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Expression of regulatory T cells in driver-gene-negative advanced non-small cell lung cancer as well as its effect on the therapeutic efficacy and prognosis of immune checkpoint inhibitors

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► ***Original article***

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ABSTRACT

Background: The To measure regulatory T cells (Tregs) expression in driver-gene-negative advanced non-small cell lung cancer (NSCLC) as well as its effect on the therapeutic efficacy and prognosis of immune checkpoint inhibitors (ICIs). ***Materials and Methods:*** Fifty patients with advanced non-small cell lung cancer without driving genes who were receiving treatment with a monoclonal antibody targeting the programmed death receptor-1 (PD-1) made up the study group. 30 healthy subjects in the same period were chosen into the control group. Flow cytometry was used to identify $CD4^{high}$CD25+Foxp3+Treg cells in peripheral blood of all participants. Relation between $CD4^{high}$CD25+Foxp3+Treg cells and tumor markers were explored, and efficacy and prognosis in patients before and after therapy was analyzed. ***Results:*** The fraction of $CD4^{high}$CD25+Foxp3+Treg cells in the study group was higher ($P<0.05$). Following three rounds of PD-1 monoclonal antibody treatment, patients' $CD4^{high}$CD25+Foxp3+Treg cells proportion was lower than before treatment ($P<0.01$), and showed a positive correlation with tumor markers ($P<0.05$). The fraction of $CD4^{high}$CD25+Foxp3+Treg cells in the CR+PR group decreased in both the second and third cycles after treatment compared to the SD+PD group ($P<0.01$), but no change was found before or during the first cycle ($P>0.05$). $CD4^{high}$CD25+Foxp3+Treg cells proportion in the death group presented higher relative to the survival group ($P<0.05$). $CD4^{high}$CD25+Foxp3+Treg cells predicted the area under the ROC curve was 0.8134, with significant difference ($P<0.05$). ***Conclusion:*** $CD4^{high}$CD25+Foxp3+Treg cells proportion in peripheral blood of NSCLC patients shows increased, and has predictive value for therapeutic efficacy of ICIs and prognosis of driver-gene-negative advanced NSCLC patients.

INTRODUCTION

Lung cancer possesses the highest death rate all around the world [1]. Non-small cell lung cancer (NSCLC) is the most frequent kind of lung cancer, accounting for more than 80% of all occurrences [2]. The majority of lung cancer patients in China have advanced stage diagnoses because of the high aggressiveness of n NSCLC and the absence of efficient early screening programs [3]. The usual standard of care for advanced NSCLC is platinum-based dual-agent chemotherapy, although the 5-year survival rate is less than 5% [4]. Immunotherapy based on immune checkpoint inhibitors (ICIs) and immunocombination treatment are primarily employed at this stage for patients with driver-gene-negative advanced NSCLC [5].

The combination of immune checkpoint molecules and ligands can block the activation of immune cells, maintain the body's autoimmune tolerance, inhibit immune overactivation and protect its own tissues [6]. Tumors can escape immune clearance by abnormally expressing immune checkpoint molecules [7]. Programmed death receptor-1 (PD-1) is expressed majorly by helper and effector T cells while its ligand, programmed death ligand-1 (PD-L1) is expressed by tumor, immune, or stromal cells in the tumor microenvironment [8]. The activation of PD-1/PD-L1 signaling pathway acts on the effector stage of T cells, inhibits T cell proliferation, cytokine secretion, along

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with cytotoxicity of effector cells, and promotes peripheral immune tolerance of tumor cells [9]. Anti-PD-1/PD-L1 antibodies can reactivate suppressed immune responses and produce long-lasting anti-tumor effects [10].

Tumor occurrence, development, invasion, and metastasis are caused by a variety of circumstances, but immunological tolerance and autoimmune control disorders are two of the fundamental causes of tumor formation [11]. The discovery of CD4+CD25+ regulatory T cells (Tregs) has recently led to a new understanding of immune control and tolerance. CD25+CD4+ Treg cells are essential to the immune system's ability to maintain immunological homeostasis and are derived from the thymus gland [12]. $CD4^{+}CD25^{+}$ Treg cells have the two major functional characteristics of immune incompetence and immunosuppression, and repress the activation of potential autoreactive T cells in normal organisms through direct contact between effector cells, which have a vital role in regulating tumor immunity and autoimmunity [13]. Foxp3 is a key transcriptional suppressor that can affect the development of human regulatory T cells. It is a new member of the fork-head wingspan spiral transcription factor family and is involved in the mechanism of tumor escape [14]. Foxp3 is considered to be a critical factor in the differentiation and function of Treg [15]. Foxp3 protein is the product of Foxp3 coding and is the specific marker of $CD4^{high}CD25^{+}$ Treg cells, and it mediates the growth, development and function of $CD4^{high}CD25^{+}$ Treg cells, and participates in maintaining the stability of immune tolerance and immune response [16]. However, there are few reports on the relationship between $CD4^{high}CD25^{+}Foxp3^{+}$Treg cells and driver-gene-negative advanced NSCLC patients.

