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Correlation between circulating tumor cells EGFR expression and T cell subsets in advanced non-small cell lung cancer patients after tyrosine kinase inhibitor treatment

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Research indicates that after EGFR-tyrosine kinase inhibitors (EGFR-TKIs) treatment of non-small cell lung cancer (NSCLC), patient immune function significantly improved, and that circulating tumor cells (CTCs) measurements and peripheral blood epidermal growth factor receptor (EGFR) mutation data can guide TKIs treatment. Sixty-six advanced NSCLC patients treated with TKIs were enrolled and CTCs, EGFR expression, T cell subsets and natural killer (NK) cells in peripheral blood were measured using flow cytometry before and after treatment and assessed with respect to patient prognosis. CTCs and EGFR expression were negatively correlated with cellular immune function and immune recovery after EGFR-TKI treatment. Thus, CD4+/ CD8+ ratios and NK cells may be useful prognostic indicators for advanced NSCLC patients who receive TKIs treatment.

Key words: non-small cell lung cancer, circulating tumor cells, EGFR expression, cellular immune function

Lung cancer is one of the most prevalent cancers worldwide and non-small cell lung cancer (NSCLC) accounts for 85% of these cancers. Platinum-doublet chemotherapy is a standard, first-line treatment for advanced NSCLC, but its efficacy plateaus [1]and such chemotherapy damages non-cancerous cells, compromising immune function and subsequently reduces chemotherapeutic efficacy [2]. For patients with an EGFR mutation, efficacy of EGFR-TKIs is 65–83% so these are currently first-line treatment for this subtype of advanced NSCLC patient.

Circulating tumor cells can be shed from primary or metastatic foci and subsequently enter the circulation via peripheral blood vessels [3]. Thus, counting CTCs may be a valid marker for the diagnostic workup for the patient [4]. In addition, CTCs can be used to identify EGFR mutations [5]. For example, EGFR expression studies of isolated CTCs response may allow molecular characterization of selected tumor targets [6].

The blood stream is a hostile environment for tumor cells, and although a tumor presumably sheds millions of tumor cells into the blood daily [7], few can be detected at any given time. This may be due to the rapid clearance of CTCs from the blood, likely mediated by natural killer cells, T cell subsets, monocytes, macrophages and neutrophils. CTCs are therefore under evolutionary pressure to develop a phenotype that can protect or hide them from the immune system [8].

The relationship among cellular immune function, CTCs, and EGFR expression is unclear at this time. Changes in T cell subsets and NK cells activity are immune consequences for NSCLC patients and as such may be used to evaluate therapeutic effects [9]. Thus, to better understand the correlation between CTCs, EGFR expression and cellular immune function in advanced NSCLC and TKIs treatment, we measured changes in CTCs and cellular immune function and prognosis.

Patients and methods

Patients. 66 patients (median: 61 years-of-age; range: 41–78 years-of-age) histopathologically diagnosed advanced NSCLC from September 2013 to May 2015 were selected from Yunnan Tumor Hospital (N= 40 males). We identified adenocarcinomas (N=58 cases) and squamous cell carcinoma (N=8 cases). TNM staging data were as follows: 4 cases were stage IIIB, 62 cases were stage IV; 44 cases had ECOG PS scores of 0–1, 22 cases had PS scores ≥2. All patients received chest and abdominal computed tomography, ultrasound, brain MRI,

ECG, and blood biochemistry and tumor marker assessment before and after treatment. The study was approved by the Ethics Committee of Yunnan Tumor Hospital, and all patients provided written informed consent prior to any enrollment.

Treatments. Patients were given one of three EGFR-TKIs: gefitinib (AstraZeneca, London, UK), 250 mg/day, po; erlotinib (Roche, Indianapolis, IN), 150 mg/day, po; icotinib HCl (Beta Pharma Inc., Zhejiang, China), 125 mg, tid, po. Treatments lasted one month.

Reagents and equipment. CD45-PC5, CK18-FITC, EGFR-PE and NH_4CL were purchased from Beckman Coulter (USA). Mouse anti-human cytokeratin 18 (CK18/PE) mouse Isotype Control/PE kit; mouse anti-human CD45 antibody (leukocyte common antigen)/PE-Cy5 mouse Isotype Control; mouse anti-human EGFR/FIT, and mouse Isotype Control/FITC FACSTM Permeabilizing Solution 2 (Perm2) (10×) as well as lymphocyte isolation media were all purchased from Beckman Coulter. An EPICS XL flow cytometer was also purchased from Beckman Coulter.

