

Efficiency of Immunological Blood Biomarkers in Predicting Chemotherapy Response and Survival Outcome for Non-Targetable Advanced Non-Small Cell Lung Cancer Patients

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Purpose: Chemotherapy is the main therapy for non-targetable advanced non-small cell lung cancer (NSCLC). Nevertheless, few biomarkers are currently available for predicting clinical outcomes and monitoring treatment response.

Patients and Methods: The blood samples of 42 patients with non-targetable advanced NSCLC who received chemotherapy were collected before chemotherapy and after chemotherapy. The circulating immune cells and subpopulation Treg were investigated using flow cytometry, and human immune checkpoint biomarker levels were analysed by multiplex bead-based assay.

Results: After selecting the effective biomarkers for survival analysis, high pre-chemotherapy sCD25 (≥ 499.52 pg/mL; 4.52 vs 14.98 months, $p = 0.030$) and post-chemotherapy (≥ 515.23 pg/mL; 3.90 vs 21.25 months, $p = 0.004$) were associated with poorer median progression-free survival (PFS). An increase in pre-chemotherapy neutrophil to lymphocyte ratio (NLR) (ratio ≥ 6.9 ; 5.15 vs 21.54 months, $p = 0.008$) and post-chemotherapy NLR (ratio ≥ 3.1 ; 10-month OS 50.35% vs 83.57%, $p = 0.014$) was correlated with shorter overall survival (OS). Moreover, increased pre-chemotherapy %NKT cells ($\geq 6.8\%$) were linked to improved clinical benefit rate (CBR) (2.72 vs 3.97 months, $p = 0.013$), while higher post-chemotherapy %NK cells ($\geq 23.1\%$) were associated with rapid overall response rate (ORR) at 4 months (82.05% vs 25%, $p = 0.035$).

Conclusion: Our findings suggest that sCD25 and NLR show potential as indicators of PFS and OS, respectively. Additionally, pre-chemotherapy %NKT and post-chemotherapy %NK cells may provide insight into monitoring chemotherapy response. This pilot study identified potential candidate biomarkers for further investigation to confirm their clinical utility.

Keywords: non-targetable advanced NSCLC, immunological biomarkers, chemotherapy, survival outcomes, clinical response

Introduction

Advanced non-small cell lung cancer (NSCLC) is a metastatic stage of lung cancer that has spread to other parts of the body, making it no longer curable. Two forms of the diagnostic stage are locally advanced NSCLC (stages 3 and 4). In Thailand, NSCLC patients present with locally advanced or metastatic disease in more than half.^{1,2} There have been significant advances in treatment, particularly in targeted therapy, which is used to treat and monitor patients with advanced NSCLC. It is used to treat lung cancer with epidermal growth factor receptor (*EGFR*) mutations and anaplastic

lymphoma kinase (*ALK*) gene rearrangements, and it has been shown to increase the response rate by 60–70% and to significantly improve progression-free survival (PFS) and overall survival (OS) compared with chemotherapy.³ However, many patients still do not have the mutations for which targeted drugs are available.

Concurrent chemoradiation (CCRT) with traditional platinum-based doublet chemotherapy remains a cornerstone of first-line therapy for non-targetable advanced NSCLC patients. It has a response rate of only about 30%.⁴ Although chemotherapy can eliminate cancer cells, it is also toxic to normal cells and often has severe side effects. However, the patient had received CCRT, which showed a median PFS (mPFS) of approximately 8 months and a 5-year recurrence rate of 76%, along with a 5-year survival rate of 15%.^{5,6}

Molecular biomarkers in advanced NSCLC patients have been used in drug-targeted therapy to personalize treatment, which significantly increases the survival rate in lung cancer patients with several mutations or alterations, such as *EGFR* mutations, B-Raf proto-oncogene serine/threonine kinase (*BRAF*) mutations, ROS proto-oncogene 1 receptor tyrosine kinase (*ROS1*), ALK receptor tyrosine kinase (*ALK*), and neurotrophic receptor tyrosine kinase 1 (*NTRK1*) fusion genes.^{7,8} Until recently, they have had an immunotherapy treatment which has been used in combination with chemotherapy for non-targeted advanced NSCLC patients, which is most lung cancer patients. However, these non-targeted therapies have not significantly improved outcomes for some patient treatments because there is no biomarker testing that can help physicians decide which is the most effective treatment option to individual's specific condition.

The immunological biomarker is a substance of immune response to a disease or state that can be used for screening, diagnosis, prediction, monitoring, and the pharmacological responses to treatments. The liquid biopsy is a simple sample taken from circulating blood, which consists of immune cells, cytokines, chemokines, and immunological substances such as Programmed cell death protein 1 (PD-1), Programmed death-ligand 1 (PD-L1), Subpopulation T-cells, and Myeloid-derived suppressor cells (MDSCs), etc.^{9–11} The predictive biomarker testing in advanced NSCLC patients has been studied in targeted therapy more than in non-targeted treatment. However, the non-targeted advanced NSCLC patients need predictive biomarker testing to predict or monitor the treatment efficacy of chemotherapy in patients through liquid biopsy.

Currently, the treatment options for advanced NSCLC patients with no detectable targeted gene mutations in Thailand are limited to chemotherapy, a treatment with a high risk of side effects and a low response to treatment compared to targeted therapy drugs. This study aims to identify prognostic immunological biomarkers that influence survival outcomes and evaluate treatment response before and after platinum-based chemotherapy in non-targeted therapy for advanced NSCLC patients.

Material and Methods

Participants and Study Design

This prospective cohort study included 42 patients who had confirmed advanced NSCLC stage IIIB or IV according to the 8th edition of the American Joint Committee on Cancer (8th AJCC) staging and were treated with first-line platinum-based doublet chemotherapy at Ramathibodi Hospital between November 2021 and June 2024. Eligible patients were aged 18 years or older, had NSCLC confirmed by cytopathology, and had received at least two cycles of platinum-based doublet chemotherapy (platinum plus paclitaxel or platinum plus gemcitabine). The study included patients who completed at least 2 cycles of treatment, with a total of 4–6 cycles, to assess treatment effects using computed tomography (CT) scans within one month. CT scans were performed every 3–4 months to monitor disease status until disease progression or discontinuation of treatment due to toxicity or until death, whichever came first, to obtain survival data. The study tracked survival until June 2024.

After patient enrollments, peripheral blood samples were collected for immune cell and cytokine testing before the initiation of chemotherapy (baseline) and after complete chemotherapy within 2 weeks. Moreover, at the time of disease progression, blood samples were also collected again. The data collected from medical records included patient characteristics and treatment details related to lung cancer. Additionally, treatment response was assessed using RECIST 1.1 criteria.¹²

The study classified patients based on their response to treatment (complete response, CR; partial response, PR; stable disease, SD; progressive disease, PD). In chemotherapy response, the clinical benefit rate (CBR) is a measure of the percentage of patients who have achieved a CR, PR or SD for a defined period (typically 6 months or more) as responders, while the overall response rate (ORR) focuses on the percentage of patients who have a measurable tumor reduction (either CR or PR) as responders. OS was defined as the period between the date of the first day of chemotherapy until the date of death from any cause. In contrast, PFS was the time from the first chemotherapy treatment to either radiographic or clinical progression, or death from any cause.

The study protocol was reviewed and approved by the Human Research Ethics Committee of Ramathibodi Hospital, Mahidol University, Bangkok, Thailand (Institutional Review Board number COA. MURA2023/568 Ref.2024/1067). All participants were informed of the study details and provided written consent before enrollment. All methods were performed under relevant guidelines and local regulations.