In our study, we explored the expression of $CD4^{high}$ CD25 + Foxp3 + Treg cells in driver-gene-negative advanced NSCLC and its effect on the therapeutic efficacy and prognosis of ICIs. It offers fresh perspectives on the clinical management of ICIs as well as new goals and avenues for the diagnosis and treatment of NSCLC in the future.

MATERIALS AND METHODS

General data

The trial included fifty driver-gene-negative advanced NSCLC patients who received PD-1 monoclonal antibody therapy (Pembrolizumab, MCE, USA) (dosage 3 mg/kg, every 3 weeks) in our hospital from January 2019 to December 2020. Inclusion criteria (1) Patient was diagnosed with driver-gene-negative advanced NSCLC by chest and abdominal CT and pathological examination. The typical symptoms were hoarseness, limb edema, shortness of breath, chest pain, etc. (2) The test results of epidermal growth factor receptor, ROS-1 gene, anaplastic lymphoma kinase and other driver genes were negative; (3) The predicted survival period is more than 3 months. Exclusion criteria: (1) Severe hypertension. (2) Serious abnormal functions of the heart, liver, kidney along with other organs. (3) There were serious bone marrow suppression, poor physical condition, pregnancy or breastfeeding women and other chemotherapy contraindications. (4) Allergic to the drugs adopted in this study. Another 30 healthy subjects with no abnormal blood routine, chest X-ray, abdominal B-ultrasound, electrocardiogram and urine routine were chosen to be the control group. The average age of the study group's thirty male and twenty female members, ranging in age from 45 to 77 years, was (61.62±6.25) years. The mean age of the 18 male and 12 female participants in the control group, ranging in age from 48 to 75 years, was (61.57±6.27). No statistical significance was discovered in comparing general data between 2 groups ($P>0.05$), which was comparable. All patients as well as their families signed informed consent forms. All enrolled patients were followed up for 2 years, including outpatient review, telephone follow-up and community return visit.

METHODS

Detection of $CD4^{high}CD25+Foxp3+$Treg cells

2 ml of EDTA anticoagulant peripheral blood was extracted and thoroughly mixed for use. 10 μl CD4-FITC and 5 μl CD25-PE-CyTM antibodies (BD Bioscience, USA) were added into 100 μl anticoagulant peripheral blood, mixed with vortex oscillation, and incubated for 20 min away from light. 1 ml Foxp3 film breaking agent A (Invitrogen, USA) was added, vortex and mix well, avoid light for 20 min, and then 1 ml Foxp3 film breaking agent B (Invitrogen, USA) was added, centrifuged 300 × g for 5 min, discard supernatant, and 100 μl Foxp3 film breaking agent B was added to re-suspension cells. 10 μl Foxp3-APC (Invitrogen, USA) was added for incubation in darkness for 30 min, centrifuged 300 × g for 3 min. After washing, the supernatant was discarded. 500 μl buffer solution was added, a flow cytometer (Beckman Coulter Company, USA) was performed to record $CD4^{high}CD25^{+}Foxp3^{+}$Treg cells proportion in all lymphocytes.

Detection of tumor markers

The results of serum tumor markers before each treatment cycle were used in all enrolled cases. Before each medication, 5 ml of fasting venous blood could be extracted from the patient, which was then centrifuged and the upper serum was extracted. The serum level of tumor markers including CEA, CYFRA21-1 as well as SCC was detected by chemiluminescence method, respectively. A chemiluminescent microparticle immunoassay was used to measure serum levels of CEA (Architect CEA

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Reagent kit, Abbott) and SCC (SCC Reagent kit, Homabio), while an immuno radiometric assay (CYFRA21-1 IRMA Kit, Beckman Coulter) was used to detect CYFRA21-1 in accordance with manufacturer's instructions.

Therapeutic evaluation

Combined with the results of tumor markers and imaging data, the efficacy of the patient after 3 cycles of PD-1 monoclonal antibody therapy was evaluated according to the 2017 version of iRECIST, which were respectively: complete response (CR), partial response (PR), disease stabilization (SD), as well as disease progression (PD).

