Importance of FOXP3 in Prognosis and Its Relationship with p16 in Tonsillar Squamous Cell Carcinoma

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Abstract. *Aim: We examined the relationship between p16, a surrogate marker of human papilloma virus (HPV), and FOXP3, marker of regulatory T-cells, in tonsillar squamous cell carcinoma (TSCC), and assessed their prognostic potential. Materials and Methods: The tumors of 79 patients with locally advanced TSCC treated from 2000 to 2008 were subjected to immunohistochemistry (IHC) assays for p16 and FOXP3 using tissue microarrays. Results: Sixty-three tumors (80%) were p16-positive and 38 (48%) were FOXP3-positive on IHC. FOXP3 correlated positively with p16 (p=0.011). The p16-positive group had a significantly higher 5-year overall survival (OS) rate than the p16-negative group [78% vs. 63%, hazard ratio (HR)=0.347, p=0.025]. The FOXP3-positive group had a significantly higher 5-year OS rate than the -negative group (89% vs. 61%, HR=0.158, p=0.003). Multivariate analysis indicated that FOXP3 is an independent prognostic factor (HR=0.11, p=0.001) but p16 did not reach statistical significance (HR=2.17, p=0.131). Conclusion: FOXP3 expression is associated positively with p16 expression, and is a favorable prognostic factor for survival in TSCC.*

Human papillomavirus (HPV)-positive oropharyngeal squamous cell cancer (OPSCC) is currently considered a distinct tumor entity, in terms of its molecular biology, carcinogenesis, and prognosis (1-3). Tonsillar squamous cell carcinoma (TSCC) accounts for the largest proportion of OPSCCs (4), and the increase in its incidence explains why the incidence of OPSCC has also risen (5). TSCC has a higher incidence of HPV-positivity than other head and neck squamous cell carcinoma (HNSCC) subtypes, and the majority of HPV-positive OPSCCs develop in the tonsil (6, 7). HPV-positivity is associated with improved survival in TSCC (2, 3). The oncoproteins produced by HPV are non-self antigens and are thus expected to provoke an immune response. Therefore, it seems reasonable to postulate that the activation of immune surveillance mechanisms might indicate a favorable prognosis for HPV-associated TSCC.

Cancer immunology research has yielded several lines of evidence showing that a common feature of malignant diseases is an imbalance between type-1 and type-2 cytokines or T-cell subsets; this imbalance seems to be associated with the development and progress of cancer (8-11). Among $CD4^+$ T-cells, a subset of $CD4^+CD25^+$forkhead box p3 $(FOXP3)^+$ T-cells is to be an immunosuppressive T-cell subset capable of tolerizing the immune system and are therefore referred to as regulatory T-cells (Tregs). Given that Tregs are also essential for maintaining self-tolerance (12, 13), defects in them lead to severe autoimmune disease (14). This function counterbalances the detrimental effects of Tregs on tumor immune surveillance and antitumor immunity (12, 15). Tregs characteristically express CD25, cytotoxic T-lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor family-related gene, and FOXP3 (13). Experiments with *FOXP3*-overexpressing transgenic or *FOXP3* gene-depleted mice and other studies have shown that *FOXP3* is a master control gene that is required for the development and function of Tregs (16). Thus, the FOXP3 marker alone is thought to be suitable for detecting Tregs. In patients with several types of solid neoplasms, higher FOXP3 expression is associated with a higher risk of recurrence and poor overall survival (OS) (17-19). However, given that other studies have linked Tregs to a favorable prognosis (20-22), the role of FOXP3 is still contradictory, the prognostic potential of FOXP3 requires further investigation.

To address this issue, our present study examined the relationship between FOXP3 and p16, which is a surrogate marker of HPV status. We specifically investigated the prognostic potential of FOXP3 in patients with TSCC.