Liquid Biopsy Collection

About 9 mL of fresh EDTA whole blood samples are collected from the patients before the initiation of chemotherapy (N = 42) and after complete chemotherapy within 2 weeks (N = 27). After that, 0.5 mL EDTA-blood was used for circulating immune cells and measured by Flow cytometry. 2.5 mL was centrifuged for plasma and stored at -80°C until cytokines were measured, and 6 mL were used for peripheral blood mononuclear cells (PBMCs) isolation.

Circulating Immune Cells

In brief, fresh EDTA whole blood was collected. The precise volume (50 μL) was added to the counting beads tube (cat. 340334, Becton Dickinson (BD), USA). The samples were incubated with a 6-Color human TBNK reagent (cat.662967, BD, USA), which consists of mouse anti-human CD3-FITC, CD16-PE+CD56-PE, CD45-PerCP-Cy5.5, CD4-PE-Cy7, CD8-APC-Cy7, and CD19-APC antibodies for T-cells, B cells, Natural killer (NK) cells and CD66b-BV421 (cat.562940, BD, USA) for neutrophil, CD297-BV510 (cat.563076, BD, USA) for PD-1 receptor with 10 minutes in the dark. Moreover, monocyte MDSCs (Mo-MDSCs) was identified using 50 μL of whole blood incubated with mouse anti-human CD33-PE-Cy7, HLA-DR-APC, CD11b-PE, and CD14-APC-H7. After extracellular staining, red blood cells were lysed with lysis solution (BD, USA) and washed twice with phosphate-buffered saline (PBS) pH 7.4, and resuspended cells in 450 μL PBS. Isotype mouse anti-human BV510, PerCP-Cy5.5, PE, APC, APC-H7, BV421, PE-Cy7 control is another tube of cells stained with all mouse anti-human fluorochromes used in the experiment. The FACSLytic flow cytometer was employed for cell phenotype acquisition and the data were analyzed using FlowJo software.

Foxp3 Regulatory T-Cells (Treg) Subpopulation

In brief, PBMCs were isolated using a commercial isolation kit (Heparinized PBMCs isolation vacutainer tube). 1×10^6 cells were treated with mouse anti-human fluorescent dye-conjugated antibodies [CD45-APC-H7, CD4-FITC, CD25-PE, CD127-PerCP-Cy5.5, CD152-BV421, CD45RO-BV510 (BD, USA)] for extracellular staining. For intracellular staining, the cells were washed and suspended, then fixed and permeabilized using fixing/permeabilizing (fix/perm) solution and perm buffer (Affymetrix eBioscience, USA) for 45 min in the dark and followed by incubation with rat anti-human Foxp3-Alexafluor660 (ref.50477642, Affymetrix eBioscience, USA) and isotype Alexafluor660 antibodies (ref.50432182, Affymetrix eBioscience, USA) for 30 min at ambient temperature in the dark. Cells were washed twice with perm buffer, suspended in 500 μL of PBS and analyzed for sub-population Treg by FACSLytic flow cytometer. Treg is defined as $\text{CD4}^+\text{CD25}^{\text{high}}\text{FoxP3}^+$ cells. Treg cell sub-populations are defined as $\text{CD4}^+\text{CD25}^{\text{high}}\text{FoxP3}^+\text{CD127}^{-/\text{low}}$ cells (induced Treg (iTreg)), $\text{CD4}^+\text{CD25}^{\text{high}}\text{FoxP3}^{\text{low}}\text{CD127}^{-/\text{low}}\text{CD152}^-\text{CD45RO}^-$ cells (Naïve Treg), $\text{CD4}^+\text{CD25}^{\text{high}}\text{FoxP3}^+\text{CD127}^{\text{low}}\text{CD152}^+\text{CD45RO}^+$ cells (Effector Treg), and $\text{CD4}^+\text{CD25}^{\text{high}}\text{FoxP3}^+\text{CD127}^-\text{CD152}^+\text{CD45RO}^+$ cells (Terminal Effector Treg).

Cytokines Determination

The cytokines detection protocol was followed by the multiplex bead assay kit (cat. No. 740961, Biolegend, USA, Human Immune Checkpoint Panel 1-S/P (10-plex) w/FP with Filter plate; 10 human cytokines including sCD25 (IL-

2Ra), s4-1BB, B7.2 (CD86), TGF- β 1 (Free active), CTLA-4, PD-L1, PD-1, Tim-3, LAG-3 and Galectin-9) recommendation. The range limit of detection is 0–10,000 pg/mL. Briefly, the stored plasma EDTA was thawed completely at room temperature. All plasma was diluted 2-fold with assay buffer prior to assay. These experiments are done in triplicate and analyzed by flow cytometry.

Statistical Analysis

For categorical variables, results were presented as percentages. Continuous variables were illustrated as the mean ($\pm$ standard deviation (SD)) for normal distribution data, and as the median with interquartile range (IQR) for non-normal distribution data. To compare continuous variables between groups, quantile regression was performed at the 25th, 50th, and 75th percentiles. For comparative analysis of paired continuous data, a paired *t*-test was used for normal distribution and the Wilcoxon signed-rank test for non-normal distribution. The efficiency of biomarkers in predicting an event was calculated using Receiver operating characteristic (ROC) analysis. Time-to-event outcomes were analyzed using the Kaplan–Meier method for univariate analysis and Cox proportional hazards regression models for multivariate analysis. Statistical analysis was carried out using STATA software version 18. All tests were two-tailed, and a *p*-value < 0.05 was considered statistically significant.

Results

Baseline Characteristics

In this study, all 42 non-targetable advanced NSCLC patients who received CCRT or palliative chemotherapy with traditional platinum-based doublet chemotherapy, of whom only 27 patients received complete chemotherapy treatment. The baseline characteristics are listed in [Table 1](#).

Table 1 Demographic and Clinical Characteristics

Characteristics	N = 42
Age (years, mean $\pm$ sd)	66.1 $\pm$ 9.8
Height (cm, IQR)	165.2 (155.0–170.4)
Weight (kg, IQR)	55.6 (50.0–66.0)
Gender	
Female	14 (33.3%)
Male	28 (66.7%)
Smoking status	
Never smoker	14 (33.3%)
Ever smoker	28 (66.7%)
ECOG PS	
0	9 (21.4%)
1	19 (45.2%)
2	10 (23.8%)
Unknown	4 (9.5%)
Comorbidities	
Hypertension	
No	20 (47.6%)
Yes	22 (52.4%)
Dyslipidemia	
No	22 (52.4%)
Yes	20 (47.6%)

(Continued)

Table I (Continued).

Characteristics	N = 42
Diabetes Mellitus	
No	34 (81%)
Yes	8 (19%)
Histology	
Adenocarcinoma	31 (73.8%)
Squamous cell carcinoma	6 (14.3%)
Others	5 (11.9%)
Staging	
IIIA	1 (2.4%)
IIIB	2 (4.8%)
IIIC	3 (7.1%)
IVA	28 (66.7%)
IVB	8 (19%)
Distance Metastatic Site	
Pleura	
No	28 (66.7%)
Yes	14 (33.3%)
Lung	
No	27 (64.3%)
Yes	15 (35.7%)
Bone	
No	33 (78.6%)
Yes	9 (21.4%)
Brain	
No	37 (88.1%)
Yes	5 (11.9%)
Liver	
No	35 (83.3%)
Yes	7 (16.7%)
Others	
No	30 (71.4%)
Yes	12 (28.6%)
Molecular Alteration	
EGFR	
Wild type	34 (81%)
Mutation	2 (4.8%)
Unknown	6 (14.3%)
ALK	
Negative	31 (73.8%)
Positive	1 (2.4%)
Unknown	10 (23.8%)
ROSI (IHC)	
Negative	18 (42.9%)
Positive	1 (2.4%)
Unknown	23 (54.8%)
PDLI	
No	10 (23.8%)
Yes	32 (76.2%)
PDLI (IHC by 22c3)	
<1%	8 (19%)
1–49%	12 (28.6%)

(Continued)

Table 1 (Continued).

