

Heterogeneity and prognosis of programmed cell death-ligand 1 expression in the circulating tumor cells of non-small cell lung cancer

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Due to tumor heterogeneity, the consistency of programmed cell death-ligand 1 (PD-L1) expression between circulating tumor cells (CTCs) and tissue is controversial. This study aimed to establish a method for detecting CTC PD-L1 expression and exploring the impact of the same on the prognosis of lung cancer. In 32 patients with non-small cell lung cancer, lung cancer cells in the blood were enriched using CD326 immunomagnetic beads. Goat anti-mouse polyclonal CD326 antibody stained the epithelial lung cancer cells and anti-PD-L1 antibody was used to detect the expression of CTC PD-L1. The DAKO Link 48 automatic staining device detected the expression in lung cancer tissue. The consistency of PD-L1 expression was analyzed in lung cancer tissue and CTCs. The effect of plasma interferon gamma, tumor necrosis factor alpha, and interleukin-2 on PD-L1 expression and prognosis was analyzed. The number of CTCs detected in patients was 1–36, with a median of 2. There was no significant difference in PD-L1 expression fractions between CTCs and paired tumor tissue ($p > 0.05$). The correlation coefficient was 0.20. Regardless of lung cancer tissue or CTCs, there was no statistically significant difference in the blood cytokine levels between the two groups with positive or negative PD-L1 expression ($p > 0.05$). There was no correlation between CTCs and PD-L1 in 23 untreated patients. The expression of PD-L1 in CTCs and lung cancer tissue is heterogeneous and unaffected by the peripheral cytokines' levels. PD-L1 expression has no correlation between CTCs and tissues and is not related to prognosis.

Key words: non-small cell lung cancer, circulating tumor cells, programmed cell death-ligand 1, heterogeneity

Programmed cell death-ligand 1 (PD-L1) is a member of the B7 family and is expressed in B, dendritic, mesenchymal stem and T cells, and in tumors. Cellular expression of PD-L1 [1] and subsequent programmed cell death receptor 1 (PD-1) binding has a negative immunomodulatory effect, inhibits the proliferation of activated T lymphocytes and cytokine production, and promotes apoptosis [2, 3].

In non-small cell lung cancer (NSCLC), over 50% of the tumor and infiltrating immune cells express PD-L1 [4]. PD-L1 positive NSCLC is characterized by weak lymphocyte infiltration [5] and a short disease-free survival period [6]. In the KEYNOTE-001, 010, and 024 studies of pembrolizumab in patients with advanced NSCLC, higher PD-L1 expression predicted longer progression-free and overall survival. Similar results have been observed for nivolumab in patients with non-squamous NSCLC [4, 7, 8]. Although many studies have shown differences in PD-L1 expression between detec-

tion and biological heterogeneity in patients receiving PD-1/PD-L1 immune checkpoint inhibitors, immunohistochemistry (IHC) staining has quickly become a clinical stratified biomarker [9].

In the KEYNOTE-042 study, compared with chemotherapy, the increase in tumor PD-L1 positivity when using pembrolizumab was correlated with an increase in survival benefit [10]. Even for patients with PD-L1 expression $< 1\%$, pembrolizumab improved overall survival [11]. However, some report that nearly 10% of patients whose tumors did not express PD-L1 could also benefit from immune checkpoint inhibitors, and tumors that expressed PD-L1 in large numbers did not respond to treatment [12]. Therefore, detection of PD-L1 using tissue is not ideal for anti-PD-1/PD-L1 immunotherapy. In a large study of 982 patients, the expression of PD-L1 detected by immunohistochemistry was not a prognostic factor for patients with early NSCLC, nor could

it predict the benefit of adjuvant chemotherapy for these patients [13]. For this reason, when the expression of PD-L1 is not high, its predictive therapeutic value is not clear. This may be related to the small sample biopsy of the puncture tissue, intra-tumor heterogeneity, and inter-tumor heterogeneity, which cannot objectively reflect the puncture tissue and other parts of the tissue. PD-L1 expression of metastases biopsy or continuous biopsy of multiple sites during treatment can solve some of these problems, but it is limited by the invasiveness of surgery and the potential risk to patients. In the development and treatment of the disease, tumor heterogeneity, the appearance of new metastatic sites, and dynamic changes of PD-L1 are also factors that affect the expression status of PD-L1 [14]. Using the archived wax block tissue detection method cannot overall reflect the current expression status of PD-L1. In this regard, circulating tumor cells (CTCs) may enter the blood circulation after detaching from the tumor tissue of the primary tumor or metastasis [15], which may represent a larger part of the genetic and epigenetic variation spectrum within the tumor [16]. Mazel [17] was the first to report the PD-L1 detection technology of CTCs in 2015. PD-L1+ CTCs were found in the peripheral blood of 11 cases (68.8%) of breast cancer patients. The positive rate of CTC PD-L1 in a single patient ranged from 0.2% to 100%. CTCs provide a viable, non-invasive alternative specimen to monitor the expression status of PD-L1 in real time. Monitoring the expression of CTC PD-L1 in patients with NSCLC may predict the prognosis of anti-PD-1 therapy [18], but still requires further investigation.

