Human Papillomavirus Genome in Oral Carcinoma and Their Metastatic Cervical Lymph Node Tissues.

Motoaki YASUDA[1,2], Masanobu SHINDOH[1,3], Takao KOHGO[3], Akira AMEMIYA[3], Yukiharu SAWADA[1] and Kei FUJINAGA[1]

[1)] *Department of Molecular Biology, Cancer Research Institute, Sapporo Medical University School of Medicine, S1, W17, Chuo-ku, Sapporo 060*

[2)] *Department of Dental Radiology, Hokkaido University School of Dentistry, N13, W7, Kita-ku, Sapporo 060*

[3)] *Department of Oral Pathology, Hokkaido University School of Dentistry, N13, W7, Kita-ku, Sapporo 060*

ABSTRACT

Twenty cases of oral squamous cell carcinoma (SCC) with cervical lymph node metastasis were investigated. Both primary lesions and metastatic lymph nodes were analyzed for the involvement of human papillomavirus (HPV) DNAs utilizing the polymerase chain reaction (PCR) method and dot blot hybridization. HPV DNAs were detected in five cases. Four primary lesions contained HPV-16 DNA, and one contained both HPV-16 and HPV-18 DNAs out of 20 cases examined. No HPV DNAs were detected in metastatic lymph node tissues in cases where HPV DNAs could not be detected in primary cancer tissues. The same types of HPV DNAs as those found in primary lesions were detected in metastatic lymph nodes including those with HPV-16 and HPV-18.

Key words: Human papillomavirus, Oral carcinoma, Lymph node metastasis, Polymerase chain reaction

INTRODUCTION

Human papillomavirus (HPV) is a closed circular DNA virus of approximately 8 kb and can be classified into over 65 different genotypes(1). Several distinct types of HPVs are associated with human epithelial lesions, and by far the most prevalent genotypes identified in anogenital lesions are HPV-6, -11, -16 and -18(2). HPV-16 and -18 have transforming and immortalizing activities in rodent and human cells(3～6) and these genes are expressed in cell lines of cervical squamous cell carcinomas(7～10). In oral epithelial lesions, HPV-13 and -32 have been detected in epithelial hyperplasia and HPV-6 and -11, in oral squamous papillomas. HPV-16 and -18 have been demonstrated in oral malig-

nant lesions(11~16). Previous detection data from our laboratory, obtained by the polymerase chain reaction (PCR) method indicated that about 30% of primary tongue squamous cell carcinoma contained DNAs of HPV-16 and HPV-18 (17). Thus, HPV-16 and -18 are associated with approximately one-fourth or more of malignant oral lesions. Since malignant lesions in the oral region often have metastatic potential, it is of importance to analyze the association of HPV DNAs with metastatic potentials of oral carcinoma. An experiment was carried out to investigate paired oral squamous cell carcinomas and their metastatic cervical lymph nodes in parallel for the presence of HPV DNAs utilizing the PCR.

MATERIALS AND METHODS

Surgical materials

Radical neck dissection was performed for 20 cases of oral cancer at the Department of Oral Surgery of the Dental Hospital of Hokkaido University, Sapporo, Japan from July 1987 to November 1991. Clinical and pathological data are shown in Table 1. Clinical staging was determined according to the

