Expression of PD-1 on CD4+ T cells in peripheral blood associates with poor clinical outcome in non-small cell lung cancer

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ABSTRACT

Recent success of using agents inhibiting the major immune check point, programmed cell death-1 (PD-1) pathway, offers a great promise for effective cancer therapy. Two blocking antibodies for PD-1, nivolumab and pembrolizumab have recently been approved for treating advanced recurrent non-small cell lung cancer (NSCLC). Activation of PD-1 on T cells and PD-L1 on tumor cells or antigen presenting cells leads to T cell exhaustion and ultimately tumor growth. In this study, we performed flow cytometry analysis of peripheral blood samples collected from patients with advanced NSCLC at initial diagnosis. We report that surface expression of PD-1 on CD4+ T cells has a prognostic value in NSCLC patients, as high expression of PD-1 is associated with a shorter progression-free survival and overall survival. Importantly, we also found that high PD-1 expression on peripheral CD4+ T cells is associated with inferior clinical response in a subset of patients who received anti-PD-L1 treatment, indicating a potential predictive value of this marker. This work highlights the potential of a non-invasive and effective method to determine prognostic and predictive biomarkers for inhibiting the PD-1 pathway in NSCLC patients.

INTRODUCTION

Lung cancer remains the leading cause of cancer-related death in the United States, with approximately 160,000 deaths every year. The majority of these patients have non-small cell lung cancer (NSCLC). The prognosis for metastatic NSCLC is generally poor with the overall five-year survival of < 5% [1]. Platinum-based chemotherapy has been the standard treatment for advanced NSCLC in addition to targeting patient-specific tumor driving mutations (i.e. EGFR) [2, 3]. These therapies, however, are limited by significant toxicities and development of resistance. Furthermore, only a fraction of these patients have targetable mutations in their tumor.

The recent success with using reagents targeting negative regulators of the immune checkpoint proteins PD-1/PD-L1 offers great promise for effective cancer therapy [4, 5, 6, 7, 8, 9]. Programmed cell death-1 (PD-1) pathway is a major immune checkpoint [10]. The interactions between PD-1 on T cells and PD ligand-1 (PD-L1) on tumor cells or antigen presenting cells (APCs) mutes T cell activation and T-cell-mediated tumor cell killing [11, 12, 13, 14]. Agents blocking the PD-1 pathway, nivolumab and pembrolizumab, have recently been approved by the FDA for treating several solid tumors including advanced NSCLC. Antibodies blocking PD-L1 have also demonstrated clinical efficacy in patients with NSCLC [15].

It is important to identify prognostic and predictive biomarkers associated with PD-1 pathway inhibitors to guide clinical decisions. Much attention has been focused on testing PD-1 or PD-L1 expression in tumor tissues, which is challenging due to the difficulties in obtaining tissue samples and the lack of consensus among the
available antibodies for immunohistochemistry staining [16, 17, 18, 19]. Here we analyzed blood samples collected from a cohort of 42 patients with advanced stage NSCLC. We utilized flow cytometry to evaluate peripheral expression of PD-1 and further dissect their prognostic and predictive value in NSCLC.

RESULTS

PD-1 is elevated on CD4$^+$ T cells in peripheral blood of patients with NSCLC

We assessed PBMCs from 42 NSCLC patients at initial diagnosis for PD-1 expression on both CD4$^+$ and CD8$^+$ T cells. PBMCs from 25 healthy donors (HD) were tested as controls. All 42 patients evaluated in our study were diagnosed with advanced stage (stage IIIB or IV) NSCLC (Table 1). As shown in Figure 1A–1B, the frequency of PD-1$^+$ cells among CD4$^+$ T cells from NSCLC patients was significantly higher than those from HD (mean frequency 13.3$\pm$1.1$\%$ vs. 8.8$\pm$0.7$\%$, $P=0.0045$). In contrast, expression of PD-1 on CD8$^+$ T cells was comparable between NSCLC patients and HD. The data suggest that CD4$^+$, and not CD8$^+$, T cells play a pivotal role in PD-1-dependent immune tolerance in NSCLC patients.