The expression of PD-L1 in human tumor tissue rather than normal tissue is divided into constitutive expression and inducible expression [19]. Inducible expression is mainly caused by external factors, such as cytokines, the most important of which is interferon gamma (IFN- γ) [20]. In addition, tumor necrosis factor alpha (TNF- α) [21], interleukin (IL)-2 [22], IL-10 [23], IL-1 β [24], IL-6 [25], IL-27 [26], etc., can induce tumor cells to express PD-L1, potentially affecting the expression of CTC PD-L1. The consistency of PD-L1 expression between lung cancer tissue and CTCs is controversial. It has been reported that the consistency of PD-L1 expression between lung cancer tissue and CTCs is very high, roughly 93% [27]. There are also reports to the contrary; in patients with NSCLC, the percentage of PD-L1+ CTCs has no correlation to the positive percentage of PD-L1 in biopsy tissue detected by immunohistochemistry [28]. This study explored the consistency of PD-L1 expression between lung cancer tissue and CTCs, the influence of blood cytokines on the expression of CTC PD-L1, and the possibility of prognostic correlation.

Patients and methods

Experimental cells and blood samples. The enrolled 32 patients had NSCLC diagnosed by histology, and none of them received immunotherapy. This study was approved by

the Ethics Committee of Beijing Chest Hospital (reference YJS-2019004). A blood collection tube containing ethylenediaminetetraacetic acid anticoagulant was used to collect 5 ml of fasting venous blood from the patient in the morning (not the first tube) and was processed within 2 h. The human lung cancer cell line NCI-H2009 which used as PD-L1 positive control was purchased from the Cell Resource Center of the Institute of Basic Medicine, Chinese Academy of Medical Sciences, and cultured in RPMI-1640 (10% fetal bovine serum, 1% penicillin/streptomycin) in a 37°C incubator with 5% CO₂.

Lung cancer cell incorporation experiment and clinical CTC PD-L1 protein detection. Anticoagulated peripheral blood from healthy donors was spiked with NCI-H2009 cells. Red blood cells were lysed and then incubated with CD326 immunomagnetic beads (Miltenyi Biotec Inc., Bergisch Gladbach, Germany) at 4°C for 30 min. The auto MACS pro platform (Miltenyi Biotec Inc., Bergisch Gladbach, Germany) was used to sort lung cancer cells. First, 1 μ g of goat anti-mouse polyclonal antibody (Abcam, Cambridge, MA, USA) was used to stain lung cancer cells and was washed twice. Then, anti-CD45 antibody (Abcam, Cambridge, MA, USA), anti-PD-L1-PE-CF594 (M1H1) antibody (BD Biosciences, San Jose, CA, USA), and Hoechst 33342 solution (Sigma, Saint Louis, Missouri, USA) were used for the staining, and lung cancer cells were observed under a fluorescence microscope. The overall shape of the selected cells was complete, round, or oval. The identification criteria were EpCAM+/PD-L1+/Hoechst+/CD45- for lung cancer cells of PD-L1+; EpCAM+/PD-L1-/Hoechst+/CD45- is the lung cancer cell of PD-L1-. According to the above detection method, CTC enrichment and PD-L1 detection were performed on 5 ml of peripheral blood from lung cancer patients. In brief, peripheral blood was sorted by lysed red blood cells and CD326 immunomagnetic beads for the enrichment of tumor cells, then subjected to the above-mentioned immunofluorescence for identification of CD326-bead attached tumor cells using an anti-mouse polyclonal antibody, after washing, followed anti-CD45 and anti-PD-L1 staining (Wang et al., Technology in Cancer Research & Treatment, accepted paper). PD-L1 positive CTCs from each patient were expressed as a percentage of all CTCs, i.e., the tumor proportion score (TPS).

