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Immunohistochemical detection of p53 in cervical epithelial lesions with or without infection of human papillomavirus types 16 and 18

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Abstract Using formalin-fixed and paraffin-embedded cervical tissues, we examined infection with human papillomavirus (HPV) types 16 and 18 by Southern blot analysis following polymerase chain reaction (PCR), and the accumulation of p53 protein by immunohistochemistry in 30 cases of normal or metaplastic cervix, 17 cases of cervical intraepithelial neoplasia grade I (CIN I), 20 cases of CIN II, 37 cases of CIN III and 23 cases of invasive squamous cell carcinoma (ISCC). In addition, we examined the ratio of HPV-infected cells by in situ hybridization (ISH) and the alteration of *p53* gene using PCR followed by single-strand conformation polymorphism (PCR-SSCP) in 2 cases of CIN III and 12 cases of ISCC, in which overexpression of p53 was immunohistochemically detected. HPV DNA was detected in 5 cases (16.7%) of normal or metaplastic cervix, 5 cases (29.4%) of CIN I, 9 cases (45.0%) of CIN II, 26 cases (70.3%) of CIN III and 15 cases (65.2%) of ISCC. Positivity for HPV in the groups of CIN III and ISCC was significantly higher than in the normal or metaplastic cervix ($P<0.05$). The accumulation of p53 was not detected in the normal or metaplastic cervix, CIN I and CIN II. High-level p53 accumulation was identified in basal and suprabasal atypical cells in 27.0% (10/37) of CIN III and in carcinoma cells in 43.5% (10/23) of ISCC cases, and low-level accumulation was identified in atypical cells of 35.1% (13/37) of CIN III and in carcinoma cells in 30.4% (7/23) of ISCC cases. The accumulation of p53 was found to coexist with infection by HPV in 17 (46.0%) of 37 CIN III cases and 12 (52.2%) of 23 ISCC cases, and high-level p53 accumulation was more frequently detected in HPV-positive ISCC cases. Either HPV infection or accumulation of p53 was found in 16.7% (5/30) of the cases of normal or metaplastic cervix, 29.4% (5/17) of CIN I, 45.0% (9/20) of CIN II, 86.5% (32/37) of CIN III and 87.0% (20/23) of ISCC cases. These results suggest that the inactivation of p53 function by HPV infection or alteration of p53 protein itself precedes the development of tumours with a fully malignant and invasive phenotype and plays an important role in tumorigenesis in the uterine cervix. ISH study provided no correlation between the degree of immunohistochemical positivity for p53 and the ratio of HPV-positive cells in the same lesions. PCR-SSCP detected the alteration of *p53* gene in at least 4 cases of ISCC, 2 of which were accompanied by HPV infection.

Key words Human papillomavirus · p53 · Cervical neoplasm

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Introduction

Recent molecular biological studies have suggested that infection by human papillomavirus (HPV) is implicated in the pathogenesis of cervical carcinoma. Up to now, 60 and more types of HPV have been identified [16]; among them, types 16 and 18 are most frequently detected in cervical intraepithelial neoplasia (CIN) and cervical carcinoma [7, 17, 34], and these types of HPV are often integrated in the genome of the neoplasm [11, 19, 47]. In vitro studies have shown that two early genes of HPV types 16 and 18, the E6 and E7 open reading frames (ORFs), are capable of transforming cells to immortalized forms [25, 54]. Products from the E6 and E7 ORFs of HPV types 16 and 18 have been shown to form complexes with the normal cellular proteins, p53 or the retinoblastoma gene product Rb, both of which are well known as tumour suppressors, and those complexes result in the inactivation of the suppression function [18, 52]. Scheffner et al. [45] have demonstrated that E6 protein of the oncogenic HPVs binds to the p53 gene product and facilitates the degradation of the p53 protein, suggesting that the selective degradation of the tumour suppressor protein is responsible for cervical carcinogenesis. However, allelic loss or mutation of these genes of *p53* in the genome inactivates tumour suppressor activity and may also contribute directly to the progression of

Table 1 Primers for human papillomavirus (HPV) types 16 and 18 and β-globin genes. Numbers in parentheses represent the length and region of amplification (*bp* base pair)

	Sense primer	Anti-sense primer
HPV16 (140 bp, E6 region)	5′AAGGGCGTAACCGAAATCGGT3′	5′GTTTGCAGCTCTGTGCATA3′
HPV18 (140 bp, E6 region)	5′AAGGGCGTAACCGAAATCGGT3′	5′GTGTTCAGTTCCGTGCACA3′
β-globin (262 bp, exon 1)	5′GGTTGGCCAATCTACTCCCAGG3′	5′TGGTCTCCTTAAACCTGTCTTG3′

cellular transformation [36]. Several investigators have reported that genetic analysis of cervical carcinoma cell lines or primary cervical carcinomas has shown HPV-positive carcinomas to have normal tumour suppressor function but HPV-negative carcinomas contained mutations in these tumour suppressor genes. They suggested that either mutation of tumour suppressor genes or infection by HPV inactivates tumour suppression, resulting in the development of cervical carcinoma [14, 15, 38, 46, 55].

