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ORIGINAL ARTICLE

The expression and prognostic impact of proinflammatory cytokines and their associations with carcinogens in oropharyngeal squamous cell carcinoma

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Abstract

In oropharyngeal squamous cell carcinoma (OPSCC), the relationships between immune responses, carcinogens, and prognoses are not clarified yet. Here, we retrospectively reviewed the pathology samples of 46 OPSCC patients, and used p16 to determine their human papillomavirus (HPV) status. The Cancer Genome Atlas (TCGA) database was also analyzed for further comparison. The immunofluorescence staining of proinflammatory cytokines showed that high interferon gamma (IFNγ; T helper 1; Th1), low interleukin 4 (IL4; T helper 2; Th2), low thymic stromal lymphopoietin (TSLP; Th2), and low transforming growth factor beta (TGFβ; T regulatory; Treg) expressions were good prognostic factors for OPSCC. p16-positive OPSCC showed higher Th1, lower Th2/Treg proinflammatory cytokine expressions, and a better prognosis than p16-negative OPSCC. In smokers alone, although p16-positive OPSCC smokers showed weaker Th2/Treg predominant cytokine expressions than p16-negative OPSCC smokers, the prognoses of both groups were equally poor. As for p16-positive OPSCC patients alone, p16-positive nonsmokers showed a significantly better prognosis than p16-positive smokers, but the immune responses of both groups were all weakly Th2/Treg predominant. Overall, higher Th1 and lower Th2/Treg proinflammatory cytokine expressions are associated with a better prognosis for OPSCC. HPV may be related to increased Th1, decreased Th2/Treg responses, and a good prognosis, while smoking may be related to increased Th2/Treg, decreased Th1 responses, and a poor prognosis in OPSCC. The impact of smoking on immune deviation may be weaker than that of HPV, but the impact of smoking on prognosis may be stronger than that of HPV in OPSCC.

Keywords Oropharyngeal squamous cell carcinoma · Smoking · Human papillomavirus · p16 · Proinflammatory cytokines · Prognosis

Abbreviations

CCL22	C-C motif chemokine ligand 22
CXCL10	C-X-C motif chemokine ligand 10
R	Correlation coefficient
DAB	Diaminobenzidine tetrachloride
FFPE	Formalin-fixed, paraffin-embedded
HNC	Head and neck cancer
HR	Hazard ratio
IF	Immunofluorescence
OPSCC	Oropharyngeal squamous cell carcinoma
PBST	Phosphate-buffered saline with Tween 20
ROC	Receiver-operating characteristic
TCGA	The Cancer Genome Atlas
TSLP	Thymic stromal lymphopoietin

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Introduction

Oropharyngeal squamous cell carcinoma (OPSCC), a main subtype of Head and Neck cancer (HNC), is a malignant tumor arising in the oropharynx, including the tonsils, soft

palate, tongue base, and posterior pharyngeal wall [1]. Over the past decades, the incidence of OPSCC continues to rise worldwide [2, 3]. Traditionally, the causes of OPSCC are related to chemical carcinogens such as smoking and alcohol [4]. However, it has been found that human papillomavirus (HPV), especially type 16, is also a fundamental and independent cause for OPSCC and contributes to 20–72% of OPSCC [5, 6]. Accumulating evidence reveals that HPV-positive OPSCC patients have a significantly better prognosis (5-year overall survival: 82%) than HPV-negative OPSCC patients (5-year overall survival: 35%) [6, 7]. HPV-positive OPSCC seems to have higher chemoradiosensitivity, leading to a more improved treatment response than HPV-negative OPSCC [1, 6]. The reasons behind this phenomenon may be multifactorial and remain incompletely clarified [1, 6].

In clinical practice, instead of the expensive direct HPV 16 in situ hybridization, the inexpensive and easily available immunohistochemistry (IHC) of p16 has been chosen to differentiate HPV-positive and HPV-negative OPSCC in the newest HNC staging criteria (American Joint Committee on Cancer, 8th edition, 2017) [8]. p16 (cyclin-dependent kinase inhibitor 2A) is a tumor suppressor protein that serves as an accurate and robust surrogate marker for overexpressed HPV16 E7 oncoprotein [8, 9]. Only when p16 immunostaining of the tumor is diffused (≥ 75%) with moderate/strong intensity can it be categorized into p16-positive OPSCC, which is almost equal to HPV-positive OPSCC (positive predictive value: 98–100%, negative predictive value: 56–86%) [8, 10].

The proliferation and metastasis of cancer cells have been proven to be closely related to the immune status of the host and tumor microenvironment [11, 12]. The adaptive immune responses can be categorized into three main subsets: T helper 1 (Th1), T helper 2 (Th2), and T regulatory (Treg) [13]. Th1 immune responses usually antagonize Th2/Treg responses [13]. Cancers with predominant Th2/Treg responses usually carry a worse prognosis than those with predominant Th1 responses [14]. The Th1 proinflammatory cytokines, such as interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), interleukin 12 (IL12), and C-X-C motif chemokine ligand 10 (CXCL10), may play a role to inhibit cancer cell proliferation [13, 14]. By contrast, the Th2/Treg proinflammatory cytokines are thought to facilitate cancer cell growth, such as interleukin 4 (IL4; Th2), thymic stromal lymphopoietin (TSLP; Th2), interleukin 10 (IL10; Th2), C-C motif chemokine ligand 22 (CCL22; Th2), and transforming growth factor beta (TGFβ; Treg) [13, 14].