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Materials and Methods

Patients and study design. The medical records of patients with histologically-confirmed TSCC who were treated with curative intent at the Asan Medical Center between January, 2000, and December, 2008, were reviewed. This study was based only on patients with locally advanced disease who received definitive treatment. Cases where TSCC tissue samples were lacking were excluded, as were cases where the quality of the tissue was inadequate because of the small size of the material or because only frozen tissue was available. Comprehensive clinical, laboratory, and pathological data were collected and reviewed to optimize accuracy and completeness. All patients received curative treatment, which primarily involved surgery, definitive radiotherapy (RT), or chemoradiotherapy (CRT). All patients completed over 80% of the planned dosage. The treatment modality of each patient was chosen at a multidisciplinary team meeting. p16 immunohistochemistry (IHC) was used to assess the HPV status, and FOXP3 IHC was used to assess the presence of Tregs. This study was approved by the Institutional Review Board of Asan Medical Center.

Tissue microarrays. Formalin-fixed, paraffin-embedded tissue blocks of patients with TSCCs were retrieved from the archives of the Department of Pathology of the Asan Medical Center. All hematoxylin- and eosin-stained sections were reviewed to re-confirm the diagnosis and to select representative tumor areas. Tissue microarrays were manufactured for IHC by inserting punched cores from the donor blocks into recipient paraffin blocks. Duplicated 2-mm cores were arrayed to reduce sampling errors and to minimize tissue loss during processing.

Immunohistochemistry analysis of p16, FOXP3, and CD25 expression. Immunoperoxidase staining was performed on 4-μm tissue microarray sections by using a Benchmark autostainer (Ventana, Tucson, AR, USA) and an ultraView DAB detection kit (Ventana) according to the manufacturer's instructions. The following antibodies were used: anti-$p16^{INK4}$ (monoclonal, 1:10; Pharmingen, San Diego, CA, USA), anti-FOXP3 (polyclonal, 1:50; Abcam, Cambridge, UK), and anti-CD25 (monoclonal, 1:50; Novocastra, Newcastle-upon-Tyne, UK). $p16^{INK4}$ expression was deemed positive if there was strong and diffuse staining in the nuclei or cytoplasm in 70% or more of the tumor cells. Given the absence of widely-accepted standard cut-off points for FOXP3 and CD25 expression that define clinical outcomes, arbitrary cut-off points based on the numbers of positive cells were used. Thus, patients were classified into the FOXP3-positive group when more than 5% of tumor-infiltrating lymphocytes were positive for FOXP3; when fewer than 5% of the cells were positive, patients were classified into the FOXP3-negative group. For CD25, when more than one-third of the tumor-infiltrating lymphocytes were positive, the patients were classified into the CD25-positive group; when less than one-third, into the negative group.

Statistical analysis. Associations of the abundance of FOXP3 and p16 with clinicopathological features were assessed by the chi-square test, Fisher's exact test, and Student's *t*-test, as appropriate. The kappa statistic of the chi-square test was performed to ascertain agreement between FOXP3 and CD25 expression. The associations of FOXP3 and p16 with clinicopathological features were assessed by both univariate and multivariate binary logistic regression. Kaplan-Meier estimates were used to analyze the OS and progression-free survival (PFS). The C index, which was derived by extending the area under the receiver operating characteristic (ROC) curve, was applied to the discrimination ability in survival analysis. To identify clinical factors that were prognostic for OS, univariate and multivariate analyses were performed using stepwise Cox proportional hazards regression. All potential prognostic factors for which $p \leq 0.2$ on univariate analyses were entered into the multivariate Cox models. The final models were determined by backward elimination. All statistical analyses were two-sided, with $p<0.05$ being considered to indicate significance. Statistical analyses were performed using SPSS version 19.0 (SPSS Inc. Chicago, IL, USA).

Results

Baseline characteristics. Between January 2000 and December 2008, 142 patients were confirmed histologically to have TSCC, and they all received definitive treatment. Out of these, 22 cases were excluded because their disease was at an early stage (stage I or II). Another 41 patients were excluded because the TSCC tissue material was lacking or was inadequate. Ultimately, the tumors of 79 patients were evaluated by IHC for p16, FOXP3, and CD25 expression (Figure 1). The baseline characteristics of the 79 patients are presented in Table I. Their median age was 54 (range=32-76) years, males predominated (92%), and 80% of all patients had stage IV disease. Sixty-three patients (80%) were p16-positive and 38 (48%) were FOXP3-positive. The p16 status was significantly correlated with lower exposure to smoking and alcohol ($p<0.05$). FOXP3 was not associated with any of the clinicopathological features investigated, including history of smoking, and of excessive alcohol consumption.