In the present study, using formalin-fixed and paraffin-embedded tissues, we examined the infection of HPV types 16 and 18 by Southern blot hybridization following polymerase chain reaction (PCR) and the accumulation of p53 protein by immunohistochemistry. We studied the normal or metaplastic cervix, CIN, and invasive squamous cell carcinoma (ISCC) in an attempt to clarify the rate of involvement of HPV infection and p53 alteration in the population of these lesions. In addition, we examined the population of HPV-infected cells by in situ hybridization (ISH) and the alteration of *p53* gene using PCR followed by single-strand conformation polymorphism (PCR-SSCP) in several cases of CIN III and ISCC, in which overexpression of p53 was detected immunohistochemically.

Materials and methods

One hundred and ninety one cervical tissue cases fixed with 10% neutral buffered-formalin and embedded in paraffin were selected from specimen files in our department, Fukui Saiseikai Hospital, Maizuru Kyousai Hospital, Komatsu City Hospital, and Kanazawa City Hospital, taken from 1986 to 1993. Six to ten 4 μm-thick sections were cut from paraffin blocks. One section was stained with haematoxylin and eosin and examined microscopically. The other sections were used for viral detection by PCR and for immunohistochemical detection of p53. In addition, several cases of CIN III and ISCC, in which overexpression of p53 was evident immunohistochemically (see Results), were selected to examine the ratio of HPV-infected cells by ISH and the alteration of *p53* gene by PCR-SSCP.

Virus detection by PCR

DNA was extracted from four to eight sections of cervical mucosas or neoplastic lesions obtained on the basis of histological analysis, using the method of Goelz et al. [23] with some modifications. The tissues were deparaffinized with xylene, dehydrated with ethanol and dried. The tissue pellets were treated in 200 μl digestive mixture (500 μg/ml proteinase K; 2% SDS; 10 mM TRIS-HCl, pH 8.0; 150 mM NaCl; 10 mM EDTA) at 37°C overnight. Following extraction with phenol/chloroform, nucleic acids were recovered by ethanol precipitation and dissolved in 41.5 μl sterilized water. PCR was performed on 50 μl of the final reaction volume containing 10 mM TRIS-HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl; 0.001% (w/v) gelatin; 0.2 mM each of dATP, dCTP, dGTP and dTTP; 0.25 μM of each virus primer; and 2.5 U of a thermal stable DNA polymerase (Amplitaq; Perkin-Elmer Cetus, Norwalk, Conn.). The virus primers used in this study are shown in Table 1. Forty rounds of amplification were done in the DNA Thermal Cycler (Perkin-Elmer Cetus) with a 94°C denaturation step (1 min), 55°C annealing step (1 min) and 72°C extension step (1 min). A 10 μl volume of the PCR reaction mixture was applied to 2% agarose gel stained with ethidium bromide. To verify sufficient extraction of DNA from the paraffin-embedded tissues, extracts were also processed in parallel for amplification of a 262-bp segment of the human β-globin gene (β-globin primer set; Takara, Japan) (Table 1). The cases in which the PCR band for the human β-globin gene was visible on agarose gel were subsequently processed for virus DNA analysis by Southern blotting. The PCR products were blotted onto a GeneScreen Plus filter membrane (Du Pont-New England Nuclear, USA). After being fixed by ultraviolet light, the filter was prehybridized at 42°C for 2 h in 5×SSC (1×SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 5× Denhardt's solution (1× Denhardt's solution = 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin), 50% formamide, 1% SDS and 150 μg/ml sonicated salmon sperm DNA. The filter was then hybridized at 42°C for 16~18 h with the above solution containing each virus-specific ^{32}P-labelled probe (1×10^6 cpm/ml) derived from an internal sequence of the amplified product (Table 2), washed three times in a solution of 2×SSC and 1% SDS for 30 min at 42°C, and then subjected to autoradiography.