PD-L1 staining of lung cancer tissue. A DAKO Link 48 automatic staining device was used to immunostaining the newly cut tissue sections. We placed the deparaffinized sections in Antigen Retrieval Buffer (Tris-EDTA-citrate buffer, pH9) at 98°C for 10 min. We used the primary antibody PD-L1 (E1L3N, CST, San Diego, USA, 1:200) at room temperature and the EnVision Flex kit (DAKO, Glostrup, Denmark) according to the manufacturer's instructions to display the bound antibody. Positive control and negative control were set up during the staining process. Only when the positive and negative controls met the requirements can the tumor PD-L1 expression be interpreted. The

expression of PD-L1 was evaluated by professional pathologists. No staining at all was considered “negative”. Therefore, any intensity of staining was considered “positive” and was evaluated as the percentage of PD-L1 positive tumor cells accounted for all tumor cells.

Detection of cytokines. Plasma (15 μ l) was used to detect the concentrations of IFN- γ , TNF- α , and IL-2 according to the manufacturer’s instructions of AimPlex™ Analyte Kit (AimPlex Biosciences, Inc., Pomona, CA, USA) by using a flow cytometer (BD LSRFortessa X-20).

Statistical analysis. The obtained data were first tested for the normal distribution. Normally distributed data are expressed as mean \pm standard deviation and non-normally distributed data are expressed by median and non-parametric test (Mann Whitney test). The rates were analyzed by using the Wechsler paired signed-rank test, and the correlation was tested using the Spearman correlation test.

Results

Clinical characteristics of patients with non-small cell lung cancer. The clinical data of the patients are shown in Table 1. Peripheral blood CTCs and tumor tissue PD-L1 protein were detected in 32 patients with NSCLC. The main pathological type was adenocarcinoma, accounting for 75% of cases. Nine patients had genetic mutations, of which five had epidermal growth factor receptor (EGFR) mutations. The clinical stage was mainly stage III and IV, accounting for 75% of cases. All patients received EGFR tyrosine kinase inhibitor (TKI) targeted therapy, chemotherapy, and surgery, and no patient received immunotherapy. Twenty-three patients were detected for CTC PD-L1 protein before treatment, and nine patients were detected for CTC PD-L1 protein after treatment. Detection of PD-L1 protein in all tumor tissue was performed before treatments.

Detection of PD-L1 expression in CTCs and tumor tissues. According to the CellSearch system, the standard for identifying CTCs is EpCAM+/Hoechst+/CD45-. Anti-PD-L1-PE-CF594 (M1H1) antibody was used to stain the spiked NCI-H2009 cells in the peripheral blood of healthy donors. Thus, the identification and staining of lung cancer cells marked with EpCAM+/PD-L1+/Hoechst+/CD45- are shown in Figure 1A. CTCs were detected from 32 patients. The number of CTCs detected was 1–36, with a median of 2 in 32 patients (Figure 1C). The expression of CTC-PD-L1 protein showed both negative and positive (Figure 1B). The PD-L1 of tumor tissue was detected by immunohistochemical method, and the positive ratio of PD-L1 was presented by TPS (Figure 1D).

Heterogeneity and no correlation between CTCs and matched tumor tissue in PD-L1 expression. The PD-L1 expression in CTCs and matched tumor tissues of 23 untreated patients were compared. Among them, 12 patients had a positive expression of PD-L1 in CTCs, and the positive rate was 52.2%; the PD-L1 expression TPS of CTCs in a single

Table 1. Clinical data.

Patient characteristics	Number of participants (n=32), n (%)	
Age	Median	60
	Range	40–76
Gender	Male	27 (84.4)
	Female	5 (15.6)
	Adenocarcinoma	24 (75.0)
Pathological type	Squamous cell carcinoma	3 (9.3)
	Others	5 (15.6)
Gene mutations	EGFR mutation	5 (15.6)
	others	4 (12.5)
	I	6 (18.8)
Clinical stages	II	2 (6.2)
	III	7 (21.9)
	IV	17 (53.1)
	Targeted therapy	6 (18.8)
Treatment methods	Chemotherapy	20 (62.5)
	Surgery	6 (18.8)
CTCs PD-L1 detection time	Before therapy	23 (71.9)
	After treatment	9 (28.1)

Abbreviations: CTCs-circulating tumor cells; EGFR-epidermal growth factor receptor; PD-L1-programmed cell death-ligand 1.

