Study on the Role of Human Papilloma Virus in Carcinogenesis of Oral Mucosa

Ujjal Kumar Bhawal

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INTRODUCTION

Human papillomavirus (HPV) is one of virus group consisting of double-stranded circular DNA genomes with approximately 8000 base pairs, and belongs to heterogenous group of non-enveloped DNA viruses with over 100 genotypes[1]. Regions of HPV genome can be distinctly divided into two as follows: a) an early region encoding the viral proteins necessary for viral DNA replication, transcriptional regulation and cellular transformation, and b) a late region encoding the viral capsid proteins[2]. It is well known that HPV causes benign epithelial proliferations, and some types of them have been implicated in the pathogenesis of carcinomas[3]. HPV has an infectious predilection against stratified squamous epithelium. Up to date, an etiological relationship has become clear between HPV infection and human uterocervical carcinoma[4], but the decisive mechanisms of virus-cell interactions leading to tumor occurrence and development are still unknown. HPV types, such as 16 and 18, have been intensively studied as a possible etiologic agent of uterocervical cancers. In addition to HPV types 16 and 18, HPV types 31, 33, 35, 39, 52 and 58 have been identified, though less frequently in uterocervical cancers[5-11]. At present, there are reported discovery rate of HPV 16 DNA ranging from 14% to 92% in uterocervical cancers[12]. The fact surely suggest that HPV may make us consider as one of possible pathogenetic etiologies inducing oral squamous cell carcinoma (OSCC). In fact, HPV 16 DNA genotypes have been isolated also from oral lesions[13-14]. A majority of them belongs to low-risk group containing HPVs 6, 11, 13, 32, which may be associated with oral benign papillomatous lesions such as squamous papilloma, condyloma acuminatum, verruca vulgaris, and focal epithelial hyperplasia, etc., even though little potential for malignant changes. On the contrary, HPV genotypes, such as 16, 18, 31, 33 and 35, are called as high-risk group, which are associated with oral epithelial dysplasia and/or squamous cell carcinoma[15-18]. For malignant alteration, it is strongly believed to require carcinogenic factors through multi-steps towards tumorigenesis. Though the role of HPV acting for development of OSCC remains still obscure, it is necessary to obtain data using various techniques with specific sensitivity to detect viral DNA in experimental materials.

Up to date, however, HPV infection alone has appeared to be insufficient for proving processes towards malignant transformation of cells, resulting in necessity of additional cellular events. The accumulation of several alterations in proto-oncogenes and tumor suppressor genes is considered necessary for occurrence and development of human cancers[19]. Of these genes, p53 tumor-suppressor gene is the most useful one of markers detected in mutations in about half of cancers arising from a wide variety of human tissues[20]. It is thought that p53 inactivation in HPV-positive squamous cell carcinomas causes an accumulation of additional genetic abnormalities by interaction with HPV E6 oncogene products, which could be trigger for the multi-steped processes of carcinogenesis together with host factors[21]. p53 may affect the cell cycle through p21 effect on cyclin-cdk[22]. p21 protein exists in quaternary complexes with a cyclin, a cyclin -dependent

Department of Oral and Maxillofacial Surgery II, Hiroshima University Faculty of Dentistry (Chief: Prof. Takenori Ishikawa). This report was published by oral presentation in 4th Asian Congress on Oral and Maxillofacial Surgery (June, 2000, Korea), The 54th Annual Meeting of The Japanese Stomatological Society (May, 2000, Tokyo) and 85th Meeting of The Hiroshima University Dental Society (December, 1999, Hiroshima).

protein kinase (CDK) and proliferating cell nuclear antigen (PCNA). Cyclin D1 and its interaction with cyclin-dependent kinases can regulate the phosphorylation state of pRB. In its hyper-phosphorylated state, pRB releases E2F, a transcription factor that induces genes necessary for the transition from G1 to S phase[23]. PCNA is induced during the cell-cycle transition from G0 to G1 phase and peak at the G1/S interphase. As a cofactor for DNA polymerase delta and DNA replication, PCNA expression is a useful marker for cell proliferation in both of normal and neoplastic tissues[24].

The molecular mechanisms of head and neck cancers are not clear. Alike most malignancies arising in adults, multiple genetic changes may be probably involved in their development.

It is very important to know whether or not prevalence of HPV infection occurs in normal, pre-malignant and malignant lesions, and moreover to consider the relationship of HPV infection and clinical backgrounds (i.e. age, gender, tissue differences in tumor occurrence sites). Our final experimental aim is to examine whether and/or how HPV may play a role to oral carcinogenesis. In the present study, we tried to screen the presence of HPV type 16 DNA in various normal and pathologic tissues by polymerase chain reaction (PCR). Moreover, whether each expression by cell cycle regulators such as, p53, p21, cyclin D1, E2F and PCNA may be influenced by HPV infection, was discussed from immunohistochemical results in HPV-positive epithelial dysplasias and squamous cell carcinomas.

MATERIALS AND METHODS

Experimental samples:

Tissue specimens were obtained from 43 oral normal mucosae, 51 oral epithelial dysplasias, 83 oral squamous cell carcinomas (OSCCs) and 6 metastatic lymph nodes extirpated in Hiroshima University Dental Hospital (1977–1999). All specimens were fixed by 10% neutral buffered-formalin and embedded in paraffin. Four μm thick sections were prepared from each of paraffin blocks. One section was stained with haematoxylin and eosin solution for microscopical examination. The other sections were prepared for viral detection by PCR. Histologic differentiation grade was classified according to the criteria of the Japan Society for Head and Neck Cancer[25].

Established cell lines:

Nine cell lines originated from oral squamous cell carcinomas were as follows; HSC-2[26], HSC-3[27], HSC-4[28], KB[29], Ca9-22[30], HO-1-u-1[31], HO-1-n-1[32], OS-F[33] and KON[34]. All cell lines were provided from Japanese Cancer Research Resources Bank. They were usually cultivated with DMEM solution supplemented with 10% fetal calf serum in 5% CO_2 incubator at 37°C. These cultured cells were used for various examinations.

