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Clinical significance of tumor-infiltrating FOXP3+ T cells in patients with ocular adnexal mucosa-associated lymphoid tissue lymphoma

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We evaluated the association between tumor-infiltrating FOXP3+ T cells and clinical outcomes in patients with ocular adnexal lymphoma of mucosa-associated lymphoid tissue type (OAML). Pretreatment formalin-fixed paraffin-embedded tissues from 42 patients with OAML were stained with 236A/E7 anti-FOXP3 murine monoclonal antibody as well as CD3, CD4 and CD8 antibodies. The amount of FOXP3+ T cells was numerically quantified using an image analysis program. Front-line treatments were as follows: combination chemotherapy (n = 25); radiotherapy (n = 9); doxycycline (n = 6); and wait and see (n = 2). Complete response (CR) was observed in 20 (50%) of 40 evaluable patients. Median progression-free survival (PFS) was 50 months. A high number of FOXP3+ T cells (n = 21, ≥180/0.58 mm^2) showed a higher CR rate (33% *vs* 71%, P = 0.013) and tendency towards prolonged PFS (48 *vs* 67 months, P = 0.110). In the combination chemotherapy group, a high number of FOXP3+ T cells was significantly associated with a higher CR rate (29% *vs* 82%, P = 0.008) and prolonged PFS (17 *vs* 79 months, P = 0.003). A high number of tumor-infiltrating FOXP3+ T cells correlates with a favorable clinical outcome in OAML patients. (*Cancer Sci* 2011; 102: 1972–1976)

Ocular adnexal non-Hodgkin lymphoma is a rare but steadily increasing malignancy, especially in Asian/Pacific Islander patients, according to the Surveillance, Epidemiology, and End Results Program.[1] Marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) type is the most common lymphoma arising in the orbital area.[2] Although various prognostic factors have been proposed, reliable prognostic factors remain to be defined because most patients with ocular adnexal lymphoma of MALT type (OAML) initially present with favorable prognostic features.[2] Recently, several studies have suggested the significance of tumor-infiltrating lymphocytes in anti-tumor immune surveillance. Favorable outcomes for cases with a high number of intratumoral T cells have been observed in patients with solid tumors, such as melanoma, ovarian cancer, gastric cancer and colon cancer.[3–5] In contrast, regulatory T cells (Tregs) contribute to the progression of human solid tumors by suppressing tumor-specific T-cell immunity.[6–9] The role of Tregs in lymphoma is complicated by the dynamic and bilateral process between malignant lymphocytes and host immune cells. Correlations between increased tumor-infiltrating Tregs and better prognosis have been reported for several types of lymphoma.[10–14] Chronic inflammation has been known to be associated with the pathogenesis of OAML, although the role of *Chlamydia psittaci* in OAML is less established than the role of *Helicobacter pylori* in gastric MALT lymphoma.[15–17] While *H. pylori*-specific activated T cells stimulate gastric MALT lymphoma growth, the role of tumor-infiltrating T cells has not been established in OAML patients.[18] Therefore, the present study was designed to evaluate the prognostic impact of tumor-infiltrating FOXP3+ T cells and other T-cell subsets in OAML patients.

Materials and Methods

Patients and samples. Seventy-two patients were newly diagnosed with ocular adnexal extranodal marginal zone B-cell lymphoma at Seoul National University Hospital between 1989 and 2006. Among them, pretreatment formalin-fixed paraffin-embedded tissues for immunohistochemical staining were available in 42 patients. Clinical data including the primary site, presenting symptoms, Eastern Cooperative Oncology Group performance status, lactate dehydrogenase (LDH) level, B symptoms, bone marrow involvement, Ann Arbor stage, International Prognostic Index (IPI), chemotherapy regimen, response to primary chemotherapy, date of diagnosis, date of progression, last follow up and survival status were retrieved from medical records. The present study was approved by the Institutional Review Board of Seoul National University Hospital. The recommendations of the Declaration of Helsinki for biomedical research involving human subjects were followed throughout. This work was carried out at Seoul National University Hospital, Seoul, Korea.

