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PROGNOSTIC SIGNIFICANCE OF FOXP3 IN RADICALLY TREATED NON-SMALL CELL LUNG CANCER PATIENTS

O.I. Vynnychenko[1], Y.V. Moskalenko[2], A.M. Piddubnyi[3], R.A. Moskalenko[4]

[1]*Sumy Regional Clinical Oncology Center, Sumy, Ukraine*
[2]*Sumy State University, Department of Oncology and Radiology, Sumy, Ukraine*
[3]*Ukrainian-Swedish Research Center SUMEYA, Sumy, Ukraine*
[4]*Sumy State University, Department of Pathology, Sumy, Ukraine*
ORCID ID: 0000-0001-5651-0323, e-mail: vynnychenkool@ukr.net
ORCID ID: 0000-0002-5398-0298, e-mail: yl.moskalenko@med.sumdu.edu.ua
ORCID ID: 0000-0002-6508-0131, e-mail: a.piddubny@med.sumdu.edu.ua
ORCID ID: 0000-0002-2342-0337, e-mail: r.moskalenko@med.sumdu.edu.ua

Abstract. Regulatory T-cells (Tregs), which are characterized by the expression of the transcription factor Forkhead box P3 (Foxp3), play a crucial role in modulating the immune response. While Tregs are primarily recognized for their immunosuppressive functions, their influence on the survival and prognosis of patients with non-small cell lung cancer (NSCLC) presents a complex and often variable picture. This variability can manifest in a range of outcomes, influencing patient prognosis in positive, negative, or neutral ways.

Our study aims to delve into the prognostic significance of Foxp3 expression in patients undergoing radical treatment for NSCLC, seeking the relationship between Treg dynamics and patient outcomes.

This study involved a cohort of forty-two patients diagnosed with non-small cell lung cancer (NSCLC) at stages IA to IIIB, all of whom underwent radical surgical intervention followed by an adjuvant regimen of platinum-based chemotherapy. The inclusion criteria were: patients who had previously received neoadjuvant chemotherapy or radiation therapy. Those who experienced postoperative complications, or individuals with significant concurrent health conditions were systematically excluded from participation. Comprehensive clinicopathological data for each patient were extracted from their medical records to ensure accuracy and reliability. To evaluate the presence of FOXP3-positive cells, we employed immunohistochemistry techniques and established a threshold of 23 cells per 1 mm². Patients were subsequently stratified into two distinct groups based on their FOXP3 expression levels: the low expression group (<23 cells/1 mm²) and the high expression group (≥23 cells/1 mm²). We compared clinical outcomes between these two groups to ascertain any significant differences. A statistical analysis utilizing the Mann-Whitney test, Chi-squared test, receiver operating characteristic (ROC) analysis, and the Kaplan-Meier method and Log-rank test to comprehensively evaluate the data.

The density of Foxp3-positive lymphocytes within the tumor microenvironment exhibited a notable variability, ranging from 5 to as many as 72 cells per square millimeter. Interestingly, analysis revealed no significant associations between the levels of Foxp3 and the selected clinicopathological features of the patients. Moreover, a comparative evaluation showed no marked differences in Foxp3 expression between adenocarcinomas and squamous cell carcinomas with respect to the clinicopathological characteristics examined. Importantly, low versus high Foxp3 expression patients did not demonstrate significant disparities in recurrence-free survival or overall survival, as indicated by log-rank p-values of 0.1817 and 0.3944, respectively. However, a discernible trend emerged, suggesting that patients exhibiting lower levels of Foxp3 tended to experience improved RFS and OS outcomes.

Research indicates that individuals exhibiting both low and high levels of Foxp3 demonstrate no significant differences in recurrence-free survival and overall survival. Furthermore, a lack of correlation between Foxp3 expression in adenocarcinomas and squamous cell carcinomas and their associated clinicopathological features has been observed. This variability in Tregs may play a crucial role in the discrepancies noted between Foxp3 expression levels and the survival outcomes of patients.

Keywords: Foxp3, regulatory T-cells, recurrence-free survival, overall survival, non-small cell lung cancer, prognosis, tumor microenvironment, immunohistochemistry.

Introduction. The environment surrounding a tumor significantly impacts patient survival by affecting the activation or inhibition of the immune response [1]. Cells within the tumor microenvironment can serve as specific biomarkers influencing treatment outcomes for patients. Additionally, these cells may represent potential targets for therapeutic interventions [2, 3].

