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Original Article

High risk HPV detection by RNAscope in situ hybridization combined with Cdc2 protein expression by immunohistochemistry for prognosis of oropharyngeal squamous cell carcinoma

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Abstract: High risk human papillomavirus (HPV) infection is related to the development of head and neck squamous cell carcinoma (HNSCC). Oropharyngeal squamous cell carcinoma (OPSCC) is a common type of HNSCC, and its incidence has increased significantly in recent years. In this study, high risk HPV, the expression of P53, P21, and Cdc2 in OPSCC tissues was detected and the prognostic factors and clinical value of OPSCC were discussed. According to the WHO classification and diagnosis standard for head and neck tumors (2017 Edition), 49 OPSCC cases with complete clinical data were collected from Tangshan Head and Neck Disease Pathology Research Base from January 1, 2012 to December 31, 2018. The E6 and E7 mRNA of HPV 16 and HPV 18 were detected by RNAscope in situ hybridization. The expression of P53, P21, and Cdc2 protein was observed by SP immunohistochemical method and all cases were followed up for survival. Median survival time was analyzed by Kaplan-Meier method. The Log-rank test was used for single factor analysis and Cox regression model was used to analyze multiple prognostic factors. In 49 OPSCC cases the median age was 53 years; 14 were HPV-DNA positive (14/49, 28.6%) while 35 were negative (35/49, 71.4%). E6, E7 mRNA test showed that 20 cases (20/49, 40.8%) were positive for HPV-16. Among them 11 cases were positive for HPV-16 DNA. 2 cases were positive for HPV-18 mRNA (2/49, 4.08%). 27 cases were negative for mRNA16 and 18 (27/49, 55.1%). The prevalence of HPV was 68.8% (11/16) in the non-smoking group, which was higher than that of the smoking group (10/33, 33.3%), (χ^2=5.463, P=0.019). There was no significant correlation between HPV detection and gender, age, drinking, tumor differentiation degree, and clinical stage (P > 0.05). The expression rates of P53, P21, and Cdc2 in OPSCC tissues were 63.3% (31/49), 65.3% (32/49), and 67.3% (33/49), respectively. There was no significant correlation between expression of all the three proteins and gender, age, HPV, smoking, drinking, tumor differentiation, and clinical stage (P > 0.05). Cox multifactor regression analysis showed that HPV (HR=0.275, 95% CI: 0.146-0.517), tumor differentiation (HR=1.751, 95% CI: 1.231-2.492), stage (HR=3.268, 95% CI: 1.758-6.074) and expression of Cdc2 protein (HR=1.804, 95% CI: 0.990-3.286) were related to the survival time of patients (P < 0.05). Our findings support that most of the HPV-positive OPSSC patients were non-smokers. The patients with negative HPV, low differentiation, late stage, and Cdc2 positive expression have poor prognosis and need to be followed up.

Keywords: RNAscope, HPV, Cdc2, OPSCC, prognosis

Introduction

At present, oropharyngeal cancer accounts for about 2.8% of new malignant tumors, and most of them have progressed to a locally advanced stage. It was previously thought that OPSCC was related to smoking and drinking, while in recent years, it was found that the etiology of OPSCC was mostly related to HPV [1]. HPV-related tumor has been considered as a solid tumor with unique molecules, which has a high response rate to treatment, with low recurrence rate and high overall survival rate. HPV has a unique intraepithelial infection cycle, mainly infecting squamous epithelium. Types 16 and 18 are the most common HPV oncoviruses that

cause human tumors, and play an oncogenic role by silencing the cell proliferation inhibition gene through the synergistic action of E6 and E7 proteins [2]. E6 can mediate the degradation of tumor suppressor gene p53, and E7 can form complex with pRB tumor suppressor. In recent years, studies have shown that HPV can interfere with the function of downstream regulatory element antagonist (DREAM). The destruction of DREAM-dependent transcriptional inhibition can eventually lead to the early expression of cell cycle regulators and the p53-p21-DREAM pathway is an important pathway for p53 to activate cell cycle checkpoints. By activating this pathway, p53 can down regulate the gene transcription controlled by DREAM [3-5]. In this study, RNAscope technique and immunohistochemistry were used to determine the infection rate of HPV in OPSCC tissues, and to analyze the relationship between HPV and clinicopathologic features. At the same time, the expression of P53, P21, and Cdc2 proteins and their relationships with the prognosis of OPSCC patients are discussed, which provides a theoretical basis for further elucidating the occurrence, development, and treatment of HPV-related tumors.

