Abstract: Regulatory T (Treg) cells can suppress antitumor immune response, but little is known about possible age-related differences in the number of these cells in the microenvironment of oral tongue squamous cell carcinoma (OTSCC). The aim of this study was to determine the number of FoxP3+ Treg cells in the microenvironment of OTSCC in young (≤ 45 years) and older (≥ 60 years) patients, and to correlate the findings with clinicopathological parameters (sex, tumor size/extent, regional lymph node metastasis, clinical staging, and histopathological grade of malignancy). Forty-eight OTSCCs (24 diagnosed in young patients and 24 diagnosed in older patients) were selected. Lymphocytes exhibiting nuclear immunopositivity for FoxP3 were quantified at the tumor invasive front and the results were analyzed statistically using the non-parametric Mann-Whitney test. FoxP3+ lymphocytes were observed in all cases assessed. The number of FoxP3+ lymphocytes in OTSCC tended to be higher in older patients (p = 0.055). Analysis of OTSCC in males and in early clinical stages revealed a higher number of Treg cells in older patients than in young ones (p < 0.05). In older patients, the number of Treg cells tended to be higher in smaller tumors (p = 0.079). Tumors with intense inflammatory infiltrate exhibited a larger number of Treg cells, both in young (p = 0.099) and older patients (p = 0.005). The results suggest a greater participation of Treg cells in immunoinflammatory responses in the microenvironment of OTSCC in older patients, particularly in males and in early stages.

Keywords: Carcinoma, Squamous Cell; Tongue; Young adult; T-Lymphocytes, Regulatory; Immunohistochemistry.

Introduction

Squamous cell carcinoma (SCC) is the most frequent malignancy of the oral cavity, accounting for 200,000 new cases per year worldwide. Oral SCC (OSCC) is typically diagnosed in male individuals in their fifties to seventies, and tobacco and alcohol are the major risk factors for its development. Among several anatomic sites of the oral cavity, the tongue is involved at a higher frequency. Data from cancer registries worldwide reveal an increase in the incidence of oral tongue SCC (OTSCC), ranging from 0.4% to 3.3% per year.
Even though OSCC is rare in individuals younger than 40 years, studies have pointed out a remarkable change in the demographic tendency of this neoplasm, with substantial increase in the number of cases among younger individuals (aged 18 to 45 years). In addition, while research has suggested important differences in molecular bases and the biological behavior of OSCC among young and older individuals, the findings are still contradictory. Some studies have described a more aggressive clinical course of OSCC in younger individuals, whereas others have not found significant age-related differences in the biological behavior of these neoplasms.

Interactions between neoplastic cells and immune cells can modulate tumor growth. Through molecules such as the transforming growth factor β (TGF-β), neoplastic cells can recruit different cell types that are capable of suppressing the immune response in the tumor microenvironment, including myeloid-derived suppressor cells, tumor-associated macrophages (TAMs) with M2 profile and T regulatory (Treg) cells. Treg cells can suppress the action of several cells of the immune system, such as natural killer cells, CD4 T and CD8 T cells, B lymphocytes, and dendritic cells, and derive from the lineage of lymphocytes that constitutively express CD4, CD25, and the forkhead box P3 (FoxP3) transcription factor. Studies have underscored the importance of FoxP3 for the differentiation of T lymphocytes in Treg cells, and for the activation of their functions; in addition, it is one of the best markers for the identification of these cell types.

Based on the immunosuppressive activity of Treg cells, studies have demonstrated some relationship between the large amount of Treg cells in the microenvironment of malignant neoplasms and worse prognosis. In OSCC, a larger number of Treg cells in the tumor microenvironment have been associated with the presence of regional lymph node metastasis, advanced staging, low disease-free survival rates, and unfavorable immunotherapy outcomes. Conversely, other studies have suggested an association between the large number of Treg cells and better prognosis in OSCC, as well as better locoregional control in head and neck cancer. Despite these important findings, little is known, to date, about the presence of Treg cells in the microenvironment of OTSCC in young individuals.

Therefore, the aim of this study was to assess the amount of Treg cells (FoxP3+) in the tumor microenvironment of OTSCC in young (≤ 45 years) and older (≥ 60 years) individuals, and to correlate the findings with clinicopathological parameters (sex, tumor size/extent, regional lymph node metastasis, clinical staging, and histopathological grade of malignancy). The main hypothesis of the study was that the number of Treg cells would be higher in OTSCC in older individuals than in young ones. In addition, it was also hypothesized that the higher number of Treg cells in OTSCC would be related to more aggressive clinicopathological characteristics.

