

The correlation between tumor-infiltrating Foxp3+ regulatory T cells and cyclooxygenase-2 expression and their association with recurrence in resected head and neck cancers

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Abstract The aim of this study was to investigate the correlation between tumor-infiltrating CD4+ CD25^{high} Foxp3+ naturally occurring regulatory T cells (Foxp3+ nTregs) and cyclooxygenase-2 (COX-2) expression and their association with local recurrence in resected head and neck cancers. Intratumoral COX-2 and Foxp3+ nTregs expressions were retrospectively assessed using immunohistochemistry. Associations between the clinicopathological characteristics and either intratumoral COX-2 expression or number of Foxp3+ nTregs were tested using the Chi-square test. The correlation between the number of Foxp3+ nTregs and COX-2 expression was tested using Spearman's rank correlation test. Associations between recurrence-free survival (RFS) and either intratumoral COX-2 expression or number of Foxp3+ nTregs were calculated using the Kaplan–Meier method, and factors that may influence the RFS were analyzed by Cox regression. The five-year RFS for all

patients was 35.09%. Patient clinicopathological characteristics had no relationship with intratumoral COX-2 expression or the number of Foxp3+ nTregs. However, a positive correlation between intratumoral COX-2 expression and the number of Foxp3+ nTregs was observed ($P < 0.001$). The RFS of patients with elevated COX-2 expression was significantly worse than that of patients without intratumoral COX-2 expression ($P = 0.0228$). The RFS of patients with tumors containing >6 Foxp3+ cells was significantly worse than that of patients with tumors containing ≤ 6 Foxp3+ cells ($P = 0.0020$). However, by Cox regression analysis, the RFS of all patients was not influenced by intratumoral COX-2 expression ($P = 0.100$) or the number of Foxp3+ nTregs ($P = 0.071$). Tumor-infiltrating CD4+ CD25^{high} Foxp3+ nTregs were positively correlated with intratumoral COX-2 expression and were associated with a worse RFS in univariate analysis.

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Introduction

Head and neck cancers include all epithelial malignancies that occur in the lips, oral cavity, oropharynx, nasopharynx, hypopharynx, larynx, nasal cavity (sinus), and salivary glands. They are phenotypically and biologically heterogeneous diseases with widely variable patterns of behavior that represent a paradigm for cancer prevention. Surgical resection still plays an important role in the treatment of early-stage head and neck cancers, but the relapse rate is high. Despite having identical radiological and histological features, many patients with presumed localized disease may have undetectable metastases at the time of diagnosis. This

implies that some biological features may influence the risk of disease relapse.

Advances in the molecular biology of head and neck cancers have led to increased interest in the study of their prevention. Most head and neck cancers are accompanied by overexpression of cyclooxygenase-2 (COX-2), which is an inducible enzyme responsive to cytokines, growth factors, oncogenes, or tumor promoters during cancer [1]. Its rapid induction results in enhanced synthesis of prostanoids, especially prostaglandin E2 (PGE2), in neoplastic tissues [2]. Elevated levels of PGE2 at the tumor site have several procarcinogenic effects. One of the effects is to promote expansion of CD4+ CD25^{high} Foxp3+ naturally occurring regulatory T cells (Foxp3+ nTregs) [3] in the COX-2-positive microenvironment. Foxp3+ nTregs are a subset of CD4+ T cells, which can express the transcription factor forkhead box protein-3 (Foxp3), and may accumulate in the tumor microenvironment where they suppress tumor-specific T-cell responses, thereby hindering tumor rejection. In cancer patients, accumulation of Foxp3+ nTregs in the tumor microenvironment has been associated with a significant reduction in survival [4, 5].

In this study, we investigated the prognostic value of intratumoral COX-2 expression and tumor-infiltrating Foxp3+ nTregs and determined whether a correlation existed between the expression of COX-2 and Foxp3+ nTregs in head and neck cancers.

