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Migração de células T regulatórias em carcinoma de células escamosas de cavidade bucal e de lábio: fatores de prognóstico clínico e microscópico**.**

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Dissertação apresentada ao Programa de Pós-Graduação da Faculdade de Odontologia da Universidade Federal de Minas Gerais, como requisito parcial para obtenção do título de mestre em Odontologia.

Área de concentração: Patologia bucal

Orientador (a): Prof (a): Dra. Tarcília Aparecida da Silva

Belo Horizonte

Faculdade de Odontologia da UFMG

2008

Ao meu bom Deus, por me proporcionar mais uma conquista.

Aos meus pais, que vivem como se fossem seus os meus sonhos, dificuldades e vitórias.

Ao meu namorado pelo incentivo, amor e paciência.

À minha madrinha, que agora como anjo protetor, foi e sempre será motivo de orgulho e inspiração.

Agradecimentos

À professora Tarcília Aparecida da Silva, por ter me acolhido como orientanda concedendo-me subsídios para a realização deste trabalho.

Aos professores Maria Cássia Ferreira de Aguiar, Maria Auxiliadora Vieira do Carmo, Ricardo Santiago Gomez, Ricardo Alves de Mesquita pela competência e estímulo.

Ao professor Alfredo Maurício Batista de Paula, exemplo de dedicação e amizade por ter sido o primeiro incentivador pela vida acadêmica.

Aos professores do departamento de patologia bucal da Faculdade de Odontologia da Universidade Federal de Goiás (UFG) e Hospital Araújo Jorge, Associação de combate ao câncer de Goiás, pela contribuição na seleção dos casos e na coleta de dados dos pacientes.

Aos amigos da patologia, em especial Bruna, Aline, Adriana, Inês, Heloísa, Patrícia, Tânia, Jeane e Daniela pela amizade e inestimável ajuda.

Aos alunos de iniciação científica Fernanda e Lívia pela amizade e grande contribuição na execução deste trabalho.

A minha família e amigos que foram fonte de estímulo durante este período.

Sumário

Lista de Abreviaturas e Siglas

CEC: Carcinoma de células escamosas

CCEB: Carcinoma de células escamosas de boca

CCEL: Carcinoma de células escamosas de lábio

Treg: Células T regulatórias

CD4: *Cluster of differentiation 4*

CD25: *Cluster of differentiation 25*

CTLA-4: *Cytotoxic T lymphocyte antigen-4*

Foxp3: *Forkhead transcription factor*

INCA: Instituto Nacional do Câncer

NK: Células *natural killer*

MHC: *Major histocompatibility complex*

IL-2: Interleucina-2

IL-10: Interleucina-10

CTLA-4: Gene que codifica a proteína CTLA-4

UFMG: Universidade Federal de Minas Gerais

UFG: Universidade Federal de Goiás

RESUMO

As células T Regulatórias (Treg) representam uma subpopulação de linfócitos T $CD4^+$ que desempenham importante papel na regulação e supressão da resposta imune. O objetivo deste trabalho foi investigar a expressão das células Treg através dos seus marcadores CD4, CD25, CTLA-4 e Foxp3 utilizando a técnica de imunoistoquímica em amostras de carcinoma de células escamosas de cavidade bucal (CECB) e carcinoma de células escamosas de lábio (CCEL). A relação dos marcadores das células Treg com os dados de sobrevida também foi avaliada. Foi observado um maior percentual de células $CD4^+$ ($p=0.019$) e $Foxp3^+$ ($p=0.04$) em amostras de CECB quando comparadas com CCEL. CECB mostrou um menor percentual de células CTLA-4^+ que CCEL ($p<0.0001$). Além disso, a relação entre CD4/Foxp3, CD4/CD25 e CD4/CTLA-4 foi significativamente maior no CECB, indicando um maior número de células Treg no CECB. O teste log-rank mostrou que pacientes com altas contagens de CD4; CD4/Foxp3; CD4/CD25 e CD4/CTLA-4 tiveram menor sobrevida em relação aos pacientes com baixa contagem celular ($p<0.05$). De acordo com estes resultados, amostras com maior número de células CD4 ($p=0.015$) e Foxp3 ($p=0.018$) exibiram maior índice de proliferatividade. Nossos achados sugerem uma associação do fenótipo de células Treg com um pior prognóstico; isso talvez resulte da supressão da resposta imune anti-tumoral pelas células Treg no CECB.

Descritores: Carcinoma de células escamosas; células T regulatórias; resposta imune.

ABSTRACT

T Regulatory (Treg) cells represent a T $CD4^+$ lymphocytes subpopulation that displays important roles in the regulation and suppression of immune responses. We investigated the expression of Treg cell markers CD4, CD25, CTLA-4 and FoxP3 by immunohistochemistry in samples of oral cavity squamous cell carcinoma (OCSCC) and lip squamous cell carcinoma (LSCC). The relationship of Treg markers with survival data was also evaluated. We observed a higher percentual of CD4 ($P=0.019$) and FoxP3 ($P=0.040$) positive cells in OCSCC samples when compared with LSCC. OCSCC showed lower percentual of CTLA-4^+ cells than LSCC ($P<0.0001$). Moreover, CD4/FoxP3, CD4/CD25 and CD4/CTLA-4 ratio was significantly greater in OCSCC, indicating higher numbers of Treg cell phenotype in OCSCC. A log-rank test showed that patients with high counts of CD4; CD4/FoxP3, CD4/CD25 and CD4/CTLA-4 showed a decrease of survival in relation to patients with low cell counts ($P<0.05$). In line with this findings, samples with high numbers of CD4 ($P=0.015$) and FoxP3 ($P=0.018$) exhibited greater proliferative index. Our findings suggest an association of Treg cells phenotype with poor prognosis; this might result from suppression of anti-tumor immune responses by Treg cells in OSCC.

Key words: squamous cell carcinoma; T regulatory cells; immune response.

Síntese Bibliográfica

O carcinoma de células escamosas (CEC) é o câncer de boca mais comum no Brasil. O Instituto Nacional do Câncer (INCA) estima uma incidência de 14160 novos casos de neoplasias malignas de cavidade bucal no Brasil em 2008, sendo 10380 para o sexo masculino e 3780 para o sexo feminino [1].

O Carcinoma de células escamosas de cavidade bucal (CECB) acomete principalmente indivíduos entre a quinta e oitava décadas de vida, do sexo masculino e de raça branca [2]. Os principais locais de aparecimento das lesões incluem região posterior da língua e o soalho bucal, seguido por palato mole, gengiva, mucosa jugal e palato duro [3]. Os principais fatores ambientais de risco para o CECB são os consumos de tabaco e álcool. No entanto, essa condição é conhecida por também se desenvolver na ausência desses fatores, o que sugere a importância de eventos relacionados ao hospedeiro [4].

