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Detection of Human Papillomavirus DNA in Esophageal Carcinomas by Polymerase Chain Reaction and in Situ Hybridization, and Its Relation to an Overexpression of p53 Antigen

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Abstract : Human papilloma viruses (HPVs) are associated with various epithelial lesions, especially squamous cell carcinomas. We studied 95 formalin-fixed, paraffin-embedded esophageal surgical sections to elucidate the relationship between HPV and esophageal carcinoma, using 6 cases of non-neoplastic benign lesions for comparison purposes. The presence of HPV-16, 18 and 33 was analyzed using DNA sequencing with type-specific primers containing E6 regions by polymerase chain reaction (PCR) and In situ hybridization (ISH). No HPV DNA was found in the benign lesions. HPV DNA was detected in 18 cases of esophageal carcinoma using the PCR method ; HPV-16 in 9 cases (50%), HPV-18 in 16 cases (88.9%), and HPV-33 in 3 cases (16.7%). HPV-16 and 18 were detected together in 6 of 18 cases (33.3%), and all 3 types of the HPVs were detected together in 2 of 18 cases (11.1%) within the same tissue specimens. A total of 17 cases with amplified HPV genomes showed pan HPV DNA signals by ISH. The HPV was diffusely detected within the solid nests of the carcinoma and within the dysplastic lesions. When using ISH, however, utilizing type-specific probes, only a small number of positive cases (3 cases of type-16, 3 cases of type-18 and 2 cases of type-33) were found. The expression of p53 protein was detected in 56 out of 95 cases (58.9%). In addition, 31 out of 77 cases (40.5%) without any HPV DNA infection showed a strong p53 expression and all 21 cases of dysplastic lesions without HPV showed a strong p53 expression. However, only 2 out of 18 HPV DNA-positive cases showed a diffuse expression of p53 protein. The other 16 cases showed a weak p53 expression and 4 dysplastic lesions with the HPV genome showed a negative expression for p53 protein. We investigated the relationship between the HPVs and p53 expression in the esophageal carcinoma. Although in our series, no significant correlation was observed between HPV DNA and the mutant p53 expression, a strong p53 expression was observed in cases without HPV DNA infection. It was suggested that the relationship between HPV and p53 in the carcinogenesis.

Key words : esophageal carcinoma, Human papilloma virus, in situ hybridization, p53

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Introduction

Human papilloma viruses (HPVs) are known to be the cause of papillomas, condylomatous lesions, and koilocytosis in the genital tract and epidermis. They are members of a group of small DNA viruses and can be classified into over 73 different subtypes. It has recently become possible to detect even a few copies of the target sequences in given samples by using polymerase chain reaction (PCR), which is the most sensitive method so far developed. Using progressive DNA detection techniques such as PCR, Southern blot hybridization (SBH), and in situ hybridization (ISH), experimental data have suggested a close association between HPVs and malignant neoplasms, namely human epithelial lesions at various sites.[1)-5)] In particular, the DNA of HPV-16, 18, 33 and 35 has frequently been detected in carcinomas. Over 90% of the carcinoma tissue specimens of the uterine cervix show such viruses.[2)-6)] Although in 1982 Syrjaeen observed the morphological changes associated with HPVs in 40% of esophageal carcinomas and papillomas,[7)] it was not possible to confirm the presence of HPVs within the carcinoma cells until the DNA of HPVs was detected recently in esophageal carcinomas by PCR and/or ISH methods.

In South Africa and China, which are both high-risk areas for esophageal carcinoma, HPV DNA has been detected in approximately 70% of fixed esophageal carcinoma tissue specimens.[8)-14)] In other countries, the rate lies between 10% and 60%.[10)15)-18)] As a result, substantial evidence has now been accumulated to support the concept that an infection is involved in the pathogenesis of esophageal squamous cell carcinoma. The E6 and E7 open reading frame (ORF) proteins encoded by the so-called high-risk types of HPV, HPV-16 and 18, are regularly expressed in HPV-positive carcinoma. E6 in particular is able to combine with p53 protein, and this pathway helps promote p53 dissolution.[17)18)] This complex does not have the normal function of p53 protein in regulating cell proliferation. Because of this association, p53 degeneration may be associated with tumor genesis. This study thus attempted to investigate both the ratio of HPV DNA involvement in esophageal carcinoma, by PCR screening, as well as the localization of HPV DNA, by ISH. In addition, p53 protein was also immunohistochemically studied, in order to further elucidate the relationship between HPV infection and p53 expression.