Materials and methods

Inclusion criteria of the patients

All the patients had undergone a primary lesion resection and lymph node dissection and had been followed up for at least 2 years. All the patients presented with local disease without any distant metastases at the time of diagnosis, and first received surgical resection without any induction therapy. The baseline demographics, recurrence-free survival (RFS) period, and pathological specimens preserved in paraffin were available for all the patients. Pathological and surgical records of the primary surgery were obtained for all patients. The primary surgery was radical resection, the surgical margins were microscopically negative and there was no residual tumor. Recurrence included primary tumor site and regional lymph nodes relapse. Patients who first presented with distant metastases were not considered to be recurrent cases. A written informed consent was obtained from each patient before surgery.

Patient characteristics

Eighty-three patients with head and neck cancers who underwent resection from January 2004 to January 2008

were retrospectively studied. There were 48 men and 35 women. The median age of the patients was 57 years (range, 36–78 years). The median time of follow-up was 43 months (range, 24–75 months). The stage of postoperation was defined according to the 2002 American Joint Committee on Cancer (AJCC) classification system. Forty-three patients received postoperative adjuvant radiotherapy and the remaining 40 patients did not receive adjuvant radiotherapy. All patient characteristics are listed in Table 1.

Radiotherapy

Radiotherapy is one of the most important means to improve local control of head and neck cancers. Thus, in this study, the influence of radiotherapy on RFS was taken into account. For patients who received postoperative radiotherapy, the irradiation techniques included conventional radiotherapy, 3-D conformal radiation therapy, and intensity-modulated radiation therapy. Patients received a tumor dose of 50–60 Gy by 1.8–2.0 Gy daily fractions for 5 days a week. The irradiation target volume included the primary tumor bed and likely involved structures and regional lymph nodes.

Immunohistochemical staining

Immunohistochemical analyses were performed on resected, paraffin-embedded head and neck cancer tissues. After microtome sectioning (5 µm), the slides were processed for COX-2 and Foxp3 staining by an experienced operator. The streptavidin–biotin–peroxidase detection technique using diaminobenzidine as a chromogen was applied. The procedure was as follows: The sections were first deparaffinized in xylene followed by 100% ethanol and rehydrated with graded ethanol solutions. The sections were pretreated to promote antigen retrieval by steaming at 90°C for 20 min in DAKO Target Retrieval Solution (DAKO Corp., Carpinteria, CA, USA), followed by a 20-min cooldown at room temperature in the retrieval solution. Samples were then quenched in 3% hydrogen peroxide solution for 5 min. Incubation with the primary antibody was performed at room temperature for 1 h. The primary antibodies were used according to the manufacturer's instructions (COX-2: Cayman Chemical, Ann Arbor, Michigan, USA; Foxp3: Abcam, Cambridge, UK). The slides were then incubated with horse anti-mouse secondary antibody, labeled with avidin–biotin complex streptavidin–peroxidase (DAKO Corp.), incubated with the chromagen diaminobenzidine tetrahydrochloride, lightly counterstained with hematoxylin, and mounted.

The slides were examined by an experienced pathologist, with no knowledge of the corresponding clinicopathological

