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Prognostic and predictive role of FOXP3 positive tumor infiltrating lymphocytes (TILs) in curatively resected non small cell lung cancer other than stage IA

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ABSTRACT

Lung cancer is the leading cause of cancer-related mortality and responsible for 1.6 million deaths per year through world-wide. Surgical resection with negative margin combined with the adjuvant therapy [except for stage IA and IB (<4 cm)] is the Standard treatment for early-stage Non-small cell lung cancer (NSCLC). Early-stage NSCLC, however, has relapse rate over 40% mostly at distant sites. Therefore, high relapse rate necessitates urgent novel biomarker for these patients. In this study, we aim to evaluate the predictive and prognostic role of FOXP3+ Treg cells along with well defined Clinicohistopathological factors in early-stage non-small cell lung cancer (NSCLC). FOXP3 expression in tumor infiltrating lymphocytes (TIL) was examined by immunohistochemical staining from resected early-stage 48 NSCLC patients. Data of patients and FOXP3 expression status along with common clinicohistopathological prognostic factors were evaluated retrospectively. Median age of patients was 62 years-old (range 43–78). Mean follow-up, median overall survival (OS), and disease-free survival (DFS) were 49, 49 and 30 months, respectively. FOXP3 expression was positive in 23 (47.9%) patients. Adjuvant chemotherapy (4 cycles of cisplatin-vinorelbine) was given to 16 patients (33.3%) at physician discretion. Patients with a FOXP3 expression of 25% or higher significantly lower OS and DFS when compared with patients with a FOXP3 staining lower than 25% with p-value of 0.016 and 0.032, respectively. In the patients with high FOXP3 expression, platin-based adjuvant chemotherapy had showed a detrimental effect on DFS and OS. These results suggest that FOXP3 expression may be used as useful prognostic biomarker in resected NSCLC. Our findings also suggest that resected NSCLC patients with FOXP3 expression of 25% or higher staining intensity may not get any benefit even disfavor from adjuvant platin chemotherapy.

1. Introduction

Lung cancer is the most common cause of cancer-related deaths in both sexes, even though the incidence of breast cancer is higher than lung cancer in women.[1] Only curative treatment is the complete surgical resection but even in early stage disease recurrence after surgery occurs in 40% of patients.[2] LACE metanalysis showed % 5.4 absolute benefit at 5 years favoring cisplatin-based chemotherapy with moderate treatment-related toxicity.[3] On the other hand, more recent metanalysis reported 4% (95% CI 3–6) at 5 years (from 60% to 64%).[4] Though, some predictive markers can be used, like ERCC, which show who would not benefit from cisplatin-based adjuvant therapy, no proven predictive biomarker has shown whether one would need adjuvant therapy or not, other than disease stage.

The immune system can invade, interact and destroy tumor cells, which this phenomenon called as 'immune surveillance.[5] The cellular immune system, specifically CD8 positive cytotoxic T

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lymphocytes form the most active part of immune surveillance.[6,7] In the experimental model with mice, a subset of $CD4^+$ T cells, FOXP3 positive T regulatory (Treg) cells can effectively block the immune response of CD8 + cytotoxic cells by expressing CTLA-4 and PDL-1 on their surfaces.[8] The rate of Treg/CD3+ cells are high in the lung, ovarian, breast, pancreatic, and hepatocellular cancer tumors that provide local immune suppression for the further development of the tumor, smoldering inflammation, the increase in angiogenesis and metastases.[9,10]

In this study, we aimed to investigate the role of Treg cells in the development of metastases in early stage non-small cell lung cancer (NSCLC) and possible correlation of Treg cells with disease stage, histopathology, disease-free survival (DFS), and overall survival (OS) time.

2. Material and methods

Charts of 70 non-small cell lung cancer patients who were treated with curative surgical resection plus adjuvant treatment at physician discretion at Baskent University were included. Those patients with stage IA disease were excluded and a total of 48 patients were included in this study. Demographic and clinicohistopathological characteristics of patients were recorded.