Treatment and recurrence. Out of the 79 patients assessed, 71 (90%) underwent surgical treatment. The remaining patients received non-surgical treatments, namely RT alone or induction CT followed by RT. Neither p16 ($p=0.087$) nor FOXP3 ($p=0.414$) status were significantly associated with the primary treatment (Table I). After completing their primary treatment, 48 (61%) patients showed no evidence of disease, 27 (34%) experienced disease progression, and four (5%) were lost to follow-up. Out of the 27 patients with progression, 17 (63%) exhibited recurrences in the tonsil and neck (locoregional), three (11%) had recurrences at a distant site, and seven (26%) developed a second primary tumor. Neither the p16 nor FOXP3 status were found to be associated with particular recurrence types ($p=0.831$ and 0.698, respectively) nor with different salvage treatment ($p=0.413$ and 0.854, respectively).

Agreement between the FOXP3 and CD25 status, and correlations between the FOXP3, CD25, and the p16 status. Analysis of the agreement between the FOXP3 status and CD25 status revealed that 30 tumors (38%) were positive for both markers, 30 (38%) were negative for both markers, 12 (15%) were negative for FOXP3 but positive for CD25, and seven (9%) were positive for FOXP3 but negative for CD25. The concordance rate between the two methods was thus

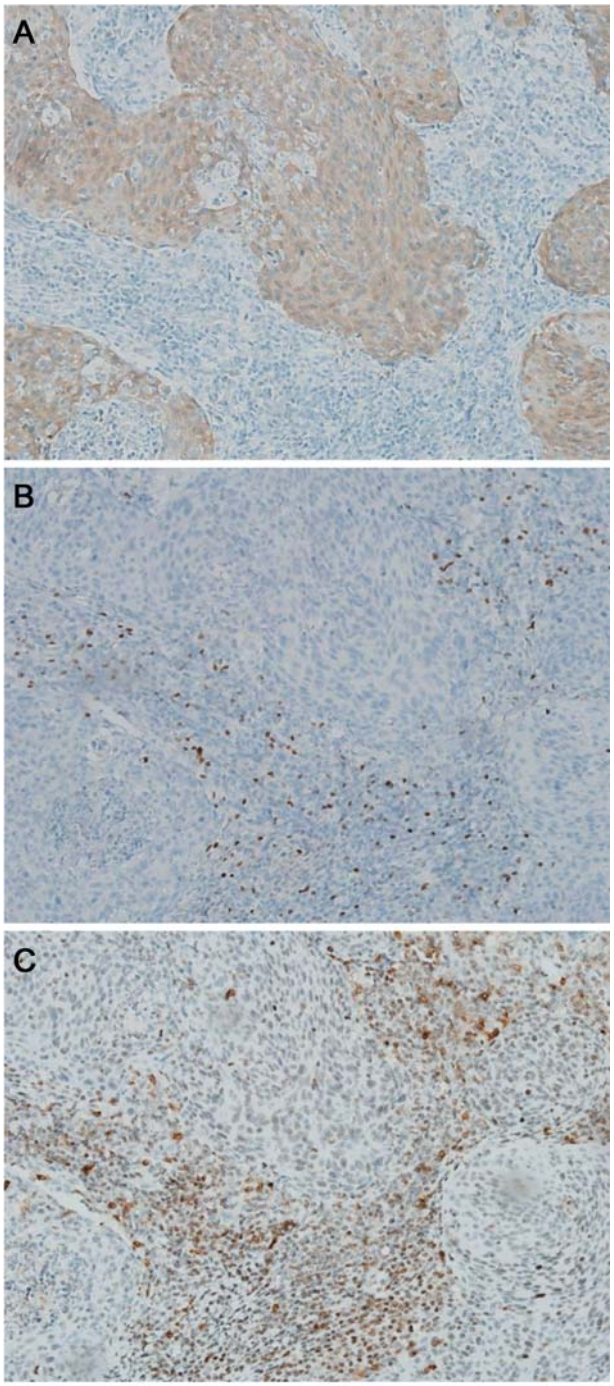

Figure 1. *Representative photographs of human papillomavirus (HPV)-positive tumor tissue. A: p16 immunohistochemical staining revealing strong nuclear and cytoplasmic staining. FOXP3 (B) and CD25 (C) expression in the nuclei of infiltrating lymphocytes between the tumor nests. Original magnification ×200.*

