Does the expression of HPV16/18 E6/E7 in head and neck squamous cell carcinomas relate to their clinicopathological characteristics?

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Abstract. Human papilloma virus (HPV) has been recently proposed to be implicated in the development of head and neck squamous cell carcinoma (HNSCC) in patients without cancer risk. We examined the expression of HPV16/18 E6/E7 in 71 cases of HNSCCs and investigated abnormalities of the p53 gene in 62 of these 71 cases. Expression of HPV16 E6/E7 was observed in 11 of the 71 cases (15.5%), while expression of HPV18 E6/E7 was not observed in any of the cases. Most of the HPV16 E6/E7-positive cases were histopathologically characterized by their verrucous or papillary structure and koilocytosis of the adjacent mucosa. There was no clear relationship between expression of HPV16 E6/E7 and tumor stage, prognosis or the positive rate of p53 abnormality. These results suggest that approximately 15% of HNSCCs are caused by HPV16 infection and the subsequent constitutive expression of E6 and E7, and that some HPV-initiated tumors lose their original characteristics during tumor progression.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy and is a major cause of cancer morbidity and mortality worldwide (1). The overall survival rate has remained unchanged in recent years, despite extensive research on the biological and molecular aspects of HNSCC (2-9). Although most HNSCC is etiologically linked with smoking and/or alcohol drinking or betel chewing, clearly there are patients who develop HNSCC despite the absence of exposure to these agents, and in the absence of any obvious predisposing genetic defect (10). Human papilloma virus (HPV) has recently been proposed to be implicated in the development of HNSCC in patients without elevated cancer risk, i.e., those with no exposure to smoking, alcohol drinking or betel chewing (10,11).

The genome of HPV is a circular double-stranded DNA molecule of approximately 8000 base pairs, and more than 100 HPV genotypes have been described (12). HPVs are classified into low-risk or high-risk types according to their presence in malignant lesions of the uterine cervix. The high-risk types, 16 and 18, are associated with more than 90% of uterine cervical cancers (13-15). Of these, HPV16 accounts for approximately half of all cervical cancers while HPV18 is involved in another 10 to 20% (12). The HPV genome contains several genes, including E1, E2, E3, E4, E5, E6, E7, L1 and L2 (16). Of these, E6 and E7 are known to be important in inducing malignant transformation of squamous cells. E6 binds to the tumor suppressor gene product, p53, and acts as a ubiquitin ligase for p53 thereby inactivating p53 function (17). E7 binds to the tumor suppressor gene product, RB, and inactivates RB function (17,18). After infection of squamous cells by high-risk HPV, the virus will act in one of two different ways. When the HPV genome exists as an episome, expression of E6 and E7 is very limited, and thus only induction of transient proliferation, not malignant transformation of the cells, is possible (12,16). However, when the HPV genome is cleaved in the E2 region, the linealized HPV genome is occasionally integrated in the chromosome of the cells (12,16). Under these circumstances the transcription-suppressor function of the E2 protein on the E6 and E7 genes is disrupted, resulting in up-regulation of E6 and E7 expression. Constitutive expression of E6 and E7 induces immortalization and proliferation of cells and finally malignant transformation of the cells (12,16). The presence of the HPV genome in HNSCCs has been reported to range from 0 to 100%; the rate depends on the area, the ethnicity of the patient, the type of specimen, and the detection method (10,19). The presence of polymerase chain reaction (PCR)-detectable HPV genome seems not to be synonymous with an HPV-related etiology, and causes an over-estimation of HPV-related carcinogenesis of oral mucosa as described above. Recent reports (20,21) have indicated that HPV E6, E7

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transcripts are less commonly found in HNSCC than the DNA of the respective HPV type.

