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Original Article

Human papillomavirus infection and its biomarkers' expressions in laryngeal basaloid squamous cell carcinoma

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Abstract: Aims: To investigate the frequency and transcriptional activity of HPV and its correlation to p16 and p21 expression in basaloid squamous cell carcinoma (BSCC) of the larynx. Methods: We evaluated tissues from 29 patients with BSCC of the larynx for the expressions of p16 and p21 proteins by immunohistochemistry (IHC) and for HPV E6 and E7 mRNA by RNA in situ hybridization (ISH). The presence of genotype-specific HPV DNA was evaluated using PCR-RDB in formalin-fixed paraffin-embedded tissues. P16 and p21 expression and HPV DNA status were correlated with clinicopathological features. Results: HPV DNA was detected in 8 of 29 (27.59%) patients, with HPV-16 being the predominant genotype. P16 and p21-positivity were observed in 7/29 (24.14%) and 8/29 (27.59%) patients, respectively. HPV was not correlated with p16 expression ($P > 0.05$). However, p21 expression was significantly higher in HPV-positive tumors than in HPV-negative tumors ($P < 0.05$). No cases exhibited transcriptionally active HPV in our series. Conclusion: Our findings suggest that a small fraction of BSCC of the larynx is HPV DNA-positive in this Chinese population, p21 expression was significantly higher in HPV-positive tumors, and no cases were HPV transcriptionally active in this small cohort. Further research of HPV and its role in BSCC of the larynx are warranted.

Keywords: Human papillomavirus, BSCC, HPV DNA, HPV mRNA, p16, p21

Introduction

Human papillomaviruses (HPV) are small, double-stranded DNA viruses and are divided into mucosal and cutaneous types, based on the tissue tropism. HPV 16 is the most oncogenic type and is responsible for approximately 55% of cervical cancer cases worldwide [1]. Recent studies have shown that a subset of head and neck carcinoma is associated with HPV [2, 3], especially oropharyngeal carcinomas. However, the oncogenic role of HPV infection in the larynx has not yet been fully evaluated. Several studies have reported the presence of high-risk HPV in a minority of laryngeal cancers; however, it is currently unknown whether there is a causal association between HPV and laryngeal squamous cell carcinoma (LSCC), or if a significant fraction of LSCCs are attributable to HPV. The reported prevalence of HPV DNA in LSCCs varies from 0% to 75% [4, 5]. This wide range may be due to the geographical differences of the study populations, tumor site misclassification, or an admixture of LSCCs with other tumor sites, such as oropharyngeal carcinomas [6]. However, the presence of HPV DNA alone cannot demonstrate causality. The existence of HPV in a transcriptionally active form has been identified as the gold standard biomarker for tumorigenesis. In addition, the concept for the causal involvement of HPV in the pathogenesis of SCC includes the following: (a) the presence of at least 1 viral genome copy per tumor cell; (b) active transcription of the viral oncogenes E6 and E7; (c) interaction of the viral oncoproteins with central cellular regulator proteins, such as p16 [7, 8].

P21, a tumor suppressor protein in the TP53 cascade, was reported as a potential biomarker

of HPV in tonsillar squamous cell carcinoma by Hafkamp et al. [9]. And P21 overexpression was reported in HPV-related cervical carcinoma, suggesting that p21 may play an important role in HPV-mediated tumorigenesis [10].

Previous studies showed HPV is associated with basaloid morphology of the head and neck SCC [11, 12], but it is now accepted that not all basaloid squamous cell carcinoma (BSCC) cases are HPV positive. BSCC of the larynx is a rare variant with distinct pathologic characteristics [13], but few studies had reported the relationship between HPV and this rare type of LSCC. In the present study, we investigated the frequency and transcriptional activity of HPV and the correlation to p16 and p21 in BSCC of the larynx.

Materials and methods

Cases of primary squamous cell carcinoma of the larynx diagnosed between 2005 and 2011 were identified from the archive of the Institute for Pathology, Beijing Tongren Hospital (Beijing, China). A total of 332 formalin-fixed and paraffin-embedded tissue blocks were collected, including 303 usual SCC and 29 BSCC. None of the patients had previously undergone adjuvant radiotherapy or chemoradiotherapy. Patient characteristics, including sex, age, tumor subsite, a history of alcohol consumption, and smoking status were obtained from clinical records. The pathological information collected included tumor histological types, lymph node metastasis, T classification, and TNM stage, which were reviewed in each case and confirmed independently by 3 pathologists. The TNM stage was classified according to the 2009 American Joint Committee on Cancer (AJCC) system as confirmed by the Head and Neck Oncology Group.