Follow-up visit

The prognosis of the patients within two years was followed up, and the patients were separated into two groups: the survival group and the death group, following their survival status within two years.

Statistical analysis

The results were statistically analyzed by help of SPSS 22.0 software package. The measurement data were represented by mean ± standard deviation (x±s), independent sample t test was adopted for comparison between groups. The relationship between CEA, CYFRA21-1, and NSE and CD4high-CD25+Foxp3+Treg cells was determined using the Pearson correlation coefficient. In driver-gene-negative advanced non-small cell lung cancer (NSCLC) patients treated with PD-1 monoclonal antibody, the prognostic significance of CD4high-CD25+Foxp3+Treg cells was examined using the Receiver Operating Characteristic Curve (ROC curve).

RESULTS

$CD4^{high}CD25^{+}Foxp3^{+}$Treg cells proportion in different groups

Fifty driver-gene-negative advanced NSCLC patients who received PD-1 monoclonal antibody therapy were chosen into the study group. Thirty healthy subjects in the same period were chosen into the control group. Flow cytometry detected the proportion of $CD4^{high}CD25^{+}Foxp3^{+}$Treg cells in control group and study group. The finding revealed that compared with control group, $CD4^{high}$-$CD25^{+}Foxp3^{+}$Treg cells proportion in the study group presented higher (figure 1) (P<0.05).

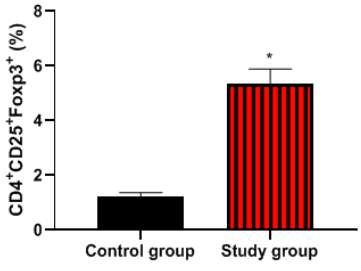

Figure 1. $CD4^{high}CD25$ +Foxp3+Treg cells proportion in different groups. *P<0.05.

$CD4^{high}CD25^{+}Foxp3^{+}$Treg cells proportion before and after PD-1 monoclonal antibody therapy

In study group fifty patients received PD-1 monoclonal antibody therapy, then we measured the proportion of $CD4^{high}CD25^{+}Foxp3^{+}$Treg cells by flow cytometry during 3 cycles of treatment. Flow cytometry revealed that after 3 cycles of treatment with PD-1 monoclonal antibody, $CD4^{high}CD25$ +Foxp3+Treg cells proportion presented lower than that before treatment (figure 2) (P<0.01).

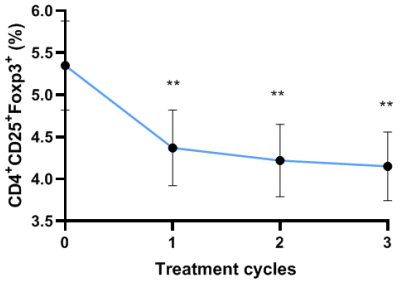

Figure 2. CD4highCD25+Foxp3+Treg cells proportion before and after PD-1 monoclonal antibody therapy. **P<0.01.

Serum level of tumor markers before and after PD-1 monoclonal antibody therapy

To explore the relationship between changes in $CD4^{high}CD25$+Foxp3+Treg cells proportions and tumor cell progression, we tested tumor markers, such as CEA, CYFRA21-1 and SCC during 3 cycles of treatment. Chemiluminescence method showed that after 3 cycles of therapy with PD-1 monoclonal antibody, serum levels of CEA, CYFRA21-1 along with SCC presented lower relative to before treatment (figure 3) (P<0.01).

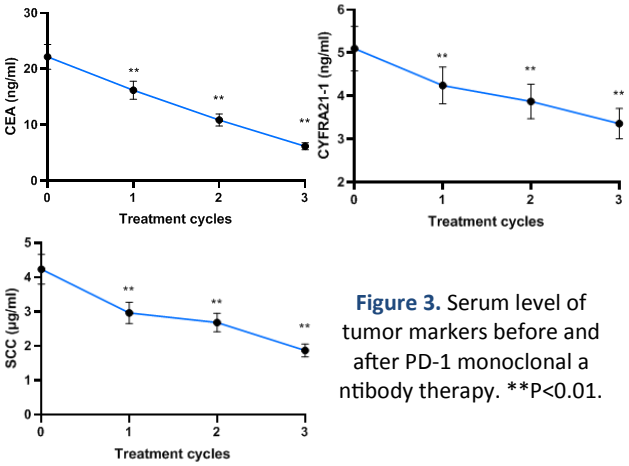

Figure 3. Serum level of tumor markers before and after PD-1 monoclonal a ntibody therapy. **P<0.01.

