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Foxp3 overexpression in tumor cells predicts poor survival in oral squamous cell carcinoma

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Abstract

Background: Forkhead Box P3 (Foxp3) is a regulatory T cells marker, and its expression correlates with prognosis in a number of malignancies. The aim of this study is to determine the relationship of Foxp3 expression with clinicopathological parameters and prognosis in oral squamous cell carcinoma (OSCC).

Methods: Foxp3 expression was examined using immunohistochemistry (IHC) in paraffin-embedded tissue samples from 273 OSCC patients. Statistical analysis was performed to evaluate the associations between Foxp3 expression, the clinicopathologic characteristics and prognostic factors in OSCC.

Results: Foxp3 protein expression was significantly associated with lymph node metastasis ($P < 0.01$). Both univariate and multivariate analyses revealed that Foxp3 was an independent factor for both 5 years overall survival (OS) and relapse-free survival (RFS) (both $P < 0.01$). Patients with Foxp3 overexpression had shorter OS and RFS.

Conclusions: Our results determined that elevated Foxp3 protein expression was a predictive factor of outcome in OSCC and could act as a promising therapeutic target.

Keywords: Oral squamous cell carcinoma (OSCC), Foxp3, Five years overall survival, Relapse-Free Survival(RFS)

Background

Consisting 90 % of oral malignancies, oral squamous cell carcinoma (OSCC) is one of the leading causes of cancer-related death worldwide due to poor prognosis. The annual estimated occurrence of lip and oral cavity malignancies is around 400,000 cases [1]. Despite a rapidly growing number of treatment options, the 5-year survival rate of OSCC still remains under 60 %. Apart from classification of differentiation and TNM stage there are as yet hardly any predictive factors for prognosis in patients with OSCC. In this situation it is of utmost importance to identify potential molecular markers and targets which provide the necessary condition of accurate prediction of 5 years overall survival (OS) and relapse-free survival (RFS) [2].

Forkhead Box P3 (Foxp3) has played a key role in the immunosuppressive functions in Tregs and a marked molecule of Tregs as well [3]. The presence of Foxp3 expression in cancer cells has been proved to be associated with tumor biological behaviors regulation [4]. A series of studies have illustrated that the mutation or loss of Foxp3 might lead to a high rate of tumor formation, such as developing in mammary [5] or prostatic [6] epithelial tissues, which indicated Foxp3 as an oncosuppressive factor. Moreover, high tumor Foxp3 levels in tumor cells are also found to be related to better outcome of cancer patients of gastric cancer [7] and Her2-positive breast carcinoma [8]. In contrast, only two retrospective studies in patients with tongue squamous cell carcinoma (TSCC) [9] and oropharynx squamous cell carcinoma (OHSCC) [10] have demonstrated accordant results that high Foxp3 expression was associated with poor overall survival. Until now, no further studies have been conducted to examine the prognostic significance of Foxp3 expression in OSCC. In the present study, we investigated

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the tumor cell Foxp3 expression in a large 273 case OSCC cohort and determined the correlation of Foxp3 with clinicopathologic factors, relapse, and prognostic significance of OSCC.

Methods

Study population

Patients included in the study were 188 males and 85 females with OSCC treated at the Department of Oral-Maxillofacial Surgery, Stomatology Hospital Affiliated to Sun Yat-sen University between 2002 and 2009. Determination of TNM classification is on basis of the Union for International Cancer Control 2002 standard (UICC2002). The end point of this study is that of 5 year overall survival (OS) and relapse-free survival (RFS). Pathological examination was performed by two independent pathologist according to the 2005 revised World Health Organization classification of tumors.