Tumor-infiltrating lymphocytes are a crucial element of the tumor microenvironment. Regulatory T cells (Treg) constitute a subset of T lymphocytes that dampen antitumor immunity, thereby fostering an immunosuppressive setting. These cells are characterized by the surface markers CTLA-4 and CD25, along with the expression of the transcription factor Forkhead box P3 (FOXP3) in their nuclei.

Research rationale. Numerous researchers have explored the significance of FOXP3 in various cancer patients and its impact on survival rates. Nevertheless, the findings have been inconsistent [4, 5, 6]. It has been demonstrated that FOXP3 is found in both Treg and other T-cell types within areas of inflammation. Furthermore,

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the Treg population can exhibit heterogeneity under certain conditions, altering FOXP3 expression [7].

Some researchers indicate that FOXP3 serves as a prognostic biomarker linked to poor outcomes [8, 9]. Conversely, other studies have found no detrimental effect of FOXP3 on survival for patients with non-small cell lung cancer (NSCLC) [10]. Hence, the objective of this research was to evaluate the prognostic significance of FOXP3 in patients with NSCLC who underwent radical treatment.

Methods. ***Patient characteristics.*** The research involved 42 individuals diagnosed with stages IA-IIIB of NSCLC who were treated with radical surgical procedures at the Sumy Regional Clinical Oncology Center. Patients diagnosed with stages IB-IIIB were provided with platinum-based adjuvant chemotherapy. Those categorized as N2 underwent distant gamma therapy (administered at a total dose of 30 Gray) in addition to adjuvant chemotherapy. The inclusion criteria specified that participants must be over 18 years old, have stages IA-IIIB of NSCLC, and should not have severe cardiovascular or pulmonary conditions, autoimmune diseases, and they must have available postoperative tumor tissue preserved as a paraffin block. Exclusion criteria included having received neoadjuvant chemotherapy or radiation therapy, suffering from postoperative complications, or having serious concomitant diseases that could potentially result in the patient's death within the following years. The pathological stages of the participants were assessed according to the 8th edition of the TNM classification. All information regarding the clinicopathological features of the patients (including age, gender, disease stage, T category, N category, performance status, histological type, tumor differentiation, and driver mutations) was extracted from their medical records.

Follow-up period. The average follow-up duration was 57.9±4.2 months, with a range from 2 to 106 months. The interval for recurrence-free survival (RFS) was defined as the time from the surgery date until the occurrence of disease recurrence. Overall survival (OS) was defined as the time from the date of surgery until death due to disease progression or other reasons. Patient survival was evaluated as of July 1, 2024. Mortality data were obtained from the cancer registry of the Sumy Regional Clinical Oncology Center.

Histology and Immunohistochemistry. Lung cancer tissue samples were preserved in a 10% neutral formalin solution for 24 hours. The preparation of paraffin blocks was performed following standard procedures [11]. For immunohistochemical analysis (IHC) of NSCLC tissues, serial sections with a thickness of 4 μm were placed on SuperFrost adhesive slides (Thermo Scientific, Waltham, MA, USA) and allowed to dry overnight at 60º. Deparaffinized sections underwent antigen unmasking by being heated for 20 minutes in 0.1 M citrate buffer (pH 6.0) at temperatures of 95–98 °C. After this step, we rinsed the sections three times with distilled water and then allowed them to cool at room temperature for 20 minutes. An endogenous peroxidase blocking procedure was conducted over a period of 10 minutes at ambient temperature using a peroxidase solution (MAD-021540Q-125). Rabbit anti-human monoclonal antibodies against Foxp3 (anti-FOXP3, Clone SP97, Master-Diagnostica, Spain, ready to use, MAD-000536-QD – 12) were utilized. Primary monoclonal antibodies were incubated for 10 minutes. The "In Vitro" detection system (Master-Diagnostica, Spain) was employed to visualize the IHC results. For each sample, we carefully selected six regions within the tumor tissue that had the highest concentrations of Foxp3-positive cells. Additionally, stroma, areas surrounding the bronchi, and blood vessels, where clusters of lymphocytes and lymphoid follicles were present, were included in this selection. The quantity of Foxp3+ cells in these “hot spot” areas per high-power field (200×) was evaluated visually. The density of FOXP3 was assessed by counting the number of FOXP3-positive cells per 1 mm² over six fields, and then average values were calculated [12]. The cut-off value for FOXP3 was determined to be 23 (cells/1 mm²) based on ROC analysis. Consequently, patients were categorized into two groups: low (<23 cells/1 mm²) and high (≥23 cells/1 mm²) FOXP3 expression. Tonsil tissue was utilized as a positive control for IHC following the manufacturer's guidelines.