Immunophenotype of FOXP3, CD3, CD4, and CD8. Immunohistochemical staining for T-cell subsets was performed using CD3 (DakoCytomation, Copenhagen, Denmark), CD4 (Lab Vision, Fremont, CA, USA), CD8 (Lab Vision) and 236A/E7 anti-FOXP3 murine monoclonal antibody (eBioscience, San Diego, CA, USA). After heat-induced antigen retrieval by microwave, sections were incubated with CD3 (1:300 dilution), CD4 (1:50 dilution), CD8 (1:100 dilution) and FOXP3 (1:50 dilution) antibodies overnight at 4°C or for <1 h at room temperature. The DAKO Real Envision Detection system (DakoCytomation) was used throughout.

The number of tumor-infiltrating lymphocytes was counted in a representative tumor area using a microscope (Olympus, Tokyo, Japan) connected with a digital camera and an image analysis program (ImageJ 1.37a; http://rsb.info.nih.gov/ij). Briefly, a representative area of an immune-stained slide was selected and a picture was taken at high magnification using a ×20 objective with a digital camera by experienced pathologists (W. Y. K., Y. K. J. and C. W. K.). In representative tumor areas, tumor cells covered more than 80% of the area. The pictures taken at ×20 magnifications covered an area of 0.58 mm^2. The number of tumor-infiltrating FOXP3+ T-cells was counted automatically by ImageJ 1.37a.

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Fluorescence *in situ* hybridization (FISH) to detect MALT1 translocation. To detect MALT1 translocation, an interphase FISH assay was performed using 3-μm-thick sections of formalin-fixed paraffin-embedded tissues and LSI MALT1 dual-color breakapart probe (Abbott-Vysis, Downers, Grove, IL, USA). Briefly, following deparaffinization and dehydration, slides were immersed in 0.2N HCl, boiled in a microwave in citrate buffer (pH 6.0), incubated in pretreatment reagent for 40 min at 80°C and immersed for 40 min in a protease solution. The DNA probe set was applied to the slides, incubated in a humidified chamber at 75°C for 5 min to denature the target DNA and probe, and subsequently incubated overnight at 37°C to achieve hybridization. Following post-hybridization washing, a 4′, 6-diamidino-2-phenylindole (DAPI)/Antifade compound (*p*-phenylenediamine) was applied to the slide as a counterstain. The cells were analyzed using an Olympus BX51TRF microscope (Olympus Corp., Tokyo, Japan) equipped with the appropriate filter sets.

Statistical analysis. Correlations between clinicopathological parameters and the number of tumor-infiltrating lymphocytes were investigated using the Chi-squared test or Fisher's exact test after transformation into categorical values. Receiver operator characteristic (ROC) curves were plotted to determine an optimal cut-off value for the number of tumor-infiltrating lymphocytes that predicted a complete response (CR). Progression-free survival (PFS) was calculated from the date of first-line chemotherapy to the date of disease progression, death or last follow-up visit. Survival analysis was performed using the Kaplan–Meier method using the log-rank test. A value of $P < 0.05$ by two-sided test was considered statistically significant. Statistical analysis was performed using SPSS versions 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Patients and treatment outcomes. The clinicopathological characteristics of the 42 patients are presented in Table 1 and those of the 25 patients who received combination chemotherapy as a front-line treatment are also presented in Table 1. None of the patients had evidence of autoimmune diseases. Most patients initially presented with favorable features such as limited disease, good performance status and low IPI score. Twenty-two patients had measurable lesions using a radiological image and the median tumor size was 22 mm (range, 13–55 mm). Unmeasurable lesions were evaluated by ophthalmic examination with photography. Front-line treatment was administered as follows: combination chemotherapy in 25 patients; radiotherapy in nine patients; and doxycycline in six patients. There were no patients who were treated with rituximab. Twenty (50%) of 40 evaluable patients achieved CR. After a median follow up of 30 months, median PFS was 50 months (range, 1–79 months).

Quantification of tumor-infiltrating T-cell subsets and MALT1 translocation. Expression of FOXP3, CD3, CD4 and CD8 was not observed in the tumor cells of the 42 study subjects. The median number of tumor-infiltrating FOXP3-, CD3-, CD4- and CD8-positive T cells per 0.58 mm^2 was 180 (range, 41–742), 1813 (range, 522–3258), 1401 (range, 42–2957) and 1004 (range, 79–2677), respectively. A frequency-based histogram of the number of FOXP3+ T cells is shown in Figure 1. MALT1 translocation was not observed in 36 patients with available tumor tissues (Fig. 2).

