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Original Article

Correlation of HPV16 infection and p16 expression with prognosis in patients with hypopharyngeal carcinoma

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Abstract: The aim of this study was to explore the relationship between HPV16 infection, p16 protein expression and prognosis in patients with hypopharyngeal carcinoma. Totally 46 cases of hypopharyngeal carcinoma specimens were selected from January 1, 2004 to December 31, 2011 in Tangshan Union Hospital. PCR-reverse dot blot hybridization was used to detect infection of HPV16, and SP immunohistochemical method was adopted to detect the expression of p16 protein in hypopharyngeal carcinoma tissues. Clinical data were collected and all the patients were followed up. Results showed that the infection rate of HPV16 in hypopharyngeal carcinoma tissues was 26.1% (12/46). No significant difference was found between HPV16 infection and patients' age, gender, primary tumor site, histological differentiation and TNM classification ($P > 0.05$). The expression rate of p16 protein in hypopharyngeal carcinoma tissues was 39.1% (18/46). Expression of p16 proteins was not significantly correlated with patients' age, gender, primary tumor site, histological differentiation and TNM classification ($P > 0.05$). However, HPV16 infection was positively correlated with the p16 expression in hypopharyngeal carcinoma tissues, with a correlation coefficient of 0.437 ($P = 0.002$). Kaplan-Meier analysis showed that HPV16-positive patients had a higher median overall survival than HPV16-negative ones (75 months vs. 51 months, respectively. $P = 0.001$). Median progression-free survival was virtually the same for both HPV16-positive and HPV16-negative patients (77 months vs. 49 months, respectively. $P = 0.002$). P16-positive patients had a higher median overall survival than p16-negative ones (69 months vs. 53 months, respectively. $P = 0.001$). Median progression-free survival was also almost the same for both p16-positive and p16-negative patients (68 months vs. 43 months, respectively. $P = 0.003$). In conclusion, there is a positive correlation between HPV16 infection and positive expression of p16 protein in hypopharyngeal carcinoma. Patients with HPV16 infection might have favorable prognosis compared with HPV16-negative ones. P16 positive expression predicates a better prognosis in patients with hypopharyngeal carcinoma.

Keywords: Hypopharyngeal carcinoma, HPV16, p16, polymerase chain reaction, immunohistochemistry, prognosis

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth largest common malignant tumor in the world, which is the eighth cause of cancer-related deaths [1]. It was estimated that about 600,000 new occurrence cases of HNSCC per year worldwide according to GLOBOCAN statistics in 2012, approximately 375,665 cases of deaths each year [2]. There are about 81,149 of new cases and about 48,505 cases of death per year in China. Male patients accounted for about 3/4 of all the cases. Studies have shown that long-term smoking, alcohol consumption is closely related with the occurrence of HNSCC, but about 20% to 25% of the HNSCC patients without smoking or drinking habits [3, 4]. Studies have shown that high-risk types of human papillomavirus 16/18 subtypes (HPV16/18) infection are closely associated with HNSCC [5, 6]. Hypopharyngeal squamous cell carcinoma is the worst prognosis of HNSCC.

Here, we analyzed the HPV16 DNA by PCR-DNA reverse dot blot and examined the expression of p16 by immunohistochemistry in the hypopharynx carcinoma tissues, to investigate the relationship between HPV16 infection, the expression of p16 protein and prognosis of