Both smoking and HPV can affect the balance between Th1 and Th2/Treg responses. Previous studies have implied that smoking may suppress the innate immune responses and promote chronic inflammation (mainly Th2) in the upper airway [15]. On the other hand, some studies have indicated that HPV in cancer cells may serve as tumor-specific antigens to facilitate immune system identifications [7, 16]. In cervical cancers, the immune responses induced by HPV are more likely deviating toward Th2/Treg [17]. However, in laryngeal cancers, the immune responses mediated by HPV may tend to deviate toward Th1 [18]. Regarding OPSCC, the relationships between immune responses, carcinogens, and prognoses are not clarified yet [7, 19]. Therefore, the aims of this study were to explore the expression and prognostic impact of proinflammatory cytokines, and to clarify their associations with HPV and smoking in OPSCC. The Cancer Genome Atlas (TCGA) database was also analyzed for further comparison in this study.

Materials and methods

Patient population

In this study, we retrospectively enrolled 46 OPSCC patients diagnosed from January 2011 to December 2015 at National Taiwan University Hospital, Yun-Lin Branch. Their formalin-fixed, paraffin-embedded (FFPE) pathology samples of the primary tumors were retrieved, and their survivals were recorded until December 2018. The exclusion criteria were patients with a history of cancer other than HNC, patients who did not receive p16 IHC at the time of diagnosis, patients who did not have medical records concerning smoking status, and immunocompromised patients who received long-term steroid treatment. The TNM (Tumor, Node, and Metastases) status of OPSCC was classified according to the 2010 criteria of the American Joint Committee on Cancer (AJCC) [20].

p16 IHC

p16 IHC was performed on FFPE tumor sections with 5-μm thickness. Rehydration and antigen retrieval of the FFPE sections were performed according to a standard protocol [21]. We used the G175-405 clone of the p16 antibody (51-1325GR; BD Biosciences, San Diego, CA, USA) to detect the expression of p16 and chose the diaminobenzidine tetrachloride (DAB) chromagen to elucidate the antibody-antigen complex. Positive p16 staining was interpreted when it was diffused (≥ 75%) with moderate/strong intensity in cancer cells [8].

Immunofluorescence (IF) staining of proinflammatory cytokines

FFPE tumor samples were sectioned with 3-μm thickness. Dewaxing and rehydration of the FFPE sections were performed with xylene and ethanol according to a standard

protocol [21]. Antigen retrieval was achieved using diluted Antigen Retrieval Buffer (Ab93678; Abcam, Cambridge, UK) by microwave heating. 0.3% hydrogen peroxide (Ab64218; Abcam) was then used to block all endogenous peroxidase activity. Nonspecific binding sites were blocked with Antibody Diluent (S080983-2; Dako, Santa Clara, CA, USA). The primary antibodies of 2 different proinflammatory cytokines were then double-stained simultaneously to the slides with proper dilutions according to the manufacturer's instructions at room temperature for 1 h. After washing the slides with phosphate-buffered saline with Tween 20 (PBST), the secondary antibodies of green and red fluorescence were applied to the slides using Goat Anti-Mouse IgG (Alexa Fluor® 488) (A150113; Abcam) and Goat Anti-Rabbit IgG (Alexa Fluor® 594) (A150080; Abcam) at room temperature for another 1 h. Finally, the nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI) (14285; Cayman Chemical, Ann Arbour, MI, USA) for 30 min.

We performed IF staining on 4 different slides for each patient. The working concentration of each primary antibody was determined according to the manufacturer's suggestion and was pretested for several times. The primary antibodies and their final working concentrations were as follows. On the first slide, Purified Mouse IgG2a, κ Isotype Control Antibody (401502; Biolegend, San Diego, CA, USA) (5 μg/ml) and Purified Rabbit Polyclonal Isotype Control Antibody (910801; Biolegend) (5 μg/ml) were used as background controls. On the second slide, Mouse Monoclonal Anti-IFNγ Antibody (OACD00112; Aviva Systems Biology, San Diego, CA, USA) (10 μg/ml) and Rabbit Monoclonal Anti- IL4 Antibody (Ab62351; Abcam) (1:100 dilution) were applied to compare Th1 with Th2 cytokines. On the third slide, Mouse Monoclonal Anti-IFNγ Antibody (OACD00112; Aviva) (10 μg/ml) and Rabbit Polyclonal Anti-TSLP Antibody (Ab47943; Abcam) (5 μg/ml) were used to compare Th1 with Th2 cytokines again. Finally, on the fourth slide, Mouse Monoclonal Anti- TGFβ Antibody (OACD00156; Aviva) (5 μg/ml) and Rabbit Polyclonal Anti-TSLP Antibody (Ab47943; Abcam) (5 μg/ml) were used to compare Treg with Th2 cytokines.

We captured the IF staining images using a fluorescence microscope (IX 73; Olympus, Tokyo, Japan) 5 min later after mounting the slides. The Olympus cellSens software was used to calculate the sum of the gray intensity value in each snapshot, which was adopted to represent the IF intensity. The setting of fluorescence strength was fixed to 50%, the gain was fixed to 1×, and the exposure time for DAPI was fixed to 7 ms for all patients. To compare the green and red fluorescence fairly, we used the same exposure time for both green and red fluorescence over the 4 slides of the same patient, ranging from 100 to 300 ms. We used DAPI as the baseline to calculate the mean florescence intensity per cell (IFNγ/DAPI, IL4/DAPI, TSLP/DAPI, TGFβ/DAPI) after deducting the background fluorescence intensity. We also calculated the green/red fluorescence intensity ratio per snapshot (IL4/IFNγ, TSLP/IFNγ, and TGFβ/TSLP) and used IFNγ as the baseline to obtain the ratios: IL4/IFNγ, TSLP/IFNγ, and TGFβ/IFNγ.