CTCs measurements. For the 10 cases mentioned above, 20 ml of peripheral blood was divided into 20 aliquots of 1 ml and loaded into a 5 ml sterilized tube. Into each tube 200 µl of CD45-PC5 and 20 µl of CK18-FITC were added for every 100 µl of peripheral blood, and the tube was kept in the dark for 30 min. Then, 4 ml of NH_4CL was added to initiate hemolysis until the liquid was transparent, and the sample was centrifuged and culture medium was added. CD45-CK18+ cells were sorted with FS PEAK as the threshold, and a flow speed of 10,000-25,000/s. Oscillation was carried out during sorting and observed every 30 min. Sorting was stopped when ~1,000 cells were obtained. Peripheral blood micrometastasis was defined as CTCs ≥ 5 /7.5 ml [10].

FCM measurement of EGFR expression in CTCs. To blood samples (5 µl) from 66 cases was added 20 µl CD45-PC5 20 µl EGFR-PE and 20 µl CK18-FITC and subsequently 100 µl heparin anticoagulated whole blood. The tube was shaken and placed in the dark at room temperature for 30 min. Finally, 2 ml of NH_4CL was added for hemolysis and samples were assayed 15 min later. CD45-CK18+ cells were counted as CTCs, and CD45-CK18+EGFR+ were considered to be CTCs positive for EGFR.

Flow cytometry. Peripheral blood samples were collected from patients before and one month after treatment and assayed within 2 h of collection. To 100 µl blood, heparin was added and then either 10 µl CD45-FITC/ CD4-RD/ CD8-ECD/ CD3-PC5 was added to samples for measuring T lymphocyte subsets. To another 100 µl blood, heparin was added and then either 20 µl of CD45-PC5/CD3-FITC/CD16+56-PE was added to the sample for measuring NK cells. Samples were kept at room temperature in the dark for 30 min, and then assessed with flow cytometry. CD3+ T cells were the total of peripheral blood T lymphocyte which was expressed as a percentage of peripheral blood lymphocyte. CD4+ T cells, CD8+ T cells were expressed as a percentage of peripheral blood T lymphocyte subsets. CD4+/CD8+ <1 was classified as "low", and CD4+/CD8+ ≥1 as "high". For NK cells, percents >15.7% were considered "high" and NK cells <15.7% were "low".

Efficacy evaluation. Evaluation of treatment efficacy against solid tumors according to WHO response assessment standards was assessed as CR (complete response), PR (partial response), SD (stable disease), and PD (disease progression). CR+PR was considered effective, and CR+PR+SD was considered under control. Progression-free survival (PFS) was the length of time from the first day of treatment to the day of progression. Overall survival (OS) was the length of time from the first day of treatment to the day of death or loss to follow-up. Survival was measured in months, and the last follow-up was conducted in June 2015.

Statistical analysis. All analyses were conducted by using SPSS v16.0 software (SPSS, Inc., Chicago, IL). Individual variables were assessed using a paired rank sum test. A Kaplan-Meier and a log rank test were used for survival analysis and p <0.05 was considered statistically significant.