Relationships between the number of tumor-infiltrating FOXP3+ T cells and clinical outcome. The optimal cut-off value for the number of tumor-infiltrating FOXP3+ T cells was equal to the median value with sensitivity of 68% and specificity of 70% to predict CR using the ROC curve. The area under the ROC curve was 0.636. Representative captured images of FOXP3-stained cells are illustrated in Figure 3. The numbers of CD3-, CD4- and CD8-positive T cells were not found to have the ability to predict CR by ROC analysis (data not shown). The number of tumor-infiltrating FOXP3+ T cells was correlated

Table 1. Clinical characteristics of ocular adnexal lymphoma of mucosa-associated lymphoid tissue-type patients and 25 of these patients who received combination chemotherapy

Characteristics	All patients (n = 42)	Chemotherapy group (n = 25)
Age (years)		
≤60	30 (71)	17 (68)
>60	12 (29)	8 (32)
Gender		
Male	27 (64)	20 (80)
Female	15 (36)	5 (20)
ECOG PS		
0	34 (81)	24 (96)
1	8 (19)	1 (4)
Anatomical location		
Conjunctiva	17 (41)	6 (24)
Orbit	13 (31)	11 (44)
Lacrimal gland	9 (21)	6 (24)
Eyelid	3 (7)	2 (8)
LDH		
Normal	27 (87)	13 (81)
Elevated	4 (13)	3 (19)
Bone marrow involvement		
Not involved	15 (94)	9 (90)
Involved	1 (6)	1 (10)
Ann Arbor stage		
IE	30 (71)	17 (68)
IIE	11 (26)	7 (28)
IVE	1 (3)	1 (4)
IPI		
Low	40 (95)	15 (94)
Low–intermediate	2 (5)	1 (6)

ECOG PS, Eastern Cooperative Oncology Group performance status; IPI, International Prognostic Index; LDH, lactate dehydrogenase. Values in parenthesis are expressed in percentage.

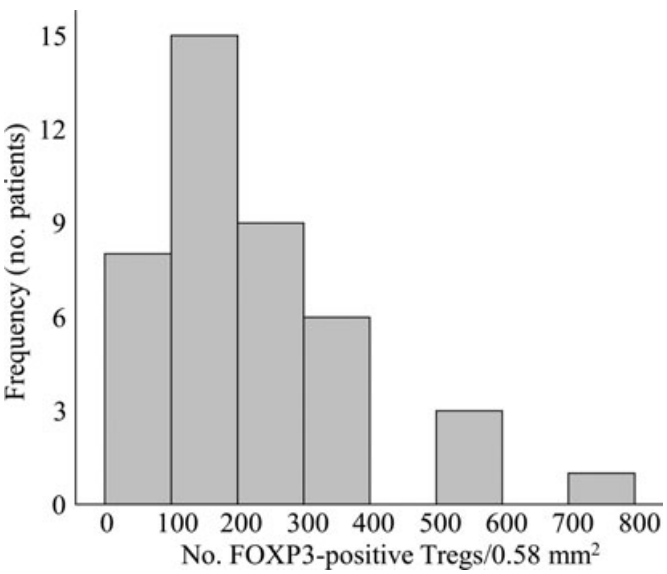

Fig. 1. Distribution of tumor-infiltrating FOXP3-positive regulatory T-cell (Treg) counts in ocular adnexal lymphoma of mucosa-associated lymphoid tissue type as shown on a frequency-based histogram.

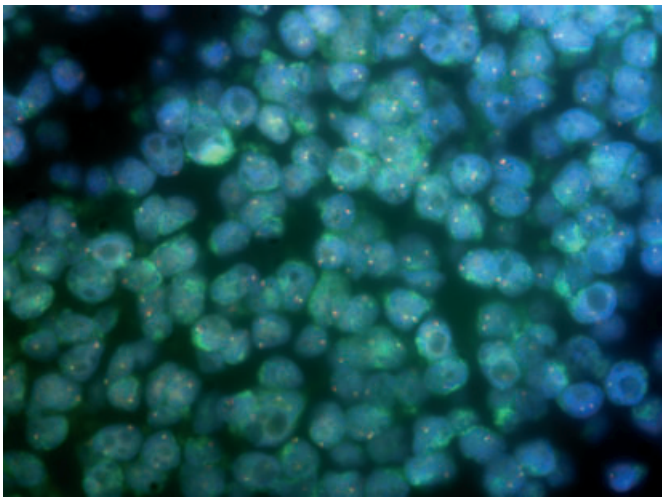

Fig. 2. Fluorescence *in situ* hybridization to detect MALT1 translocation showed negative signals.