Table 1 The patient characteristics by COX-2 and Foxp3

| Characteristics | <i>n</i> | COX-2 expression | | <i>P</i> | Foxp3+ nTreg count | | <i>P</i> |
|-------------------------------------|----------|------------------|----------|----------|--------------------|----|----------|
| | | Positive | Negative | | >6 | ≤6 | |
| Patients, number | 83 | 41 | 42 | | 38 | 45 | |
| Median age | 57 | 56 | 58.5 | 0.531 | 56 | 57 | 0.865 |
| Sex | | | | | | | |
| Male | 48 | 22 | 26 | 0.447 | 18 | 30 | 0.076 |
| Female | 35 | 19 | 16 | | 20 | 15 | |
| Primary tumor site | | | | | | | |
| Larynx | 23 | 11 | 12 | 0.581 | 9 | 14 | 0.350 |
| Hypopharynx | 16 | 7 | 9 | | 5 | 11 | |
| Oral cavity | 25 | 11 | 14 | | 13 | 12 | |
| Others | 19 | 12 | 7 | | 11 | 8 | |
| Pathological type | | | | | | | |
| Squamous cell carcinoma | 71 | 36 | 35 | 0.562 | 30 | 41 | 0.116 |
| Non-squamous cell carcinoma | 12 | 5 | 7 | | 8 | 4 | |
| Primary tumor size(T) | | | | | | | |
| T1 | 12 | 5 | 7 | 0.340 | 6 | 6 | 0.747 |
| T2 | 48 | 27 | 21 | | 23 | 25 | |
| T3 | 23 | 9 | 14 | | 9 | 14 | |
| Lymph node involvement(N) | | | | | | | |
| N0 | 38 | 17 | 21 | 0.435 | 15 | 23 | 0.289 |
| N+ | 45 | 24 | 21 | | 23 | 22 | |
| Clinical staging(TNM) | | | | | | | |
| I/II | 26 | 13 | 13 | 0.941 | 11 | 15 | 0.668 |
| III | 57 | 28 | 29 | | 27 | 30 | |
| Postoperative adjuvant radiotherapy | | | | | | | |
| No | 40 | 21 | 19 | 0.586 | 15 | 25 | 0.144 |
| Yes | 43 | 20 | 23 | | 23 | 20 | |

COX-2 cyclooxygenase-2, *Foxp3* transcription factor forkhead box protein-3

data. To evaluate the COX-2 immunostaining, the reactions in smooth muscle and vascular endothelial cells, which were present in all the specimens, were used as internal controls. Cases with tumor cells that presented a significantly more intense staining pattern than the internal control cells were recorded as positive (Fig. 1a). To evaluate Foxp3 immunostaining, 10 high-power field (HPFs) digital images of the tumor areas were selected, and the absolute number of Foxp3+ lymphocytes in these 10 HPF digital images was determined. The number of immunostained Foxp3 cells was then determined by averaging the 10 HPF digital image cell counts (Fig. 1b).

Evaluation methods

Since the immunohistochemical staining of tumor-infiltrating Foxp3+ nTregs has seldom been reported in previous studies, there are no widely accepted standard cutoff points for defining the clinical outcome according to the number of

immunopositive Foxp3+ nTregs. Shimizu et al. [6] selected the median number of intratumoral Foxp3+ nTregs of the entire group as the cutoff value when they evaluated intratumoral Foxp3+ nTregs expression. In this study, we used the same approach and so all the patients were divided into two groups according to the cutoff value. When we analyzed the associations among COX-2 expression, number of Foxp3+ nTregs, clinicopathological features, and RFS, the following patient characteristics were investigated: sex, age, primary tumor site, pathological type, primary tumor size, lymph node involvement, clinical staging, and postoperative adjuvant radiotherapy.

Statistical analysis

The RFS was calculated from the end of the primary therapies (including surgery and/or adjuvant radiotherapy) to the appearance of tumor recurrence using the Life Table Method. The associations between the clinicopathological