Genomic DNA extraction

Four paraffin sections (4 μm thickness) were prepared from each block of formalin-fixed, paraffin-embedded tissues and transferred into a 1.5 ml DNase-free microtube. After xylene was added to each tube for dewaxing purposes, 100 μL of lysis solution was mixed together well, and then incubated in 100°C water for 10 minutes, immediately followed by centrifugation for 10 minutes at 13,000 RPM. Finally, the middle solution was used as the amplification template DNA.

HPV genotyping

A HPV Genotyping Kit for 15 types (Yaneng Biotech) was used to perform PCR-RDB, which can identify 13 high risk-HPV types (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68) and 2 low risk-HPV types (HPV-6, -11). The total cellular DNA was extracted from specimens according to the manufacturer's instructions. 5 μL of the extracted HPV DNA was used as a template, and then the HPV was amplified in a thermal cycler under the following conditions: 50°C for 15 minutes, 95°C for 10 minutes; 40 cycles of 94°C for 30 seconds, 50°C for 90 seconds, 72°C for 30 seconds; and final extension at 72°C for 5 minutes. After amplification, the HPV genotyping was performed by hybridization and RDB on the strips.

Interpretation of HPV infection

The final results were directly judged by the naked eye. If only "PC" showed a blue spot, the sample was considered negative or below the limit of detection ($< 1.0 \times 10^3$ copies/ml). A blue spot observed on both "PC" and any site indicated a positive result, especially for a single HPV infection, while multiple sites showing blue dots represented a multi-type HPV infection. The numbers on the strip represented the HPV genotypes. "PC" was considered positive based on an internal control. If the "PC" site was negative, the result was considered invalid.

Tissue microarray (TMA) construction

Tumor-rich areas were selected and circumscribed on H&E stained slides. Each slide was then overlaid on the original paraffin block to determine the donor area. A Beecher manual tissue microarrayer MTA-1 (Beecher Instruments, Inc., Sun Prairie, WI) was used to construct TMAs. A 2 mm tissue cylinder was punched out and transferred into the recipient block. A map specifying the exact position of each case was made. After construction, a cut section of the TMA was again stained with H&E to ensure accurate tumor sampling.

Immunohistochemical analysis of TMA

We measured expression patterns of p16 and p21 by immunohistochemistry (IHC). The tissue sections were cut at 5 μm, heated to 60°C, deparaffinized in xylene, and hydrated in a

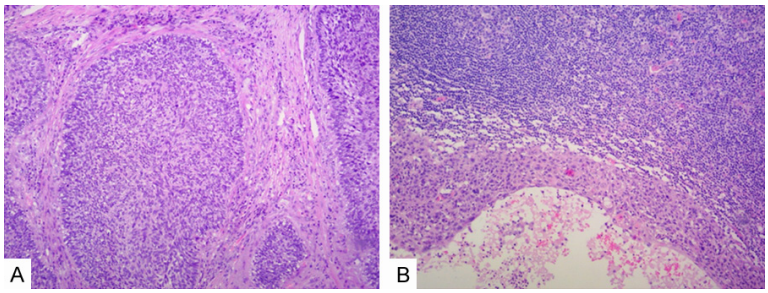

Figure 1. A. Characteristics of BSCC include solid nesting, and the surrounding cells are arranged in a palisade (× 100). B. Cystic lesion in lymph node (× 100).

Table 1. Clinicopathologic characteristics of patients with BSCC of the larynx

Variables	BSCC	SCC	Statistic	*P*
Sex				
Male	27	280	χ^2 = 0.018	0.892
Female	2	23		
Age	62.55	59.00	*t* = -1.801	0.073
Alcohol				
Yes	16	188	χ^2 = 0.528	0.468
No	13	115		
Smoking				
Yes	24	266	χ^2 = 0.606	0.436
No	5	37		
Tumor sites				
Supraglottis	15	108	χ^2 = 2.934	0.087
Glottis+subglottis	14	195		
TNM stage				
I	3	31	Z = -0.614	0.539
II	10	75		
III	8	111		
IV	8	86		
Tumor size				
T1	3	34	Z = -0.195	0.845
T2	12	102		
T3	7	106		
T4	7	61		
lymph node metastasis				
Yes	7	60	χ^2 = 0.309	0.578
No	22	243		
No. of lymph node metastasis	0.79	0.59	*t* = -0.570	0.569
Type of Lymph node metastasis				
Cyst	1	21	χ^2 = 1.164	0.281
Solid	6	40		