Characteristics	N = 42
≥50%	12 (28.6%)
Unknown	10 (23.8%)
Chemotherapy treatment	
Platinum-based	34 (81%)
Others	8 (19%)
Response	
Complete Response	1 (2.4%)
Partial Response	7 (16.7%)
Stable Disease	14 (33.3%)
Progressive Disease	15 (35.7%)
Not Evaluated	5 (11.9%)
PD Status	
PD	31 (73.8%)
Non-PD	6 (14.3%)
Unknown	5 (11.9%)
Dead	
Dead	24 (57.1%)
Alive	18 (42.9%)

Effects of Chemotherapy Treatment on Peripheral Blood Biomarkers

The final analysis for blood biomarkers was performed on 27 out of the total 42 participants (N = 27). Fifteen patients were excluded due to death during chemotherapy or loss to follow-up before post-chemotherapy sample collection. A comparison of pre- and post-chemotherapy samples revealed significant changes in 12 of the 29 biomarkers ([Supplementary Table 1](#)). Among the Cellular markers, the %PD1⁺ CD8⁺ T-cells, %T-cells, %CD8⁺ T-cells, and %NK cells were significantly higher after a complete cycle of chemotherapy treatment (p = 0.044, p = 0.028, p = 0.003, p = 0.045; respectively). In the Treg markers, the % naïve Treg, %effector Treg, and %terminal effector Treg showed significant changes after chemotherapy (p = 0.036, p = 0.014, p = 0.005; respectively). Similarly, the plasma levels of sCD25, s41BB, Tim-3, LAG-3, and Galectin-9 were all found to be significantly different between before and after chemotherapy treatment (p = 0.028, p = 0.029, p = 0.018, p = 0.015, p = 0.003; respectively) ([Supplementary Table 1](#)).

Compared between the PD groups, there are no significant differences between the non-PD (N = 6) and PD (N = 21) groups before and after chemotherapy. Within the non-PD groups, white blood cells (WBC) count and %MDSCs were increased after chemotherapy (p = 0.043, p = 0.043, respectively), while LAG-3 was decreased after chemotherapy (p = 0.043). Within the PD groups, %T-cells, %CD8⁺ T-cells, %PD1⁺ CD8⁺ T-cells, %Naïve Treg, %Effector Treg, %Terminal Effector Treg, sCD25, Tim-3, Galectin-9 were elevated after chemotherapy (p = 0.042, p = 0.021, p = 0.046, p = 0.044, p = 0.020, p = 0.009, p = 0.048, p = 0.035, p = 0.016; respectively) ([Table 2](#)).

Comparing between patients who achieved a CBR (CR+PR+SD, N=17) and non-responder (PD, N=10), the WBC count was higher in the non-responder, while Galectin-9 levels were lower in the non-responder compared to the responders before chemotherapy (p = 0.047, P = 0.036). Only PD-L1 was higher in non-responder compared to responder after chemotherapy (p = 0.022). When comparing biomarkers before and after chemotherapy in the responder, the %T-cells (p = 0.024), %CD8⁺ T-cells (p = 0.018), %Natural killer T-cells (NKT) (p = 0.049), %Naïve Treg (p = 0.036), and %Terminal Effector Treg (p = 0.026) showed significantly increased, while s41BB (p = 0.022) and LAG-3 (p = 0.035) were decreased after chemotherapy. In contrast, non-responders showed significant elevations in %PD1⁺ CD8⁺ T-cells (p = 0.028), %Effector Treg (p = 0.028), Tim-3 (p = 0.028), and Galectin-9 (p = 0.015) after chemotherapy ([Supplementary Table 2](#)).

Table 2 Change in Blood Biomarker Levels Before and After Chemotherapy Within and Between PD Groups

Characteristics	Before Chemotherapy		After Chemotherapy		P-value ^a	P-value ^b	P-value ^c	P-value ^d
	Non-PD (N=6)	PD (N=21)	Non-PD (N=6)	PD (N=21)				
Cellular Markers								
WBC count	7800 (7300–8900)	8900 (6500–10,900)	5200 (4700–6000)	7150 (5100–10,000)	0.655	0.346	0.0431*	0.4599
Neu/Lym ratio	2.5 (2.3–6.7)	6.8 (4.5–10.6)	2.6 (2.3–2.9)	5.0 (1.8–8.4)	0.438	0.618	0.1380	0.6968
%T cells	67.18 ± 18.53	64.86 ± 12.92	71.64 ± 11.31	69.50 ± 10.34	0.577	0.772	0.4757	0.0418*
%CD4 ⁺ T cells	34.08 ± 16.98	35.85 ± 10.80	27.4 ± 12.14	36.94 ± 11.87	0.784	0.209	0.2643	0.6358
%CD8 ⁺ T cells	27.8 (15.8–39.3)	23.8 (15.7–31.3)	36.2 (32.9–45.4)	25.6 (21.3–33.2)	0.562	0.244	0.0796	0.0212*
%PD1 ⁺ CD8 ⁺ T cells	12.2 (10.5–19.1)	17.8 (13.3–29.8)	15.7 (11.5–18.3)	24.4 (15.4–34.8)	0.594	0.423	0.6858	0.0457*
%NK cells	13.2 (12.4–18.1)	19.4 (14.2–30.0)	13.8 (13–25.6)	18.1 (12.6–22.3)	0.451	0.423	0.8927	0.0500
%NKT cells	8.4 (6–9)	4.5 (3.2–9.3)	10.9 (10.2–11.9)	5.7 (3.8–7.6)	0.415	0.177	0.1380	0.1885
%B cells	10.5 (9.8–17.9)	9.3 (8–12.6)	10.9 (10.8–11)	9.3 (6.3–13.2)	0.785	0.781	0.5002	0.1191
%MDSCs	5.1 (4–9.8)	16.8 (7–19.2)	10.1 (6.5–29.2)	16.3 (10.6–26.2)	0.089	0.548	0.0431*	0.2959
Absolute CD3 ⁺ T cells	1300 (400–1700)	750 (500–1400)	1100 (1000–1200)	850 (600–1400)	0.610	0.870	0.7865	0.8706
Absolute CD4 ⁺ T cells	750 (200–800)	450 (300–800)	400 (300–700)	500 (300–900)	0.512	0.555	0.8927	0.6584
Absolute CD8 ⁺ T cells	450 (100–900)	300 (200–500)	600 (400–800)	300 (200–400)	0.096	0.288	0.3363	0.8057
Absolute NK cells	300 (200–400)	300 (200–400)	300 (200–300)	250 (100–400)	1.000	1.000	0.5637	0.2118
Absolute NKT cells	100 (30–200)	100 (20–100)	200 (100–200)	100 (30–200)	1.000	0.270	0.0897	0.4546
Absolute B cells	200 (130–280)	120 (50–300)	190 (100–280)	100 (60–160)	0.552	0.436	0.5879	0.2469

(Continued)

Table 2 (Continued).

