

Abstract

Over 50% of patients diagnosed with oral cavity squamous cell carcinoma (OSCC) develop recurrent or metastatic disease following multimodality treatment. The immune cell milieu is a significant component of the stromal niche in cancers and instrumental in governing tumor cell behavior during cancer progression and treatment. Anti-PD1 checkpoint inhibitor immunotherapy is an option for recurrent/metastatic OSCC; however, clinical response is only about 20%. The challenges in checkpoint inhibitor therapy are primarily the overlapping and synergistic immune escape pathways that tumor cells utilize to survive immune surveillance and therapy, resulting in tumor progression. It is believed that immunosuppression elicited by the tumor, secondary to microenvironmental changes, plays a significant role in disease progression and clinical outcomes. Profiling cells in OSCC for pro-tumorigenic immune cells (Tregs, immunosuppressive monocytes) and for cells that can facilitate improved immune response to tumors, is essential to evaluate the feasibility of anti-cancer immunotherapy. Interplay among CD4+ and CD8+ cytotoxic T cells, transcription factor FoxP3, and other primary immune cell types dictates the balance between tumor progression and immune response. In OSCC, CD8+ cells are associated with low metastatic incidence and better survival. The presence of intra-tumoral CD8+ T cells suggest that an anti-tumor immune response may have initiated but been suppressed via PD-1:PD-L1/L2 checkpoint. Therefore, such patients may be responsive to anti-PD-1 immunotherapy. However, the prevalence and activity of regulatory T cells in patients with several types of solid neoplasms, including OSCC, correlates with poor prognosis. Forkhead box protein-3 (*FoxP3*) is a master transcription factor required for Treg development and function. Higher FoxP3 expression is associated with a greater risk of recurrence and poor overall survival. We previously established a large panel of patient samples representing the progression from oral dysplasia to carcinoma and characterized CD4 and CD8 T cell infiltration. We hypothesized that the infiltrating CD4+ population was primarily Treg that could suppress an effective anti-tumor response. Immune marker profiling (FoxP3, CD4, CD25, CD8, PD1, PD-L1) was carried out on patient samples (n=275) with tissue histology ranging from normal to mild dysplasia, moderate dysplasia, severe dysplasia and carcinoma. Immunohistochemistry scoring showed strong correlation between disease grade and immunological marker staining levels for each of these markers. Furthermore, many of the immune markers correlated with each other suggesting increasing immune complexity with OSCC cancer progression. The correlated staining of CD25 and CD4 (Pearson Correlation coefficient $p=0.961$, $p<0.001$) suggested the presence of Tregs. Direct staining for FoxP3 showed tight correlations with CD4 and CD25 ($p=0.690$ & 0.705 , $p<0.005$) validating the presence of Treg in the OSCC microenvironment. Additionally, Treg presence correlated with disease progression ($p=0.55$, $p<0.001$) further supporting our thesis that suppressive immune components accumulate in the tumor during progression and facilitate immune evasion. The correlation between FoxP3 expression and overall survival was -0.262 ($p=0.043$) which is statistically significant for a negative association between FoxP3 staining and patient survival. Kaplan-Meier analysis demonstrated a survival advantage for patients with low FoxP3 expression ($p=0.022$). Targeting FoxP3 is a potential strategy to improve OSCC immunotherapy outcomes.

Introduction

Regulatory T cells (T reg) were initially characterized as CD4+ T cells with high levels of CD25. Subsequently, the transcription factor FOXP3 was identified as a master regulator of T reg generation and maintenance. While the normal role of T regs is to prevent autoimmunity, these mechanisms can also interfere with anti-tumor responses and contribute to tumor immune evasion. T regs influence immune function through both cell-cell interactions and release of immunosuppressive cytokines. To determine the abundance of T regs in OSCC, we stained oral premalignant and malignant tissue sections individually with CD4, CD25 and FoxP3 antibodies. After characterizing T regs in the tumor microenvironment we found their presence correlates with OSCC grade and may have prognostic value.