Statistical analysis. Data were analyzed using Stata software version 18.0 (StataCorp, TX, USA; https://www.stata.com; 2024). The Shapiro-Wilk test was utilized to evaluate the normality of the distribution. To assess the significance of differences between the studied groups, the Mann-Whitney test was applied to continuous variables. A chi-squared test was used to analyze the differences in proportions of categorical variables between groups with high and low levels of FOXP3 expression. Kaplan-Meier method was used to visualize survival curves. The Log-rank test was applied to determine the significance of survival difference between the two groups under study. All findings were regarded as statistically significant when $p < 0.05$.

Results. ***Histology and Immunohistochemistry***

The tissue of squamous cell lung cancer is characterized by layers of abnormal flat epithelium exhibiting pathological mitoses and varying cell forms. The cancer cells and clusters are enveloped by fibrous tissue along with a lymphohistiocytic inflammatory infiltrate surrounding the tumor, occasionally forming inflammatory shafts (Fig. 1, column 1, upper row). Adenocarcinoma is identified by atypical glandular formations that infiltrate the interstitial lung tissue. The tumor cells displaying glandular and pseudo-glandular patterns demonstrate signs of cellular irregularity. Some tumors exhibited a papillary arrangement and contained mucin (Fig. 1, column 2, upper row).

To identify the presence of Treg lymphocytes within non-small cell lung cancer tissue, we conducted immunohistochemistry using antibodies against FOXP3. We observed FOXP3-positive cells in the tumor microenvironment of the stroma associated with squamous cell lung cancer (Fig. 1, column 1, bottom row). FOXP3-positive cells, featuring deeply stained nuclei, were situated among the glandular complexes of adenocarcinoma (Fig. 1, column 2, bottom row).

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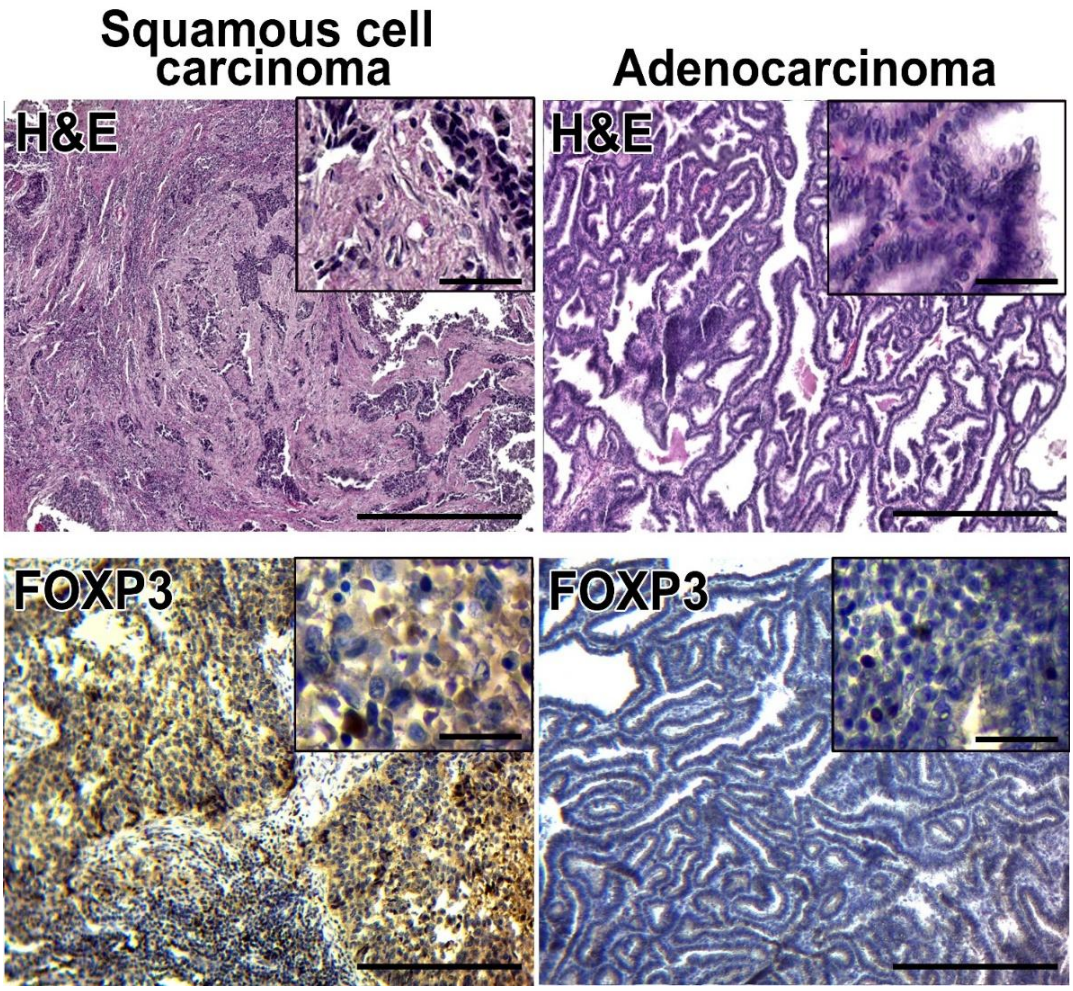

