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HPV16 DNA and integration in normal and malignant epithelium: implications for the etiology of laryngeal squamous cell carcinoma

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Background: Molecular evidence suggests that human papillomavirus (HPV) has a role in the etiology of oropharyngeal squamous cell carcinoma. However, the role of HPV in laryngeal squamous cell carcinoma (LSCC) is unclear.

Patients and methods: We conducted a case–control study using tumor tissue specimens from 300 LSCC patients and vocal cord polyp specimens from 300 cancer-free controls. HPV genotype, HPV16 viral load and viral integration status, and p16 expression were determined.

Results: The prevalence of HPV (all types) was higher in cases than controls [21 (7.0%) versus 10 (3.3%), adjusted odds ratio (aOR) 2.37, 95% CI 1.08–5.21]. The prevalence of HPV16 was higher in cases than controls [20 (6.7%) versus 8 (2.7%), aOR 2.84, 95% CI 1.21–6.68]. The risk of LSCC associated with HPV16 DNA positivity was even higher in patients aged 55 years or younger (aOR 3.52, 95% CI 1.07–11.54), males (aOR 4.74, 95% CI 1.33–16.90), never-smokers (aOR 5.57, 95% CI 1.41–22.10), and never-drinkers (aOR 3.72, 95% CI 1.09–12.72). HPV DNA was partly or fully integrated in all 20 HPV16-positive cases but was episomal in all 8 HPV16-positive controls; however, the HPV16-positive cases and controls had similar viral loads ($P = 0.28$). P16 immunostaining was positive in 31 of the 300 cases (10.3%) and negative in all 300 controls.

Conclusion: These results suggest that prior infection with HPV16 may play a role in the etiology of some LSCC. This larger case–control study will offer for the first time the possibility to address in depth the understanding of a tissue-specific role of HPV in laryngeal carcinogenesis. Further studies with larger samples are needed to confirm these findings.

Key words: human papillomavirus, head and neck cancer, laryngeal cancer, risk, viral load, physical status

Introduction

Approximately 600 000 people develop some form of head and neck cancer and ~300 000 people die of this cancer each year. Most head and neck cancers are classified as squamous cell carcinoma, which is currently the sixth most common cancer in the world [1]. More than 30 years ago, Gissmann et al. first reported a link between infection with certain types of human papillomavirus (HPV) and head and neck neoplasms [2]. Since then, numerous studies have confirmed that infection with high-risk HPV types, especially HPV16, has an important role in the etiology of oropharyngeal squamous cell carcinoma. HPV-driven oropharyngeal carcinomas overexpress p16, primarily occur in the tonsils and base of tongue, have distinct clinical features, and are associated with better patient outcomes than HPV-unrelated oropharyngeal carcinomas [3].

The link between HPV infection and laryngeal squamous cell carcinoma (LSCC) is less clear. The major risk factors for laryngeal cancer are tobacco smoking and alcohol drinking. Several studies have reported the presence of high-risk HPV in a minority of laryngeal cancers, but whether there is a causal association between HPV and LSCC or whether a significant fraction of LSCCs are

attributable to HPV remains unknown. The reported prevalence of HPV DNA in LSCCs varies from 0% to 75% [4, 5]. This wide range may be due to geographical differences of the study populations, tumor site misclassification, or admixture of LSCCs with oropharyngeal carcinomas [6]. In addition, serum-based ELISA and tumor-based PCR for HPV detection have different sensitivities and specificities. Serum-based assays detect the presence of antibodies against HPV, an indirect and systematic measure of prior HPV infection; tissue specimen-based techniques are direct and more reliably implicate a role for the virus in carcinogenesis.

We sought to clarify the association of HPV with LSCC. We systematically analyzed fresh-frozen tumor tissue specimens from patients with LSCC and vocal cord polyp specimens from cancer-free controls. We chose to study vocal cord polyps from cancer-free individuals because vocal cord polyps are considered to represent non-precancerous reactive change predominantly within the superficial layer of the lamina propria [7]. Furthermore, to our knowledge, there are no reports of an association of HPV infection with vocal cord polyps, and our own research showed no relationship between HPV prevalence and vocal cord polyps compared with normal laryngeal mucosa.