DNA extraction:

Genomic DNA was extracted from formalin-fixed and paraffin-embedded surgical tissues with proteinase K-phenol-chloroform, though with some modifications of reported reference[35]. Microdissected samples were collected from each specimen with a microscope and in 1.5 ml microfuge tubes. The instruments were sterilized with 5% sodium hypochlorite solution and ultraviolet exposure, and care was taken to avoid cross-contamination of samples. They were de-paraffinized with xylene solution (3 times), rinsed with 100% ethanol (2 times), and dried in a dessicator. After then, they were suspended in 500 μl of lysis buffer [50 mM Tris-hydrochloride (pH 9.0) containing proteinase K to a final concentration of 0.5mg/ml] at 55°C for 8 hours. DNA was extracted and purified by equilibrated phenol with proportional rate of chloroform to isoamyl alcohol showing ratio of 24 to 1 (twice), precipitated with ethanol, and dissolved in distilled water. From established cell lines, genomic DNA was also prepared by proteinase K-phenol-chloroform extraction method.

Polymerase Chain Reaction (PCR):

To check whether human DNA was present within samples tested, PCR was carried out for all the samples using the primers from a cellular gene, HGPRT (forward CTTGCTGGTGAAAAGGACCC, reverse GTCAAGGGCATATCCTACAA) produce a DNA amplification product of 267 bps[19]. Amplification of DNA was carried out with 3 pairs of type-specific primers were sold from Takara, Japan, on the basis for HPV 16, 18 and 33 according to published data[36]. The primer pF (5'-AAGGGCGTAACCGAAATCGGT-3') was located close to the 5' end of the E6 sense sequence of HPV 16,

which was available as a common primer of HPV 16, 18 and 33. The primers p16R (5'-GTTTGCAGCTCTGTGCATA-3'), p18R (5'GTGTTCAGTTCCGTGCACA-3') and p33R (5'-GTCTCCAATGCTTGGCACA-3'), corresponding to type-specific E6 anti-sense sequences of HPV 16, 18 and 33 respectively, were located in the middle of E6 open reading frame. To make sure the reproducibility and reliability of results, at least one positive and negative control were considered in each PCR. Cellular DNA was first denatured at 94°C for 10 minutes. The DNA was placed in 100 μl of 1×PCR buffer (10 mM Tris-hydrochloride, pH8.3, 50 mM potassium chloride, 1.5 mM MgCl2, 0.01% [weight/volume] gelatin), dATP, dGTP, dCTP, dTTP (0.2 mM for each), primers (0.2 μM each), and 2.5 units of Taq-polymerase. Thirty-five cycles of amplification were performed with DNA Thermal Cycler (M.J. Research, Watertown, MA, USA) with 1 min. denaturation at 94°C, 2 min. annealing at 55°C, and 3 min. extension at 72°C. Ten-microliter aliquots of the amplified reaction mixture were subjected to electrophoretic analysis on 3% NuSieve and 1% Seakem agarose (FMC, Rockland, ME) and stained with ethidium bromide.

Immunohistochemistry:

Antibodies used were summarized in the Table 1. HPV 16 positive samples consisting of 35 epithelial dysplasias and 35 squamous cell carcinomas were investigated by immunohistochemistry. These sections were stained using the avidin biotin streptavidin peroxidase immunostaining method (Vector Laboratories). Deparaffinized sections in 10mM citrate buffer of pH 6.0 were pre-treated with microwave (800 W, 3× 5 min.) to remove an antigenicity and immersed in methanol containing 0.03% hydrogen peroxide for 30 min. to block endogenous peroxidase activity. The sections were then incubated with normal horse serum for 30 min. to eliminate non-specific binding sites of antibody. The sections were treated consecutively with the primary antibodies (dilutions shown in Table 1) at 4°C overnight, biotinylated anti mouse IgG serum for 30 min. and streptavidin-peroxidase for 30 min. Peroxidase staining was performed for 3–5 min. using a solution of 3, 3'-diaminobenzidine tetrahydrochloride in 50mM Tris-HCl (pH 7.6) containing 0.001% hydrogen peroxide. The sections were slightly counterstained with Mayer's hematoxylin. Negative controls corresponded to substitution of the monoclonal antibodies with mouse IgG and concentration as the monoclonal antibodies.

Table 1 Antibodies used for Immunohistochemistry

Antibodies	Source	Dilution
p53 mouse monoclonal	Novocastra	1 : 200
p21 mouse monoclonal	Snata Cruz	1 : 400
Cyclin D1 mouse monoclonal	MBL	1 : 100
E2F-1 mouse monoclonal	Santa Cruz	1 : 100
PCNA mouse monoclonal	Dako	1 : 200

Grading criteria by immuno staining

G1 : 0–5% positive cells
G2 : 6–25% positive cells
G3 : 26–50% positive cells
G4 : 51–75% positive cells
G5 : >76% positive cells

For the judgement, three independent observers evalutated all immunostaining results and reviewed any discripancies. All analyses for immunostainings were performed without previous knowledge of the patients' clinical history and HPV infection status. Staining ability of nucleus was judged as positive regardless of cytoplasmic staining. For large tumors, at least five fields including superficial, central and deep areas were observed, and numbers of stained cells were evaluated. In each field, we counted at least 400 cells using eyepiece graticule to prevent recounting. To assess the number of stained nuclei, each slide was observed with ×400 magnifications and scoring on a semi-quantitative analyses of stained nuclei was as follows; grade 1 (patchy staining; 0–5% positive cells), grade 2 (weak staining; 6–25% positive cells), grade 3 (moderate staining; 26–50% positive cells), grade 4 (strong staining; 51–75% positive cells), and grade 5 (diffuse staining; >76% positive cells).

Statistical Analysis:

Statistical analysis was confirmed with Mann-Whitney's U test and Scheffe's F test for the results. A p-value of less than 0.05 was judged to be significant.

RESULTS

HPV detection:

All samples gave positive results for the presence of cellular DNA (Fig. 1).

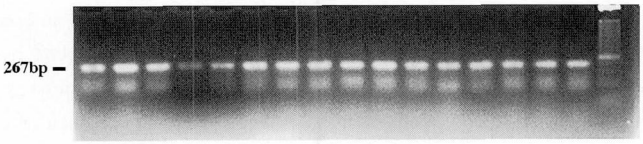

Fig. 1 PCR detection of HGPRT gene. 267 bp bands were detected in all samples.

