

The biomarkers of human papillomavirus infection in tonsillar squamous cell carcinoma—molecular basis and predicting favorable outcome

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Presence of human papillomavirus (HPV) in variable proportions in tonsillar squamous cell carcinoma tissues has been demonstrated by several worldwide studies. Some reports emphasized the significance of HPV in predicting a better prognosis, as well as ethnic differences between Chinese and Caucasians. In order to understand the biological role of HPV and find out clinically accessible methods to determine its prognostic significance in primary tonsillar squamous cell carcinoma, we collected 92 patients with primary tonsillar squamous cell carcinoma diagnosed or treated in National Taiwan University Hospital, for whom archival tumor tissue were available. Immunohistochemical stains of p16^{INK4A}, high-risk HPV *in situ* hybridization, and nested polymerase chain reaction (PCR)-based genechips were performed to detect HPV infection and determine its genotype. Clinical data were compared with HPV infection detected by the different methods mentioned above. Real-time PCR was also performed on the HPV16-positive [HPV16(+)] lesions to understand viral integration status. The positive rates of nested PCR-based genechips, overexpression of p16^{INK4A}, and high-risk HPV *in situ* hybridization were 75% (69/92), 53% (49/92), and 44% (40/92), respectively. Both overexpression of P16^{INK4A} and high-risk HPV *in situ* hybridization positivity were associated with favorable prognoses ($P=0.004$ and 0.001 , respectively) and also independent prognostic factors in multivariate analyses ($P=0.01$ and 0.01 , respectively). The positivity of nested PCR-based genechips was not statistically significant. From our data, primary tonsillar squamous cell carcinoma with positive immunohistochemical stains of p16^{INK4A} and/or high-risk HPV *in situ* hybridization is associated with a better outcome, and both methods may serve as clinically accessible markers.

Modern Pathology (2008) 21, 376–386; doi:10.1038/modpathol.3800979; published online 25 January 2008

Keywords: tonsillar squamous cell carcinoma; human papillomavirus (HPV); p16^{INK4A}; *in situ* hybridization; real-time PCR

In the United States, the annual incidence of head and neck cancer is approximately 30 900 cases;¹ the worldwide annual incidence of head and neck cancer also makes it the fifth most common cancer in the world.² In Taiwan, it ranks as the fifth most

prevalent cancer in both sexes, and accounts for the fourth most common cancer in males.³ These tumors have diverse clinical features and distinctive natural histories, and their treatment involves surgery, radiotherapy or chemotherapy alone, or in different sequential combinations. Prognosis has not really improved during the past two decades, although there have been improvements in our understanding of the spreading patterns of invasive tumors, leading to the increased use of combined modality treatment. Clinical staging currently guides treatment but still remains an uncertain predictor of outcome.

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Received 21 May 2007; revised 4 October 2007; accepted 8 October 2007; published online 25 January 2008

Although oral habits like betel nut chewing, cigarette smoking, and alcohol drinking are thought to be major risk factors responsible for the development of oral squamous cell carcinomas in Taiwan, there is a small proportion of patients who do not use betel nut, tobacco or alcohol, yet still develop oral cancer.^{4,5} Several other reports have indicated that patients without oral habits or who are young adults may also develop oral squamous cell carcinoma.^{6–8} Such evidence implies that carcinogenesis by environmental chemical carcinogens is not the only cause of oral squamous cell carcinoma. Presence of human papillomavirus (HPV), especially those genotypes with known high oncogenic potential in the uterine cervix (such as HPV16 and 18), in variable proportions in the oral squamous cell carcinoma tissues has been demonstrated by several worldwide studies.^{9–25} Recent studies suggest that HPV, especially type 16, may be responsible for a small subgroup of oral squamous cell carcinoma and up to 50% of oropharyngeal squamous cell carcinomas, especially the tonsillar squamous cell carcinoma,^{26–33} despite an ethnic difference between Chinese and Caucasians, as noted in one report.³⁴ Some reports emphasized the influences of HPV in predicting a better disease prognosis in oropharyngeal and tonsillar cancers.^{28,29} Furthermore, a strong association between the presence of HPV and expression of key cell cycle proteins has been recently reported in a series of tonsillar squamous cell carcinoma.^{26,27} These data seem to strongly suggest that HPV-positive tonsillar squamous cell carcinoma represents a distinct molecular, clinical and pathologic entity. In the present study, we will focus on this distinct subgroup of head and neck cancers.