Table 2 Probes for polymerase chain reaction (PCR) products of HPV types 16 and 18

HPV16	5′CATTTTATGCACCAAAAGAGAACTGCAATG3′
HPV18	5′TGAGAAACACACCACAATACTATGGCGCGC3′

The specificity of the primers and probes used in this study (Tables 1, 2) has been confirmed by other workers [44, 48]. To avoid false positives, we carefully handled all specimens and reagents according to the standard set of recommended protocols [20, 33].

p53 immunohistochemistry and staining evaluation

p53 was detected by the avidin-biotin-peroxidase complex (ABC) method of Hsu et al. [27] using an ABC kit (Dako, Glostrup, Denmark). Briefly, deparaffinized sections were treated for 10 min with 0.3% H_2O_2 to block endogenous peroxidase. The sections were incubated with a polyclonal rabbit antibody to p53 oncoprotein, CM1 (diluted at 1:200, Novocastra Laboratories, Newcastle upon Tyne, UK), at 4°C overnight and sequentially with a biotinylated anti-rabbit antibody (diluted at 1:200; Vector Laboratories, Burlingame, Calif., USA) at room temperature for 30 min, followed by incubation with avidin and biotinylated horseradish peroxidase complex. After development of peroxidase reaction, the tissue was counterstained with methyl green. Rectal carcinoma tissues were used as positive controls. Negative controls were stained simultaneously, using normal rabbit IgG.

Table 3 Primers of exons 5–8 of human *p53* gene. Numbers in parentheses represent the length of amplification

Exon	Sense primer	Anti-sense primer
5 (211 bp)	5′CTCTTCCTGCAGTACTCCCCTGC3′	5′GCCCCAGCTGCTCACCATCGCTA3′
6 (185 bp)	5′GATTGCTCTTAGGTCTGGCCCCTC3′	5′GGCCACTGACAACCACCCTTAACC3′
7 (139 bp)	5′GTGTTGTCTCCTAGGTTGGCTCTG3′	5′CAAGTGGCTCCTGACCTGGAGTC3′
8 (200 bp)	5′ACCTGATTTCCTTACTGCCTCTGGC3′	5′GTCCTGCTTGCTTACCTCGCTTAGT3′

Table 4 Detection of HPV types 16 and 18 in cervical tissues by PCR (*CIN* cervical intraepithelial neoplasia, *ISCC* invasive squamous cell carcinomas, + positive, – negative, (%) percentage of β-globin-positive case, <%> percentage of HPV-positive case

Histological diagnosis	Number of β-globin-positive cases	Number of HPV-positive cases		HPV16+ HPV18–	HPV16– HPV18+	HPV16+ HPV18+
Normal or metaplastic cervix	30	5 (16.7%)		3<60.0%>	1 <20.0%>	1 <20.0%>
CIN I	17	5 (29.4%)	*	1 <20.0%>	3 <60.0%>	1 <20.0%>
CIN II	20	9 (45.0%)	**	7 <77.8%>	0	2 <22.2%>
CIN III	37	26 (70.3%)		18 <69.2%>	3 <11.5%>	5 <19.2%>
ISCC	23	15 (65.2%)		9 <60.0%>	0	6 <40.0%>
Total	127	60 (47.2%)		38 <63.3%>	7 <11.7%>	15 <25.0%>

* Significant difference ($P<0.01$)
** Significant difference ($P<0.05$)

Immunostaining was evaluated by two independent observers (M.A. and Y.O.), and any discrepancies were resolved by joint review. All analysis for p53 immunostaining was performed without knowledge of the patients' clinical history or HPV infection status. Nuclear stainability regardless of cytoplasmic staining was regarded as positive. The intensity of nuclear staining was graded as either absent (0), weak (1), moderate (2) or strong (3). The frequency of nuclear staining was also graded as absent (0), focal (1; <10% of the epithelial cells staining), regional (2; between 10 and 50% of the epithelial cells staining), or diffuse (3; >50% of the epithelial cells staining). By the sum of intensity and frequency grades, cervical epithelial lesions were roughly divided into three groups in terms of p53 accumulation. Lesions with four to six points were categorized as neoplasms with high-level p53 accumulation, epithelial lesions with two or three points were categorized as having neoplasms with low-level p53 accumulation, and lesions with no points were categorized as having no accumulation. This system has been known to correlate reliably with the presence of mutations in exons 5–8 of the *p53* gene [5, 53]. High-level p53 accumulation correlates with mis-sense mutations in exons 5 to 8 of the *p53* gene, whereas low-level p53 accumulatn correlates with deletions, splicing mutants, non-sense mutants or mis-sense mutations outside of exons 5–8 [5], although the biological significance of low-level p53 accumulation is not completely understood [53].

Virus detection by ISH

Twenty-three cases of CIN III and 16 cases of ISCC were analysed for HPV infection using a non-isotopic ISH kit (Patho Gene DNA Probe Assay for Identification of HPV, Enzo, New York, USA). According to the manufacturer's protocol, a dewaxed tissue section was treated in a proteinase K solution at 37°C for 15 min. The tissue section was heat-denatured at 95°C for 10 min and incubated in the hybridization solution containing a biotin-labelled probe cocktail of HPV types 16 and 18. After being washed three times, the slide was processed for detection of DNA-DNA hybrids with a streptavidin-biotynylated horseradish peroxidase complex. HPV 16 probe control slides were used as a positive control.

