J Oral Pathol Med (2007) 36: 18–24
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Highly sensitive detection of HPV-DNA in paraffin sections of human oral carcinomas

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BACKGROUND: Although human papillomavirus (HPV) infection has been shown to be a significant carcinogen in cervical squamous cell carcinoma (SCC), its significance in oral SCC remains unclear.
METHODS: We developed highly sensitive detection methods for HPV to elucidate the prevalence and localization of HPV in paraffin sections from human oral SCC using modified *in situ* polymerase chain reaction (PCR) and *in situ* hybridization AT tailing (ISH-AT). Analyses revealed a high prevalence of several HPV types (HPV-16, -18, -22, -38 and -70) under optimal conditions. The ISH-AT method can be used as an alternative to *in situ* PCR.
RESULTS: Various staining patterns were observed in the 20 cases examined, and HPV-positive cells were localized within the surface epithelium as well as in neoplastic cells. We demonstrated that HPV-DNA could be detected in paraffin sections using either the method of *in situ* PCR or ISH, providing an appropriate primer and probe are used.
CONCLUSION: These results suggest that HPV infection could be one of several risk factors being involved in oral SCC.
J Oral Pathol Med (2007) **36**: 18–24

Keywords: human papillomavirus; *in situ* polymerase chain reaction; oral squamous cell carcinoma

Introduction

More than 130 different human papillomavirus (HPV) genotypes infecting human skin and mucosa have been identified in various lesions (1, 2). HPV infection is well known for its association with benign and malignant tumors of the genital tract (3), and certain types of HPV, such as high-risk or oncogenic HPV-16, -18 and -31, have been detected in up to 96% of cases of cervical squamous cell carcinoma (SCC; 4). Recently, it has been reported that high-risk HPVs are frequently detected in oral pre-malignant lesions and SCCs (5–15), and at least one high-risk type, such as HPV-16, -18 or -33, has been detected in 90% of such oral lesions as hyperplastic lesions, dysplastic lesions, and SCCs (5). On the other hand, high prevalence of HPVs has also been found in up to 81% of normal oral mucosa (16), and the positive rate of high-risk type HPV-16 and -18 was 55% (7). Furthermore, the results of these studies show greatly differing frequencies, ranging from 0% to 100% of HPV infection in oral tissues, mainly due to the varying sensitivity of the applied methodologies and the examined patient groups (15).

Many past studies were limited owing to the fact that HPV is present in a low copy number in oral SCC and most researchers relied on Southern blot analysis or *in situ* hybridization (ISH) technique, which is not sensitive enough to demonstrate the presence of HPV genome (17). Recently, several methods of highly sensitive detection of DNA or RNA genomes in paraffin sections, including *in situ* polymerase chain reaction (PCR), *in situ* hybridization AT tailing (ISH-AT; 18, 19), and *in situ* PCR ISH (20), have been developed. The method of ISH has become an important tool for the pathologist with respect to the detection and precise localization of nucleic acid targets within cells and tissues without the destruction of morphology.

We previously investigated the detection and localization of HPV in oral tissue using the *in situ* PCR method (8, 21) from a pathologic viewpoint. However, the prevalence of HPV infection, such as HPV-22, -38 and -70, and their localization have not been detected using highly sensitive detection methods. In this study, we examined the prevalence and localization of HPV infection (HPV-16, -18, -22, -38 and -70) in paraffin sections of human oral carcinomas using the highly sensitive detection methods and evaluated the efficiency of the different *in situ* methods in detecting HPV infection.

Materials and methods

Tissue samples

Formalin-fixed, paraffin-embedded oral tissue blocks, which had been stored at room temperature for a period of 3 months to 5 years, were collected from the archives

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Accepted for publication June 27, 2006

at the Osaka Dental University Hospital. We used the archival blocks to evaluate whether or not old paraffin blocks were useful for detection of HPV. Comparison of these results with those obtained using new materials revealed that archival tissues were fully useful. About 4-μm-thick sections were placed on silane-coated slides and stored at 4°C until use for *in situ* detection to prevent the oxidation. In addition, the microtome blades were changed frequently during preparation of sections for each paraffin block to reduce contamination. Cases were reviewed histopathologically and 20 SCCs classified as well differentiated (grade 1) according to the WHO grading (22), 11 leukoplakias, and 11 epulides were selected for the investigation. All leukoplakias and epulides were mild dysplasia and fibrous epulides, respectively. The clinical details for the patients are summarized in Tables 1 and 2. No HIV patients were included in the present study. Commercially available HPV-infected sections of human anogenital lesions (Dako Cytomation, Carpinteria, CA, USA) were used as positive controls. As negative controls, HPV-negative epulides were examined by PCR and *in situ* PCR detections.