patient was 0–100%, of which 7 patients had \geq 50%. There were 13 patients with positive PD-L1 expression in tumor tissues, and the positive rate was 56.5%; the PD-L1 expression TPS of a single patient’s tissue was 0–95%, of which 6 cases were \geq 50%. Seven patients were positive for PD-L1 expression with CTCs, but the positive rate of PD-L1 was not completely consistent with the matched tumor tissue. Six patients had only tumor tissue PD-L1 positivity, and five patients had only CTC PD-L1 positivity (Figure 2A). The positive expression rate of PD-L1 between CTCs and paired tumor tissues in 23 patients was not significantly different according to Wicker’s paired signed-rank test ($p>0.05$) (Figure 2B), and the correlation coefficient was 0.045 according to Pearson’s correlation test. Very weak or no correlation was observed (Figure 2C).

Blood cytokine concentration does not affect the expression of PD-L1 in CTCs and tumor tissues. The cytokines IFN- γ , TNF- α , and IL-2 were detected in the peripheral blood of 32 patients. Among them, 17 cases were PD-L1 positive and 15 cases were PD-L1 negative in tumor tissue. There were 16 cases with CTC PD-L1 positivity and 16 cases with CTC PD-L1 negativity. The cytokine concentrations in the PD-L1+ CTC and PD-L1- CTC groups were as follows: IFN- γ (median 3.800 vs. 4.890, $p>0.05$), TNF- α (median 2.395 vs. 3.040, $p>0.05$), and IL-2 (median 4.710 vs. 6.245, $p>0.05$). There was no statistically significant difference in the concentration of peripheral blood cytokines between the two groups (Figure 3A). The cytokine concentrations in tumor tissue PD-L1+ and PD-L1- groups were as follows: IFN- γ (median 4.575 vs. 4.890, $p>0.05$), TNF- α (median 2.110 vs. 3.040, $p>0.05$), and IL-2 (median 4.400