76% (60/79) and a positive association was recorded (kappa value=0.49). FOXP3 and CD25, which are markers of Tregs, were consistently detected in this study cohort.

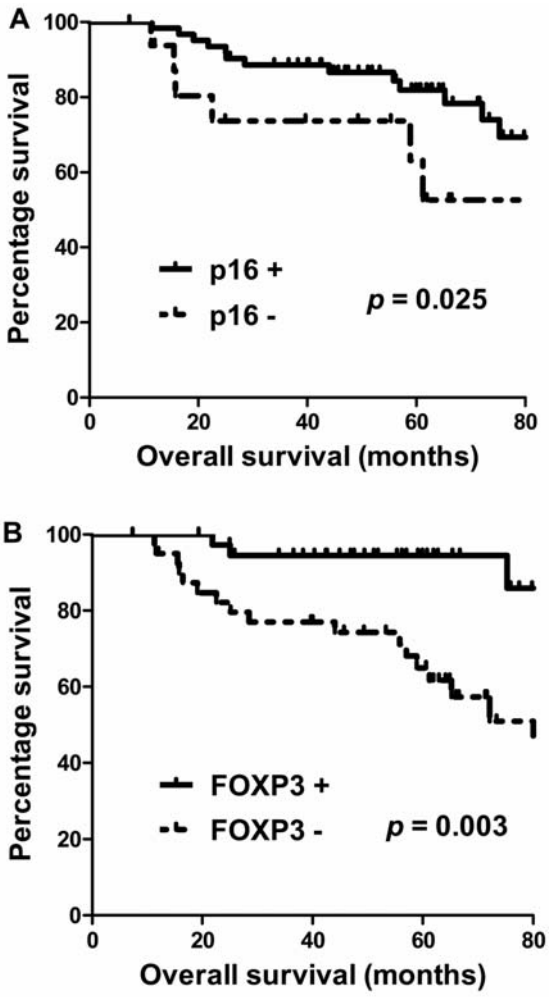

Figure 2. *Kaplan-Meier survival curves showing that the HPV status (A) and the FOXP3 status (B) is significantly associated with overall survival.*

With regard to the relationships between Tregs and HPV, the FOXP3 status positively correlated with the p16 status (p=0.011). CD25 tended to correlate with p16 (p=0.057). Univariate analysis using logistic regression indicated that the p16 status was the only clinicopathological feature associated with FOXP3 [odds ratio (OR)=5.42, p=0.014). This correlation became more significant after adjustments for age, sex, and tumor differentiation (OR=6.75, p=0.008).

Prognosis according to the p16 and FOXP3 status. The median duration of follow-up after the initial treatment was 62.9 months [95% confidence interval (CI)=59.15-66.65 months]. At the time of analysis, 29% (23/79) of the patients had died and 34% (27/79) had a disease that progressed. The median survival duration of patients overall was 93.9 (95% CI=66.0-121.1) months and the 5-year survival rate was 78%. The 5-year OS rate of the p16-positive group was significantly higher than that of the p16-negative group [78% *vs*. 63%; hazard ratio

Table I. *Associations of p16 and FOXP3 positivity with demographic and clinicopathological variables.*

