Original Article

Detection of Human Papilloma Virus Type 16 E6 mRNA in Laryngeal Squamous Cell Carcinoma by In Situ Hybridization

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ABSTRACT

Objective: Laryngeal squamous cell carcinoma (LSCC) is a common malignant tumor in Northeast China and is frequently associated with well-established risk factors like smoking and alcohol abuse. Human papilloma virus (HPV) is an epitheliotropic oncogenic virus that has been detected in a variety of head and neck tumors including LSCC. This retrospective study was to investigate the prevalence of HPV infection in patients with LSCC.

Methods: In situ hybridization was performed in 99 patients with LSCC to detect the expression of HPV-16 E6 mRNA.

Results: The positive rate of HPV16 E6 mRNA was 36.36% (36/99) in laryngeal squamous cell carcinoma (LSCC), whereas only 3 of 50 (6%) specimens of the normal laryngeal mucosa as a control group showed positive results ($P<0.05$).Additionally, there was no corelation between HPV16 and age, gender, clinical stage, nodal status and tumor site ($P>0.05$).

Conclusion: The results suggest that the increased prevalence of HPV infection compared to normal laryngeal mucosa and the fact that high-risk HPV types (especially type 16) were the most frequently identified do not allow the exclusion of HPV as a risk factor in laryngeal squamous cell carcinoma. However, their clinical value remains to be further investigated.

Key words: Laryngeal squamous cell carcinoma; Human papilloma virus; In situ hybridization

INTRODUCTION

Human papillomavirus (HPV) is a small double-stranded circular DNA molecule of approximately 8000 base pairs, belonging to the Papillomaviridae family, small DNA viruses, of which the genetic organization is very similar[1, 2]. It is believed to be an important contributing factor in the etiology of certain benign and malignant tumorous lesions in human. In cervical carcinomas different types of HPV have been detected in up to 96% of all cases[3]. HPV is also associated with benign tumors of the upper aerodigestive tract, such as laryngeal papillomas[4]. With the resemblance of HPV induced genital condylomas and laryngeal papillomas, and the frequent association between HPV and genital carcinomas, HPV may be expected to be associated with laryngeal squamous cell carcinoma (LSCC) as

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well.

Over the past 20 years, high-risk human papillomavirus (HPV) infection has been established as a risk factor for developing head and neck tumors including LSCC, independent of tobacco and alcohol use[5]. Current data suggest that approximately 20%–25% of head and neck squamous cell carcinomas (HNSCC) are linked to HPV infection[2], which has also been found in 0 to 63% of LSCC using different techniques of HPV detection[6–13]. These patients may display a clinical course different from those patients with HPV negative LSCC and may be helped by additional therapeutic modalities including vaccine immunotherapy. Due to these considerations, the detection of HPV in patients with LSCC is of clinical importance[2, 14]. Hence, to contribute to the understanding of the role of HPV in LSCC, in this study we detected the expression of HPV16 E6 mRNA in laryngeal squamous cell carcinoma and normal laryngeal mucosa tissues by in situ hybridization (ISH); and evaluated the association between HPV status and clinical/pathological parameters of LSCC.

MATERIALS AND METHODS

Patients and Laryngeal Carcinoma Tissue Specimens

From December 1995 to November 2008, a total of 99 paraffin-embedded tissue samples of diagnosed primary laryngeal carcinoma were obtained from the Beijing Cancer Hospital and Shougang Hospital for this study. All tumor specimens were reviewed independently by two pathologists for verification of the diagnosis. Also, 50 cases of laryngeal mucosa with no, or very minor, histological alterations were included in this study as a control group. From the formalin-fixed and paraffin-embedded laryngeal biopsy specimens, 5-μm sections were obtained and placed on organosilane-pretreated slides to be submitted to ISH analysis. The entire study was conducted according to ethical permissions from the Ethical Committee of the Hospital.

Patients' ages ranged from 36 to 78 years (median age, 65 years). Sixty-nine patients were males (69.7%) and thirty were females (30.3%). Tumor staging was done according to TNM classification after histological studies. By histologic analysis on haematoxylin-eosin-stained sections, 39 tumors were graded as well differentiated, 42 as moderately differentiated, and 18 as poorly differentiated/undifferentiated. Special care was taken not to contaminate the samples. Disposible or sterile instruments were used to harvest and store the samples.

In Situ Hybridization (ISH)