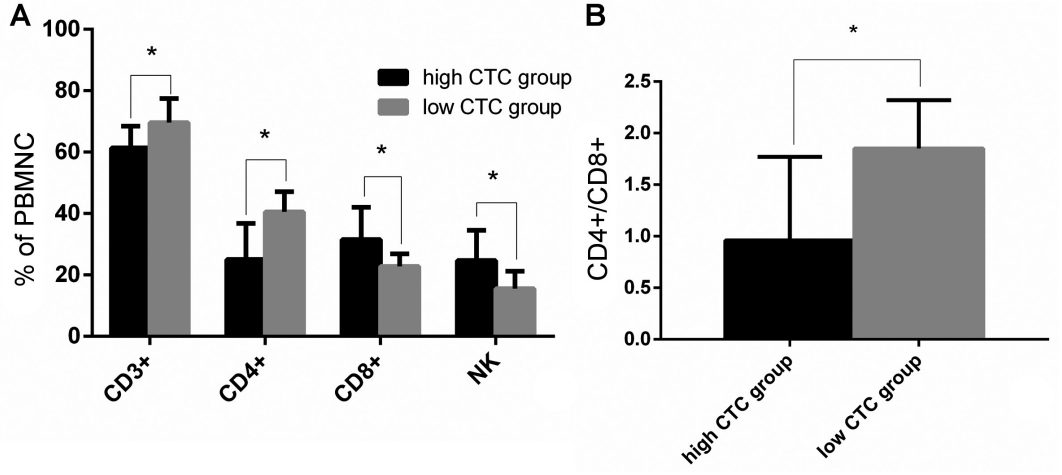

Figure 1. Relationship between CTC count and immune function. Percent CD3+, CD4+ T cells and CD4+/CD8+ ratios in the high CTCs group were lower than the low CTCs group, but CD8+ and NK cells were greater (p< 0.001).

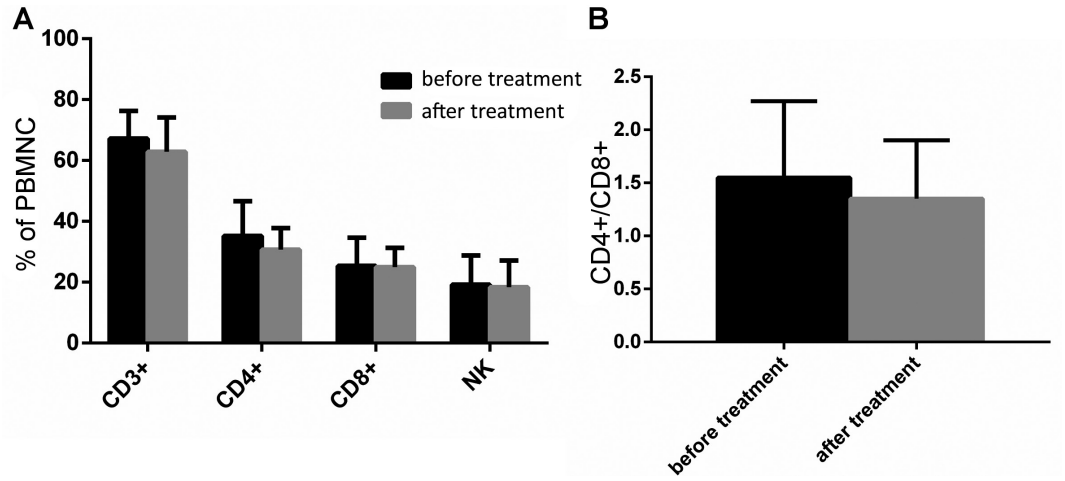

Figure 2. Changes in high CTC group immune function before and after treatment. Changes in T cell subsets and NK cells before and after treatment were not statistically significant (p>0.05).

Results

CTCs were associated with T cell subsets and NK cells. A median CTCs count of 68.5 was the threshold and patients with CTCs counts exceeding this were considered "high" whereas those below this were the "low" group. The percent of CD3+, CD4+,CD8+ T cells, and NK cells and CD4+/CD8+ ratios in the two groups were 61.39±7.04 *vs.* 69.61±7.87, 25.08±11.73 *vs.* 40.55± 6.60, 31.53 ±10.50 *vs.* 22.76±4.12, 24.68±9.88 *vs.* 15.50±5.72 and 0.96± 0.81 *vs.* 1.85±0.47, respectively ($p<0.001$). The percent of CD3+,CD4+ T cells and CD4+/CD8+ ratios in the high CTCs group were lower than in the low CTCs group, but CD8+ and NK were greater ($p<0.001$, Figure 1).

Changes in T cell subsets and NK cells were statistically different before and after TKIs treatment in patients with low CTCs. In the high CTCs group, T cell subset and NK cell changes before and after treatment were not statistically significant. The percents of CD3+, CD4+, CD8+ T cells, and NK cells and CD4+/CD8+ ratios before and after TKIs treatment were 67.21±9.10 *vs.* 62.84±11.25 , 35.24±11.40 *vs.* 30.69 ± 7.08, 25.34±9.26 *vs.* 24.93±6.48, 19.20± 9.56 *vs.*18.40±8.73 , and 1.55±0.72 *vs.* 1.35±0.55 , respectively ($p > 0.05$, Figure 2).