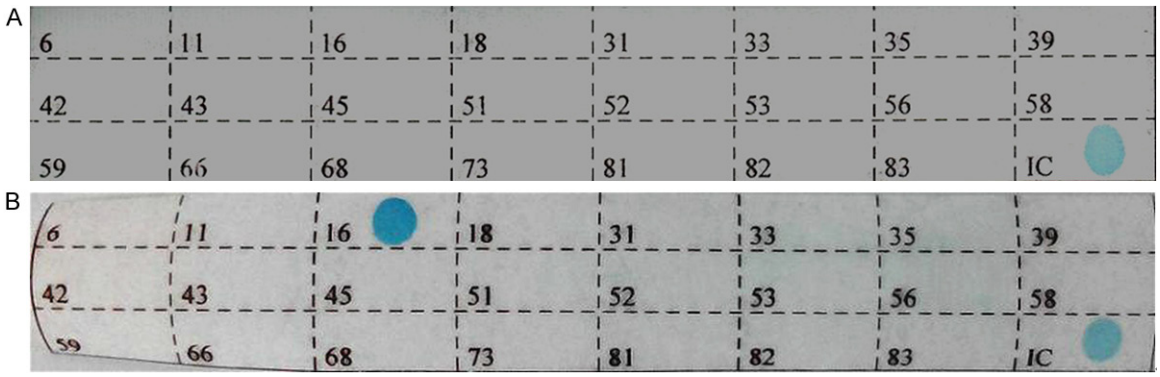

Figure 1. HPV genotype in hypopharynx carcinoma tissues. PCR-DNA reverse dot blot hybridization method. A. Negative control. B. HPV16 is positive.

Table 1. Correlation between expression of p16, HPV infection and clinical features N (%)

Clinical features	Total No.	HPV16+	χ^2/P	p16+	χ^2/P
Age					
< 56 y	21	4 (19.0%)	0.435/0.510	11 (52.4%)	2.848/0.091
≥ 56 y	25	8 (32.0%)		7 (28.0%)	
Gender					
Male	35	7 (20.0%)	1.647/0.199	16 (45.7%)	1.633/0.201
Female	11	5 (45.4%)		2 (18.2%)	
Smoking Status					
Non-smoker	20	8 (40.0%)	2.390/0.122	10 (50.0%)	1.755/0.185
Smoker	26	4 (15.4%)		8 (30.8%)	
Site					
Sinus piriformis	27	6 (22.2%)	0.520/0.771	11 (40.7%)	0.786/0.675
Postcricoid area	13	4 (30.8%)		5 (38.5%)	
Lateral and back wall of pharynx	6	2 (33.3%)		2 (33.3%)	
Differentiation					
Well-differentiated	20	6 (30.0%)	0.303/0.859	9 (45.0%)	0.552/0.759
Well-moderately differentiated	18	4 (22.2%)		6 (33.3%)	
Poorly-differentiated	8	2 (25.0%)		3 (37.5%)	
TNM staging					
Stages I+II	9	4 (44.4%)	2.076/0.354	5 (55.6%)	2.146/0.342
Stage III	16	3 (18.8%)		7 (43.8%)	
Stage IV	21	5 (23.8%)		6 (28.6%)	

hypopharyngeal squamous cell carcinoma patients.

Material and methods

Cases description

Our study involved 51 cases of hypopharyngeal resection specimens selected at Tangshan Union Hospital from January 1, 2004-December 31, 2011. 46 cases underwent HPV DNA detection, including 35 males and 11 females, aged 48 to 75 years, with median age 56 years. Lesional location was in the pyriform (27 cases), pharyngoesophageal junction (13 cases) and sidewall and rear wall (6 cases), respectively. According to the AJCC Cancer Staging, they were all divided into four stages: including 2 cases in stage I, 7 cases in stage II, 16 cases in stage III and 21 cases in stage IV. Tumor grade was classified into well-differentiated (20 cases), moderately differentiated (18 cases) and poorly differentiated (8 cases). All the 46 patients involved in the study have been informed content and approved by the Ethics Committee of Tangshan Union Hospital. All the patients were followed up every 3 months by