graded alcohol series. When necessary, antigen retrieval was performed in a citrate buffer (PH = 6.0) using a pressure cooker for 1 minute, 30 seconds. Endogenous peroxidase activity was inactivated by incubation in 3% hydrogen peroxide for 10 minutes. The tissue sections were then incubated with primary antibodies against p16 (predilution, ZM-0205, OriGene, USA) or p21 (1:50, ZM-0206, OriGene, USA), at 37°C for 2 hours. All sections were sequentially treated with biotinylated anti-mouse immunoglobulin for 20 minutes at 37°C and then peroxidase-labeled streptavidin for 20 minutes at 37°C. A negative control was replaced by PBS. All sections were counterstained with 3, 3'-diaminobenzidine tetra-hydrochloride (DAB).

Immunohistochemical staining was assessed in cancer cells only and evaluated by 2 investigators. Protein expression was semiquantified according to the IRS described by Antonsson et al. [14]. Briefly, the p16 antibody stained both the nucleus and cytoplasm of each of the tumor cells, and each slide was given a score by multiplying the positivity proportion score by the intensity score, ranging from 0~12, and scores ≥ 4 were considered p16-positive. p21 expression was assessed as a nuclear pattern, using the same scoring system; scores ≤ 2 were considered negative, and scores ≥ 3 were regarded as positive (p21 overexpression). The positivity proportion of the tumor cells was graded as follows: 0 = 0%, 1 = 1~10%, 2 = 11~50%, 3 = 51~80%, 4 = 81~100% and the staining intensity: 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining.

RNA in situ hybridization

RNA ISH for HPV 16/18 E6/E7 mRNA was performed manually using the RNAscope® HPV kit

Table 2. The relationship between HPV DNA and clinicopathological features

Variables	HPV+	HPV-	Statistics	*P* value
Age	62.62	62.52	t = -0.025	0.980
Alcohol consumption				
No	2	11	χ^2 = 1.756	0.238
Yes	6	10		
tumor sites				
Supraglottis	5	10	χ^2 = 0.514	0.682
Glottis+subglottis	3	11		
TNM stage				
I-II	4	9	χ^2 = 0.12	1.000
III-IV	4	12		
Tumor size				
T1-T2	4	11	χ^2 = 0.013	0.909
T3-T4	4	4		

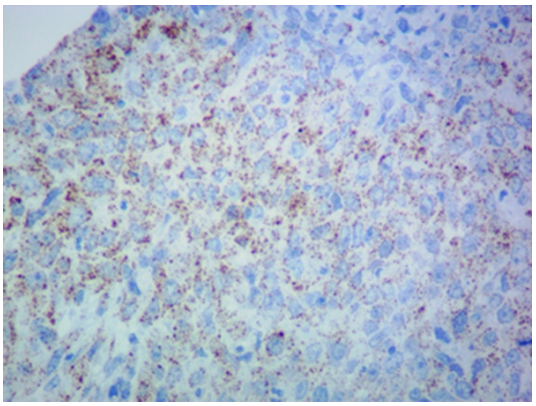

Figure 2. Strong staining signals of HPV mRNA were identified in the cytoplasms of tumor cells (positive control) (× 400).

(Advanced Cell Diagnostics, Inc., Hayward, CA) according to the manufacturer's instructions. Briefly, 4 μm formalin-fixed and paraffin-embedded sections from the TMA were dewaxed and pretreated with heat and protease prior to hybridization with a target probe to the HPV 16 and 18 genotypes. The preamplifier, amplifier, and HRP-labeled probes were then hybridized sequentially, followed by color development with DAB. Probes to the bacterial gene dapB and the endogenous UBC mRNA were used as negative and positive controls, respectively, for each case. Positive staining was identified as brown, punctate dots present in the cytoplasm. All 3 stained slides of each case (HPV, UBC and dapB) were examined together to determine the HPV status. HPV positive cases had definitive punctate brown staining present in at least 1 subset of tumor cells.