with clinical outcome. A high number of FOXP3+ T cells (n = 21, ≥180/0.58 mm^2) showed a tendency towards prolonged PFS (48 *vs* 67 months, P = 0.110). In addition, it was significantly related to a higher CR rate (33% *vs* 71%, P = 0.013). Clinicopathological variables and chemotherapy regimens were evenly distributed between two groups according to the number of tumor-infiltrating FOXP3+ T cells (Table 2). When the analysis was limited to patients who had received combination chemotherapy, a high number of FOXP3+ T-cells was significantly associated with both higher CR rate (29% *vs* 82%, P = 0.008, Table 3) and prolonged PFS (17 *vs* 79 months, P = 0.034). The PFS according to the number of FOXP3+ T cells is plotted in Figure 4.

Table 2. Association between the number of tumor-infiltrating FOXP3+ T cells and clinicopathological features in 25 ocular adnexal lymphoma of mucosa-associated lymphoid tissue-type patients who received combination chemotherapy

Variables	FOXP3+ T cells		
	<180/0.58 mm^2, patients (%)	≥180/0.58 mm^2, patients (%)	*P**
Age (years)			
≤60	9 (64)	8 (73)	1.000
>60	5 (36)	3 (27)	
Gender			
Male	12 (86)	8 (73)	0.623
Female	2 (14)	3 (27)	
Lacrimal gland			
Not involved	11 (79)	8 (73)	1.000
Involved	3 (21)	3 (27)	
Tumor size (mm)			
<22	5 (36)	3 (27)	1.000
≥22	9 (64)	8 (73)	
LDH			
Normal	7 (100)	6 (67)	0.213
Elevated	0 (0)	3 (33)	
Ann Arbor stage			
IE–IIE	13 (93)	11 (100)	1.000
IIIE–IVE	1 (7)	0 (0)	
Chemotherapy			
CVP	9 (64)	8 (73)	1.000
Non-CVP	5 (36)	3 (27)	
Total	14 (100)	11 (100)	

**P*-values are of the interactions between the number of tumor-infiltrating T-cell subsets and clinical variables. CVP, cyclophosphamide, vincristine and prednisolone; LDH, lactate dehydrogenase.

Discussion

The present study shows that a high number of tumor-infiltrating FOXP3+ T cells is associated with a higher CR rate and prolonged PFS in patients with OAML, and this association is evident in patients who received combination chemotherapy as a front-line treatment. Patients with a high number of FOXP3+ T cells in the initial tumor tissue had a CR rate of 71% and a median PFS of 67 months.

Because the relationship between the extent of immune cell infiltration and prognosis was first identified in 1949 in cases of breast cancer, several studies have demonstrated the role of tumor-infiltrating T cells in antitumor immune surveillance and the role of Tregs in immune escape in patients with solid tumors.[3–5,7,19] A high number of tumor-infiltrating T cells has been related to prolonged survival whereas a high number of tumor-infiltrating Tregs has been associated with reduced survival in melanoma, colorectal and ovarian cancer.[3–5,7,19] However, the role of the T cells is more complex for lymphoma. Autoimmunity, chronic infection, antigenic stimulation and T cells play a pivotal role in the pathogenesis of some lymphomas.[20–22] Lymphomas that arise according to this model commonly originate from marginal zone B cells.[21,23] Furthermore, *in vitro* studies on gastric MALT lymphoma support the interaction between T cells and lymphoma cells. Hussell *et al.*[18] showed that the neoplastic B cells of gastric MALT lymphoma are not immunoresponsive to *H. pylori*, but that their proliferation is dependent on cognate help from *H. pylori*-specific T cells. In addition, one study revealed that Tregs can direct the