TCGA database analysis

OPSCC patients were identified from all HNC patients in the publicly available TCGA database. The clinical characteristics of these patients and the RNA-sequencing data of the cancer specimens were then analyzed. The RNA expression data of proinflammatory cytokines was measured by fragments per kilobase of transcript per million mapped reads (FPKM).

Statistical analysis

All statistical analyses were performed using the SPSS software package, version 18.0 (SPSS Inc., Chicago, IL, USA). Chi-square tests, Fisher's exact tests, Independent T tests, and the Mann–Whitney *U* tests were used to determine differences in the clinical features and proinflammatory cytokine intensities of the OPSCC patients. Spearman's rank-order correlation coefficient (*R*) was used to delineate the relationships between different proinflammatory cytokines. ROC curves and Youden's index were adopted to determine the optimal cut-off points of proinflammatory cytokine intensities. Finally, Kaplan–Meier survival analyses were plotted and the survival differences for various factors were examined using the log-rank test and hazard ratio (HR).

Results

Patient demographics

46 eligible OPSCC patients were enrolled in this study, including 41 male and 5 female patients. The clinical characteristics of all patients are listed in Table 1. Among these 46 patients, 20 patients were p16-positive and 26 patients were p16-negative. Age, sex, tumor site, T and N classification, stage and primary treatment did not differ significantly between the p16-positive and p16-negative groups. However, there were significantly more smokers in the p16-negative group (25/26, 96.15%) than in the p16-positive group (9/20, 45%) ($P < 0.001$). To eliminate the differences, all smokers (34/46, 73.91%) were analyzed alone. Among these 34 smokers, 9 patients were p16-positive and 25 patients were p16-negative. Age, sex, tumor site, T and N classification, stage and treatment did not differ significantly between the 2 groups. To specify the influence of smoking, we further analyzed p16-positive OPSCC patients (20/46, 43.48%) alone.

Table 1 Clinical characteristics of the patients with oropharyngeal squamous cell carcinoma

	Overall			Smokers alone			p16-positive alone		
	p16-positive ($n=20$)	p16-negative ($n=26$)	*P* value	p16-positive ($n=9$)	p16-negative ($n=25$)	*P* value	Smoker ($n=9$)	Nonsmoker ($n=11$)	*P* value
Age (years)	55.85 ± 10.83	51.77 ± 8.29	0.599	58.22 ± 12.24	52.04 ± 8.34	0.103	58.22 ± 12.24	53.91 ± 9.69	0.39
Sex									
Male	16/20 (80%)	25/26 (96.2%)	0.081	9/9 (100%)	24/25 (96%)	1.000	9/9 (100%)	7/11 (63.64%)	0.094
Female	4/20 (20%)	1/26 (3.8%)		0/9 (0%)	1/25 (4%)		0/9 (0%)	4/11 (36.36%)	
Smoking (ever)	9/20 (45%)	25/26 (96.15%)	< 0.001*	9/9 (100%)	25/25 (100%)	1.000	9/9 (100%)	0/11(0%)	< 0.001*
Tumor site									
Tonsil	13/20 (65%)	17/26 (65.38%)	0.171	5/9 (55.56%)	17/25 (68%)	0.533	5/9 (55.56%)	8/11 (72.73%)	0.336
Tongue base	5/20 (25%)	2/26 (7.7%)		2/9 (22.22%)	2/25 (8%)		2/9 (22.22%)	3/11 (27.27%)	
Soft plate/ Pharyngeal wall	2/20 (10%)	7/26 (26.92%)		2/9 (22.22%)	6/25 (24%)		2/9 (22.22%)	0/11 (0%)	
T stage									
T1, 2	11/20 (55%)	14/26 (53.85%)	1.000	6/9 (66.67%)	14/25 (56%)	0.704	6/9 (66.67%)	5/11 (45.45%)	0.406
T3, 4	9/20 (45%)	12/26 (46.15%)		3/9 (33.33%)	11/25 (44%)		3/9 (33.33%)	6/11 (54.55%)	
N stage									
N0, 1	6/20 (30%)	11/26 (42.31%)	0.54	4/9 (44.44%)	10/25 (40%)	1.000	4/9 (44.44)	2/11 (18.18)	0.336
N2, 3	14/20 (70%)	15/26 (57.69%)		5/9 (55.55%)	15/25 (60%)		5/9 (55.56)	9/11 (81.82)	
Overall stage									
I, II	1/20 (5%)	5/26 (15.4%)	0.262	0/9 (0%)	5/25 (20%)	0.293	0/9 (0%)	1/11 (9.09%)	1.000
III, IV	19/20 (95%)	21/26 (84.6%)		9/9 (100%)	20/25 (80%)		9/9 (100%)	10/11 (90.91%)	
Treatment									
OP + CCRT	17/20 (85%)	24/26 (92.3%)	0.303	6/9 (66.67%)	23/25 (92%)	0.058	6/9 (66.67%)	11/11 (100%)	0.074
OP only	0/20 (0%)	1/26 (3.85%)		0/9 (0%)	1/25 (4%)		6/9 (66.67%)	0/11 (0/11%)	
CT only	3/20 (15%)	1/26 (3.85%)		3/9 (33.33%)	1/25 (4%)		3/9 (33.33%)	0/11 (0/11%)	

OP operation, *CCRT* chemoradiotherapy, *CT* chemotherapy

*Statistical significant (Fisher's exact test)

Among these 20 p16-positive patients, 9 patients were smokers and 11 patients were nonsmokers. No significant differences were found between the 2 groups in age, sex, tumor site, T and N classification, stage and primary treatment.