Methods

Samples were obtained from patients with histologically confirmed oral potentially malignant disorders (OPMD) and oral cancer presented to the Department of Head and Neck Surgery, Roswell Park Cancer Institute, New York, USA (2006 to 2013). Patient demographics, outcome and survival data were provided with de-identified samples, along with answers to a health questionnaire which addressed tobacco and alcohol usage. All samples (n=270) in the selection came from the oral cavity. Samples were selected based on their clinical pathology assessment, then independently reviewed and scored by a pathologist. Samples were stratified into risk groups as well as pathology assessment. Pathology assessment was given an ordinal score between 1-6. Sections were classified as 1 for normal tissues, 2 for parakeratosis, 3 for mild dysplasia, 4 for moderate dysplasia, 5 for severe dysplasia and 6 for all stages of cancer (including carcinoma in situ). For survival analyses, each patient's pathology designation was based on the sample with the most severe pathology. Standard Immunohistochemistry (IHC) method were performed by deparaffinization, rehydration and antigen retrieval followed by incubation with the mouse monoclonal anti-FOXP3 (Abcam, 236A/E7, ab20034) diluted to 10ug/ml primary antibody overnight at 4°C in a humidified chamber. Sections were then developed with Mouse and Rabbit Specific HRP/DAB (ABC) secondary antibody detection kit (Abcam - ab64264) following the manufacturer's protocol, counterstained using Mayer's-Hematoxylin, and mounted using DPX mountant. All slides were scanned (Aperio Scanscope XT 1509, AT2, Leica biosystems, IL, USA) at 20x magnification. Quantification of FoxP3 stained cells was analyzed by two independent reviewers blind to the sample's pathology. Each image from the Aperio scanscope (20x magnification) was raised to 20x in imagescope analysis software (Leica biosystems, IL, USA) resulting in a total magnification of 400x measured as one high power field (hpf)(20x X 20x). The number of positive FoxP3 stained cells (brown) was then counted throughout the hpf. Scores from all hpf were combined and an average value of FoxP3+ cells per hpf was used as the final score for each section. The scores from both reviewers were then averaged, and samples with large differences between scores were reviewed and re-scored together. All data were entered in MS Excel and IBM SPSS Statistics v26 where analyses included standard frequency and descriptive assessments as well as bivariate correlation analysis and multivariate analysis. The expression of FoxP3 was also correlated with CD4 and CD25 expression in the same individuals. Depending on the nature of the variables, both Pearson (scale) and Spearman (ordinal) correlations were analyzed. Significance was determined with Chi-square test (2-tailed sigma <0.05). Survival analysis was carried out on a limited patient set for which overall survival time was available. Survival analysis used the Kaplan-Meier algorithm with significance determined by Mantel-Cox Log Rank test $p<0.05$. Power analyses were carried out in G*Power 3.1.