In the low CTCs group, the percent of CD3+, CD4+,CD8+ T cells, NK cells and CD4+/CD8+ ratios were 64.03±7.83 *vs.*71.32±5.10, 30.62±12.72 *vs.* 39.83±8.68, 28.68±8.92 *vs.* 23.45±5.13, 20.09± 9.16 *vs.* 12.50±3.84, 1.28±0.84 *vs.* 1.77±0.4. The percents of CD3+, CD4+ T cells and CD4+/CD8+ ratios increased after a month of TKIs treatment, while CD8+ T cells and NK cells decreased ($p <0.05$, Figure 3).

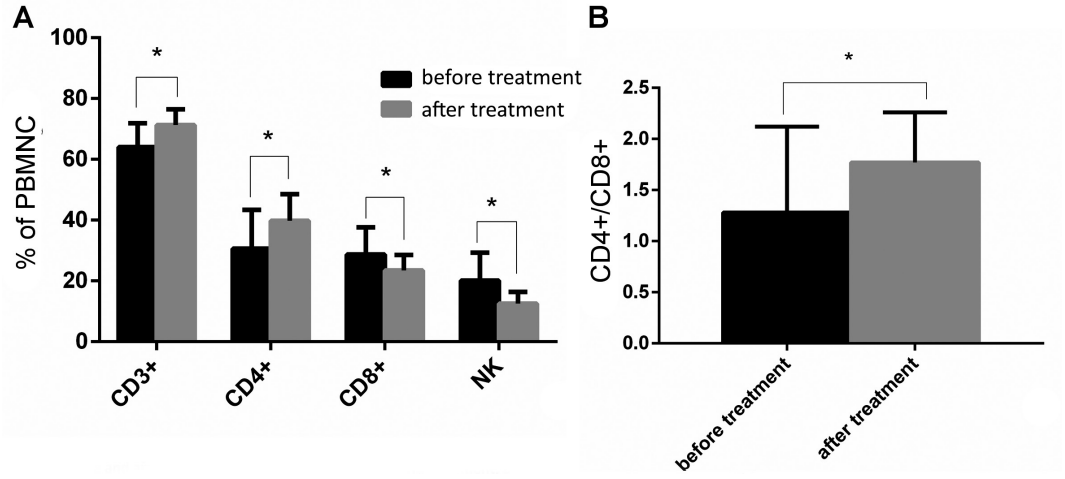

Figure 3. Changes in low CTC group immune function before and after treatment.
Percent of CD3+, CD4+ T cells and CD4+/CD8+ ratios increased after a month of TKI treatment, and CD8+ T cells, NK cells decreased ($p <0.05$).