Methodology

For this study, we selected 48 cases of OTSCC (24 diagnosed in young individuals [≤ 45 years] and 24 in older individuals [≥ 60 years]), obtained from the Pathological Anatomy Division of Napoleão Laureano Hospital. The sample size was defined by the number of available institutional archival cases. We only selected cases derived from surgical resections and whose clinical records contained information about tumor size/extent, regional lymph node metastasis, distant metastasis, and clinical staging. In addition, OTSCC of patients undergoing radiation therapy, chemotherapy, or any other treatment prior to surgery were excluded. Patient sex and age were collected for all cases. The parameters of the sixth edition of the TNM Classification of Malignant Tumors were used for clinical staging of OTSCC. The study was approved by the institutional Research Ethics Committee (Process: 39076214.4.0000.5187).

Morphological analysis

Five-micrometer thick sections were obtained from paraffin-embedded biological material. The histological sections were stained with hematoxylin and eosin and examined under a light microscope (Leica DM 500; Leica Microsystems Vertrieb GmbH, Wetzlar, Germany) by two experienced oral pathologists (PMA and CFWN). The histopathological
grade of malignancy of OTSCC was analyzed at the invasive front using the system proposed by Bryne et al.,\textsuperscript{22} in which scores (1–4) are attributed to the following parameters: degree of keratinization, invasion pattern, nuclear pleomorphism, and inflammatory infiltrate. The scores attributed to each parameter were summed to obtain the final score of malignancy for each case.\textsuperscript{22} Tumors with a final score ≤ 8 were classified as low grade of malignancy and tumors with a final score ≥ 9 as high grade of malignancy.\textsuperscript{23} If the examiners disagreed on the analysis of the histopathological grade of malignancy, the slides were reexamined until a consensus was reached.

**Immunohistochemistry**

Three-micrometer thick sections were mounted on glass slides prepared with organosilane adhesive. The histological sections were deparaffinized, rehydrated, and subjected to antigen retrieval with citrate buffer, pH 6.0, at 90 °C in a steamer for 60 min. The sections were then immersed in 3% hydrogen peroxide to block endogenous tissue peroxidase. After incubation with the primary monoclonal anti-FoxP3 antibody (dilution 1:150, Ref. Ab22510; Abcam, Cambridge, UK) in a humidity chamber for 60 min, the sections were washed with Tris-HCl buffer and treated with a polymer-based complex (Reveal\textsuperscript{TM}; Spring Bioscience Corp., Pleasanton, USA). Peroxidase activity was visualized by immersing the sections in diaminobenzidine (DAB Substrate System; Spring Bioscience Corp., Pleasanton, USA). Finally, the sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted on slides with a coverslip. Tonsillar tissue sections were used as positive control. The negative control consisted of omission of the primary antibody in the protocol described above.

**Immunohistochemical analysis**

The histological sections were analyzed in a blind fashion under a light microscope (Leica DM 500; Leica Microsystems Vertrieb GmbH, Wetzlar, Germany) by two previously trained evaluators. The analysis of Treg cells was performed quantitatively, adapting the methodology used by Schwarz et al.\textsuperscript{24} at 100× magnification, and 10 fields along the invasive front of OTSCC with the highest immunoreactivity to the FoxP3 antibody were selected. Each field was photomicrographed (ICC 50HD; Leica Microsystems Vertrieb GmbH, Wetzlar, Germany) at 400× magnification and the images were transferred to the ImageJ® program (Imaging Processing and Analysis in Java; National Institute of Mental Health, Bethesda, USA). Lymphocytes in the stromal compartment of the invasive front exhibiting nuclear immunostaining for FoxP3 were counted in each photographed field, regardless of the intensity of brown staining. The total number of FoxP3+ lymphocytes was established by summing the values obtained in each of these fields. This number was used to calculate the mean number of Treg cells per case.