The present study was approved by the Ethics Committee of Osaka Dental University (Approval No. 3).

ISH and in situ *PCR*

Both ISH and *in situ* PCR experiments were performed according to the procedure described by Uobe et al. (8). Type-specific L1 region primer pairs for HPV-16, -18, -22, -38 and -70 were synthesized according to the published sequences (23, 24), and the amplification lengths were 158, 158, 224, 154 and 204 bp, respectively. Commercially available consensus primers for MY09 and 11 (450 bp), a wide spectrum HPV FITC-labeled DNA probe, and HPV-16- and -18-specific probes (Dako Cytomation) were used. HPV-16 E2 N-terminal region, hinge region, and C-terminal region-specific primers (25), HPV-16 E6 specific primer pairs (7), and HPV-16 E7 primers (26) were the published sequences and were synthesized by an advanced order and their expected sizes were 107, 279, 190, 120 and 132 bp, respectively. Control procedures were as described in our previous reports. Specifically, a hot start PCR experiment was carried out on the tissue sections using QIAGEN OneStep RT-PCR Kit (Qiagen GmbH, Hilden, Germany) including Hot-StartTaq DNA polymerase for control procedure for *in situ* PCR (8, 21).

Table 1 *In situ* PCR using MY09/11 primer pairs and ISH

Lesions	*Sites*	*Cases*	*Male/female*	*HPV-positive, N (%) in total*	
				In situ PCR	*ISH*
Condyloma	Perineum	10		10/10 (100)	10/10 (100)
SCC	Tongue	11	6/5	11/11 (100)	0/11 (0)
	Gingiva	7	4/3	7/7 (100)	0/7 (0)
	Cheek	2	1/1	2/2 (100)	0/2 (0)
	Total	20	11/9	20/20 (100)	0/20 (0)
Epulis	Gingiva	11	5/6	4/11 (36)	0/11 (0)
Leukoplakia	Gingiva	5	4/1	4/5 (80)	0/5 (0)
	Cheek	4	3/1	3/4 (75)	0/4 (0)
	Tongue	2	1/1	1/2 (50)	0/2 (0)
	Total	11	8/3	8/11 (73)	0/11 (0)

SCC, squamous cell carcinoma; PCR, polymerase chain reaction; ISH, *in situ* hybridization.
HPV-positive cells in SCC were observed in both epithelia and tumor islands.
The number of positive cells in covering non-neoplastic squamous epithelium included in the tumor samples exceeded that in tumor islands and some of them in the deeper region did not show the presence of HPV-DNA at all.
Epulis means fibrous epulis.
Severity of leukoplakia is mild.

Table 2 *In situ* polymerase chain reaction (PCR) using type-specific L1 primer pairs

Case	*Age (years)*	*Sex*	*Site*	*HPV types (number of types)*
1	65	Male	Tongue	16, 18, 22, 38, 70
2	58	Male	Tongue	18, 70
3	78	Male	Tongue	16, 70
4	56	Male	Tongue	16, 18, 22, 38
5	74	Male	Tongue	18, 38, 70
6	52	Male	Tongue	22
7	52	Male	Gingiva	16, 22, 70
8	60	Male	Gingiva	16, 18, 22, 70
9	75	Male	Gingiva	18, 22, 38, 70
10	77	Male	Gingiva	16, 22, 38, 70
11	53	Male	Cheek	18, 38
12	74	Female	Tongue	18, 38, 70
13	64	Female	Tongue	16, 18, 70
14	74	Female	Tongue	16, 22, 38, 70
15	79	Female	Tongue	18, 22, 70
16	51	Female	Tongue	22, 38
17	77	Female	Gingiva	18, 38, 70
18	70	Female	Gingiva	18, 38, 70
19	37	Female	Gingiva	18, 22, 38
20	60	Female	Cheek	22, 38, 70

Average age 64 years, male/female 11/9 and HPV-16 8/20 (40%), HPV-18 13/20 (65%), HPV-22 12/20 (60%), HPV-38 13/20 (65%), and HPV-70 15/20 (75%).