Patients and methods

Study participants

Three hundred patients with newly diagnosed histopathologically confirmed, untreated LSCC were consecutively recruited at Peking Union Medical College Hospital from January 2013 to December 2015. Patients were excluded if they had any of the following: second primary tumor; primary tumor of the sinonasal tract, oral cavity, nasopharynx, oropharynx, or hypopharynx or outside the upper aerodigestive tract; cervical metastasis of unknown origin; or histopathologic diagnosis other than squamous cell carcinoma. A pool of cancer-free patients with vocal cord polyps who were admitted to the same hospital at the same time were recruited as controls. All individuals in both groups provided demographic and epidemiologic information, including age, sex, ethnicity, smoking status, and alcohol consumption. None of the controls had a previous personal history of cancer. The study was approved by the Ethics Committee of Peking Union Medical College Hospital, and all cases and controls provided written informed consent for participation in this study.

The 300 controls were selected from the pool of potential controls by use of matching to cases with regard to sex, age (5-year groups), ethnicity, and consumption of tobacco and alcohol. These variables were further adjusted in multivariable logistic regression to account for residual confounding.

DNA isolation

Tissue specimens were collected from cases and controls at surgical resection and divided into two parts. One part was frozen at $-80\,^{\circ}$C, and the other part was embedded in paraffin. All tumor specimens were microdissected to ensure that most of the DNA was from the tumor cells. DNA was extracted using a commercial kit (QIAamp DNA Mini Kit, Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA quality and the absence of PCR inhibitors in all clinical samples were tested by PCR for a 268-bp fragment of the human β-globin gene using PCO4 and GH2O primers [8]. Extracted DNA was stored at $-20\,^{\circ}$C until PCR analysis.

HPV detection and genotyping

HPV DNA was detected using the short PCR fragment 10 (SPF10) line probe assay (LiPA) 25 DNA enzyme immunoassay system (version 1,

Labo Bio-Medical Products, Rijswijk, The Netherlands). All the amplifications were carried out in a laboratory physically separated from the location used for DNA isolation.

Real-time PCR

The physical status and viral load of HPV16 was determined by HPV16 E2/E6 real-time PCR on the HPV16 DNA–positive samples using the Rotor Gene 6000 instrument and the SYBR Premix Ex Taq kit (Perfect Real Time) (Takara DRR063A) as described previously [9]. Serial dilution of the full-length HPV16 (ATCC 45113 DTM), which contained equal numbers of E2 and E6 genes and from 419 to 41.9 million copies per reaction, served as a standard control. Threshold cycle numbers obtained from E2 and E6 PCR were equivalent in each run. Linear plots of the $\log_{10}$ of copy number and the threshold cycle number were consistently obtained for both genes, and the correlation coefficient was between 0.99 and 1.0 in each run.

P16 immunohistochemistry

P16 immunohistochemical staining was carried out on 4-μm-thick formaldehyde-fixed, paraffin-embedded (FFEP) tissue sections using the CINtec-p16 kit (Roche mtm laboratories AG, Germany). Staining was scored as positive if it was strong and diffuse and if nuclear and cytoplasmic staining was present in more than 80% of malignant cells. Staining was scored as negative if it was focal and weak. Two trained pathologists who were blinded to HPV status and clinical information independently evaluated all specimens and reviewed discordant cases to reach agreement.

Statistical analysis

The significance of differences between cases and controls in demographic variables, tobacco smoking, alcohol consumption, and HPV16 status was determined using the χ^2 test. We estimated the association of HPV16 status with risk of LSCC by computing odds ratios (ORs) and associated 95% CIs using univariate and multivariate logistic regression analysis, with stratification by age, sex, smoking status, and drinking status. In the multivariable logistic regression model, the ORs and relevant 95% CIs were adjusted for age, sex, smoking, and drinking for calculation of adjusted ORs (aORs). All statistical tests were two-sided, P values <0.05 were considered statistically significant. Data were analyzed using SPSS (SPSS for Windows version 21.0; SPSS, Chicago, IL).

Results

Table 1 shows the demographic and clinical characteristics of LSCC patients and controls. Because of matching, the cases and controls were similar with regard to age, sex, tobacco smoking, and alcohol drinking. We further adjusted for these parameters in subsequent multivariate logistic regression analysis to account for any residual effects (see below).

The human β-globin gene was positively amplified in all specimens, indicating that the DNA was adequate for molecular analysis (data not shown). The prevalence of HPV16 DNA was higher in cases than controls [20 (6.7%) versus 8 (2.7%), $P = 0.02$].