Statistical analysis

The statistical analysis was performed using Student's *t* test, the Mann-Whitney U test, and χ^2 test or Fisher's exact test for small samples when the expected number in any cell was < 5 (SPSS, version 19.0). All statistical tests were 2-sided, and a $P < 0.05$ was considered statistically significant.

Results

Clinicopathologic characteristics of BSCC

A total of 303 patients with conventional SCC and 29 (8.73%, 29/332) patients with BSCC were identified (**Figure 1A**). **Table 1** summarizes the clinicopathologic characteristics of patients with BSCC of the larynx. The patients were between 46~82 years old, with a median age of 62 years. Most patients were male (27/29, 93.10%) and had a history of smoking (16/29, 82.76%) and alcohol consumption (16/29, 55.17%). More than half of BSCC tumors were in the supraglottis (51.72%), and advanced stage disease (stages III and IV together) accounted for 55.17% of the cases. Neck dissection showed 7 (7/29, 24.13%) patients with nodal involvement, among which 1 patient was found to have cystic nodal metastasis (**Figure 1B**). Compared with conventional SCC, the mean age at diagnosis, the number of lymph node metastases, and the rate of supraglottic location were slightly higher (62.55 *vs* 59.00, 0.79 *vs* 0.59, 51.72% *vs* 35.64%, respectively), but the differences were not significant ($P > 0.05$).

HPV findings

Overall, 27.59% (8/29) of the BSCC tumors in this series were HPV-positive, and HPV16 was identified in all the positive cases. Interestingly, all HPV-positive patients were male and had a history of smoking. There was no lymph node metastasis in the HPV-positive cases, while 7 HPV-negative cases were identified with lymph node metastasis. The presence of HPV DNA was not significantly associated with any clinicopathological features ($P > 0.05$) (**Table 2**). HPV RNA ISH was performed on all patient samples. All cases showed strong signals on the positive control (**Figure 2**), but none of the

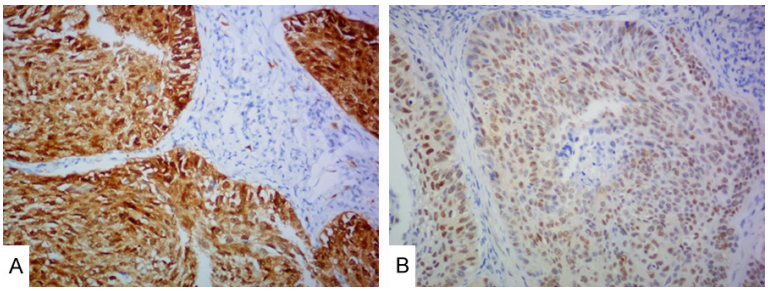

Figure 3. A. p16 exhibits strong nuclear and cytoplasmic staining in a diffuse pattern (× 200). B. Strong nuclear staining of p21 protein (× 200).

Table 3. The relationship between p16 and p21 proteins and clinicopathological features

Variables	p16		*P*	p21		*P*
	+	-		+	-	
Sex						
Male	5	22		8	19	
Female	2	0		0	2	
Age	59	63.68	0.211	62.75	62.47	0.946
Alcohol						
Yes	6	10	0.238	4	12	1.000
No	2	11		4	9	
Smoking						
Yes	8	16		7	17	1.000
No	0	5		1	4	
Tumor sites						
Supraglottis	6	9	0.08	5	10	0.682
Glottis+subglottis	1	13		3	11	
TNM stage						
I-II	2	11	0.410	5	8	0.406
III-IV	5	11		3	13	
Tumor size						
T1-T2	3	12	0.682	5	10	0.682
T3-T4	4	10		3	11	
LNM						
Yes	3	4	0.311	2	5	1.000
No	4	18		6	16	
No. of LNM	0.6	1.3	0.440	1.25	0.6	0.433

LNM: lymph node metastasis.

cases had positive signals for HPV16/18 RNA in our series.

Immunohistochemical findings

The expression of p16 was detected in 7 (24.14%) of 29 cases (**Figure 3A**). Two women in this series were p16-positive, while 5 men (5/27, 18.52%) were p16-positive. Lymph node metastasis was slightly higher in the p16-positive cases compared to the p16-negative cases (42.86% *vs* 18.18%), but without a significant difference ($P > 0.05$). P21 positivity was seen in 8 cases (8/29, 27.59%) (**Figure 3B**). There were no statistically significant differences in age, smoking/alcohol history, tumor stage or lymph node metastasis by p21 tumor status ($P > 0.05$) (**Table 3**). In addition, 3 cases (3/8, 37.50%) were HPV+/P16+, while 5 cases (5/8, 62.50%) were HPV-/P16+. P21 expression was significantly higher in HPV-positive tumors than in HPV-negative tumors ($P < 0.05$) (**Table 4**).