Correlation between $CD4^{high}CD25^{+}Foxp3^{+}$Treg cells proportion and serum level of tumor markers

Following three rounds of PD-1 monoclonal antibody therapy, there was a decrease in blood levels of SCC, CYFRA21-1, and CEA. We investigated the correlation between CD4highCD25+Foxp3+Treg cells proportion and tumor markers during 3 cycle's treatment. Table 1 revealed that after 3 cycles of PD-1 monoclonal antibody treatment, $CD4^{high}CD25$ + Foxp3 + Treg cells proportion showed a certain degree of

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positive correlation with CEA, CYFRA21-1 as well as SCC (P<0.05), but no significant correlation with CEA, CYFRA21-1, SCC in other stages (P>0.05).

Table 1. Pearson correlation coefficient between $CD4^{high}CD25$ +Foxp3+Treg cells proportion and CEA, CYFRA21-1 and SCC.

	Before treatment (n=50)	1 cycle after treatment (n=50)	2 cycles after treatment (n=50)	3 cycles after treatment (n=50)
CEA	0.047 (P>0.05)	-0.87 (P>0.05)	0.265 (P>0.05)	0.735 (P<0.05)
CYFRA 21-1	-0.158 (P>0.05)	-0.368 (P>0.05)	0.146 (P>0.05)	0.658 (P<0.05)
SCC	0.243 (P>0.05)	0.042 (P>0.05)	0.134 (P>0.05)	0.652 (P<0.05)

Short-term effects after 3 cycles PD-1 monoclonal antibody therapy

To further observe the short-term clinical effects of 3 cycles PD-1 monoclonal antibody therapy, we investigated the clinical response rate in 50 patients. The result displayed that among the 50 patients, there were 8 cases of CR, 10 cases of PR, 25 cases of SD, as well as 7 cases of PD, and the clinical response rate (CR+PR) was 36.00% (figure 4).

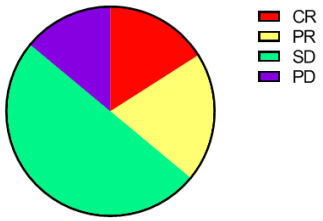

Figure 4. Short-term effects after 3 cycles PD-1 monoclonal antibody therapy, complete response (CR), partial response (PR), disease stabilization (SD), as well as disease progression (PD).

Correlation between $CD4^{high}CD25^{+}Foxp3^{+}$Treg cells proportion and short-term effects

Flow cytometry detected the proportion of CD4highCD25+Foxp3+Treg cells in CR+PR group and SD+PD group. Flow cytometry findings showed that $CD4^{high}CD25^{+}Foxp3^{+}$Treg cells proportion in CR+PR group presented lower after the second and third cycles treatment relative to SD+PD group (P<0.01), but no change was discovered before and in the first cycle after treatment between 2 groups (figure 5) (P>0.05).

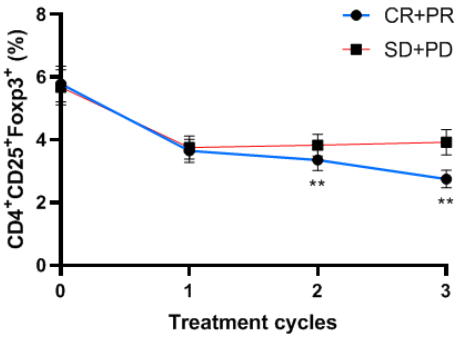

Figure 5. Correlation between $CD4^{high}CD25$+Foxp3+Treg cells proportion and short-term effects. **P<0.01.

$CD4^{high}CD25^{+}Foxp3^{+}$Treg cells proportion in patients with different prognosis

To observe the relationship between $CD4^{high}CD25$ +Foxp3+Treg cells proportion and the survival status of NSCLC patients, we compared the proportion between the death group and survival group. The result indicated that among the 50 patients, 16 died within 2 years and 34 survived. $CD4^{high}CD25$ +Foxp3+Treg cells proportion in the death group presented higher when comparing with the survival group (figure 6) (P<0.05).