All patients had adequate bone marrow, kidney and liver function. The patients with autoimmune disease and significant comorbidities were excluded. All results were presented as the rate for categorical values or mean and median for continuous variables. Survival curves were estimated according to the Kaplan-Meier method, and log-rank tests were used for univariate statistical comparisons. Adjusted Hazard Ratio (HR) and 95% confidence interval (95% CIs) were used for estimation. All statistical data were analyzed using the SPSS version17.0, and a p value of <0.05 was considered statistically significant.

3. Pathological evaluation

Formalin-fixed, paraffin-embedded specimens were obtained from 48 non-small cell lung cancer patients. Immunohistochemical staining with FoxP3, and CD3 was performed to successive sections. FOXP3 (clone 236A/E7), CD3 (clone IR503), were used at diluted concentrations of 1: 175, 1: 100. The expression of the CD3, and Foxp3 positive cells was evaluated on the TILs in intra-tumoral part by using an optical microscope (BX50; Olympus, Tokyo, Japan). The counting of positive cell was performed by a pathologist who had no previous knowledge of the clinical stage of patients, and the results were noted. Foxp3 expression was evaluated semi-quantitatively in 10 HPF. Cases were scored negative if no staining of the cytoplasm of lymphocytes was found, and positive if nuclear staining of lymphocytes was presented and the positive cases were subdivided into three groups based on the intensity of staining (I = 0–25%, II= >25%3) (Fig. 1a, b and c). Since Foxp3 expression can be seen in (cd25+) activated B lymphocytes. T lymphocytes in the evaluation area was highlighted by immunohistochemically CD3 application in order to ensure the evaluated cell population was composed entirely of T cells. The density (cells/mm^2) of infiltrating Foxp3+ lymphocyte numbers in ten fields divided by the observed area.[11]

4. Results

Patient and tumor characteristics are summarized in Table 1. A total of 48 patients had adequate tumor samples and full patient data evaluated for CD3 and FOX-P3 expression in tumor tissue. Out of the 48 patients, 42 patients undergone lobectomy with adequate mediastinal lymph node dissection. Of note, patients with other than RO resection were excluded from the study. Mean follow-up time for the whole group was 49 months(range 6–128). Adjuvant chemotherapy was given to 16 patients (33.3%) at physician discretion.