According to earlier investigations,^{35–38} high-risk HPVs and two HPV-related oncoproteins E6 and E7 can immortalize and transform oral keratinocytes *in vitro*. These cumulated evidences suggest that HPVs may play a crucial role in the carcinogenesis of head and neck cancer. When the host epithelial cell is infected with the HPV, the HPV genome integrates into the host cell's DNA. It will typically disrupt the HPV *E1/E2* open reading frame, resulting in the loss of function of *E2*, a physiological regulatory protein for the HPV *E6* and *E7* oncogenes.^{39,40} The E2F–pRb complex dissociates in the presence of the HPV oncoprotein E7; this, in turn, activates E2F, thereby initiating the transcription of genes required for DNA replication and inappropriately forcing the cell past the G1/S restriction point into the S phase.^{41–43} Due to positive feedback, the functional inactivation of pRb by HPV E7 results in the reciprocal overexpression of p16^{INK4A}.^{40,44–47} Therefore, p16^{INK4A} has been considered a complimentary surrogate biomarker for HPV-related uterine cervical and vulvar neoplasia.^{48–50} The same phenomenon was also noted in HPV-related tonsillar squamous cell carcinoma.²⁶ In accordance with the mechanism described above, previous reports demonstrated a

unique region of the *E2* open reading frame that is most often deleted during HPV16 integration. Using real-time polymerase chain reaction (PCR), if *E2* open reading frame is targeted by one set of PCR primers and a probe, and another set targets the *E6* open reading frame, both targets should theoretically be equivalent in episomal form, while in integrated form, the copy numbers of *E2* would be less than those of *E6*.⁵¹

We used three methods, including nested PCR-based genechips, immunohistochemical staining of p16^{INK4A}, and high-risk HPV *in situ* hybridization, to detect HPVs and determine their genotypes in 92 primary tonsillar squamous cell carcinoma cases. We then performed real-time PCR to detect DNA products of *E2* and *E6* open reading frame, in order to calculate the *E2/E6* ratio in HPV16-positive cases. Comparing the clinical and pathologic data of the patients (including staging, survival, oral habits, and histological type, etc.), we hoped to further understand the biological effects of HPV in these tonsillar squamous cell carcinoma cases.

Materials and methods

Patients

Formalin-fixed, paraffin-embedded tissue blocks of 92 Taiwanese patients, 79 male (median age 51, 29–79) and 13 female (median age 49, 42–79), were obtained from patients diagnosed with primary tonsillar squamous cell carcinoma based on histological examination of hematoxylin and eosin-stained tissue sections according to the World Health Organization Classification of Tumors.⁵² Keratinizing tumor cells could be found in 47 of 92 (51%) cases. All patients received biopsy or surgical operation between 1997 March and 2005 March at the National Taiwan University Hospital, Taipei, Taiwan. The clinical data were abstracted from the patient's medical charts and the survival data were obtained from the Bureau of Health Promotion, Taiwan.

Immunohistochemical and *In Situ* Hybridization

For immunohistochemical staining, a 5- μ m thick section of the tumor tissue was deparaffinized, rehydrated, and then underwent antigen retrieval (DAKO target retrieval solution, high pH, S3307, autoclaved, 10 min). After cooling for 20 min at room temperature, decant retrieval solution was washed 2–3 times in room temperature using PBS solution. The primary antibody p16^{INK4A} (neomarker; JC-8, 1:50 dilution) was added and the specimen was incubated at 4°C overnight. Staining was performed using the Ventana autostainer (DAB detection kit; iVIEW, Ventana Medical Systems, SA, USA). The cases with more than 50% of tumor cells showing strong nuclear

staining with/without cytoplasmic staining were considered positive⁵³ (Figure 1a). The *in situ* hybridization of high-risk HPV was performed using Ventana autostainer (INFORM HPV II Family 16 Probe; *in situ* hybridization iVIEW™ Blue Plus Detection kit; Ventana Medical Systems). According to the manual provided by the manufacturer, each dispenser of the probe we used contains a cocktail of 10 ml of approximately 2 µg/ml of DNP-labeled genomic probes in a formamide-based diluent. This probe cocktail has an affinity to HPV genotypes 16, 18, 31, 33, 35, 45, 51, 52, 56, 58, and 66. Using this method of *in situ* hybridization, cases with more than 10% of tumor cells containing the integrated form (nuclear dots) of HPV were considered positive (Figure 1b).