Patients and methods

Case description

From January 1st, 2012 to December 31st, 2018, 49 OPSCC cases from patients aged 37 to 75 years, with a median age of 53 years were selected at Tangshan Head and Neck Disease Pathology Research Base, including 29 males and 20 females. All sections were reviewed by two senior pathologists.

PCR-DNA reverse dot blot hybridization to detect HPV DNA

The tissue specimens were fixed with 4% neutral formalin, then H&E stained sections were made according to the routine method and 10 consecutive sections were observed. Then, detection of HPV DNA and HPV 16/18 E6 and E7 mRNA were carried out respectively. Human Papillomavirus Subtype Nucleic Acid Detection Kit (Chaozhou Kaipu Biotechnology Co., Ltd., China) was used to detect the HPV infection with PCR-DNA reverse dot blot hybridization. A positive result showed blue signals at the corresponding HPV genotype and IC membrane sites, while other sites were not colored. Negative results were only colored at IC sites. In accordance with the order of the probe sequence and the color on the membrane, the HPV genotypes were determined.

RNAscope to detect HPV E6, E7 mRNA

The E6 and E7 mRNA of HPV16/18 were detected by RNAscope Kit (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., China). Sample preparation, probe hybridization, signal detection and slide re-staining was carried out according to the kit instructions. The positive signal was dot-shaped brown-yellow granules in cytoplasm. HPV16 mRNA positive laryngeal carcinoma was used as a control.

P53, P21 and Cdc2 expression by immunohistochemistry

The specimens were sectioned at 4 μm, stained with hematoxylin and eosin (H&E) and evaluated by immunohistochemistry according to the protocol described in the manufacturer's guide accompanying the kit. The mouse anti-human P53 (1:100), P21 (1:100), and Cdc2 (1:300) monoclonal antibody were all purchased from Santa Cruz Biotechnology (USA). SP immunohistochemistry kit was purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. Known positive samples of P53, P21, and Cdc2 were used as positive controls. For the negative control, the primary antibodies were replaced with phosphate-buffered saline (PBS). Positive signals were brown or brownish yellow in color. Nuclear staining was considered positive by immunostaining for P53, while cytoplasmic and/or nuclear immunostaining were considered positive for P21 and Cdc2. 10 high magnification fields were selected randomly in each stained section and were observed and the brown-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 24.0 (SPSS Inc., Chicago, IL, USA). Differences in gender, age, smoking, drinking, differentiation degree, and TNM stage between HPV, P53, P21, and Cdc2 were analyzed by chi-square test or nonparametric rank test. Kaplan-Meier model, log-rank test, and univariate analysis were used for survival analysis. Cox regression model was used to calcu-