Characteristic		p16			FOXP3		
	All (n=79)	+ 63 (80%)	– 16 (20%)	*p*-Value	+ 38 (48%)	– 41 (52%)	*p*-Value
Age, years							
Median (range)	54 (32-76)	53.2	56.0	0.361	52.6	57.1	0.317
<50	26 (26)	20 (32)	6 (36)	0.768	10 (26)	16 (39)	0.244
≥50	53 (67)	43 (68)	10 (64)		28 (74)	25 (61)	
Gender							
Male	73 (92.4)	57 (90)	16 (100)	0.338	36 (95)	37 (90)	0.676
Female	6 (7.6)	6 (10)	0 (0)		2 (5)	4 (10)	
Smoking							
<20 pack-years	35 (44.3)	32 (51)	3 (19)	0.026	16 (42)	19 (46)	0.821
≥20 pack-years	44 (55.7)	31 (49)	13 (81)		22 (58)	22 (54)	
Alcohol use[a]							
None	28 (35.4)	23 (31)	5 (31)	0.047	12 (32)	16 (39)	0.596
Moderate	40 (50.6)	35 (56)	6 (38)		22 (58)	19 (46)	
Severe	10 (12.7)	5 (8)	5 (31)		4 (10)	6 (15)	
Histological differentiation							
Well	8 (10.1)	5 (8)	3 (19)	0.299	3 (8)	5 (12)	0.257
Moderate	45 (57.0)	38 (60)	7 (44)		19 (50)	26 (63)	
Poor	26 (32.9)	20 (32)	6 (38)		16 (42)	10 (25)	
AJCC Stage							
III	16 (20)	13 (21)	3 (19)	1.000	8 (21)	8 (20)	1.000
IV	63 (80)	50 (79)	13 (81)		30 (79)	33 (80)	
Tumor stage							
T 1-3	63 (79.7)	50 (79)	13 (81)	1.000	30 (79)	33 (80)	1.000
T 4	16 (20.3)	13 (21)	3 (19)		8 (21)	8 (20)	
Nodal stage							
N 1-2a	23 (29.1)	18	5	1.000	12 (32)	11 (25)	0.805
N 2b-3	56 (70.9)	45	11		26 (68)	30 (75)	
Primary treatment							
Surgery alone	6 (8)	5 (8)	1 (6)	0.087	3 (8)	3 (5)	0.414
Surgery and adjuvant (C)RT	65 (82)	54 (86)	11 (69)		32 (87)	33 (80)	
Primary (C)RT	8 (10)	4(6)	4 (25)		2 (5)	6 (15)	

RT: Radiation therapy, (C)RT: chemo-radiation therapy. [a]Alcohol use scored on: 1) None, no history of alcohol use; 2) moderate, 14 or fewer drinks per weeks; and 3) severe, more than 15 drinks per weeks (a drink equivalent is one 12oz beer, one 6oz glass of wine, one 3oz Korean spirit, or one 1.5oz of liquor).

(HR)=2.88, p=0.025] (Figure 2A). The 5-year OS rate of the FOXP3-positive group was significantly higher than that of the FOXP3-negative group (89% *vs*. 61%; HR=0.158, p=0.003) (Figure 2B). The associations between OS and different p16 and FOXP3 status combinations were also assessed (Table II). Other than CD25, all factors were significantly associated with the OS, particularly FOXP3 alone (HR=6.34, p=0.003). Analyses of the C index revealed that the FOXP3 status alone was the best discriminator for OS (C index=0.683, 95% CI=0.592-0.775). Multivariate analyses revealed that the FOXP3 status was also an independent prognostic factor after adjustment for age and T stage (HR=7.47, p=0.001), whereas the p16 status did not have a significant impact on prognosis (HR=2.17, p=0.131) (Table III). Multivariate analyses were performed separately for FOXP3 status and p16 status because of the correlation between p16 and FOXP3 expression.

The median PFS was not reached, and the 5-year PFS was 64%. The p16-positive and FOXP3-positive groups tended to have a better PFS than the corresponding negative groups, although these differences did not achieve statistical significance (HPV, HR=1.75, p=0.206; and FOXP3, HR=1.72, p=0.169).

Discussion

The present study aimed to determine whether p16 and FOXP3 expression in TSCCs correlated and whether the FOXP3 status could serve as a prognostic factor for TSCC. The relationship between p16 and FOXP3 expression was significantly correlated (p=0.008). Moreover, FOXP3 positively correlated with a favorable survival outcome. This correlation was strengthened further after adjustments using multivariate analysis.