DNA Extraction, Nested PCR and Genechips

Four 8-µm tissue sections were cut and deparaffinized, and DNA was extracted using a commercial kit (QIAamp DNA Mini Kit). In order to control the quality and quantity of the isolated DNA, a 300-bp sequence of the *β-actin* gene was amplified by PCR as internal control to monitor the genomic DNA extraction procedure. Each PCR amplification reaction was carried out in a total volume of 50 µl containing a PCR master mixture (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 800 µM each of dATP, dCTP, dGTP, 600 M dUTP, 10 pmol of each primer set, 1.25 IU/l AmpliTaq Gold DNA polymerase; Applied Biosystems, San Francisco, California, USA). Each PCR was carried out in the Geneamp PCR System 9700 (Applied Biosystems), with the first denaturation step at 94°C for 3 min and final extension step at 72°C for 5 min. General consensus primers *MY09/GP6+* were used for the first PCR round to amplify the corresponding part of the HPV *L1* gene in accordance with methods reported by others,⁵⁴ with

some modifications.^{55–57} A nested PCR was then carried out with the primers *GP5+/GP6+* according to previously published protocols.⁵⁸ A 15-µl volume of the resultant amplified product was then hybridized with an HPV genechip (Easychip HPV Genotyping Array; King Car, Taipei, Taiwan), which offers a revert-blot hybridization to detect 39 subtypes of HPV DNA in a single reaction, as reported by Huang and co-workers.^{5,59}

Real-Time PCR

Real-time PCR was performed in all HPV16-positive [HPV16(+)] cases with the ABI Prism 7900 Sequence Detection System and the TaqMan Universal PCR Master Mix (Applied Biosystems). Primer and probes used were designed in a previous study⁵¹ and synthesized by Purigo and Prisma. The probes were synthesized with the fluorescent reporter dye FAM (6-carboxy-fluorescein) attached to the 5' end and a non-fluorescent quencher with minor groove binder (Table 1). The amplification program included a preincubation step at 95°C for 10 min to activate the DNA polymerase, and a two-step cycle of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 60 s for a total of 40 cycles. The sizes of the *E2* and *E6* amplimers were 76 and 81 bp, respectively. The final primer and probe concentrations, in a total volume of 25 µl, were 0.3 and 0.1 M, respectively. A 40-ng weight of genomic DNA from the specimens was added to each reaction mixture. Standard curves were obtained by amplification of a dilution series of a plasmid (King Car, Taipei, Taiwan) solution, which carried the complete HPV16 genome from 10⁷ to 10² copies. The sensitivity of this method was 10² viral copies. Any data outside the standard curves (less than 10² copies) were viewed as representing an absence of viral load. No-template reactions were included as

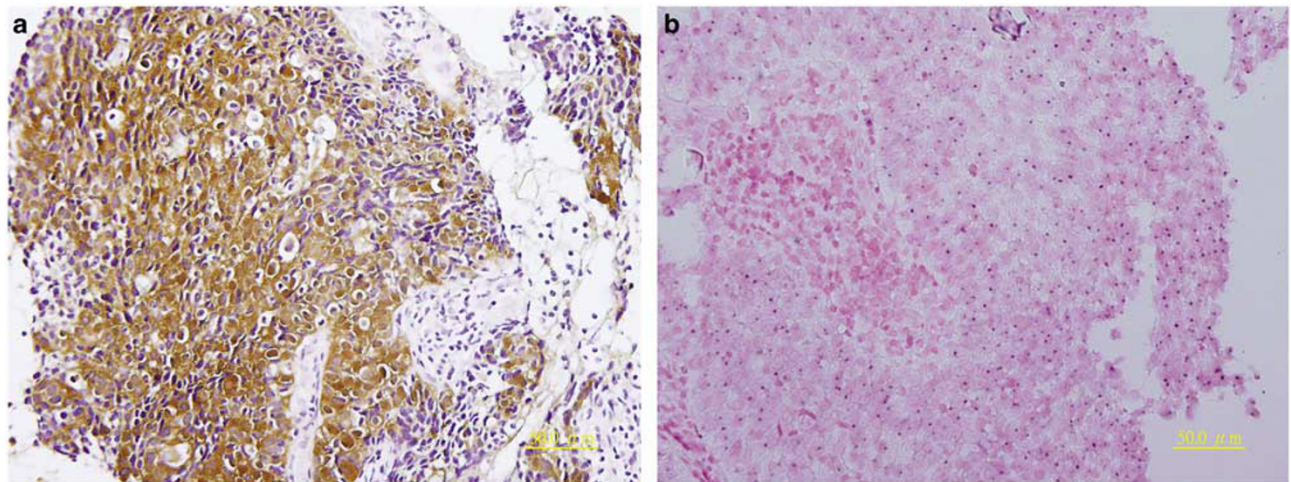


Figure 1 (a) Positive case of overexpression of p16^{INK4A} in tonsillar squamous cell carcinoma. (b) Positive case of high-risk HPV *in situ* hybridization in tonsillar squamous cell carcinoma.