ISH mRNA expression of HPV16 E6 gene in LSCC tissues was carried out as previously described[15]. The HPV16 E6 gene was cloned by PCR using primers (forward, 5'-ATGCACCAA-AAGAGAACTG C-3' and reverse, 5'-ACAAGACA-TACATCGACCGG-3') with plasmid containing full-length HPV 16 as template. PCR products, 438 bp in length (82 to 520 on HPV16 genome), were purified and ligated into PGEM-T-easy vector (Promega, Madison, WI). After Sal I digestion, the antisense probes of HPV16 E6 were digoxin-labeled by *in vitro* transcription with T7 RNA polymerase according to the manufacturer's instructions (a Kit purchased from Boehringer Mannheim, Germany). Meanwhile, 4 μm-thick paraffin-embedded slices were dewaxed, rehydrated and treated with 0.2 mol/L HCl for 10 min, followed by digestion with 100 μg/ml proteinase K for 15 min at 37°C. The slides were washed with 1×PBS, dehydrated, and then hybridized with 50 ng digoxin-labeled HPV16 E6 probe in 20 μl hybridization solution containing 50% formamide, 4×SSC, 5% dextran sulfate, 5×Denhardt's solution and 200 mg/ml denatured salmon sperm DNA. After hybridization at 42°C overnight, the slides were washed with 2×SSC and 1×SSC for 30 min each. Antidigoxin antibody conjugated with alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany. Cat No.1175041) was added to the samples for 30 min at 37°C. After washing, the slides were developed by the nitroblue tetrazolium chloride/5-bromo-4-indoly phosphate system (Boehringer Mannheim, Mannheim, Germany. Cat. No.1175041). The reaction was stopped by washing the slides with PBS (pH 7.4). The purple-blue signals located in the cytoplasm represent positive staining for HPV16 E6 mRNA. Slides with HPV16 positive CaSki cells were used as positive control. The hybridization solution with digoxin-labeled sense probes of HPV16 E6 was used as negative control. The staining was scored on a scale from 0 to III as follows: 0, less than 5% cells were stained; 1+, 5%–25% cells were stained; 2+, 25%–50% cells were stained; and 3+, more than 50% cells were stained. Scores 1+ to 3+ were classified as positive, while score 0 was negative.

Table 1. *HPV-16 E6 mRNA expression by ISH in normal larynx and laryngeal carcinoma*

Samples	Cases (n)	Expression intensity				Positive rate (%)	P^*
		-	+	++	+++		
Normal	50	47	1	2	0	6	<0.01
Tumor	99	63	9	13	14	36.36	

$^*\chi^2$ test

Table 2. *Association between clinicopathological features and HPV-16 E6 mRNA expression in99 patients with LSCC*

Patient or tumor characteristic	N	HPV-16 E6 mRNA expression		*P* value
		N	%	
Gender				
Male	69	25	25.25	0.570
Female	30	11	11.11	
Age				
< 60	40	15	15.15	0.506
≥ 60	59	21	21.21	
Tumor status				
T0	4	0	0	0.302
T1	10	4	4.04	
T2	25	9	9.09	
T3	37	17	17.17	
T4	23	6	6.06	
Localization				
Supraglottic	31	11	11.11	0.967
Glottic	58	21	21.21	
Subglottic	10	4	4.04	
Grade				
Well	39	12	12.12	0.282
Moderate	42	19	19.19	
Poor/un	18	5	5.05	
Stage				
I	7	1	1.01	0.095
II	18	6	6.06	
III	30	18	18.18	
IV	44	11	11.11	
Lymph nodes				
Positive	64	27	27.27	0.078
Negative	35	9	9.09	

Statistical Analysis

Statistical analysis was performed using the SPSS statistical software. The relationship of HPV16 E6 mRNA detected by ISH in cancer and normal samples was statistically evaluated by χ^2 test. HPV-positive and HPV-negative patients were compared by using Fisher exact test or χ^2 test between different clinical/pathological parameters of LSCC. All statistical tests were two-sided and a P value of 0.05 or less was considered statistically significant.

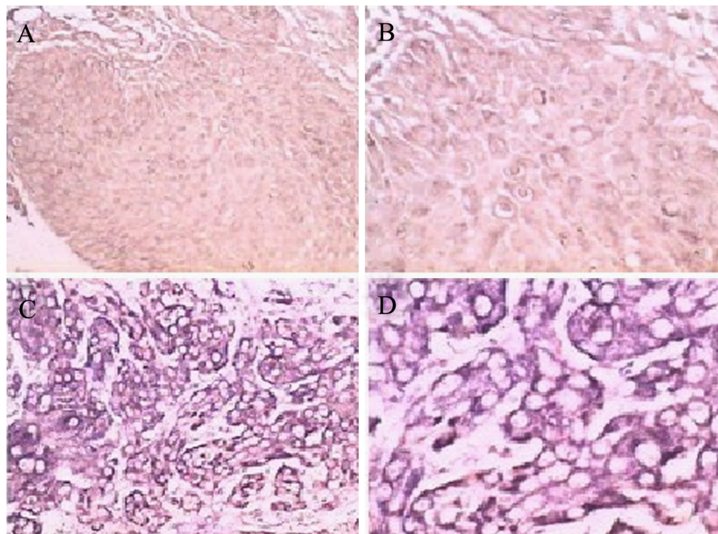

Figure 1. ISH staining of HPV16 E6 mRNA in LSCC tissues. A, B: ISH negative control using digoxin-labeled sense probes of HPV16 E6 in a sample with LSCC. C, D: ISH positive staining of HPV16 E6 mRNA in a sample with LSCC. The positive signal is located in the cytoplasm and inner side of cell membrane. Magnification, (A, C) 200× and (B, D) 400×, respectively.