Fig. 1. Histology and immunohistochemistry of NSCLC. The first column is squamous cell lung cancer. The second column is lung adenocarcinoma. Upper row - staining of NSCLC with hematoxylin and eosin. Bottom row - immunohistochemistry of NSCLC tissue with antibodies against FOXP3. Magnification is indicated in each image's lower right corner as a marker corresponding to 200 µm in photomicrographs and 50 µm in insets

Patient characteristics and assessment of FOXP3 expression. The research involved 42 patients who underwent radical surgery followed by 2-4 cycles of platinum-based adjuvant chemotherapy. Additionally, eight patients with N2 classification received adjuvant gamma therapy. The average age of the participants was 58 years, with an age range of 29 to 75 years. Among these patients, 19 % were female while 81 % were male. Driver mutations were identified in 11 out of the 42 patients (26.2 %), which included 2 instances of EGFR mutation, 7 instances of KRAS mutation, 1 instance of ALK mutation, and 1 instance of BRAF mutation. Based on FOXP3 expression, patients were categorized into two groups using a cut-off of 23 FOXP3-positive lymphocytes per square millimeter. No statistically significant difference in FOXP3 expression was observed between the patient groups (see Table 1).

We additionally assessed the differences between groups based on tumor histology. The count of FOXP3-positive lymphocytes within the tumor microenvironment ranged from 5 to 72 cells per 1 mm². There were no statistically significant differences observed in FOXP3 expression between adenocarcinomas and squamous cell carcinomas (Table 2).

Survival analysis. The average follow-up period was 57.9±4.2 months. During the data analysis, disease relapse occurred in 19 patients. In total, 19 patients from the cohort passed away. Eighteen patients died due to the progression of lung cancer, while one succumbed to other causes. No significant difference in recurrence-free survival (RFS) was observed between patients with low (<23 cells/1 mm²) and high (≥23 cells/1 mm²) FOXP3 expression (Log-rank P=0.1817). Nonetheless, there was a tendency for improved RFS among patients with low FOXP3 levels (Fig. 2).

Comparable findings were observed regarding the influence of FOXP3 expression on overall survival (OS). There was no notable difference in OS between patients exhibiting low (<23 cells/1 mm²) and high (≥23 cells/1 mm²) FOXP3 expression (Log-rank P=0.3944). Nonetheless, there was a tendency indicating improved OS in patients with low FOXP3 levels (Fig. 3).

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Table 1

Correlation between clinicopathological characteristics of patients and FOXP3 expression