negative control. All experiments were performed in triplicate and the mean of them was calculated. In order to demonstrate the status of infection (episomal, mixed, and integrated), *E2/E6* ratio was calculated. Integration was defined as absence of the *E2* signal or a *E2/E6* ratio less than 0.003.⁶⁰ A ratio between 0.004 and 0.999 indicates mixed episomal and integrated form, and a ratio not smaller than 1 means the episomal form is predominant.⁶⁰

Statistical Analysis

Data on clinical and pathological characteristics were compared by the Mann–Whitney test for continuous variables and the χ^2 -test for categorical variables. For analyses of survival rate, death from any cause was considered an event and data on patients who were alive at the last follow-up contact were censored. The Kaplan–Meier method was used to estimate the probabilities of survival. The multivariate analysis was used for finding out independent variables. The proportional hazards model was used with stepwise backward selection to evaluate independent prognostic factors for survival. These analyses were performed using SPSS for Windows version 11.0 software.

Results

Patient Characteristics and Treatment

The characteristics, treatment, and overall survival of 92 patients are summarized in Table 2. Data on specific treatment modality were lacking in 16 cases. The treatment modalities included surgery alone ($n=6$), surgery followed by radiotherapy ($n=13$), surgery followed by concurrent chemoradiation ($n=25$), radiotherapy alone ($n=7$), concurrent chemoradiation ($n=20$), chemotherapy followed by radiotherapy ($n=4$), and chemotherapy followed by surgery ($n=1$). We divided the treatment modalities into two groups (definitive surgery and definitive radiotherapy). The overall survival rates between these groups did not differ significantly (data not shown, $P=0.94$).

Data Associated with HPV Infection Status

Sixty-nine (75%) cases were positive for HPV nested PCR, and HPV DNA types 16, 18, 33, 35, 58, 66, and 69 can be detected in 58, 2, 2, 1, 3, 1, and 2 cases, respectively. The cases with HPV genotypes 35, 58, and 69 were positive for both p16^{INK4A} immunostainings and high-risk HPV *in situ* hybridization,

Table 1 Primers and probes used for real-time PCR

Name	Sequence (5' → 3')	T_m (°C)	Amplimer length (bp)
Probe 16E2PRO	FAM-CACCCCGCCGCGACCCATA-MGB	70	
Primer 1, 16E2F	AACGAAGTATCCTCTCCTGAAATTATTAG	59	
Primer 2, 16E2R	CCAAGGCGACGGCTTTG	60	76
Probe 16E6PRO	FAM-CAGGAGCGACCCAGAAAGTTACCACAGTT-MGB	69	
Primer 1, 16E6F	GAGAACTGCAATGTTTCAGGACC	59	
Primer 2, 16E6R	TGTATAGTTGTTTCAGCTCTGTGC	60	81

PCR, polymerase chain reaction.

Table 2 Characteristics, treatments, and survival of patients with primary tonsillar squamous cell carcinoma

Characteristics	All patients	HPV-PCR			p16 ^{INK4A}			HPV <i>in situ</i> hybridization		
		Positive	Negative	P-value	Positive	Negative	P-value	Positive	Negative	P-value
Patient no. (%)	92	69 (75%)	23 (25%)		49 (53%)	43 (47%)		40 (43%)	52 (57%)	
Age (years)				0.49			0.99			0.77
Median	51	52	48		51	51		52	51	
Range	29–79	29–79	39–77		29–79	37–79		29–79	37–79	
Male–female ratio	79:13	56:13	23:0	0.03	40:9	39:4	0.25	32:8	47:5	0.23
Keratinizing–non-keratinizing ratio	47:45	32:37	15:8	0.12	18:31	29:14	0.003	15:25	32:20	0.022
Alcohol (yes/no)	48/44	28/41	20/3	<0.001	14/35	34/9	<0.001	9/31	39/13	<0.001
Betel nut (yes/no)	41/51	23/46	18/5	<0.001	13/36	28/15	<0.001	9/31	32/20	<0.001
Smoking (yes/no)	63/29	40/29	23/0	<0.001	23/26	40/3	<0.001	17/23	46/6	<0.001
Staging (II/III/IV)	15/59/18	14/44/11	1/15/7	0.10	9/32/8	6/27/10	0.65	7/27/6	8/32/12	0.63
Definitive treatment				0.81			0.04			0.12
Surgery	45	33	12		30	15		24	21	
Radiotherapy	31	23	8		13	18		12	19	
Unknown	16	13	3		6	10		4	12	
5-year survival rate (%)	73	75	69	0.31	84	59	0.004	89	61	0.001
Median follow-up time (months)	28.7	36.1	18.1	0.01	36.1	19.7	0.06	36.1	19.7	0.17

HPV, human papillomavirus; PCR, polymerase chain reaction.