Table 2 shows the association of selected variables with HPV-associated laryngeal carcinoma. Following adjustment for age, sex, tobacco smoking, and alcohol drinking, HPV16 DNA positivity was associated with a significantly increased risk of laryngeal carcinoma (aOR 2.84, 95% CI 1.21–6.68). We also determined the effects of different factors on the risk of HPV16-associated laryngeal tumors in different subgroups. This analysis showed that HPV16 positivity was significantly associated with laryngeal carcinoma in individuals who were 55 years of age or

Table 1. Demographic and clinical characteristics of LSCC patients and controls recruited from The Peking Union Medical College Hospital from 2013 through 2015					
Variable	Cases (N = 300)		Controls (N = 300)[a]		P-value[b]
	No.	%	No.	%	
Age (years)					
≤40	14	4.7	18	6.0	0.41
41–55	111	37	115	38.3	
56–70	153	51	154	51.3	
>70	22	7.3	13	4.3	
Sex					
Male	220	73.3	209	69.7	0.32
Female	80	26.7	91	30.3	
Tobacco smoking					
Ever	230	76.7	226	75.3	0.70
Never	70	23.3	74	24.7	
Alcohol drinking					
Ever	190	63.3	184	61.3	0.61
Never	110	36.7	116	38.7	
HPV16 infection					
Yes	20	6.7	8	2.7	0.02
No	280	93.3	292	97.3	

[a]The controls were matched with cases for age, sex, and consumption of alcohol and tobacco.
[b]Two-sided χ^2 test.

younger (aOR 3.52, 95% CI 1.07–11.54), males (aOR 4.74, 95% CI 1.33–16.90), never-smokers (aOR 5.57, 95% CI 1.41–22.10), and never-drinkers (aOR 3.72, 95% CI 1.09–12.72).

Analysis of the HPV16-positive samples by real-time PCR indicated that the cases and controls had similar HPV16 viral loads ($P = 0.28$) (Table 3). However, whereas all of the cases had partially or entirely integrated HPV16 DNA, all of the controls had episomal HPV16 DNA.

Thirty-one of the 300 cases (10.3%) had positive p16 staining, while none of the 300 controls had positive p16 staining (Table 4). Among 20 HPV16 positive tumor specimens, 19 were found p16 overexpressed. Only one tumor specimen that tested negative for p16 was positive for high-risk HPV DNA.

Discussion

The results of this case–control study indicated that HPV was significantly associated with LSCC. Moreover, HPV16 positivity had a greater effect on risk of LSCC in never-smokers than ever-smokers and in never-drinkers than ever-drinkers. There was no significant difference in viral load between HPV16-positive cases and HPV16-positive controls; however, HPV16 DNA was partially or entirely integrated in all HPV-positive cases but episomal in all HPV-positive controls. These results suggest that prior infection with HPV16 may play a role in the etiology of a fraction of LSCC in China.

To the best of our knowledge, the present study is the largest case–control study to examine the relationship between HPV and

Table 2. Association of selected variables with HPV-associated laryngeal carcinoma						
Variable	HPV16 status	Cases (N = 300)		Controls (N = 300)		Adjusted OR (95% CI)[a]
		No.	%	No.	%	
All subjects	−	280	93.3	292	97.3	1.00
	+	20	6.7	8	2.7	2.84 (1.21–6.68)
Age (y)						
≤55	−	114	90.5	129	97.0	1.00
	+	12	9.5	4	3.0	3.52 (1.07–11.54)
>55	−	166	95.4	163	97.6	1.00
	+	8	4.6	4	2.4	2.15 (0.62–7.40)
Sex						
Male	−	205	93.2	203	98.5	1.00
	+	15	6.8	3	1.5	4.74 (1.33–16.90)
Female	−	75	93.8	89	94.7	1.00
	+	5	6.2	5	5.3	1.46 (0.40–5.46)
Smoking						
Never	−	57	81.4	69	95.8	1.00
	+	13	18.6	3	4.2	5.57 (1.41–22.10)
Ever	−	223	97.0	223	97.8	1.00
	+	7	3	5	2.2	1.17 (0.81–1.70)
Drinking						
Never	−	98	89.1	110	96.5	1.00
	+	12	10.9	4	3.5	3.72 (1.09–12.72)
Ever	−	182	95.8	182	97.8	1.00
	+	8	4.2	4	2.2	2.06 (0.61–7.00)

[a]HPV-positive versus HPV-negative in each stratum; ORs were adjusted for age, sex, smoking, and drinking in logistic regression models.