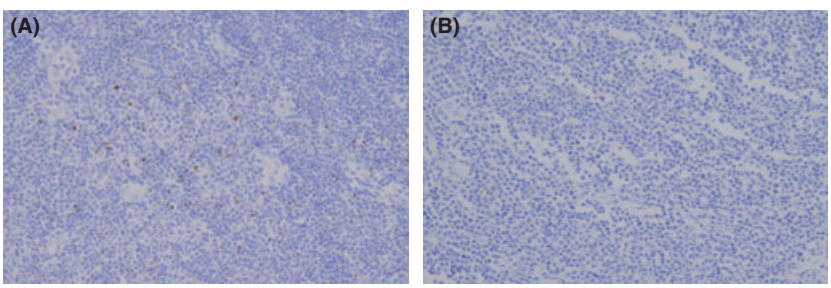

Fig. 3. Immunohistochemistry for FOXP3 in ocular adnexal lymphoma of mucosa-associated lymphoid tissue type. (A) Example of tumor tissue with a high number of tumor-infiltrating FOXP3-positive regulatory T-cells (Tregs) ≥180/0.58 mm^2. (B) Example of tumor tissue with a low number of tumor-infiltrating FOXP3-positive Tregs <180/0.58 mm^2 (original magnification, ×200).

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Table 3. Association between the number of tumor-infiltrating FOXP3+ T cells and complete remission

Response	FOXP3+ T cells		
	<180/0.58 mm^2, patients (%)	≥180/0.58 mm^2, patients (%)	*P**
CR (*n* = 13)	4 (29)	9 (82)	0.008
PR, SD, PD (*n* = 12)	10 (71)	2 (18)	
Total	14 (100)	11 (100)	

**P*-value for the test of the interaction of the treatment by the number of tumor-infiltrating T-cell subsets. CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease.

differentiation of conventional CD4-positive helper T cells toward an additional population of Tregs that inhibit the activation of conventional, freshly isolated CD4-positive helper T cells.[24] This suppressive activity is partially mediated by soluble transforming growth factor-β. It has also been shown that Tregs potently suppress follicular helper T cells and follicular helper T-cell-mediated B-cell functions such as immunoglobulin production and B-cell survival.[25,26] Thus, we hypothesized that Tregs would have antitumor activity in OAML by suppressing antigen-stimulated T cells. Consequently, it was found that the number of FOXP3+ T cells was significantly associated with clinical outcomes, whereas the number of CD3-, CD4- and CD8-positive T cells was not associated with either the number of FOXP3+ T cells or clinical outcome. The literature provides an explanation for the direct relationship between FOXP3+ T cells and B cells. Lim *et al.*[27] revealed that Tregs can directly suppress and even kill B cells. In this study, FOXP3+ T cells are present in B-cell areas where T–B cell interaction and humoral immune responses are believed to occur and can directly suppress the B-cell immunoglobulin response without having to suppress helper T cells. Zhao *et al.*[28] also reported that preactivated Tregs suppress B-cell proliferation and induce B-cell apoptosis in a cell-contact-dependent but cytokine-independent manner. Taken together, it might be considered that the lack of a significant effect of CD4- or CD8-positive T cells on clinical outcome is a result of the direct suppressive effect of FOXP3+ T cells on neoplastic B cells. Tregs have been consistently associated with improved overall survival in patients with several types of lymphomas such as follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL), Hodgkin lymphoma, T-cell and natural killer-cell lymphoma.[10,11,13,14,29] Carreras *et al.*[13] demonstrated that high numbers of FOXP3+ Treg improve the 5-year overall survival in FL patients. Similarly, Lee *et al.*[29] showed that FOXP3+ cells in a perifollicular location are more frequently found in FL patients with long-term survival. In addition, Alvaro *et al.*[14] demonstrated that a small number of FOXP3+ cells and a high proportion of TIA-1+ cells independently reduce event-free survival and disease-free survival in classic Hodgkin lymphoma. Recently, Tzankov *et al.*[11] performed a large-scale analysis using tissue microarray in 1019 patients with various types of lymphomas. High numbers of intratumoral FOXP3+ Treg correlates with improved survival in germinal center B cell like DLBCL, FL and classical Hodgkin lymphoma. Higher numbers of FOXP3+ T cells are associated with longer overall survival in patients with DLBCL.[30] Accordingly, our results produced a conclusion that corresponded with the literature on other types of lymphoma. In the current study, the association between the number of tumor-infiltrating FOXP3+ T cells and clinical outcome was more significant in patients treated with chemotherapy. This result might have meaning despite the small sample size. Replenishment of Tregs by conversion and proliferation has been extensively reviewed in the literature.[31] Although cyclophosphamide might target proliferation of Treg, replenishment by conversion would result in expansion of Tregs. Hypothetically, cyclophosphamide-containing chemotherapy used in the present study might selectively affect lymphoma cells, providing a way for Tregs to repopulate the tumor microenvironment. However, caution is warranted in interpreting these results due to the admixture of FOXP3+ effector T cells or Tregs as well as activated or resting Tregs. Furthermore, the unknown results of tumor-infiltrating FOXP3+ T cells after chemotherapy might weaken the repopulation hypothesis.