Table 1. Clinical and Pathological data

Case	Sex	Age	Site	Stage	P.D.	HPV(Primary)	HPV(L/N)
1	M	54	T	T2N1M0	SCC Gr.II	Negative	Negative
2	M	66	T	T2N0M0	SCC Gr.II	Negative	Negative
3	F	68	T	T4N0M0	SCC Gr.II	HPV-16	HPV16
4	F	40	F	T4N2M0	SCC Gr.III	HPV-16, -18	HPV-16, -18
5	F	71	T	T2N0M0	SCC Gr.II	Negative	Negative
6	F	64	T	T1N0M0	SCC Gr.II	HPV-16	HPV16
7	F	76	T	T1N2M0	SCC Gr.II	Negative	Negative
8	M	63	T	T2N1M0	SCC Gr. I	HPV-16	HPV16
9	M	47	T	T2N2M0	SCC Gr.II	Negative	Negative
10	M	48	T	T2N0M0	SCC Gr.II	Negative	Negative
11	M	60	T	T2N0M0	SCC Gr. I	HPV-16	HPV16
12	F	34	T	T1N0M0	SCC Gr.II	Negative	Negative
13	F	26	T	T1N0M0	SCC Gr.II	Negative	Negative
14	M	57	FM	T4N2M0	SCC Gr.II	Negative	Negative
15	F	65	G	T3N0M0	SCC Gr. I	Negative	Negative
16	M	81	T	T1N0M0	SCC Gr. I	Negative	Negative
17	M	56	T	T3N1M0	SCC Gr.II	Negative	Negative
18	M	65	G	T2N0M0	SCC Gr.II	Negative	Negative
19	M	68	FM	T2N0M0	SCC Gr.II	Negative	Negative
20	M	62	G	T2N1M0	SCC Gr.II	Negative	Negative

T: Tongue
G: Gingiva
FM: Floor of the mouth

UICC criteria (1989).

Histopathological examination

Surgical specimens from both primary sites and lymph nodes were fixed in 10% neutral-buffered formalin, embedded in paraffin and examined histopathologically. Histopathological estimation of tumor differentiation was undertaken following the WHO classification. Only tumor-cell-positive specimens were selected for the study.

Extraction of DNA

Genomic DNA was extracted from each paraffin-embedded specimen. Three 10μm thick paraffin sections were deparaffinized with xylen (twice), rinsed with 100% ethanol (twice) and dried in a desiccator. They were then digested overnight with 50μg/m*l* proteinase K at 37℃. DNAs were purified by phenol/chloroform extraction, precipitated with ethanol and dissolved in 50 μl of distilled water.

PCR and dot blot hybridization

PCR was carried out according to the method described by Shimada et al. (15). We used one sense primer (p16-1) which is capable of amplifying E6 sense sequences of HPV-16, -18 and -33, and three anti-sense primers (p16-2R, p18-2R, p33-2R) specific to the E6 anti-sense sequence of HPV-16, -18 and -33, respectively (see Table 2). Cellular DNA was denatured at 94℃ for 10 min, and placed in 100 μl of PCR buffer (10 mM Tris-HCl, pH8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% [w/v] gelatin), containing 0.2 mM each of dATP, dGTP, dCTP, dTTP, primers (all 0.2 μM) and 2.5 unit of Taq polymerase. To amplify HPV DNA, 40 cycles of denaturation (94℃, 1 min), annealing (55℃, 2 min) and extension (72℃, 2 min) were performed on a DNA Thermal Cycler (Perkin-

Table 2. Primers and Probes

Olgonucleotide probe sequenses	Base Position
p16-1 5'-AAGGGCGTAACCGAAATCGGT-3'	(26-46)
p16-2R 5'-GTTTGCAGCTCTGTGCATA-3'	(147-165)
p18-2R 5'-GTGTTCAGTTCCGTGCACA-3'	(154-172)
p33-2R 5'-GTCTCCAATGCTTGGCACA-3'	(152-170)
Olgonucleotide probe sequenses	**Base Position**
pB16-1 5'-CATTTTATGCACCAAAAGAGAACTGCAATG-3'	(77-106)
pB18-1 5'-TGAGAAACACACCACAATACTATGGCGCGC-3'	(84-113)
pB33-1 5'-CATTTTGCAGTAAGGTACTGCACGACTATG-3'	(82-111)

Elmer Cetus). After amplification, 10 μl aliquots of reaction mixture were subjected to gel electrophoresis on 3% NuSieve and 1% Seakem agarose (FMC) and the gel was stained with ethidium bromide. One-μl aliquots of the reaction mixture were transferred to a nylon filter (Schleicher & Schuell) and hybridized to [^{32}P] 5' end-labeled HPV type specific oligonucleotide probes (Table 2). Filters were washed twice in 2X SSC (SSC: 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS for 10 min at room temperature and washed twice in 0.2X SSC, 0.1% SDS for 30 min at 55℃. Finally the filters were autoradiographed with Fuji RXOH X-ray film for 3-12 h at -70℃.