The p53 tumor suppressor gene is reported to be mutated in more than 60% of HNSCCs, and 20-30% of the cases without mutation show an abnormality in the p53 signaling pathway, such as MDM2, ING1 or ARF1 (7). Balz *et al* (22) reported that p53 mutations are absent in human HPV-positive HNSCCs. Cheng *et al* (23) reported that HPV16/18 E6 expression correlated inversely with p53 expression, although p53 expression was not associated with mutant p53 in lung tumors. Thus, an association between p53 gene mutation and HPV16/18 infection or expression of E6 and E7 remains controversial.

Based on the data from a clinical study and the basic study concerning HPV and p53 in HNSCC, we conducted the present experiment to clarify the role of HPV16/18 E6/E7 in the carcinogenesis of HNSCC, and to examine whether the expression of HPV16/18 E6/E7 in HNSCC is related to clinicopathological characteristics.

Materials and methods

Patients and histopathological diagnosis. Seventy-one patients with HNSCC (men, 44; women, 27) participated in this study. Each patient underwent surgical resection of their tumor at our hospital between 1996 and 2005. The patients did not receive any previous radiation therapy or chemotherapy. The primary sites of the HNSCCs are shown in Table I. More than half of the tumors were taken from the tongue (37 cases), while 11 were from the upper gingiva, 8 from the lower gingiva, 5 from the mouth floor and buccal mucosa, 3 from the hard and soft palates, and 1 from the lower lip and maxillary sinus (Table I).

Tissue samples were formalin-fixed and paraffin-embedded, and sections (4-μm) were stained with hematoxylin and eosin for histopathological diagnosis. All tumors were diagnosed as squamous cell carcinoma or verrucous carcinoma.

Cell lines. HeLa and CaSki cells were derived from human cervical cancer cell lines, and these cells were maintained at 37˚C in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) and RPMI-1640 (Nissui), respectively, in a humidified atmosphere of 95% air and 5% CO_2. Both media were supplemented with 10% fetal calf serum (Sigma, St. Louis, MO), 100 μg/ml of streptomycin, 100 units/ml of penicillin, and 0.25 μg/ml amphotericin B (Life Technologies, Inc., Gaithersburg, MD). CaSki cells have been reported to be positive for HPV16 (24), while HeLa cells have been reported to be positive for HPV18 (25). Both cell types were used as controls in subsequent PCR analysis.

RNA extraction. All 71 cancer tissues were snap-frozen and subjected to molecular analysis. Total RNA from the cell lines and the tissues was extracted using a modified acid-guanidinium-thiocyanate-phenol-chloroform method with Isogen RNA extracting mixture (Nippon Gene, Toyama, Japan) according to the manufacturer's recommendations. Tissue was resected from the central part of the tumors without necrosis, and half was used for RNA preparation and half for histopathological diagnosis. The samples were confirmed to contain active cancer cells with minimum contamination by normal tissue. RNA integrity was confirmed by visualizing intact 28S and 18S rRNAs on a formaldehyde denaturing agarose gel.

Table I. Human HNSCC examined in this experiment.

Primary site	No. of cases (%)	Male	Female
Tongue	37 (52.1)	23	14
Upper gingiva	11 (15.5)	4	7
Lower gingiva	8 (11.3)	5	3
Mouth floor	5 (7.0)	4	1
Buccal mucous	5 (7.0)	4	1
Soft palate	2 (2.8)	1	1
Palate	1 (1.4)	1	0
Lower lip	1 (1.4)	1	0
Maxillary sinus	1 (1.4)	1	0
Total	71 (100.0)	44	27

Reverse transcriptase-polymerase chain reaction (RT-PCR). The RNA from HeLa and CaSki cells and tissue specimens was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) at 42˚C for 60 min using a random primer (5 μm; Life Technologies, Inc.) in 20 μl of reaction mixture (26). Subsequently, 1 μl of the products was subjected to quantitative PCR amplification (TaqMan PCR) as described below.