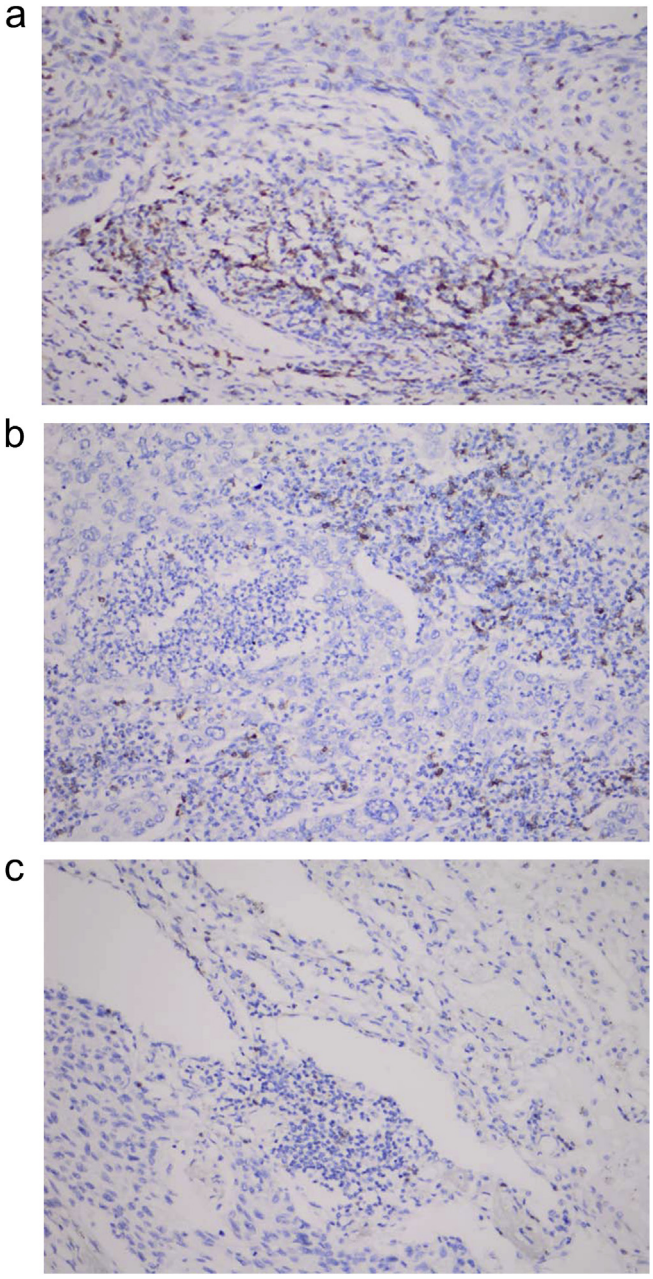

Fig. 1. a, b,c: FOXP3positive cases were subdivided into three groups based on the intensity of staining (I = 0–25%, II= >25%3).

Overall, CD3 expression in tumor tissue was positive for all patients (showing lymphocyte infiltration as expected), staining intensities of I and II were found in 27 (56.2%) and 21(43.8%) patients, respectively. However, FOXP3 expression was positive in 23 (47.9%) patients, staining intensity of I and II were found in 25 (52.1%) and 23 (47.9%) patients, respectively. There are no patients over 50% staining intensity on FOXP3 expression. Statistical analyses failed to show any significant correlation between primary tumor size, pathological stage, histopathology, sex, CD3, and FOXP3 expression, respectively.

Univariate analysis showed that ECOG performance, disease

Table 1
Patient characteristics.

Clinicopathological features	All cohort, n (%)	Relapse, n (%)	Death, n (%)	Median DFS (95%CI)	p-value	Median OS (95%CI)	p-value
Sex (male)	40(83.3)	24 (60)	17(42.5)	28 months (6.9–49.1)	0.997	87 months (82.3–91.7)	0,616
ECOG					0.025		0,008
0	32(66.7)	18(56.2)	17(53.1)	54 months (9.7–98.3)		68 months (31.5–104.5)	
1	14(29.2)	10(71.4)	10(71.4)	14 months (0–47.8)		42 months (29.9–54.1)	
2	2(4.2)	2(100)	2(100)	1 months		1 months	
Operation type							
Lobectomy	42(87.5)	24(57.1)	23 (54.7)	40 months (1.2–78.8)	0.003	62 months (34.0–90.0)	0,006
Adjuvant Chemotherapy (yes)	16(33.3)	14 (87.5)	14 (87.5)	23 months (8.1–37.9)	0.277	42 months (13.6–70.4)	0.295
Stage					0.029		0.030
IB	21(43.8)	11(52.3)	10 (47.6)	75 months (0–170.6)			
II	14(29.2)	7(50)	7(50)	60 months (21.0–99.0)		69 months (59.7–78.3)	
III	13(27.2)	12(92.3)	12(92.3)	20 months (13.1–26.9)		21 months (0–42.1)	
Histopathology					0.211		0.262
Non-Squamous	24(50)	19 (79.1)	18 (75)	23 months (7.4–38.6)		40 months (17.9–62.1)	
Squamous	24(50)	11 (45.8)	11 (45.8)	30 months		86 months	
CD-3 staining intensity					0.778		0,967
I (0–50%)	27(56.2)	19 (70.3)	18 (66.6)	30 months (9.6–50.4)		55 months (16.0–94.0)	
II (over %50)	21(43.8)	11 (52.3)	11 (52.3)	34 months (0,0–87.6)		49 months (12.6–85.4)	
FOX P3 staining intensity					0.032		0.016
I (0–25%)	25(52.1)	12 (48)	11 (44)	not reached		not reached	
II (over %25)	23(47.9)	18 (78.2)	18 (78.2)	22 months (14.9–29.1)		33 months (18.2–47.8)	