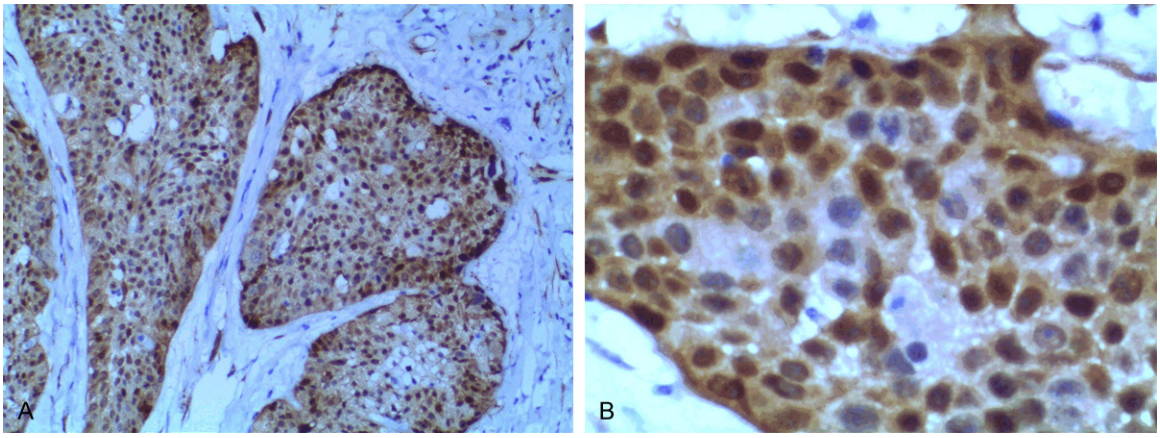

Figure 2. Expression of p16 in hypopharynx carcinoma tissues. SP method. A. Immunohistochemical detection of p16 in laryngeal carcinoma tissue. Magnification ×100. B. Immunohistochemical detection of p16 in laryngeal carcinoma tissue. Original magnification, ×400.

Table 2. Correlation between HPV infection and p16 expression in hypopharyngeal carcinoma tissues

	Total No.	HPV16+	n (%)	χ^2	*P*
p16+	18	9	50.0%	6.851	0.009
p16-	28	3	10.7%		

telephone to record the survival outcomes. Follow-up rate was 100% and date was up to January 6, 2015.

HPV16 DNA detection by PCR-DNA reverse dot blot hybridization

Human HPV16 Subtype Nucleic Acid Amplification Detection Kit (Shenzhen Yaneng Biotechnology Co., Ltd., China) was used to detect the HPV16 infection with PCR-DNA reverse dot blot. The paraffin-embedded hypopharyngeal squamous cell carcinoma specimens were cut to extract HPV-DNA, PCR amplification, hybridization, filter washing and color development according to kit instructions. The positive quality control was colored (blue spots) at the corresponding and the IC membrane sites and other sites were not colored. In accordance with the order of the probe sequence and the color on the membrane the HPV genotypes were determined.

P16 expression by immunohistochemistry

All the surgically removed tissue specimens were fixed in 10% neutral formalin, embedded in paraffin, and one representative block from each patient was sectioned at 4 μm, stained with hematoxylin and eosin (HE) and evaluated by immunohistochemistry according to the protocol described in the manufacturer's guide accompanying the kit. Mouse anti human p16 monoclonal antibody and SP immunohistochemistry kit were all purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd (Clone Number: ABM51100-10, the titer of monoclonal antibody was 1:100). Positive reaction of the immunohistochemical staining showed brown or brownish yellow in color. Known positive samples of p16 were used as positive control. For the negative control, the primary antibodies were replaced with phosphate-buffered saline (PBS). Positive signals of p16 located in nucleus. 10 high magnification visions were selected randomly in each stained section and 10 high power field representatives were observed, the brown nuclear staining cells were counted. Positive staining in more than 10% of the cells was considered positive, while less than 10% or colorless were defined as negative.

Statistical analysis

The statistical analyses were performed with PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA). The correlation between HPV infection and expression of p16 with the various clinicopathological findings were evaluated using the Chi-square test, and the Kaplan-Meier method was used to analyze survival rate. *P* values less than 0.05 were considered to be statistically significant.