O Carcinoma de células escamosas de lábio (CCEL) ocorre principalmente em homens, da raça branca entre a quinta e sétima décadas de vida [5]. A mucosa do lábio inferior é a localização anatômica mais comum do carcinoma epidermóide de lábio [6]. A exposição crônica à radiação solar é apontada como o principal fator de risco para o CCEL apesar de se admitir uma etiopatogenia multifatorial para esta condição [5].

Apesar dos recentes avanços terapêuticos, a sobrevivência de pacientes com CEC não sofreu modificações. Desta forma, se faz necessário identificar marcadores bem definidos e clinicamente aplicáveis da agressividade do tumor

[7]. Neste sentido, as imunidades inata e adaptativa desempenham um importante papel na vigilância imunológica e destruição tumoral. A imunidade inata é a primeira linha de defesa do hospedeiro contra patógenos e células tumorais. Os tipos celulares da imunidade inata incluem células *natural-killer* (NK), macrófagos e neutrófilos que desempenham um papel crítico na proteção do hospedeiro contra o câncer. Já a imunidade adaptativa está envolvida na eliminação de patógenos e na defesa do hospedeiro em fases mais tardias do crescimento tumoral. O linfócito é o tipo celular mais predominante na imunidade adquirida e pode também ocorrer produção de anticorpos. A imunidade adaptativa também é a responsável por uma resposta mais específica e pela memória imunológica [10].

A presença de um infiltrado imune/inflamatório em contato com as células neoplásicas pode ser um sinal indicativo de uma resposta imunológica favorável por parte do hospedeiro, contra o câncer [7]. Entretanto, os diferentes tipos celulares da resposta inata e adaptativa podem apresentar efeitos que favorecem ou antagonizam o crescimento tumoral [7, 8].

Existem muitos fatores que concorrem para a falha do sistema imune do hospedeiro no controle do crescimento tumoral. Algumas dessas razões incluem o desenvolvimento de variantes tumorais que escapam do reconhecimento imunológico ou uma menor regulação da classe de moléculas MHC *(major histocompatibility complex)*, supressão imune mediada pelas células T regulatórias (Treg) e outras células supressoras da imunidade inata [9-13].

As células Treg constituem 5-10% do total populacional de células T $CD4^+$ em camundongos e em seres humanos e estão principalmente envolvidas na repressão de doenças auto-imunes [9-13]. Estas células são constitutivamente

caracterizadas pela expressão da proteína transmembrana CD25 que é a cadeia α do receptor da interleucina-2 (IL-2); CTLA-4 *(cytotoxic T lymphocyte antigen-4)* e Foxp3 *(forkhead transcription factor)* [9-15].

Estudos prévios revelaram que as células T regulatórias são potentes inibidores da resposta imune anti-tumoral e estão associadas com prognóstico desfavorável em diferentes tipos de câncer [12, 16-29]. Estes trabalhos demonstraram que a depleção de células Treg $CD4^+$ $CD25^+$ resultou em uma menor taxa de crescimento do tumor [19]. Além disso, uma alta prevalência de células T regulatórias tem sido observada no estroma do adenocarcinoma pancreático ductal e estas células estão fortemente correlacionadas com diversos fatores de malignidade como: presença de metástases à distância, grau de proliferação celular avançado e estágio precoce de metástase linfonodal [24]. Alguns estudos também têm observado crescente número de células Treg no sangue periférico de pacientes portadores de CEC de cabeça e pescoço [18, 30, 31].

O CTLA-4 é um membro da família das imunoglobulinas que participa da ligação das moléculas co-estimulatórias B 7.1 e B 7.2 [9-14]. O gene *CTLA-4* é o responsável por codificar o receptor transitoriamente expresso nas células T ativadas, que desempenham um papel principal na regulação imunológica, por fornecer um sinal negativo para a célula T uma vez que a resposta imune foi iniciada e completada [14]. O CTLA-4 está envolvido na atividade supressora de células Treg CD4 CD25 contra células T CD4 ou CD8 [12, 14]. Anticorpos específicos que bloqueiam o CTLA-4 têm sido utilizados como um agente anti-

tumoral resultando em um acionamento da resposta imune contra o tumor [16, 17, 20, 22, 26, 27].

O Foxp3 é considerado o marcador mais específico das células Treg. É um membro da família *forkhead* de fatores transcripcionais que está criticamente envolvido no desenvolvimento e função das células Treg $CD25^{+}$ [13, 15, 32, 33]. No câncer humano, a expressão do Foxp3 está sendo usualmente correlacionada com um curso desfavorável da doença. Dessa forma, talvez, essa expressão possa representar, no futuro, uma variável prognóstica independente em termos de sobrevida geral e sobrevida livre da doença [21, 24, 29, 23].

A importância dos marcadores das células Treg no carcinoma de células escamosas de cavidade bucal e lábio ainda não tem sido determinada. Assim, o objetivo deste estudo foi investigar a expressão do CD4, CD25, CTLA-4 e Foxp3 no CECB e CCEL e sua implicação com a agressividade tumoral e prognóstico da doença.

Optou-se por redigir esta dissertação em forma de artigo científico, (seguindo as normas preconizadas pelo periódico de escolha), considerações finais e referências.

Original article, submitted May 2008, to Oral Oncology

T regulatory cell markers in oral squamous cell carcinoma: relationship with survival and tumor agressiveness

Short title: T regulatory cell markers in squamous cell carcinoma

Geane Moreira[a]

Fernanda Oliveira Ferreira[a]

Lívia Bonfim Fulgêncio[a]

Rita de Cássia Gonçalves Alencar[b]

Elismauro Francisco de Mendonça[c]

Cláudio Rodrigues Leles[c]

Aline Carvalho Batista[c]

Tarcília Aparecida da Silva[a*]

[a]Department of Oral Surgery and Pathology, Dental School, Federal University of Minas Gerais, Belo Horizonte, Brazil;

[b]Anatomopathology and Cytopathology Division of Araújo Jorge Hospital, Association of Cancer Combat of Goiás, Goiânia, Brazil;

[c]Department of Oral Medicine (Oral Pathology), Dental School, Federal University of Goiás, Goiânia, Brazil;

[*] Corresponding author: Tarcília Aparecida da Silva Mailing address: Departamento de Clínica, Patologia e Cirurgia Odontológicas, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, CEP 31.270-901, Belo Horizonte, Minas Gerais, Brazil. Phone: 55 31 3409-2476 (voice); 55 31 3409-2430 (Fax). E-mail: tarcilia@.ufmg.br

Original article, submitted May 2008, to Oral Oncology

Abstract

Background: T Regulatory (Treg) cells represent a T $CD4^+$ lymphocytes subpopulation that displays important roles in the regulation and suppression of immune responses.