Materials and Methods

Patients

Ninety-five surgical specimens from cases of primary esophageal carcinoma were obtained from the Gastrointestinal Tract Registry of the First Department of Pathology, Fukuoka University. All cases had undergone an .esophagectomy between 1987 and 1996. In all cases, the histological and clinical classification was made using the Guidelines for the 8th edition of Clinical and Pathologic Studies on Carcinoma of the Esophagus established by the Japanese Society for Esophageal Disease, 9th edition (1999). All obtained specimens were fixed in 20% formalin and embedded in paraffin wax. Routine histological examinations, including hematoxylin and eosin staining were performed and the representative blocks of central and peripheral regions were selected for further study. Six cases of benign lesions, in which the patients had all undergone surgery because of stenosis and/or ulcerative lesions, were used as a control.

DNA extraction from the clinical samples

DNA was extracted using the same method as described in a previous report[19)]: formalin-fixed, paraffin embedded specimens were cut into 10-μm thick slices, deparaffinized and then precipitated with xylene and alcohol. The samples were incubated with buffer (Tris-EDTA, pH 9.0) containing 0.1% proteinase K (Wako, Japan) at 55℃ for several days. After the tissue specimens were dissolved, they were incubated at 95℃ for 5 min. The extracted DNA was preserved at 4℃. Positive control DNA was extracted from Hela cells containing HPV-18 DNA.

Polymerase chain reaction

The same oligonucleotide primers and probes as previously described by Shimada et al.[6] were used (Table 1). Amplification of target HPV DNA from the esophageal tissue specimens was performed by using the Heat Soak and Hot Start PCR method. These primers and probes corresponded to sequences homologous to the E6 open reading frames of HPV-16, 18, and 33.[6] The extracted cellular DNA was denatured at 95℃ for 30 min, and then incubated PCR buffer was added: the PCR buffer contained 0.2 mMol of dNTP (i.e. dATP, dCTP, dGTP, and dTTP) mixture, 10 μl of 10× extender buffer, 5 U of Thermus aquaticus (Taq) extender, and 5 U of and 0.5 μMol of each primer. To amplify the HPV DNA, 40 cycles of denaturation (94℃, 1 min.), annealing (45℃, 2 min), and extension (72℃, 2 min) were performed. To analyze the DNA amplification, electrophoresis was performed on 4% agarose and samples were stained with ethidium bromide. After electrophoresis the DNA was examined by UV light, before being transferred to a nylon filter (Pall Biosupport Division, Port Washington, NY, USA) and hybridized to 32P end-labeled HPV type-specific oligonucleotide probes. The filters were then washed and autoradiographed with X-ray film for either 24 hrs or 1 week at −80℃ (Figure 1).

In situ hybridization

In situ hybridization was performed using digoxigenin-labeled probes for pan-HPV DNA (AP-4010-pHPV) and type specific proves for HPV-16 (AP-4016-pHPV), 18 (AP-4018-pHPV) and 33 (AP-4033-pHPV) (Kreatec Diagnostics, Amsterdam, Netherlands. 2- or 3-μm thick paraffin sections were deparaffinized in xylene, dehydrated in ethanol, and air dried. Next, the sections were predigested with 0.8% pre-warmed pepsin/0.01 N HCl for 10 min. at 37℃. After pre-hybridization with Pre-hybridization solution (Kreatec Diagnostics, Amsterdam, Netherlands), simultaneous denaturation of the cellular DNA and of each of the

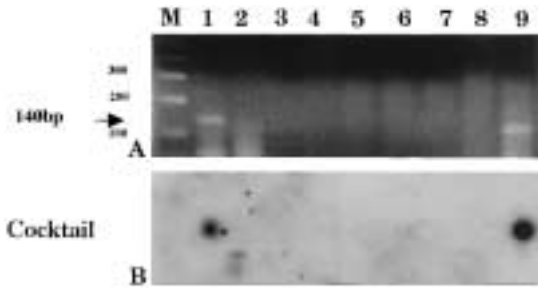

Fig. 1. Amplification and detection of HPV DNA in esophageal carcinomas. PCR was carried out using cocktail primers (Type-16, 18 and 33). Cellular DNA was isolated from Hela cells (lane 1), normal placenta (lane 2), non-neoplastic esophageal mucosa (lanes 3-5), and esophageal carcinoma (lanes 6-9).
(A) After amplification, a 10-μl aliquot of the reaction mixture was subjected to electrophoretic analysis on 4% agarose and stained with ethidium bromide.
(B) After an electrophoretic analysis, the amplified DNA was transferred to a nylon filter and hybridized to 32P end-labeled HPV cocktail probes (16, 18 and 33).