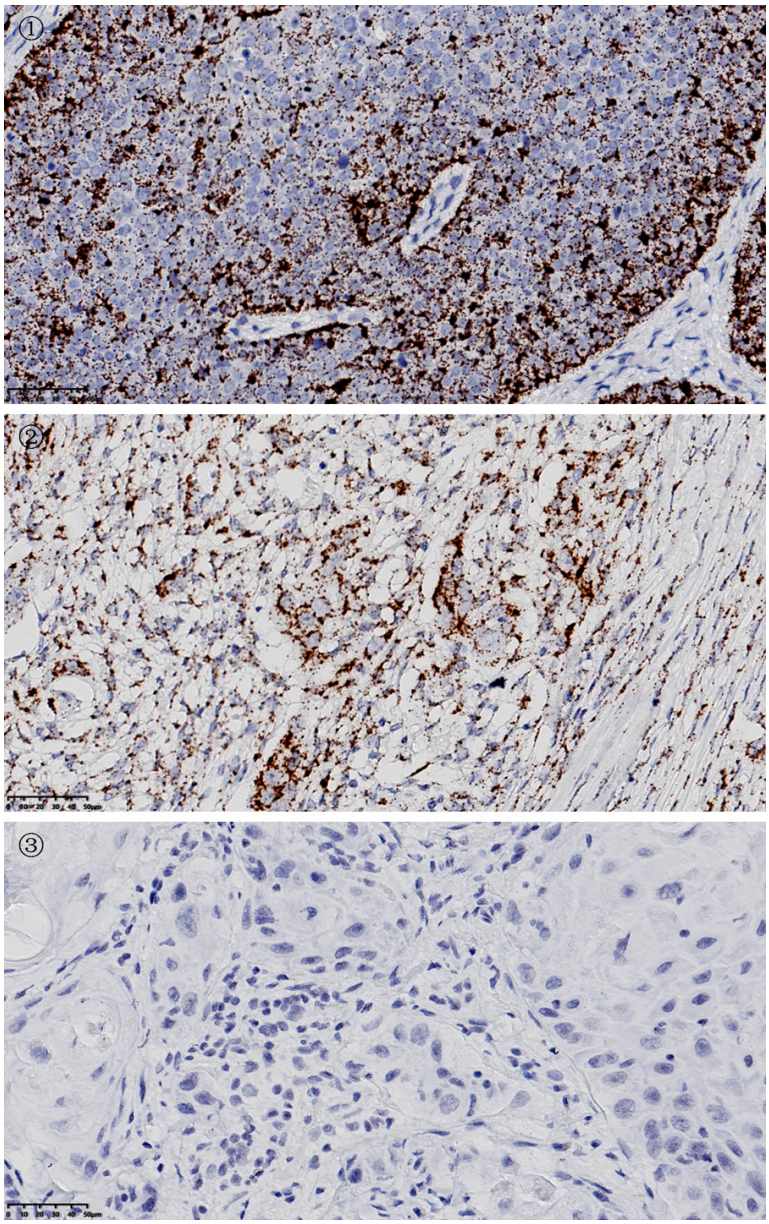

Figure 1. Expression of high-risk HPV mRNA in OPSCC. ① Brown granules in the cytoplasm of HPV 16-positive cells ② Positive control. ③ Negative control. ×100 RNA Scope method.

late hazard ratio (HR) and 95% confidence interval (CI) to evaluate the relationship between the markers and the survival of OPSCC patients. *P* values < 0.05 were considered significant.

Results

HPV16/18 DNA detection by PCR-DNA reverse dot blot hybridization

In 49 OPSCC patients, HPV DNA was positive in 14 cases (14/49, 28.6%), including 11 cases that were positive for HPV DNA 16/18, 2 cases for HPV DNA 11, and 1 case for HPV DNA 52. HPV DNA was negative in 35 cases (35/49, 71.4%).

HPV E6/E7 mRNA in situ hybridization by RNAscope

In the 49 OPSCC specimens, HPV 16/18 E6/E7 mRNA was positive in 22 cases (22/49, 44.9%, including 11 cases showed HPV DNA positive), 20 cases of which were positive for HPV 16 mRNA and 2 cases were positive for HPV-18 mRNA. 27 specimens showed HPV mRNA negative (27/49, 55.1%), in which 3 cases were HPV DNA positive (**Figure 1**).

Expression of P53, P21 and Cdc2 in OPSCC tissues

Immunohistochemistry showed that expression rate of P53, P21, and Cdc2 in OPSCC tissues were 63.3% (31/49), 65.3% (32/49), and 67.3% (33/49), respectively (**Figure 2**). The detection rate of HPV in the non-smoking group (68.7%) was higher than that of the smoking group (33.3%, *P*=0.019). The detection rate of HPV was irrelevant to the patient gender, age, alcohol consumption, differentiation, and clinical stage (*P* > 0.05). The expression of P53, P21, and Cdc2 were all irrelevant to patient's gender, age, smoking, alcohol consumption, tumor differentiation degree, and clinical stage (*P* > 0.05, **Table 1**). The detection of HPV was irrelevant to the expression of P53, P21, and Cdc2 (*P* > 0.05).

Prognosis of patients with OPSCC

Kaplan-Meier survival analysis showed that the median overall survival (OS) of HPV16 negative and positive patients were 24 months and 34 months respectively (*P* < 0.001). The median