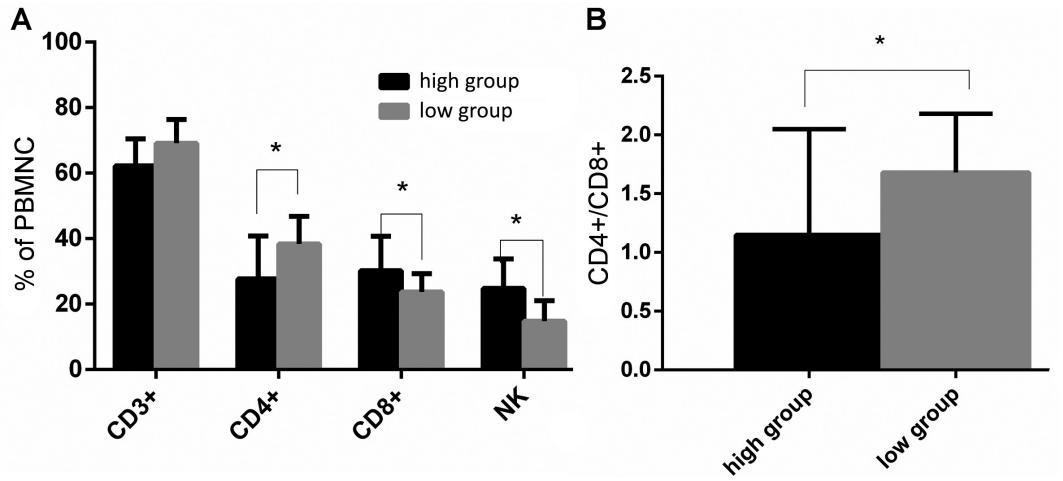

Figure 4. Relationship between CTCs EGFR expression and immune function. Percent of CD8+ T cells and NK cells were higher in the high EGFR expression group ($p<0.05$). Percent of CD4+ T cells and CD4+/CD8+ ratios were higher in the low EGFR expression group($p<0.05$). Percent of CD3+ cells was not significantly different between the two groups ($p> 0.05$).

CTCs EGFR expression was statistically associated with T cell subset and NK cells. The median CTCs EGFR expression count was 21.4, which was the threshold. Patients with EGFR expression count above the median were considered to be in the high group, and patients with EGFR expression count lower than the median were considered the low group. The percent of CD3+, CD4+, CD8+ T cells, and NK cells and CD4+/CD8+ ratios in two groups were 62.23±8.20 *vs*. 69.15±7.26, 27.73±13.02 *vs*. 38.40±8.39, 30.20±10.50 *vs*. 23.74±5.56, 24.75±9.05 *vs*.14.81± 6.27 , 1.15±0.90 *vs*. 1.68±0.54, respectively.

CD8+ T cells and NK cells were greater in the high EGFR expression group ($p<0.05$) and CD4+ T cells, and the CD4+/CD8+ ratios were greater in the low EGFR expression group ($p<0.05$). CD3+ T cells was not significantly different between the two groups ($p>0.05$, Figure 4).

CD4+/CD8+ ratios and NK cells were statistically different before and after TKIs treatment in the high CTCs EGFR expression group. In high EGFR expression group, the percents of CD3+, CD4+, CD8+ T cells, and NK cells and CD4+/CD8+ ratios before and after TKIs treatment were 66.94±7.8 *vs.* 65.66±10.69, 36.42±10.28 *vs.* 37.83±7.44, 25.16±7.66 *vs.* 22.81±7.21, 16.48±6.92 *vs.* 18.49±6.37, 1.65±0.77 *vs.* 1.84± 0.65, respectively. CD4+/CD8+ ratios and NK cells increased after treatment ($p<0.05$). Changes in CD3+, CD4+,CD8+ T cells were not significantly different between the two groups (p> 0.05). (Figure 5)

T cell subsets and NK cells changes before and after treatment were not statistically significant in the low EGFR

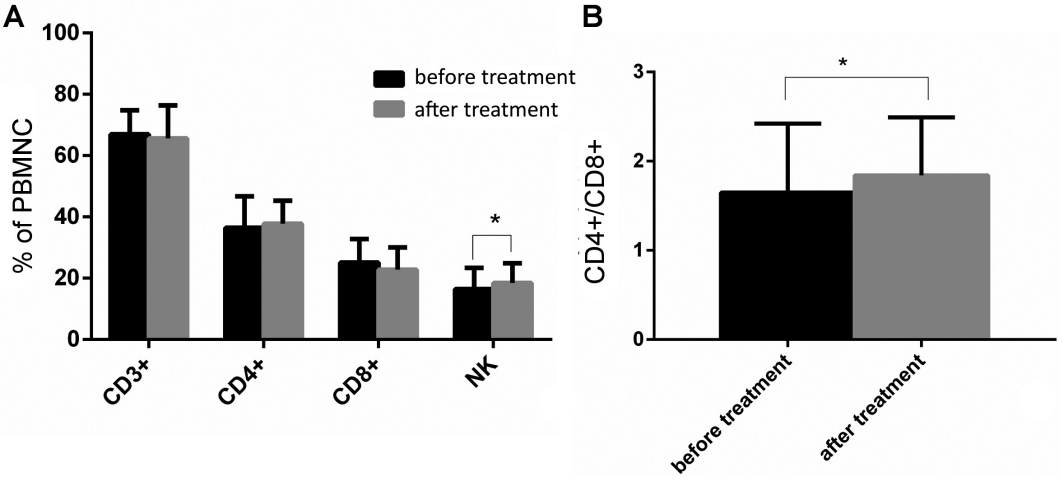

Figure 5. Changes in high EGFR group immune function before and after treatment. CD4+/CD8+ ratios and NK cells increased after treatment ($p<0.05$). Changes of CD3+, CD4+, CD8+ T cells were not significantly different between the two groups ($p> 0.05$).