**Statistical analysis**

The results were analyzed using the IBM SPSS Statistics 20.0 program (IBM Corp., Armonk, NY, USA). Descriptive statistics was used for characterization of the sample. Analysis of the number of FoxP3+ lymphocytes by the Kolmogorov-Smirnov test revealed absence of a normal distribution. Therefore, the non-parametric Mann-Whitney test was used to compare the median number of immunopositive cells for FoxP3 according to age group and different clinicopathological data. The significance level (p < 0.05) was set at 5% for all tests.

**Results**

**Clinical and morphological data**

The present sample showed a higher frequency of OTSCC in males, both among young (n = 15; 62.5%) and older (n = 13; 54.2%) individuals, with a mean age of 37.4 ± 7.3 (range: 21–45) for young patients and of 70.6 ± 7.4 (range: 61–91) for older patients. Regarding tumor size, cases classified as T1 (n = 10; 41.7%) were more frequent in young individuals and those classified as T2 (n = 13; 54.2%) were more frequent in older individuals. Absence of regional lymph node metastasis was observed at a higher frequency in older individuals (n = 21; 87.5%). There was high predominance of absence
of distant metastasis both in young (n = 23; 95.8%) and older (n = 23; 95.8%) individuals. The clinical staging revealed higher frequency of cases classified as stage II in older individuals (n = 11; 45.8%), and as stages I (n = 8; 33.3%) and IV (n = 8; 33.3%) in young individuals. As for the histopathological grade of malignancy, there was predominance of high-grade lesions both in young (n = 18; 75.0%) and older (n = 17; 70.8%) individuals (Table).

**Immunohistochemical analysis**

FoxP3+ lymphocytes were observed at the invasive tumor front in all OTSCCs assessed (Figures 1A and 1B), with a tendency towards smaller amounts in young individuals (median: 31.70; range: 4.90–75.20) when compared with older individuals (median 53.60; range: 14.90–97.10) (p = 0.055). Sex did not show statistically significant differences in the number of FoxP3+ lymphocytes in OTSCC, neither among young nor among older individuals (p > 0.05; Figure 2). On the other hand, the analysis of tumors in male individuals revealed a higher median of FoxP3+ lymphocytes in older individuals than in young ones (p = 0.020; Figure 2).

Regarding tumor size/extent, analysis of FoxP3+ lymphocytes demonstrated a relatively similar number in T1 and T2–T4 lesions among young individuals (p > 0.05; Figure 2). Older individuals tended to have a larger number of Treg cells in lesions classified as T1 than in those classified as

| Clinopathological parameters | Young patients n (%) | Older patients n (%) |
|-----------------------------|----------------------|---------------------|
| Sex                         |                      |                     |
| Male                        | 15 (62.5)            | 13 (54.2)           |
| Female                      | 9 (37.5)             | 11 (45.8)           |
| Tumor                       |                      |                     |
| T1                          | 10 (41.7)            | 10 (41.7)           |
| T2                          | 6 (25.0)             | 13 (54.2)           |
| T3                          | 3 (12.5)             | 1 (4.2)             |
| T4                          | 5 (20.8)             | 0 (0.0)             |
| Node                        |                      |                     |
| N0                          | 13 (54.2)            | 21 (87.5)           |
| N1                          | 5 (20.8)             | 2 (8.3)             |
| N2                          | 2 (8.3)              | 1 (4.2)             |
| N3                          | 4 (16.7)             | 0 (0.0)             |
| Metastasis                  |                      |                     |
| M0                          | 23 (95.8)            | 23 (95.8)           |
| M1                          | 1 (4.2)              | 1 (4.2)             |
| Clinical stage              |                      |                     |
| Stage I                     | 8 (33.3)             | 10 (41.7)           |
| Stage II                    | 4 (16.7)             | 11 (45.8)           |
| Stage III                   | 4 (16.7)             | 2 (8.3)             |
| Stage IV                    | 8 (33.3)             | 1 (4.2)             |
| Histopathological grade of malignancy | | |
| Low-grade                   | 6 (25.0)             | 7 (29.2)            |
| High-grade                  | 18 (75.0)            | 17 (70.8)           |
Figure 1. A) Many FoxP3+ lymphocytes (arrows) at the invasive front of low-grade OTSCC in older patient exhibiting marked inflammatory infiltrate (Reveal, 400×). B) Few FoxP3+ lymphocytes (arrows) at the invasive front of high-grade OTSCC in young patient exhibiting slight inflammatory infiltrate (Reveal, 400×).