Tissue microarrays and IHC testing of patient samples

Tissue microarrays (TMAs) were constructed for 273 patients cohorts using three 2 mm tissue cores collected from areas of OSCC epithelium on each tissue block. Sections were cut into 4 μ m thickness, deparaffinized, rehydrated and treated with a peroxidase block. Foxp3 staining procedures was based on manufacturers protocol. Heat-based antigen retrieval was carried out by microwave treatment in 10 mM citrate buffer (pH 6.0). The sections were incubated with mouse monoclonal anti-human Foxp3 (clone AO1042a) diluted

1:200 (Abgent, USA) at 4 °C overnight, followed by incubation with ready to use EnVision HRP-IgG secondary antibody (DAKO, Denmark) for 30 min. Staining was developed using 3, 3'-diaminobenzidine (DAB) as a chromogen substrate. The nuclei were counterstained with Mayer's hematoxylin.

Predominantly cytoplasmic staining was observed in tumor cells. The immunoreactive score (IRS) was calculated as intensity of the staining reaction multiplied by the percentage of positive cells. Based on the IRS values, Foxp3 were scored as low, medium and high [10]. Representative micrographs indicating that medium and high levels of Foxp3 were found only in malignant tissues, while healthy epithelial tissues displayed only low levels of Foxp3. Consequently according to the HLA & Cancer Component of the 12th IHW [11], the results were confirmed by two experienced pathologists who were blinded to the clinicopathologic data of the patients. The whole sections were repeatedly operated in the condition that tissue microarray staining was not typical represented. Negative controls which were treated without primary antibodies were implemented during each experimentation.

Statistical analysis

The OS rate was the primary endpoint of this study, and the secondary endpoint was the relapse-free survival (RFS) of the OSCC patients. OS was defined as the duration from the date of each patient's hospitalization to the date of death from any cause or to the censoring of the patient

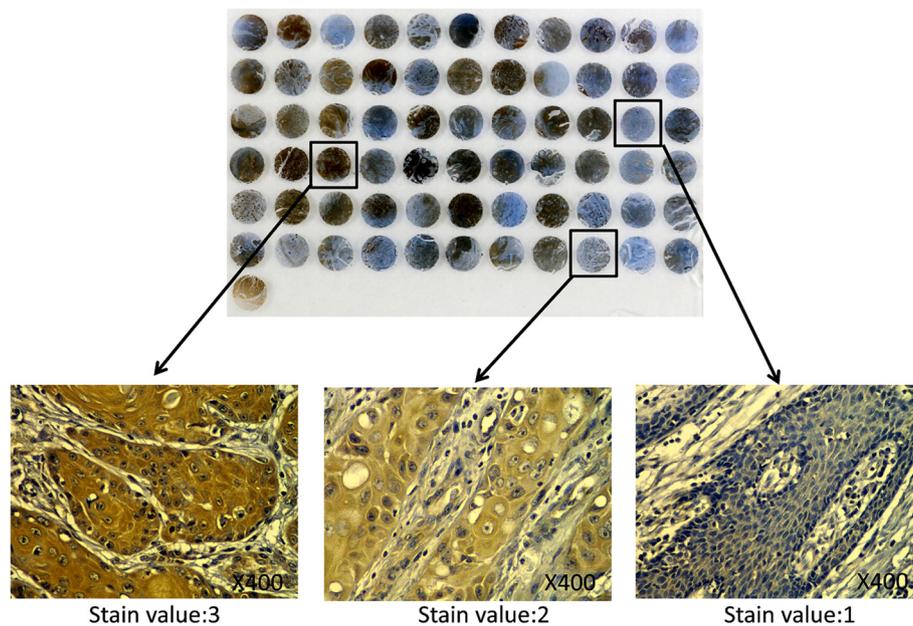


Fig. 1 Expression and scoring of Foxp3 in oral squamous cell carcinoma (OSCC) tissue. Representative micrographs from tissue microarray (TMA) cores indicating the low, medium and high cytoplasmic expression in tumor nest

Table 1 Correlation between the Foxp3 expression and clinicopathological characteristics of OSCC