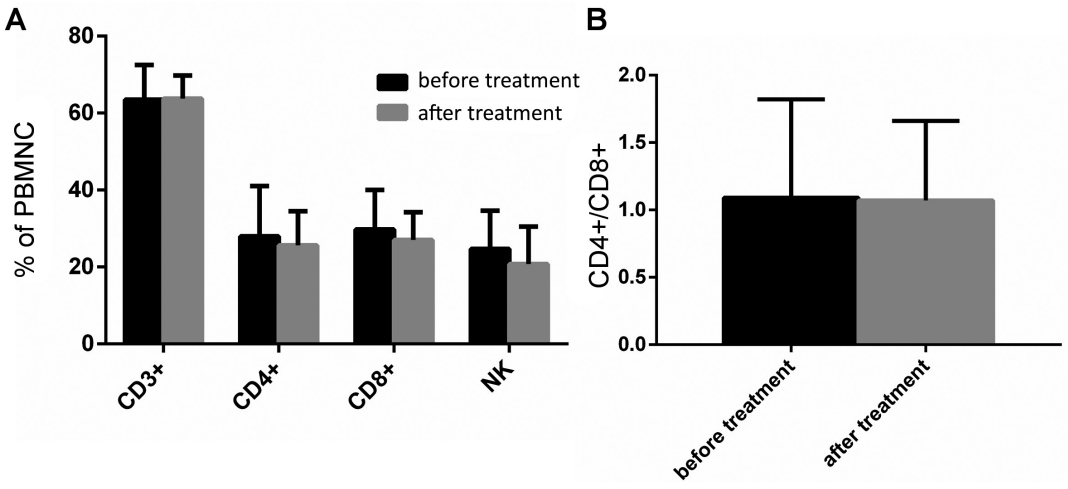

Figure 6. Changes in low EGFR group immune function before and after treatment. T cell subsets and NK cells changes before and after treatment were not statistically significant in the low EGFR expression group (p> 0.05).

expression group. The percent of CD3+, CD4+, CD8+ T cells, and NK cells and CD4+/CD8+ ratios in the two groups were 63.50±8.98 *vs.* 63.74±6.04, 27.97±13.09 *vs.* 25.60±8.92, 29.79±10.23 *vs.* 27.00±7.12, 24.73±9.89 *vs.* 20.74±9.75, and 1.09±0.73 *vs.* 1.07±0.59 (p>0.05, Figure 6).

Relationship between CD4+/CD8+ and survival were statistically significant. For low and high CD4+/CD8+ ratio groups, median overall survival (OS) was 25 and 13 months, respectively. Also, 1- and 2-year survival was 75.0% and 54.2% for the low-expressing group and 52.4% and 28.6% for the high-expressing group. Three-year survival was 20.8% and 7.1% for the low- and high-expressing groups, respectively and these differences were statistically significant (p=0.025) according to a log rank test (Figure 7). The median PFS for all patients was 6 months. The median PFS of CD4+/CD8+ low and high expression groups was 14 and 2 months, respectively and these differences were statistically significant (p=0.013) according to a log rank test (Figure 8).

Relationship between NK Cells and survival were statistically significant.

For patients with high and low NK expression, median OS was 25 and 12 months, respectively. For the high NK expression group, 1-, 2- and 3- year survival was 77.8%, 55.6%, and 22.2%, and for the low NK expression group, 1-, 2- and 3- year