1) Oral normal mucosae

Forty-three oral normal mucosa samples consisted of 21 male and 22 female cases. Sixteen (37%) were positive for HPV 16 DNA. All these positive incidences were relatively higher (16/32; 50%) in 40's and above in age (Table 2). HPV 16 DNA detection rate of gingiva and buccal mucosa were 39% and 33%, respectively (Table 3). But, HPV 18 and 33 DNA could not be found in normal mucosae (Fig. 2).

2) Oral epithelial dysplasias

For the frequency of HPV DNA in oral dysplasias, specimens taken from 14 patients with mild dysplasia, 29 with moderate one, and 8 with severe one were investigated. Of 51 samples, 31 (61%) were positive for HPV 16 DNA and all of them were in 50's and above in age (Table 4). HPV 16 DNA was detected in mild, moderate and severe dysplasias, with rate of 9 of 14 (64%), 16 of 29 (55%) and 6 of 8 (75%), respectively (Table 5).

Table 2 Correlation Among Age, Sex and HPV 16 Detected in Normal Oral Mucosae

Classification		HPV(+)	HPV(–)	Total
Age (yrs)	– 39	0	11	11
	40 – 49	6	7	13
	50 – 59	5	4	9
	60 – 69	3	3	6
	70 – 79	2	2	4
Sex	Male	7	14	21
	Female	9	13	22

Table 3 Correlation Between Anatomical Site and HPV 16 Detected in Normal Oral Mucosae

Site	HPV(+)	HPV(–)	Total
Gingiva	11	17	28
Buccal Mucosa	3	6	9
Lip	0	1	1
Palate	2	2	4
Oral Floor	0	1	1
Total	16	27	43

Table 4 Correlation Among Age, Sex and HPV 16 Detected in Epithelial Dysplasias

Classification		HPV(+)	HPV(–)	Total
Age (yrs)	– 39	0	0	0
	40 – 49	0	4 (1)	4 (1)
	50 – 59	9 (4)	6 (2)	15 (6)
	60 – 69	7 (5)	3 (0)	10 (5)
	70 – 79	6 (4)	5 (1)	11 (5)
	80 –	9 (5)	2 (0)	11 (5)
Sex	Male	17 (9)	7 (1)	24 (10)
	Female	14 (9)	13 (3)	27 (12)

( ): change to carcinoma

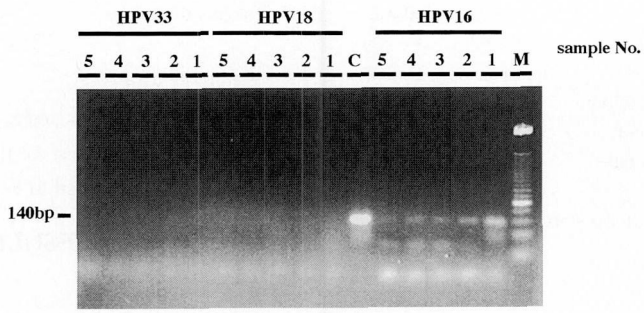

Fig. 2 PCR detection of HPV 16, 18 and 33 DNA in normal mucosae.
Samples 1: gingiva, 2: gingiva, 3: oral floor, 4: buccal, 5: palate, M: marker, C: control.
HPV 16 was detected in samples 1–5, but HPV 18 and 33 were not detected.

Table 5 Correlation Among Anatomical Site, Grading and HPV 16 Detected in Epithelial Dysplasias

Site	Mild	Moderate	Severe	Total
Tongue	4/ 7	5/ 9	3/4	12/20
Gingiva	3/ 4	6/ 9	2/3	11/16
Buccal Mucosa	1/ 1	1/ 4	0/0	2/ 5
Lip	0/ 0	0/ 1	0/0	0/ 1
Palate	1/ 2	1/ 2	0/0	2/ 4
Oral Floor	0/ 0	3/ 4	1/1	4/ 5
Total	9/14	16/29	6/8	31/51

Table 6 Correlation Among Age, Sex and HPV 16 Detected in OSCCs

Classification		HPV(+)	HPV(–)	Total
Age (yrs)	– 39	0	1	1
	40 – 49	1	9	10
	50 – 59	6	10	16
	60 – 69	7	9	16
	70 – 79	7	15	22
	80 –	7	11	18
Sex	Male	15	38	53
	Female	13	17	30

By anatomical sites, HPV 16 DNA detection rate was as follows; tongue (12/20), gingiva (11/16), buccal mucosa (2/5), oral floor (4/5) and palate (2/4). Interestingly, HPV DNA was detected in 18 (82%) of 22 dysplasias having changed to carcinoma, whereas 45% (13/29) of dysplasias without having changed to carcinoma were HPV 16 DNA positive. There was a statistically significant difference between these two groups (Mann Whitney's U test; p value 0.0248). Moreover, there was a higher tendency for HPV 16 positive epithelial dysplasias (18/31; 58%) in comparison to HPV 16 negative samples (4/20; 20%). We could not find any HPV 18 and 33 DNA positive dysplasias (Fig. 3).

3) Oral squamous cell carcinomas

The number of HPV positive and HPV negative patients by different decades was shown in the Table 6. The patients ranged from 31 to 89 old years. Of 83 OSCCs, 53 were male and 30 were female. HPV positive OSCCs showed significantly similar tendency in older-aged patients in 50's and above in age as well as oral dysplasias did. These occurrence sites of cancer were at tongue, gingiva, oral floor, buccal, palate and lip, 24, 31, 13, 7, 2 and 6 in number, respectively. There were seen some differences in the detection rate by occurrence sites, but as a whole, HPV 16 DNA was detected in approximately 34% of them. HPV 16 DNA positive OSCCs were relatively common in tongue (33%), gingiva (32%), buccal area (57%), oral floor (31%), but less common in lip. The detection rate showed comparatively higher in palate, though the number of patients examined was small. Histologically, HPV DNA was detected in 36% of well differentiated ones and 24% of moderately differentiated ones, but relatively higher also in poorly differentiated OSCCs, although the number of cases examined was small (Table 7).