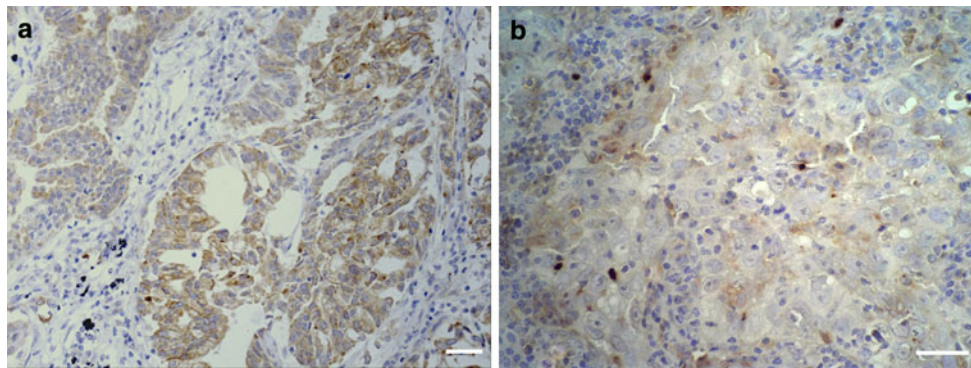


Fig. 1 Immunohistochemical staining for COX-2 (a) and Foxp3 (b). Bar:200 μ m

characteristics and either intratumoral COX-2 expression or number of Foxp3+ nTregs were tested using the Chi-square test. The correlation between the number of Foxp3+ nTregs and COX-2 expression was tested using Spearman's rank correlation test. The associations between RFS and either intratumoral COX-2 expression or number of Foxp3+ nTregs were calculated using the Kaplan–Meier method. Factors that may influence the RFS were analyzed by Cox regression. A difference was considered statistically significant when $P \leq 0.05$. Statistical analyses were performed using the SPSS statistical software program (SPSS® for Windows Release 15.0, SPSS Inc., Chicago, IL, USA).

Results

Associations between the clinicopathological characteristics and either intratumoral COX-2 expression or number of Foxp3+ nTregs

For all patients, the median number of intratumoral Foxp3+ nTregs of the entire group was 6(0–21)/10 HPFs. Among them, the number of intratumoral Foxp3+ nTregs in 38 patients was greater than 6/10 HPFs, whereas the number in the remaining 45 patients was equal to or less than 6/10 HPFs. A markedly more intense COX-2 immunoreactivity was found in the tumor cells of 41 patients, whereas the remaining 42 cases did not show increased intratumoral COX-2 expression. There were no significant associations between sex, age, primary tumor site, pathological type, primary tumor size, lymph node involvement, clinical staging, postoperative adjuvant radiotherapy, and either COX-2 expression or number of Foxp3+ nTregs. All data are shown in Table 1.

Correlation between intratumoral COX-2 expression and number of Foxp3+ nTregs

A positive correlation between intratumoral COX-2 expression and the number of Foxp3+ nTregs was revealed

using Spearman's rank correlation test (correlation coefficient = 0.737, $P < 0.001$). In the COX-2-positive and COX-2-negative groups, the number of intratumoral Foxp3+ nTregs was $11.05 \pm 4.159/10$ HPFs and $3.90 \pm 2.712/10$ HPFs, respectively (Fig. 2). The difference in these counts was statistically significant ($P < 0.001$).

Associations between RFS and either intratumoral COX-2 expression or number of Foxp3+ nTregs

Thirty-seven patients presented with recurrent disease during follow-up, and the five-year RFS for all patients was 35.09% (Fig. 3). The RFSs of COX-2-positive and COX-2-negative patients were 24.65 and 54.92%, respectively. The RFSs of patients with >6 Foxp3+ nTregs and ≤ 6 Foxp3+ nTregs were 20.90 and 60.19%, respectively. The RFS of patients with elevated COX-2 expression was significantly worse than that of patients without intratumoral COX-2