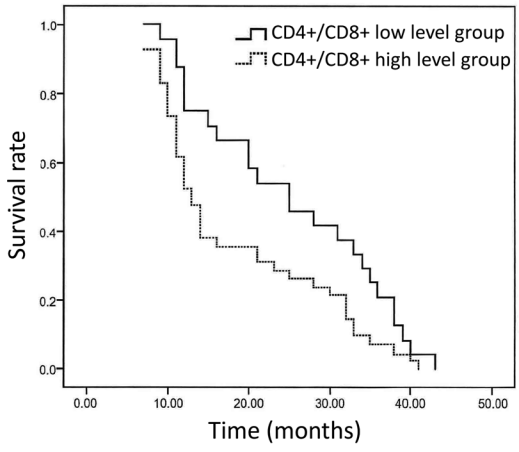

Figure 7. Kaplan-Meier plots of OS in patients with high or low CD4+/ CD8+expression. For low and high CD4+/CD8+ expressing groups, median OS was 25 and 13 months. 1- and 2-year survival was 75.0% and 54.2% for the low-expressing group and 52.4% and 28.6% for the high-expressing group. Three-year survival was 20.8% and 7.1% for the low- and high- expressing groups, respectively and these differences were statistically significant (p=0.025) according to a log rank test.

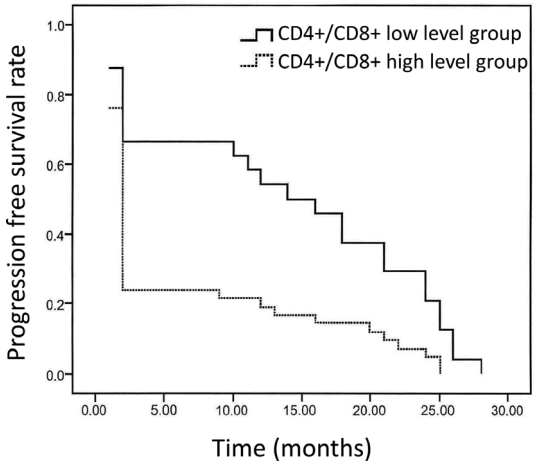

Figure 8. Kaplan-Meier plots of PFS in patients with CD4+/ CD8+ high or low expression. The median PFS of CD4+/CD8+ in low- and high- expression groups was 14 and 2 months, respectively and these differences were statistically significant (p=0.013) according to a log rank test.

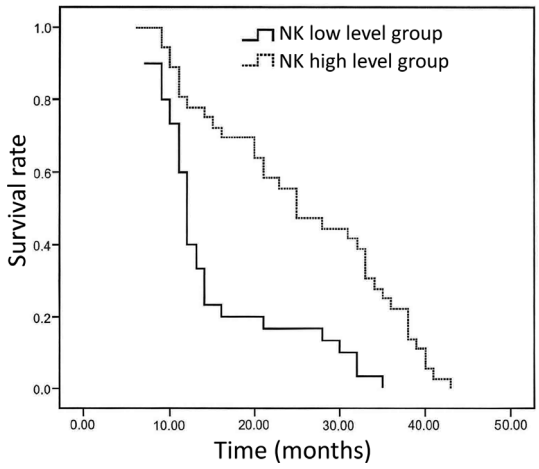

Figure 9. Kaplan-Meier plots of OS in patients with NK high or low expression. For high and low NK expression, the median OS was 25 and 12 months. For the high NK expression group, 1-, 2- and 3- year survival was 77.8%, 55.6%, and 22.2%, and for the low NK expression group, 1-, 2- and 3- year survival was 40.0%, 16.7%, and 0%.These differences were statistically significant ($p< 0.001$) according to a log rank test.

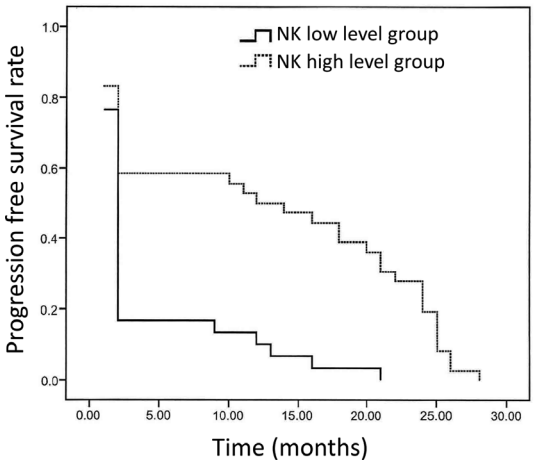

Figure 10. Kaplan-Meier plots of PFS patients with high or low NK expression. High- and low-NK expressing patients had a median PFS of 12 and 2 months, respectively, and these differences were statistically significant ($p< 0.001$) according to a log rank test.

survival was 40.0%, 16.7%, and 0%, respectively. These differences were statistically significant ($p< 0.001$) according to a log rank test (Figure 9). High- and low-NK expressing patients had median PFSs of 12 and 2 months respectively, and these differences were statistically significant ($p< 0.001$) according to a log rank test (Figure 10).