but all the cases with HPV genotypes 18, 33, and 66 were doubly negative. Among the 58 cases with HPV genotype 16, 34 cases were positive for both p16^{INK4A} immunostaining and high-risk HPV *in situ* hybridization, five cases were only positive for p16^{INK4A} immunostaining, and 19 cases were doubly negative. Combined with the data of real-time PCR in HPV16(+) cases, more than 10² viral copies were detected in 33 of 34 doubly positive cases (one integrated and 32 mixed), five of five cases positive only for p16^{INK4A} (one integrated, one episomal, and three mixed) and two of 19 doubly negative cases (two mixed). Forty of the HPV16(+) cases contained more than 10² viral copies, according to the real-time PCR method (223–688 689; median 19 909); while the remaining 18 HPV16(+) cases demonstrated less than 10² viral copies, also via the real-time PCR method (0–63; median 4). Among the 23 HPV nested PCR(–) cases, only four of them were positive for p16^{INK4A} immunostaining, but all cases had negative results for high-risk HPV *in situ* hybridization.

Comparing Clinical Data with Differentiated Methods

HPV-related tonsillar squamous cell carcinoma apparently tends to develop in patients who do not use alcohol, betel nut, or cigarettes, regardless of the method chosen to represent HPV infection (Table 2). A non-keratinizing histological type and a more

favorable 5-year survival rate seem to characterize HPV-related tonsillar squamous cell carcinoma with positive p16^{INK4A} immunostaining or high-risk HPV *in situ* hybridization (Table 2). Statistically, female patients seemed more likely to develop HPV-related tonsillar squamous cell carcinoma when using nested PCR for detection (Table 2).

Survival Analyses

Univariate analysis demonstrated that consumption of alcohol, betel nuts, and cigarettes was significantly associated with shorter survival and that patients with overexpression of p16^{INK4A} or *in situ* hybridization HPV-positive disease were significantly associated with longer survival (Table 3; Figure 2a–c). HPV-PCR, p16^{INK4A} and *in situ* hybridization were separately analyzed with all the clinical and pathological variables in multivariate analyses, which demonstrated that both p16^{INK4A} and *in situ* hybridization were independent prognostic factors, but HPV-PCR was not. In HPV16(+) cases, positive p16^{INK4A} and *in situ* hybridization were also significantly associated with longer survival (Figure 3a and b). Using real-time PCR, HPV16(+) patients with integrated and mixed forms of HPV DNA also shared significantly longer survival times than those with episomal forms of HPV DNA or lower viral DNA copies (<10²) (Figure 3c).

Table 3 Univariate and multivariate analyses of clinical and HPV markers for primary tonsillar squamous cell carcinoma patients

Variable	Category	No. of patients	5-Year survival rate (%)	P	P ^a	P ^b	P ^c
Age	<60 years	70	70	0.23		0.18	
	≥60 years	22	82				
Sex	Male	79	70	0.16			
	Female	13	91				
Histology	Keratinizing	47	69	0.35			
	Non-keratinizing	45	77				
Alcohol	Yes	48	61	0.02			
	No	44	83				
Betel nut	Yes	41	57	<0.01	0.01	0.10	
	No	51	83				
Cigarette smoking	Yes	63	67	0.03			
	No	29	86				
Stage	II	15	93	0.39			
	III	59	70				
	IV	18	69				
Definitive treatment	Surgery	45	81	0.04	0.10	0.08	0.13
	Radiotherapy	31	75				
	Unknown	16	NE				
HPV-PCR	Positive	69	75	0.31			
	Negative	23	69				
P16 ^{INK4A}	Positive	49	84	0.004		0.04	
	Negative	43	59				
<i>In situ</i> hybridization	Positive	40	89	0.001			<0.01
	Negative	52	61				

HPV, human papillomavirus; NE, not evaluable.

^aP, P-values obtained by multivariate analysis of all clinical/pathological variables and HPV-PCR.

^bP, P-values obtained by multivariate analysis of all clinical/pathological variables and p16^{INK4A}.

^cP, P-values obtained by multivariate analysis of all clinical/pathological variables and *in situ* hybridization.