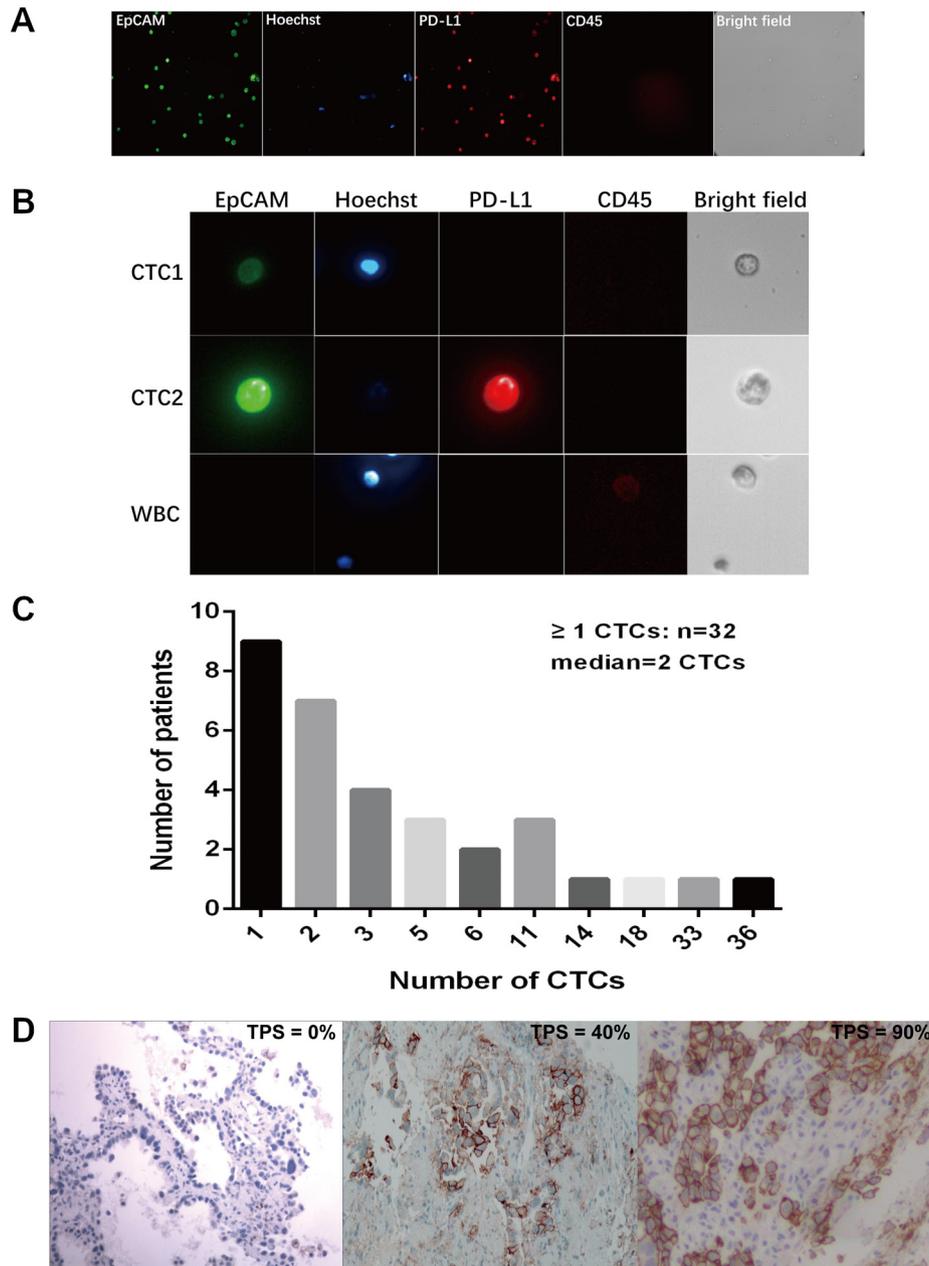


Figure 1. Detection of PD-L1 protein in blood lung cancer cells and tumor tissue. **A)** PD-L1 (Anti-Human CD274 PE-CF594) was used to stain the NCI-H2099 lung cancer cell line (PD-L1 positive) spiked into the peripheral blood. PD-L1+ lung cancer cells expressed EpCAM+/PD-L1+/Hoechst+/CD45-. **B)** The CTCs from patients were stained by EpCAM+/Hoechst+/PD-L1+/CD45-. The white blood cell (WBC) was identified by EpCAM-/Hoechst+/CD45+ staining. **C)** The obtained CTC numbers and distribution in 32 patients. **D)** The PD-L1 expression of lung cancer tissue was detected by immunohistochemical method, and the positive ratio of PD-L1 was shown by tumor proportion score (TPS).

vs 5.500, $p > 0.05$). There was no significant difference in the concentration of peripheral blood cytokines between the two groups (Figure 3B). The expression of PD-L1 in CTCs and tumor tissue was unrelated to the concentration of cytokines IFN- γ , TNF- α , and IL-2 in the peripheral blood.

The effect of treatment on CTC PD-L1 expression and prognostic analysis. Nine patients were tested for CTC

PD-L1 expression after treatment. When compared with the PD-L1 expression TPS of the tumor tissue before treatment, CTC PD-L1 expression TPS was increased in five, unchanged in two, and decreased in two cases after treatment (Figure 4A). Five patients with elevated levels of CTC PD-L1 had been treated for 0.7–2.5 months. Among them, EGFR TKI treatment was used in four cases, and pemetrexed