Quantitative evaluation of the expression of HPV16/18 E6/E7. The primers and TaqMan-probe used are listed in Table II. The PCR mixture contained 1 μl of reverse transcription product, primers (200 nM each) and TaqMan probe (100 nM) in a final volume of 25 μl (26). Amplification and detection were performed with the ABI PRISM 7700 Sequence Detection System using the following profile: 94˚C for 3 min followed by 40 cycles at 94˚C for 1 min, 55˚C for 1.5 min, and 72˚C for 2.5 min, with a final extension at 72˚C for 4 min (26). The amount of each RT-PCR product was standardized against the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal control.

p53 immunohistochemical analysis. Immunohistochemical analysis was performed using an LSAB2 Kit (Dako Japan, Tokyo, Japan) with microwave accentuation. In brief, 4-μm sections were placed onto silane-coated slides (Muto Pure Chemicals, Tokyo, Japan), deparaffinized in xylene followed by sequential washes in graded ethanol solutions, and pretreated with 3% H_2O_2 in methanol for 20 min at room temperature to quench endogenous peroxidase activity. The sections were then placed in 10 mM citrate buffer (pH 6.0) and heated in a microwave oven (400 W) for 10 min to facilitate antigen retrieval. They were subsequently incubated with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 20 min, then incubated with a polyclonal antibody against p53 protein (1:2,000; NCL-p53pCM1, Novocastra

Table II. Primers and probes used for HPV16 E6, E7 and HPV18 E6, E7.

Target gene	Upstream primer	Downstream primer	TaqMan probe
HPV16 E6	5'-gaatgtgtgtactgcaagcaacag-3'	5'-tggattcccatctctatatactatgcat-3'	5'-cgacgtgaggtatatgactttgcttttcgg-3'
HPV18 E6	5'-cagaaaccgttgaatccagca-3'	5'-tttctctgcgtcgttggagtc-3'	5'-actatagaggccaggccattcgtgctg-3'
HPV16 E7	5'-tgatctctactgttatgagcaattaaatga-3'	5'-tgtccggttctgcttgtcc-3'	5'-ctcagaggaggaggatgaaatagatggtccag-3'
HPV18 E7	5'-catcaacatttaccagcccga-3'	5'-gctcaattctggcttcacacttac-3'	5'-agccgaaccacaacgtcacacaatgtt-3'

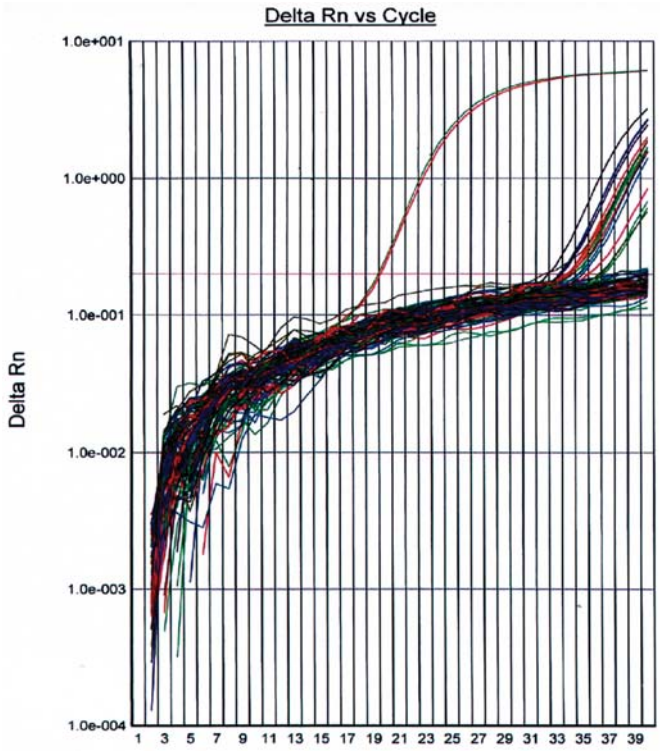

Figure 1. Expression of HPV16 E7 was observed in 11 of 71 cases (15.5%). In all HPV16 E7-positive cases, the fluorescence was detected at 33 to 37 cycles of amplification.