Clinical pathological feature	N	Expression of Foxp3			P Value
		Stain value = 1 N = 99	Stain value = 2 N = 106	Stain value = 3 N = 68	
Age					0.65
<60	133	51 (38.3 %)	49 (36.9 %)	33 (24.8 %)	
≥60	140	48 (34.3 %)	57 (40.7 %)	35 (25.0 %)	
Sex					0.29
Male	188	63 (33.5 %)	74 (39.4 %)	51 (27.1 %)	
Female	85	36 (42.4 %)	32 (37.6 %)	17 (20.0 %)	
Smoking					0.84
Yes	132	47 (35.6 %)	50 (37.9 %)	35 (26.5 %)	
No	141	52 (36.9 %)	56 (39.7 %)	33 (23.4 %)	
Drinking					0.47
Yes	131	45 (34.4 %)	49 (37.4 %)	37 (28.2 %)	
No	142	54 (38.0 %)	57 (40.1 %)	31 (21.9 %)	
Differentiation					0.30
High	200	73 (36.5 %)	82 (41.0 %)	45 (22.5 %)	
Medium	64	23 (35.9 %)	22 (34.4 %)	19 (29.7 %)	
Low	9	3 (33.3 %)	2 (22.2 %)	4 (44.5 %)	
Tumor site					0.23
Gingiva	52	16 (30.8 %)	19 (36.5 %)	17 (32.7 %)	
Tongue	131	50 (38.1 %)	47 (35.9 %)	34 (26.0 %)	
Buccal	61	26 (42.6 %)	24 (39.4 %)	11 (18.0 %)	
Others ^a	29	7 (24.1 %)	16 (55.2 %)	6 (20.7 %)	
T stage					0.63
T1-T2	186	68 (36.5 %)	74 (39.8 %)	44 (23.7 %)	
T3-T4	87	31 (35.6 %)	32 (36.8 %)	24 (27.6 %)	
N stage					0.000***
N0	183	73 (39.9 %)	82 (44.8 %)	28 (15.3 %)	
N1-N3	90	26 (28.9 %)	24 (26.7 %)	40 (44.4 %)	
Clinical Stage					0.09
I-II	142	55 (38.8 %)	59 (41.5 %)	28 (19.7 %)	
III-IV	131	44 (33.6 %)	47 (35.9 %)	40 (30.5 %)	

Stain value = 1, low level of Foxp3 expression; Stain value = 2, medium level of Foxp3 expression; Stain value = 3, high levels of Foxp3 expression

^aOthers included include hard palate, oropharynx and lips

P value was determined using the Linear-by-Linear Association test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

at the date of the last follow-up. RFS was defined as the time from hospitalization to local, regional, distant failure, or other second primary cancer. All statistical analyses were conducted using SPSS 22.0 statistical software. The relationships between Foxp3 expression and the clinicopathological characteristics were using Linear-by-Linear Association test (Age, Differentiation, T stage, N stage, and Clinical stage), and Pearson's chi-square test (Gender, Smoking, Drinking, and Tumor site). Patient survival was evaluated using the Kaplan-Meier method and compared using Log-Rank test. Univariate and multivariate Cox

regression analyses were performed to analyze the survival data. Multiple comparison (Wilcoxon Rank Sum test) was performed to analyze the mean OSCC survival time of Foxp3 staining value. P -value < 0.05 was considered statistically significant.

Results

Association of Foxp3 with lymph node metastasis and other clinicopathological variables

In a cohort of 273 patients with OSCC, Foxp3 expression was determined to be low in 99 (36.3 %), medium in 106

Table 2 Univariate Analyses of Selected Characteristics with 5 years Overall Survival (OS) and Relapse-free Survival (RFS) among Patients with Oral Squamous Cell Carcinoma (N = 273)