Aims/Methods: We investigated the expression of Treg cell markers CD4, CD25, CTLA-4 and FoxP3 by immunohistochemistry in samples of oral cavity squamous cell carcinoma (OCSCC) and lip squamous cell carcinoma (LSCC). The relationship of Treg markers with survival data was also evaluated.

Results: We observed a higher percentual of CD4 (P=0.019) and FoxP3 (P=0.040) positive cells in OCSCC samples when compared with LSCC. OCSCC showed lower percentual of CTLA-4^+ cells than LSCC (P<0.0001). Moreover, CD4/FoxP3, CD4/CD25 and CD4/CTLA-4 ratio was significantly greater in OCSCC, indicating higher numbers of Treg cell phenotype in OCSCC. A log-rank test showed that patients with high counts of CD4; CD4/FoxP3, CD4/CD25 and CD4/CTLA-4 showed a decrease of survival in relation to patients with low cell counts (P<0.05). In line with this findings, samples with high numbers of CD4 (P=0.015) and FoxP3 (P=0.018) exhibited greater proliferative index.

Conclusions: Our findings suggest an association of Treg cells phenotype with poor prognosis; this might result from suppression of anti-tumor immune responses by Treg cells in OSCC.

Key words: squamous cell carcinoma; T regulatory cells; immune response.

Introduction

It is becoming accepted that tumor-infiltrating immune cells may have a dual function: inhibiting or promoting tumor growth and progression[1, 2]. There are many reasons that account for the failure of host immune systems to control tumor growth such as development of tumor variants that escape immune recognition or downregulation of MHC (major histocompatibility complex) class molecules; immune suppression mediated by T regulatory (Treg) cells and other supressor cells of innate immune cells[3-7].

Treg cells comprise 5-10% of the total population of $CD4^+$ T cells in mice and men and were primarily thought to be critically involved in the repression of autoimmune disorders [3-7]. Treg which are characterized by the constitutive expression of a transmembrane protein (CD25) that is alpha chain of the receptor for interleukin-2 (IL-2); CTLA-4 (cytotoxic T lymphocyte antigen-4) and forkhead transcription factor (FoxP3)[3-9].

Previous results revealed that Treg are potent inhibitors of anti-tumor immune response and associated with poor prognosis in different types of cancer[6, 10-23]. It has been showed that the depletion of $CD4^+$ $CD25^+$ Treg cells results in slower growth rate of tumor[13]. Furthermore, a high prevalence of T regulatory cells has been observed in stroma of pancreatic ductal adenocarcinoma and these cells have been closely correlated with several malignant features, such as distant metastasis, high tumor grade, and advanced pathologic tumor-node-metastasis stage[18]. Significant numbers of regulatory T cells are also increased in the peripheral blood of patients suffering from squamous cell carcinoma of the head and neck[12, 24, 25].

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is a member of immunoglobulin superfamily and binds to the B7.1 and B7.2 coestimulatory molecules[3-8]. The CTLA-4 gene encodes a receptor transiently expressed on activated T-cells that plays a pivotal role in immune regulation by providing a negative feedback signal to the T cell once an immune response has been initiated and completed[8]. Cytotoxic T lymphocyte antigen-4 (CTLA-4) plays roles in the supressive activity of CD4 CD25 Treg against CD4 or CD8 T cells[6, 8]. Specific antibodies that block CTLA-4 have been used as an anti-tumor agent resulting in enhancement of anti-tumor immune response[10, 11, 14, 16, 20, 21].

FoxP3 expression has been thought to be the most specific marker of Treg cells. It is a member of the forkhead family of transcription factors critically involved in the development and function of $CD25^+$ regulatory T cells[7, 9, 26, 27]. In human cancer, FoxP3 expression has usually been correlated to an unfavorable course of disease and may even represent an independent prognostic variable in terms of overall survival and progression-free survival[15, 18, 23, 17].

The significance of T regulatory cells markers in squamous cell carcinoma of oral cavity and lip has not yet been determined. The aim of this study was to investigate the expression of CD4, CD25, CTLA-4 and FOXP3 in OCSCC and LSCC and their relationship with tumor aggressiveness and prognosis.

Materials and Methods

Patient population

Surgically-excised specimens of primary OSCC were obtained from the files of the Anatomopathology and Cytopathology Division of Araujo Jorge Hospital, Association of Cancer Combat of Goias, Goiania, Brazil. This study has been approved by Ethics Committee of Universidade Federal de Minas Gerais (UFMG) and Araujo Jorge Hospital.

Our patient population consisted of eighteen patients with primary oral OCSCC being seven patients without cervical lymph node metastasis and eleven patients with cervical lymph node metastasis, and thirty-six patients had LSCC. All the patients with oral cavity tumors of this study were submitted to surgical treatment consisting of cervical lymph node removal with microscopic evaluation and none received radiotherapy, chemotherapy or any other treatment prior to surgery. Clinical data (gender, age, ethnic group, tobacco and alcohol consumption, tumor location, extension, T and N stages) and follow up information (clinical outcome and survival time) were obtained from medical records. All specimens were fixed in 10% buffered formalin (pH 7.4) and paraffin embedded. The microscopic features were evaluated from the analysis of one 5 μm section of each sample, stained routinely with hematoxylin and eosin (H.E.). All the sections were examined by light microscopy to confirm the presence or not of lymph node metastasis and to characterize the oral SCC.

Immunohistochemistry

Secctions of 4 μm from routinely processed paraffin embedded blocks were desparaffinized and dehydrated. The sections were deparaffinized by immersion in xylene, and this was followed by immersion in alcohol and then incubation with 3% hydrogen peroxide diluted in Tris-buffered saline (TBS) (pH 7.4) for 40 minutes. Antigen retrieval was obtained as described in Table 1. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. The slides were then incubated with the primary antibodies, all from Santa Cruz Biotecnology (Santa Cruz, CA) (Table 1) 18 hours at 4ºC. After washing in TBS, the sections were treated with the EnVision® + Dual Link System-HRP (Dako Corporation, Carpinteria, CA) or using LSAB®+system, HRP Peroxidase Kit (Dako). The sections were then incubated in 3,3'-Diaminobenzidine (DAB) (Dako) for 2 to 5 minutes. Finally, the sections were stained with Mayer´s hematoxylin and were covered. Negative controls were obtained by the omission of primary antibodies, which were substituted by 1% PBS-BSA and by non-immune rabbit (X0902, Dako) or mouse (X501-1, Dako) serum.