Although translocations that involve the MALT1 gene in chromosome 18 are the most frequent abnormality in OAML, *t*(11;18) and *t*(14;18) are very low in OAML (0–10% and 0–25%, respectively).[32,33] Similarly, none harbored MALT1 translocation in the present study.

Patients with OAML generally have a favorable prognosis and follow an indolent clinical course. Several retrospective studies have identified clinical prognostic factors such as the primary site, stage, nodal involvement, age, presence of B symptoms, serum LDH levels and histopathological factors such as

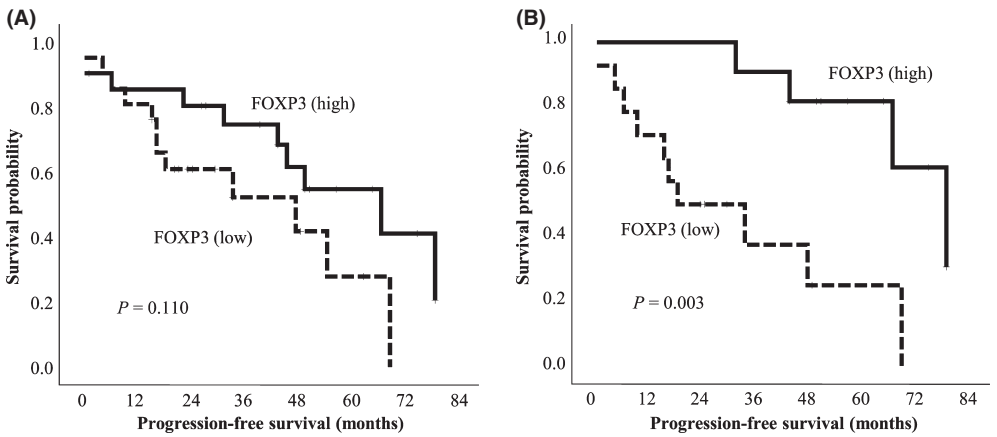

Fig. 4. (A) Kaplan–Meier survival analysis revealed that patients with a higher number of intratumoral FOXP3-positive cells (≥180/0.58 mm^2) showed a tendency to have better progression-free survival than those with a lower number of intratumoral FOXP3-positive cells (<180/0.58 mm^2; 48 *vs* 67 months, $P = 0.110$). (B) When the analysis was limited to patients who had received combination chemotherapy, a higher number of intratumoral FOXP3-positive cells was significantly related to prolonged progression-free survival (19 *vs* 79 months, $P = 0.003$).

CD5, CD43, p53 and BCL-6 in ocular adnexal lymphoma.[34] However, the results of these studies are difficult to generalize due to the heterogeneity of pathological diagnosis, treatment protocols and primary objectives. Various effective treatment modalities are available for OAML patients such as excision, radiotherapy, single-agent or combination chemotherapy, immunotherapy, *Chlamydia psittaci* eradicating antibiotics and even observation.[34] Most of these strategies are well tolerated, and re-treatment with the same regimens is an option. Although the present study is limited by its retrospective nature and small cohort size, it is noteworthy that it demonstrates significant prognostic discrimination on PFS in a homogeneous subgroup of OAML patients who underwent chemotherapy. In the present study, PFS after front-line treatment was assessed to exclude possible biases.

In conclusion, a high number of tumor-infiltrating FOXP3+ T cells is associated with a favorable clinical outcome in patients with OAML. Future efforts should be directed towards finding a network between the tumor microenvironment and tumor cells in extranodal marginal zone B-cell lymphoma of MALT type.

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Disclosure Statement

All authors have no potential conflict of interest.

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