Characteristics	5-years OS rate (95 % CI)	P Value	5-year RFS rate (95 % CI)	P Value
T stage				
T1-T2	0.415 (0.256–0.574)	0.212	0.652 (0.540–0.764)	0.034*
T3-T4	0.252 (–0.101–0.605)		0.713 (0.448–0.978)	
N stage				
N0	0.470 (0.290–0.650)	0.000***	0.671 (0.548–0.794)	0.584
N1-N3	0.252 (–0.101–0.605)		0.733 (0.623–0.843)	
Clinical stage				
I-II	0.437 (0.249–0.625)	0.012*	0.627 (0.502–0.752)	0.043*
III-IV	0.303 (0.052–0.554)		0.727 (0.527–0.927)	
Foxp3 staining value				
1	0.474 (0.268–0.680)	0.000***	0.701 (0.562–0.840)	0.003**
2	0.611 (0.499–0.723)		0.795 (0.711–0.879)	
3	0.162 (0.074–0.250)		0.330 (0.133–0.793)	

Abbreviations: 95 % CI 95 % confidence interval

P value was determined using the Log-rank test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

(38.8 %) and high in 68 (24.9 %) patients (Fig. 1). The median age of patients at diagnosis was 60 (range 24–85) and was followed up for an average of 3.58 years. Our analyses showed that the level of Foxp3 in OSCC was significantly correlated with lymph node metastasis ($P < 0.001$), but was not associated with age, gender, smoking, drinking, grade of differentiation, tumor site, T stage, Clinical stage ($P > 0.05$) (Table 1). Notably, the correlation of Foxp3 with prominent lymph node metastasis positivity suggested a potential role of Foxp3 in increased invasion and metastasis of OSCC.

Relationship between Foxp3 expression and prognosis in OSCC patients

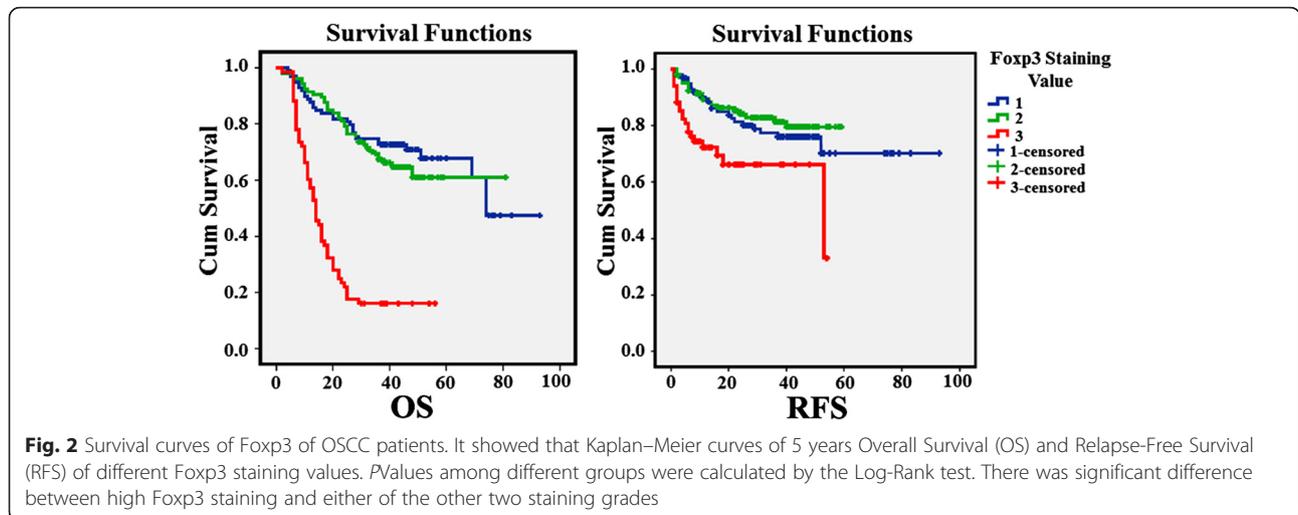
To determine whether Foxp3 expression might be a prognostic predictor in OSCC, we examined Foxp3 expression levels and the clinical follow-up information in all 273 patients of OSCC by Kaplan-Meier analysis and Log-Rank test. 126 patients died during the follow-up period, whereas 147 patients were still alive at the end of follow-up. The crude and adjusted relative risks of all-cause mortality in these 273 patients were assessed by univariate and multivariate analyses. As univariate