Characteristics	Before Chemotherapy		After Chemotherapy		P-value ^a	P-value ^b	P-value ^c	P-value ^d
	Non-PD (N=6)	PD (N=21)	Non-PD (N=6)	PD (N=21)				
Treg Markers								
%iTreg	9 (4.0–10.9)	6.1 (5.3–8.4)	5.8 (5.8–13.1)	7.1 (3.9–8.1)	0.380	0.583	0.8927	0.8076
%Naïve Treg	4.4 (3.9–6.6)	1.7 (0.8–4.7)	5.7 (2.3–9)	5.5 (2.1–8.9)	0.245	0.963	0.5930	0.0438*
%Effector Treg	1.5 (1.1–8.9)	4.5 (2.5–9)	6.2 (6–9.5)	11 (3.6–14.9)	0.377	0.341	0.5002	0.0196*
%Terminal Effector Treg	8.9 (6.9–38.2)	28.5 (9.3–44.3)	33.5 (17.9–33.7)	35.6 (32.2–48)	0.331	0.887	0.3452	0.0087†
Soluble Markers								
sCD25	372.6 (300.5–412.2)	668.12 (377.4–1178.5)	385.1 (285.0–478.5)	928.9 (515.2–1352.2)	0.291	0.222	0.1730	0.0475*
s41BB	19.1 (7.3–50.7)	26.2 (12.5–46.3)	10.9 (4.8–20.4)	18.9 (7.8–37.4)	0.904	0.826	0.0679	0.1075
B7.2	199.2 (189.3–239.0)	186.8 (142.0–235.1)	224.5 (179.5–250.4)	214.2 (142.1–281.2)	0.671	0.695	0.6002	0.3052
CTLA-4	0.6 (0.0–0.64)	0.4 (0.0–1.7)	0.0 (0.0–1.0)	0.3 (0.0–1.4)	0.297	0.504	0.3991	0.8202
PDL-1	29.3 (11.8–55.0)	45.8 (14.6–56.6)	14.4 (10.4–42.3)	54.0 (12.0–67.8)	0.522	0.343	0.5002	0.0990
PD-1	7.8 (3.7–9.1)	7.7 (3.4–14.9)	8.7 (3.1–9.5)	7.4 (3.3–14.7)	0.861	0.903	0.3452	0.8583
Tim-3	446.8 (178.3–568.7)	501.6 (247.7–832.3)	625.8 (374.1–1864.5)	1102.0 (592.1–2304.0)	0.410	0.718	0.3454	0.0347*
LAG-3	2826.4 (2273.7–3590.8)	2537.6 (1624.4–4054.2)	1749.0 (1630.7–1843.2)	1978.2 (1049.7–3387.3)	0.844	0.858	0.0431*	0.0987
Galectin-9	15,340.9 (14,357.5–18,031.6)	13,530.0 (8750.0–19,484.4)	19,791.3 (15,762.4–40,467.0)	18,883.6 (15,552.5–27,276.9)	0.540	0.732	0.0747	0.0157*

Notes: ^a Quartile regression, * $p < 0.05$, † $p < 0.01$ (Non-PD vs PD in Before Chemotherapy). ^b Quartile regression, * $p < 0.05$, † $p < 0.01$ (Non-PD vs PD in After Chemotherapy). ^c Wilcoxon signed-rank test or Paired t-test, Two-tailed, * $p < 0.05$, † $p < 0.01$ (Non-PD before vs after chemotherapy). ^d Wilcoxon signed-rank test or Paired t-test, Two-tailed * $p < 0.05$, † $p < 0.01$ (PD before vs after chemotherapy). %PD1⁺ CD8⁺ T Cells was reported as % of CD8⁺ cells. % MDSCs was reported as % of CD14⁺ cells. All absolute cellular markers were reported as cells/ul. All Treg markers were reported as % of CD4⁺ cells. All soluble markers were reported as pg/mL.

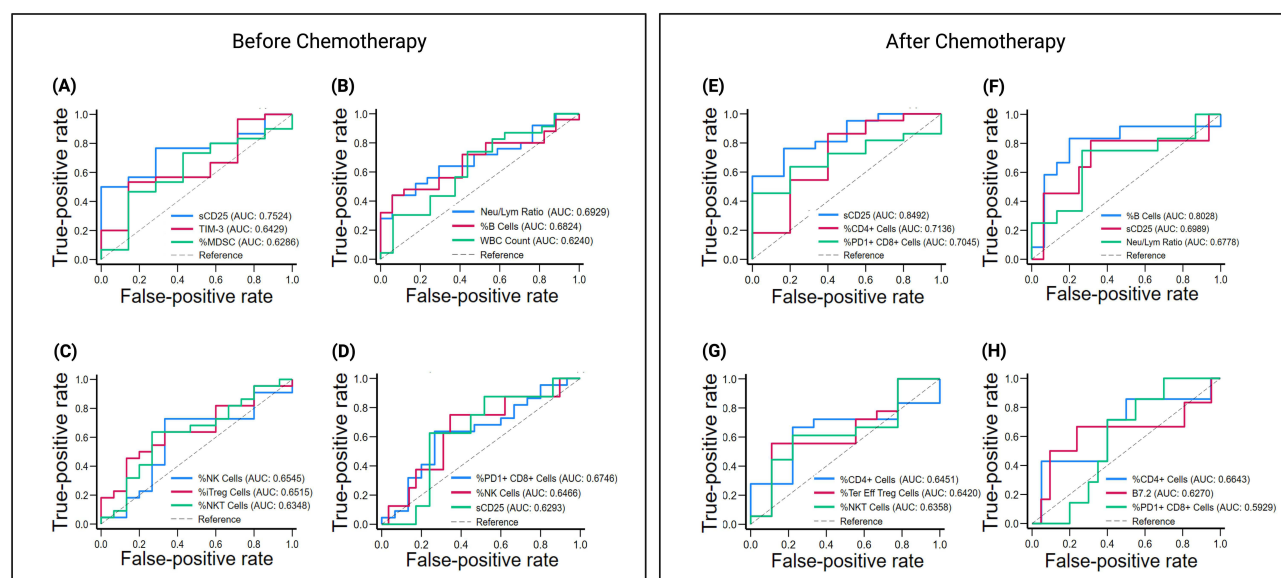


Figure 1 ROC Curves for Clinical Outcomes Across Different Selected Blood Biomarkers. (A) ROC Curve for Progression Free Disease (PD Status) before Chemotherapy (B) ROC Curve for Overall Survival before Chemotherapy (C) ROC Curve for Clinical Benefit Rate (CBR) before Chemotherapy (D) ROC Curve for Overall Response Rate (ORR) before Chemotherapy (E) ROC Curve for Progression Free Disease (PD Status) after Chemotherapy (F) ROC Curve for Overall Survival after Chemotherapy (G) ROC Curve for Clinical Benefit Rate (CBR) after Chemotherapy (H) ROC Curve for Overall Response Rate (ORR) after Chemotherapy.

Next, we compared the biomarkers between patients who achieved ORR (CR+PR; N = 6) and non-responder (PD+SD; N = 21) groups ([Supplementary Table 3](#)). Before chemotherapy, only PD-1 levels were significantly higher in responders ($p = 0.003$), while no differences were observed between the two groups after chemotherapy. When comparing the biomarkers before and after chemotherapy in the same responder group ([Supplementary Table 3](#)). On the other hand, in the responder group, %T-cells, %CD4⁺ T-cells, and %NK cells were higher after chemotherapy ($p = 0.03$, $p = 0.038$, $p = 0.028$; respectively). Conversely, the non-responder group showed that the %Naive Treg ($p = 0.035$), %Effector Treg ($p = 0.018$), %Terminal Effector Treg ($p = 0.012$), Tim-3 ($p = 0.011$), and Galectin-9 ($p = 0.003$) were significantly increased after chemotherapy. Additionally, absolute NKT cells also showed a significant difference between pre- and post-chemotherapy measurements within this group ($p = 0.038$). However, only LAG-3 level ($p = 0.037$) was significantly decreased after chemotherapy in the non-responder group ([Supplementary Table 3](#)).