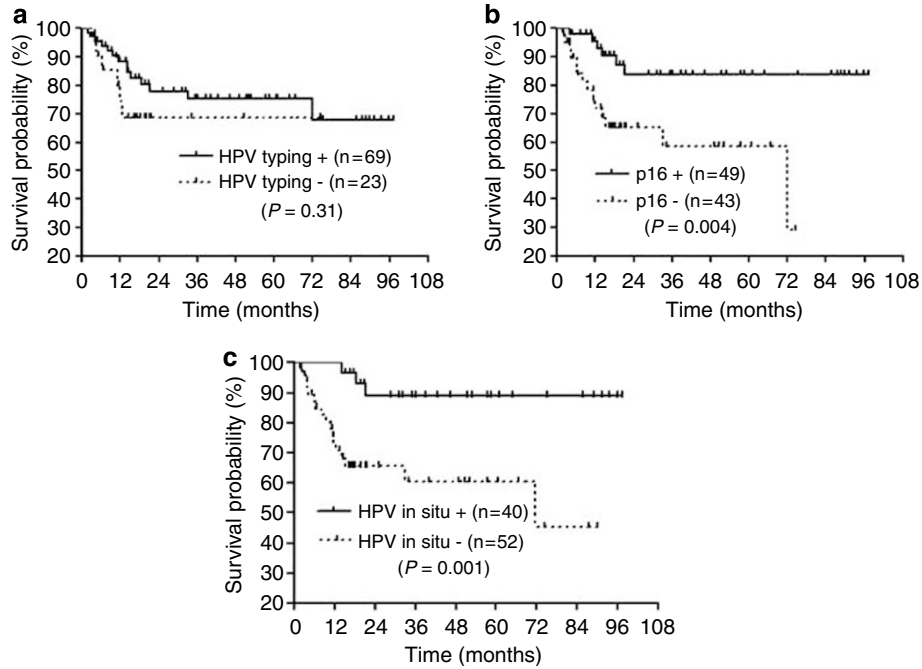


Figure 2 (a) Relationship between overall survival and HPV-PCR positivity in 92 patients with tonsillar squamous cell carcinoma (hazard ratio: 0.61; 95% CI: 0.19–1.69). (b) Relationship between overall survival and p16^{INK4A} immunostaining positivity in 92 patients with tonsillar squamous cell carcinoma (hazard ratio: 0.27; 95% CI: 0.10–0.64). (c) Relationship between overall survival and high-risk HPV *in situ* hybridization positivity in 92 patients with tonsillar squamous cell carcinoma (hazard ratio: 0.17; 95% CI: 0.10–0.57).

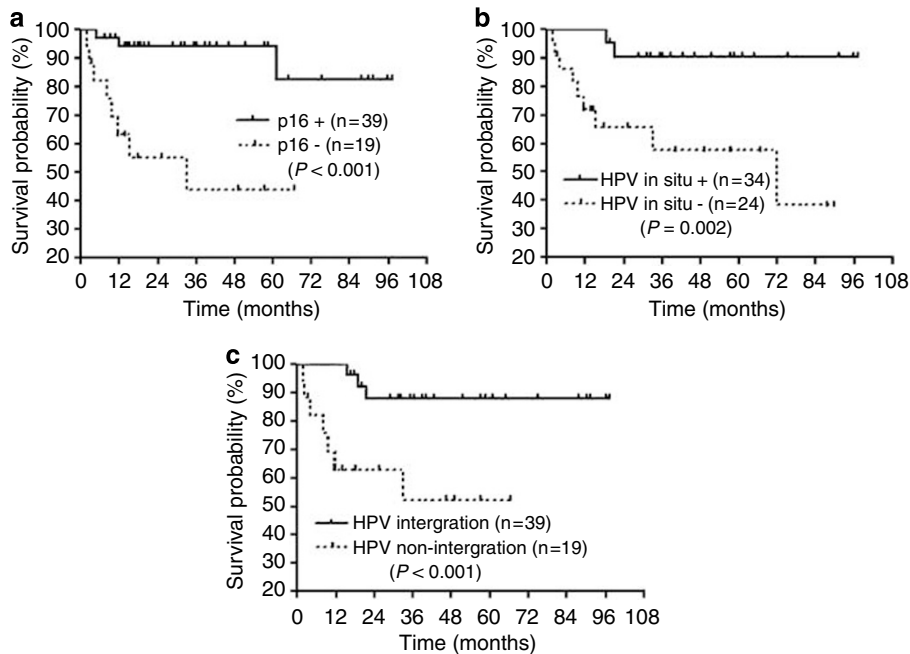


Figure 3 (a) Relationship between overall survival and p16 positivity in 58 patients with HPV16(+) tonsillar squamous cell carcinoma. (b) Relationship between overall survival and high-risk HPV *in situ* hybridization positivity in 58 patients with HPV16(+) tonsillar squamous cell carcinoma. (c) Relationship between overall survival and HPV integration in 58 patients with HPV16(+) tonsillar squamous cell carcinoma.