Staining of the pathology samples

For each patient, we performed hematoxylin and eosin (H&E) staining, IHC staining of p16, and IF staining of IFNγ, IL4, TSLP, and TGFβ on FFPE tumor sections. In IF staining, we performed double-stained background IgG (green/red) on the first slide, IFNγ (green)/IL4 (red) on the second slide, IFNγ (green)/TSLP (red) on the third slide, and TGFβ (green)/TSLP (red) on the fourth slide. DAPI was used to mark the tumor cell nucleus. Figures 1 and 2 show examples of the staining pictures in p16-negative and p16-positive OPSCC.

Intensity of proinflammatory cytokine expression

To focus on the changes in cancer cells and diminish the influences of normal cells, we calculated the IF intensity under the largest magnification power (400×) of our microscope. 3 different tumor sites were chosen in each slide to reduce the selection bias, and the mean sum of the gray intensity value was calculated as representative of the IF intensity. Table 2 shows the results of IF intensities in our series. Among the 46 patients, the intensities of IFNγ/DAPI were significantly higher in the p16-positive group (0.074 ± 0.06) than in the p16-negative group (0.038 ± 0.05)

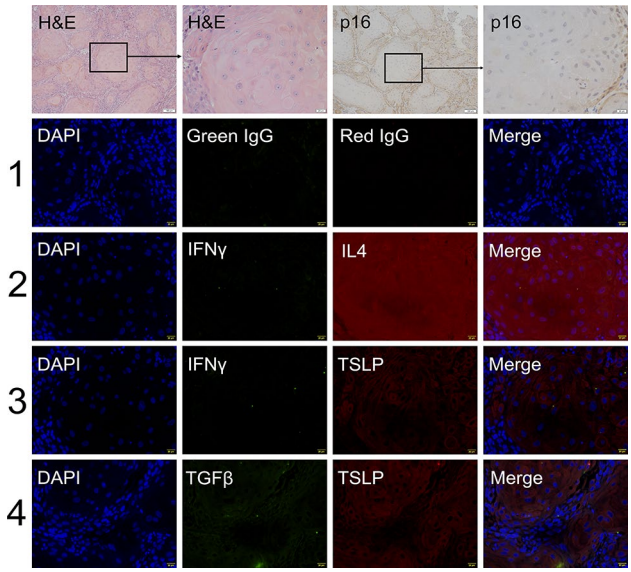

Fig. 1 Pathology images of a 63-year-old male patient with p16-negative, stage IV tonsillar cancer. H&E: hematoxylin and eosin; p16: p16 immunohistochemistry; No. 1–4: immunofluorescence double staining; No. 1: background; No. 2: IFNγ (interferon γ) and IL4 (interleukin 4); No. 3: IFNγ and TSLP (thymic stromal lymphopoietin); No. 4: TGFβ (transforming growth factor β) and TSLP; *DAPI* 4′-6-diamidino-2-phenylindole

($P=0.029$). By contrast, the intensities of IL4/DAPI, IL4/IFNγ, and TGFβ/IFNγ were significantly higher in the p16-negative group (0.92 ± 0.47, 183.48 ± 501.31 and 4.88 ± 5.92, respectively) than in the p16-positive group (0.59 ± 0.41, 25.61 ± 48.07 and 2.21 ± 1.44, respectively) ($P=0.016$, 0.010 and 0.035, respectively). These results showed that p16-negative OPSCC had strongly Th2/Treg predominant immune responses, while p16-positive OPSCC had only weakly Th2/Treg predominant immune responses. Namely, HPV may be associated with increased Th1 and decreased Th2/Treg responses in OPSCC.

Among the 34 smokers, IFNγ/DAPI was still significantly higher in the p16-positive group (0.083 ± 0.082) than in the p16-negative group (0.036 ± 0.046) ($P=0.043$). IL4/IFNγ, TSLP/IFNγ, and TGFβ/IFNγ, although not statistically significant, were relatively weaker in the p16-positive group (27.61 ± 33.71, 3.46 ± 2.45 and 2.34 ± 1.56, respectively) than in the p16-negative group (190.64 ± 510.29, 9.38 ± 17.43 and 5.02 ± 6.00, respectively) ($P=0.120$, 0.335 and 0.050, respectively). These results showed that, though the effect of HPV on immune deviation was some part hampered by smoking, HPV was still associated with increased Th1 and decreased Th2/Treg responses in OPSCC smokers. Namely, smoking may be associated with increased Th2/