The correlation between HPV 16 detection and clinical aspects in OSCCs were summarized in Table 8. HPV 16 DNA was relatively often seen by naked eye appearances as follows; verrucous (1/2; 50%), papillomatous (6/8; 75%) and leukoplakia-like (9/15; 60%), etc. But it was less common in granulomatous and ulcerative ones. There was statistically significant dif-

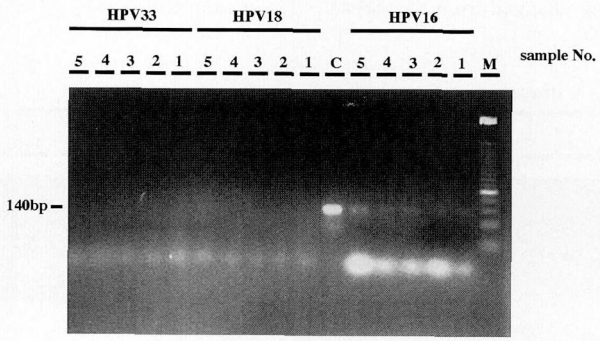

Fig. 3 PCR detection of HPV 16, 18 and 33 DNA in epithelial dysplasias.
Samples 1: moderate, 2: severe, 3: severe, 4: mild, 5: moderate, M: marker, C: control.
HPV 16 was detected in samples 1–5, but HPV 18 and 33 were not detected.

Table 7 Correlation Among Anatomical Site, Differentiation and HPV 16 Detected in OSCCs

Site (cases)	Mild	Moderate	Severe	Total
Tongue (24)	5/15	1/ 6	2/3	8/24
Gingiva (31)	6/16	4/15	0/0	10/31
Buccal Mucosa (7)	3/ 5	1/ 2	0/0	4/ 7
Lip (6)	0/ 5	0/ 1	0/0	0/ 6
Palate (2)	2/ 2	0/ 0	0/0	2/ 2
Oral Floor (13)	2/ 7	1/ 5	1/1	4/13
Total	18/50	7/29	3/4	28/83

Table 8 Correlation Between Clinical Appearance and HPV 16 DNA Detected in OSCCs

Site (cases)	U	G	L	P	V
Tongue (24)	2/11	2/ 5	4/ 7	0/1	0/0
Gingiva (31)	2/ 8	2/15	4/ 6	1/1	1/1
Buccal Mucosa (7)	1/ 2	1/ 2	0/ 1	2/2	0/0
Lip (6)	0/ 3	0/ 1	0/ 0	0/1	0/1
Palate (2)	1/ 1	0/ 0	0/ 0	1/1	0/0
Oral Floor (13)	0/ 7	1/ 3	1/ 1	2/2	0/0
Total (83)	6/32	6/26	9/15	6/8	1/2

U: Ulcerative, G: Granulomatous, L: Leukoplakia-like, P: Papillomatous, V: Verrucous

ference by Scheffe's F test between leukoplakia-like and ulcerative groups.

HPV 16 DNA was detected in 22 (30%) of 73 single primary SCCs. Of 7 patients suffering from multi-centric carcinomas, 3 were HPV 16 positive. Three non-decisive oral multi-centric SCCs were also HPV 16 positive (Table 9). All HPV 16 positive multi-centric carcinomas were in passive form at each time during follow-up. Moreover, HPV 16 DNA was found in 16 (72%) of 22 OSCCs having changed from dysplasias.

Table 9 HPV 16 DNA Detected in Single and Multi-centric Carcinomas

Cases	HPV(+)	HPV(–)	Total
Single Carcinoma	22	51	73
Multi-centric Carcinoma			
decisive	3	4	7
non-decisive	3	0	3
Total	28	55	83

Table 10 HPV 16 DNA Detected in Epithelial Dysplasias Changing to OSCCs

HPV 16 detected Dysplasia/OSCC	Cases (%)
+/+	16 (73%)
+/–	2 (9%)
–/–	4 (18%)
–/+	0 (0%)

HPV 16 DNA was not found only in 2 cases after cancerization (Table 10). HPV 18 DNA was positive in 2 decisive multi-centric SCCs that were also positive for HPV 16 DNA (Fig. 4). HPV 33 DNA was not present in all OSCC samples. Of 6 metastatic SCC cases investigated, HPV 16 DNA was not detected in metastatic lymph nodes, whereas 2 HPV-positive SCCs were found in primary sites. HPV 18 and 33 DNA were not found in metastatic lymph nodes (Table 11).

4) Cell lines examined

HPV 16, 18 and 33 DNA could not be detected in all of cell lines established from oral squamous cell carcinomas (Table 11).

Immunohistochemistry:

Immunohistochemical results were obtained in epi-

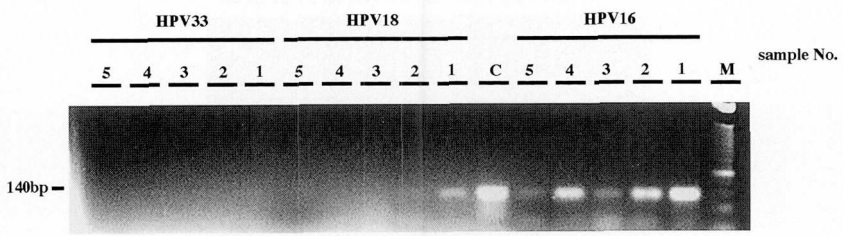

Fig. 4 PCR detection of HPV 16, 18 and 33 DNA in OSCCs.
Samples 1: multicentric, 2: multicentric, 3: tongue, 4: gingiva, 5: oral floor, M: marker, C: control. HPV 16 was detected in samples 1–5 and HPV 18 was detected in sample 1, but HPV 33 was not detected.

Table 11 Detection of HPV 16, 18 and 33 DNA

Site	HPV 16	HPV 18	HPV 33
Normal Gingivae	16/43	0/43	0/43
Epithelial Dysplasias	31/51	0/51	0/51
OSCCs	28/83	2/83	0/83
Metastatic lymph nodes	2/ 6	0/ 6	0/ 6
Cell lines	0/ 9	0/ 9	0/ 9

thelial dysplasias and squamous cell carcinomas, as follows:

1) p53 protein expression (Table 12 and 13)

All cases except one dysplasia and OSCC in each showed more than 5% immunoreactivity. Two different staining patterns were classified. In some samples, p53 was detectable only in occasional basal cells, while in others a strong staining throughout the epithelial cell layer was seen (Fig. 5). No significant correlation was detected between HPV infection and p53 protein expression.