Table 1. Sequences of Oligonucleotide Primers and Probes

Primer	sequences (5'-3')	genomic location
p16-1	AAGGGCGTAACCGAAATCGGT	26-46
p16-2R	GTTTGCAGCTCTGTGCATA	147-165
p18-2R	GTGTTCAGTTCCGTGCACA	154-172
p33-2R	GTCTCCAATGCTTGGCACA	152-170
Probe		
pB16-I	CATTTTATGCACCAAAAGAGAACTGCAATG	77-106
pB18-I	TGAGAAACACACCACAATACTATGGCGCGC	84-113
pB33-I	CATTTTGCAGTAAGGTACTGCACGACTATG	82-111

digoxigenin-labeled HPV probes was carried out for 5 min at 96℃, and then the sections were hybridized overnight at 37℃. Next, hybridization was detected by anti-digoxigenin antibody using the alkaline phosphatase-anti-alkaline phosphatase method. Finally, the sections were immersed in nitroblue tetrazolium chloride (NBT) -5-bromo-4-chloro-3- indolylophosphate (BCIP) substrate for 2-4 hrs in a dark box at room temperature. The reaction was terminated by washing in Tris-HCL/EDTA (pH 9.5), and then the sections were counter-stained with methyl green.

Immunohistochemichal staining for p53 antibody

Anti-p53 protein monoclonal antibodies of Do-7 (DAKO, Kyoto, Japan) and PAb-1801 (Oncogene Science, NY, USA) were used to detect p53-protein. Deparaffinized tissue sections in citrate acid buffer were autoclaved for 10 min at 121℃, and the avidin-biotin complex method was used to detect the antigen. The nuclei were stained in the cells which showed a positive reaction. We classified specimens as negative if less than 5% of the cells showed positivity for p53 protein, and we classified the positive patterns into 3 types; 1) sporadic pattern (+); a small number of isolated positive cells were scattered throughout the lesion, 2) focal pattern (++); the positive cells were aggregated in the focal area(s) of the lesion, and 3) diffuse pattern (+++); nuclear positive cells existed in most areas of the lesion.[20]

Statistical analysis

A statistical analysis was performed using StatView-J 5.0 statistical software package for Power Macintosh (SAS Institute). Differences the between groups were analyzed using the Chi square test and Fisher's exact test. Significance was assumed when P values were <0.05.

Results

Clinicopathological findings

We investigated 95 cases of primary esophageal carcinoma (80 males, 15 females, ranging in age from 44 to 81 years, mean 63.9 years) with 6 benign control cases (3 males, 3 females, ranging in age from 16 to 78 years, mean 49.7 years) (Table 2). The carcinomas consisted of well differentiated squamous cell carcinoma (SCC) in 17 patients, moderately differentiated SCC in 49, poorly differentiated SCC in 21, adenosquamous carcinoma in 4, basaloid-squamous carcinoma in 2, mucoep-idermoid carcinoma in 1, and small cell carcinoma in 1.

The histological stage was stage 0 in 10 patients, stage Ⅰ in 7, stage Ⅱ in 3, stage Ⅲ in 39, stage Ⅳ in 30, and unknown in 7. The foci of epithelial dysplastic lesions in adjacent areas were found in 25 out of 95 cases (26.3%). These dysplastic lesions were positive for the HPV DNA sequence in 4 out of 25 cases (16.0%) and positive for p53 protein in 21 out of 25 cases (84.0%) (Table 3).

PCR analysis

We first performed a control study of PCR with HPV-18 DNA extracted from Hela cells. The results are shown in Figure 2. Based on these results a fragment consisting of approximately 140 base pair (bp) fragments was amplified by PCR. We next investigated 95 cases of esophageal carcinoma and 6 cases of benign lesions. 18.9% (18/95) of these cases had amplified HPV genomes, but the benign lesions had no HPV genomes. The positive cases with type-specific oligonucleotide probes are summarized in Table 4. HPV-16 was detected in 9 out of 18 (50%), HPV-18 was found in 16 out of 18 (88.9%), while HPV-33 was observed in 3 out of 18 (16.7%) HPV-positive cases. In 8 cases, 2 or 3 types of HPV DNA were detected within a single tissue specimen; both HPV-16 and 18