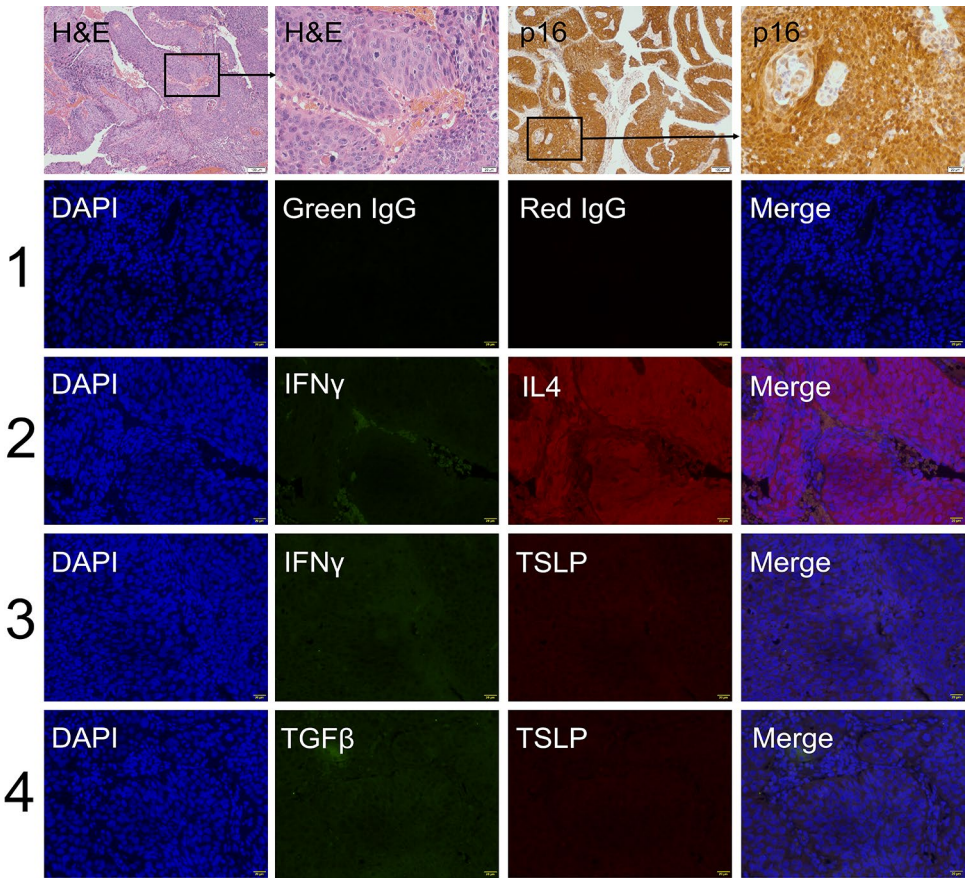

Fig. 2 Pathology images of a 78-year-old female patient with p16-positive, stage IV tongue base cancer. H&E: hematoxylin and eosin; p16: p16 immunohistochemistry; No. 1–4: immunofluorescence double staining; No. 1: background; No. 2: IFNγ (Interferon γ) and IL4 (interleukin 4); No. 3: IFNγ and TSLP (thymic stromal lymphopoietin); No. 4: TGFβ (transforming growth factor β) and TSLP; *DAPI* 4′-6-diamidino-2-phenylindole

Table 2 Immunofluorescence intensities of oropharyngeal squamous cell carcinoma pathology sections

	Overall			Smokers alone			p16-positive alone		
	p16-positive ($n=20$)	p16-negative ($n=26$)	*P* value	p16-positive ($n=9$)	p16-negative ($n=25$)	P value	Smoker ($n=9$)	Nonsmoker (n = 11)	*P* value
Mean intensity per cell									
IFNγ/DAPI	0.074 ± 0.06	0.038 ± 0.05	0.029*	0.083 ± 0.082	0.036 ± 0.046	0.053	0.083 ± 0.08	0.066 ± 0.04	0.563
IL4/DAPI	0.59 ± 0.41	0.92 ± 0.47	0.016*	0.73 ± 0.53	0.93 ± 0.47	0.299	0.73 ± 0.53	0.48 ± 0.25	0.168
TSLP/DAPI	0.19 ± 0.14	0.20 ± 0.20	0.938	0.24 ± 0.15	0.20 ± 0.21	0.650	0.24 ± 0.15	0.16 ± 0.11	0.200
TGFβ/DAPI	0.15 ± 0.09	0.13 ± 0.12	0.492	0.16 ± 0.09	0.13 ± 0.12	0.517	0.16 ± 0.09	0.15 ± 0.09	0.804
Intensity ratio per snapshot									
IL4/IFNγ	25.61 ± 48.07	183.48 ± 501.31	0.010※	27.61 ± 33.71	190.64 ± 510.29	0.120	27.61 ± 33.71	23.97 ± 58.94	0.295
TSLP/IFNγ	3.10 ± 2.45	9.08 ± 17.15	0.080	3.46 ± 2.45	9.38 ± 17.43	0.335	3.46 ± 2.45	2.81 ± 2.53	0.456
TGFβ/IFNγ	2.21 ± 1.44	4.88 ± 5.92	0.035#	2.34 ± 1.56	5.02 ± 6.00	0.050	2.34 ± 1.56	2.11 ± 1.40	0.782

IFNγ interferon γ, *IL4* interleukin 4, *TSLP* thymic stromal lymphopoietin, *TGFβ* transforming growth factor β, *DAPI* 4′,6-diamidino-2-phenylindole

*Statistical significant (independent *T* test, *P* value of Levene > .05)

※Statistical significant (Mann–Whitney *U* test)

#Statistical significant (independent *T* test, *P* value of Levene < .05)

Treg and decreased Th1 responses in OPSCC, but its impact on immune deviation seems to be weaker than HPV.

Among the 20 p16-positive patients, the intensities of all proinflammatory cytokines did not differ significantly between the smoker and nonsmoker groups. Furthermore, the immune responses of both groups were all weakly Th2/Treg predominant, indicating that the effect of smoking on immune deviation may be weaker than that of HPV in OPSCC.

Correlations between different proinflammatory cytokines

The correlation coefficients (*R*) between different proinflammatory cytokines in our series are shown in Table 3. Regarding the mean intensity per cell, except for the modest correlation between IFNγ/DAPI and IL-4/DAPI ($R=0.199$; $P=0.184$), the relationships between other proinflammatory cytokines were all moderately correlated ($R=0.3\sim0.7$; $P<0.05$). As for the intensity ratio per snapshot, the relationship between TSLP/IFNγ and TGFβ/IFNγ was moderately correlated ($R=0.688$; $P<0.001$), while the relationship between IL4/IFNγ and TSLP/IFNγ and that between IL4/IFNγ and TGFβ/IFNγ were all highly correlated ($R=0.745$ and 0.707, respectively; both $P<0.001$). These results showed that, in the OPSCC microenvironment, the proinflammatory cytokines of Th2 and Treg were strongly synchronized, while the proinflammatory cytokines of Th1 and Th2 were only weakly related. Namely, the antagonism between Th1 and Th2/Treg immune response still maintains, to a certain extent, in the tumor microenvironment.