Cell counting and statistical analysis

In primary oral SCC (lip and oral cavity) samples the density (per mm^2) and percentage (%) of CD4, CD25, CTLA-4 and FOXP3 cells in inflammatory infiltrate adjacent to the tumor front area (stroma) were determined. The counts were performed in 15 alternate microscopic high power fields using an integration graticule (4740680000000-

Netzmikrometer 12.5x, Carl Zeiss, Göttingen, Germany). The percentage of positive cells in the stroma was calculated as the proportion of the total of immune/inflammatory cells.

The cell densities/proportions were expressed as density per mm^2 and percentages (mean ± SD). A P value of less than 0.05 was considered to be statistically significant. The comparative analyses between experimental groups were performed using the non-parametric Kuskall Wallis, followed by Dunn test, and/or Mann-Whitney.

The influence of tumor-associated FOXP3, CTLA-4, CD4 and CD25 on the prognosis of oral SCC patients was evaluated by the Kaplan-Meier and Cox Regression tests. The survival time was calculated from surgical resection until their last follow up appointment or the death of the patient. FOXP3, CTLA-4, CD4 and CD25 were dichotomized by median value and differences in survival between groups were evaluated by the log rank test. Significance was set at 0.05.

Results

The main clinical features of our series of 18 patients with oral OCSCC and 36 patients with LSCC are summarized in Table 2.

In OCSCC and LSCC, CD4, CD25, CTLA-4 and FoxP3 positive cells were distributed throughout the tumoral stroma. All stained cells had a mononuclear appearance in both groups (Fig. 1A-1D).

We observed higher percentual of CD4 (P=0.019) and FoxP3 (P=0.040) positive cells in OCSCC samples when compared with LSCC (Fig. 2A and 2D). On the other hand, lower percentual of CTLA-4^+ cells were observed in OCSCC group (P<0.0001) (Fig. 2C). Similar numbers of $CD25^+$ cells were observed in both groups (Fig. 2B). When OCSCC samples were dicotomized in metastatic and non-metastatic groups, no statistical significance was observed for all cells markers comparing these two groups. Moreover, positive correlations were observed when analyzing CD4 and FoxP3 cells population (P=0.027); CD25 and CD4 (P=0.099) and CD25 with FoxP3 (P=0.089) (Pearson Chi-square test).

The CD4/FoxP3 (P<0.0001), CD4/CD25 (P=0.007) and CD4/CTLA-4 (P<0.0001) ratio was significantly greater in OCSCC in relation to LSCC (Fig. 3), suggesting higher numbers of Treg cell phenotype in OCSCC. Furthermore, these data indicate that major cell population within CD4 positive cells is CD25 for LSCC and equally CD25 and CTLA-4 in OCSCC. Thus, the double FoxP3 CD4 positive cells are considerable smaller than CTLA-4 CD4 or CD25 CD4 in both lesions (P<0.0001)(Fig. 3).

To analyze the relationship of T regulatory cell markers and proliferative index of tumoral cells, the values were dicotomized in high and low groups by using the median. We obtained that samples with high counts of CD4 (12.23 ± 2.01) (P=0.015) and FoxP3 (12.50 ± 1.99) (P=0.018) exhibited greater proliferative index than samples with low counts (6.39 ± 1.03; 7.27 ± 1.04; respectively for CD4 and FoxP3). No significant differences in the proliferative index were observed when high and low CD25 and CTLA-4 groups were compared.

With regard to the last follow up, mean survival time of patients with LSCC was 49.75 months (95% CI= 37.61 – 61.89), of OCSCC without lymph node metastasis was 43.88 months (95% CI= 23.70 – 64.05) and of patients with OCSCC with lymph node metastasis was 34.50 months (95% CI= 17.49 – 51.51). For survival analysis, values obtained for each cell marker were dichotomized by median values (high and low groups). A log-rank test showed no difference in survival between high and low FoxP3 and CD25 groups. However, we observed a tendency of groups with high counts (70 ± 7 and 76 ± 7 months; respectively FoxP3 and CD25) to have lower survival compared with groups with low counts (124 ± 12 and 109 ± 15 months; respectively FoxP3 and CD25). In addition, patients with low counts of CD4 showed a significant increase of survival (P=0.019) (133 ± 11 months) in relation to patients with high CD4 counts (59 ± 10 months) (Fig. 4A). In line with these results, the survival analysis only of metastatic patients showed a significant reduced survival for patients with high CD4 counts (6 ± 2 and 26 ± 10 months, respectively for high and low groups; P=0.034). In contrast, patients with high counts of CTLA-4

showed significant increase of survival (144 ± 6 and 31 ± 4 months, respectively for high and low groups; P=0.006).

When analyzing the CD4/Treg cells markers ratio, we obtained that patients with high counts of double positive CD4/CD25 (P=0.029) (Fig. 4B); CD4/CTLA-4 (P=0.0083) (Fig. 4C) and CD4/FoxP3 (P=0.0002) (Fig. 4D) showed significant decrease of survival.

Discussion

Innate and adaptive immunity play important roles in immunosurveillance and tumor destruction. Both types of effectors responses are regulated by a heterogeneous family of cells, which are known as T Regulatory (Treg) cells[3-7]. Human tumor-derived Treg supresses tumor-specific T cell immunity and may therefore contribute to growth of human tumors[6, 10-23]. In the present study we found a higher percentual of CD4 and FoxP3 positive cells in OCSCC samples when compared with LSCC. Furthermore, the CD4/FoxP3, CD4/CD25 and CD4/CTLA-4 ratio was significantly greater in OCSCC, indicating higher numbers of Treg cell phenotype in OCSCC. Similar results have showed an increase of T regulatory cell population in others types of cancers, such as pancreas[18], ovarian tumors[17], metastatic melanoma[23] and head and neck cancer[12, 24, 25]. Our data also indicate that major cell population within CD4 positive cells is CD25 for LSCC and equally CD25 and CTLA-4 in OCSCC suggesting a different cell profile in OCSCC and LSCC. The double FoxP3 CD4 positive cells are considerable smaller than CTLA-4 CD4 or CD25 CD4 in both lesions. FoxP3 expression is correlated with development and function of Treg. FoxP3 is a member of the forkhead family of transcription factors critically involved in the development and function of $CD25^{+}$ regulatory T cells[7, 9, 27]. The small proportion of double positive CD4/FoxP3 cells suggests that others cell types would account for the expression of FoxP3 in both SCC types. In fact, expression of FoxP3 can be transiently induced in human non-Treg cells by activation through the T-cell receptor[26, 27]. Until recently, FoxP3 expression was thought to be restricted to the T-cell lineage, but recently

FoxP3 expression was detected in the melanoma cells[28] and various types of tumor cells[29]. However, we did not verified FoxP3 expression in neoplastic epithelial cells. It is also important to consider that the evaluation of the number of positive cells does not necessarily reflect the level of expression of these molecules at each cell. Indeed, FoxP3 expression can be influenced by different cytokines such as TGF-β, IL-10 or IL-2[7, 9, 27]. We have observed a slightly increase in the IL-10 concomitant with FoxP3 expression in OSCC samples (data not shown).