Results

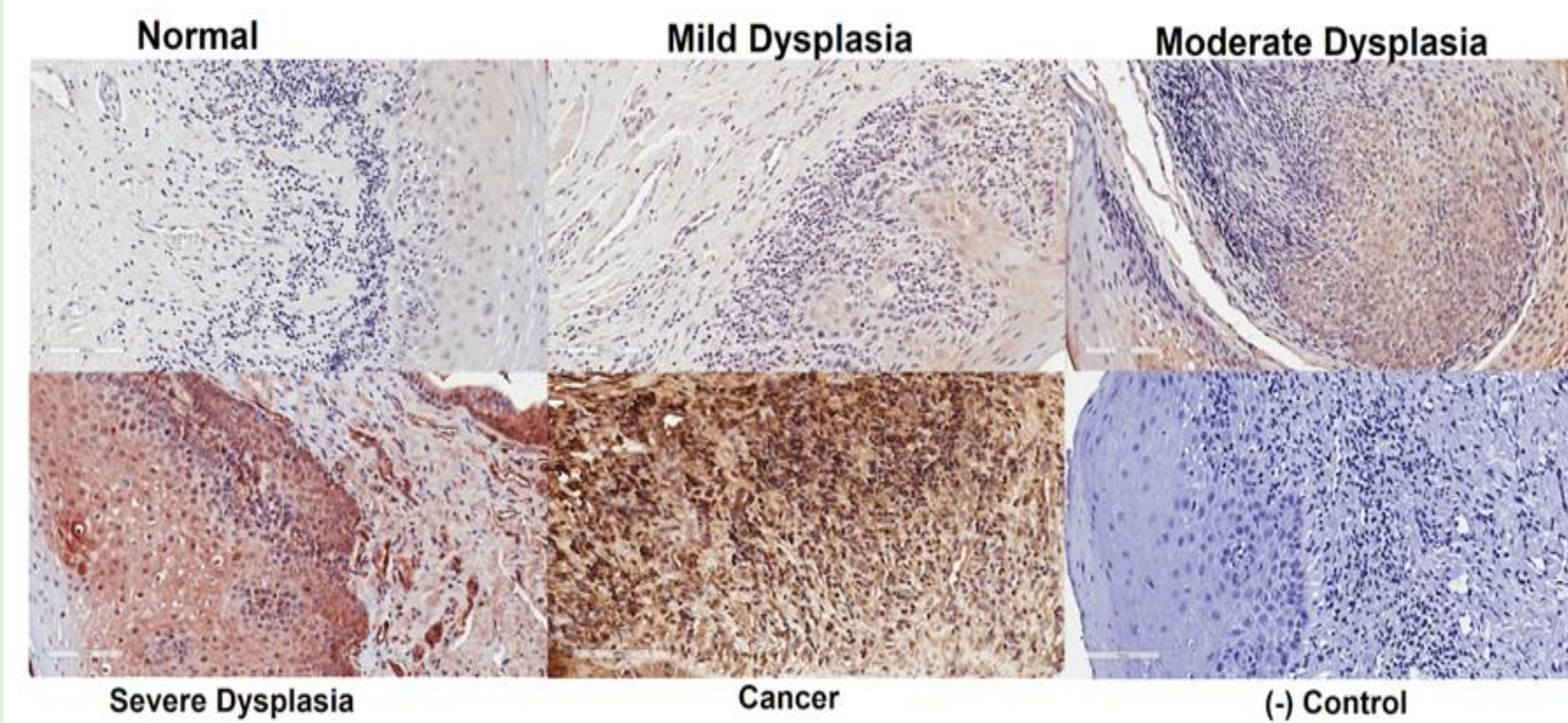


Figure 1: FOXP3 detection in OPMD samples and association with advanced pathology. Oral cancer patient biopsy FFPE sections were stained for FOXP3 expression (brown) and counterstained with Hematoxylin

FOXP3 staining intensity increased along the spectrum of oral cavity dysplasia and carcinoma (**Figure 1**). Independently, we stained the same samples for CD4 and CD25 and demonstrated correlations between each of these markers and the FOXP3 staining (**Table 1**), suggesting that our visualized FoxP3+ cells are T regs and indicating increasing presence of T regs over the progression of dysplastic oral cavity tissues to OSCC. The mean number of positively stained Treg per high power field correlated with increasing dysplasia – Spearman's correlation coefficient 0.53, $p<0.01$ (**Figure 2**). In a limited set of patients whose outcomes were available, overall survival was negatively correlated with FOXP3 staining intensity ($p=-0.262$, $p=0.043$). Kaplan-Meier survival analysis distinguished survival in high and low FoxP3 cohorts, but statistical significance was not reached. In the log rank chi square test, the difference in survival between low and high FoxP3 cohorts was found to be 2.958 ($p=0.085$) (**Figure 3**).