In situ *hybridization combined with tyramide signal amplification (ISH-Tyramide)*

ISH-Tyramide was performed using the commercial DNA probes listed above according to the recommendations of the manufacturer. The optimal conditions were identified by the procedure described in Table 3.

Table 3 Optimal conditions of *in situ* PCR

1. Organosilane-coated glass slide
2. Deparaffinization with xylene
3. Pepsin treatment (1–30 min at room temperature)
4. Amplification for 30 cycles at 94 (1 min), 55 (2 min) and 72°C (2 min)
5. Stringent wash at 50°C for 60 min
6. Immunostaining with AP-labeled anti-DIG and BCIP/NBT
7. Counterstaining with nuclear fast red

AP, alkaline phosphatase; DIG, digoxigenin; BCIP, 5-bromo-4-chloro-3-indoxyl phosphate; NBT, nitroblue tetrazolium; PCR, polymerase chain reaction.

In situ *hybridization AT tailing combined with tyramide* ISH-AT was performed using ISH-AT probe as described by Nakajima et al. (18, 19). The ISH-AT probe consists of two regions, i.e. hybridization and elongation regions. The hybridization region, a 40-base-long complementary sequence, is located on the 5′-side, and the AT tailing region for elongation, consisting of 10 repeats of AT, on the 3′-side. Each sequence of the hybridization region was checked for absence of cross homologies with human sequences. One biotin molecule was conjugated on the 5′-side of the probes for control experiments. The GC% of the hybridization region ranged between 50% and 60%. The probes were synthesized at Nippon Gene Research Laboratories, Inc. (Sendai, Japan) by an advanced order. The optimal conditions were found by basically the same procedure as shown in Table 3. The sequences and positions of HPV-16 L1-specific oligonucleotides used in this study; sense: 5′-GCC TGT GTA GGT GTT GAG GTA GGC CGT GGT CAG CCA TTA G ATATATATATATATATATA-TAT-3′; antisense: 5′-CTA ATG GCT GAC CAC GGC CTA CCT CAA CAC CTA CAC AGG C ATATA-TATATATATATATATAT-3′; length: 40-mer plus 20-mer (60-mer); GC%: 58; 5′-position: 301.

Results

When 1% or more of the cells showed positive reaction under the microscope, it was judged that HPV positivity was present. *In situ* PCR experiment using MY09/11 showed high prevalence of HPV in SCC (100%), leukoplakia (73%), and epulis (36%; Table 2), in agreement with previous observations (8) and other reported data (15).

The prevalence of type-specific HPV-16, -18, -22, -38 and -70 L1 regions in oral SCCs was eight of 20 (40%), 13 of 20 (65%), 12 of 20 (60%), 13 of 20 (65%) and 15 of 20 (75%), respectively, under optimal conditions. Four of the SCCs were HPV-16 and -18 dual-positive, and 19 were multi-positive. On the other hand, *in situ* PCR using HPV-16 E2 (80%, 16 of 20), E6 (80%, 16 of 20) and E7 (80%, 16 of 20) primer pairs showed higher prevalence than that using L1 primer. Among HPV-16-positive SCCs, all of the C-terminal, N-terminal, and hinge DNA regions of HPV-16 E2 showed positive reactions (100%, 16 of 16).

ISH, ISH-Tyramide, ISH-AT, and *in situ* PCR experiments showed 0% (zero of eight), 15% (two of eight), 75% (six of eight) and 100% (eight of eight) in detection rates using HPV-16-specific L1 primer pairs. ISH and ISH-Tyramide experiments yielded very low detection of HPV-16 L1, whereas ISH-AT and *in situ* PCR methods showed high detection of HPV. The ISH-AT was an almost equivalent alternative to the more destructive *in situ* PCR on routinely processed paraffin sections.