RESULTS

Using digoxin-labelled HPV16 E6 specific cRNA probe, 36 of 99 samples (36.36%) showed positive ISH signals in the cytoplasm of cancer cells, while 3 out of 50 samples (6%) from the normal laryngeal mucosa were positive for HPV16. The difference of the expression of HPV16 E6 mRNA between cancer and normal samples was significant ($P<0.05$, Table 1). ISH negative control was plain in color using digoxin-labeled sense probes of HPV16 E6 in a sample with LSCC.

Among those positive samples, the intensity of the nuclear hybridization signal for HPV in tumor and normal cell nuclei was scored as 1+ for 9 tumors and 1normal tissue, 2+ for 13 tumors and 2 normal tissues, and 3+ for 14 tumors (Figure1, Table 1).

In addition, we analyzed the association of patient variables (eg, age, gender, clinical stage, nodal status, and tumor site) and tumor HPV status. No significant statistical differences between HPV16 E6 mRNA -positive and HPV16 E6 mRNA -negative patients were observed in the subset of patients with laryngeal tumors (Table 2).

DISCUSSION

At least 120 subtypes of HPV have been identified and can be characterized by their predilection for distinct mucosal or dermal lesions, 33% of which are known to infect the human genital tract[16]. Certain virus types are frequently found associated with cancers (such as cervical cancer, anal cancer, vulvar cancer, penile cancer[17] and lung cancer[18, 19]) and therefore considered high-risk HPVs (the most frequently detected being HPV 16, 18, 31 and 45). In particular, type 16 has been found to be associated with oropharyngeal squamous-cell carcinoma, a form of head and neck cancer[20], whereas others usually give rise to benign lesions or warts and are thus classified as the low-risk HPV types (the most common of these being HPV 6 and 11). HPV-induced cancers often have viral sequences integrated into the cellular DNA. Some of the HPV "early" genes, such as E6 and E7, are known to act as oncogenes that promote tumor growth and malignant transformation. The E6 protein of high-risk HPV types has been shown to bind the tumor suppressor p53 protein and to promote its degradation[21], and the E7 protein to bind the Rb tumor suppressor protein[22]. In cervical cancer, the knowledge has been firmly established that HPV infection could interfere with normal cell cycles by degrading tumor suppressor protein P53 and Rb and cause host genomic instability through its DNA random integrating into host genome accompanied with increased centrosome number[23, 24].

As for the prevance of HPV in larynx tissues, a significant number of recent studies have undoubtedly confirmed etiologic association between infection with certain HPV types (mainly HPV types 6 and 11) and laryngeal squamous cell papillomas, the most frequent benign neoplasia of the upper respiratory tract[7, 25]. Using PCR, HPV infection was detected in more than 90% of these

benign tumors, including both juvenile and adult forms[26]. For laryngeal squamous cell carcinoma, however, there is still controversy over the incidence of HPV infection and its possible significance as an oncogenic cofactor. Previous series using Southern-blot, dotblot and in situ hybridization techniques found a low prevalence of HPV in laryngeal carcinoma, ranging from 0 to 12.9%[9]. Using the more sensitive PCR method, 8%−54% of laryngeal cancers were found to harbor HPV DNA[9, 12, 27]. These studies were performed on different populations using heterogeneous HPV diagnostic procedures. However, they consequently produced conflicting results, precluding any definitive conclusion.

To address more clearly the involvement of HPV in laryngeal carcinogenesis, we have reported that the presence of HPV-16 DNA was found in 52.03% laryngeal carcinoma patients using highly sensitive PCR technique with specific primer targeting E6 gene of HPV-16[28]. In this further study, the E6 mRNA expression was detected in 36 (36.36%) of 99 tumor samples among those viral DNA positive patients by ISH. It is confirmed that high-risk HPV 16 may be a most frequent risk factor for laryngeal carcinoma. Furthermore, the prevalence of HPV infection in relation to clinical/ pathological parameters is shown in Table 2. We found that HPV presence was not significantly associated with age, gender, clinical stage, nodal status, indicating that HPV is likely not to play a role in the progression of malignant transformation in laryngeal carcinoma. Previous studies have similarly reported clinical stage and TNM status not to be associated with HPV presence[29, 30]. Up to now, the follow-up study is being undertaken to observe the length of the disease-free interval between HPV positive and negative patients.

Since the first description of a potential link between HPV infection and head and neck cancer[31], several studies have strongly supported an etiologic role for HPV in cancers arising from specific mucosal sites within the head and neck[32−34]. HPV genomic DNA has been found in approximately 25% of all HNSCC using sensitive PCR-based methods[35]. The association is strongest for oropharynx cancers, with detection rates of 50% or more[36−39] possibly because of a facilitated viral access to basal mucosal cells in the tonsillar crypts and an apparent predilection of this anatomic site to transformation by HPVs, analogous to the cervical transformation zone. However, in our present study, there was no evidence to demonstrate the association of tumor HPV status and tumor's primary site, such as supraglottis, glottis and subglottis in laryngeal carcinoma (P>0.05).

In conclusion, from our previous study and this study, as well as others, HPV infection should be considered as a risk factor for LSCC, at least in high incidence area of China, and in order to further explore the role of viral DNA infection during the carcinogenesis of LSCC, more works are needed in the future.

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