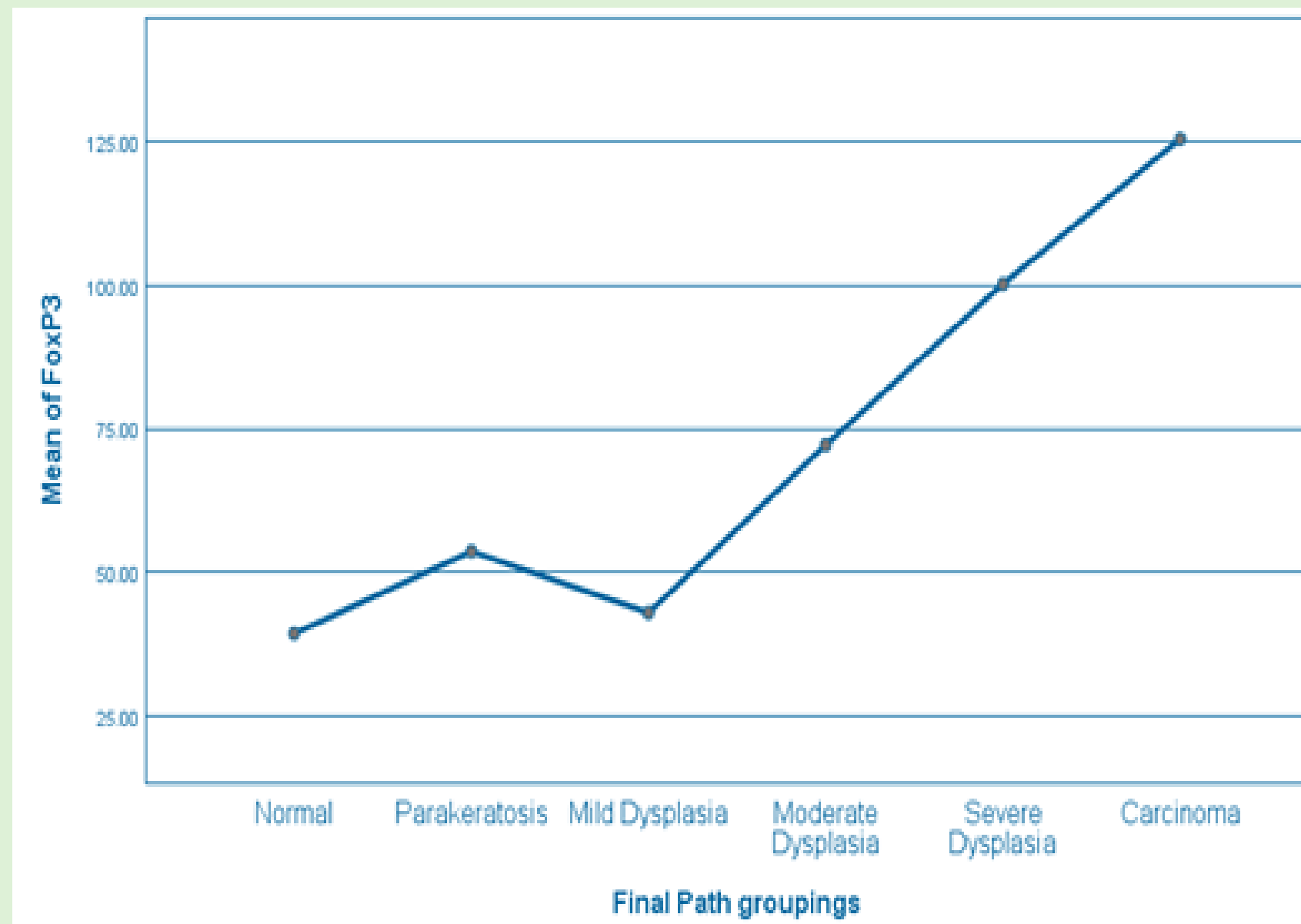


Figure 2: FOXP3 Expression in patient cohorts

Results

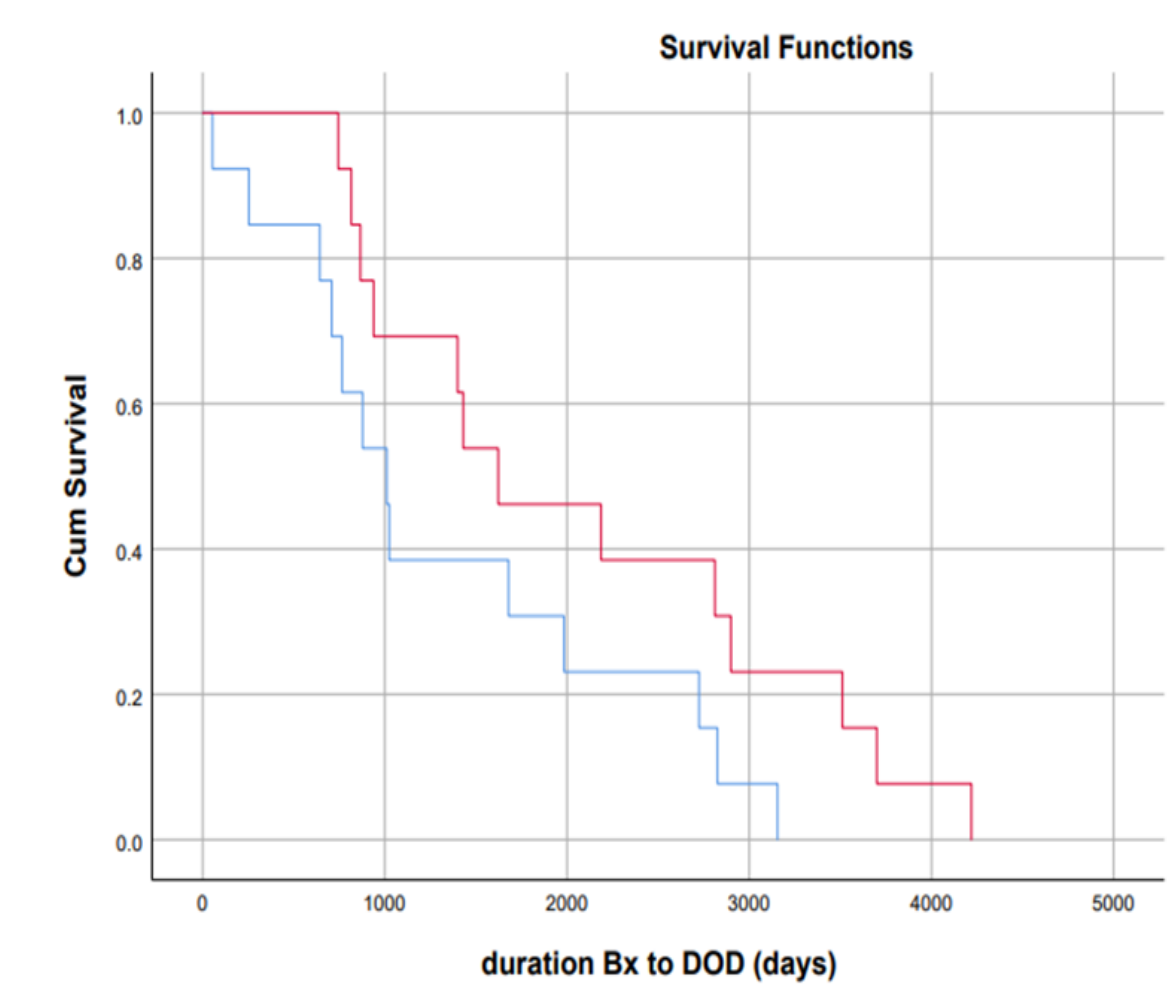


Figure 3: Kaplan-Meier analysis of overall survival for high and low FoxP3 cohorts.

The bimodal distribution of FoxP3 IHC scores allowed designation of high and low FoxP3 cohorts; their overall survival was analyzed by the Kaplan-Meier function in SPSS (Log rank test $p=0.085$).

Table 1: FOXP3 IHC score correlation with other Treg markers and disease progression.

	Correlation coefficient	p value
FOXP3:CD4	0.690	<0.005
FOXP3:CD25	0.705	<0.005
FOXP3:Risk group	0.440	<0.005
FOXP3:Pathology	0.420	<0.005

Conclusions

- Our study validated the hypothesis that Treg are recruited to the oral cancer TME, found that FoxP3 is significantly correlated with disease progression.
- Furthermore, FoxP3 correlation with overall survival approached significance.
- These data suggest the role of FoxP3 as a potential prognostic marker and support studies to target Tregs in oral cancer therapy. A higher FoxP3 level indicates an increased infiltration of Tregs into the tumor microenvironment, leading to a more immunosuppressed tumor microenvironment. Highly suppressive TME reduce the immune response to the tumor contributing to poorer prognosis.