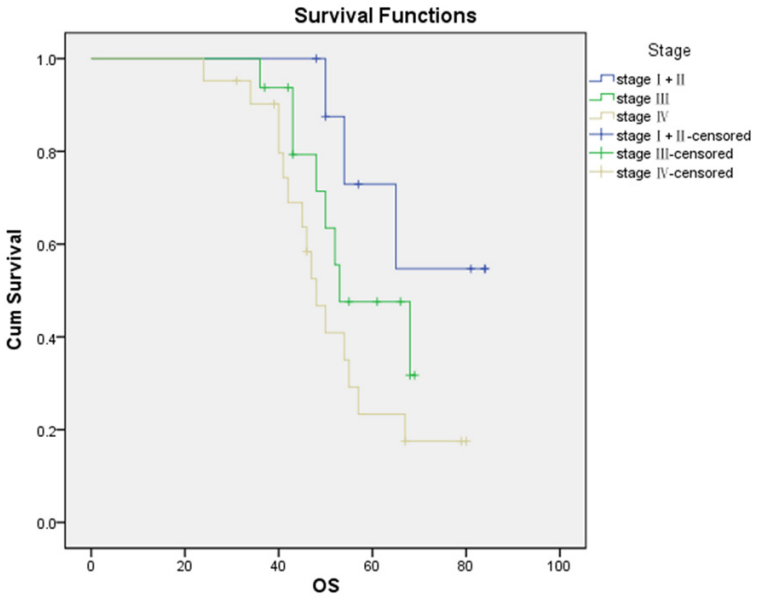

Figure 3. Relationship between tumor staging and OS. $\chi^2 = 6.048$, $P = 0.014$.

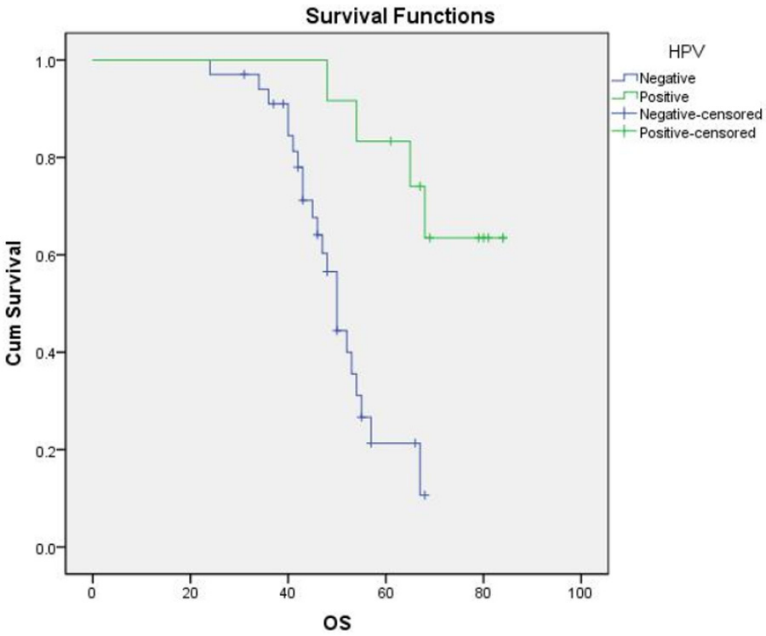

Figure 4. Relationship between HPV16 infection and OS. $\chi^2 = 11.404$, $P = 0.0001$.

Results

Infection rate of HPV16 and expression rate of p16 in the hypopharyngeal squamous cell carcinoma tissues

PCR-DNA reverse dot blot hybridization showed that the infection rate HPV16 was 26.1% (12/46) in the hypopharynx carcinoma tissues (**Figure 1**; **Table 1**). HPV16 infection in the hypopharynx carcinoma tissues was irrelevant to the patient's age, sex, disease location, differentiation and TNM stage. Immunohistochemistry showed that expression rate of p16 in the hypopharynx carcinoma tissues was 39.1% (18/46), and the positive rate of p16 was irrelevant to the patient's age, sex, disease location, differentiation, TNM stage ($P > 0.05$) (**Figure 2**; **Table 1**).