Biomarker Performance of Clinical Outcome Before and After Chemotherapy Treatment

At baseline before chemotherapy (N = 42), the top three highest-performing biomarkers for each outcome, including PFS, CBR, ORR, and OS, were identified based on their respective area under the curve (AUC) values ([Supplementary Table 4](#)). An optimal cut-off value for each biomarker was determined by comparing the sensitivity and specificity. For predicting PFS, the highest-performing biomarkers were sCD25 levels (AUC = 0.7524, cut-off ≥ 499.52 pg/mL), Tim-3 levels (AUC = 0.6429, cut-off ≥ 697.82 pg/mL), and %MDSCs (AUC = 0.6286, cut-off $\geq 16.4\%$ of CD14⁺ Cells). Neutrophil to lymphocyte ratio (NLR) (AUC = 0.6929, cut-off ≥ 6.9), %B cells (AUC = 0.6824, cut-off $\geq 11.6\%$ of lymphocytes), and WBC count (AUC = 0.6413, cut-off 8200 cells/ul), were the most predictive for OS. In predicting CBR, the most effective biomarkers were Absolute NK Cells (AUC = 0.6545, cut-off ≥ 300 cells/ul), %iTreg cells (AUC = 0.6515, cut-off $\geq 7.19\%$ of lymphocytes), and %NKT cells (AUC = 0.6348, cut-off $\geq 5\%$ of lymphocytes). For ORR, the highest-performing biomarkers were %PD1⁺ CD8⁺ T-cells (AUC = 0.6552, cut-off $\geq 27.6\%$ of CD8⁺ cells), %NK cells (AUC = 0.6466, cut-off $\geq 23.1\%$ of lymphocytes), and sCD25 levels (AUC = 0.6293, cut-off ≥ 742.68 pg/mL) ([Figure 1](#), [Table 3](#), [Supplementary Table 4](#)).

Table 3 Cut-Off Value for Outcomes Predictors

Before Chemotherapy (n = 42)					After Chemotherapy (n = 27)				
Variables	Cut-off	Sensitivity (%)	Specificity (%)	AUC (95% CI)	Variables	Cut-off	Sensitivity (%)	Specificity (%)	AUC (95% CI)
Progression-Free Survival (PFS)					Progression-Free Survival (PFS)				
sCD25	499.52	76.67	71.43	0.7524 (0.5815–0.9231)	sCD25	515.23	76.19	83.33	0.8492 (0.6854–1.0000)
Tim-3	697.82	85.71	59.46	0.6429 (0.4113–0.8744)	%CD4 ⁺ T Cells	27.8	86.36	60.00	0.7136 (0.3971–1.0000)
%MDSs Cells	16.4	46.67	85.71	0.6286 (0.3875–0.8697)	%PD1 ⁺ CD8 ⁺ T Cells	19.5	63.64	80.00	0.7045 (0.4989–0.9102)
Overall Survival (OS)					Overall Survival (OS)				
Neu/Lym Ratio	6.9	64.00	70.59	0.6929 (0.5331–0.8528)	%B Cells	10.6	83.33	80.00	0.8028 (0.6105–0.9951)
%B-Cells	11.6	56.00	70.59	0.6824 (0.5204–0.8444)	sCD25	701.05	81.82	68.75	0.6989 (0.4689–0.9289)
WBC	8200	73.91	56.25	0.6413 (0.4583–0.8243)	Neu/Lym Ratio	3.1	75.00	73.33	0.6778 (0.4587–0.8969)
Clinical Benefit Rate (CBR)					Clinical Benefit Rate (CBR)				
Ab NK Cells	300	72.73	66.67	0.6545 (0.4627–0.8464)	%CD4 ⁺ T Cells	32.5	66.67	77.78	0.6451 (0.4231–0.8670)
%iTreg	7.19	50.00	80.00	0.6515 (0.4703–0.8327)	%Terminal Effector Treg	35.6	55.56	77.78	0.6420 (0.4076–0.8764)
%NKT Cells	5	63.64	73.33	0.6348 (0.4442–0.8255)	%NKT Cells	6.8	61.11	77.78	0.6358 (0.4010–0.8706)
Overall Response Rate (ORR)					Overall Response Rate (ORR)				
%PD1 ⁺ CD8 ⁺ T Cells	27.6	50.00	75.86	0.6552 (0.4587–0.8517)	%CD4 ⁺ T Cells	35.3	71.43	60.00	0.6643 (0.3995–0.9291)
%NK Cells	23.1	75.00	65.52	0.6466 (0.4231–0.8700)	B7.2	258.98	66.67	76.19	0.6270 (0.2929–0.9611)
sCD25	742.68	62.50	75.86	0.6293 (0.4217–0.8369)	%PD1 ⁺ CD8 ⁺ T Cells	24.4	71.43	60.00	0.5929 (0.3755–0.8102)

Notes: %PD1⁺ CD8⁺ T Cells was reported as % of CD8⁺ cells. %MDSs was reported as % of CD14⁺ cells. All absolute cellular markers were reported as cells/ul. All Treg markers were reported as % of CD4⁺ cells. All soluble markers were reported as pg/mL.