Discussion

HPV infection, like alcohol and tobacco, is now recognized to play a role in the pathogenesis of head

and neck squamous cell carcinoma, especially in the tonsillar area, which appears uniquely susceptible to transformation by the virus.^{5,9–28,31–33} HPV is known not to work via a hit-and-run mechanism;

therefore, demonstration of HPV genomic DNA in the tumor is essential for it to play a role in carcinogenesis.⁶¹

In this study, we used three methods to detect or represent HPV directly or indirectly in the tonsillar squamous cell carcinoma tissue, and the positive rate ranged from 43 to 75%. In comparison with previous reports,^{26–30} our findings are consistent with these reports and it seems that there are no differences between Caucasian and non-Caucasian patients, which is different from the results of Li *et al*.³⁴ Comparing the HPV-positive rate detected by similar PCR methods, it is significantly different in oral and tonsillar malignancies (41.7 vs 75.0%, $P < 0.001$).⁵ This difference was also noted in previous articles^{27,29–31} and in a more recent review article.⁶² This finding may be associated with anatomic site specificity. Like the cervix and anus,⁶³ where squamous cell carcinomas are also highly associated with HPV, transitional zones between different epithelial linings exist in the tonsillar area. Perhaps under these circumstances, the basal cell and the basal extracellular matrix (which are thought to be HPV receptors⁶⁴) are more likely to be exposed and infected by environmental HPV. According to our data and previous articles, we therefore believe that the anatomic site rather than ethnicity may influence the biological pathways of HPV-induced mucosal cancer.

In our study, irrespective of the method we used, HPV-positive tonsillar squamous cell carcinoma was more likely to occur in patients without alcohol, cigarette, and betel nut usage than HPV-negative tonsillar squamous cell carcinoma. Similar results have been proven by other reports.^{30,65,66} This may imply that in tonsillar squamous cell carcinoma patients who live relatively 'healthier' lifestyles, carcinogenesis may be associated with HPV. Although some studies have found associations between advanced TNM staging and HPV positivity,

these findings have been somewhat inconsistent. HPV-positive tumors tend to have non-keratinizing histology (Figure 4a and b),^{30,65,67,68} a point also demonstrated in our study. Using the methods of high-risk HPV *in situ* hybridization and immunostaining of p16^{INK4A}, our data, like previous reports,^{28–30,69} disclosed a better 5-year survival in HPV-positive tonsillar squamous cell carcinoma than HPV-negative tumors. Multivariate analysis also showed positive high-risk HPV detection (*in situ* hybridization), or expression of related proteins, (immunostaining of p16^{INK4A}) to be one of the most important independent prognostic factors. The reason for the favorable prognosis in patients with HPV-positive tumors is still unclear. Some studies proved E6-related degradation of p53 in HPV-positive cancers may be functionally inequivalent to HPV-negative p53 mutations,^{70,71} and therefore, HPV-positive cancers may have an intact apoptotic response to radiation and chemotherapy.⁷² On the other hand, *in vitro* cell line studies have revealed that HPV oncoproteins E6 and E7 mediate suppression of NF- κ B transcriptional activity; this may contribute to HPV escape from the immune system in cases of HPV-related carcinogenesis and poor tumor cell recovery after radiation and chemotherapy.⁷³

A prophylactic vaccine composed of the HPV16 viral capsid protein has recently been shown to prevent persistent HPV16 infection and development of cervical dysplasia in phase three randomized controlled trials,^{74,75} but the trials have not included the evaluation of head and neck HPV infection. Data on the influence of head and neck HPV infection under immunization are limited to canine and hamster models,^{76,77} which did show protective effects and a reduction in the development of HPV-positive lesions. If vaccines can give HPV-related tonsillar squamous cell carcinoma the same protective effect, we then have to tackle the