stage and FOXP3 staining intensity had the significant effects on DFS with the p values of 0.025, 0.029 and 0,032 respectively. Univariate analysis showed that ECOG performance, disease stage and FOXP3 staining intensity had the significant effect on OS with the p values of 0.008, 0.03 and 0,018 respectively (Fig. 2a, b and c).

When we use staining intensity of FOXP3 as a stratum, independent from pathological disease stage, statistical analysis showed that adjuvant chemotherapy had detrimental effect in FOXP3 high group on DFS and OS with $p = 0.029$ and $p = 0.154$, respectively (Fig. 3a and b).

5. Discussion

NSCLC is a lethal tumor, and only curative treatment is total surgical resection. However even in early stage lung cancer with successful surgical resection, relapse rate exceeds 40%. Cisplatin-based chemotherapy significantly decreases relapse rate for patients with stage IB to III and increase five years OS and DFS.[3] Theoretically, adjuvant chemotherapy destroys micrometastatic foci and prevent further development of symptomatic metastases.[3] However, in practice, tumor cells can overcome the cytotoxic effect of chemotherapy and present with overt metastases. Some studies suggested that Treg cells are an important contributor to local suppression of anti-tumor immunity[12] and may have the role in chemotherapy resistance. Here, our study showed that high staining intensity of FOXP3 (over 25%) significantly correlated with shorter OS and DFS in early stage resected NSCLC along with low ECOG scale and high pathological disease stage. Our study indicated that effect of FOXP3 on survival rates was independent of adjuvant chemotherapy and intensity of FOXP3 did not correlate with primary tumor size, CD3 staining intensity, and absence or presence of lymph node metastasis. Patients with the high staining intensity of FOXP3 had shorter DFS and OS when treated with adjuvant chemotherapy compared with patients not treated with adjuvant chemotherapy. However, adjuvant chemotherapy did not have the detrimental effect on OS and DFS in patients with the low staining intensity of FOXP3. These effects were independent of pathological stage.

Immune system and tumor interaction start at the very early period of cancer development. Primary management of these complex and flexible interactions by host or tumor is largely unknown. However, this interaction is strongly related to angiogenesis and metastasis.[13] Besides, this interaction can be changed by treatment modalities. Immunogenic chemotherapeutics (platin), VEGF inhibitors (sunitinib, bevacizumab), anti-CTLA 4 antibody (ipilimumab), anti-PD1 and anti-PDL1 antibodies (pembrolizumab, nivolumab, atezolizumab, durvalumumab) can significantly change the immune contexture of tumors.[13,15] Although, exact percent is unclear, some therapeutic effects of chemotherapeutics and targeted therapeutic agents occur via changing the tumor immune contexture.[13] So, defining the enigma of tumor-immune system communication is not academic interest, it can change our vision, identify new prognostic and predictive markers.

The association of intrat-tumoural TILs and clinical outcome was demonstrated in different types of tumors. Bates et al. reported that elevated Treg cells in primary breast cancer confer a significantly shorter OS and DFS.[14] Results of other studies supported this finding by reporting increased intensity of Treg cells correlated with decreased survival rates in ovarian, HCC, gastric, and esophageal cancer.[16,17] However, studies with lung cancer showed prognostic value of TILs may vary with distribution (tumor stroma vs nest) and types ($CD8^+$, $CD3^+$, Treg lymphocytes).[15] In addition, PDL-1 expression on tumor cells and immune cells with tumor mutational load proved to be predictive marker for treatment of anti-PD-1 and anti-PDL-1 drugs.[18] In this study, our results showed FOX-P3 positive TILs over 25% in early-stage lung cancer was a strong prognostic biomarker. Though the prognostic role of FOX-P3 positive TILs parallel to ECOG performance and pathological stage, there was no statistically important correlation between staining intensity of FOX-P3 positive TILs and primary tumor size, CD3 staining intensity, and absence or presence of lymph node metastasis. Therefore, these results suggested that development of immune contexture of tumor or relation between host immune system and the tumor not be parallel with the anatomic evolution of the primary tumor, even beyond this concept.