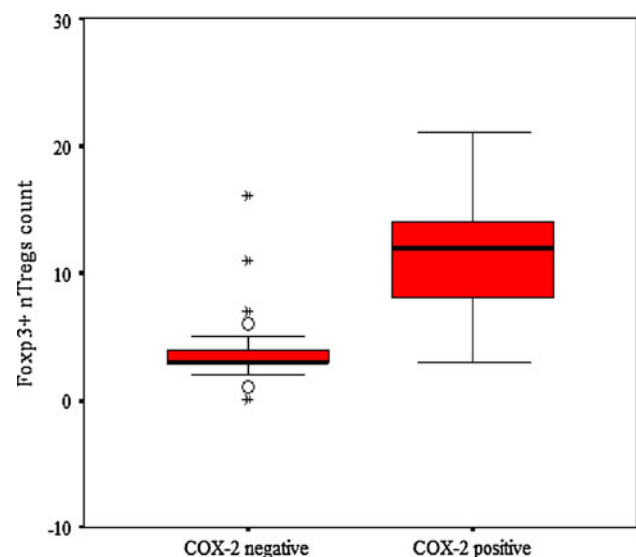


Fig. 2 The correlation of COX-2 expression and Foxp3+ nTregs count. $P < 0.001$

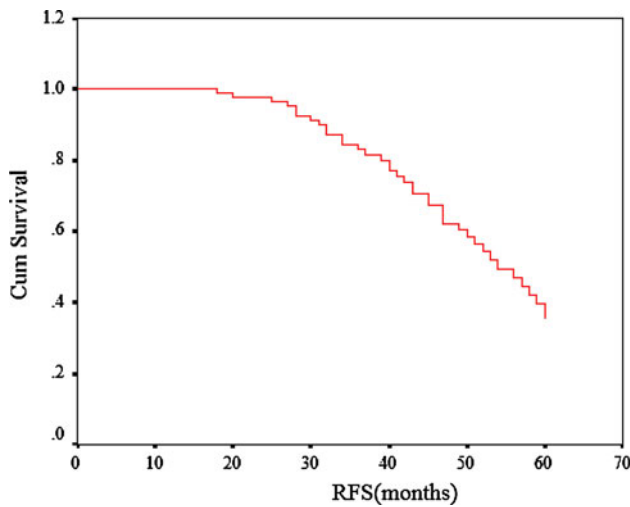


Fig. 3 Life tables 5-year RFS curve for all patients. The 5-year RFS was 35.09%

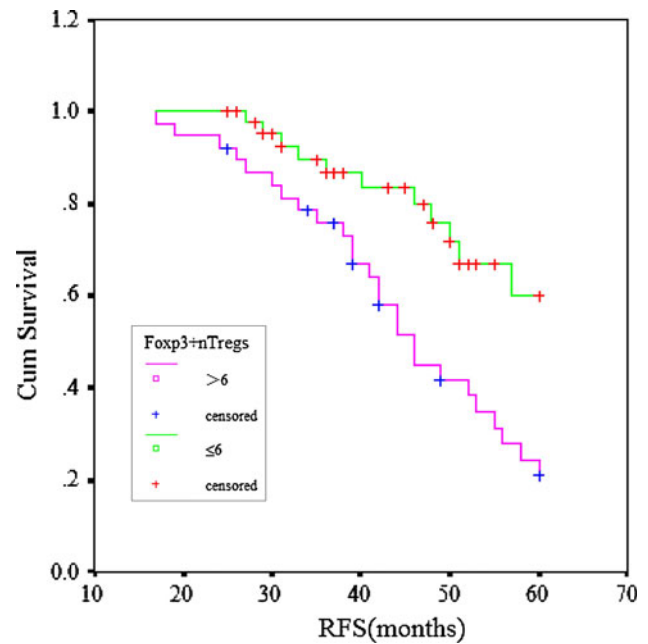


Fig. 5 Kaplan–Meier 5-year RFS curve for the Foxp3+ nTregs> 6/10HPFs and Foxp3+ nTregs ≤6/10HPFs groups. Log-rank $P = 0.0020$

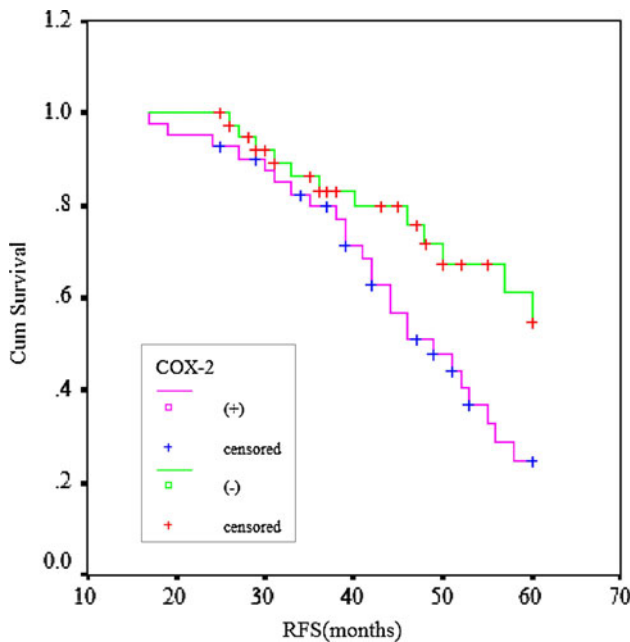


Fig. 4 Kaplan–Meier 5-year RFS curve for the COX-2(+) and COX-2(-) groups. Log-rank $P = 0.0228$