The histopathologic sections stained with hematoxylin–eosin (H & E) showed a Grade 1 well-differentiated SCC with infiltrating nests and keratinizing sheets (Fig. 1a). HPV-DNA using *in situ* PCR method was detected in the nuclei of invasive neoplastic cells and stratified squamous epithelium in all of the SCCs examined and in some of the epulides and leukoplakias examined. Strong reaction for HPV-22, -38 and -70 was

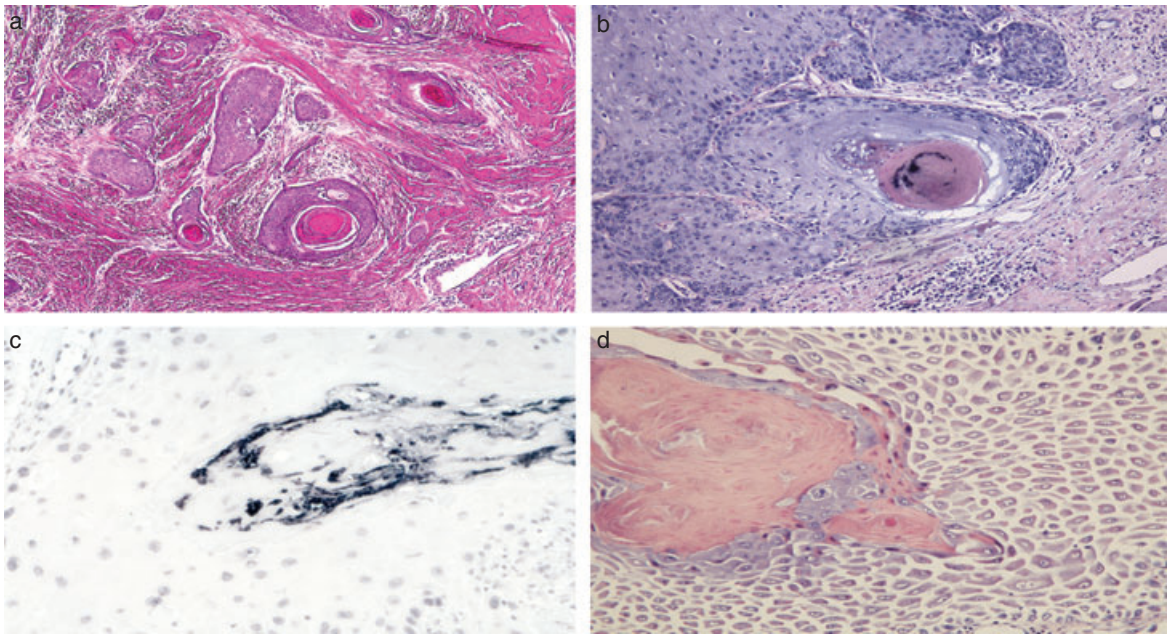

Figure 1 Well-differentiated squamous cell carcinoma. (a) Hematoxylin–eosin stain (original magnification ×33). (b) HPV-22-DNA is detected in the cancer pearl using *in situ* polymerase chain reaction (PCR; original magnification ×66). (c) HPV-38-DNA is observed in the center of the tumor nest using *in situ* PCR (original magnification ×132). (d) HPV-70-DNA is detected in the tumor cells neighboring the center of the tumor nest using *in situ* PCR (original magnification ×132).