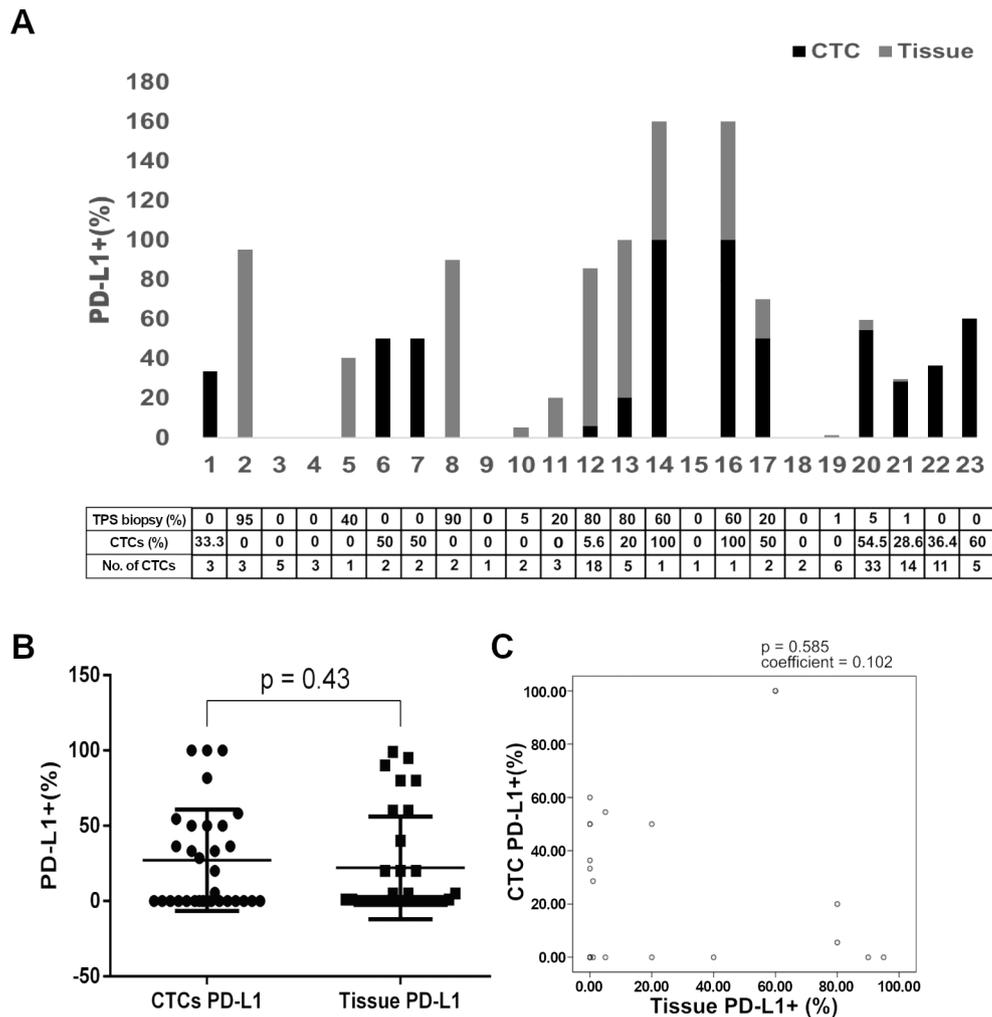


Figure 2. Heterogeneity and no correlation between the patient’s CTC and matched tumor tissue PD-L1 expression. A) TPS of 23 patients with CTC and tumor tissue PD-L1 expression. B) The comparison of CTC and paired tumor tissue PD-L1 expression TPS ($p > 0.05$). C) The correlation between CTCs and paired tissues in PD-L1 protein TPS. A p-value indicated in each panel.

combined with cisplatin chemotherapy was used in one case. The other two patients with not changed CTC PD-L1 expression had been treated with EGFR TKI for 1–6 months. The remaining two patients with decreased CTC PD-L1 were treated for 1.7–8 months (Figure 4B), one with crizotinib and one with pemetrexed combined with nedaplatin chemotherapy when the CTC PD-L1 was detected again.

The median progression-free survival (PFS) duration of the 23 patients in the CTC PD-L1 positive group (12 cases) and negative group (11 cases) before treatment were 5.9 months and 7.3 months, respectively. There was no statistically significant difference between the two groups ($p > 0.05$; Figure 4C). The median PFS of the PD-L1 positive and negative groups of tumor tissue before treatment were 3 months and 12 months, respectively. There was no statistically significant difference between the two groups ($p > 0.05$; Figure 4D).

Discussion

Despite different reports, patients with high PD-L1 expression in tumor tissues are more likely to benefit from anti-PD-1/PD-L1 treatment [7]. This study detected the expression of PD-L1 in lung cancer CTCs and assessed its correlation with PD-L1 expression in tumor tissue.

Our study found that the positive rate of PD-L1 expression in CTCs was 51.6%, and the proportion of PD-L1 expression in CTCs was between 5.6% and 100%. Previous reports detected PD-L1 in CTCs using the CellSearch system and ISET filtering platform. PD-L1 positive CTCs were detected in 8–95% of CTCs [18, 27, 29, 30]. The difference in the positive rate of PD-L1 expression may be explained by the use of different platforms for the detection of CTCs, different antibodies for PD-L1 staining, and different patient