The main mechanism of the antitumor response depends on T-cell receptor engagement by major histocompatibility complex (MHC) antigens as well as CD28 ligation by B7. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4; CD152) is a second receptor of the co-stimulating factors B7-1 (CD80) and B7-2 (CD86), which have structures similar to CD28. CTLA-4 inhibits T-cell activation and terminates the T-cell response by blocking signals stimulated via CD28. In contrast to resting $CD4^+CD25^-$ T cells, Tregs express CTLA-4 constitutively. In general, CTLA-4 is not expressed by resting $CD4^+$ $CD25^-$ T cells but is induced upon T-cell stimulation by de novo transcription[5, 7, 8]. Lot of evidence accounts the importance of CTLA-4 blockade for prevention of malignancy and metastases spread[10, 11, 14, 16, 20, 21]. Furthermore, in oral squamous cell carcinoma, the A/A polymorphism of CTLA-4 gene which results in a high producer phenotype, is associated with poor survival[30]. Unexpectedly, our results showed a higher expression of CTLA-4 in LSCC and a significant increase of survival in patients with high counts of

CTLA-4. It is under known that LSCC patients usually have a good prognosis and a low rate of regional lymph node metastasis and mortality when compared with oral cavity

SCC[31, 32]. These results could be in part explained by recently demonstrated the function of CTLA-4 on destruction of tumor cells in vivo via interaction with B7[33]. Moreover, the CTLA-4 expression seems not to be exclusive of Treg cells[3, 6, 8]. On the hand, we consider the double positive CD4/CTLA-4 population; we observed a poor prognosis in patients with high counts of these cells, corroborating the potential role of CTLA-4 blockade in the cancer immunotherapy[10, 11, 14, 16, 20, 21].

We obtained that patients with high counts of CD4 and FoxP3 exhibited greater proliferative index. Consistent with these results patients with low counts of CD4 showed a significant increase of survival in relation to patients with high CD4 counts. Furthermore, we observed a tendency of groups with high counts of FoxP3 and CD25 to have lower survival compared with groups with low counts of these cells. When evaluating the double positive CD4/CD25 and CD4/FoxP3 cells we obtained that patients with high counts showed significant decrease of survival. Our results are corroborated by previous data showing an association of Tregs with inhibition of anti-tumoral immunity and consequently poor prognosis[10-23].

Our findings suggest an association of $CD4^+$ $CD25^+$ CTLA-4^+ $FoxP3^+$ T cells with poor prognosis which might result from suppression of anti-tumor immune responses by Treg cells in OSCC.

Acknowledgments

The authors thank Araújo Jorge Hospital, Association of Cancer Combat of Goiás, Goiânia, Brazil. This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grant 401305/2005-8). T.A.S. is a research fellow of the CNPQ.

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Table 1: Antibodies and protocol of immunohistochemical reaction

Antibody (clone)	**Dilution**	**Antigen retrieval**	**Secondary antibody**
Anti-CD4[1] (0.N.52)	1:100	EDTA buffer (pH=8.0 for 30 minutes at 98ºC)	Envision
Anti-CD25[1] (N-19)	1:50	EDTA buffer (pH=8.0 for 30 minutes at 98ºC)	Envision
Anti-CTLA-4[1] (C-19)	1:1200	Citrate buffer (pH=6.0 for 30 minutes at 95ºC)	Kit-LSAB
Anti-FoxP3[1] (236A/E7)	1:400	Citrate buffer (pH=6.0 for 30 minutes at 95ºC)	Kit-LSAB
Cyclin B1[2] (7A9)	1:40	Citrate buffer (pH=6.0 for 30 minutes at 95ºC)	Kit-LSAB

[1]FromSanta Cruz Biotechnology (Santa Cruz, CA); [2]From Novocastra (New Castle, UK).

Table 2 Main clinical findings of patients with OSCC (oral cavity and lip):

Clinical features		OCSCC (%)		LSCC (%)
Age	≤ 60 years	38.8		47.2
	> 60 years	61.2		52.8
Gender	Male	61.1		72.2
	Female	38.9		27.8
Ethnic group	Caucasian	55.6		52.8
	Non Caucasian	44.4		47.2
Location	Tongue	44.4		-
	Floor of the mouth	27.8		-
	Superior lip	-		11,1
	Inferior lip	-		88.9
	Others	27.8		-
Tobacco	Yes	93.75		73.9
	No	6.25		26.1
Alcohol	Yes	50		66.7
	No	50		33.3
T stage	T1-T2	0		78.5
	T3-T4	100		21.5
Clinical outcome	Dead	44.4		2.8
	Alive (overall survival)	55.5		97.2