Table 12 Summarized Results of p53, p21, CCND1, E2F and PCNA expression

Cases	HPV 16	p53	p21	CCND1	E2F	PCNA
1	+/−	G3/G5	G4/G3	nd/G2	nd/G1	G4/G5
2	−/−	G2/G4	G4/G3	G2/G2	G2/G1	G4/G5
3	−/−	G2/G5	G4/G2	G0/G2	G0/G1	G4/G3
4	−/−	G2/G2	G3/G3	G2/G3	G2/G1	G4/G3
5	+/−	G3/G3	G4/G4	G0/G3	G0/G3	G3/G3
6	+/+	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd
7	−/−	nd/G2	nd/G3	nd/G3	nd/G1	nd/G2
8	+/+	G3/G3	G3/G3	G1/G1	G1/G1	G3/G3
9	+/+	G3/G2	G3/G3	G1/G1	G2/G2	G3/G3
10	+/+	G2/G3	G3/G2	G1/G1	G1/G2	G3/G4
11	+/+	G3/G4	G3/G2	G0/G1	G2/G2	G3/G4

nd: not done Epithelial Dysplasia/OSCC

Table 13 Summarized Results of p53, p21, CCND1, E2F and PCNA expression

Cases	HPV 16	p53	p21	CCND1	E2F	PCNA
12	+/+	G2/G3	G3/G3	G1/G1	G1/G2	G4/G4
13	+/+	G3/G2	G4/G3	G1/G1	G1/G1	G3/G3
14	+/+	nd/G4	nd/G4	nd/G1	nd/G2	nd/G4
15	+/+	nd/G3	nd/G2	nd/G1	nd/G1	nd/G2
16	+/+	G3/G5	G4/G4	G1/G1	G2/G2	G3/G5
17	+/+	nd/nd	nd/nd	nd/nd	nd/nd	nd/nd
18	+/+	nd/G3	nd/G3	G1/G1	G1/G2	G3/G5
19	+/+	G3/G2	G2/G3	nd/G1	nd/G1	nd/G4
20	+/+	G1/G3	G4/G2	G1/G3	G1/G1	G2/G4
21	+/+	G1/G1	G4/G2	G1/G1	G1/G1	G4/G3
22	+/+	G2/G3	G3/G2	G1/G1	G1/G1	G2/G4

nd : not done Epithelial Dysplasia/OSCC

2) p21 protein expression (Table 12 and 13)

In epithelial dysplasias and carcinomas, p21 immunoreactivity was confined to epithelial cells, especially at the basal cell layer (Fig. 6). No significant correlation was detected between HPV infection and p21 protein expression.

3) Cyclin D1 protein expression (Table 12 and 13)

Nuclear staining of cyclin D1 was preferebly detected in HPV 16 DNA negative dysplasias and carcinomas in comparison with that in HPV 16 DNA positive cases (Fig. 7). Mean score of cyclin D1 protein expression in HPV-positive and HPV-negative cases were 1.1 ± 0.5 and 2.5 ± 0.5, respectively. There was a significant correlation in cyclin D1 protein expression between

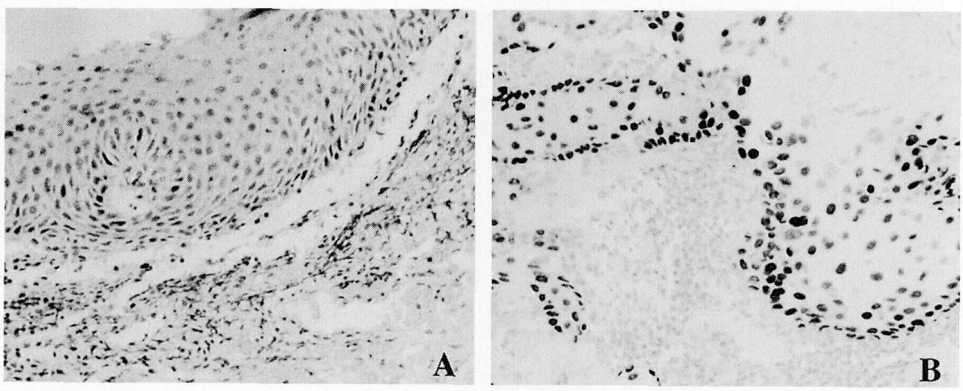

Fig. 5 Immunostaining of p53 in epithelial dysplasia (A) and OSCC (B).
A; A strong reactivity is seen throughout the epithelial layer (×200). B; Basal-layered cells are stained clearly in OSCC (×200).

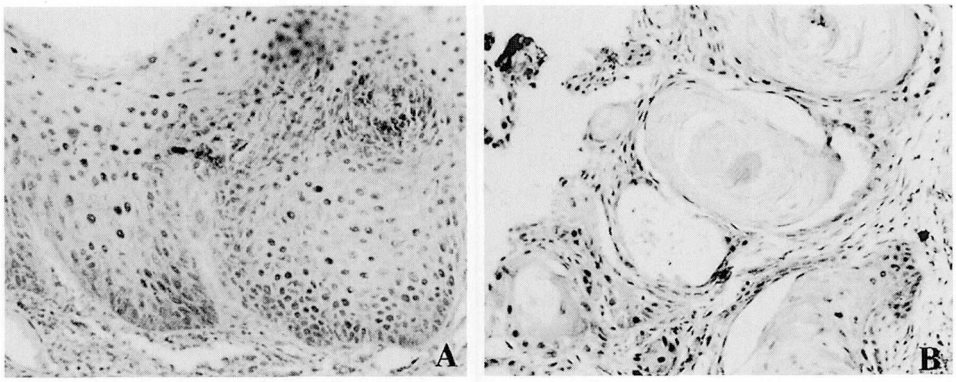

Fig. 6 Immunostaining of p21 in epithelial dysplasia (A) and OSCC (B).
A and B; p21 is strongly detected in both of affected epithelial cells (×200).