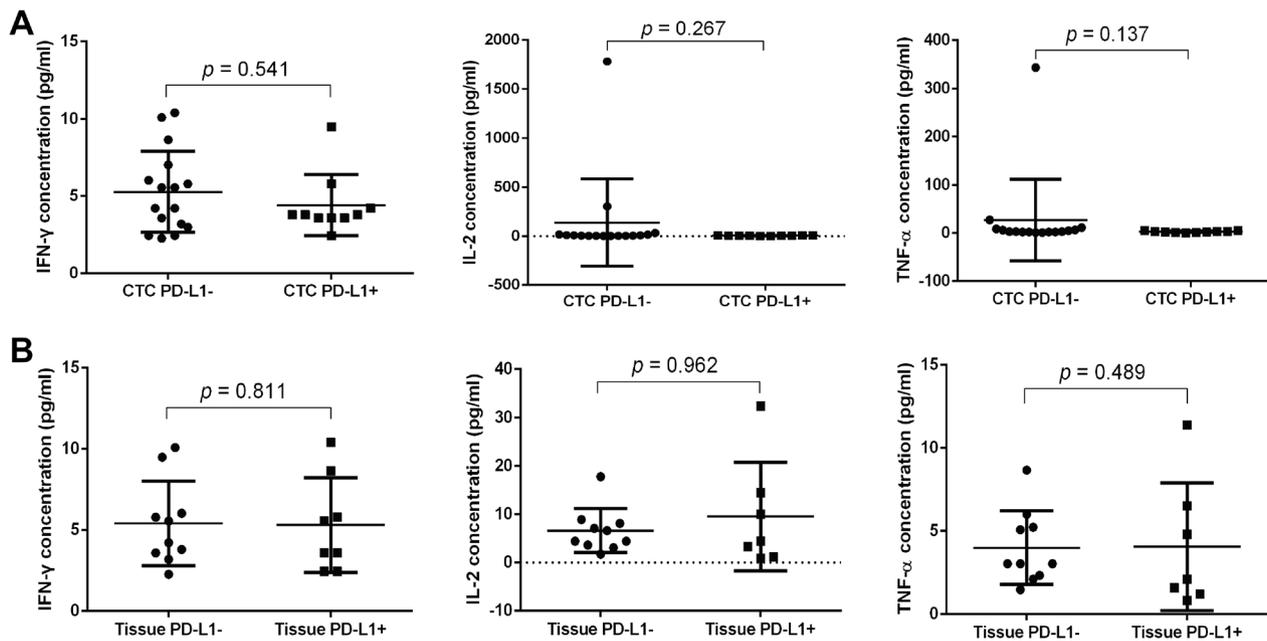


Figure 3. The correlation between the expression of PD-L1 and the blood interferon gamma (IFN- γ), tumor necrosis factor alpha (TNF- α), and interleukin (IL)-2. A) The relationship between the expression of PD-L1 of CTCs and the concentration of IFN- γ , TNF- α , and IL-2 in the peripheral blood. B) The relationship between the expression of PD-L1 in the lung cancer tissue and the concentration of IFN- γ , TNF- α , and IL-2 in the peripheral blood. A p-value indicated in each panel.