Predictive role of immune contexture in chemotherapy response was shown in some cancers. In breast cancer and melanoma, high lymphocyte infiltrate is associated with high response rate and better survival parameters.[19,20] In colorectal cancer, high expression of $CD8^+$ T cells and T_H1 cells correlates with better survival rates.[21] According to the best of our knowledge, there is no study in lung cancer that evaluates predictive role of FOX-P3

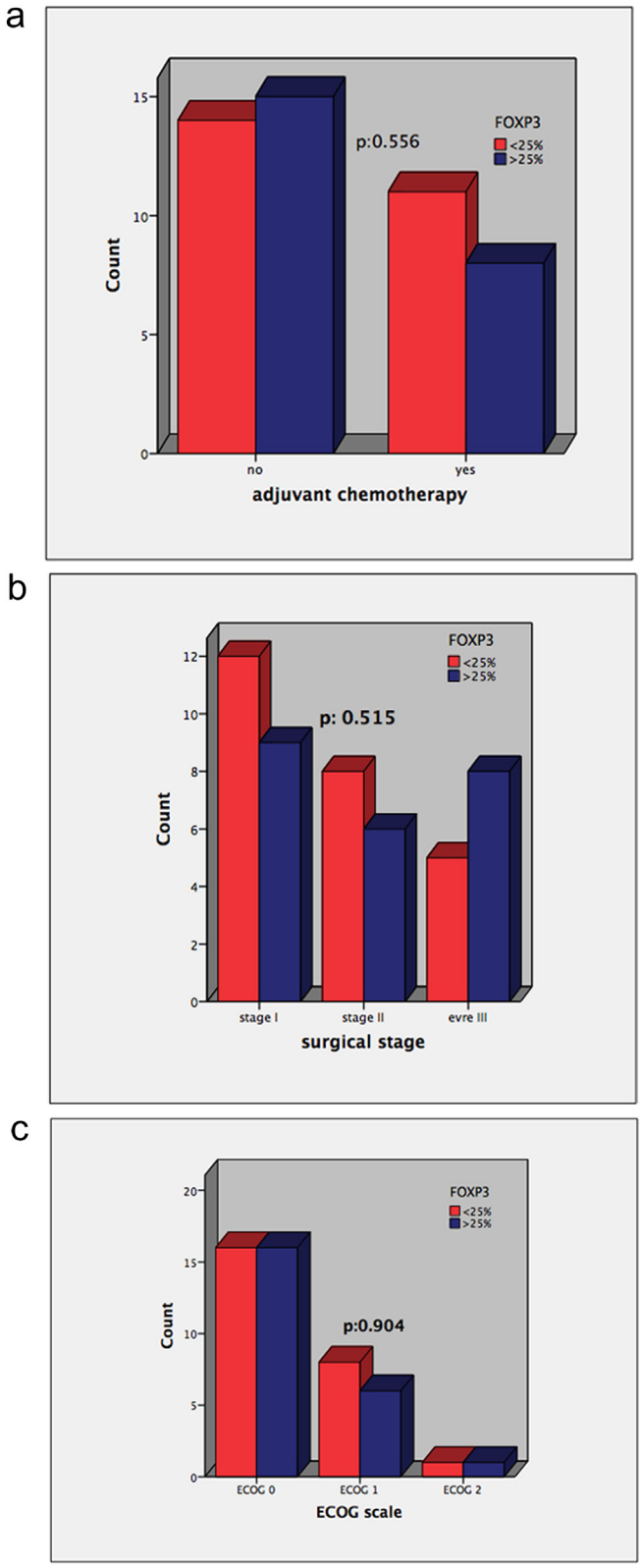

Fig. 2. a, b, c: The association of FOXP3 and other important prognostic factors; adjuvant chemotherapy, stage, and ECOG scale ($p > 0.05$ for all of parameters).