expression ($P = 0.0228$; Fig. 4). Furthermore, the RFS of patients with tumors containing >6 Foxp3+ nTregs was significantly worse than that of patients with tumors containing ≤ 6 Foxp3+ nTregs ($P = 0.0020$; Fig. 5).

Prognostic value of intratumoral COX-2 expression and number of Foxp3+ nTregs

In the above univariate analysis, intratumoral COX-2 expression and number of Foxp3+ nTregs were positively

Table 2 The results of cox regression analysis

| Factors | <i>P</i> | Factors | <i>P</i> |
|-------------------------------------|----------|--------------------|----------|
| Primary tumor size | 0.000 | Primary tumor site | 0.714 |
| Lymph node involvement | 0.001 | Pathological type | 0.499 |
| Postoperative adjuvant radiotherapy | 0.001 | Clinical staging | 0.832 |
| COX-2 expression | 0.100 | Sex | 0.712 |
| Foxp3+ nTregs count | 0.071 | Age | 0.248 |

COX-2 cyclooxygenase-2

Foxp3 transcription factor forkhead box protein-3

associated with the worse RFS of patients. However, the Cox regression analysis revealed that only primary tumor size, lymph node involvement, and postoperative adjuvant radiotherapy were positively correlated with the RFS. The remaining factors, including intratumoral COX-2 expression and number of Foxp3+ nTregs were not correlated with the RFS (P values are shown in Table 2). For analysis of the factors which may affect the RFS, postoperative adjuvant radiotherapy was found to be a protective factor (OR 0.297, 95%CI 0.145–0.606), whereas primary tumor size and lymph node involvement were independent risk factors (OR 3.369, 95%CI 2.015–5.632 and 3.886, 95%CI 1.781–8.480, respectively).

Discussion

nTregs are generated in the thymus and are defined as CD4+ CD25^{high} Foxp3+ nTregs. Foxp3+ nTregs, upon T-cell receptor (TCR) engagement [7, 8], exert their immunosuppressive effects in a contact-dependent fashion. However, it has been shown that the expression of major histocompatibility complex-II (MHC-II) and inducible co-stimulator (ICOS) characterizes Foxp3+ nTregs that are functionally different [9, 10]. These latest data suggest that Foxp3+ nTregs can be further differentiated into discrete subsets that are distinct in their reliance on cell-to-cell contact or on interleukin 10 (IL-10) and tumor growth factor- β (TGF- β) production for their functional activities. These studies have also implicated Foxp3+ nTregs in the suppression of the immune response against tumors [11, 12]. However, accumulating evidence has demonstrated a significant increase in the number of Foxp3+ nTregs in the tumor microenvironment of patients with various types of cancer [13, 14]. Moreover, a higher accumulation of Foxp3+ nTregs is often associated with advanced disease stages and is inversely correlated with favorable prognosis and overall survival [4, 5]. In our study, we also found that a higher number of Foxp3+ nTregs in the tumor microenvironment was associated with a worse RFS of patients with head and neck cancers.