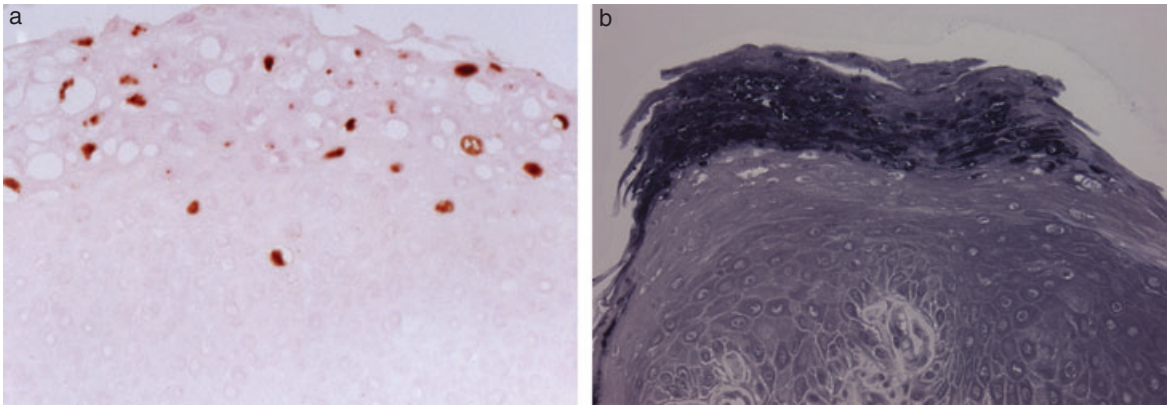

Figure 2 HPV-DNA is detected in the non-neoplastic epithelium using *in situ* polymerase chain reaction (PCR) with consensus primer. (a) In some case, HPV-DNA is localized in scattered cells of upper spinous and parakeratinized layer (original magnification ×132). (b) In other case, almost all cells in the parakeratinized layer are positive for HPV-DNA (original magnification ×132).

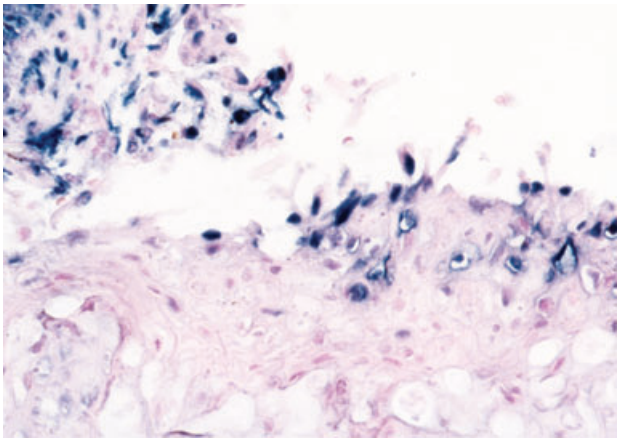

Figure 3 HPV-16-DNA-positive cells are scattered in the parakeratinized layer cells of the non-neoplastic epithelium using *in situ* polymerase chain reaction (PCR; original magnification ×132).

observed in the center of the tumor nest (Fig. 1b,c) and its vicinity (Fig. 1d) using *in situ* PCR. HPV-DNA was localized to the cells in the upper spinous and parakeratinized layers of the epithelium, and positive cells were observed not only in koilocytes, but also in morphologically normal cells (Fig. 2a). Almost all cells in the parakeratinized layer were positive (Fig. 2b), whereas HPV-16-positive cells were scattered in the parakeratinized layer of the non-neoplastic epithelium (Fig. 3). HPV-16 and -18 showed dual infections (Fig. 4a,b) using *in situ* PCR. ISH-AT using sense and antisense HPV-16 probes shows the localization of HPV-DNA in the parakeratinized, upper spinous, and basal and parabasal layers (Fig. 5a–c).

Discussion

Although further investigation is necessary to test this assertion in larger studies, our present study clearly demonstrates that high prevalence of certain HPV types, such as HPV-16, -18, -22, -38 and -70 is frequently detected in human oral SCCs, suggesting that HPV infection might be associated with the occurrence of oral carcinomas. Despite the numerous studies on HPV over the past two decades showing the presence of HPV-DNA in oral SCC, the reported prevalence rates of