Survival time	≥ 48 months	0		37.5
	< 48 months	100		62.5

Legends of Figures

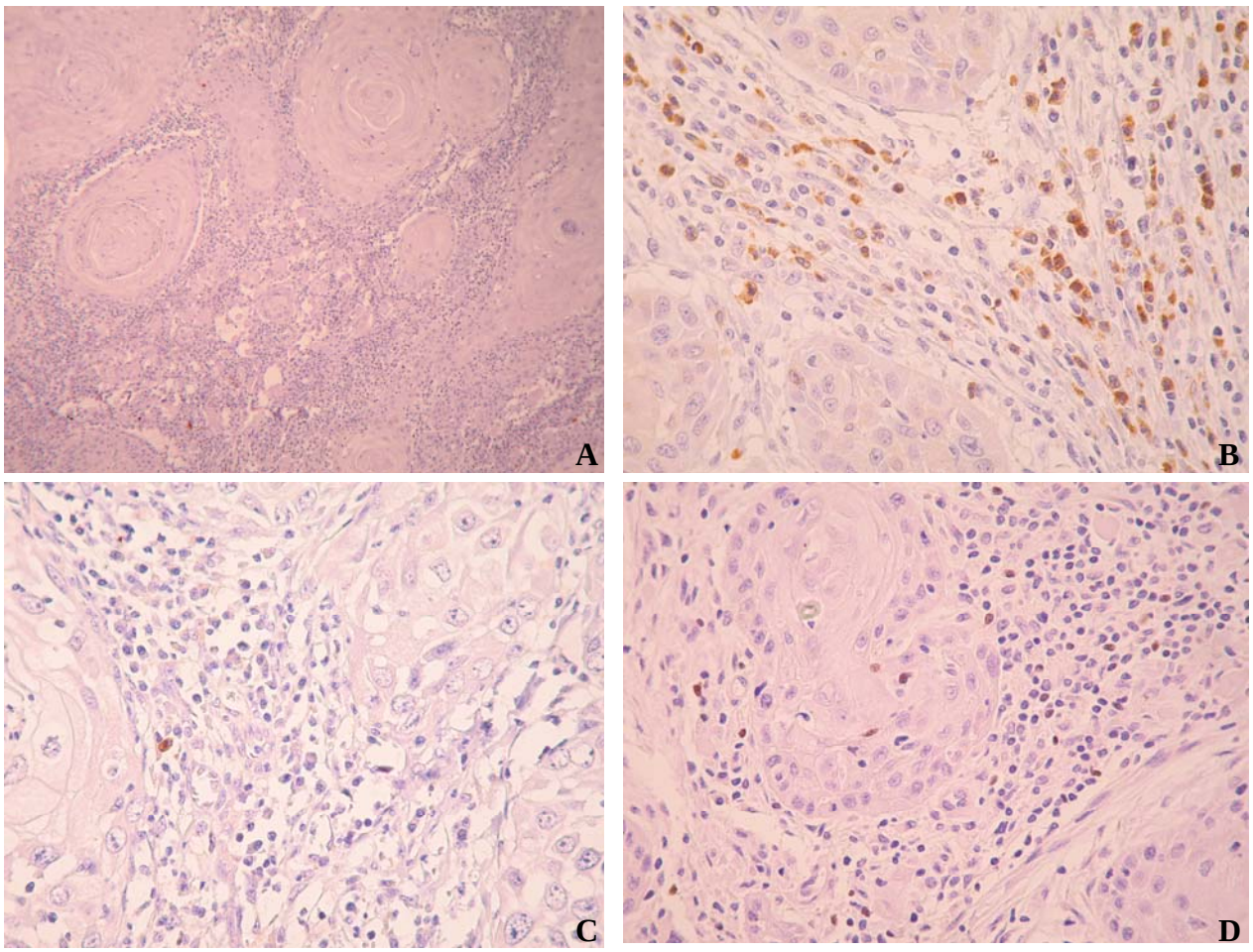

Figure 1 Representative immunostaning for CD4 (A), CD25 (B), CTLA-4 (C) and Foxp3 (D) in oral squamous cell carcinoma of OSCC. Positive cells for CD4 (100X), CD25 (100X), CTLA-4 (100X) and Foxp3 (X400) distributed throughout the tumoral stroma.

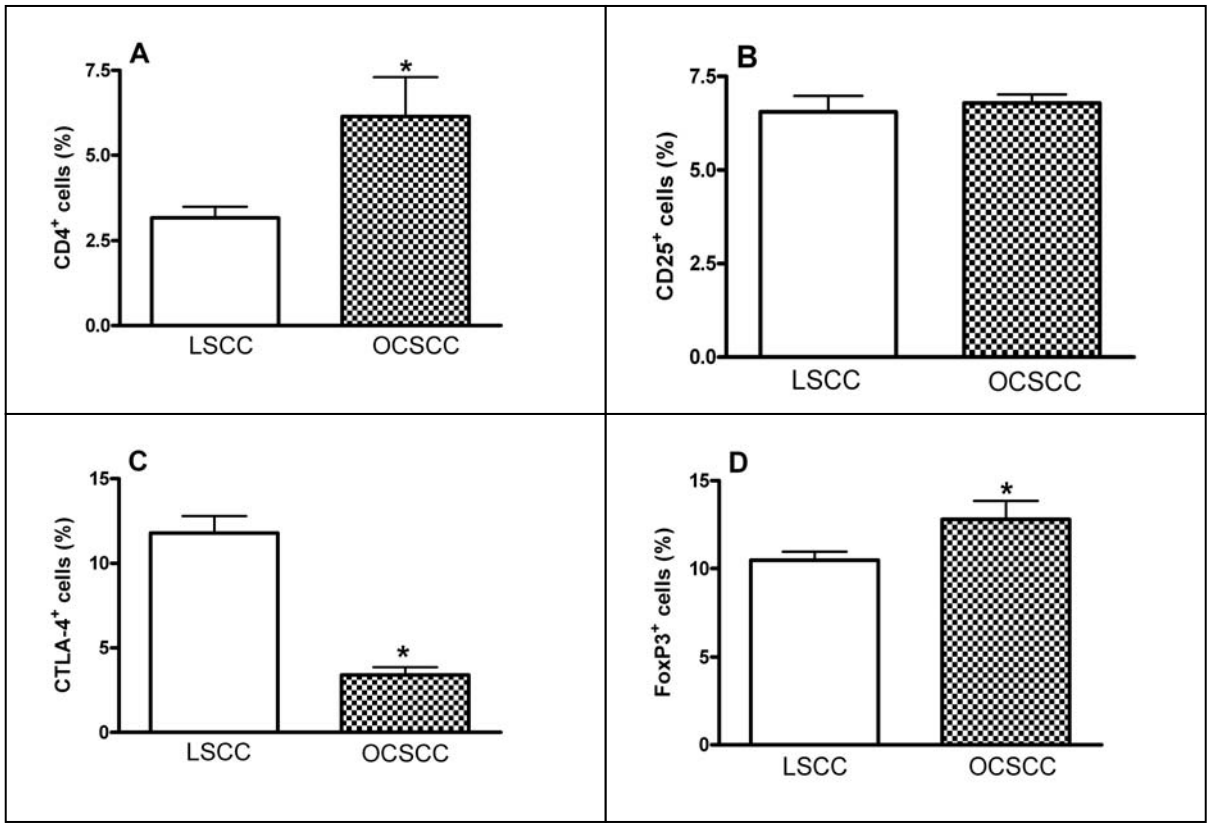

Figure 2 Densities of CD4 (A), CD25 (B), CTLA-4 (C) and FoxP3 (D) positive cells in stroma of primary squamous cell carcinoma of lip (LSCC) and oral cavity (OCSCC). Results are expressed as the mean of percentage of positive cells ± SD. All counts were performed in 15 alternate microscopic high power fields as described in Material and methods.