Table 2. Clinical Findings

Age (years old)	Control	Carcinomas
mean	49.7	63.9
range	16-78	44-81
Sex		
Male	3	80
Female	3	15

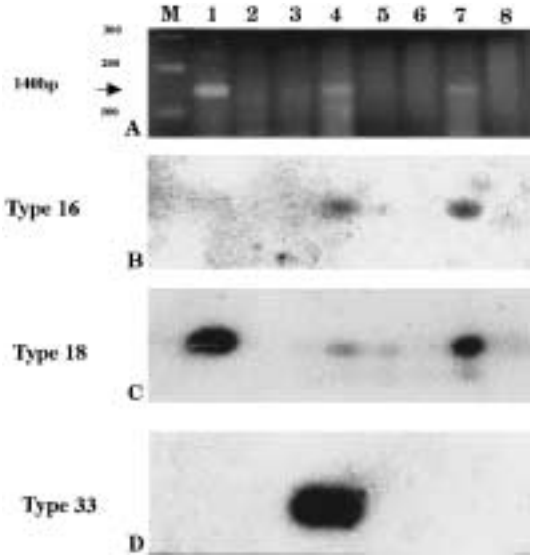

Fig. 2. Type-specific detection of HPV DNA. (A) After PCR analysis using cocktail primers and probes, the positive cases were selected. Cellular DNA was isolated from Hela cells (lane 1) and esophageal carcinomas (lanes 2-8). The amplified DNA was hybridized by specific probes, pB16-I (B), pB18-I (C), and pB33-I (D).

were detected together in 6 cases (33.3%), and HPV-16, 18, and 33 were all detected together in 2 cases (11.1%).

In situ hybridization with HPV DNA probes

In cases with amplified HPV genomes by PCR., we used the ISH method to investigate the localization of HPV DNA. A total of 94.4% (17/18) cases were positive for HPV DNA with the pan-HPV probe. In these cases, 3 cases were positive for HPV-16, 3 were positive for HPV-18, and 2 were positive for HPV-33 utilizing type-specific probes (Table 5). In 2 cases, 2 or 3 types of HPV DNA coexisted within a single tissue specimen; HPV-16 and 18 were both detected in one case, and HPV-16, 18, and 33 were all detected in one case. These findings were con-

Table 4. Results of PCR

HPV-positive	
Total	18 (=n)
Type-16	9 (50.0%)*
Type-18	16 (88.9)*
Type-33	3 (16.7)*

*Including double or multiple infectious cases.

Table 3. Histological Findings

Histologic type		HPV-positive	p53 protein-positive
Well	17	3 (17.6%)	10 (58.8)
Moderately	49	8 (16.3)	28 (57.1)
Poorly	21	6 (28.6)	13 (61.9)
Adenosquamous	4	1 (25.0)	2 (5.0)
Basaloid-squamous	2	0 (0.0)	1 (5.0)
Mucoepidermoid	1	0 (0.0)	1 (1.0)
Small	1	0 (0.0)	1 (1.0)
Dysplasia*	25	4 (16.0)	21 (84.0)
Clinical stages			
Unknown	6	0 (0.0)	3 (5.0)
0	10	2 (2.0)	5 (5.0)
I	7	0 (0.0)	2 (28.6)
II	3	0 (0.0)	2 (66.7)
III	39	10 (25.6)	27 (69.3)
IV	30	6 (2.0)	17 (56.7)

*Epithelial dysplasia existed in the adjacent carcinoma.

sistent with the results of a PCR analysis. A positive signal was strongly observed within the nuclei of the carcinoma cells, but was never detected in the normal esophageal mucosa, although it was detected in the dysplastic mucosa either overlying or adjacent to the HPV-DNA positive carcinoma (Figure 3). The distribution of positive-signal patterns of the pan-HPV probe was diffuse within the solid nests of carcinoma cells, as were the patterns of the other type-specific probes (HPV-16, 18, and 33).