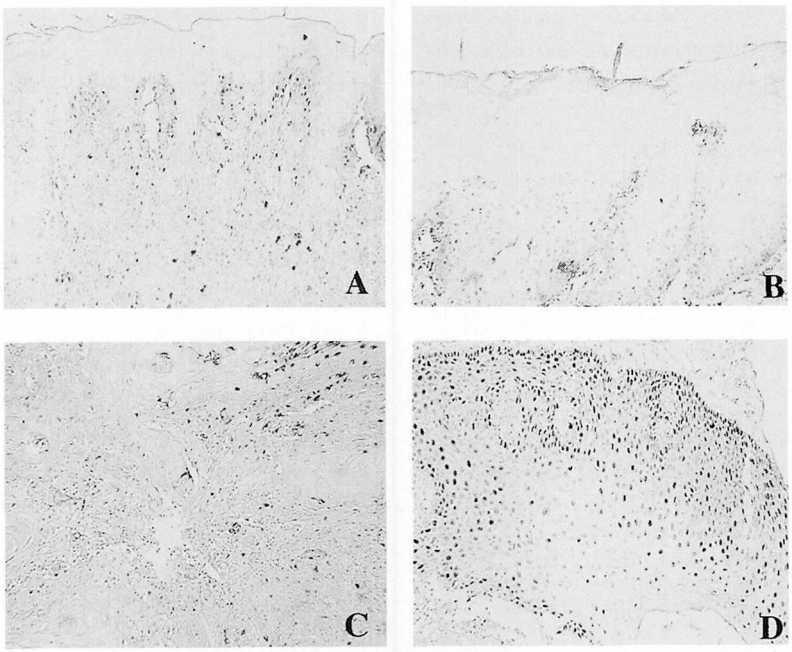

Fig. 7 Immunostaining of CyclinD1 in HPV 16 DNA positive dysplasia (A), HPV 16 DNA negative dysplasia (B), HPV 16 DNA positive OSCC (C) and HPV 16 DNA negative OSCC (D).
Nuclear immunoreactivity shows in HPV 16 DNA negative cases (B and D) more preferable than that of positive cases (A and C) (×100).

HPV-positive and HPV-negative cases ($p=0.0016$ in carcinomas).

4) E2F protein expression (Table 12 and 13)

Most of dysplasias and OSCCs showed grade 1 or grade 2 of immunoreactivity (Fig. 8). There was no significant correlation between HPV infection and E2F protein expression.

5) PCNA protein expression (Table 12 and 13)

Most of dysplasias and OSCCs showed grade 3 and grade 4 of immunoreactivity, although mean score of OSCCs was slightly higher than that of dysplasias was (Fig. 9). There was no significant correlation between HPV infection and PCNA protein expression.

DISCUSSION

Up to date, it has been well known that HPV is detected with etiologic correlation to some human lesions. Especially, HPV rates detected in utero-cervi-

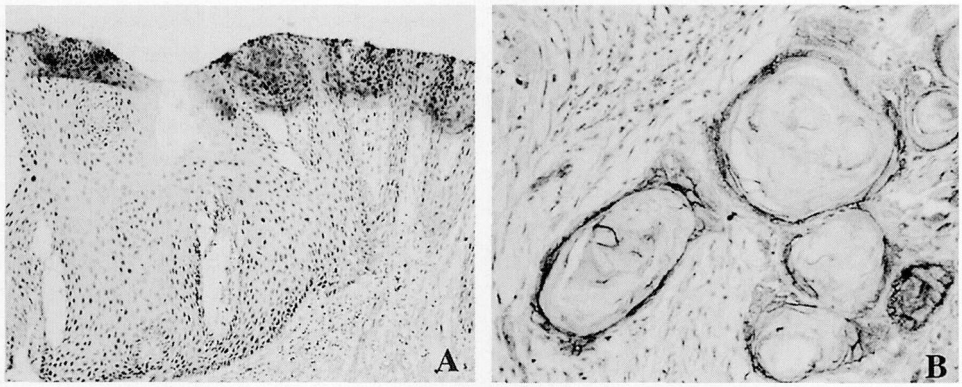

Fig. 8 Immunostaining of E2F in epithelial dysplasia (A) and OSCC (B).
A and B; Most of dysplastic epithelia and cancer cells show grade 1 or 2 of immnoreactivity (×100).

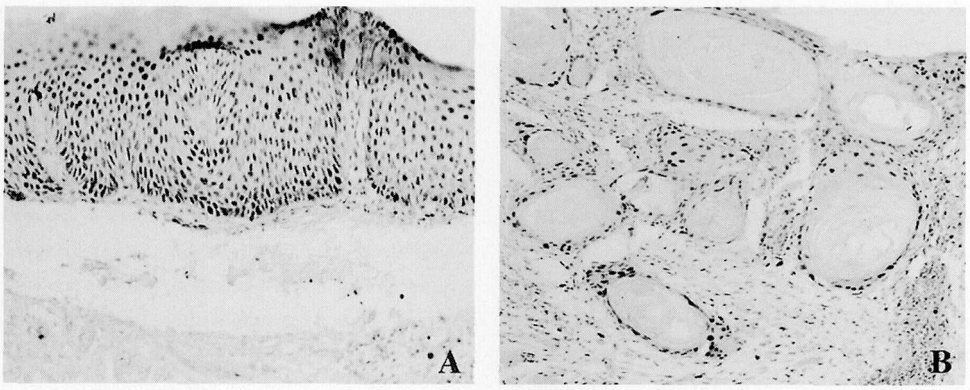

Fig. 9 Immunostaining of PCNA in epithelial dysplasia (A) and OSCC (B).
A and B; Most of dysplastic epithelia and cancer cells show grade 3 or 4 of immunoreactivity (×100).

cal cancers make us strongly consider HPV as etiologic cause for such a special disease. Moreover, various technical advancements let HPV detection easier, resulting in providing good proof for relationship between HPV and occurrence lesions. In addition to classical methods like immunohistochemical staining, at present Dot blot, Southern blot, and in situ hybridization, and further advanced polymerase chain reaction (PCR) method can be applied with extreme sensitivity. Also, advanced molecular biology has provided us further new approach technique for more rapid detection of viral infections in paraffin-embedded tissue sections. Now a days, sensitive PCR methods are available to allow easily the detection of this broad spectrum of HPV genotypes from clinical specimens. For example, within DNA extracted from biopsy materials of apparently healthy mucosae of normal adults, HPV 16 could be succesfully detected in more than 40% of samples using Southern blot hybridization[37]. Recently, PCR with extremely high sensitivity has such a fine capability to detect even only one copy of viral gene per cell. Moreover, in the recent time PCR can help HPV to detect even in the latent form of HPV infection. Moreover, immunohistochemistry and in situ hybridization were useful also in this experiment. However, specific E6 protein or capsid protein expression was not found out.