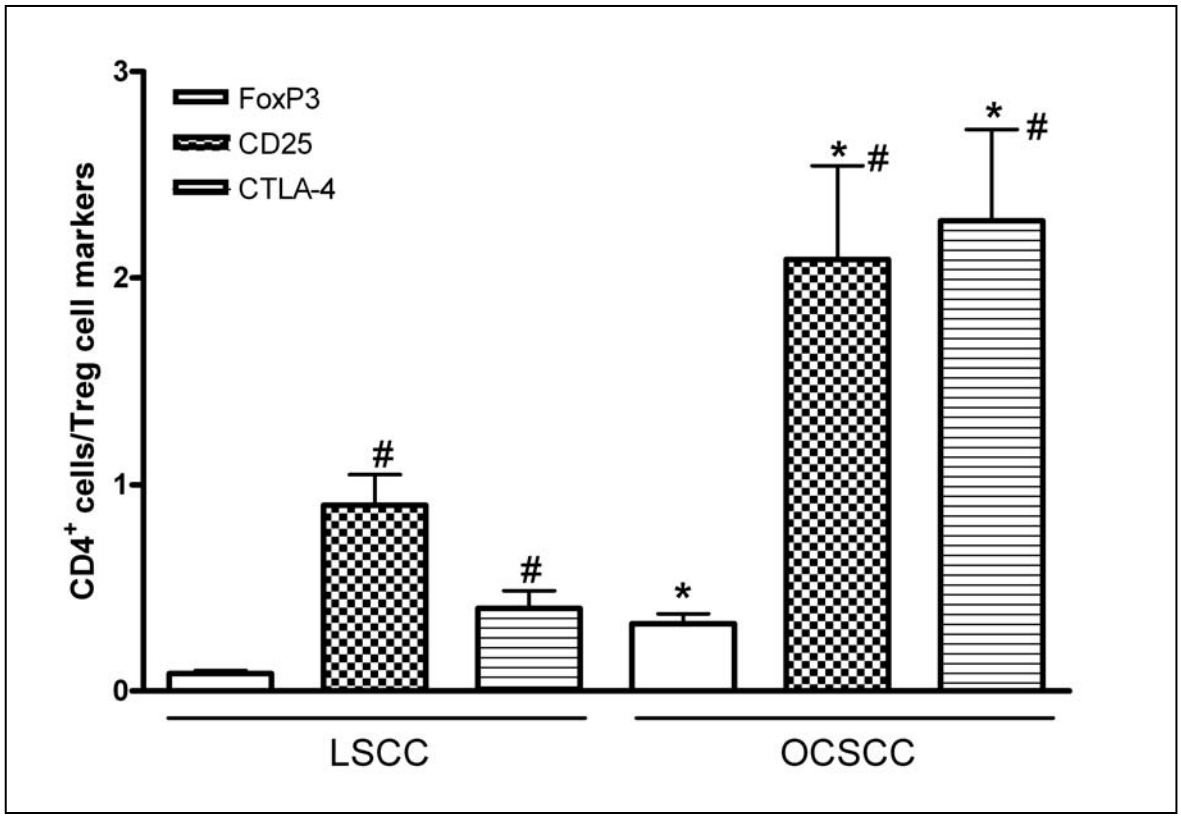

Figure 3 CD4/Treg markers ratio in primary squamous cell carcinoma of lip (LSCC) and oral cavity (OCSCC). The total number of CD4 positive cells (mm^2) was divided by number of CD25, CTLA-4 and FoxP3 positive cells (mm^2) to obtain CD4/Treg markers ratio in each respective group.

*$p<0.05$ when comparing LSCC and OCSCC for each respective marker ≠ $p<0.05$ when comparing Foxp3 in relation to other markers.