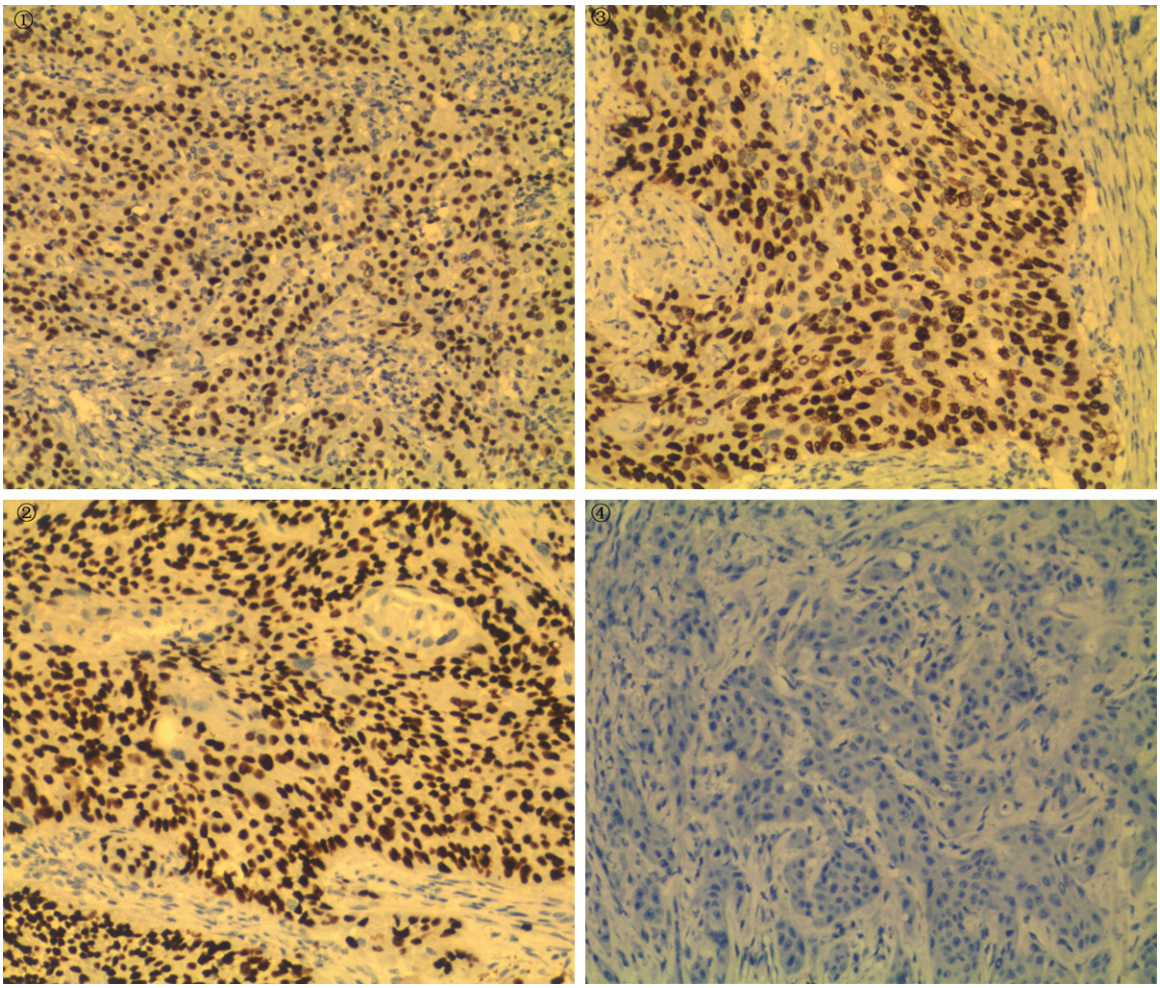

Figure 2. Immunohistochemical expression of P53, P21, and Cdc2 in OPSCC tissues: ① Immunohistochemical detection of P53 in OPSCC tissue. ② Immunohistochemical detection of P21 in OPSCC tissue. ③ Immunohistochemical detection of Cdc2 in OPSCC tissue. ④ Negative control, SP method ×100.

OS of well-moderately differentiated patients and poorly differentiated ones was 25 months and 31 months respectively ($P < 0.05$). The median OS of stages I and II, stage III and stage IV were 32 months and 24 months respectively ($P < 0.001$). The median OS of Cdc2-negative and positive patients were 34 months and 25 months respectively ($P < 0.001$, **Figure 3**).

Taking survival time as a dependent variable; sex, age, smoking, drinking, differentiation degree and tumor stage as independent variables, the indexes were regressed by a single factor, and statistically significant variables ($P < 0.1$) were selected for multivariate Cox regression analysis. The results showed that the detection coefficient was negative, HR < 1, indicating that the death risk of HPV-positive patients was lower than that of HPV negative ones, 95% CI=0.146-0.517.