Correlation between HPV16 infection and p16 expression in hypopharyngeal squamous cell carcinoma tissues

Chi-square test showed that there was positive correlation between HPV infection and p16 expression (coefficient R = 0.437, $P = 0.002$) (**Table 2**).

Prognosis of patients with laryngeal carcinoma

Follow-up to January 6, 2015, in total 46 hypopharyngeal squamous cell carcinoma patients, 26 cases were dead, the total mortality rate was 56.5%. The median overall survival (OS) was 60 months in all patients. The 5-year OS rate was 43.5% (20/46). Kaplan-Meier survival analysis showed that the median OS of stages I and II, stage III and stage IV was 72 months, 58 months and 52 months, respectively, with statistically significant difference ($P = 0.014$, **Figure 3**). The median OS of HPV16 negative and positive patients was 51 months and 75 months respectively, with statistically significant difference ($P = 0.001$, **Figure 4**). The median OS of p16-negative and positive patients was 53 months and 69 months respectively, and these difference was also statistically significant ($P = 0.001$,

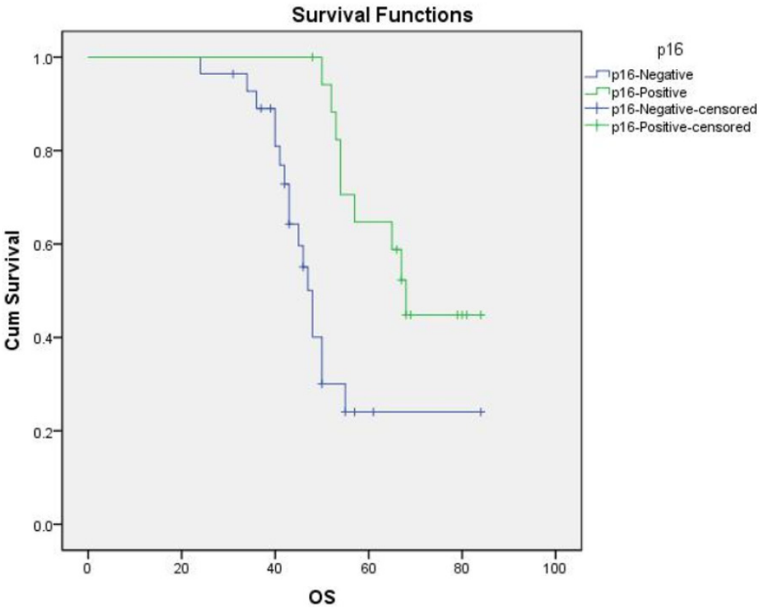

Figure 5. Relationship between expression of p16 and OS. χ^2 = 10.212, *P* = 0.001.