Table 3. HPV genotype (determined by INNO-LiPA), HPV16 viral load, and HPV physical status determined by real-time PCR in patients with laryngeal squamous cell carcinoma and controls

Sample ID	Genotype	Viral load		Physical status	
		E6 copies per 10^4 cells	E2 copies per 10^4 cells	E2/E6	Status
Laryngeal cancer (LC)					
LC-10	16	1140	490	0.43	Mixed
LC-11	16	588	488	0.83	Mixed
LC-15	16	961	820	0.85	Mixed
LC-48	16	6230	0	no E2	Integrated
LC-74	16	1230	756	0.62	Mixed
LC-78	16	874	340	0.39	Mixed
LC-92	16	980	546	0.56	Mixed
LC-96	16	600	476	0.79	Mixed
LC-133	16	886	760	0.86	Mixed
LC-151	16	1080	498	0.46	Mixed
LC-163	16	936	820	0.88	Mixed
LC-167	16	530	476	0.90	Mixed
LC-175	16	694	512	0.74	Mixed
LC-183	16	640	234	0.37	Mixed
LC-186	16	924	735	0.80	Mixed
LC-191	16	1420	362	0.25	Mixed
LC-192	16	780	590	0.76	Mixed
LC-211	16	873	620	0.71	Mixed
LC-246	16	970	492	0.51	Mixed
Controls (C)					
C-27	16	712	613	≈1	Episomal
C-33	16	582	570	≈1	Episomal
C-64	16	700	680	≈1	Episomal
C-86	16	890	912	≈1	Episomal
C-125	16	745	730	≈1	Episomal
C-211	16	560	566	≈1	Episomal
C-224	16	795	790	≈1	Episomal
C-267	16	640	652	≈1	Episomal

Table 4. P16 staining and HPV16 status in patients with laryngeal squamous cell carcinoma and controls

	Cases (N = 300)		Controls (N = 300)	
	HPV16(+)	HPV16(−)	HPV16(+)	HPV16(−)
p16(+)	19	12	0	0
p16(−)	1	268	8	292

risk of LSCC in a Chinese population. Our findings are consistent with the findings of a study of Central European and Latin American individuals that included 2214 cases and 3319 controls; that study showed an OR of 2.91 (95% CI 1.43–5.95) for HPV16 E6 antibody [10]. Our findings are also consistent with results of a German hospital-based study that indicated a causal role of HPV16 in a small fraction (<5%) of LSCCs [11]. A recent meta-analysis reported that the overall prevalence of HPV in LSCC was 28% (95% CI 23.5%–32.9%) in patients with LSCC [12]. However, other studies have reported no association between HPV16 infection and LSCC, although some of these studies suffered from poor tumor site classification [13].

These discrepant results may be explained by several factors, including different demographics of the study populations, variable quality of tested specimens, and different sensitivities and specificities of the testing methods. In this study, we used a highly sensitive and broad-spectrum SPF10 PCR followed by a general hybridization and INNO-LiPA genotyping assay to detect active and inactive HPV infection. Use of RNA in situ hybridization or RT-PCR to detect transcriptionally active HPV (i.e. active translation of HPV oncoproteins supporting an HPV-driven tumor) could lead to even lower false-positive rates. The HPV detection method we used in our study is likely more accurate than other methods that have been used to detect HPV. Previous studies showed that serum prevalence of antibodies to HPV proteins was neither a good biomarker of oral/laryngeal infection nor a sufficient marker for genital infection in women, and infection status and serum antibodies failed to predict each other sufficiently [14]. Retrospective studies tend to use FFEP tissues for detection of HPV DNA, but samples prepared this way have several defects, including DNA–DNA and DNA–protein cross-linking and the presence of PCR inhibitors [15]. Moreover, a biopsy-based method is more accurate for the detection of HPV than brush sampling [16].