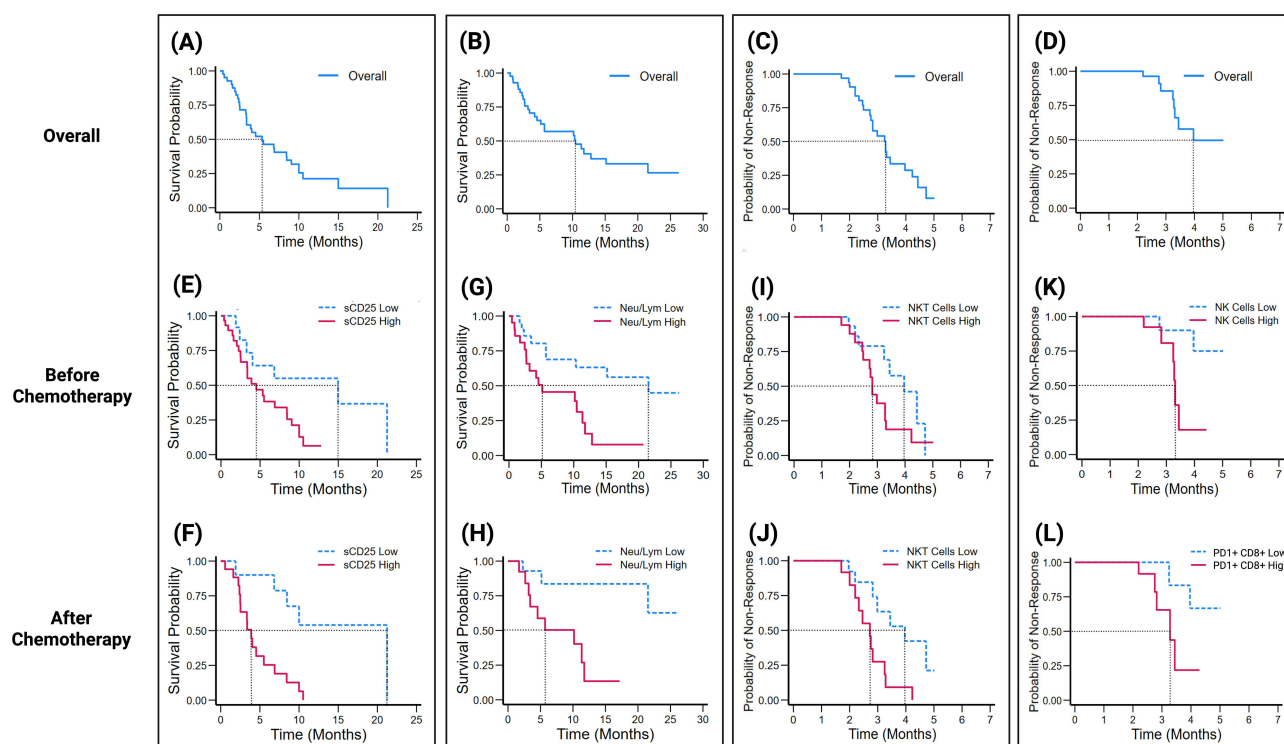


Figure 2 Cox Proportional Hazard Ratios for Different Clinical Outcomes Based on Biomarker Group Stratification. **(A)** Progression-free survival for all patients **(B)** Overall survival for all patients **(C)** Clinical benefit rate for all patients **(D)** Overall response rate for all patients **(E)** Comparison of PFS in patients before chemotherapy between sCD25 high (sCD25 ≥ 499.52 pg/mL) and sCD25 low (sCD25 < 499.52 pg/mL) groups **(F)** Comparison of PFS after chemotherapy between sCD25 (sCD25 ≥ 515.23 pg/mL) high and sCD25 low (sCD25 < 515.23 pg/mL) **(G)** Comparison of OS in patients before chemotherapy between neutrophil to lymphocyte ratio high (NLR ≥ 6.9) and low (NLR < 6.9) groups **(H)** Comparison of OS in patients after chemotherapy between neutrophil to lymphocyte ratio high (NLR ≥ 3.1) and low (NLR < 3.1) groups **(I)** Comparison of CBR in patients before chemotherapy between NKT cells high percentage (%NKT cells $\geq 5\%$) and low percentage (%NKT cells $< 5\%$) groups **(J)** Comparison of CBR in patients after chemotherapy between NKT cells high percentage (%NKT cells $\geq 6.8\%$) and low percentage (%NKT cells $< 6.8\%$) groups **(K)** Comparison of ORR in patients before chemotherapy between NK cells high percentage (%NK cells $\geq 23.1\%$) and low percentage (%NK cells $< 23.1\%$) groups **(L)** Comparison of ORR in patients after chemotherapy between %PD1⁺ CD8⁺ high (%PD1⁺ CD8⁺ cells $\geq 24.4\%$) and low percentage (%PD1⁺ CD8⁺ cells $< 24.4\%$) groups.

After chemotherapy (N = 27), the top three biomarkers for predicting PFS were sCD25 levels (AUC = 0.8492, cut-off ≥ 515.23 pg/mL), %CD4⁺ T-cells (AUC = 0.7136, cut-off $\geq 27.8\%$ of lymphocytes), and %PD1⁺ CD8⁺ T-cells (AUC = 0.7045, cut-off $\geq 19.5\%$ of CD8⁺ T-cells). %B cells (AUC = 0.8028, cut-off $\geq 10.6\%$ of lymphocytes), sCD25 levels (AUC = 0.6989, cut-off ≥ 701.05 pg/mL), and NLR (AUC = 0.6778, cut-off ≥ 3.1) demonstrated the highest predictive power for OS. In CBR, the most predictive biomarkers were %CD4⁺ T-cells (AUC = 0.6451, cut-off $\geq 32.5\%$ of lymphocytes), %Terminal Effector Treg (AUC = 0.6420, cut-off $\geq 35.6\%$ of lymphocytes), and %NKT cells (AUC = 0.6358, cut-off $\geq 6.8\%$ of lymphocytes). Lastly, ORR was best predicted by %CD4⁺ T-cells (AUC = 0.6643, cut-off $\geq 35.3\%$ of lymphocytes), B7.2 levels (AUC = 0.6270, cut-off ≥ 258.98 pg/mL), and %PD1⁺ CD8⁺ T-cells (AUC = 0.5929, cut-off $\geq 24.4\%$ of CD8⁺ T-cells) (Figure 1, Table 3, [Supplementary Table 4](#)).

Survival Analysis Outcomes

Follow-up data were available for all participants. The median follow-up duration in this cohort study was 5.34 months for PFS (Figure 2A), 10.46 months for OS (Figure 2B), 3.28 months for CBR (Figure 2C), and 3.97 months for ORR (Figure 2D). Comparing the best hazard ratio (HR) of candidate biomarkers in each clinical outcomes between high and low groups, the data showed that patients with elevated sCD25 levels (≥ 499.52 pg/mL) had significantly shorter mPFS, mPFS 4.52 vs 14.98 months before chemotherapy (HR = 2.96, 95% CI: 1.11–7.86, $p = 0.030$) (Figure 2E), and mPFS 3.90 vs 21.25 months after chemotherapy (HR = 5.35, 95% CI: 1.73–16.53, $p = 0.004$) (Figure 2F). For OS, the survival rate was lower in the high NLR (ratio ≥ 6.9), showing shorter survival before chemotherapy, median OS (mOS) 5.15 vs 21.54 months (HR = 3.27, 95% CI: 1.36–7.87, $p = 0.008$) (Figure 2E). The 10-month survival rate was lower in the high

NLR group (ratio ≥ 3.1) after chemotherapy, 50.35% vs 83.57% (HR = 7.03, 95% CI: 1.49–33.19, $p = 0.014$) (Figure 2H). For CBR, patients with higher NKT percentages ($\geq 6.8\%$ of lymphocytes) had shorter median CBR (mCBR), mCBR 2.72 vs 3.97 months after chemotherapy (HR = 3.68, 95% CI: 1.32–10.26, $p = 0.013$) (Figure 2I). However, no significant differences were observed between low and high NKT cell groups before chemotherapy (Figure 2J). For ORR, a high percentage of NK cells ($\geq 23.1\%$ of lymphocytes) showed a significant difference before chemotherapy, with 4-month response rates of 82.05% vs 25% (HR = 5.95, 95% CI: 1.13–31.20, $p = 0.035$) (Figure 2K). In contrast, no difference was found between %PD1⁺ CD8⁺ T-cells groups (Figure 2L) (Table 4).

The multivariable analysis was performed, which included age (≥ 60 years old), sex, and smoking status (never vs ever-smoker). The high sCD25 levels were confirmed as a prognostic factor of PFS in both before chemotherapy (≥ 499.52 pg/mL; HR = 3.62, 95% CI: 1.22–10.71, $p = 0.020$) and after chemotherapy (≥ 515.23 pg/mL; HR = 6.50, 95% CI: 1.85–22.84, $p = 0.004$). For OS, patients with high NLR (≥ 6.9 ; HR = 3.36, 95% CI: 1.39–8.15, $p = 0.007$) and elevated WBC count (≥ 8200 cells/ul; HR = 3.03, 95% CI: 1.06–8.63, $p = 0.038$) before chemotherapy had a shorter OS. Moreover, the high %B-cells ($\geq 10.6\%$ of lymphocytes; HR = 9.90, 95% CI: 2.05–47.85, $p = 0.004$), high sCD25 levels (≥ 701.05 pg/mL; HR = 11.05, 95% CI: 1.46–83.79, $p = 0.020$), and high NLR (≥ 3.1 ; HR = 6.82, 95% CI: 1.39–33.44, $p = 0.018$) were independently associated with shorter OS after chemotherapy. In addition, a high %NK cells ($\geq 23.1\%$ of lymphocytes) were an independent prognostic factor for shorter ORR (HR = 13.00, 95% CI: 1.39–121.13, $p = 0.024$) (Table 4).

Discussion

Recently, combining immunotherapy with chemotherapy has become one of standard first-line treatments for non-targetable advanced NSCLC.¹³ Thus, immunological biomarker detection plays an important role in NSCLC management, including diagnosis, severity prognosis, monitoring treatment, and selecting the optimal treatment. However, there are inadequate biomarkers used in real-world clinical practice to assist physicians in managing non-targetable advanced NSCLC, such as PD-L1 expression.^{13,14} Therefore, our study aims to identify the biomarkers that can predict the OS, PFS, and treatment response of patients after chemotherapy, which is the main treatment for non-targetable advanced NSCLC in clinical practice nowadays. To complete the objective, we investigated various peripheral blood immunological biomarkers before and after chemotherapy, such as cellular markers, Treg markers, and plasma soluble markers. The ROC analysis showed the top three biomarkers that have the potential to predict the clinical events, including PFS, OS, CBR, and ORR (Table 4).