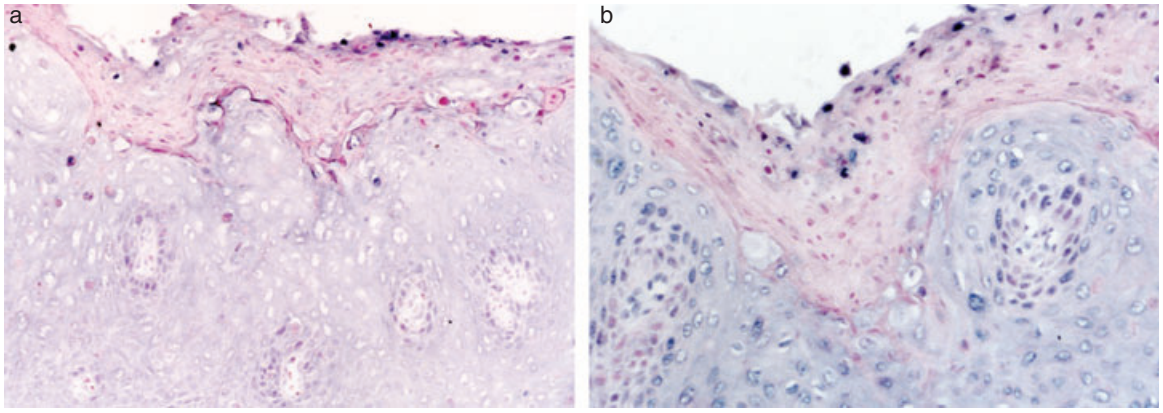

Figure 4 Dual infections with HPV-16 and -18 are observed in the non-neoplastic epithelium from serial sections of the same case. (a) HPV-16-DNA is positive in parakeratinized and upper spinous cells using *in situ* polymerase chain reaction (PCR; original magnification ×132). (b) HPV-18-DNA is also positive in parakeratinized and upper spinous cells from the serial section of Fig. 4a using *in situ* PCR (original magnification ×132).

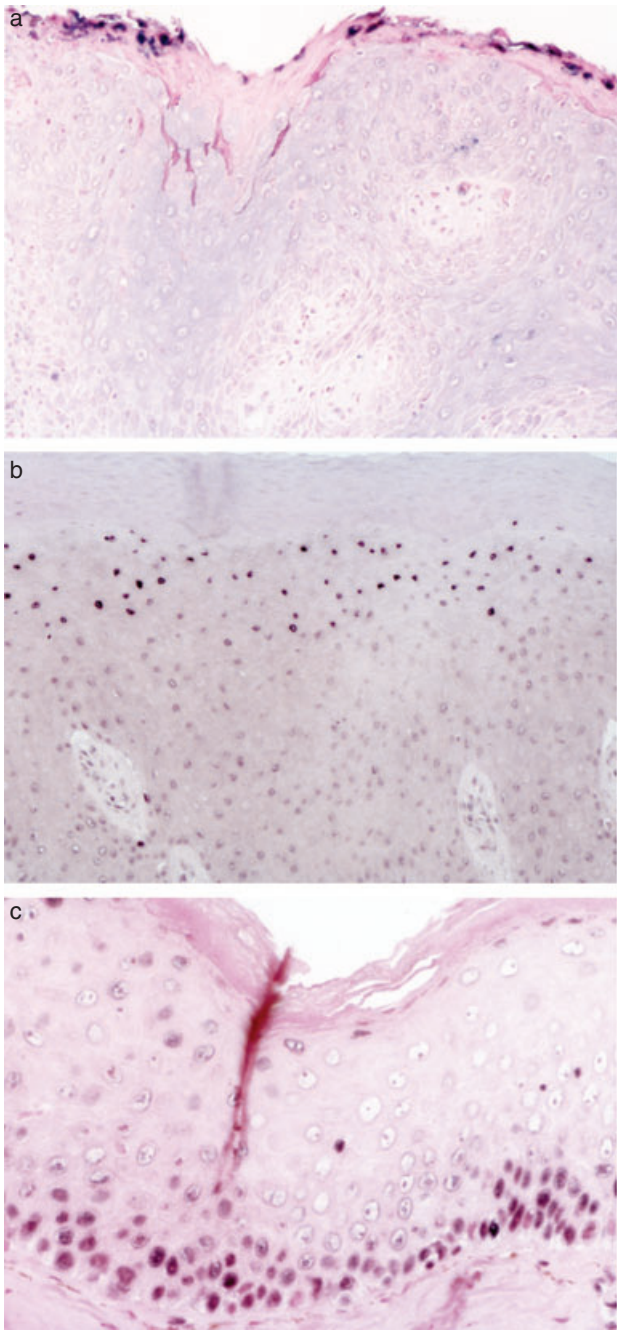

Figure 5 Different staining patterns of HPV-16-DNA are seen in cells of non-neoplastic epithelium from different cases using ISH-AT. (a) Parakeratinized cells are positive (original magnification ×132). (b) Spinous cells beneath the parakeratinized layer are positive (original magnification ×132). (c) Basal and parabasal cells are positive (original magnification ×132).