Immunohistochemical study for p53

Of the 95 cases, 58.9% (56/95) showed a positive reaction with PAb 1801 or Do-7 antibody and the signals were observed within the nucleus of the carcinoma cells. The findings are summarized in Table 6. This positive nuclear reaction was observed in the basal layer of dysplastic lesions or occasionally in the non-neoplastic lesions around the carcinoma (Figure 4). 40.3% of the lesions (31/77) were diffusely positive, 13.0% (10/77) were focally positive, 6.5% (5/77) were sporadically positive, and 40.3% (31/77) were negative in the HPV DNA-negative cases. In the 18 HPV DNA positive- cases, 44.4% of the lesions (8/18) were negative for an overexpression of p53 protein. The distribution of positive cells was sporadic in 5.5% (1/18), focal in 38.9% (7/18), and diffuse in only 2 cases (11.1%). In these cases, HPV DNA-positive carcinoma cells were negative for the expression of p53 protein. A statistical analysis was performed between the cases where the HPV DNA sequence was detected and p53 overexpression, but no significant association was observed. Dysplastic lesions adjacent to a carcinoma showing the HPV DNA sequence were negative or weakly positive for p53 protein, whereas the dysplastic lesions adjacent to a carcinoma without the HPV DNA sequence focally or even more strongly positive for p53 protein (Figure 5).

Table 5. Results of In Situ Hybridization

Types	Total
pan HPV	17 (94.4%)
16*	3
18*	3
33*	2

n=18 : HPV DNA sequence-positive cases by PCR method.

*Including double or multiple infectious cases.

Table 6. Relationship between HPV DNA and p53 Protein expression in 95 cases of Carcinoma

p53	HPV-negative	HPV-positive
negative	31 (40.3%)	8 (44.4)
positive	46 (59.7)	10 (55.6)
sporadic	5 (10.9%)	1 (10.0)
focal	10 (21.7)	7 (70.0)
diffuse	31 (67.4)	2 (20.0)
	(n=77)	(n=18)

Discussion

Previous studies have suggested that HPVs play an important role in the proliferation of lesions in the squamous epithelium, including papillomas, condylomas, and squamous cell carcinomas in various organs.[1)-5)] The molecular mechanisms between HPV infection and carcinogenesis have not yet been clarified. Some investigators have described the transforming proteins produced by HPV, in particular ORF E6 and E7 proteins which interact with two known tumor-suppressor gene products, Rb and p53 proteins.[10)22)] The HPV detection rate has been reported to be as high as 91% in squamous cell carcinomas and 100% in cases of intra-epithelial neoplasia of the uterine cervix.[2)] Regarding esophageal lesions, Syrjaeen described the histologic changes characterized by koilocytosis, which were identical to the effect of HPV seen in dysplastic and carcinomatous lesions of the squamous epithelium.[7)]

In this study, we used the PCR method for screening in formalin-fixed, paraffin-embedded tissues utilizing a cocktail of type-specific primers ; E6 regions containing HPV type 16, 18, and 33. In addition, we also performed a PCR analysis with type-specific probes and showed the presence of HPV DNA in 18.9% (18/95) of esophageal carcinomas by PCR.