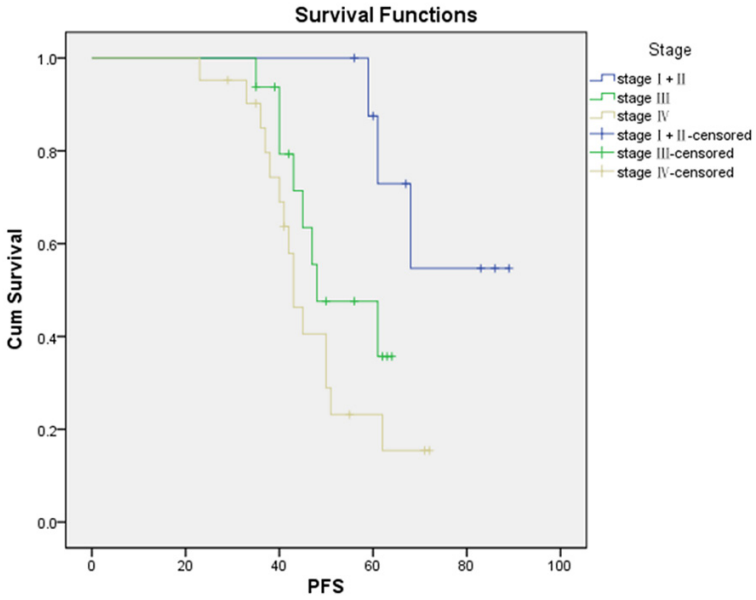

Figure 6. Relationship between tumor staging and PFS, χ^2 = 8.748, *P* = 0.003.

Figure 5). Median OS was irrelevant to patients' gender, age, smoking status, differentiation and lesion sites (*P* > 0.05). The overall median progression-free survival (PFS) of patients was 60 months, Kaplan-Meier survival analysis showed that the PFS of stages I and II, stage III and stage IV was 77 months, 53 months and 48 months, respectively (*P* = 0.003, **Figure 6**). The median PFS of HPV16 negative and positive patients was 49 months and 77 months, respectively (*P* = 0.002, **Figure 7**). The median PFS of p16-negative and positive patients was 43 months and 68 months (*P* = 0.003, **Figure 8**). Median PFS is irrelevant to patients' gender, age, smoking status, differentiation and lesion sites (*P* > 0.05).

Discussion

Hypopharyngeal carcinoma is the tumor with high degree of malignancy, prone to early metastasis through cervical lymph node and often invades the throat, oropharynx, cervical esophagus and parapharyngeal space. The 5-year survival rate of hypopharyngeal carcinoma is about 20%~48% [7]. The related factors of the poor prognosis of the carcinoma are still under study. A report in analyzing 5046 HNSCC patients displayed that the infection rate of HPV (most for HPV16 subtype) was 25.9% [8]. HNSCC induced by HPV infection gradually increased, especially in oral cancer and oropharyngeal cancer. The conclusion about the HPV in the process of inducing the hypopharyngeal carcinoma was different between the studies of China and other countries. Gillison et al [9] reported that the positive rate of HPV 16 was 9.5% (2/21) in the hypopharyngeal carcinoma tissues, while Huang et al [10] reported that in 20 patients of stage IV was 10% (2/20), 8.3% (1/12) in the 12 cases of metastatic cervical lymph node. Rodrigo et al [11] showed that the overall positive rate of HPV was 29% (6/21), and those in stage II, III and IV patients were 4.8% (1/21), 14.3% (3/21) and 9.5% (2/21), respectively. Ma et al [12]

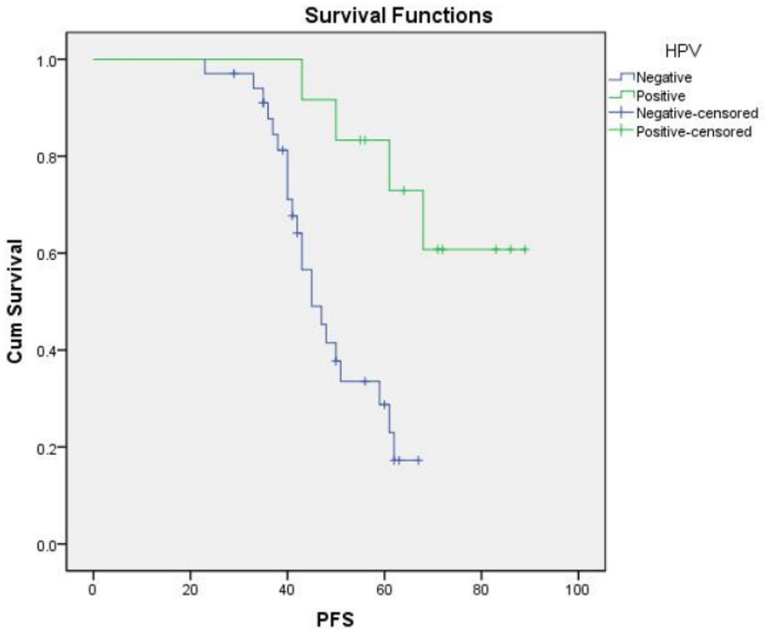

Figure 7. Relationship between HPV16 infection and PFS, χ^2 = 9.860, *P* = 0.002.