Cyclooxygenase (COX) is the key enzyme in the metabolism of prostaglandin (PG). To date, two isoforms of this enzyme have been characterized: COX-1 and COX-2. COX-2 can be induced by a wide spectrum of growth factors and cytokines in pathophysiological states [15]. An increased expression of COX-2 is commonly found in both premalignant and malignant tissues [16, 17]. Overexpression of COX-2 in epithelial cells has been shown to inhibit apoptosis and increase the invasiveness of tumor cells. In head and neck cancers, COX-2 is expressed in both the tumor tissue and the adjacent epithelium, with a higher expression level in invasive carcinoma compared to normal epithelium [18]. Furthermore, COX-2 overexpression correlates with tumor aggressiveness and poor prognosis [19–21]. The following COX-2 procarcinogenic effects are carried out by elevated levels of PGE₂ at the tumor site: (1) direct stimulation of tumor growth and inhibition of immune surveillance [22, 23], (2) induction of tumor angiogenesis and promotion of metastases of cancer cells [24, 25], (3) prevention of apoptosis induced by anticancer drugs [26, 27], (4) indirect suppression of dendritic cell functions and antitumor T-cell responses [18, 28], and (5) induction and accumulation of different types of immune suppressor cells at the tumor site, which facilitate expansion of CD4+ CD25^{high} Foxp3+ nTregs [29]. Similar findings were observed in this study. Here, we found that COX-2 overexpression correlated with a worse RFS of patients with head and neck cancers, which implies poor prognosis.

Notably, in our study, we found that COX-2 overexpression was positively correlated with the number of Foxp3+ nTregs in the local microenvironment of head and neck cancers. This could be because COX-2 facilitated expansion of CD4+ CD25^{high} Foxp3+ nTregs in the COX-2-positive tumor microenvironment through PGE₂. As for the correlation between COX-2 expression and number of Foxp3+ nTregs in the tumor microenvironment of patients, only Li et al. [30] and Shimizu et al. [6] have reported that an increase in the number of peritumoral Foxp3+ nTregs was associated with a worse prognosis and was positively correlated with intratumoral COX-2 expression in patients with renal cell carcinoma and non-small cell lung cancer. Here, we present the first report of a similar phenomenon in head and neck cancer. These findings might provide us with a new strategy and evidence for the application of anticancer treatment using COX-2 inhibitors, which could inhibit the production of PG and reduce the accumulation of Foxp3+ nTregs in the COX-2-positive tumor microenvironment. Thus, both the suppression of the immune response against tumors and the prognosis of patients could be improved. In fact, a recent clinical trial by the Cancer and Leukemia Group B demonstrated that patients with increased COX-2 expression receiving a COX-2 inhibitor had a better survival rate than COX-2-expressing patients who did not receive the drug [31].

Cox regression analysis revealed that intratumoral COX-2 expression and number of Foxp3+ nTregs had no significant influence on the RFS of patients. In contrast, postoperative adjuvant radiotherapy, primary tumor size and lymph node involvement were important influential factors on the RFS of patients. In fact, the relationship between these factors and prognosis of head and neck cancers has been recognized for many years, especially the protective function of radiotherapy on patient prognosis, which has resulted in radiotherapy becoming an important treatment modality of head and neck cancers. In this study, perhaps just because of the protective function of radiotherapy, the prognostic values of intratumoral COX-2 expression and number of Foxp3+ nTregs were covered. If there were a large cohort of patients without radiotherapy, different results (power calculation for COX-2/Treg as prognostic markers) might be displayed, and so further studies would be significant. Lissoni et al. [32] reported a dramatic statistically significant decrease in the number of both total lymphocytes and CD4+ cells in peripheral circulation after radiotherapy, whereas no substantial change was observed in the number of Foxp3+ nTregs. As for the influence of radiotherapy on tumor-infiltrating Foxp3+ nTregs, no studies were available.

Taken together, our results show that tumor-infiltrating Foxp3+ nTregs were positively correlated with intratumoral COX-2 expression and were associated with a worse

RFS in univariate analysis. Since there is a significant correlation between intratumoral COX-2 expression and number of Foxp3+ nTregs, a COX-2 inhibitor might be beneficial for the improvement of the antitumor immune response of patients that overexpress COX-2. Further studies examining the relationship between COX-2 expression and number of Foxp3+ nTregs in other types of cancer are required.

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