Table 3 Multivariate COX Regression analysis on factors for OSCC survival

Characteristics	5-years OS rate		5-year RFS rate	
	HR (95 % CI)	P Value	HR (95 % CI)	P Value
T Stage				
T1 or T2	0.547 (0.331–0.904)	0.019*	1.188 (0.483–2.923)	0.707
T3 or T4	Reference		Reference	
N Stage				
N0	0.238 (0.143–0.398)	0.000***	0.614 (0.280–1.347)	0.223
N1-N3	Reference		Reference	
Clinical Stage				
I-II	2.503 (1.348–4.649)	0.004**	2.249 (0.865–5.852)	0.097
III-IV	Reference		Reference	
Foxp3 Staining Value				
1	0.197 (0.123–0.315)	0.000***	0.451 (0.239–0.849)	0.014*
2	0.271 (0.174–0.422)	0.000***	0.371 (0.192–0.714)	0.003**
3	Reference		Reference	

Abbreviations: OS overall survival, RFS, relapse free survival, HR hazard ratio, 95 % CI 95 % confidence interval

P value was determined using Cox proportional-hazards model. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$



analyses shown in Table 1, the clinicopathological parameters such as N stage, Clinical stage and Foxp3 expression were related to 5-year OS rate, whereas T stage, Clinical stage and Foxp3 expression were related to 5-year RFS rate. Multivariate analyses were used to reveal that T stage, N stage, Clinical stage and Foxp3 expression were the independent risk factors for OS, and Foxp3 expression were the most important risk factor for relapse (Tables 2 and 3; Fig. 2).

The results showed that the median overall survival time in patients with high levels of Foxp3 expression (stain value = 3) (14, 2–56 months, *n* = 68) was significantly shorter than that in patients with a low level of Foxp3 expression (stain value = 1) (43, 4–93 months, *n* = 99) (*P* = 0.000) and medium level of Foxp3 expression (stain value = 2) (38, 2–81 months, *n* = 106) (*P* = 0.000). Furthermore, the median relapse-free survival time was markedly longer in the low Foxp3 expression group (39, 2–93 months, *n* = 99) (*P* = 0.000) and medium Foxp3 expression group (36, 2–59 months, *n* = 106) (*P* = 0.000) compared to that in the high Foxp3 expression group (11, 1–54 months, *n* = 68). These results indicated that patients with high levels of Foxp3 expression have a worse prognosis than those with low levels of Foxp3 expression (Table 4). The hazard ratio of 5 years OS for low and

medium Foxp3 expression group are 0.197 (*P* = 0.000) and 0.271 (*P* = 0.000), respectively, while that of relapse-free survival (RFS) for low and medium Foxp3 expression group are 0.451 (*P* = 0.014) and 0.371 (*P* = 0.003) taking high Foxp3 expression group as reference (Table 3).

Discussion

In the present study, the expression of Foxp3 was investigated in 273 OSCC tissues by immunohistochemistry. We found that Foxp3 expression was significantly associated with lymph node metastasis and clinical outcome in OSCC. Considering these findings, we suggest that Foxp3 is a potential novel marker for prognosis and represents a therapeutic target for the treatment of OSCC patients.

Continuous effort has been made to identify molecular biomarkers that could provide accurate information regarding OSCC prognosis. While the potential role of tumor Foxp3 as prognostic biomarker of overall survival has been previously investigated, only two studies addressed this issue and draw accordant conclusion that high Foxp3 expression was associated with poor overall survival in patients with TSCC and OHSCC respectively [9, 10]. But the relationship with RFS is not clear. And no association was found with lymph node metastasis.