We found a greater risk of HPV16-associated LSCC in never-smokers than ever-smokers and in never-drinkers than ever-

drinkers. In particular, HPV16-positive never-smokers had approximately five times the risk of LSCC of HPV16-negative never-smokers, but the risk was similar in HPV16-positive ever-smokers and HPV16-negative ever-smokers. The risk was also similar for HPV16-positive ever-drinkers and HPV16-negative ever-drinkers. This finding is consistent with a previous study that reported a stronger association of HPV16 with LSCC among never-smokers and never-drinkers [17]. The findings of our study and the previous study imply that HPV16 and tobacco and alcohol consumption may not be cofactors in the development of LSCC and that the fraction of LSCC attributable to HPV would be higher if limited to LSCC occurring in nonsmokers.

Integration of high-risk HPV DNA into the human genome appears to be a critical event during malignant transformation and neoplastic progression, but whether HPV integration is a necessary step in the development of LSCC is still unclear. Studies of cervical cancer indicated that integration of HPV DNA into the host leads to a selective growth advantage [18]. However, data on the physical status of HPV DNA in head and neck squamous cell carcinoma are limited and confusing. Our real-time PCR results showed that all 20 HPV16-positive tumors showed evidence of integration (either partly or fully integrated) whereas all HPV16-positive controls showed no evidence of integration based on the E2/E6 ratio. Thus, integration seems to provide a selective advantage in terms of progression of premalignant laryngeal precursors to LSCC. Another study also showed that HPV was present in mixed (predominantly integrated) form in head and neck cancers [19]. Several *in vitro* studies attributed the growth advantage to an enhanced expression of E6 and E7 due to disruption of E2 (a negative regulator of the E6/E7 gene) [20]. Similarly, Andersson et al. reported that integration of HPV correlated with high and stable expression of full-length E6 protein [21]. Of note, the viral load in the present study was relatively low compared with the viral loads previously reported for oropharyngeal and anogenital carcinomas.

Overexpression of p16 has been accepted as surrogate biomarker for an HPV-driven oropharyngeal cancer and is present in the vast majority of cervical cancers [22, 23]. However, overexpression of p16 is not considered a reliable surrogate of HPV status in carcinomas of the head and neck region outside the oropharynx. In the present study, only 19 of the 31 p16-positive LSCCs were also HPV16 positive, though 19 of the 20 HPV-positive LSCCs overexpressed p16. One HPV16 positive laryngeal cancer was tested negative for p16 expression, it is likely that the one HPV16 positive laryngeal tumor that failed to express p16 was not driven by HPV, but HPV was just an incidental bystander in that tumor. In contrast, among all 300 controls, the 8 HPV16 positive polyp samples all failed to express p16. This is likely since these are probably not caused by HPV.

This discordance could occur between HPV status and p16 expression since some events (e.g. mutation, deletion, other epigenetic changes, etc.) may impact the p16. Therefore, some HPV positive cases may fail to express p16 because the tumor or polyp are not being driven by HPV and therefore are not overexpressing E7. Some p16 positive tumors may be positive because Rb is mutated and inactivated or E2F is amplified.

Our study has both strengths and limitations. The major strengths are the case–control design, the focus on a single type of cancer (LSCC), and the large number of patients. Another strength is that the study specimens were fresh-frozen tissues instead of exfoliated cells or serum samples. Moreover, all of the cases and controls had paraffin-embedded tissue samples. The limitations of our study include the possibility of selection bias as this was a hospital-based case–control study and the controls might not be representative of the same population from which the LSCC patients arose. In addition, our stratified analysis included a limited number of individuals. Moreover, because most of the participants in our study were of Han ethnicity, it is uncertain whether these results are generalizable to other ethnic groups. Finally, it is the inability of the assay to measure a single copy of integrated DNA in the presence of multiple copies of non-integrated DNA (either episomal or concatenated) because in that case the E2/E6 ratio would be close to 1. This would make it appear that there was no integration despite the tumor being HPV-driven with just one copy of integrated DNA. However, in our study, there were no tumors that did not have either integrated or mixed integrated/episomal DNA. In future studies, we will use more robust methods to measure HPV integration for more reliable determination.

In conclusion, the prevalence of HPV16 was higher in LSCC patients than in controls (6.7% versus 2.7%, aOR 2.84, 95% CI 1.21–6.68). HPV16 was the most common HPV subtype detected in LSCC. HPV infection was independently associated with LSCC, particularly in never-smokers and never-drinkers. Further studies with larger samples size and other populations are necessary to verify our findings.

Disclosure

The authors have declared no conflicts of interest.

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