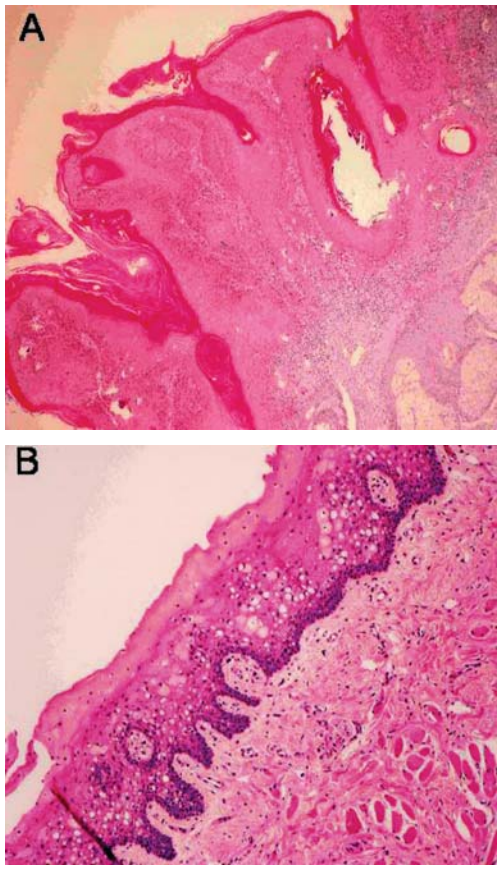

Figure 2. Most of the HPV16 E6- and E7-positive cases showed verrucous (or papillary) structure (A) and koilocytosis of the adjacent mucosa (B).

Laboratories Ltd., Newcastle, UK). Biotinylated anti-rabbit immunoglobulin G was used as the secondary antibody. Following treatment with peroxidase-conjugated streptavidin, the sections were washed in cold PBS, allowed to react with 3,3'-diaminobenzidine tetrahydrochloride solution and counter-stained with hematoxylin. As a positive control, sections of a colon cancer carrying a mutated p53 gene and strongly expressing the p53 protein in the nucleus, were used. Sections that were not exposed to the primary antibody formed the negative control (26).

Statistical analysis. Statistical analysis was carried out using the Stat View. The χ^2 test, Fisher exact test (two-tailed) and the Mann-Whitney test were applied for statistical analysis.

Ethical standards. The Ethics Committee of Dokkyo University School of Medicine approved this study. All patients gave their informed consent prior to their inclusion in this study.

Results

Expression of HPV16/18 E6/E7. Expression of HPV16 E6/E7 was observed in 11 of 71 cases (15.5%) (Fig. 1). In all HPV16 E6-positive cases, fluorescence was detected at 27 to 34 cycles of amplification. As expected, the HPV16 E7-positive cases were completely identical to the HPV16 E6-positive cases (data not shown). In all HPV16 E7-positive cases, the fluorescence was detected at 33 to 37 cycles of amplification (Fig. 1). On the other hand, expression of HPV18 E6/E7 was not observed in any of the cases (data not shown).

Clinicopathological characteristics of the HPV16-positive cases. We examined the relationship between HPV16 E6/E7 expression and the primary site of the tumor, TNM

Table III. Characteristics of the HPV16-positive cases.

Case	Primary site	TNM	Gender	Age	Histopathological characteristics		
					Verrucous	Koilocytosis	p53
1	Lower lip	T1N0M0	M	56	+	+	+
2	Maxillary sinus	T2N0M0	M	60	-	-	+
3	Upper gingiva	T2N0M0	F	79	+	+	+
4	Upper gingiva	T4N2bM1	M	80	+	-	NI
5	Tongue	T2N0M0	M	61	+	+	+
6	Tongue	T2N1M0	M	63	-	NI	+
7	Tongue	T2N1M0	F	43	+	+	-
8	Mouth floor	T4N2aM0	M	68	+	+	-
9	Tongue	T2N0M0	M	70	+	NI	+
10	Tongue	T2N2cM0	M	58	-	-	-
11	Tongue	T2N0M0	M	27	-	+	-

+, positive; -, negative; NI, not informative. M, male; F, female.