Discussion

CTCs were first described based on their similarity to cancer cells [11]. During malignant proliferation and development, tumor cells may be modified at the level of DNA or protein to alter cellular phenotype via epithelial-mesenchymal transition (EMT) and invade the peripheral circulation to form metastatic CTCs [12].

Most CTCs are eradicated via host immune function, blood flow shear force or apoptosis. CTCs were observed in the peripheral blood of patients with advanced NSCLC but patients' immune function was inhibited and remove tumor cells could not be removed. Thus a few active, potentially metastatic CTCs survived , adhered to the vascular endothelium and passed through lymphatic vessels to colonize in secondary or distant organs as metastases [13]. T lymphocyte subsets and NK cell activity are important players in anti-tumor immunity. CD3+ T cells usually represent total T cells, and CD4+ T cells represent T helper cells as a component of anti-tumor immunity. They can directly kill tumor cells via an interferon-g-mediated mechanism [14] and positively regulate immunity. CD8+ T cells, on behalf of T cells, inhibit the immune response, as well as kill target cells and hence, have a negative regulatory role. The CD4+/CD8+ ratio can reflect immune function and this ratio decreased as immune function decreased and as resistance to tumors decreased.

For advanced NSCLC patients for whom primary chemotherapy is not successful, oral EGFR-TKIs is an alternative option. Tumor cells can regulate EGFR through mutation or overexpression of EGFR to inhibit tumor cell apoptosis and promote tumor growth [15]. Thus, EGFR-TKIs inhibit tumor cell overexpression by blocking EGFR and promote apoptosis [16]. Because EGFR-TKIs treatment increases PFS and OS for patients with EGFR mutations, EGFR-TKIs is a first-line treatment for patients with advanced NSCLC with EGFR mutations. Research indicates that after TKIs treatment, NSCLC patient immune function and regulation significantly improved.

NK cells are non-specific components of cellular immunity acting independently of antibodies and complement factors. They do not require tumor cell activation and instead promote cytokine secretion for immune regulation. NK cells can also adapt as needed during immunity [17]. NK cell killing of target cells is chiefly instigated by target cell cleavage by perforation and granular proteolytic enzyme B, which is also activated by TNF-α is an effector cell that participates in early anti-tumor immune mechanisms [18].

In this study, patients with high CTCs had CD3+ and CD4+ T cells than patients with low CTCs, but CD8+ T cells were greater than for patients with low CTCs and the CD4+/CD8+ ratios decreased ($p< 0.001$); NK cells were higher in high CTCs patients compared to those with low CTCs ($p<0.001$). CTCs were negatively correlated with patient immune function. In the low CTC group, CD3+ and CD4+ T cells increased after a month of TKI treatment, and CD8+ T cells decreased ($p<0.05$). Patients with low CTCs expres-

sion had significantly improved cellular immune function after treatment.

CD4+ T cells and CD4+/CD8+ ratios in the high EGFR expression group were lower than those in the low EGFR group, but CD8+ T cells and NK cells in the high EGFR expression group were greater than those in the low EGFR group ($p<0.05$). In the high EGFR expression group, CD4+/CD8+ ratios and NK cells increased after treatment ($p<0.05$). Thus, EGFR expression was positively correlated with patient immune function.

Patients with low expression of CD4+/CD8+ ratios but high expression of NK cells had a greater OS and PFS. Because TKIs can improve immune cell function for patients with late stage NSCLC, efficacy may be related to enhancing cellular immune function. Molecularly targeted drugs for treating NSLCL may improve existing therapies, and better therapeutic targets and prognostic and predictive markers will be important to these improvements. We report that CTCs were negatively associated but EGFR expression was positively correlated with cellular immune function and immune recovery in advanced NSCLC patients after EGFR-TKIs treatment. CD4+/CD8+ ratios and percent NK cells may be useful prognostic factors for these patients.

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