Figure 2. Box plot charts illustrating the number of FoxP3+ lymphocytes in OTSCC in young (≤ 45 years) and older (≥ 60 years) patients according to sex, tumor size, clinical stage, and histopathological grade of malignancy.

Figure 2. Box plot charts illustrating the number of FoxP3+ lymphocytes in OTSCC in young (≤ 45 years) and older (≥ 60 years) patients according to sex, tumor size, clinical stage, and histopathological grade of malignancy.
T2–T4, but no statistically significant differences were observed (p > 0.05; Figure 2). When only those lesions classified as T1 were compared, there was a tendency towards an increased number of FoxP3+ lymphocytes in older individuals than in young ones (p = 0.059; Figure 2).

Regarding regional lymph node metastasis, the non-parametric Mann-Whitney test did not reveal any statistically significant difference in the number of FoxP3+ lymphocytes in young individuals (p = 0.505). In older individuals, possible differences in the number of Treg cells were not assessed because of the low number of cases with regional lymph node metastasis. Likewise, the low number of cases with distant metastasis, both in young and older individuals, did not allow comparing the number of Treg cells when this clinical parameter was used.

Clinical staging showed a slight tendency towards a smaller number of FoxP3+ lymphocytes in stage I lesions in young individuals, when compared with stage II–IV lesions (p = 0.178; Figure 2). However, there was a tendency towards a larger number of Treg cells in stage I lesions in older individuals, when compared with stage II–IV lesions, without any statistically significant differences (p = 0.079; Figure 2). When only stage I lesions were compared, the number of FoxP3+ lymphocytes was higher in older individuals than in young ones (p = 0.010; Figure 2).

As for the histopathological grade of malignancy, the non-parametric Mann-Whitney test did not reveal any statistically significant difference in the number of FoxP3+ lymphocytes between low-grade and high-grade OTSCC, neither in young nor in older individuals (p > 0.05; Figure 2). By taking into consideration the individual grading parameters, no statistically significant difference was observed in the number of Treg cells regarding the degree of keratinization and nuclear pleomorphism among young and older individuals (p > 0.05; Figure 2).

By assessing the invasion pattern, OTSCC arranged into small cell groups or with diffuse infiltration showed a larger number of FoxP3+ lymphocytes when compared with tumors that exhibited clearly defined infiltrative borders or that were arranged as cord-like, band-like, or solid lesions, both for older and young individuals, with a tendency towards statistical significance in the latter (p = 0.052; Figure 3). A higher median number of FoxP3+ lymphocytes was found in OTSCC with intense inflammatory infiltrate, both among young and older individuals, with statistically significant difference in the latter (p = 0.005; Figure 3). Additionally, by considering only lesions with intense inflammatory infiltrate, OTSCC in older individuals showed a larger number of Treg cells than did OTSCC in young individuals (p = 0.034; Figure 3).

Discussion

There is a paucity of information on qualitative and/or quantitative age-related differences in cells of the immune system in the microenvironment of OSCC. Accordingly, this study aimed to determine the number of Treg cells in the microenvironment of OTSCC in young and older individuals and to correlate the findings with clinicopathological parameters. The main hypothesis was that the number of Treg cells would be higher in OTSCC in older patients than in young ones. It was also hypothesized that a higher number of Treg cells would be related to more aggressive clinicopathological features in OTSCC. The findings of this study present novel aspects related to antitumor immunity in OTSCC, suggesting a larger participation of Treg cells in immunoinflammatory responses in the microenvironment of these tumors in older patients, especially in males and in early stages.

Kouketsu et al. assessed 82 cases of OSCC and did not observe any significant differences in the number of FoxP3+ lymphocytes in the tumor stroma when comparing age groups (27 to 93 years). Likewise, Ryu et al. analyzed 552 head and neck SCCs (HNSCCs), including cases of OTSCC, and did not observe significant differences in the number of FoxP3+ lymphocytes in the microenvironment of lesions in young individuals (≤ 45 years) and in those aged over 45 years. Those authors noted, however, that the ratio between Treg cells and CD8 T lymphocytes was significantly higher in the microenvironment of HNSCC in young individuals (85.3%) than in older individuals (46.6%) (p < 0.001). These findings, combined with programmed death ligand-1 (PD-L1)
expression in neoplastic cells, might suggest an important change in immunosurveillance in the microenvironment of HNSCC in young individuals.\textsuperscript{26} Studies on the participation of Treg cells in the development and progression of OSCC have shown contradictory results. A larger number of these cell types in the microenvironment of OSCC have been associated with more advanced stages and worse prognosis\textsuperscript{16,17,27,28} or with initial stages and higher survival rates.\textsuperscript{16,29} The reasons for these discrepancies remain unclear, but the findings of the present study suggest that patient age could be an important confounding factor. In line with this suggestion, previous studies that have correlated the number of Treg cells in the microenvironment of OSCC with the progression and prognosis of these malignancies, have used samples from young and older individuals, aged 26 to 92 years.\textsuperscript{16,17,28,29}