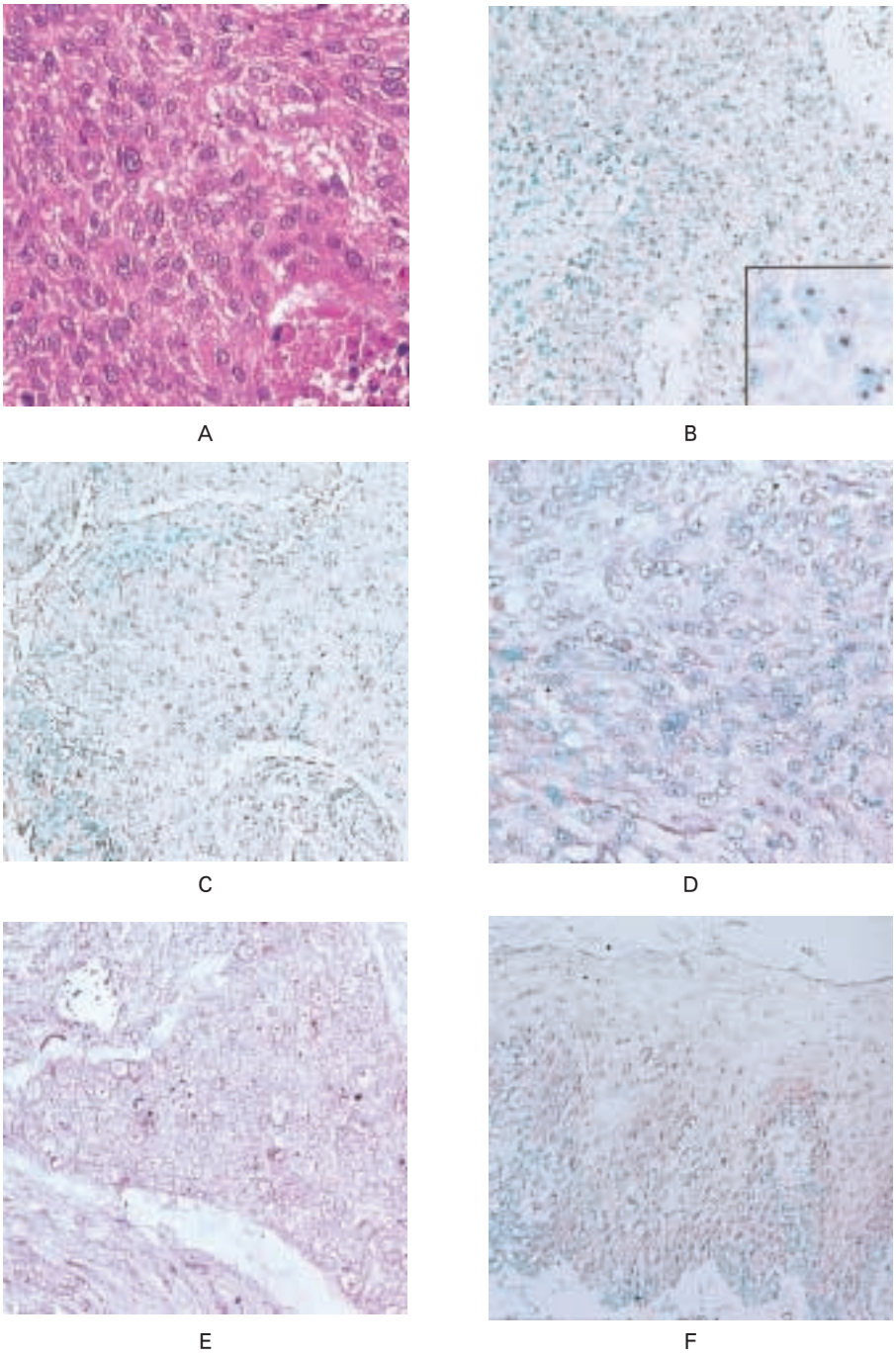

Fig. 3. Distribution patterns of HPV DNA by ISH. Histological features, H & E stain (A); a dense and strong reaction was observed in most of the carcinoma cell nuclei with a pan-HPV probe (B); a type-16-specific probe, original magnification X50 (C); a type-18-specific probe, original magnification X100 (D); and a type-33-specific probe, original magnification X100 (E); HPV DNA also helped to detect a dysplastic lesion adjacent to the carcinoma using a pan HPV probe, original magnification X50 (F).