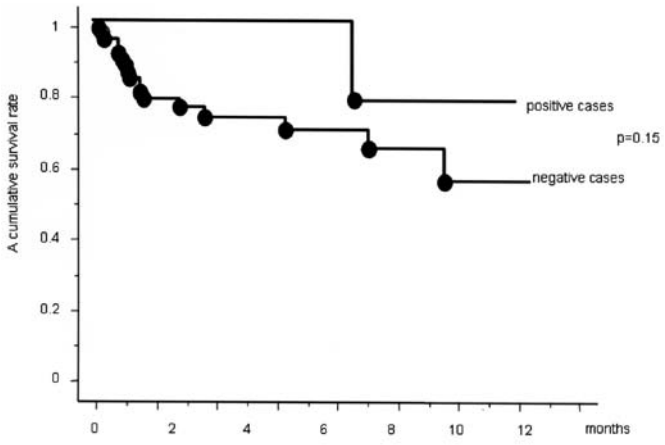

Figure 3. The cumulative survival rates of HPV16 E6/E7-positive and -negative cases are shown. There was no clear relationship between expression of HPV16 E6/E7 mRNAs and patient prognosis.

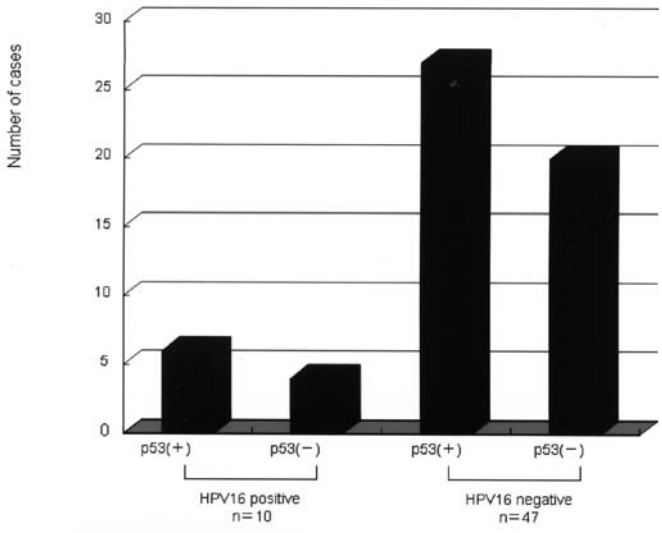

Figure 4. Abnormal nuclear accumulation of p53 gene products was detected by immunohistochemistry.

classification, gender and age (Table III). However, there was no relationship between HPV16 E6/E7 expression and any of these factors. The age distribution of HPV16 E6/E7-positive cases was identical to that of all cases examined.

The histopathological features of the HPV16 E6- and E7-positive cases included the characteristic verrucous (or papillary) structure (7 of 11 cases) and koilocytosis of the adjacent mucosa (6 of 9 cases) (Table III, Fig. 2). However, cases 2 and 10 did not exhibit either the verrucous (or papillary) structure or koilocytosis.

We also examined the relationship between HPV16 E6/E7 expression and T and N classifications and clinical stage of the tumors (Table IV), and compared the cumulative patient survival rates of HPV16 E6/E7-positive and -negative cases (Fig. 3). There was no clear relationship between expression of HPV16 E6/E7 mRNAs and tumor stage or patient prognosis.

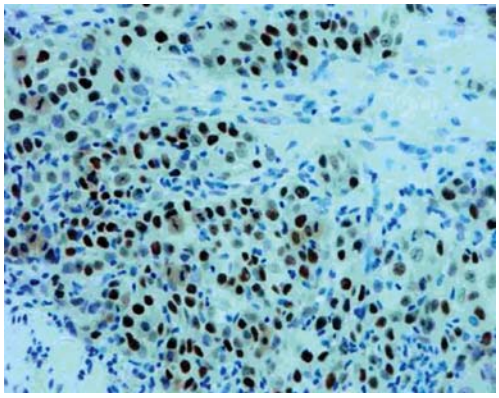

Figure 5. p53 abnormality was observed in 6 of 10 cases (60.0%) with HPV16 E6/E7 expression, and in 29 of 52 cases (55.7%) without HPV16 E6/E7 expression.