RESULTS

Clinical data are shown in Table 1. The age distribution of patients was 26 to 81 years old. All cases were diagnosed as squamous cell carcinoma (SCC). The primary sites were the tongue (15 cases), gingiva (2 cases) and floor of the mouth (3 cases). Lymph node metastasis was confirmed histopathologically in all 20 cases. All cases who had had no nodal involvement at the first consultation later showed secondary lymph node metastasis; one case showed skin metastasis. The majority of primary tumors were relatively advanced in size (4/20 cases were classified as T1).

Primary lesions indicated several degrees of keratinization, however, the majority of the cases showed a relatively low tendency to keratinization tendency. Four cases were well differentiated SCC (grade I), 15 were moderately differentiated SCC (grade II) and one was poorly differentiated (grade III).

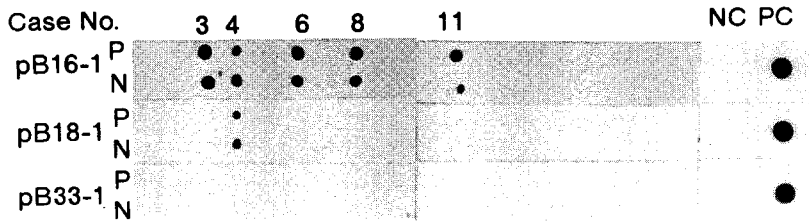

Fig. 1 Evaluation of human papillomavirus (HPV) by dot blot hybridization. The results of dot blot analyses of HPV DNAs from 20 cases are shown. P means primary specimen and LN means lymph node specimen. PC is positive control PCR products amplifying 0.1 μg of HPV DNA plasmid. NC is negative control distilled water. Cases 3, 4, 6, 8, and 11 show positive reactions against the pB16-1 probe specific for HPV-16. Case 4 is also positive for pB18-1 probe to HPV-18. Note that both primary and lymph node specimens are positive for the same probe simultaneously.

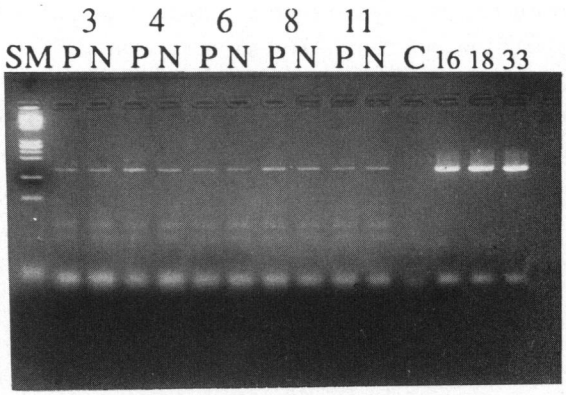

Fig. 2 Agarose gel electrophoresis of positive cases. A10 μl aliquot of reaction mixture was subjected to gel electrophoresis on 3% NuSieve and 1% Seakem agarose (FMC) and the gel stained with ethidium bromide. SM is a size marker (ϕX174 Hae III digest). 140bp bands were visible in all cases.

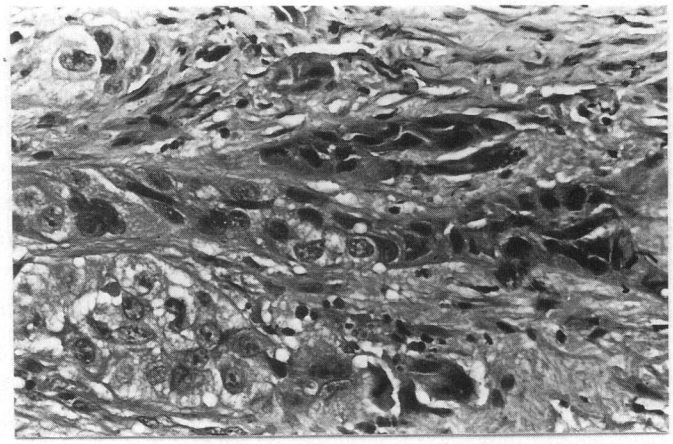

Fig. 3 Histopathological appearance of case 4. Hematoxylin and eosin (X50). Distinct cellular atypia and mitotic figures are observed. Cancer cells indicate infiltrative growth. Keratinization is inconspicuous.