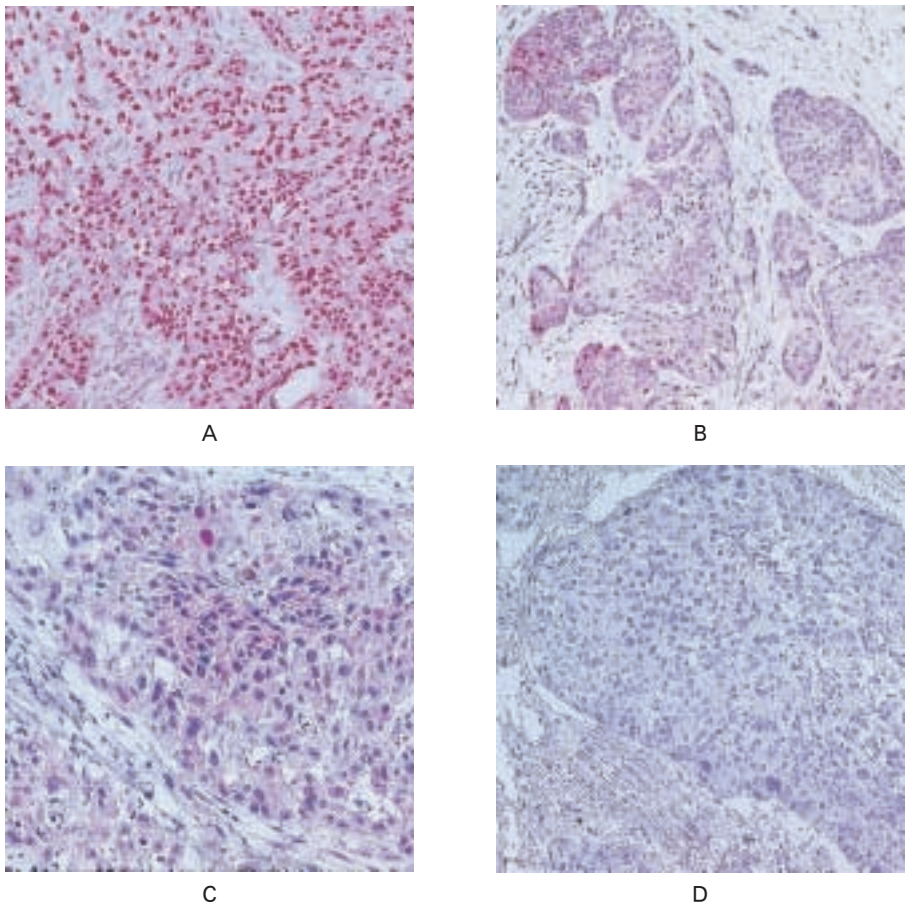

Fig. 4. Patterns of expression of p53 protein in the carcinoma. (A) Diffuse positive, (B) Focal positive, (C) Sporadic positive., (D) Negative : Positive reaction of carcinoma cell nuclei with p53 antibody (Do-7) by immunohistochemistry. Original magnification X100.

Type-16 was present in 50% (9/18) of the HPV-positive cases, type-18 was present in 88.9% (16/18), but type-33 was present in only 16.7% (3/18). The limit of PCR to detect the previously described HPV DNA was one copy in 105-106 cells.[23] This method is highly sensitive for detecting the target DNA. On the other hand, ISH is a good method for investigating the specific localization of target sequences within the tissue section. However, this method is less sensitive than either PCR or Southern blot hybridization. The previously described sensitivity of the conventional ISH of HPV DNA was about 20 copies/cell.[24] Numerous investigators have reported the most detectable DNA sequence to be HPV-16 in various organs, e.g. cervix, head and neck, and esophagus.[6)8)-17)23)24)] In the esophagus, HPV type 16 was detected in about 10 to 60% of all carcinomas.[8)15)-18)] In this study, HPV DNA genomes were identified in 18.9%. These findings generally agreed with those of previous papers, but the most detectable HPV DNA was type-18, and this result was different from that reported in previous papers.

In some cases, two or three types of HPV DNA were detected in a single tissue specimen. Both HPV-16 and 18 were detected together in 6 cases (33.3%), while HPV-16, 18,