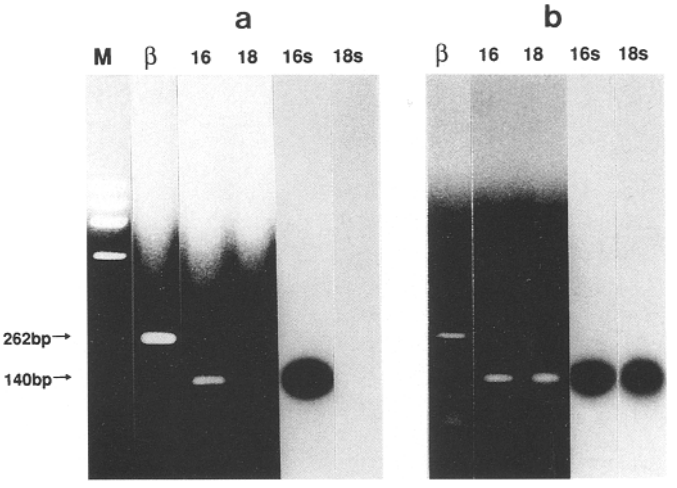

Fig. 1 Detection of human papillomavirus (HPV) types 16 and 18 DNA in cervical intraepithelial neoplasia (CIN) III (**a**) and invasive squamous cell carcinoma (ISCC) (**b**) by Southern blot hybridization following polymerase chain reaction (PCR). *β*, *16* and *18* indicates β-globin, HPV type 16 and HPV type 18 on agarose gel electrophoresis after PCR amplification. *16s* and *18s* indicate Southern blot analysis of the lanes 16 and 18, respectively. *M* indicates a size marker (ØX174 DNA digested with *Hae* III); *bp* base pair

Polymerase chain reaction/single-strand conformation polymorphism

PCR-SSCP [35] of exons 5–8 of *p53* gene was performed in 2 cases of CIN III and 12 cases of ISCC. Two cases of CIN III and 9 cases of ISCC presented high-level p53 accumulation, and 3 cases of ISCC presented low-level accumulation (see Results). DNA (0.1 μg) was applied to the PCR in the mixture (5 μl) described above using a set of two appropriate oligonucleotide primers (Clontech Laboratories, Calif., USA) labelled with γ-^{32}P-ATP

Table 5 Accumulation of p53 in cervical tissues by immunohistochemistry (*n* number of *β*-globin positive cases)

Histological diagnosis	*n*	Number of p53-positive cases		
		Total	Low-level	High-level
Normal or metaplastic cervix	30	0	0	0
CIN I	17	0	0	0
CIN II	20	0	0	0
CIN III	37	23 (62.1%)	13 (35.1%)	10 (27.0%)
ISCC	23	17 (73.9%)	7 (30.4%)	10 (43.5%)

(7000 Ci/mmol, 160 mCi/ml) by polynucleotide kinase. The nucleotide sequences of the primers used are shown in Table 3. The PCR product (5 µl) with 5 µl formamide dye mixture (95% formamide:20 mM EDTA:0.05% bromphenol blue:0.05% xylene cyanol) was heated to 80°C for 5 min, and then 2 µl of the preparation was loaded on a 6% polyacrylamide gel to which 5% glycerol was added. Electrophoresis was performed at 40 W for 3 h at 4°C. The gel was dried on filter paper and exposed to X-ray film at –80°C for 1–12 h with an intensifying screen. Human genomic DNA obtained from colonic mucosa was used as a normal control.

Statistical analysis

Statistical analysis was performed on results of virus infection and p53 accumulation using the chi-square test.

Results

Virus detection by PCR

The *β*-globin gene was amplified in 127 of 191 cervical tissue samples (66.5%): 30 cases of normal or metaplastic tissues, 17 cases of CIN I, 20 cases of CIN II, 37 cases of CIN III, and 23 cases of ISCC. Because these positive tissues were considered to contain DNA of sufficient quality and quantity for amplification, they were examined further and analysed to detect HPV. The results of virus detection in these cases are summarized in Table 4. HPV DNA was detected in 5 cases (16.7%) of normal or metaplastic cervix, 5 cases (29.4%) of CIN I, 9 cases (45.0%) of CIN II, 26 cases (70.3%) of CIN III and 15 cases (65.2%) of ISCC (Fig. 1). There was a tendency for the positive rate in each lesion to become higher with progression. The positivity rates of HPV in the groups of CIN III and ISCC were significantly higher than those in the group of normal or metaplastic tissues ($P<0.01$ and $P<0.05$, respectively). HPV type 16 was more often detected than type 18. Both types were detected in the same tissues of approximately a quarter of the HPV-positive cases, especially in cases of ISCC.