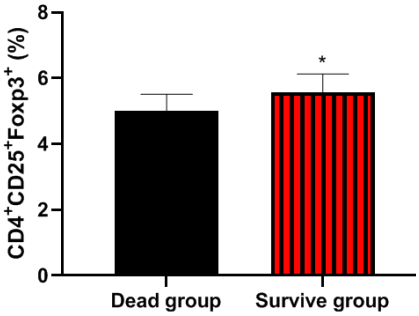

Figure 6. $CD4^{high}$CD25+Foxp3+Treg cells proportion in patients with different prognosis. *P<0.05.

ROC curve analysis results

It was displayed in figure 7 that $CD4^{high}CD25$ +Foxp3+Treg cells predicted the area under the ROC curve was 0.8134, with significant distinction (P<0.05). The result indicated that $CD4^{high}CD25$ +Foxp3+Treg cells had high predictive value in the prognosis of NSCLC patients.

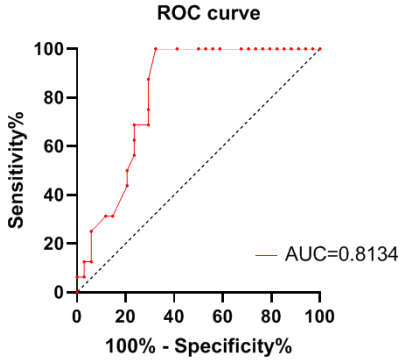

Figure 7. ROC curve analysis results.

DISCUSSION

$CD4^{high}CD25$+Foxp3+Treg cells belong to a special subset of $CD4^{+}$ T cells and have a crucial role in modulating immune response by repressing the activation or proliferation of other T cell subsets, thereby maintaining immune system homeostasis [17]. $CD4^{high}CD25^{+}Foxp3^{+}$Treg cells not only possess a vital role in the development of autoimmune diseases, but also inhibit the cellular immune function of the body, causing tumor cells to evade the anti-tumor potential of the body [18]. It has been documented that $CD4^{high}CD25$+Foxp3+Treg cells proportion in

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advanced NSCLC patients presented higher when comparing with the control group [19]. In our study, we proved similar results, which implied that abnormal high $CD4^{high}CD25+Foxp3+$Treg cells proportion in driver-gene-negative advanced NSCLC patients.

Literatures have demonstrated that monoclonal antibody blocking against PD-1 or PD-L1 can induce the proliferation of $CD8^+$T cells as well as the production of cytokines to negatively regulate the number of Treg cells [20]. Immunotherapy led by PD-1/PD-L1 inhibitors has changed the therapy landscape, efficacy, and prognostic benefits of advanced NSCLC, and the 5-year survival rate of advanced NSCLC patients has increased from 5% to 16% [21]. PD-1 inhibitor monotherapies have also become the new standard of second-line therapy for advanced NSCLC [22]. On the basis of the efficacy of second-line treatment, PD-1 inhibitor monotherapy or combined chemotherapy has successively emerged to be the first-line therapy [23]. Based on the latest CSCO guidelines [24], pemetrexel and platinum in combination with pembrolizumab or carrilizumab or sindellizumab or tirellizumab are recommended to be grade I first-line therapy for non-squamous NSCLC in advanced NSCLC with driver negative genes. Standard platinum-containing chemotherapy combined with pabolizumab or tirellizumab or sindellizumab was recommended for grade I lung squamous cell carcinoma, and carrilizumab was recommended for grade II. Pabolizumab monotherapy is the first-line therapy for advanced NSCLC. Therefore, this study used PD-1 monoclonal antibody treatment to explore its effect on the efficacy and prognosis of NSCLC, as well as its relationship with Treg cells.

In our study, $CD4^{high}CD25^+Foxp3^+$Treg cells proportion in patients after PD-1 monoclonal antibody treatment was lower than that before PD-1 monoclonal antibody treatment. We consider that this may be related to the response produced by immunotherapy, which indicated that effective PD-1 monoclonal antibody treatment could reduce the tumor load of patients, thus greatly reducing the ability of tumors to induce Treg cells, so that the level of immunosuppressive cells in tumor patients was significantly reduced, and the immunosuppressive state of the body was improved, which was in accordance with previous study [25].

Besides, our study revealed that $CD4^{high}CD25+Foxp3+$Treg cells proportion of patients with PD and SD efficacy was significantly elevated relative to that of patients with PR and CR efficacy at 2 and 3 cycles after treatment, with statistical significance. These results implied that $CD4^{high}CD25+Foxp3+$Treg cells proportion after treatment had certain suggestive value in the therapeutic effect of NSCLC, and the change of the proportion of $CD4^{high}CD25+Foxp3+$Treg cells may be linked to the therapeutic effect of tumor treatment. However, no significant difference was discovered in $CD4^{high}CD25+Foxp3+$Treg cells proportion in CR and PR patients compared with SD and PD patients before treatment and after 1 cycle of treatment, which may be caused by the insignificant influence of PD-1 on the change of $CD4^{high}CD25^+Foxp3^+$Treg cells proportion and efficacy in the early stage of treatment. It also showed that PD-1/PD-L1 inhibitors can produce relatively durable effects in some patients.