Baseline clinicopathological characteristics	FOXP3 <23 cells/1 mm^2 (%) n=18	FOXP3 ≥23 cells/1 mm^2 (%) n=24	χ^2 (p)*
Age, n (%): Average Interval < 60 ≥ 60	 61 51–69 7 (38.9) 11 (61.1)	 57 29–75 15 (62.5) 9 (37.5)	 2.2989 (0.129)
Sex, n (%): Female Male	 5 (27.8) 13 (72.2)	 3 (12.5) 21 (87.5)	 1.5570 (0.212)
Stage, n (%): IA-IIA IIB-IIIA	 6 (33.3) 12 (66.7)	 9 (37.5) 15 (62.5)	 0.0778 (0.780)
Category T, n (%): T1a-2a T2b-4	 8 (44.4) 10 (55.6)	 11 (45.8) 13 (54.2)	 0.0080 (0.929)
Category N, n (%): N0 N1-2	 10 (55.6) 8 (44.4)	 14 (58.3) 10 (41.7)	 0.0324 (0.857)
Histology, n (%): Adenocarcinoma Squamous cell carcinoma	 10 (55.6) 8 (44.4)	 10 (41.7) 14 (58.3)	 0.7955 (0.372)
Differentiation, n (%): Low-grade High-grade	 11 (61.1) 7 (38.9)	 15 (62.5) 9 (37.5)	 0.0084 (0.927)
Smoking history, n (%): Never smokers Current or former smokers	 5 (27.8) 13 (72.2)	 3 (12.5) 21 (87.5)	 1.5570 (0.212)
Performance status, n (%): 0 1	 1 (5.6) 17 (94.4)	 2 (8.3) 22 (91.7)	 0.1197 (0.729)
Surgery type, n (%): Lobectomy Pneumonectomy	 11 (61.1) 7 (38.9)	 14 (58.3) 10 (41.7)	 0.0329 (0.856)
Driver mutations (EGFR, KRAS, BRAF, or ALK), n (%): Present Absent	 4 (22.2) 14 (77.8)	 7 (29.2) 17 (70.8)	 0.2566 (0.612)

Table 2

Number of enumerated FOXP3+ cells in different clinical categories, paired comparisons

Basic clinicopathological characteristics	Adenocarcinoma	p-value*	Squamous cell carcinoma	p-value*
Age: < 60 ≥ 60	 26 (5–74) 25 (9–72)	0.8491	 31 (18–49) 27 (18–50)	0.4886
Sex: Female Male	 29 (7–74) 24 (5–43)	0.3827	 27 (22–36) 30 (18–50)	1.0000
Stage: IB-IIA IIB-IIIA	 23 (7–72) 29 (5–74)	0.2464	 29 (18–49) 29 (18–50)	0.8842
Category T: T1a-2a T2b-4	 29 (9–74) 18 (5–29)	0.3858	 29 (18–50) 30 (18–49)	0.8382
Category N: N0 N1-2	 24 (5–72) 29 (11–74)	0.4047	 29 (18–50) 29 (18–49)	0.7864
Differentiation: Low-grade High-grade	 27 (5–74) 23 (11–43)	0.7509	 29 (18–49) 32 (18–50)	0.7949
Smoking history: Never smokers Current or former smokers	 27 (5–74) 25 (11–43)	0.1420	 26 (21–36) 30 (18–50)	0.9555
Surgery type: Lobectomy Pneumonectomy	 27 (5–74) 23 (7–38)	0.6340	 31 (18–50) 28 (18–49)	0.2430
Driver mutations (EGFR, KRAS, BRAF, or ALK): Present Absent	 20 (9–33) 28 (7–72)	0.6477	 40 (30–49) 28 (18–49)	0.1025

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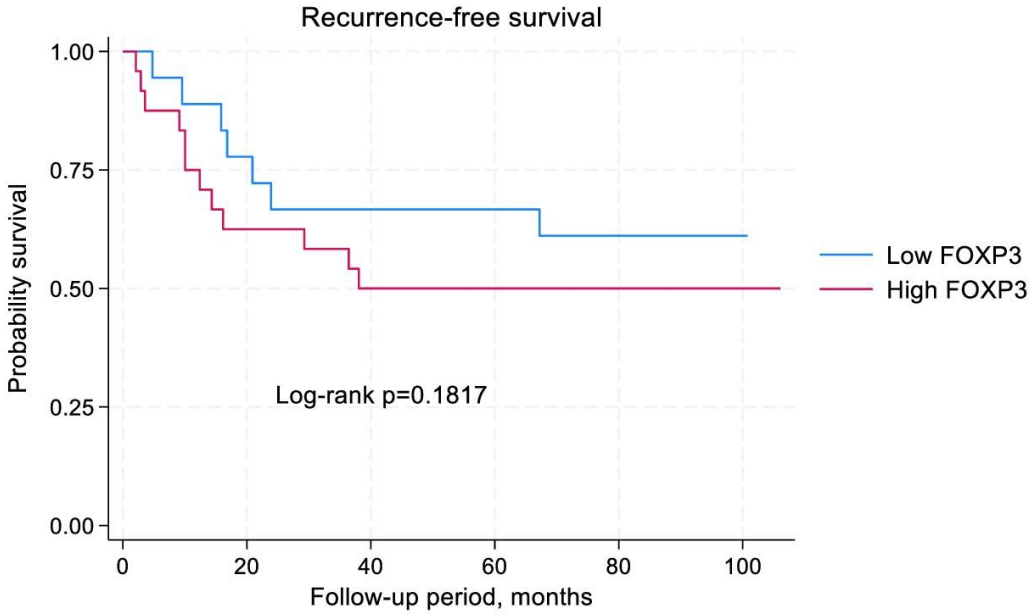