The correlation coefficient of differentiation was positive, HR > 1, which indicated that the death risk in patients with poor differentiation was higher than that of patients with well-moderate differentiation, 95% CI=1.231-2.492. The correlation coefficient of tumor stage was positive, HR > 1, which indicated that the death risk in patients with stage III and stage IV was higher than that of patients with stage I and stage II, 95% CI=1.758-6.074. The correlation coefficient of Cdc2 expression was positive, HR > 1, which indicated that the death risk in Cdc2 positive patients was higher than that of Cdc2 negative ones, 95% CI=0.990-3.286 (**Tables 2-4**).

Table 1. Clinicopathologic characteristics of 49 cases of OPSCC and distribution frequency of measured markers [n (%)]

Clinical Feature	HPV16/18 mRNA+	*P*	P53+	*P*	P21+	*P*	Cdc2+	*P*
Gender								
Male	12 (41.4)	0.551	18 (62.1)	0.834	18 (62.1)	0.566	20 (69.0)	0.771
Female	10 (50.0)		13 (65.0)		14 (70.0)		13 (65.0)	
Age								
≤ 50 y	9 (52.9)	0.409	11 (64.7)	0.879	14 (82.4)	0.131	12 (70.6)	0.839
> 50 y	13 (40.6)		20 (62.5)		18 (56.3)		21 (67.7)	
Smoking status								
Smoker	11 (33.3)	0.019	22 (66.7)	0.478	22 (66.7)	0.774	25 (75.8)	0.071
Non-smoker	11 (68.7)		9 (56.3)		10 (62.5)		8 (50.0)	
Drinking								
Drinker	11 (40.7)	0.517	16 (59.3)	0.519	18 (66.7)	0.825	16 (59.3)	0.181
Non-drinker	11 (50.0)		15 (68.2)		14 (63.6)		17 (77.3)	
Differentiation								
Well	4 (26.7)	0.222	10 (66.7)	0.115	9 (60.0)	0.733	11 (73.3)	0.405
Moderate	10 (55.60		14 (77.8)		13 (72.2)		10 (55.6)	
Poor	8 (50.0)		7 (43.8)		10 (62.5)		12 (75.0)	
TNM staging								
I and II	11 (45.8)	0.897	15 (62.5)	0.913	16 (66.7)	0.845	17 (70.8)	0.610
III and IV	11 (44.0)		16 (64.0)		16 (64.0)		16 (64.0)	

Discussion

The incidence of head and neck squamous cell carcinoma (HNSCC) has been increasing in recent years. Smoking and drinking are the main causes of the development of HNSCC [6]. Studies found that the incidence of non-smoking and non-drinking HNSCC may be related to HPV. Stephen *et al.* [7] through systematic retrospective analysis of HPV-related HNSCC, found that the detection rate of HNSCC related to HPV was 25.9%. Among them, the incidence rate of OPSCC is 35.6%, higher than HPV-associated oral squamous cell carcinoma (23.5%) and laryngeal squamous cell carcinoma (24%). Ethnic and geographic differences in HNSCC patients are known to contribute to different infection rates of HPV. The incidence rate of HPV-positive HNSCC is lowest in Africa, while in Asian countries, especially Japan, the incidence rate is highest in the world. The high prevalence of HPV in Asian patients with oral cancer suggests that viral infection may be an important cause of disease, and that eating habits and potential genetic predisposition may also cause malignant transformation of cells [8]. A study by Kulkarni *et al.* [9] found that HPV was detected in 96% of cervical cancer tissues and in 70.59% of oral squamous cell carcinoma tissues in Karnataka, India. The results showed that the prevalence of HPV18 was higher than that of HPV16 in the general population. Elango *et al*. [10] found 48% of tongue squamous cell carcinoma was positive for HPV16 by PCR. In this study, the detection rates of HPV DNA and HPV mRNA was 28.6% and 44.9% in 49 patients with OPSCC. The sensitivity of RNAscope in situ hybridization was higher than that of PCR reverse dot hybridization. The statistical results showed that HPV positity occurred mostly in nonsmokers, and was irrelevant to patient's gender, age, alcohol consumption, tumor differentiation and clinical stage, which was consistent with the conclusions of Cardin [11] and Liu *et al.* [12]. Cox multivariate regression analysis showed that HPV detection was related to the survival time, and the death risk in HPV-positive patients was lower than that of HPV-negative ones (HR= 0.275, 95% CI: 0.146~0.517). The results are consistent with those of Hoppe *et al.* [13]. The prognosis of HPV-positive OPSCC is different from that of other patients. The difference is mainly due to their different diffusion patterns and the lower incidence rate of primary tumors. This may be one of the reasons why HNSCC