Table 4 Multiple comparison with the median OSCC survival time of Foxp3 staining value

Foxp3 Staining Value	OS		RFS	
	median time (range) (months)	<i>P</i> Value	Mean time (range) (months)	<i>P</i> Value
1 (<i>n</i> = 99)	43 (4–93)	0.000***	39 (2–93)	0.000***
2 (<i>n</i> = 106)	38 (2–81)	0.000***	36 (2–59)	0.000***
3 (<i>n</i> = 68)	14 (2–56)		11 (1–54)	
	Reference		Reference	

Abbreviations: OS overall survival, RFS relapse free survival
P value was determined using Wilcoxon Rank Sum test. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001

Takenaka et al. observed that tumor cytoplasm Foxp3 expression was associated with worse relapse-free survival in breast cancer [12]. In small cell lung cancer, relapse-free survival in patients with Foxp3-positive tumor was better with earlier follow-up [13]. Whether Foxp3-positive cancer cells are relevant to recurrence is controversial. For all the head and neck squamous cell carcinoma (HNSCC) types, the relationship of Foxp3 expression with RFS is not clear. This is the first study to describe the association of Foxp3 expression with both five years overall survival (OS) and relapse-free survival (RFS) in OSCC patients. It helps to define the possible link between the biological function of Foxp3 and the progression of OSCC.

Moreover, we provided evidence that Foxp3 expressed by OSCC cells might play a role in the metastasis of OSCC. We found that OSCC patients with high tumor Foxp3 expression had significantly shorter survival time and more lymph nodes involvement than other groups. Although no association was found with lymph node metastasis in the previous TSCC and OHSCC cohort studies, there is still considerable amount of evidence available which indicates Foxp3 expression in breast cancer [14], gastric cancer [6], non-small cell lung cancer [15] and esophageal squamous cell carcinoma [16] is correlated with tumor metastasis. The molecular mechanism of Foxp3 about metastasis remained unclear. One study demonstrated that Foxp3 inhibited breast cancer cell adhesion, invasion and metastasis, while study on the molecular mechanism revealed that Foxp3 inhibited breast cancer metastasis by down-regulating CD44 expression directly [17]. Further studies are required to elucidate its mechanism in OSCC.

Conclusions

In summary, this study demonstrated that Foxp3 expressed in OSCC cells were significantly correlated with worse prognosis of both overall survival and relapse-free survival in OSCC. Ultimately, these findings provided evidence that Foxp3 expressed by OSCC cells might play a role in the invasion of OSCC, and tumoral Foxp3 might be suitable as a prognostic marker in OSCC. More studies are needed to further explore how tumor cells regulating the metastasis behavior during this interaction, especially in light of the current anticancer efforts to interfere with Foxp3 expression.

Abbreviations

Foxp3, Forkhead Box P3; HNSCC, head and neck squamous cell carcinoma; IHC, immunohistochemistry; IRS, immunoreactive score; OHSCC, oropharynx squamous cell carcinoma; OS, overall survival; OSCC, oral squamous cell carcinoma; RFS, relapse-free survival; TMAs, tissue microarrays; TSCC, tongue squamous cell carcinoma

Acknowledgements

We sincerely thank Dr Vivian Lui (HongKong University) for thoughtful editing of this manuscript. We sincerely thank the patients for their participation in this study.

Funding

This project was supported by grants from National Natural Science Foundations of China (No. 81272954, 81472524). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Availability of data and materials

Data supporting the findings are included within the manuscript.

Authors' contributions

ZW and BC conceived of the study, and participated in its design and coordination and helped to draft the manuscript. JJS and SJZ carried out the IHC experiments and drafted the manuscript. JF and XQL performed the statistical analysis. DM, XBC and YW participated in the pathological evaluation and helped to draft the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interest.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Ethical approval was obtained from the Scientific and Ethical Committee of Sun Yat-sen University, China (ERC-2013-11). Written informed consent was obtained from all patients. The data were analyzed anonymously.

Endnotes

Not applicable.

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Received: 10 December 2015 Accepted: 27 June 2016

Published online: 26 July 2016

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