Fig. 2. Kaplan-Meier curves show the difference in RFS of patients with low and high FOXP3

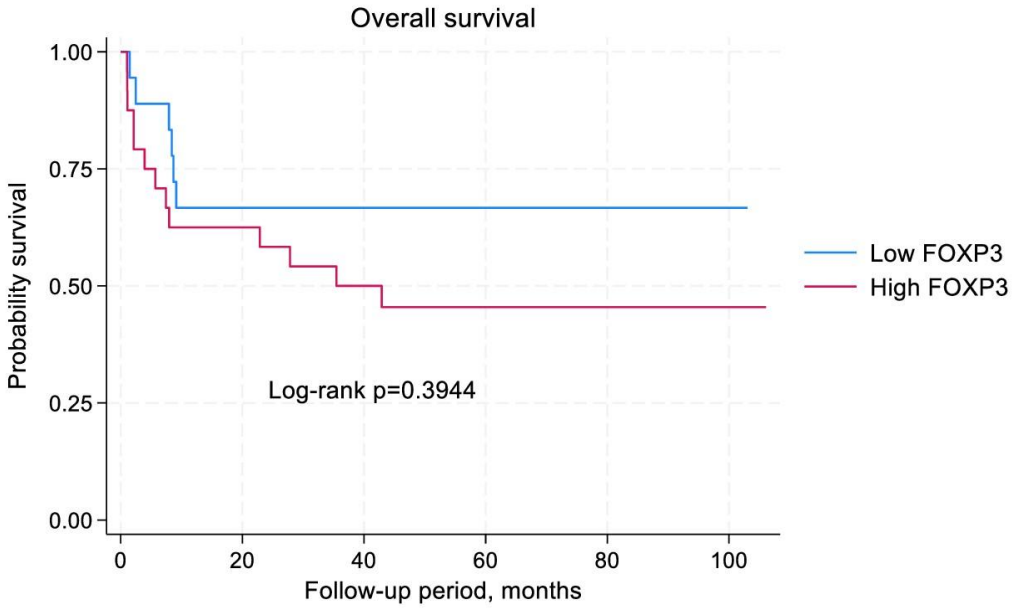

Fig. 3. Kaplan-Meier curves show the difference in the OS of patients with low and high FOXP3

Discussion. The present research indicated that FOXP3 expression does not have prognostic importance in individuals with radically treated NSCLC. Patients exhibiting low levels of Foxp3 expression demonstrated a tendency towards improved disease-free survival and overall survival; however, this difference was not statistically significant. FOXP3 expression is strongly linked to Tregs, positioning it as a potential survival predictor and a promising avenue for biomarker-driven targeted therapy for NSCLC. Mutations in FOXP3 result in disrupted peripheral tolerance and the onset of immune dysregulation polyendocrinopathy X-linked (IPEX) syndrome, along with severe autoimmune responses. Patients with lung cancer have elevated Treg levels in both peripheral blood and the tumor microenvironment when compared to healthy individuals. Although Tregs possess immunosuppressive characteristics, their effect on survival and prognosis in NSCLC patients can be positive, negative, or neutral.

Jackute et al. [17] explored the expression levels of Foxp3, CD4, and CD8, along with the CD8/Foxp3 and CD4/CD8 ratios in the tumor stroma and tumor islets of patients with stages I-III NSCLC. The researchers concluded that a high presence of Foxp3 in the tumor stroma correlates with improved overall survival. Kinoshita et al. [18] also took into account CD8 expression while assessing the prognostic significance of Foxp3 in patients with stage IA lung adenocarcinoma. Their findings indicated that patients exhibiting low levels of CD8 alongside high Foxp3 had significantly worse recurrence-free survival and overall survival rates. In line with our research, the authors mentioned above evaluated the prognostic importance of Foxp3 in patients with

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NSCLC who underwent surgical treatment. Nevertheless, their patient sample sizes were larger, and their study design included a comparison of Foxp3 expression with other tumor-infiltrating lymphocytes, particularly CD8.