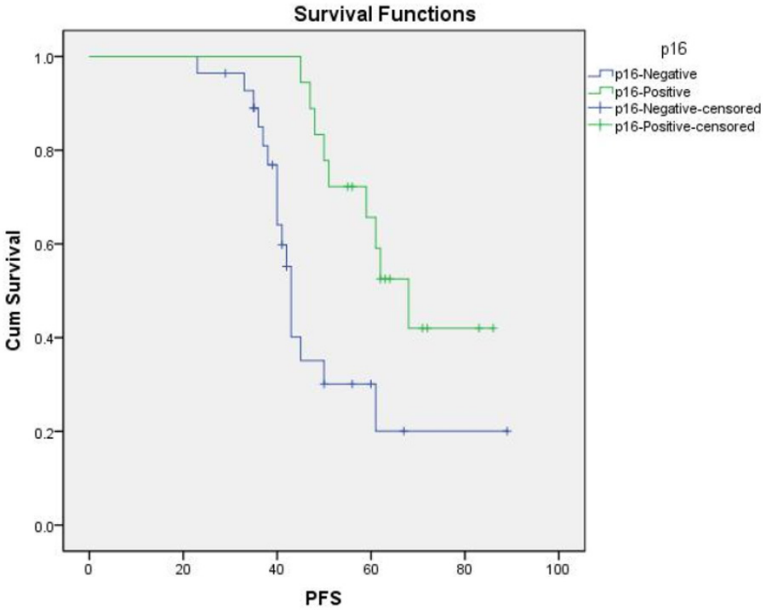

Figure 8. Relationship between expression of p16 and PFS, χ^2 = 8.565, *P* = 0.003.

reported that the positive rate of HPV was 26.7% (4/15) in 43 cases of HNSCC. Ang et al [13] retrospectively analyzed accelerated segmentation and conventional radiotherapy combined with concurrent cisplatin chemotherapy in patients with stage III and IV squamous cell carcinoma of the oropharynx and showed that the positive rate of HPV was 63.8% (206/323) and 96.1% (198/206), respectively. Our study demonstrated that the positive rate of HPV16 in hypopharyngeal carcinoma patients was 26.1% (12/46), and the results were similar to the finding of Ma et al. Ernoux-Neufcoeur et al [14] reported that the positive rate was 82%, which was higher than that of other research results, which may be related to the high sensitivity of real-time fluorescence PCR method, and possibly false positive existed in a certain degree. Ma et al [12] reported that the 3 year survival rates of HPV positive and negative HSCCN patients were 60% and 59.7%, respectively, with statistically significant difference (*P* = 0.789). Garbuglia [5] showed that HPV positive patients had better 5 years DFS (*P* = 0.026) and 5 year DSS (*P* = 0.047). Our study also showed that the median OS of HPV16 negative and positive hypopharyngeal carcinoma patients were 51 months and 75 months, respectively, with statistically significant difference (*P* = 0.001), and the median PFS of HPV16-negative and HPV16-positive patients were 49 months and 77 months, respectively (*P* = 0.002). Both OS and PFS in patients with HPV16 positive were better than those of HPV16 negative ones. To sum up, the difference of HPV infection rate in the hypopharyngeal carcinoma tissues, which may be related to different regions and countries and different detection methods. Part of hypopharyngeal carcinoma patients with HPV infection prompted that HPV may only be one of the contributing factors in

development of the hypopharyngeal carcinoma, but its occurrence mechanism is to be elucidated in the further study of genomic medicine.