As the primers for PCR, viral regions such as E6, E7 or L1 have been often effectively used. E6 and E7 proteins encoded by HPV 16 and 18 may contribute to cellular transformation by inactivating p53 and Rb proteins. For above reasons, HPV positive cancers could be found out relatively with ease. On the contrary, L1 primer can recognize a wider spectrum of HPV sequences[38]. However, one of limitations of the L1 consensus primer-mediated PCR may show a tendency

of HPV nucleic acids to become integrated in the host chromosomal DNA. Considering together with the above reasons and convenience for experiment, we used the primers in E6 region, which was useful for HPV detection.

Firstly, it is important to find out incidence of HPV 16 infection in normal tissues in comparison to that by different oral lesions, resulting in decisive speculation of the role of HPV as pathogenetic agent. Furthermore, preference sites of the viral infections were investigated by different oral sites, i.e. buccal mucosa, palate, gingiva, etc. Detection rate of HPV 16 DNA showed 37% of oral normal mucosa specimens, which was consistent with the result of Anwar et al.[39]. Moreover, HPV 16 DNA was detected relatively higher (16/32; 50%) in 40's and above in age (Table 2). The HPV detection rate in gingiva and buccal mucosa were 39% and 33%, respectively, even though samples were small in number compared with one of other sites (Table 3). Other PCR-based studies by oral mucosae have demonstrated variable rates (<1-43%) as for HPV 16[19,40-41]. However, Adler-Storthz et al.[42] reported HPV 16 DNA detection in 60% of normal buccal mucosa specimens, which was inconsistent with the result of this study. The highest data of HPV infection was obtained from fresh oral scrapes in normal volunteers[19]. Ostwald et al.[41] and Mao et al.[43] studied biopsy tissues from normal individuals, resulting in lower levels of HPV infection. Nevertheless it is surprised to be able to detect HPV 16 also in normal mucosa. Here may be differences in fixation of specimen and tissue handling among different institutions and also they may account for some differences of a variety of districts concerning HPV infection.

By the way, oral leukoplakia is a so-called white patch or plaque of oral mucosa that cannot be characterized clinically or pathologically as any other diagnosable disease. It is generally accepted as one of precancerous lesions; that is patients with oral leukoplakia have a higher risk being able to change to oral cancer with time. According to recent reports, approximately 36% of these lesions can change to cancers[44]. Lind et al.[45] reported 13 cases of HPV-positive leukoplakias. Of them, 7 progressed to oral carcinomas within a 10-year period. However, the HPV genotype was not reported in these cases. Moderate or severe degrees of dysplasia can be apparently changeable to epithelial malignancies, resulting in development to oral cancers. Of 22 leukoplakias of this study, HPV DNA was detected in 18 (82%) having changed to carcinoma, but most of them were accompanied with moderate or severe degrees of dysplasia. Moreover, 61% of epithelial dysplasias were positive for HPV 16 DNA and all of them were in 50's and above in age (Table 4). Of 22 OSCCs, 16 showed HPV 16 DNA positive, having changed from dysplasias. Two cases were HPV 16 DNA negative also after cancerization (Table 5). These results suggest that HPV 16 E6 may become a possible potential marker helpful for early detection of oral cancer.

An argument whether HPV play a single role for occurrence of oral cancer, may be supposed due to high prevalence rate in the normal control[19], but inconsistent with HPV infection rate in oral cancer tissues (2.5-76%)[40]. In a recent comprehensive review of the head and neck squamous cell carcinoma, McKaig et al.[46] reported that HPV 16 rate was overall assessed with 34.5% by PCR. The wide differences in primer choice, amplification conditions, hybridization stringency, and other technical variables among various studies may be able to account for such scattered different results. In the present study, HPV 16 DNA sequences were seen in 34% of oral squamous cell carcinomas, even though in agreement with prevalence levels reported by Steinberg et al.[47] using PCR-based sensitive methods. Mao et al.[43] found out HPV 16 DNA in 20 (31.3%) of 64 malignant oral lesions by PCR amplification using early (E6/E7) primers, whereas Mineta et al.[48] reported HPV 16 DNA in 21% (3/14) of oral cancers. Our result was consistent with previous reports[40,43] on the basis of PCR-based examinations. It might suggest that HPV infection of the oral cavity was not due to a unique factor linked with malignant transformation of normal epithelium. The role of HPV to oral carcinogenesis is more likely to be involved to a synergetic effect between viral infection and influence of other factors.

In this study, a verrucous carcinoma having changed from dysplasia was HPV 16 DNA positive. This clinical finding may agree with the report indicating that HPV is detected less frequently in the oral cavity, whereas HPVs are mainly associated with verrucous carcinoma of the larynx[47]. There were some differences in the detection ratio by occurrence sites of OSCCs, but, HPV 16 DNA was detected overall in ap-

proximately 34% of OSCCs and HPV 16 was detected most frequently, in accordance with previous reports, whereas HPV 33 was never detected (Table 11).

After several years of course without clinical symptoms, there were possible patients recurred by the same HPV infection, in which re-infection might be endogenous or probably due to reactivation of the latent virus[49]. In a case of single carcinoma and the same case having changed to multi-centric carcinoma with 20 years' course, HPV 16 DNA was found in both tissues in the same case before and after cancerization. Here were 7 cases with decisive multi-centric carcinomas and 3 of them were HPV 16 positive. Three non-decisive multi- centric carcinomas were also HPV 16 positive. Our result was very similar to that of Shindoh et al.[50]. All of HPV 16 positive multi-centric carcinomas were in passive form at each time during follow-up. This suggests that HPV 16 DNA may play as one potential role of multi-centric carcinogenetic factors. Of 6 metastatic SCC cases, HPV 16 DNA positive cases were not found in metestatic lymph nodes, whereas only 2 HPV 16 positive primary SCCs were found. It was suggested that HPV 16 DNA infection might not exist homogenously in primary cancer cells and that HPV negative cells might have only metastasized to lymph nodes. HPV DNA often remains in an episomal state in oral carcinomas, as well as in carcinomas of the cervix uteri.