Yan et al. [19] examined the prognostic significance of tumor-infiltrating lymphocytes in a meta-analysis that included 60 studies with a total of 15,829 NSCLC patients. The authors discovered that an increase in CD8, CD4, and CD3 infiltration is associated with a better prognosis. However, elevated levels of Foxp3 are linked to a poorer prognosis and decreased overall survival among NSCLC patients. Shang et al. [20] found in their meta-analysis that Foxp3 negatively influences survival rates. Nevertheless, the prognostic impact is heavily reliant on the disease's molecular subtype, stage, and the interactions with other tumor-infiltrating lymphocytes.

The immunosuppressive characteristics of Tregs are evident in the diminished activity of cytotoxic T cells and other effector cells, resulting in the suppression of antitumor immunity and progression of the disease [21]. Furthermore, the expression mechanisms of checkpoint suppressor molecules (PD-1, CTLA-4, TIGIT, LAG-3, and TIM-3) become activated, enabling the immune response to be evaded [22]. Consequently, the overexpression of FOXP3 in the tumor microenvironment is linked to a poor prognosis for individuals with NSCLC [23].

In the present study, we obtained results that differ from the conclusions of many researchers. We did not find a correlation between the expression of FOXP3 and the clinical and pathological characteristics examined. The quantity of FOXP3-positive cells in adenocarcinomas and squamous cell carcinomas did not show significant differences and was not associated with the clinicopathological features. Moreover, progression-free survival (PFS) and overall survival (OS) are not influenced by the number of FOXP3-positive cells. Nevertheless, there was an observed trend toward reduced survival in patients exhibiting high FOXP3 levels.

Recent studies reveal the diversity and variability present in Tregs [24, 25]. In this context, the level of FOXP3 expression does not always align with the quantity of Tregs. Sakaguchi [26] demonstrated that in an inflammatory setting, there is an increase in the number of cells exhibiting low FOXP3 expression, which is associated with a favorable prognosis. This phenomenon arises from the existence of two subpopulations among tumor-infiltrating lymphocytes; one exhibiting high FOXP3 levels and the other showing low FOXP3 levels, resulting in differing capacities to suppress antitumor immunity. Phillips et al. [27] noted the pro-inflammatory characteristics of Tregs in patients diagnosed with NSCLC. Furthermore, FOXP3 can also be present in non-Treg cells, potentially leading to an inaccurate assessment of Treg numbers [28]. Hatzioannou et al. [29] suggest that the primary indication of Treg plasticity is the alteration in FOXP3 expression and the emergence of "ex-Treg" and "fragile" Treg cell types. In these cells, FOXP3 expression is partially maintained, but their immunosuppressive functions are greatly diminished, resulting in a shift toward pro-inflammatory behavior. Mortezaee [30] indicated that Tregs may originate from various sources, which influences the sensitivity of FOXP3 stability regulators. The downregulation and depletion of Tregs are linked to FOXP3 abnormalities caused by methylation, ubiquitination, and acetylation processes. Consequently, different subpopulations of Tregs display varying levels of FOXP3 expression, capable of both enhancing and diminishing antitumor immunity.

According to our findings, the results are consistent with those reported by the authors, which indicate a notable plasticity and variability within the Treg population. Given the existence of various Treg subpopulations capable of transforming into other cell types, it can be inferred that FOXP3 expression does not accurately represent the level of immunosuppression.

The adaptability of Tregs presents considerable therapeutic possibilities. Enhancing the stability of Tregs could lead to a decrease in chronic inflammation, while destabilizing these cells may help activate antitumor immunity. Nevertheless, FOXP3 should not be regarded as a biomarker that indicates the quantity and functionality of Treg cells.

Our research faced several notable limitations, such as a small sample size, a retrospective design, and being conducted at a single institution. We did not account for the differing distribution of Tregs within the tumor stroma as opposed to tumor clusters, nor did we assess the influence of cytotoxic T cells.