The sCD25 is the shedding of IL-2R α upon T-cell activation. Several studies have discovered the negative-feedback role of sCD25 by binding IL-2, leading to reduced IL-2 levels. This mechanism impacts immune regulation. Therefore, increased sCD25 levels are correlated with poor outcomes in various malignancies.¹⁵ The study in advanced NSCLC patient groups found that elevated plasma sCD25 levels were associated with rapid progression and short survival after anti-PD-1/PD-L1 immunotherapy.¹⁶ Another study in acute myeloid leukemia (AML) demonstrated the efficiency of plasma sCD25 for treatment prognosis.¹⁷ These results are consistent with our data that patients with high sCD25 levels before chemotherapy (≥ 499.52 pg/mL) and after chemotherapy (≥ 515.23 pg/mL) experienced faster PFS. Additionally, high sCD25 levels ≥ 701.05 pg/mL after chemotherapy are associated with shorter OS 6.3 times in the lower group. We also found elevated levels of sCD25 after chemotherapy in PD groups.

Leukocytosis in pre-chemotherapy increases the risk of thrombosis, which leads to mortality. A large study on advanced NSCLC patients shows the strong association of leukocytosis with thrombosis and mortality rate.¹⁸ Our univariate survival analysis results did not find a significant difference in OS between patients with high and low WBC counts. However, after adjustment using Cox survival analysis, we discovered that leukocytosis (≥ 8200 cells/ul) was independently associated with earlier mortality (HR = 3.03, $p = 0.038$). Furthermore, NLR has been widely reported as an indicator of systemic inflammation, which drives cancer progression.¹⁹ Elevated neutrophil levels enhance inflammatory cytokine production, angiogenesis, and cancer cell proliferation, whereas lymphocytopenia reduces tumor-destructive immune response. Both mechanisms promote tumor growth and disease progression.²⁰ High NLR has been suggested as a prognostic factor for shorter OS in various cancers, including ovarian, colorectal, pancreatic, breast, and lung cancer.^{19–21} The efficiency of NLR was also shown as a prognostic factor for OS in our study. High NLR before

Table 4 Univariable and Multivariable Cox Regression Analysis of Predictors for Different Clinical Outcomes

Before Chemotherapy (n = 42)					After Chemotherapy (n = 27)				
Variables	Crude HR (95% CI)	P-value	aHR [§] (95% CI)	P-value	Variables	Crude HR (95% CI)	P-value	aHR [§] (95% CI)	P-value
Progression-Free Survival (PFS)					Progression-Free Survival (PFS)				
sCD25 ≥ 499.52	2.96 (1.11–7.86)	0.030*	3.62 (1.22–10.71)	0.020*	sCD25 ≥ 515.23	5.35 (1.73–16.53)	0.004 [†]	6.50 (1.85–22.84)	0.004 [†]
Tim-3 ≥ 697.82	1.15 (0.55–2.39)	0.710	1.04 (0.47–6.81)	0.920	%CD4 ⁺ T Cells ≥ 27.8%	1.71 (0.50–5.82)	0.392	0.58 (0.11–3.13)	0.527
%MSCDs ≥ 16.4%	1.67 (0.80–3.47)	0.173	1.87 (0.83–4.23)	0.131	%PD1 ⁺ CD8 ⁺ T Cells ≥ 19.5%	1.84 (0.76–4.48)	0.179	1.11 (0.25–4.93)	0.891
Overall Survival (OS)					Overall Survival (OS)				
Neu/Lym Ratio ≥ 6.9	3.27 (1.36–7.87)	0.008 [†]	3.36 (1.39–8.15)	0.007 [†]	%B-Cells ≥ 10.6%	6.71 (1.47–30.78)	0.014*	9.90 (2.05–47.85)	0.004 [†]
%B-Cells ≥ 11.6%	1.62 (0.73–3.59)	0.231	2.24 (0.88–5.67)	0.090	sCD25 ≥ 701.05	6.30 (1.35–29.34)	0.019*	11.05 (1.46–83.79)	0.020*
WBC ≥ 8200	2.38 (0.93–6.09)	0.070	3.03 (1.06–8.63)	0.038*	Neu/Lym Ratio ≥ 3.1	7.03 (1.49–33.19)	0.014*	6.82 (1.39–33.44)	0.018*
Clinical Benefit Rate (CBR)					Clinical Benefit Rate (CBR)				
Ab NK Cells ≥ 300	1.98 (0.77–5.14)	0.158	2.78 (0.99–7.85)	0.053	%CD4 ⁺ T Cells ≥ 32.5%	1.24 (0.47–3.35)	0.658	1.63 (0.52–5.11)	0.398
%iTreg ≥ 7.19%	1.61 (0.69–3.75)	0.270	2.04 (0.79–5.28)	0.143	%Terminal Effector Treg ≥ 35.6%	1.82 (0.71–4.67)	0.212	4.48 (1.17–17.20)	0.029*
%NKT Cells ≥ 5%	2.03 (0.84–4.91)	0.118	2.67 (0.81–8.81)	0.106	%NKT Cells ≥ 6.8%	3.68 (1.32–10.26)	0.013*	4.54 (1.36–15.12)	0.014*
Overall Response Rate (ORR)					Overall Response Rate (ORR)				
%PD1 ⁺ CD8 ⁺ T Cells ≥ 27.6%	2.79 (0.68–11.42)	0.154	2.52 (0.38–16.90)	0.341	%CD4 ⁺ T Cells ≥ 35.3%	2.10 (0.41–10.86)	0.377	1.75 (0.16–18.53)	0.643

(Continued)

Table 4 (Continued).

Before Chemotherapy (n = 42)					After Chemotherapy (n = 27)				
Variables	Crude HR (95% CI)	P-value	aHR [§] (95% CI)	P-value	Variables	Crude HR (95% CI)	P-value	aHR [§] (95% CI)	P-value
%NK Cells ≥ 23.1%	5.95 (1.13–31.20)	0.035*	13.00 (1.39–121.13)	0.024*	B7.2 ≥ 258.98	1.62 (0.29–9.00)	0.577	1.68 (0.30–9.54)	0.557
sCD25 ≥ 742.68	3.32 (0.79–13.96)	0.101	4.30 (0.78–23.76)	0.095	%PD1 ⁺ CD8 ⁺ T Cells ≥ 24.4%	4.32 (0.78–23.85)	0.093	N/A	N/A

Notes: All soluble markers were reported as pg/mL. [§] Hazard Ratio estimated by COX proportional hazard regression model adjusted for age (≥60 years old), sex, and smoking status (never smoke and ever smoke). * P-value < 0.05. † P-value < 0.01. %PD1⁺ CD8⁺ T Cells was reported as % of CD8⁺ cells. %MDSCs was reported as % of CD14⁺ cells. All absolute cellular markers were reported as cells/ul. All Treg markers were reported as % of CD4⁺ cells.

Abbreviations: HR, Hazard Ratio; sCD25, soluble CD25; MDSC, Myeloid-derived suppressor cells; WBC, White blood cells; Ab NK Cells, Absolute Count NK Cells.

chemotherapy (≥ 6.9) and after chemotherapy (≥ 3.1) was associated with worse OS with HR of 3.27 and 6.71 than the lower group, respectively. The multivariate analysis further confirmed that high NLR was independently associated with shorter OS, showing 3.36-fold and 6.82-fold higher risks compared to the lower groups.