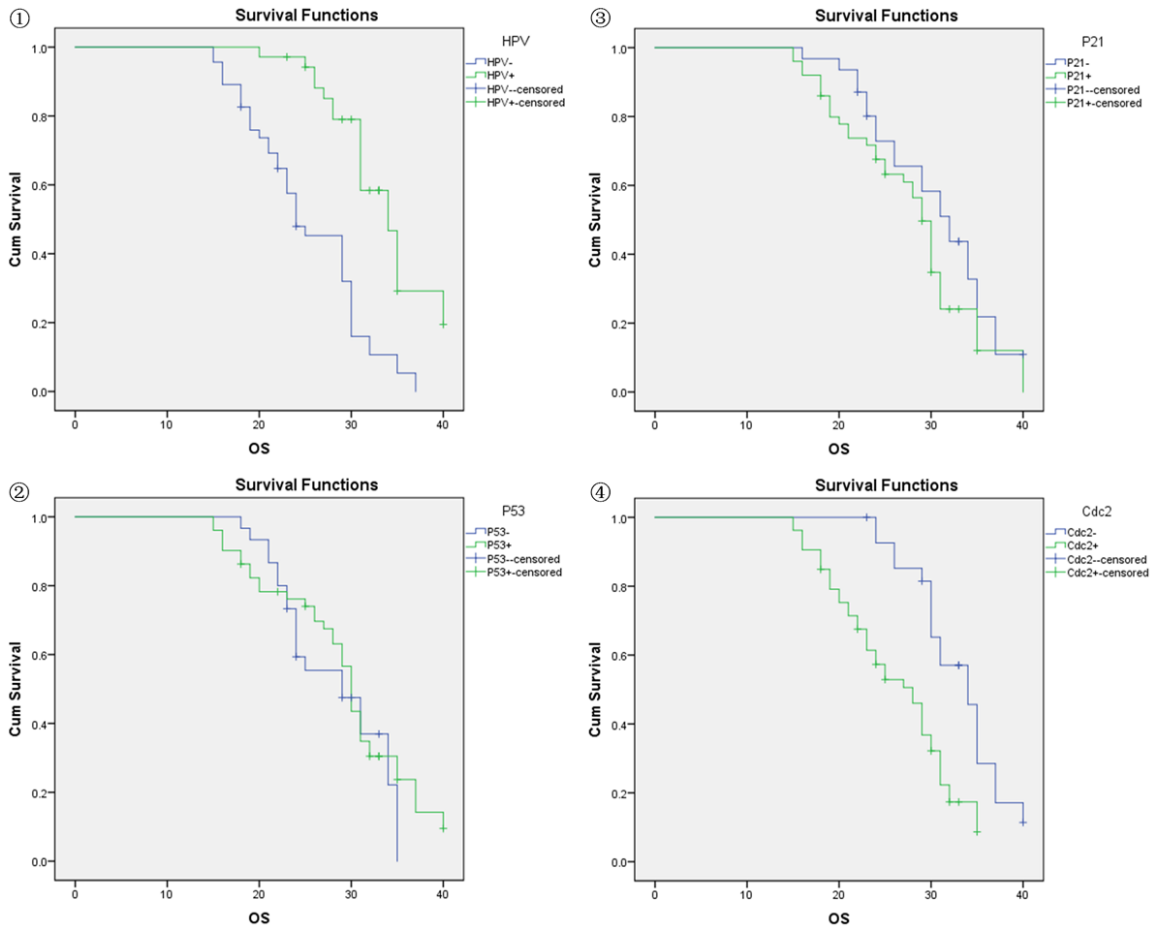

Figure 3. Correlation of the immunohistochemical expression with overall survival of patients with OPSCC: ① Relationship between HPV infection and OS. χ^2=24.340, $P < 0.001$. ② Relationship between P53 expression and OS. χ^2=0.912, P=0.340. ③ Relationship between P21 expression and OS. χ^2=1.656, P=0.198. ④ Relationship between Cdc2 expression and OS. χ^2=13.106, $P < 0.001$.

with positive HPV is sensitive to chemotherapy and radiotherapy. Therefore, the follow-up of patients with HPV negative OPSCC should be strengthened.