p53 immunohistochemistry

The immunohistochemical results for p53 are summarized in Table 5. p53 was not detected in normal or metaplastic cervix, CIN I or CIN II. However, p53 was detected granularly or diffusely in nuclei of basal and suprabasal atypical cells in 23 (62.1%) of 37 CIN III (Figs. 2, 3) and in nuclei of tumour cells in 17 (73.9%) of 23 ISCC cases (Fig. 4). High-level p53 accumulation was identified in 27.0% (10/37) of CIN III and 43.5% (10/23) of ISCC cases and low-level accumulation was identified in 35.1% (13/37) of CIN III and 30.4% (7/23) of ISCC cases. Extensive positivity for p53 was more frequently observed in cases of ISCC than in CIN III (not statistically significant).

Relation between HPV infection detected by PCR and p53 accumulation detected by immunohistochemistry

The results on accumulation of p53 with relation to HPV infection defected by PCR are shown in Table 6. The accumulation of p53 without HPV infection was detected in 16.2% (6/37: high-level, 2 cases; low-level, 4 cases) of CIN III and 21.8% (5/23: high-level, 2 cases; low-level, 3 cases) of ISCC cases. The accumulation of p53 with HPV infection was identified in 46.0% (17/37: high-level, 8 cases; low-level, 9 cases) of CIN III and 52.2% (12/23: high-level, 8 cases; low-level, 4 cases) of ISCC cases. The number of cases where the accumulation of p53 was detected tended to be higher for those with than for those without HPV infection. However, statistically there was no significant relationship between the HPV infection and the positivity rate or degree of accumulation of p53 in the CIN III and ISCC groups.

Fig. 2a,b p53 immunostaining in CIN III (high-level accumulation). **a** Haematoxylin and eosin stain. **b** Section close to **a**, p53-immunostained. Positive atypical cells extend from the basal layer to the superficial layer of the epithelium. ×280, *bar* = 50 µm

Fig. 3a,b p53 immunostaining in CIN III (low-level accumulation). **a** Haematoxylin and eosin stain. **b** Section close to **a**, p53-immunostained. An atypical cell is positive among the entire thickness of the atypical epithelium. ×280, *bar* = 50 µm

Fig. 4a,b p53 immunostaining in ISCC (high-level accumulation). **a** Haematoxylin and eosin stain. **b** Section close to **a**, p53-immunostained. Positive carcinoma cells are diffusely distributed in tumour cell nests. ×170, *bar* = 50 µm

Fig. 5a,b Detection of HPV DNA by in situ hybridization (ISH). **a** HPV DNA (types 16/18) is observed within the nucleus of superficial epithelial cells. A CIN III case with low-level accumulation of p53. ×280. **b** HPV-positive cells are scattered. An ISCC case with high-level accumulation of p53. ×170, *bar* = 50 µm