Table II. *Ability of FOXP3, CD25, and p16 positivity to discriminate between good and poor overall survival.*

Feature	Overall survival		C index (95% CI)
	HR (95%CI)	*p*-Value	
p16 (negative *vs*. positive)	2.88 (1.14-7.26)	0.025	0.594 (0.486-0.702)
FOXP3 (negative *vs*. positive)	6.34 (1.88-21.42)	0.003	0.683 (0.592-0.775)
CD25 (negative *vs*.positive)	1.79 (0.75-4.28)	0.189	0.573 (0.458-0.688)
FOXP3/p16 (negative *vs*. positive)[a]	3.88 (1.53-9.82)	0.004	0.612(0.505-0.719)
FOXP3/p16 (negative *vs*. positive)[b]	5.56 (1.65-18.77)	0.006	0.665 (0.574-0.757)

HR: Hazard ratio, CI confidence interval. [a]Positive if the result of one or both methods was positive, and negative if both methods yielded negative results. [b]Positive if the results of both methods were positive, and negative if one or both results were negative.

Table III. *Identification of prognostic factors for tonsillar squamous cell cancer.*

Feature	Overall survival	
	HR (95% CI)	*p*-Value
Univariate		
Age (≥50 years *vs*.<50 years)	2.55 (0.92-7.12)	0.073
Sex (male *vs*.female)	2.72 (0.36-20.3)	0.329
Smoking (≥20 pack-years *vs*. <20 pack-years)	1.08 (0.47-2.46)	0.861
Alcohol use (yes *vs*. none)	0.91 (0.39-2.10)	0.906
Tumor stage (T4 *vs*. T2-3)	1.84 (0.76-4.48)	0.179
Nodal stage (N2b-N3 *vs*. N0-N2a)	1.33 (0.49-3.61)	0.572
Differentiation (poor *vs*. mild-moderate)	0.52 (0.18-1.54)	0.247
Anemia (Hb<13 g/dl *vs*.Hb≥13 g/dl)	0.96 (0.22-4.16)	0.954
Treatment (non-surgery *vs*.surgery)	3.45 (1.25-9.53)	0.017
p16 (negative *vs*. positive)	2.88 (1.14-7.27)	0.025
FOXP3 (negative *vs*. positive)	6.34 (1.88-21.42)	0.003
Multivariate (including FOXP3)[a]		
Age (≥50 years *vs*.<50 years)	3.75 (1.27-11.01)	0.017
Treatment (non-surgery *vs*.surgery)	2.29 (0.81-6.43)	0.118
FOXP3 (negative *vs*. positive)	7.47 (2.17-27.75)	0.001
Multivariate (including HPV)[a]		
Age (≥ 50 years *vs*.<50 years)	2.41 (0.86-6.73)	0.093
Treatment (non-surgery *vs*. surgery)	2.39 (0.78-7.27)	0.127
p16 (negative *vs*. positive)	2.17 (0.79-5.96)	0.131

Hb: Hemoglobin. [a]Multivariate analyses were performed separately for FOXP3 status and p16 status because of the correlation between p16 and FOXP3 expression.

Immune responses are mounted to many antigens from various tumors (23). Inadequate responses to these tumor antigens is considered to be one of the causes of tumor progression and of poor prognosis (10). Tregs are potent inhibitors of antitumor immune responses (12), which may explain why higher FOXP3 expression is associated with a higher risk of recurrence and poor OS in patients with various types of solid neoplasms (17-19). If the favorable prognosis of HPV-associated TSCC results from HPV-mediated activation of immune surveillance, one would expect to see low levels of Tregs (as indicated by low levels of FOXP3 expression) in HPV-associated TSCC. However, our present findings revealed a positive association between HPV status (as measured by p16 IHC) and Tregs. Moreover, increased levels of Tregs correlated with a favorable survival outcome. Notably, although p16 IHC does not depict direct existence of HPV infection, it is widely known to be a good surrogate marker of HPV (24).