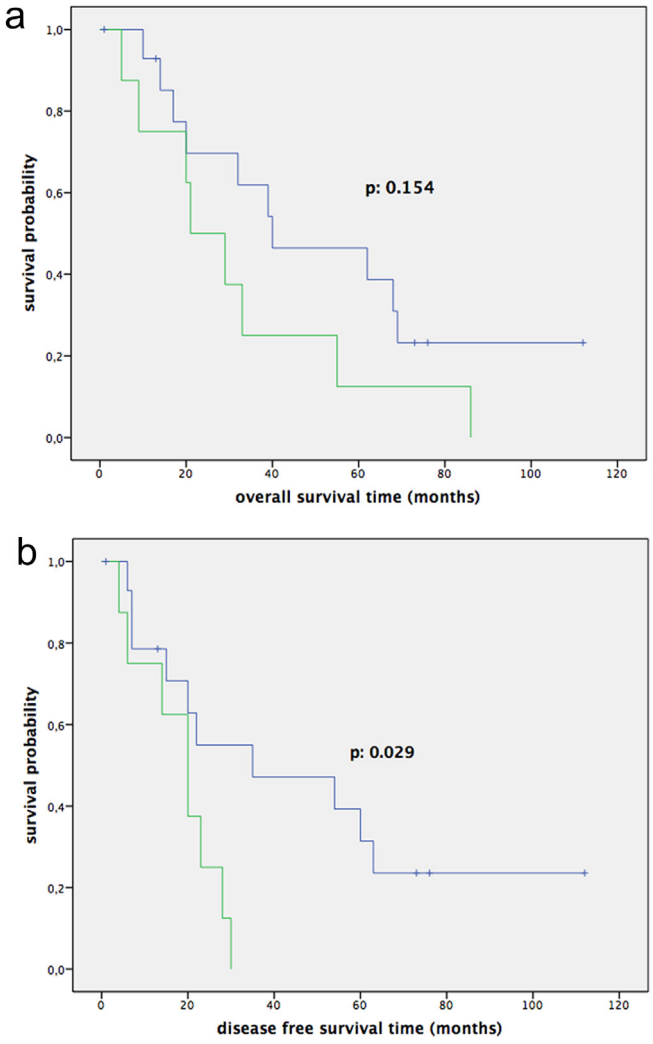

Fig. 3. a, b: Kaplan-Meier survival analysis of OS and DFS for the FOXP3 high group (over 25%) patients those who treated with adjuvant chemotherapy(blue line) or not(green line), adjuvant chemotherapy had detrimental effect on OS (40 vs 21 month, p: 0.154) and DFS (35 vs 20 months, p: 0.029).

positive TILs in patients treated with chemotherapy. In our study, we showed that platin-based adjuvant chemotherapy had the detrimental effect on survival parameters in the group of patients with high FOXP3 staining intensity.

We acknowledged that current study has a number of crucial limitations that need to be considered. First, as a retrospective study, it is subject to all design related the inherited biases. Secondly due to relatively small group of patients in our study, our analyses were limited in statistical power. Thirdly we didn't account location of TIL expression in statistical analysis.

In conclusion, we found that early stage NSCLC patients with high FOXP3 staining intensity had significantly shorter DFS and OS. Besides, the adjuvant cisplatin-based chemotherapy had the detrimental effect on DFS and OS in patients with curatively resected non-small cell lung cancer. Our findings highlighted the very high-risk group in resected early-stage NSCLC patients with high FOXP3 staining intensity who did not get any benefit even disfavor from adjuvant platin chemotherapy.

Conflict of interest

We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the manuscript and agreed with submission to 'Journal of Oncological Science'. The authors have no conflicts of interest to declare.

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