In summary, there are no significant distinctions in progression-free survival (PFS) and overall survival (OS) between patients with low and high FOXP3 levels. FOXP3 expression in both adenocarcinomas and squamous cell carcinomas shows no variation and lacks correlation with clinicopathological features. The variability and adaptability of Tregs account for the divergence between FOXP3 expression and patient survival.

Prospects for further research. We plan to investigate the mutual influence of FOXP3 and CD8+ expression on the patients` survival. In addition, the expression of PD-L receptors will be considered.

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ПРОГНОСТИЧНЕ ЗНАЧЕННЯ FOXP3 У РАДИКАЛЬНО ПРОЛІКОВАНИХ ПАЦІЄНТІВ, ХВОРИХ НА НЕДРІБНОКЛІТИННИЙ РАК ЛЕГЕНЬ

О.І. Винниченко[1], Ю.В. Москаленко[2],
А.М. Піддубний[3], Р.А. Москаленко[4]

[1]Сумський обласний клінічний онкологічний центр, Суми, Україна
[2]Сумський державний університет, кафедра онкології та радіології, Суми, Україна
[3]Україно-Шведський дослідниький центр SUMEYA, Суми, Україна
[4]Сумський державний університет, кафедра патологічної анатомії, Суми, Україна
ORCID ID: 0000-0001-5651-0323,
e-mail: vynnychenkool@ukr.net
ORCID ID: 0000-0002-5398-0298,
e-mail: yl.moskalenko@med.sumdu.edu.ua
ORCID ID: 0000-0002-6508-0131,
e-mail: a.piddubny@med.sumdu.edu.ua
ORCID ID: 0000-0002-2342-0337,
e-mail: r.moskalenko@med.sumdu.edu.ua

Резюме. Пухлинне мікрооточення відіграє ключову роль у протипухлинній імунній відповіді. Особливе значення мають регуляторні Т-клітини, які експресують фактор транскрипції Forkhead box P3 (Foxp3). Незважаючи на імуносупресивні властивості, їх вплив на прогноз і виживаліть при недрібноклітинному раку легень (НДКРЛ) може бути неоднозначним.

Метою нашого дослідження було оцінити прогностичну цінність Foxp3 у пацієнтів, які пройшли радикальне лікування НДКРЛ.

У дослідженні взяли участь 42 пацієнти з НДКРЛ (стадії ІА-ІІІВ), які перенесли радикальне хірургічне втручання та отримали ад'ювантну хіміотерапію. Критерії включення: вік >18 років, відсутність важких супутніх патологій та наявність післяопераційної пухлинної тканини. Виключалися пацієнти, які отримували неоад'ювантну терапію або мали серйозні післяопераційні ускладнення. Імуногістохімічним методом в тканині НДКРЛ оцінювали FOXP3-позитивні клітини. Пацієнтів поділили на групи з низькою (<23 клітин/1 мм²) та високою (≥23 клітин/1 мм²) експресією FOXP3. Порівняння між групами проводили з використанням критеріїв Манна-Уітні, Хі-квадрат, ROC-аналізу, методу Каплана-Мейєра та тесту Log-rank.

Кількість Foxp3-позитивних лімфоцитів у пухлинному мікрооточенні варіювала (5 – 72 клітин/1 мм²). Низьку експресію Foxp3 мали 18 пацієнтів, високу – 24 пацієнти. Кореляцій між рівнем Foxp3 і клініко-патологічними характеристиками не виявлено. Також не спостерігалося відмінностей в експресії Foxp3 між аденокарциномами та плоскоклітинними карциномами. Середня тривалість спостереження становила 57,9±4,2 місяці. Рецидив хвороби і смерть зафіксовано у 19 (45,2 %) пацієнтів. Встановлено, що рівень експресії Foxp3 не впливає на безрецидивну (БРВ) та загальну виживаліть (ЗВ) (Log-rank p=0,1817 та p=0,3944 відповідно). Однак відзначалася тенденція до кращої БРВ і ЗВ у пацієнтів із низькою експресією Foxp3.

Отже, пацієнти з різними рівнями експресії Foxp3 не мали суттєвих відмінностей у БРВ та ЗВ. Не виявлено зв'язку між рівнем Foxp3 та клініко-патологічними характеристиками пухлини.

Ключові слова: Foxp3, регуляторні Т-клітини, безрецидивна виживаліть, загальна виживаліть, недрібноклітинний рак легень, прогноз, пухлинне мікрооточення, імуногістохімія.

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