Similar to the many previous studies on numerous types of solid cancers (17-19), Nasman *et al*. recently reported that increased abundance of infiltrating $CD8^+$ T-cells and the $CD8^+$/FOXP3 cell ratio were associated with a favorable

prognosis in patients with TSCC (25). Similar to the design used for our present investigation, that specific study investigated Tregs within the cancer microenvironment. However, several other studies have also shown that increased levels of Tregs are associated with a favorable disease course and outcome. In follicular and Hodgkin's lymphoma, head and neck cancer, and colorectal cancer, high levels of Tregs are associated with longer disease-free survival and event-free survival (20-22). In particular, Badoual *et al*. showed that the level of tumor-infiltrating Tregs in head and neck cancer is positively-correlated with locoregional control (20). It was thus suggested that Tregs may promote locoregional control by down-regulating harmful inflammatory reactions (20, 26). However, given that Tregs have been associated with poor prognosis in numerous cases, including the only prior report on TSCC (25), possible reasons for the discrepancies between the different studies are further discussed below.

Firstly, the discrepancies might reflect heterogeneity of the phenotype of $FOXP3^+$ T-cells. To validate the identification of Tregs using FOXP3, we also measured CD25 expression *via* IHC and searched for a correlation between FOXP3 and CD25 expression. The characterization of 'suppressive' cells has been improving over the past 10 years, with the initial focus on CD25 expression and eventually on FOXP3 expression. At present, the majority of Tregs can be better identified as $CD4^+CD25^+CD127^{low/neg}FOXP3^+$ T-cells (13). FOXP3 on its own therefore cannot be regarded as an absolute functional marker of Tregs. For example, Helios was recently found to be a new marker of Tregs, and Kim *et al*. showed that the Tregs only have an immunosuppressive function when both FOXP3 and Helios are expressed (27). Similarly, in our present study, when we investigated CD25 and FOXP3 expression separately, we found a discordance between FOXP3 positivity and CD25 positivity in 24% of the cases. To address these issues, several surface markers should be measured simultaneously, using double or triple immunofluorescence staining.

The second possible reason for the differences in the findings of earlier studies relates to the unique immunological function of the tonsils. The main function of the tonsils is to discriminate between potentially harmless and harmful pathogens, thus promoting adaptive immunity or tolerance (7, 28). Tregs in the tonsils have been found to have a unique level of functional plasticity, given that they are believed to contribute not only to induction of immune tolerance but also to the development of an effective immune response. Siegmund *et al*. have shown that there is preferential accumulation or local differentiation of $CD8^+$ $FOXP3^+$ T-cells in tonsillar tissue, whereas such T-cells are rarely detected in the peripheral blood (28). $CD8^+$ $FOXP3^+$ T-cells can be converted by multiple cytokines into various types of T-helper cell, such as T-helper 17 cells, which are effector T-cells (29, 30). Thus, $FOXP3^+$ T-cells in the tonsils are not simple immunosuppressive effector cells, but have also other functions that could explain the apparent discrepancies in the roles played by these cells in various types of cancer.

A third possible reason for the different results is that we only measured the frequency of $FOXP3^+$T-cells in the lymphocyte infiltrate; the frequency of effector cells was not measured. It cannot be excluded that the frequency of effector T-cells increases with the abundance of the Tregs. To adjust for this phenomenon, Tregs numbers are often expressed relative to the number of effector T-cells, such as $CD4^+$ or $CD8^+$ T-cells, rather than to the number of tumor-infiltrating lymphocytes (18, 31). Moreover, some previous studies have reported that the pattern of Tregs localization correlates more strongly with the prognosis than with increased Tregs numbers (32, 33). In particular, Mizukami *et al*. showed that compared with a peritumoral distribution of Tregs, a diffuse intratumoral distribution predicts for short survival time (32). There was evidence in the present study that the $FOXP3^+$ T-cells had a peritumoral pattern (shown in Figure 1B). Thus, different patterns of Tregs localization might explain differences in the effects of these cells.

Our present findings demonstrate that Tregs are up-regulated in HPV-associated TSCC, although further research is needed to explore the underlying mechanisms. Moreover, FOXP3 was found to be related to a favorable prognosis in TSCC.

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