee CL, Munger K, Howley PM: The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. *Cell 53: 539-547, 1988*

Presti JC, Reuter TG, Fair WR, Cordon-Cardo C: Molecular genetic alterations in superficial and locally advanced human bladder cancer. *Cancer Res 50: 7081-7083, 1990*

Reich NC, Levine AJ: Two distinct mechanisms regulate the level of a cellular tumor antigen. *Nature 308: 199-201, 1984*

Resnick RM, Cornelissen MTE, Wright DK, Eichinger GH, Fox HS, Schegget J ter, Manos MM: Detection and typing of human papillomavirus in archival cervical cancer specimens by DNA amplification with consensus primers. *J Natl Cancer Inst 82: 1477-1484, 1990*

Sambrook J, Fritsch EF, Maniatis T: *Analysis of genomic DNA by Southern Hybridization.* In Sambrook J, Fritsch EF, Maniatis T eds. Molecular Cloning, A Laboratory Manual, 2nd ed.

New York, Cold Spring Harbor Laboratory Press, *1989, 9.31-9.62*

Scheffner M, Munger K, Byrne JC, Howley PM: The state of p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc Natl Acad Sci USA 88: 5523-5527, 1991*

Scheffner M, Munger K, Huibregste JM, Levine AJ, Howley PM: The E6 oncoprotein encoded by human papillomavirus type 16 and 18 promotes the degradation of p53. *Cell 63: 1129-1136, 1990*

Schwarz E, Freese UK, Gissmann L, Mayer W, Roggenbuck B, Stremlau A, zur Hausen H: Structures and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature 314: 111-114, 1985*

Spruck III CH, Rideout III WM, Olumi AF, Ohneseit PF, Yang AS, Tsai YC, Nicholas PW, Horn T, Hemann GG, Steven K, Ross RK, Yu MC, Jones PA: Distinct pattern of p53 mutation in bladder cancers: Relationship to tobacco usage. *Cancer Res 53: 1162-1166, 1993*

Srivastava S, Tong YA, Devadas K, Zou ZQ, Chen Y, Pirollo KF, Chang EH: The status of the p53 gene in human papillomavirus positive or negative cervical carcinoma cell lines. *Carcinogenesis 13: 1273-1275, 1992*

Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL: Genetic alterations during colorectal tumor development. *N Engl J Med 319: 525-532, 1988*

Yaginuma Y, Westphal H: Analysis of the p53 gene in human uterine carcinoma cell lines. *Cancer Res 51: 6506-6509, 1991*

Yokota J, Wada M, Shimosato Y, Terada M, Sugimura T: Loss of heterozygosity on chromosome 3, 13 and 17 in small cell carcinoma and on chromosome 3 in adenocarcinoma of the lung. *Proc Natl Acad Sci USA 84: 9252-9256, 1987*

zur Hausen H, Schneider A: *The role of papillomaviruses in human anogenital cancer.* In Howley PM and Salzman N, eds. *The papovaviridae: The papillomaviruses.* New York, Plenum press, *1987, 245-263*

zur Hausen H: Viruses in human cancers. *Science 254:1167-1173, 1991*