Discussion

BSCC was first described in 1986 [15] and has been reported in the larynx, hypopharynx, oral cavity, oropharynx, and in other sites in the upper aerodigestive tract as well as other anatomical sites. Although approximately one-fourth of the cases reported in the upper aerodigestive tract are in the larynx [16], they comprise less than 1% of all laryngeal cancers [17]. Microscopically, BSCC has a biphasic pattern which includes a basaloid component and often areas with typical squamous differentiation [18]. The basaloid cells are pleomorphic with minimal cytoplasm and hyperchromatic nuclei, and form demarcated nests of small basaloid cells with peripheral palisading and show numerous mitotic figures. Comedo necrosis is common. In our report, the median age of the patients at diagnosis was 62 years, 93.1% of patients were men, and more than half of the reported tumors were located in the supraglottis (51.7%) and diagnosed at an advanced stage (55.2% III/IV). Our data is largely consis-

Table 4. The relationship between HPV DNA and cell cycle related proteins

Cell cycle proteins	HPV+	HPV-	Statistic	*P* value
p16				
+	3	4	$\chi^2 = 1.077$	0.357
-	5	17		
p21				
+	5	3	$\chi^2 = 6.741$	0.019
-	3	18		

tent with previous studies [19]. Fritsch et al. [20] compared 145 cases of BSCC to 20,866 conventional SCC cases and found that patients with BSCC were more likely to harbor node metastases, but in our study, the 2 groups had statistically similar node metastasis rates ($P > 0.05$). The authors suggest that our small cohort did not include enough patients and therefore could not detect a significant difference between the groups.

The overall prevalence of HPV DNA in our study was 27.59%, with HPV16 being the predominant genotype. This is comparable with a previous study that reported an HPV positivity rate of 32% in BSCC [21]. Of note, we focused on a single site of the larynx only, while other studies often mixed different subsites of head and neck cancers. However, the presence of HPV DNA alone cannot demonstrate causality, and HPV mRNA is a better approach than DNA-based methods for providing evidence of clinically relevant HPV infection. Recent studies reported that HPV mRNA positivity rates in LSCC ranged from 1.6%~6.5% [22, 23], suggesting that only a small fraction of LSCC cases are HPV-driven tumors. In our series, none of the cases were HPV mRNA positive. The presence of HPV DNA in tumors may represent a very low viral burden that is incidental or "bystander" detectable only by a technique as sensitive as PCR. This seems plausible, since HPV DNA has been found in a significant proportion of benign laryngeal lesions [24].

The correlation between p16 and HPV infection in oropharyngeal cancer is well established; nonetheless, the relationship between the 2 indicators in LSCC remains controversial. Some have reported that p16 may act as a surrogate marker for HPV infection in LSCC [25], whereas others discouraged the use of p16 expression [26, 27]. Our results suggest that p16 is not correlated with HPV DNA and the specificity of p16 is poor (37.5%). In addition, none of the cases overexpressing p16 had a transcriptionally active HPV infection, suggesting that mechanisms other than HPV infection are responsible for p16 overexpression in BSCC of the larynx.

We identified p21 overexpression in a proportion of tumors (28%) in our series, a rate slightly lower than in other reports [28]. The cutoff point used for p21 overexpression may be the reason Jeannon [28] defined increased expression of p21 as > 50% positive cells, whereas we used another criteria (described above). Our results showed that p21 expression was significantly higher in HPV-positive tumors than in HPV-negative tumors. Some evidence indicated that HPV E7 may be responsible for increased p21 protein levels. However, in our cases, none was HPV mRNA positive, and there may be other factors leading to p21 overexpression.

Conclusions

In summary, we found that a small subset of BSCC of the larynx was HPV DNA positive, and p21 expression was significantly higher in the HPV-positive tumors. However, p16 was not found to be a surrogate marker for HPV infection. Given that none of our cases exhibited transcriptionally active HPV, studies with much larger cohorts would be required to definitively establish whether BSCC of the larynx is HPV-driven.

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Disclosure of conflict of interest

None.

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