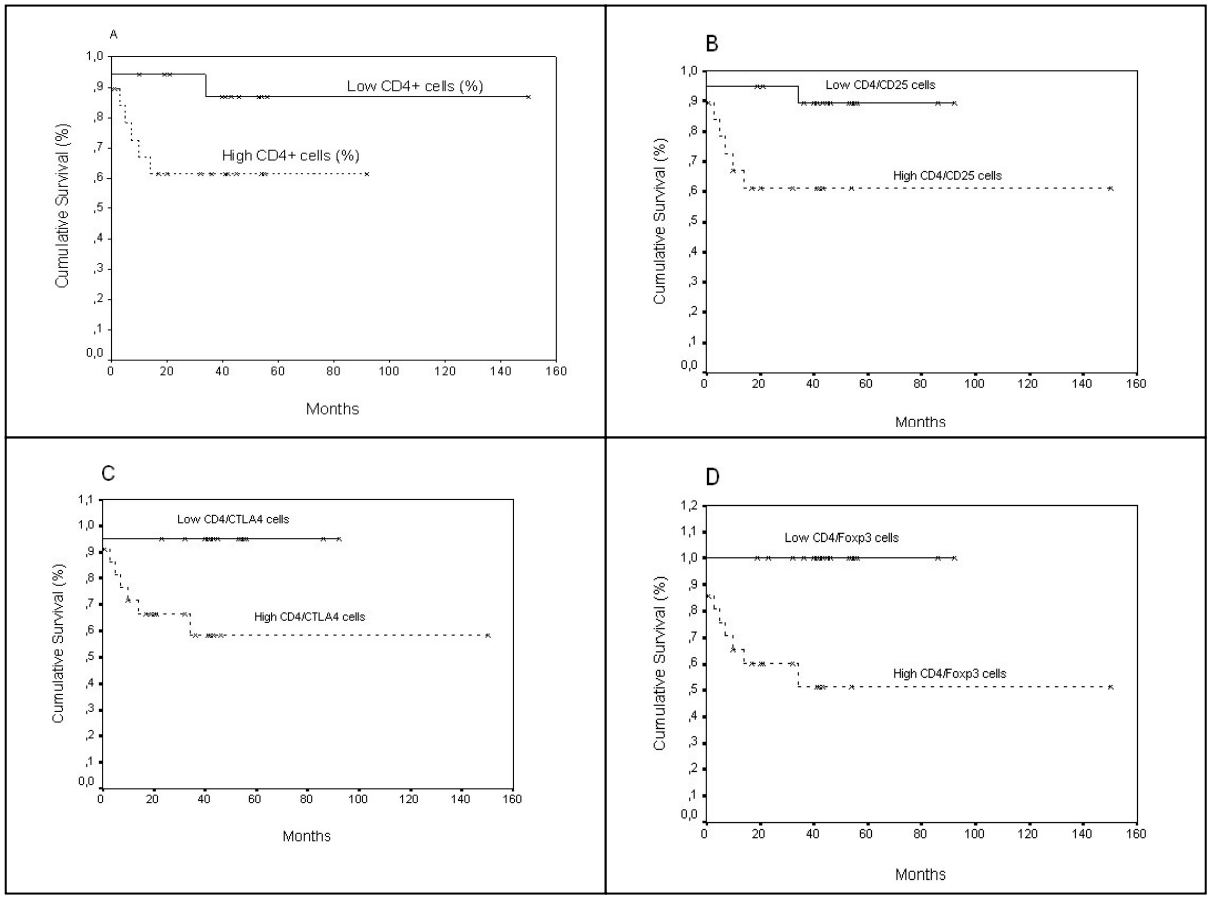

Figure 4 Kaplan-Meier survival curves, according to density status of CD4 (A), CD4/CD25 (B), CD4/CTLA-4 (C) and CD4/FoxP3 (D) in primary oral squamous cell carcinoma of lip and oral cavity. CD4, CD25, CTLA-4 and FoxP3positive cells were dichotomized by median values (high groups n=24 and low groups n=22).

Considerações Finais

A imunidade inata e adaptativa desempenha um papel essencial na vigilância imunológica e destruição tumoral. Ambos os efeitos das respostas imunológicas são regulados por diferentes tipos celulares, dentre os quais se incluem as células T regulatórias (Treg) [9-13]. Em tumores humanos, as células Treg estão relacionadas à supressão da resposta imunológica podendo então contribuir para o crescimento tumoral [12, 16-29].

No presente estudo foi encontrado um elevado percentual de células positivas para CD4 e Foxp3 em amostras de CECB quando comparado ao índice de marcação no CCEL. Além disso, a relação CD4/Foxp3, CD4/CD25, e CD4/CTLA-4 foi significativamente maior no CECB o que indica uma maior expressão fenotípica desta célula nesta lesão. Resultados semelhantes têm sido encontrados revelando aumento da população de células Treg em outros tipos de câncer, tais como pâncreas [24], tumores ovarianos [23], melanoma metastático [29] e neoplasias malignas de cabeça e pescoço [18, 30,31]. Nossos dados, também indicam que a maior população celular dentro das células CD4 positivas são as células CD25 para CCEL e igualmente CD25 e CTLA-4 para o CECB sugerindo um diferente perfil celular nestas lesões.

A proporção encontrada de células positivas para Foxp3 foi menor que CD4/CTLA-4 e CD4/CD25 em ambas as lesões. O Foxp3 é um membro da família de fatores transcripcionais que está relacionado com o desenvolvimento e função das células Treg [13, 15,33]. Uma menor proporção de células positivas

CD4/Foxp3 sugere que outros tipos celulares poderiam contribuir para a expressão do Foxp3 em ambas as lesões. De fato, a expressão do Foxp3 foi transitoriamente induzida em células humanas através da ativação do receptor T celular [32,33]. Recentemente, a expressão do Foxp3 foi atribuída estar restrita a linhagem de células T. No entanto, alguns trabalhos observaram a expressão do Foxp3 em células neoplásicas do melanoma [34] e vários outros tipos de células tumorais [35]. Entretanto, no presente trabalho, não foi observado a expressão deste marcador em células epiteliais neoplásicas. Também é importante considerar que a avaliação do número de células positivas não reflete necessariamente o nível de expressão das moléculas de cada célula. Soma-se a isso, o fato da expressão do Foxp3 poder ser influenciada por diferentes citocinas tais como TGF-B, IL-10 ou IL-2 [13, 15, 33].

O CTLA-4 inibe a ativação da célula T e também finaliza a resposta da célula T pelo bloqueio de sinais estimuladores via CD28. Em geral, o CTLA-4 não está expresso em células T $CD4^+$ $CD25^-$ mas a sua estimulação sobre as células T pode ser induzida por diferentes mecanismos [11, 13 14]. Muitas evidências apontam para a importância do bloqueio do CTLA-4 para a prevenção de malignidades e invasão metastática [16, 17, 20, 22, 26, 27]. Além disso, no CECB, o polimorfismo A/A do gene *CTLA-4*, que resulta em alto fenótipo produtor, foi associado a uma menor sobrevida [36].

Inesperadamente, nossos resultados mostraram uma maior expressão do CTLA-4 em CCEL e um aumento significativo da sobrevida em pacientes com maiores contagens de CTLA-4. Sabe-se que pacientes com CCEL usualmente

apresentam bom prognóstico e baixo índice de mortalidade e de metástases em linfonodos regionais quando comparado com CECB [37,38]. Estes resultados podem em parte ser explicados pela recente demonstração da função do CTLA-4 sobre a destruição das células tumorais *in vivo* via interação com B7. Além disso, a expressão do CTLA-4 não é exclusiva de células Treg [9, 12, 14]. Neste sentido, foi considerado que em pacientes com elevadas contagens de CD4/CTLA-4 observou-se um prognóstico ruim, o que vai ao encontro do potencial papel do bloqueio do CTLA-4 na imunoterapia para o câncer [16, 17, 20, 22, 26, 27].

Os pacientes com elevadas contagens de CD4 e Foxp3 exibiram um maior índice de proliferatividade. Em acordo com estes resultados, pacientes com baixas contagens de CD4 revelaram um aumento significativo da sobrevida em relação aos pacientes com altas contagens. Além disso, observou-se uma tendência dos grupos com elevadas contagens de Foxp3 e CD25 apresentarem menor sobrevida quando comparados com grupos com baixas contagens destas células. Quando foi avaliada a dupla positividade para CD4/CD25 e CD4/Foxp3 observou-se que pacientes com maiores contagens mostraram redução significativa da sobrevida. Tais achados são semelhantes a estudos prévios que descreveram uma associação de células Treg com a inibição da imunidade anti-tumoral e conseqüente prognóstico desfavorável [16-29].

Os achados deste trabalho sugerem uma associação das células T $CD4^+$ $CD25^+$ CTLA-4^+ $Foxp3^+$ com prognóstico ruim o que pode resultar da supressão da resposta imune anti-tumoral pelas células Treg no CECB.

Conclusões

Com base nos achados obtidos, podemos concluir que existe uma relação das células T regulatórias com a inibição da imunidade anti-tumoral no carcinoma de células escamosas de boca e lábio conseqüente com prognóstico clínico desfavorável em pacientes com elevada população de células com este fenótipo.

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