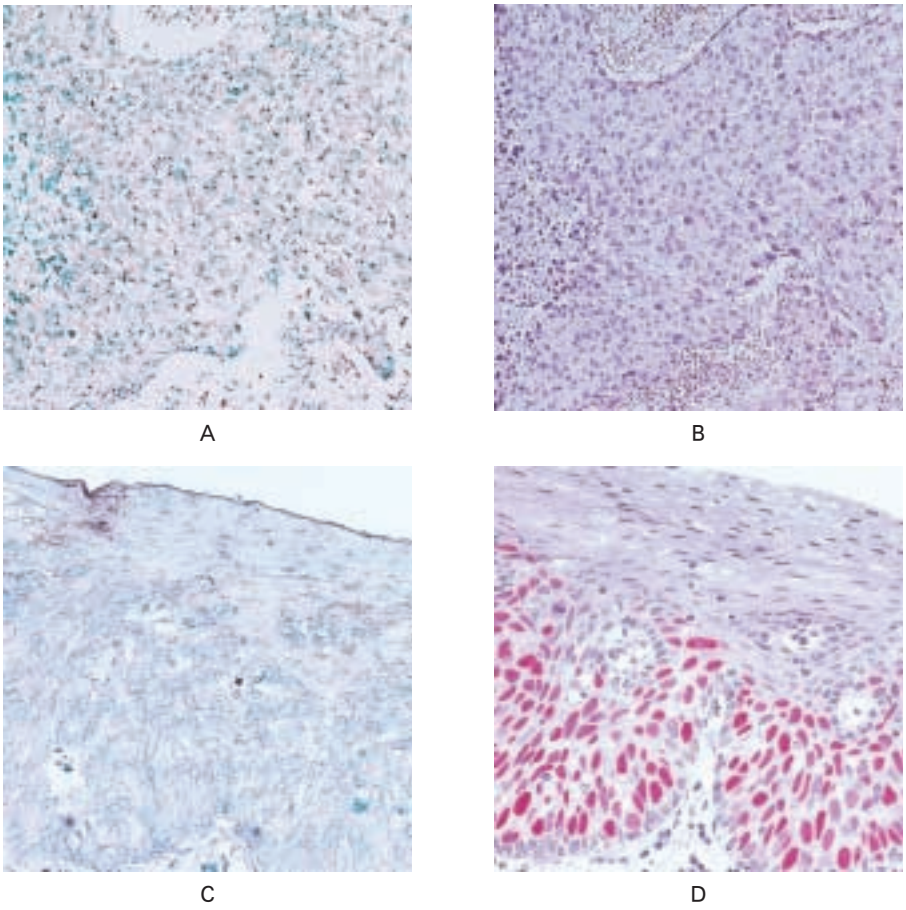

Fig. 5. Relationship between the localization of HPV DNA and p53 protein overexpression. A and B were taken from same specimen.: (A) In an HPV DNA−positive carcinoma case, (B) negative staining was observed for p53 protein in the same place. C and D were taken from same specimen: (C) In the HPV DNA−negative dysplastic lesion, (D) positive staining was observed for p53 protein in the basal layer. Original magnification X100.

and 33 were all detected together in 2 cases (11.1%). Mixed infections of HPV were seen in previous studies.[13] Mixed infections might be synergic effects in the enhancement of cell proliferation and transformation.[12]

We performed ISH to investigate the localization of HPV DNA in carcinoma tissue. In addition, 94.4% (17/18) of the cases with amplified HPV genomes, also showed positive signals in ISH with a pan−HPV probe. However, using type-specific probes, the localization was difficult to detect. Only 3 cases of HPV-16, 3 cases of HPV-18, and 2 cases of HPV-33 were detected. In 8 HPV DNA−positive cases, either 2 types (6/18 cases) or 3 types (2/18 cases) of HPV DNA were observed. The limit of ISH to detect HPV DNA was at a level of about 20 copies. The total number of HPV DNA copies might be more than 20 copies, however the each type of DNA copies might be at less than detectable numbers. The difference in these results would thus seem to reflect the different sensitivities among the probes used, or a small number of DNA copies for each subtype.

We used anti-p53 protein antibody which has the ability to react with both wild-type and mutant protein. In our series, 58.9% of all cases (56/95) showed a positive reaction in carcinoma cells and in the dysplastic epithelium adjacent to the carcinoma. Wild-type p53 protein has been described as a cell-cycle-control protein and its expression was thus found to be restricted during the quite short period of the late G1 and early S phases of the cell cycle.[24] The loss of p53 protein function generally follows mutations or deletions.[20)26)] The mutation of p53 is a common abnormality in neoplasms of various organs and it promotes tumor genesis. In contrast to wild-type p53, mutant p53 acts as a proto-oncogene. It has a long life span and is usually expressed in a number of cells in which the p53 gene sequence is mutated.[20)27)] described a small number (less than 4%) of immunohistochemically positive cells for p53 antigen in the carcinoma tissue, which rarely demonstrate point mutations. However, a large number (more than 5%) of positive cells in the specimen, which often demonstrate point mutations. Previous studies revealed that some mutations in the p53 gene do not result in a protein sufficiently stable to permit immunohistochemical detection and that some stabilized p53 represents wild-type protein rather than mutant protein.[29)30)]

Crook et al.[28] were described that HPV EORF (Early Open Reading Frame) E6 oncoprotein both combined with and helped to promote a dissolution of the cellular tumor suppressor protein p53. Some investigators.[22)23)31)] have reported that carcinomas of the cervix with HPV DNA sequence show a rare p53 gene mutation. This complex has a short half-life, so the absence of p53 protein is frequently observed in positive cases with HPV DNA. Crook et al. and Furihata et al. reported that the relationship between E6 protein and p53 protein might be associated with carcinogenesis.[17)18)28)] As a result, HPV-positive and p53 mutations were not mutually exclusive in esophageal carcinoma.

In our series, there was no significant correlation between HPV DNA and mutant p53 expression. However, 8/18 of HPV18 DNA positive cases were not seen p53 expression. HPV DNA was detected only in the carcinoma tissue, indicating that infection may play a more important role in the carcinogenesis. Our results suggest that HPV may be one of the risk factors in the development of this tumor.

Further investigations into the concomitant presence of mutant p53 and HPV in esophageal carcinoma are therefore urgently needed.

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