HPV is a double-stranded DNA virus with more than 100 genotypes, more than 24 of which can be detected in HNSCC. HPV can induce malignant transformation by producing E6 and E7 proteins that lock cells in the forward replication phase. The E6 protein of HPV binds to the tumor suppressor protein p53 and E3 ubiquitin ligase of E6AP cells, which leads to the ubiquitination of p53, the degradation of the proteasome, and the impairment of its antitumor function. As a result, the G1/S and G2/M checkpoints mediated by p21 are out of control; thus the function of DNA repair is reduced, the cell cycle is inhibited, and the expression of Bax and Puma are reduced, thus cell death is also reduced [14]. Jung *et al*. [15] found that most HPV-related HNSCCs contain wild-type p53, and their sensitivity to cytotoxic drugs may be due to the activation of wild-type p53. In the culture of HNSCC cell line containing HPV and adding E6 protein inhibitor, p53 protein expression was stable and cell death was increased. Further study found that proteasome inhibitor bortezomib can increase the expression levels of p53 and p21, leading to the dose-dependent death of HPV-positive cells. HPV E7 protein also binds to retinoblastoma protein (pRb) and induces its degradation. pRb inhibits cell cycle progression by inhibiting E2F family transcription factors. The loss of pRb can activate E2F and promote cell proliferation.

Cell cycle is a cascade event. Among all the factors that control cell mitosis, cyclin and cyclin dependent kinase (CDK) are two important

Table 2. Single factor analysis of prognosis in 49 patients with OPSCC

Factor	n	Median overall survival (Months)	Statistics	*P*
Gender				
Male	29	30	0.010	0.920
Female	20	29		
Age				
≤ 50 y	17	25	0.366	0.545
> 50 y	32	30		
Smoking Status				
Smoker	33	30	2.434	0.119
Non-smoker	16	35		
Drinking				
Drinker	27	30	0.104	0.747
Non-drinker	22	30		
HPV				
positive	22	34	24.340	< 0.001
negative	27	24		
Differentiation				
Well	15	31	6.164	0.046
Moderate	18	31		
Poor	16	25		
TNM staging				
I and II	24	32	13.489	< 0.001
III and IV	25	24		
P53				
P53+	31	25	0.912	0.340
P53-	18	30		
P21				
P21+	32	29	1.656	0.198
P21-	17	32		
Cdc2				
Cdc2+	33	25	13.106	< 0.001
Cdc2-	16	34		

molecules. These proteins form a heterodimer, in which cyclin is the regulatory subunit and CDK is the catalytic subunit. When the complex is activated from the external signal, CDKs activate or inactivate the downstream target protein to coordinate the next stage of the cell cycle. CDKs can be divided into two categories: one is activated by CDK binding protein, the other is activated by CDK inhibitor CKI. CKI inhibitors can be divided into two families: one is the INK4 family, including $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$, which can specifically bind CDK4 and CDK6. The other is protein kinase inhibitors (KIP) or CDK interacting protein family (CIP), including p21, $p27^{kip1}$ and $p57^{kip2}$. All of these CKIs bind to and inhibit cyclin binding [16]. p21 is one of the members of CKI/CIP family. The relationship between p21 gene and tumorigenesis is mainly the result of a synergistic effect with other related genes [17]. Whether p21 is a tumor suppressor gene or an oncogene remains controversial, and its expression is not consistent with clinical reports of tumor prognosis. In this study, the expression of P53 and P21 protein was irrelevant to gender, age, smoking, drinking, tumor differentiation or clinical stage of the patients, $P > 0.05$. The results of the survival curve and Cox multivariate regression analysis showed that expressions of P53 and P21 protein were irrelevant to overall survival time. They showed no significant effect on the prognosis of 49 patients with OPSCC in this study. At present, it is thought that the expression of p53 and p21 in tumor tissue is complicated; p21 expression has two pathways, a p53-dependent pathway and p53-independent pathway, and the expression of P53 and P21 in different cells and tumors is heterogeneous. Kapranos *et al.* [18] have proven that P21 expression is a good prognostic factor for HNSCC. Liu *et al.* [19] found that the recurrence rate in P21-positive laryngeal cancer patients was higher than that of negative ones, with a statistically significant difference. In laryngeal squamous cell carcinoma, the expression of P21 protein may be through the p53 independent pathway, which may increase the stability of P21 protein, rather than increasing the transcription activity. In addition, biochemical and genetic studies have shown that p21 is independent of the classical p53 signaling pathway and plays a role as an anti-oncogene in a variety of anti-proliferative activities. Although the main function of p21 is to inhibit cell proliferation, and promote cell differentiation and aging, new research shows that under certain conditions, p21 can promote cell proliferation and play a carcinogenic role. Therefore, the expression of p21 depends on the cell environment. In different cell environments, p21 may play different roles as a tumor suppressor gene or oncogene.