Information on possible sex-related differences in the number of Treg cells in the microenvironment of OSCC is scarce.\textsuperscript{27} Kouketsu et al.\textsuperscript{27} in a study on OSCC diagnosed in individuals aged 27 to 93 years, did not observe significant differences in the number of FoxP3+ lymphocytes in the microenvironment of lesions associated with patient sex. Similar results were obtained for OTSCC in the present study, both for young and older individuals (p > 0.05). When lesions in male individuals were compared, however, there were a larger number of Treg cells in older than in young individuals (p = 0.020). These findings underscore the necessity of further investigation to assess possible age-related differences in immunoinflammatory...
responses in the microenvironment of OTSCC in male individuals.

In the present study, no statistically significant differences in the number of FoxP3+ lymphocytes were observed between low-grade and high-grade OTSCC, neither for young nor for older individuals (p > 0.05). Nevertheless, the assessment of individual grading parameters showed a larger number of Treg cells in OTSCC with intense inflammatory infiltrate, both among young and older individuals, with significant difference in the latter (p = 0.005). Previous studies on OSCC and lower lip SCC also revealed a larger number of Treg cells in lesions with intense inflammatory infiltrate.

Da Cunha-Filho et al. highlight that lower lip SCC in initial stages often have intense inflammatory infiltrate and, therefore, neoplastic cells would require a larger number of Treg cells for the tumor microenvironment so as to evade the antitumor immune response. Based on the findings of the present study, it may be suggested that neoplastic cells of OTSCC in initial stages, especially in older individuals, also recruit a larger number of FoxP3+ lymphocytes in order to reduce immunosurveillance in the tumor microenvironment. On the other hand, according to Da Cunha-Filho et al., neoplastic cells would be less able to stimulate immune responses in more advanced stages of OSCC and, consequently, the number of Treg cells in the tumor microenvironment would decrease. Notwithstanding, the possibility of other cells, such as M2 TAMs and myeloid-derived suppressor cells, taking part in immune response suppression in the OTSCC microenvironment should not be ruled out, especially in advanced stages.

It has been reported that changes in the balance of different types of cells of the immune system could be more important for the modulation of antitumor immunity than quantitative alterations in single cell types. Coherently, Feng et al. found that multiparametric immune profiling in cases of HPV-negative OSCC, which includes the simultaneous analysis of antigen-processing machinery and characterization of CD3+, CD8+, FoxP3+, CD163+, and PD-L1+ cells, correlates with clinical and prognostic parameters. These findings highlight the complexity of the tumor microenvironment in OSCC and, in addition, suggest that the assessment of multiple parameters could be important to identify patients in need of treatment intensification. In line with these findings, the results of the present study suggest possible age-related differences in antitumor immunity in the OTSCC microenvironment, which could affect the immunotherapy of these tumors.

Some limitations of the present study need to be acknowledged. One important limitation is the lack of data about tobacco, alcohol consumption, and tumor HPV status in the sample. This is relevant as OTSCC in young individuals is believed to be etiopathogenetically distinct from OTSCC in older individuals. The relatively small sample size and the absence of follow-up data also represent limiting factors in this study. Further research with larger samples, which should evaluate potential confounding factors and long follow-up periods, is required to shed further light on possible age-related differences in cells of the immune system in OTSCC microenvironment and their probable influence on the progression and prognosis of these tumors.

**Conclusion**

The findings of the present study suggest a larger participation of Treg cells in immunoinflammatory responses in the OTSCC microenvironment in older patients, especially in male individuals and in initial stages. Thus, these findings underscore the importance of further characterization of immune responses in the OTSCC microenvironment based on patient age.

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