Table IV. Relationship of HPV16 E6, E7 expression with T, N classifications and clinical stage.

T classification			
T1	1/6	(16.7%)	p=0.0606
T2	8/36	(22.2%)	
T3	0/8	(0.0%)	
T4	2/21	(9.5%)	
N classification			
N0	6/41	(14.6%)	p=0.2623
N1	2/16	(12.5%)	
N2a	1/2	(50.0%)	
N2b	1/5	(20.0%)	
N2c	1/6	(16.7%)	
N3	0/0	(0.0%)	
Clinical stage			
I	1/6	(16.7%)	p=0.9344
II	5/27	(18.5%)	
III	2/13	(15.4%)	
IV	3/25	(12.0%)	

p53 abnormality in the HPV16-positive cases. Abnormal nuclear accumulation of p53 gene products was detected by the immunohistochemical study (Fig. 4). In previous studies, we demonstrated that all of the cases which showed abnormal nuclear accumulation of p53 gene products had a point mutation in the p53 gene (26,27). In the present study, p53 abnormality was observed in 6 of 10 cases (60.0%) with HPV16 E6/E7 expression, and in 29 of 52 cases (55.7%) without HPV16 E6/E7 expression (Fig. 5). There was no clear relationship between expression of HPV16 E6/E7 and the positive rate of p53 abnormality.

Discussion

In the present study the expression of HPV16 E6/E7 was detected in 11 of 71 HNSCCs (15.5%). The HPV16 E6-positive cases were identical to the HPV16 E7-positive cases, indicating not only that the experiment was properly conducted but also that constitutive expression of E6 and E7 in HNSCC might be controlled by the same regulatory mechanism as described above (12,16). On the other hand, expression of HPV18 E6/E7 was not observed in any of the cases, which is compatible with the results of Balz *et al* (22), who reported that HPV18 infection is very rare in oral squamous cell carcinomas.

It is well known that the HPV16/18 oncoprotein E6 binds to the p53 protein and inactivates its function (17). Thus, p53 gene mutation and consequent loss of function is not necessary in cells with constitutive expression of HPV16/18 E6. In fact, several investigators have reported that the expression of HPV16 E6 and p53 mutation appear to be mutually exclusive (20-22). However, in the present study, there was no clear relationship between the expression of HPV16 E6/E7 mRNA and the positive rate of p53 abnormality. These results suggest that in these patients i) HPV16 infected cancer cells which already had a p53 mutation, or ii) HPV16 infection of normal oral squamous epithelium occurred during the early stage of carcinogenesis, and subsequently several genetic alterations including p53 mutation developed during the progression of the cancer.

Most of our HPV16 E6/E7-positive cases showed characteristic clinical and histopathological features, such as verrucous (or papillary) growth of the tumors, and koilocytosis of the adjacent mucosa. However, two of the HPV16 E6/E7-positive cases, 1 from the maxillary sinus and 1 from the tongue, did not exhibit these histopathological phenotypes. These findings suggest that in these patients i) HPV16 infected cancer cells that developed independently of the HPV infection, or ii) HPV-initiated cancer cells lost their phenotypic characteristics during tumor growth and progression.

Our observations suggest that approximately 15% of HNSCCs may be caused by HPV16 infection and subsequent constitutive expression of E6 and E7. These tumors might be prevented by the control of HPV16 infection in normal squamous epithelium. Recently, vaccines for high-risk HPV were developed (28,29) and used for prevention of uterine cervix cancer (30). These vaccines may also be applicable to preventing the development of HPV-associated HNSCCs.

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