In this study, the various subpopulations of the lymphocytes show performance as prognostic factors. B-cells have two main functions in lung cancer, including anti-tumor and pro-tumor, depending on their subtypes. An imbalance between two sides of their roles causes the tumor progression.^{22,23} As shown in a recent study, after treatment with nivolumab and chemotherapy in stage IIIA NSCLC, an increase of tumor-infiltrated total B-cells, naïve B-cells, memory B-cells, and transitional B-cells was associated with longer survival, while the elevated CD19⁺CD20^{low}CD25^{low}CD27^{low} B-cells were correlated to worse PFS.²⁴ Another study on stage I–III lung adenocarcinoma showed that an elevated tumor-infiltrated B-cell percentage ($>10\%$) was associated with improved OS.²⁵ In our cohort, an increase in the % B-cells was linked to shorter survival (HR = 6.71, $p = 0.014$). The different results of B-cells may be caused by distinct sample types. Most studies investigate %B-cells in the tumor tissue, whereas our study uses the peripheral blood. Another possible reason is that our cohort consisted of patients with non-targetable advanced-stage (III–IV) NSCLC, which differs from other studies. Furthermore, the B-cell subtypes analysis is needed to confirm which subtypes of B-cells are dominant in non-targetable advanced-stage NSCLC.

Another interesting finding in our study was that NKT cells, NK cells, and terminal effector Tregs might serve as prognostic factors for better chemotherapy response. Our study showed that %NKT cells was elevated in CBR responders, while %NK cells was decreased in ORR responders after chemotherapy. Patients with higher %NKT cells after chemotherapy achieved CBR more rapidly than those with lower %NKT cells. Additionally, an elevated percentage of NK cells was associated with a higher ORR. By multivariable analysis, NKT cells and NK cells were confirmed as the independent predictors of CBR and ORR events, respectively. They play a role in anti-tumor cells through cytotoxic function.^{26–28} A previous study showed that the increase of infiltrated NK cells and NKT cells in lung tumors is associated with patients' survival.^{28,29} In addition, the high percentage of NK cells in the blood before and after immunochemotherapy is linked to prolonged PFS.³⁰ These underscore the importance of the roles of NKT and NK cells in improving chemotherapy response to cancer diseases.

Tregs play a role in immune regulation through their suppressive function, including cytokine secretion and cell contact-dependent mechanisms. Elevated Treg was found to be highly associated with staging, metastasis, and recurrence of NSCLC.³¹ The high ratio of Treg to CD8⁺ T-cells was independently associated with poor response to platinum-based chemotherapy.^{31,32} Additionally, a low Treg number was associated with long OS, PFS, and recurrence-free survival in a meta-analysis of NSCLC.³³ Conversely, terminal effector Treg is the subset of effector Treg that has a rapid suppressive function through secreting high levels of IL-10 and TGF- β .³⁴ Our results show that the %Terminal Effector Treg was significantly increased after chemotherapy in the ORR responder groups. We also found that %Terminal Effector Treg were independently associated with faster CBR, although it was not significant in the univariable analysis. Consistent with our findings, the study of the Treg subset in NSCLC reported that a high population of terminal effector Treg was associated with better CBR compared with the PD group. In the same studies, higher levels of terminal effector Tregs were also associated with prolonged OS and PFS in NSCLC.³⁴ This study investigated the subset of Treg, which differs from other studies that report only total Treg. Our results highlight the crucial role of terminal effector Treg as a prognostic factor for chemotherapy response. This may be explained by the terminal effector Treg reduces inflammation in the tumor, which is associated with NSCLC development. Thus, elevated terminal effector Treg percentage could improve the response rate with chemotherapy.³⁴ Nonetheless, the mechanisms of terminal effector Tregs in NSCLC remain poorly investigated.

These biomarkers in our study show potential as candidate tools for prognosing clinical outcomes and monitoring treatment of non-targetable advanced NSCLC. Future studies are needed to determine whether these biomarkers can support chemotherapy or alternative treatment decision-making. Furthermore, evaluating integrated multi-biomarker models may improve the prognostic power for OS, PFS, and treatment response.

Our study has some limitations. Firstly, the small sample size of our study may affect the statistical power and bias the statistical analysis, resulting in a wide range of data. The analysis of %PD1⁺ CD8⁺ T-cells in the Cox regression model was excluded because the sample size was too small, resulting in excessively wide 95% CI. A large sample size is

needed to further investigate. Although we adjusted for some confounding factors using Cox regression models, the possibility of residual confounding cannot be completely excluded. Due to the limitations of our cytokine measurement method, we excluded TGF- β marker because its levels were below the lower limit of detection. Moreover, this study was conducted in a single center, which may limit the generalizability to real-world practice. This cohort reflects the real-world situation. Although we focused on the “non-targetable” group, some individuals with oncogenic drivers received only chemotherapy due to clinical reasons and limited drug access, as determined by the clinicians’ decision. Another limitation of this study is the short follow-up period, which may affect the evaluation of survival endpoints.

Conclusion

In conclusion, our findings suggested pre- and post-chemotherapy sCD25 levels as the prognostic factor for PFS. Pre- and post-chemotherapy NLR, along with post-chemotherapy %B-cells and sCD25 levels, were associated with longer OS. Post-chemotherapy %NKT cells and %Terminal Effector Treg were associated with better CBR. Additionally, pre-chemotherapy %NK cells can predict better ORR. These markers have the potential to support future personalized management strategies in non-targetable advanced NSCLC.

Abbreviations

8th AJCC, 8th American Joint Committee on Cancer; ALK, Anaplastic Lymphoma Kinase; AML, Acute Myeloid Leukemia; AUC, area under the curve; BRAF, B-Raf Proto-Oncogene Serine/Threonine Kinase; CBR, Clinical Benefit Rate; CCRT, Concurrent Chemoradiation; CR, Complete Response; CT, Computed Tomography; ECoG, Electrocardiography; EGFR, Epidermal Growth Factor Receptor; HR, Hazard Ratio; iTreg, Induced Regulatory T-cells; mCBR, Median Clinical Benefit Rate; MDSCs, Myeloid-derived suppressor cells; Mo-MDSCs, Monocyte Myeloid-derived suppressor cells; mOS, Median Overall Survival; mPFS, Median Progression-Free Survival; NK cells, Natural Killer Cells; NKT cells, Natural Killer T-cells; NLR, Neutrophil to Lymphocyte Ratio; NSCLC, Non-Small Cell Lung Cancer; NTRK, Neurotrophic Receptor Tyrosine Kinase 1; ORR, Overall Response Rate; OS, Overall Survival; PBMCs, Peripheral Blood Mononuclear Cells; PBS, Phosphate-Buffered Saline; PD, Progressive Disease; PD-1, Programmed Cell Death Protein 1; PD-L1, Programmed Death-Ligand 1; PFS, Progression-Free Survival; PR, Partial Response; ROC, Receiver operating characteristic; ROS1, ROS Proto-Oncogene 1 Receptor Tyrosine Kinase; SD, Stable Disease; Treg, Regulatory T-cells; WBC, White Blood Cells.

Data Sharing Statement

The data are available from the corresponding author [Kantapat Simmalee] on reasonable request.

Ethics Approval and Informed Consent

This article is approved by the Human Research Ethics Committee of Ramathibodi Hospital, Mahidol University, Bangkok, Thailand (Institutional Review Board number COA. MURA2023/568, as a continuation of project COA. MURA2021/956). All procedures were conducted in accordance with the ethical standards of the 2013 Declaration of Helsinki and local regulations. All participants provided written informed consent prior to enrollment. Participants were informed that they could withdraw from the study at any time without affecting their treatment or care. All data were de-identified, and no personal information was collected without consent.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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