Table 3 Correlation coefficients between different proinflammatory cytokines

Mean intensity per cell	IFNγ/DAPI	IL4/DAPI	TSLP/DAPI	TGFβ/DAPI
IFNγ/DAPI	–	0.199 ($P=0.184$)	0.330* ($P=0.025$)	0.581* ($P<0.001$)
IL4/DAPI	0.199 ($P=0.184$)	–	0.499* (P < 0.001)	0.372* ($P=0.011$)
TSLP/DAPI	0.330* ($P=0.025$)	0.499* ($P<0.001$)	–	0.681* ($P<0.001$)
TGFβ/DAPI	0.581* ($P<0.001$)	0.372* ($P=0.011$)	0.681* ($P<0.001$)	–
Intensity ratio per snapshot	IL4/IFNγ	TSLP/IFNγ	TGFβ/IFNγ	
IL4/IFNγ	–	0.745* ($P<0.001$)	0.707* ($P<0.001$)	
TSLP/IFNγ	0.745* ($P<0.001$)	–	0.688* ($P<0.001$)	
TGFβ/IFNγ	0.707* ($P<0.001$)	0.688* ($P<0.001$)	–	

IFNγ interferon γ, *IL4* interleukin 4, *TSLP* thymic stromal lymphopoietin, *TGFβ* transforming growth factor β, *DAPI* 4′,6-diamidino-2-phenylindole

*Statistical significant (Spearman's rank-order correlation)

Prognostic impact of p16, smoking status, and proinflammatory cytokines

First, we established the receiver-operating characteristic (ROC) curves of proinflammatory cytokines to predict the prognosis of OPSCC patients, and then the optimal cut-off points for the intensity of proinflammatory cytokines were determined based on the Youden's index as follows: IFNγ/DAPI = 0.0444, IL4/DAPI = 0.6113, TSLP/DAPI = 0.1343, TGFβ/DAPI = 0.0696, IL4/IFNγ = 21.705, TSLP/IFNγ = 4.755, and TGFβ/IFNγ = 2.845.

After obtaining the optimal cut-off points, Kaplan–Meier overall survival plots (Fig. 3) were then created according to the expression of p16, smoking status, and proinflammatory cytokines. The overall survival did not differ significantly between the p16-postive and p16-negative groups ($P = 0.162$) (Fig. 3a). However, there was still a trend of a better prognosis in the p16-postive group (3-year overall survival: 60%) than in the p16-negative group (3-year overall survival: 34.62%). Among the 34 smokers, no significant survival differences were noted between the p16-positive and p16-negative groups ($P = 0.589$) (Fig. 3b). Among the 20 p16-positive patients, the nonsmoker group displayed significantly better survival than the smoker group ($P = 0.022$) (Fig. 3c). These results showed that the good prognostic impact of positive p16 would be handicapped by smoking.

Regarding the proinflammatory cytokines, high expression of TSLP/DAPI, TGFβ/DAPI, IL4/IFNγ, TSLP/IFNγ, and TGFβ/IFNγ were significantly related to a worse overall survival (Fig. 3f–J). High expression of IFNγ/DAPI, although it did not influence the survival significantly, still showed a trend to be associated with a better prognosis (Fig. 3d). Likewise, high expression of IL4/DAPI was correlated with a worse prognosis, although not statistically significant (Fig. 3e). These results showed that high expression of Th2/Treg cytokines and low expression of Th1 cytokines would worsen the prognosis in OPSCC.

Taken together, p16-positive OPSCC showed weaker Th2/Treg predominant proinflammatory cytokine expressions and a relatively better prognosis than p16-negative OPSCC. p16-positive OPSCC smokers showed weaker Th2/Treg predominant proinflammatory cytokine expressions than p16-negative OPSCC smokers, but the prognoses of both groups were equally poor. p16-positive nonsmokers showed a significantly better prognosis than p16-positive smokers, but the immune responses of both groups were all weakly Th2/Treg predominant. These results indicated that, although the impact of smoking on immune deviation was weaker than that of HPV, the impact of smoking on prognosis was stronger than that of HPV in OPSCC.

TCGA OPSCC database analysis

A cohort of 70 OPSCC patients was found from 528 HNC patients in the publicly available TCGA database. HPV statuses of the OPSCC were identified in 37 patients. The RNA expression data of proinflammatory cytokines were shown in Supplementary Table 1. The gene expressions of IFNγ were significantly higher in the HPV-positive group than in the HPV-negative group. In contrast, the gene expressions of TGFβ were significantly higher in the HPV-negative group than in the HPV-positive group. For smokers alone, the gene