Cyclin-dependent Kinase 1 (CDK1) is a member of the Silk/Threonine kinase family encoded by

Table 3. Factors influencing survival rate of OPSCC patients and their evaluation

Variable	Assignment	
X_1	Gender	Male=0, Female=1
X_2	Age	≤ 50 y=0, > 50 y=1
X_3	Smoking	No=0, Yes=1
X_4	Drinking	No=0, Yes=1
X_5	HPV	HPV-=0, HPV+=1
X_6	Differentiation	Well=1, Moderate=2, Poor=3
X_7	Stage	I+II=0, III+IV=1
X_8	P53	P53-=0, P53+=1
X_9	P21	P21-=0, P21+=1
X_{10}	Cdc2	Cdc2-=0, Cdc2+=1
T	Survival time	Month
Y	Survival outcome	Delete or Survival=0, Death=1

Table 4. Cox Regression analysis of survival factors in 49 OPSCC patients

Factor	*B*	S.E.	Wald	*P*	Exp(B)	HR (95% *CI*)
HPV	-1.292	0.323	16.012	< 0.001	0.275	0.146~0.517
Differentiation	0.560	0.180	9.695	0.002	1.751	1.231~2.492
Staging	1.184	0.316	14.014	< 0.001	3.268	1.758~6.074
Cdc2	0.590	0.306	4.202	0.040	1.804	0.990~3.286

the gene 2 of the Cell Division Cycle (Cdc2); its main function is to monitor the assembly of spindle microtubules and the correct attachment of motile DNA at the G2/M checkpoint. If the regulatory mechanism of Cdc2 is wrong, it will directly lead to disorders of cell differentiation, cell cycle process, abnormal cell proliferation or malignant transformation and then promote the occurrence and development of tumor [20]. In the late G2, Cdc2 and cyclin B combine to form a Cdc2 cyclin B kinase complex, known as mitotic promoting factor or maturation promoting factor (MPF), which can promote cells from G2 to M phase. Cdc2 is one of the important regulatory kinases in cell cycle. Its activity is not only positively regulated by cyclin B, but also negatively regulated by CKI [21]. Cdc2 is overexpressed in many HNSCC tissues. Yang *et al*. [22] found that the positive rate of Cdc2 protein was 70.6% in laryngeal carcinoma tissues, 57.1% in the recurrent group, and 23.9% in non-recurrent group, suggesting that the expression of Cdc2 was closely related to recurrence and poor prognosis of laryngeal cancer. In this study, we found that the expression of Cdc2 protein was irrelevant to gender, age, smoking, drinking, tumor differentiation, and clinical stage (*P* > 0.05). Cox regression analysis showed that the expression of Cdc2 protein, differentiation, and clinical stage were related to the overall survival time of patients, and the death risk of Cdc2 positive patients was higher than that of Cdc2 negative ones (HR=1.804, 95%, CI: 0.990-3.286). The death risk in patients with poor differentiation and late stage was higher than that in patients with high and moderate differentiation (HR=1.751, 95% CI: 1.231-2.492) and early stage (HR= 3.268, 95% CI: 1.758-6.074).

In conclusion, routine detection of HPV and expression of Cdc2 may be useful in predicting the prognosis of OPSCC patients. The sensitivity of RNAscope in situ hybridization was higher than that of PCR reverse dot hybridization. The patients with negative HPV, poor differentiation, late stage, and Cdc2 expression suggest poor prognosis and clinical follow-up should be strengthened.

Disclosure of conflict of interest

None.

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