Serological biomarkers such as CEA, CYFRA21-1 and SCC have been studied to be prognostic or predictive indicators in NSCLC patients received chemotherapy [26]. CYFRA21-1 is mainly used as a tumor marker, and the alteration of its level is closely linked to the stage of NSCLC, which is of great importance in the diagnosis of NSCLC [27]. Meanwhile, the detection of CYFRA21-1 level in patients can effectively evaluate the therapeutic effect of patients [28]. CEA is a human embryonic antigen-like acidic glycoprotein that is expressed on the surface of cancer cells that have undergone differentiation from endoderm cells [29]. The progress of tumor tissues are closely linked to CEA level expression [30]. SCC is a kind of glycoprotein associated with tumor cell division and proliferation that may be useful in the early detection of lung cancer [31]. To investigate the relationship between $CD4^{high}CD25^+Foxp3^+$Treg cells changes and NSCLC prognosis after PD-1 monoclonal antibody treatment, we measured the levels of tumor markers. In our study, we found atht after 3 cycles of treatment, $CD4^{high}CD25^+Foxp3^+$Treg cells proportion showed a certain degree of positive correlation with CEA, CYFRA21-1 and SCC, but no significant correlation with CEA, CYFRA21-1, SCC in other stages. It was suggested that Treg levels decreased with the decrease of CEA, CYFRA21-1 along with SCC after 3 cycles of treatment, and no significant correlation was observed in other stages, which may be related to the slow and lasting effect of immunotherapy on tumor burden.

The studies observed so far can confirm that tumor development is inextricably linked to an imbalance between immunity and immunosuppression, and it is precisely because of the loss of Treg cells that this imbalance between immunity and immunosuppression is corrected, resulting in an anti-tumor immune response [32]. Other studies have shown that tumor-invasive Treg cells are linked to clinical outcomes in NSCLC patients at different stages [33]. Elevated peripheral Treg cells levels are also linked to poor prognosis of NSCLC [34]. Kotsakis *et al.* investigated the role of peripheral Treg subpopulations in patients received chemotherapy for advanced NSCLC, and they found that normal levels of Treg cells were linked to longer OS relative to high levels of Treg cells, while a high proportion of end-effect Treg cells were linked to longer OS and PFS [35]. Consistently, our study also

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revealed that $CD4^{high}CD25+Foxp3+$Treg cells proportion in the death group presented elevated when comparing with the survival group, which indicated that $CD4^{high}CD25^{+}Foxp3^{+}$Treg cells also had certain prognostic significance for advanced NSCLC patients. The increase in the number or function of Treg cells can lead to tumor immune tolerance [36]. Foxp3 induces initial $CD4^{+}$T cells to develop into Treg cells, and Treg cells can express Foxp3 specifically, further up-regulate the differentiation of $CD4^{+}$T lymphocytes into Treg cells, and then reduce the immune response of the immune system to NSCLC and promotes the development of NSCLC [37]. Notably, our study also performed ROC curve to further validate that $CD4^{high}CD25^{+}Foxp3^{+}$Treg cells had high predictive value in the prognosis of driver-gene-negative advanced NSCLC patients.

CONCLUSION

$CD4^{high}CD25+Foxp3+$Treg cells proportion in driver-gene-negative advanced NSCLC patients shows an increasing trend, and has certain predictive value for therapeutic efficacy of immune checkpoint inhibitors and prognosis of driver-gene-negative advanced NSCLC patients.

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***Conflicts of interests*:** No potential conflict of interest was reported by the authors.

***Ethical consideration*:** All patients provided their written, voluntarily informed consent. All procedures were carried out in accordance with the guidelines outlined in the Helsinki Declaration and this study was approved by the Ethics Committee of our institution.

***Authors' contribution*:** Yan Chen conceived and designed the experiments. Silin Chen, Yaping Li, Xiaoguang Guo, Qingsong Liang contributed significantly to the experiments and arranging data. Lixia Yang and Cui Lei performed data analyses. Yan Chen and Silin Chen wrote the draft manuscript. Yan Chen revised the manuscript. All authors read and approved the final manuscript.

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