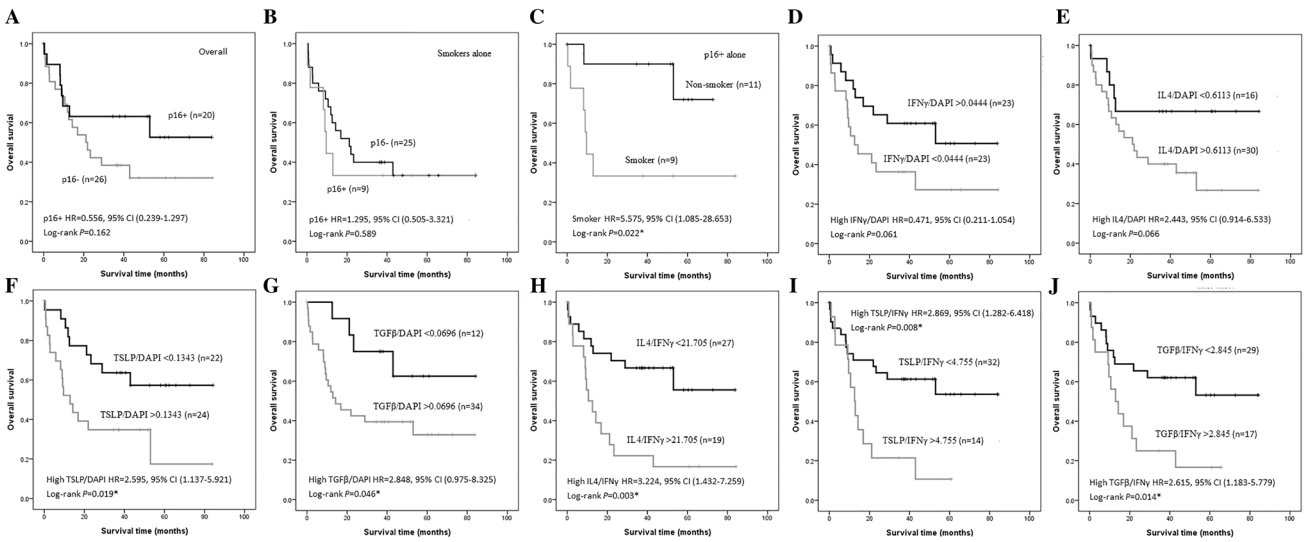

Fig. 3 Kaplan–Meier overall survival plots for different factors. **a** p16 in overall patients, **b** p16 in smokers alone, **c** smoking in p16-positive alone, **d** IFNγ/DAPI, **e** IL4/DAPI, **f** TSLP/DAPI, **g** TGFβ/DAPI, **h** IL4/IFNγ, **i** TSLP/IFNγ, **j** TGFβ/IFNγ. *IFNγ* interferon γ, *IL4* interleukin 4, *TSLP* thymic stromal lymphopoietin, *TGFβ* transforming growth factor β, *DAPI* 4′,6-diamidino-2-phenylindole; *statistically significant ($P < 0.05$)

expressions of IFNγ were still significantly higher in the HPV-positive group. The Kaplan–Meier overall survivals based on carcinogens and cytokine expressions are shown in Supplementary Fig. 1. Consistent with our IF staining data, high expression of TGFβ was significantly related to a worse overall prognosis.

Discussion

It is found that HPV-positive and HPV-negative OPSCC patients have various distinct clinical characteristics [22]. In previous studies, HPV-positive OPSCC patients comprised more nonsmokers and were associated with several advantageous prognostic factors, including younger age, better performance status, and smaller tumor sizes [22]. In our study, although we did not directly check the HPV status of our patients, due to p16 IHC, a reliable surrogate marker for overexpressed HPV 16 E7 oncoprotein, we could still identify the HPV status of OPSCC patients very precisely and inexpensively [10]. In our patient series, the p16-negative OPSCC group comprised significantly more smokers (96.15%) than the p16-positive group (45%), compatible with previous literature findings (HPV-negative OPSCC smokers: 73.6%; HPV-positive OPSCC smokers: 65.1%, $P < 0.001$) [22]. However, smoking may also interfere with the expressions of immune responses. Therefore, we further analyzed smokers alone and p16-positive patients alone and found that the effects of smoking and HPV on the proinflammatory cytokines and prognosis seemed to be opposite. In OPSCC, HPV may be associated with increased Th1, decreased Th2/Treg cytokines, and a good prognosis, while smoking may be associated with increased Th2, decreased Th1 cytokines, and a poor prognosis. Additionally, high expression of Th1 and low expression of Th2/Treg proinflammatory cytokines are related to a better prognosis in OPSCC. Similarly, The TCGA analysis also supports that HPV may be related to increased Th1 and decreased Treg cytokines, and supports that low expressions of Treg cytokines are related to a better prognosis in OPSCC. The significant findings of our data and TCGA database analysis were summarized in Supplementary Table 2.

Existing studies imply that smoking may suppress the innate host anti-microbial responses (mainly reduce Th1) and promote chronic inflammation (mainly increase Th2) in the upper airway [15]. In HNC, if a patient displays molecular smoking signatures, there will be less IFNγ expression and $CD8^+$ T-cell infiltration in the tumor microenvironment [23]. These findings support that smoking is associated with increased Th2 and decreased Th1 responses, which are compatible with our studies in OPSCC.

Increasing evidence indicates that, in OPSCC, HPV can serve as tumor-specific antigens to facilitate cytotoxic $CD8^+$ T-cell and $CD4^+$ T-cell infiltration and reduce regulatory T-cell accumulation [7, 21]. However, the role of HPV in the deviation of proinflammatory cytokines remains controversial. HPV may promote Th2/Treg proinflammatory cytokines in cervical cancer but facilitate the Th1 response in laryngeal cancer [17, 18]. In OPSCC, a recent study suggested that intratumoral HPV-specific T-cells may construct a Th1-oriented tumor microenvironment [7]. In our study, although HPV was also associated with increased Th1 and decreased Th2/Treg cytokines in OPSCC, a finding that was compatible with the above study, the final tumor microenvironment of p16-positive OPSCC was still weakly Th2/Treg predominant. One of the explanations may be related to the effect of cancer cells on immune responses. In most cases, cancer cells tend to promote Th2/Treg and reduce Th1 responses to facilitate tumor proliferation and metastasis [24, 25]. The effect of HPV to increase Th1 and decrease Th2/Treg responses in OPSCC may be overwhelmed by that of cancer cells plus smoking.