Metastatic cancer cells resembled their primary sites morphologically and indicated the same differentiation pattern, however, some of them degenerated after irradiation.

PCR was carried out for both primary lesions and metastatic lymph nodes.

HPV-16 DNA was detected in four primary tumors, and both HPV-16 and HPV-18 DNA were detected in one case. The same type of HPV DNA sequence was detected in the corresponding metastatic lymph nodes (Fig. 1 and 2). No HPV DNAs were detected in metastatic lymph node tissues in cases where HPV DNAs could not be detected in primary cancer tissues (Fig. 1). HPV-16 DNA was detected in 2 of 4 cases (50%) with well differentiated SCC. Surprisingly, in the one poorly differentiated squamous cell carcinoma (Fig. 3), two types of HPV DNAs were detected simultaneously both in primary and metastatic lymph nodes. In none of the cases was HPV-33 DNA detected.

DISCUSSION

The detection of HPV DNA in both primary and metastatic tumors has suggested an etiologic role for HPV in cervical cancers(19~22). These reports demonstrated HPV DNA persisted in lymph node metastasis and supported the role of HPV in the pathogenesis of cervical cancer. Several investigators have detected HPV DNA in metastatic sites of other lesions. Hoshikawa *et al.*(23) detected HPV-16 sequences in 17.6% of metastatic tumors of laryngeal cancer patients and Wiener *et al.*(24) found the same DNA in both primary and metastatic sites in penile cancer patients. However, a study of a group patients with oral carcinoma, those developed into metastases has not been performed.

In the present study, we examined both primary and metastatic lymph nodes of the same patients utilizing the PCR method and dot blot hybridization. HPV DNAs were detected in metastatic lymph nodes of all five HPV-positive cases. These results strongly suggest that HPVs are associated with oral carcinogenesis and are stable in cancer cells during metastatic events. It is noteworthy that one of our cases showed the presence of both HPV-16 and -18 DNAs in metastatic lymph nodes.

Recently several studies have been performed to determine the prognostic value of HPV DNA detection. Walker *et al.*(26) described that the HPV-18-containing tumors had a higher recurrence rate and worse prognoses than those containing HPV-16 or no. Girardi *et al.*(27) reported that HPV-16-positive invasive cervical cancers had significantly higher rates of parametrial and pelvic lymph node involvement. Previous investigation of HPV DNAs in oral lesions utilizing PCR showed that DNAs of HPV, HPV-16 or both of HPV-16 and -18 DNAs were found in approximately 30% of oral carcinomas(17). In the 20 cases of oral carcinoma with cervical lymph node metastasis analyzed in this report, the HPV detection rate was 25% (5/20), showing no significant difference from previous detection rates. Since several other types of HPV DNAs (such as -6, -11,-31,-52 and 58) were detected in other malignant epithelial lesions, the

remaining 75% of HPV-negative cases should be investigated utilizing another consensus primer system which can detect more broad range of HPVs(28~30). On the other hand our histopathological analysis indicated a relatively low tendency to differentiation in most of the present cases (16 of 20) which accords with the histopathological theory that poorer differentiated tumors have worse prognoses. However, the present HPV-16 positive cases showed high keratinization (2 of 4 cases) whereas those positive for both HPV-16 and -18 had poor differentiation. Perez et al.(31) reported that HPV-16 positive laryngeal carcinomas were most frequently poorly differentiated squamous-cell carcinomas. McCance *et al.*(32) reported that HPV-16 alters human epithelial-cell differentiation and it is possible that the transition of well- to poorly-differentiated types may involve changes in the integration of the viral genome. The correlation between HPV infection and tumor cell differentiation needs extensive study.

In conclusion, our study on 20 cases of oral squamous-cell carcinoma with lymph node metastasis has revealed HPV DNAs associated with one-fourth of the specimens, in both primary and metastatic deposits. These results suggest that HPV DNA is strongly associated with oral tumorigenesis. To our knowledge, this is the first report demonstrating both HPV-16 and -18 DNA simultaneously in metastatic lymph node as well as in primary carcinoma.

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