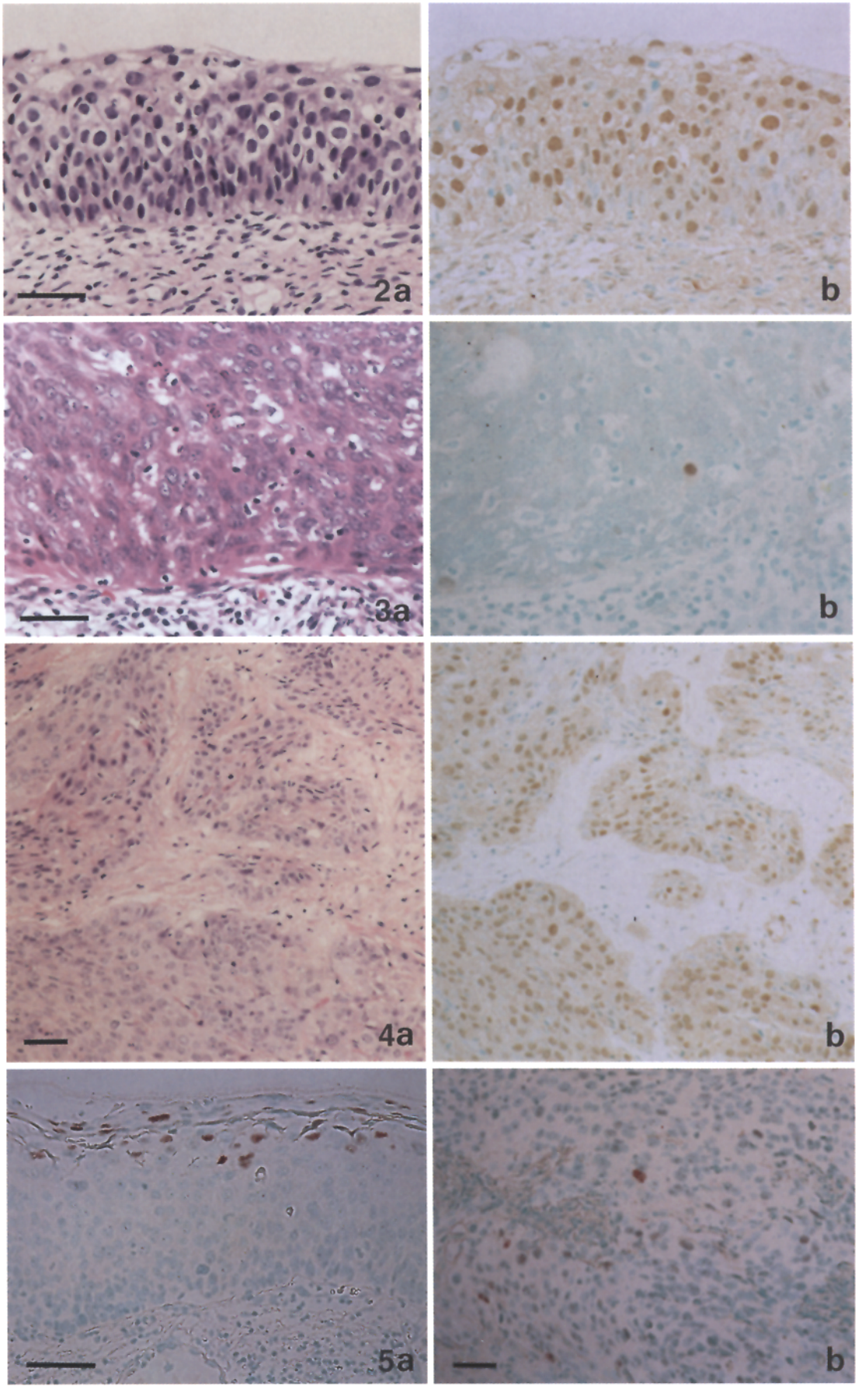
2a
b
3a
b
4a
b
5a
b

Table 6 Accumulation of p53 in relation to HPV infection detected by PCR (*n* number of β-globin positive cases, *N* number of HPV-negative or positive cases, (%) percentage of β-globin positive cases)

Histological diagnosis	(*n*)	HPV-negative cases				HPV-positive cases			
		p53 immunoreactivity				p53 immunoreactivity			
		N	No	Low	High	*N*	No	Low	High
Normal or metaplastic cervix	(30)	25	25 (100%)	0	0	5	5 (100%)	0	0
CIN I	(17)	12	12 (100%)	0	0	5	5 (100%)	0	0
CIN II	(20)	11	11 (100%)	0	0	9	9 (100%)	0	0
CIN III	(37)	11	5 (13.5%)	4 (10.8%)	2 (5.4%)	26	9 (24.3%)	9 (24.3%)	8 (21.7%)
ISCC	(23)	8	3 (13.0%)	3 (13.0%)	2 (8.8%)	15	3 (13.0%)	4 (17.4%)	8 (34.8%)

Table 7 Detection of HPV DNA by in situ hybridization (ISH) in p53-positive cases

Histological diagnosis	(*N*)	Number of p53-positive cases		Number of HPV (16/18)-positive cases	Ratio of HPV-positive cells-		
					<1%	1–10%	10%<
CIN III	(23)	Low-level	13	3	1	1	1
		High-level	10	4	3	1	0
ISCC	(16)	Low-level	7	2	1	1	0
		High-level	9	2	1	0	1

Table 8 Results of immunohistochemical and PCR-SSCP analyses of *p53* with relation to HPV infection detected by PCR and ISH (+ positive, – negative, *NA* not amplified)

Case number	Histological diagnosis	Immunohistochemistry of p53	PCR-SSCP shift of *p53*				HPV types detected by PCR	HPV (16/18) infection detected by ISH (positive cells)
			Exon 5	6	7	8		
1	CIN III	High-level	–	–	–	–	16	+ (<1%)
2	CIN III	High-level	–	–	–	NA	–	–
3	ISCC	High-level	–	–	–	–	16+18	+ (14.5%)
4	ISCC	High-level	–	–	–	–	16+18	–
5	ISCC	High-level	+	–	–	NA	16+18	–
6	ISCC	High-level	–	+	–	NA	16	+ (<1%)
7	ISCC	High-level	–	–	–	–	16	–
8	ISCC	High-level	–	–	–	NA	16	–
9	ISCC	High-level	–	–	–	NA	16	–
10	ISCC	High-level	+	–	–	NA	–	–
11	ISCC	High-level	–	+	–	NA	–	–
12	ISCC	Low-level	–	–	–	NA	16	–
13	ISCC	Low-level	–	–	–	NA	–	–
14	ISCC	Low-level	–	–	–	–	–	–