IL4 is a fundamental cytokine for Th2 differentiation [26]. In various cancers including thyroid cancer, colon cancer and leukemia, IL4 is reported to induce apoptosis resistance and to enhance tumor proliferation [27]. Although the main cellular source of IL4 has not been identified, there are several cells which are thought to have the abilities to produce IL4, including mast cells, basophils, and even cancer cells [26, 27]. It has been found that the thyroid, colon, prostate, breast, and bladder cancer cells can resist apoptosis through the elevated autocrine production of IL-4 [27]. The level of IL4 was immensely higher than other cytokines with a high standard deviation in our IF staining exams for OPSCC. However, in our TCGA analysis results, the gene expression of IL4 was extremely low in OPSCC specimens. In previous study, the expression level of IL-4 in HNC microenvironment seems to vary in a wide range. Two studies found that the level of IL4 was entirely low in the supernatants of HPV-positive OPSCC harboring HPV-specific T-cell cultures and in the supernatants of HPV-positive and HPV-negative HNC cell cultures [7, 19]. Nevertheless, one study showed that 5 of 8 HNC homogenates and 5 of 8 supernatants in primary HNC cell cultures contained significant expressions of IL-4 [28]. Another study showed that 4 of 8 HNC cell lines expressed significant level of IL-4 [29]. In the human protein atlas, it had been found that 3of 4 HNC pathology specimens showed moderate cytoplasmic staining of IL-4 [30]. Differences of detected IL-4 expression levels found by different research groups may be due to different kinetics, metabolism, and binding protein modulation in different HNC specimens [31]. Various detection tools and specimens from distinct regions (United States, Europe, Taiwan or others) may also influence the results of detected IL-4 levels. The major cellular sources of IL-4 in our study may be from the cancer cells and the innate immune cells in the OPSCC

microenvironment. Further investigation is required to clarify the situation in OPSCC.

A growing body of literature has shown that HPV-positive OPSCC patients have a better prognosis (5-year survival: 82%) than HPV-negative OPSCC patients (5-year survival: 35%) in the United States and Europe [6, 7]. However, in Taiwan, a previous study showed that the survival differences between the 2 groups was smaller (5-year overall survival: HPV-positive = 59.4%; HPV-negative = 31.2%) [32], which was compatible with our study (3-year overall survival: p16-positive = 60%; p16-negative = 34.62%). The reason behind this phenomenon remains unclear but may be related to the differences in the susceptibility to smoking exposure in different areas. Several studies have unveiled that smoking can increase the risk of oncologic failure and death for HPV-positive OPSCC patients [33], a finding that is compatible with our study results. p16-positive OPSCC smokers showed a significantly worse prognosis than p16-positive OPSCC nonsmokers, and all of the OPSCC smokers had the same poor prognosis, with or without p16 positivity. The prognostic impact of smoking may be stronger in Taiwan than in the United States and Europe, thus decreasing the survival advantages of HPV-positive OPSCC in Taiwan.

In our study, it was interesting to notice that, in p16-positive OPSCC, although smoking did not significantly change the deviation of immune responses, it significantly worsened the prognosis of p16-positive OPSCC. This suggested that, besides immune responses deviation, smoking could worsen the prognosis through other pathways, such as oxidative stress, free radical attack and direct DNA damage [15].

Our study possessed some limitations. First, this was a retrospective study with a small sample size; thus, it might not represent the whole population accurately. However, since there are seldom studies with large sample sizes regarding the cytokine expressions and their prognostic impacts in Asian OPSCC patients, our study have significant reference values for exploring the cancer immunities in unique habitats. Second, IF staining is a semiquantification method and may not be as precise as other quantification methods, such as enzyme-linked immunosorbent assay or polymerase chain reaction. Different storage conditions and durations of the FFPE specimens may also influence the final IF intensity. Nevertheless, by means of following the standard and optimized procedures of dewaxing, rehydration, antigen retrieval, permeabilization, blocking, double IF staining, and mounting, we can still obtain reliable and comparable cytokine expression levels with the method of IF staining. Third, the standard deviation of our data was high, likely obscuring the interpretations of the outcomes. Fourth, we only chose 4 proinflammatory cytokines (IFNγ, IL4, TSLP, and TGFβ), which may not thoroughly represent all proinflammatory cytokines.

In conclusion, higher Th1 and lower Th2/Treg proinflammatory cytokines are associated with a better prognosis for OPSCC. HPV may be related to increased Th1, decreased Th2/Treg responses, and a good prognosis, while smoking may be related to increased Th2, decreased Th1 responses, and a poor prognosis in OPSCC. The impact of smoking on immune deviation may be weaker than that of HPV, but the impact of smoking on prognosis may be stronger than that of HPV in OPSCC.

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Author contributions Conceived and designed the research: C-ML and Y-LY. Collected the data: C-ML and L-WL. Reviewed the pathologic slide: L-WL. Analyzed the data: C-ML, Y-WC and Y-LY. Wrote and edited the paper: C-ML, Y-WC and Y-LY. Whole correspondence: Y-LY.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval and ethical standards The Research Ethics Committee of the National Taiwan University hospital approved this study (NTUH IRB- 201711065RINC). All methods were performed in accordance with the Declaration of Helsinki.

Informed consent Written informed consent was waived by the ethics committee which approved this study.

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