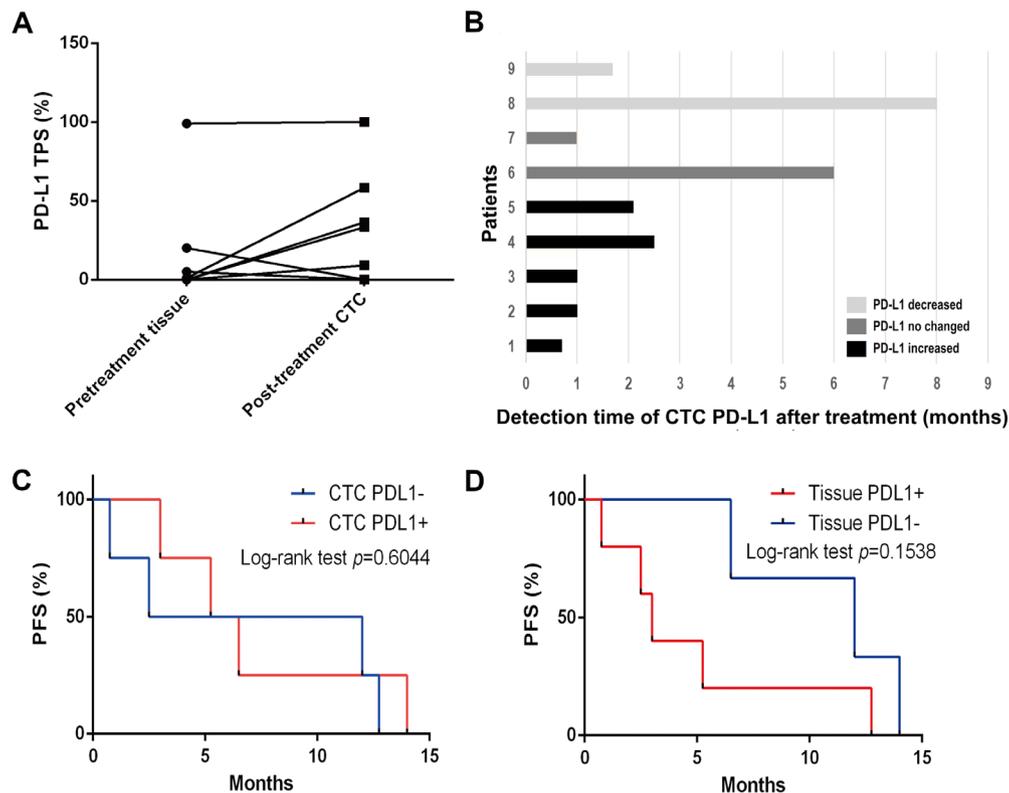


Figure 4. Effect of treatment on the expression of PD-L1 in CTC and tumor tissues and its correlation with prognosis. A) CTC PD-L1 expression TPS after treatment compared with PD-L1 expression TPS of tumor tissue before treatment. B) The CTC PD-L1 expression TPS changed before and after treatment. C) There was no statistically significant difference between the CTC PD-L1- and CTC PD-L1+ groups in PFS. D) There was no statistically significant difference between the tissue PD-L1- and tissue PD-L1+ groups on PFS. A p-value indicated in each panel.

groups at different time points. Ilie et al. reported that the consistency of the PD-L1 positive rate between tumor tissue and CTCs was 93% [27], which is in contrast with our findings. In three studies with similar results as ours, there was no correlation between CTC and tissue PD-L1 expression. A report using the ISET filter system showed that the PD-L1 positive rate was 93%, and another report using the microcavity array system showed that the PD-L1 positivity rate was 73%, and the PD-L1 expression ratio of CTCs was 3–100%. Another report using the Parsortix™ system showed that the PD-L1 positive rate was 73%. The incomplete report may be related to the different separation platforms and detection antibody clones.

In theory, CTCs originate from different tumor sites, which may reflect the heterogeneity of tumor PD-L1 expression. Patients whose tissues are negative for PD-L1 can also benefit from immunotherapy. Perhaps the results of the detection of PD-L1 expression of CTCs can provide an explanation. Several studies have been conducted to evaluate the predictive significance of PD-L1+ CTCs for immune checkpoint inhibitor therapy in lung cancer patients. Nicolazzo et al. [18] evaluated the predictive significance of PD-L1+ CTCs in nivolumab treatment. At baseline and at 3 months of treatment, they found that the presence of CTCs and the expression of PD-L1 on the surface were associated with poor prognosis, and patients with PD-L1– CTCs benefited after 6 months of treatment, whereas patients with PD-L1+ CTCs progressed. Boffa et al. [31] reported that the 2-year survival rate of patients with NSCLC >1.1 PD-L1+ CTCs/ml at the time of diagnosis was worse than those with ≤1.1 PD-L1+ CTCs/ml (31.2% vs. 78.8%, $p=0.002$). It can be seen that PD-L1 positive CTCs still have certain predictive significance for the treatment of immune checkpoint inhibitors in patients with lung cancer.

This study did not observe a correlation between the PD-L1 expression status of CTCs and prognosis possibly because some patients received drug treatment that could potentially affect the expression of CTC PD-L1. The expression of PD-L1 in tissue was also not related to prognosis. The correlation between the CTC PD-L1 expression level and prognosis needs to be analyzed in a larger sample.

The expression of PD-L1 is affected by a variety of regulatory mechanisms. Among them, the most reported are cytokines, such as IFN- γ , TNF- α , and ILs. Tumor site specificity mainly depends on the local induction of PD-L1 by cytokines, and the expression of PD-L1 is usually a sign of the anti-tumor immune response state. It is not clear whether soluble cytokines, such as IFN- γ , will spread to a wider range of tissues or even the peripheral blood to affect the expression of PD-L1 in cancer cells. We found that the expression of PD-L1 in CTCs and tumor tissue was unrelated to the concentration of cytokines IFN- γ , TNF- α , and IL-2 in the peripheral blood. It is speculated that the regulation of PD-L1 expression in cancer cells by cytokines is limited to the tumor microenvironment. We previously confirmed that IFN- γ significantly

induces PD-L1 expression [22]. This study preliminarily showed the feasibility of CTCs for detecting PD-L1 expression. CTC PD-L1 expression may be different from tissue PD-L1 expression and is not affected by the concentration of blood cells. The prognostic value of CTC PD-L1 expression needs to be verified in a larger sample in the future.

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