HPV infection have varied from 0% to 100%. This wide range may be due to a variety of reasons, such as the choice of primers used in the PCR, inherent differences in the populations being studied, and the methods used for HPV detection. Recent reports showed that the *in situ* detection techniques of HPV in paraffin-embedded tissue sections of oral SCC using *in situ* PCR (8) and *in situ* PCR ISH (20) are more powerful in detecting HPV than the liquid-phase PCR methods in terms of both sensitivity and specificity. In addition, the appearance of the new technology of ISH-AT enabled highly sensitive detection in tissue section (18, 19). In this study, we showed that ISH-AT and *in situ* PCR methods are very useful for detection of HPV-DNA in paraffin sections and that the former is a simple techniques that can be used as an alternative to *in situ* PCR, which is time-consuming and destructive.

In a recent meta-analysis including data for 4680 OSCC samples from 94 reports (9), HPV was found to be two to three times more likely to be detected in precancerous oral mucosa and 4.7 times more likely to be detected in OSCC than in non-neoplastic mucosa. These findings are consistent with our research data: the detection rates in OSCC, epulides, and mild epithelial dysplasia were 20 of 20 (100%), four of 11 (36%) and eight of 11 (73%), respectively. However, even in normal oral mucosa, high-risk HPVs have also been found at the high prevalence of 55% (7), and an understanding of the role of HPV in oral carcinogenesis is complicated by the different frequencies, ranging from 0% to 81%, of HPV infection in normal oral mucosa (15, 16). Multiple infections with 14 different types of HPV (HPV-1, -2, -4, -16, -18, -22, -36, -37, -38, -48, -52, -70, -75 and -77) in a patient with gingival carcinoma and co-infections with 2–12 different types of HPV in patients with tongue carcinomas have been reported (24). These results suggest that oral mucosal carcinogenesis proceeds through mechanisms different from those of high-risk HPV, and it is possible that when a combination of HPV types are present together, they act synergistically and cause malignancies. In our study, infections with two to five different types of HPV were found in all except one case of OSCC. In addition, as suggested by some references, other factors, such as tobacco and alcohol might cause malignant changes, although we did not examine the use of these substances by any of the 20 patients whose archived tissues were used in the present study. It remains unclear whether these factors are associated with HPV infection or not.

Miller and White (27) found that for PCR use of E gene primers was two to three times more efficient than use of L gene primers in the detection of HPV-DNA in oral SCC. On the other hand, opposite results have been reported (28). Our results showed that E gene primers of HPV-16 E2, E6 and E7 were more efficient than HPV-16 L1 gene primer pairs. However, the most important aspect of increasing the detection frequency specifically seems to be the demasking treatments, such as pepsin and proteinase K or washing conditions. For demasking, the deparaffinized sections were digested with pepsin (0.8% in 0.2 N HCl) for 5–10 min at 37°C. These procedures are described in our previous reports in detail (8, 21).

It is noted that HPV-transformed cells often contain chromosomally integrated copies of the HPV genome in which the viral E2 gene is disrupted and the HPV-16 E2 protein can induce apoptosis in both HPV-transformed and non-HPV-transformed cell lines (29). In all of the oral SCCs, we examined HPV-16 E2 genes of the C-terminal,

N-terminal, and hinge regions were stable and retained in an intact form in invasive SCC, and this seems to support the participation of this gene in oncogenesis.

Concerning the localization and distribution of HPV-DNA, the patterns observed were in almost total agreement with the published reports (8, 21) and those reported for cervical cancers. The distribution of HPV-DNA in all layers of the epithelium and in tumor islands to various extents suggests that HPV-DNA integrated into the human genome might associated with oncogenes and antioncogenes in various stages of SCC carcinogenesis. However, in many cases cancer cell nests in the deeper region were not positive for HPV-DNA at all (data not shown), as shown in our previous reports (8, 21), and the number of positive cells in covering non-neoplastic squamous epithelium included in the tumor samples generally exceeded that in the invasive neoplastic island. Therefore, HPV-DNA seems to participate in the early stage of carcinogenesis (30). These results suggest that the establishment of a highly sensitive detection system for HPV, such as *in situ* PCR or ISH-AT using an appropriate primer and probe under optimal conditions would contribute to the development of carcinogenesis research. Moreover, HPV infection could be one of several risk factors contributing to oral SCC.

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