In the HNSCC patients, the expression of p16 is often presented at high level. Studies have shown that only the HPV infection accompanied with p16 expression was the active HPV, and p16 protein expression may be used as an alternative indicator for detecting HPV status confirmed by clinical research [14]. Stephen et al [15] reported that expression of p16 was significantly different in the different parts. The positive rate of p16 in hypopharyngeal carcinoma was 5% (1/20) while in oropharyngeal cancer was 65% (13/20) ($P < 0.001$). p16 status was irrelevant to HPV infection ($P = 0.446$), and p16 positive patients had a higher survival rate, the same as HPV16 infection patients. Lassen et al [16, 17] showed that the positive rate of p16 in 156 supraglottic and pharynx cancer patients was 22% (35/156) in Denmark and the expression of p16 was closely related to HPV infection status. The p16 positive patients were associated with higher rates of 5-year local control rate (58%:28%, $P = 0.0005$), disease specific survival rate (72%:34%, $P = 0.0006$) and overall survival rate (62%:26%, $P = 0.0003$). Rischin et al [18] detected p16 and HPV simultaneously in oropharyngeal cancer patients and indicated that 86% (88/102) p16 positive cases were also positive for HPV.

The literature on the p16 expression of hypopharyngeal carcinoma patients was not quite the same. Stephen et al [15] reported that the positive rate of p16 was only 5% and Wilson et al. [19] reported that the positive rate was 33.3% (9/27) in 27 hypopharyngeal carcinoma patients who received radiation therapy in 2002-2011, including 22 patients with concurrent chemotherapy and 5 patients with radiotherapy alone, while HPV was detected in 19 patients, only 1 case showed HPV positive (p16 positive simultaneously). When p16 was used as the detection index, HPV positive predictive value was 17%, which indicated that the expression of p16 in the hypopharyngeal carcinoma patients presented with lower rate of HPV infection. Survival analysis showed that 3-year local control rates were 74.1% and 87.7% in 9 cases of p16-positive and 18 p16-negative hypopharyngeal carcinoma patients, respectively ($P = 0.52$), 3-year disease-free survival rate were 43.2% and 65.4% ($P = 0.60$) and 3-year OS rate was 64.3% and 64.6% ($P = 0.88$). It was concluded that p16 is not a predictor of the hypopharyngeal carcinoma. Ernoux-Neufcoeur et al reported that p16 positive rate was 9.3% (7/75) in hypopharyngeal carcinoma patients and no correlation existed between p16 positive and high risk HPV [17]. The 5 years DFS rate in 7 cases of p16 positive and 68 cases of p16 negative patients were 100% and 58%, with no statistically significant. High risk HPV positive patients were all expressed in p16. Christian et al [20] reported that the positive rates of HPV16/18 and p16 were 78% (11/14) and 71.4% (10/14) in hypopharyngeal carcinoma patients, HPV16/18 infection was associated with expression of p16 ($P < 0.05$). Ma et al reported that 3-year survival rate of p16-positive and p16-negative HNSCC patients were 72.2% and 43.9% respectively, with statistically significant difference ($P = 0.012$). Our study indicates that the expression rate of p16 in hypopharyngeal carcinoma patients was 39.1% (18/46), and the expression of p16 was correlated with HPV16 infection. The median OS of p16-negative and p16-positive patients were 53 months and 69 months ($P = 0.001$). The median PFS of p16-negative and p16-positive patients were 43 months and 68 months respectively ($P = 0.003$). That is to say p16-positive patients have longer OS and PFS than the negative ones.

Our study concluded that HPV16 infection was associated with p16 expression in hypopharyngeal carcinoma patients. The prognosis of HPV16 infection and p16 positive expression in hypopharyngeal carcinoma patients is better than those with negative ones. Its clinical value needs further study to confirm, in order to judge clinical prognosis, or to improve the evidence for further individualized treatment.

Disclosure of conflict of interest

None.

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