Virus detection by ISH

Table 7 shows the results of virus infection detected by ISH in available cases of CIN III and ISCC, which were immunohistochemically positive for p53. HPV (types 16/18) was detected in 7 cases of CIN III (30.4%) and in 4 cases of ISCC (25.0%). Positive signals were observed predominantly within the nucleus of superficial epithelial cells in CIN III (Fig. 5a), whereas in ISCC positive cells were scattered or focally accumulated (Fig. 5b). The proportion of HPV-positive cells was on average less than 15% in the lesions. Six of 11 cases had less than 1% of positivity. No correlation was evident between the degree of immunohistochemical positivity for p53 and the percentage of HPV-positive cells in the same lesions.

Polymerase chain reaction/single-strand conformation polymorphism

When formalin-fixed, paraffin-embedded tissue samples were used, all cases examined showed visible bands of amplification in exons 5–7 of *p53* gene, but exon 8 was amplified in 5 of 14 cases (Table 8). Samples were regarded as positive if their mobility pattern differed from that of the control. In CIN III, an abnormal mobility shift was not observed. In ISCC, two additional bands with mobility shifts were observed in exon 5 of *p53* in 2 cases and in exon 6 of *p53* in 2 cases (Fig. 6), all of which showed high-level accumulation of p53 by immunohistochemistry and 2 of which were infected with HPV.

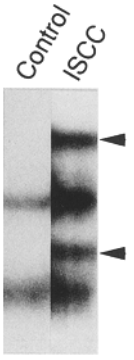

Fig. 6 PCR-SSCP analysis of *p53* gene in ISCC. Two additional bands with mobility shifts (*arrows*) are observed in exon 5 (case 5)

Discussion

Using Southern blot analysis following PCR, infection of HPV types 16 and 18 was detected in 29.4% of CIN I, 45.0% of CIN II, 70.3% of CIN III, and 65.2% of ISCC cases. The prevalence rates of HPV increased with the severity of the lesions. These results are comparable to those in recent studies using PCR as a detection system. HPV 16 and/or 18 DNAs have been detected in 20–62.5% in CIN I, 40–60% in CIN II, 48.4–77.8% in CIN III, and 53.0–93.1% in ISCC cases [1, 2, 12, 13, 29, 31, 39, 42, 48, 51, 56].

Several investigators have reported that HPV type 18 is more frequently associated with a more aggressive form of cervical carcinoma and with a greater likelihood of lymph node metastasis than tumours containing HPV type 16 [2, 3, 32]. However, our study provided no firm evidence for a close association between tumour invasiveness and the type of infection with HPV. The population of HPV type 18 among the groups of patients with CIN or ISCC did not differ significantly. Instead, ISCC contained of relatively high proportions of both types of HPV. Infection with more than one HPV type may be a high risk for tumour progression, because, as mentioned by Arends et al. [2], multiple factors derived from two or more types of HPV interact with many different sites in cellular regulatory genes, possibly inducing the deregulation of cellular differentiation and proliferation with high effectivity. However, other possibilities are not excluded; multiple HPVs might easily infect ISCC cells or multiple HPVs might be derived not only from ISCC cells but also from non-neoplastic cells adjacent to ISCC.

HPV was also detected 16.7% of cases with a normal or metaplastic cervix in this study. Several previous studies using the PCR technique have found HPV infection in women without abnormal cytology, although the viral prevalence cited in these studies varied (2–30%) [13, 49, 51, 56]. The higher sensitivity of PCR may allow the identification of HPV in many cases prior to manifestation of either cytological or histological abnormality. Practically, however, it is not clear whether this positivity is related to an inactive latent infection or viral function insufficient to cause disease. Alternatively, HPV type 16 in the cervix without association of atypical cells is stated to be a less oncogenic subtype (HPV 16b) [50]. Further study is necessary to elucidate the significance of HPV infection in such cases.

Some proportions of CIN and ISCC lesions were negative for HPV DNA. There is a possibility that these cases had HPV at copy numbers below the level of detection or infected with other or undetermined types of HPV [49]. Thus, further molecular studies such as PCR analysis using other types of HPV primers are required. Neverthless, HPV-negative cervical neoplasms exist even though the incidence is low [2, 12, 13, 29, 31, 39, 42, 48, 51, 56]. In such cases, some cellular genes such as c-*myc* and H-*ras* proto-oncogenes are presumably activated and predominantly involved in carcinogenesis, as Riou et al. [40, 41, 43] suggested.