Although a significant role of high-risk groups of HPV types, such as 16 and 18, to carcinogenesis are uncertain, but carcinogenetic possibility of synergetic effects of 2 types of HPV appears plausible. The presence of plural typed of HPVs within the same lesion has been already reported[51,52]. Using PCR, we could find plural infections with HPV 16 and 18 DNA in 2 cases of squamous cell carcinoma, but the precise mechanisms of plural viral infections are not clear. Our results are similar to those of Wen[53] et al., which indicates that dual infections with high-risk HPV types might be associated with OSCCs, but even though not dysplastic lesions.

Researches on cell cycle regulators revealed that there was a statistical difference on cyclin D1 immunoreactivity between HPV positive carcinomas and HPV negative ones. In the related literatures, cyclin D1 and G1 cyclin over-expressed in the head and neck tumors were reported[54]. However, other reports did not show over-expression of cyclin D1 in some HPV positive lesions, although levels of other cyclins (eg. cyclin A, B or E) were elevated[55,56]. The above findings suggest that cyclin D1 might be over-expressed mainly in HPV negative cases and therefore cause a statistical difference on cyclin D1 expression between HPV-positive and HPV-negative cases, as shown in this study.

PCNA expression is always associated with G1 and S phases of the cell cycle[56]. Mate et al.[57] reported that PCNA immunoreactivity in HPV-associated skin proliferative condition showed positive also in both of parabasal and in differentiated upper layers, although non-viral proliferative one showed immunoreactivity only in the parabasal layers. However, immunoreactive distribution was not investigated in this study, but this will be a future subject to be examined.

Over-expressed frequency of p53 depends on what scoring system is used. In the present study, we regarded as positive for all cases with immunoreactive nuclei of tumor cells, and used a scoring system with a cut point with less than 5% immunostained cells, according to the previous studies[58]. Immunohistochemical over-expression of p53 protein has been reported in 54–67% of oral cancers[59,60]. But the frequency of p53 over-expression was 95% in this study, which is considerably higher than those in other studies. Some investigators gave criteria of 10% for cut point. If the cut point was chosen as 10% in this study, over-expression rate might be lower.

There have been various studies about the relationship between p53 over-expression and HPV states. Tenti et al.[61] reported that less over-expression was observed in HPV-positive transitional carcinomas of the oropharynx than that of negative ones was. On the other hand, Akasofu[62] et al. reported that p53 accumulation was observed more frequently in HPV positive uterocervical carcinomas than HPV negative ones were. Moreover, Badaracco[63] et al. described that over-expression of p53 and the presence of HPV are independent events in the head and neck cancers. The result of the present study agrees with that of the previous report. In order to know sure relationship between p53 and HPV infection, further researches will be required.

p21 protein has suggested to mediate p53-induced growth arrest[64]. It blocks an initiation phase of DNA-

replication by inhibiting CDK complexes[65]. Hennig et al.[66] reported that immunoreactivity of p53 and p21 was less detectable in HPV 16 positive breast carcinomas than in negative ones. Moreover, previously in vitro study disclosed that p21 expression decreased in the cell line immortalized by HPV 16, although the reduction was not significant. In this study, similar tendency, that is, less immunoreactivity of p53 and p21 was observed in HPV-positive carcinomas than in negative ones, although there was no significant difference.

Viral oncogenes are thought to exert their effect by altering intracellular states of growth regulating proteins. A good target of viral oncoproteins is E2F as cellular transcription factor, which associates with the product of susceptibility gene of retinoblastoma[67]. Under the hypothesis that HPV E7 region is probably involved in the lesions containing HPV E6 region, the present study was performed to know the relationship between HPV positivity and E2F status. However, no statistical correlation was observed between them. In order to clarify this subject, detection of E7 region is first required. This examination is interesting, because E7 makes E2F transcription factor free by uncoupling E2F-Rb or E2F-cyclinA complexes and causes the events, such as immortalization or cell cycle acceleration.

Since HPV 16 was detected also in normal individuals, our results can exclude a possibility that HPV is only the single etiological agent responsible for human oral neoplasia. It is reasonable to hypothesize that HPV 16 DNA may play a potential role in pre-cancerous lesions, resulting in carcinogenetic processes.

Detection of HPV DNA may become a good prognostic indicator screening individuals with epithelial dysplasias who may be at risk developing to malignant oral lesions. To determine a precise role of HPV in oral neoplasia, further study will be demanded, particularly concerning a state of viral gene, its expression, and its interaction with oncogenes or cell cycle regulators. The results in this study emphasize an importance in promoting these combined studies simultaneously also in normal as well as oral cancerous tissues.

CONCLUSIONS

HPV 16, 18, and 33 in high risk group have been frequently reported as one of causal agents for uterocervical carcinomas. On the basis of such data, above HPVs were examined by normal and lesional tissues of the mouth and the results were as follows:

1) HPV 16 DNA was detected in oral normal mucosae, epithelial dysplasias and OSCCs, with the rate of 37%, 61% and 34%, respectively. Especially, HPV 16 could be detected in 82% of dysplasias having changed to carcinomas but in 45% of dysplasias without having changed to carcinomas. HPV 16 DNA showed different existence rate by naked-eye appearances of OSCCs. Moreover, HPV 16 DNA was detected with higher rate of 60% in multi-centric carcinomas. All metastatic lymph nodes showed HPV 16 negative, but of these metastatic OSCCs, primary sites were HPV 16 positive only in 2 cases.

2) HPV 18 DNA was not present in normal mucosae and dysplasias, except for only in 2 multi-centric OSCCs. But, these two were positive also for HPV 16 DNA. HPV 33 DNA was never found out in all samples.

3) In all established cell lines used, none of all typed HPVs were detected.

Above results might mean that HPVs showed different sensitivities between oral mucosa and uterocervical one, resulting in different detection rate. On the contrary, HPV 16 was more sensitive to oral mucosae, resulting in connection to possible carcinogenetic processes. Considered for related references, these results seemingly suggested possibility that HPV 16 DNA might be not a decisive etiological agent responsible for human oral carcinogenesis. It may be hypothesized, however, that HPV 16 DNA might play an important role in pre-cancerous lesions, resulting in inducing carcinogesis. Moreover, this suggests that HPV 16 DNA may play as one potential role of multicentric carcinogenetic factors.

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