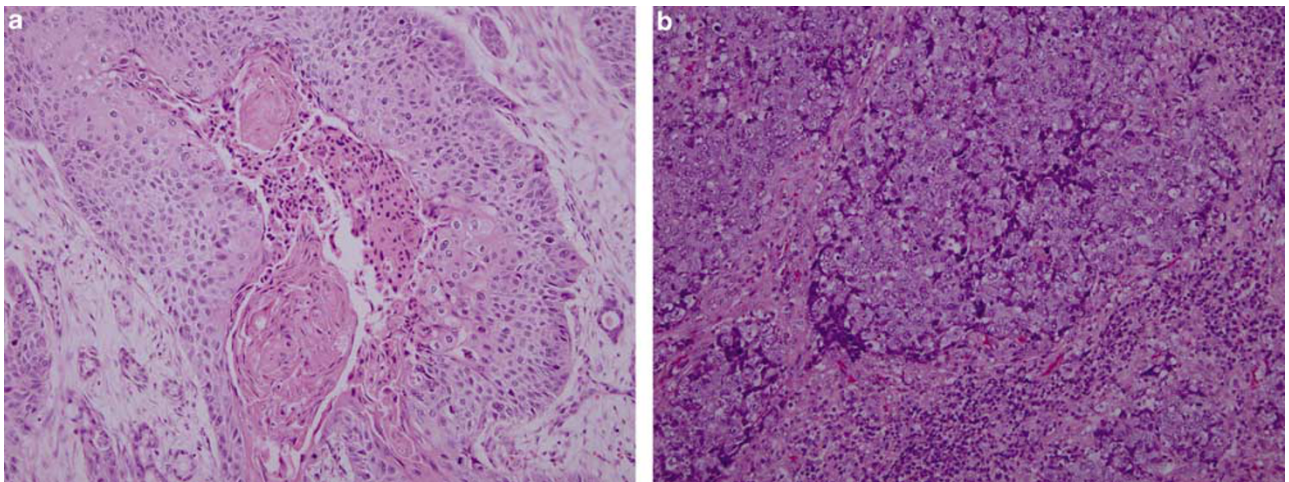


Figure 4 (a) H&E picture of a keratinizing tonsillar squamous cell carcinoma unrelated to HPV. (b) H&E picture of a non-keratinizing tonsillar squamous cell carcinoma related to HPV.

HPV-negative neoplasms with unfavorable prognoses, and the problem of finding vaccines with sustained efficacy against other high-risk HPV serotypes.

Recent reports suggest that HPV-positive tonsillar squamous cell carcinoma should be viewed as a unique subset and the diagnosis of HPV-positive tonsillar squamous cell carcinoma should be considered in all squamous cell carcinoma that arise from the head and neck.^{26,28,30,62} In this study, in addition to nested PCR, we used the immunostaining of p16^{INK4A} and commercialized autostainer for *in situ* hybridization to detect most high-risk HPV in tumor tissue, and compared the clinical and prognosis data. According to our results, although the nested PCR method had the highest HPV detection rate, it alone may be too sensitive to make a reliable prognostic prediction. Similar results are seen using real-time PCR. In 58 HPV16(+) cases detected with nested PCR, 18 cases (31%) had low viral copy numbers when real-time PCR was used to quantitate the HPV16 viral load. On the other hand, high-risk HPV *in situ* hybridization and immunostaining of p16^{INK4A} seem more clinically suited to provide us with reliable prognostic data. Weinberger and Psyrri recently reported similar results in cases of oropharyngeal squamous cell carcinoma.⁶⁹ In their series, all cases except one with overexpression of p16^{INK4A} were noted in their HPV(+) group, and the only case with overexpression of p16^{INK4A} noted in their HPV(-) group was excluded from their statistical analysis. They concluded that only the HPV +/p16 high group had a favorable prognosis.⁶⁹ We think that using real-time PCR or nested PCR alone for the detection of HPV DNA may not provide reliable results. Although not included in their statistical analysis, we believe that overexpression of p16^{INK4A} alone could served as a reliable prognostic marker in Weinberger and Psyrri's series, which had results similar to ours. p16^{INK4A} expression has been considered a surrogate biomarker for HPV-related cervical and vulvar cancers.⁴⁸⁻⁵⁰ Recent literature^{26,69} and our data (positive predict value for HPV was 91.8%) also confirmed this phenomenon in tonsillar squamous cell carcinoma, despite there being a few HPV(-) cases overexpressing p16^{INK4A}. A proportion of non-HPV-related human malignancies also overexpress p16^{INK4A}, for example, some endometrioid adenocarcinoma, endometrial and ovarian serous carcinoma, and urothelial carcinoma.⁷⁸⁻⁸⁰ This suggests that there should be some other types of control mechanisms, in addition to those described above, that can induce overexpression of p16^{INK4A}. In our data, a few cases with integrated HPV DNA did not overexpress p16^{INK4A}. A similar situation was reported in cervical cancer cells with p16 promoter hypermethylation leading to abrogation of p16^{INK4A}.⁸¹ Although a few exceptions in the association between p16^{INK4A} overexpression and HPV positivity have been noted, it does not influence the status of p16^{INK4A} as a

prognostic predictive maker for primary tonsillar squamous cell carcinoma and high-risk HPV *in situ* hybridization, as well as its importance in providing accurate prognostic information to allow for adequate treatment.

Acknowledgement

This research was supported by National Taiwan University Hospital Research Grant no. NTUH 95-309. The study has not been presented elsewhere. We thank Taiwan Molecular Diagnostic Laboratories Ltd for the kind donation of plasmid *pHPV16*.

Conflict of interest

The authors indicated have no potential conflicts of interest.

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