Our immunohistochemical study of p53 in cervical tissues showed that p53 protein was detected in 62.1% of CIN III and 73.9% of ISCC cases. No staining was observed in the normal or metaplastic cervix, CIN I or CIN II. There are diverse data on p53 immunostaining. Bosari et al. [6] examined the immunolocalization of p53 protein on formalin-fixed, paraffin-embedded cervical tissues with the monoclonal antibody PAb1801 and documented that suprabasal p53 immunoreactivity was observed in 25% of high-grade squamous intraepithelial lesions (CIN II and CIN III) and 72% of invasive squamous cell carcinomas. Surprisingly, they claimed positive p53 immunoreactivity, though confined to basal cell layer, in 74% of chronic cervicitis and in all cases of low-grade squamous intraepithelial lesions (CIN I). Holm et al. [26] identified p53 protein in 7% of squamous cell carcinoma in situ (CIN III) and 62% of ISCC using both monoclonal and polyclonal anti-p53 antibodies (PAb 1801 and CM1) and detected no staining in normal and dysplastic cells. Although the rates of p53 positivity in the cervical neoplasms differ somewhat between the previous two reports and the present study, all of them at least indicated that p53 immunoreactivity is commonly detected in cases of high-grade CIN and ISCC. The wild form of p53 protein has a very short half-life [24], so it cannot be detected immunohistochemically. However, overexpressed or mutated p53 proteins have a longer half-life [21] and can be recognized by immunohistochemistry. Burns et al. [9] investigated human primary SCCs and their cell lines, and reported good correlation between the presence of elevated immunoreactivity of p53 protein and the presence of mutations within the coding region of the gene. In all SCC cell lines where elevated levels of p53 protein were detected, mis-sense mutations or inframe deletions within the coding region were found. Other investigators have reported similar findings [4, 10, 28]. Thus, it has been widely accepted that the immunoreactive positivity of this protein indicates *p53* gene mutations. However, more recently, it was reported that the presence of p53 immunoreactivity is not always accompanied by gene

mutations. Mutations of *p53* gene occur very infrequently in cervical carcinoma [22, 30] compared with the immunohistochemical positivity for p53 protein [6, 26]. In the present study, abnormal bands with mobility shifts in *p53* gene were detected in only 4 of 11 cases which showed immunohistochemically high-level accumulation of p53, although the number of cases examined is fractionary and the aberration of exon 8 of *p53* was not completely denied in some cases. Therefore, taking recent and present studies together, the possibility remains that in CIN III and ISCC the immunoreactivity of *p53* protein results from the abnormal expression or degradation of p53 protein rather than from the alteration of *p53* gene itself.

Our finding that p53 positivity was found equally in advanced carcinoma and in severe dysplastic lesions suggests that the alteration of p53 protein precedes the development of malignant tumours of invasive phenotype. This speculation is supported by the report of Oka et al. [37] revealing that p53 positivity in cancer cells is not a predictive factor for prognosis in cervical cancer.

Several authors dealing with cervical carcinoma cell lines have reported that HPV-positive carcinomas express wild-type p53, whereas HPV-negative carcinomas express mutated p53 [14, 46, 55]. Scheffner et al. [45] have demonstrated that the E6 protein of the oncogenic HPVs binds to facilitate degradation of p53 protein and inactivates its function in tumour suppression. It is therefore postulated that loss of p53 function by either mutations of *p53* gene or binding to HPV coding proteins is important in the pathogenesis of cervical carcinomas. In the present study, either high-risk HPV infection or p53 accumulation was detected in most cases of CIN III (32/37, 86.5%) and ISCC (20/23, 87.0%) and the incidence in CIN III or ISCC was high compared with that in other groups. Our observations suggest that the inactivation of p53 function by HPV infection or altered expression of p53 protein occurs mostly in advance of the development of invasive carcinoma, which supports the hypothesis that the abnormality of p53 protein is closely linked with cervical carcinogenesis. Although no inverse correlation between the degree of p53 immunoreactivity and the ratio of HPV-positive cells was evident in the same lesions, our studies cannot deny an inverse correlation in the same cells.

The accumulation of p53 was found to coexist with infection by HPV in 45.9% of CIN III cases and 52.2% of ISCC cases. As mentioned above, the wild type of p53 protein bound with the E6 protein of oncogenic HPVs is rapidly degraded by an ATP-dependent, ubiquitin-mediated protease system [45]. However, it has been shown that mutated p53 proteins form stable complexes with heat shock protein 70 and extend their half-life [21]. Therefore, in vivo, mutated p53 proteins complexed with other proteins such as heat shock proteins may not be completely degraded by the protease system involving the gene products of HPV and can be detected in immunohistochemical studies. Alternatively, mRNA of mutated *p53* may be overexpressed in some cases of CIN III and ISCC, as demonstrated in a few SCC cell lines [10] or in primary lung carcinomas [8].

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