Tumor burden is independent of PD-1$^+$ T cells from peripheral blood in patients with NSCLC

We next assessed the correlation of PD-1 expression with clinical characteristics of NSCLC patients. Greater tumor burden is considered a negative impact on cancer prognosis. We analyzed the correlation between PD-1 expression on peripheral T cells and tumor size in our patients with NSCLC. There was no significant correlation between PD-1 expression on CD8$^+$ T cells and tumor burden (Figure 2B, $P=0.450$). There was a positive correlation among patients with higher PD-1 expression on CD4$^+$ T cells and the presence of smaller tumor burden, but this did not reach statistical significance: (Figure 2A, $P=0.861$). Consistently, no correlation was found between the PD-1 expression on peripheral blood T cells and the stage of NSCLC patients (data not shown).

Expression of PD-1 on T cells from peripheral blood does not correlate with PD-L1 expression on tumor tissues in patients with NSCLC

Although controversial, a large number of studies have demonstrated that PD-L1 expression on tumor tissues predicts clinical response to inhibitors of the PD-1 pathway. Among 42 NSCLC patients evaluated in our study, 19 patients were tested for PD-L1 expression by immunohistochemistry (IHC) in their tumor tissues. We further assessed whether the level of PD-1 expression on peripheral blood T cells associates with PD-L1 in tumor tissues. As shown is Figure 3, the percentage of PD-1 expression on T cells is comparable between PD-L1$^+$ and PD-L1$^-$ population. This occurs in both CD4$^+$ and CD8$^+$ T cells. Thus, the expression of PD-1 on T cells from peripheral blood does not associate with PD-L1 expression on tumor tissues in patients with advanced NSCLC.

High expression of PD-1 on CD4$^+$ T cells at initial diagnosis associates with poor clinical outcome in NSCLC

Based on the level of PD-1 expression on CD4$^+$ T cells, we defined high-PD-1 (PD-1$>$12.27$\%$) vs. low-PD-1 (PD-1$<$12.27$\%$) subgroups in NSCLC patients. The mean value (12.27$\%$) of PD-1 expression on CD4$^+$ T cells of the 42 NSCLC patients was the cutoff. Seventeen patients are classified in the high-PD-1 group and 25 in the low-PD-1 group. We analyzed clinical characteristics and found no significant difference between the two groups in terms of age, gender, histology, stage, smoking history, and treatments (chemotherapy, targeted-therapy or immunotherapy). We next investigated PD-1 expression on clinical outcome. We found a significantly lower rate of both overall survival (OS) and progression free survival (PFS) in the high-PD-1 group compared with the low-PD-1 group (median OS: 397 days vs. 721 days, $P=0.028$, Figure 4A; median PFS: 88 days vs. 391 days, $P=0.044$, Figure 4B). Consistent with this finding, elevated PD-1 expression on CD4$^+$ T cells was observed in patients with early progression of disease, compared with patients who had partial response and stable disease (Figure 5). These data demonstrate that PD-1 expression on peripheral CD4$^+$ T cells is associated with worse clinical outcomes in NSCLC, independent of age, stage, and histology type. In addition, the same analyses were performed for CD8$^+$ T cells and no correlation between PD-1 expression on peripheral CD8$^+$ T cells and clinical outcomes was observed (Figure 4C–4D, Figure 5B).

High expression of PD-1 on CD4$^+$ T cells at initial diagnosis associates with poor clinical outcome after anti-PD-L1 treatment

Six patient tumors were found to be PD-L1 positive by immunohistochemistry. Five of these patients were treated with a PD-L1 inhibitor while one patient received chemotherapy. We further dissected the association of PD-1 expression on CD4$^+$ T cells from peripheral blood to clinical outcome. Among the three patients who had low PD-1 expression at initial diagnosis, two had stable disease; one had disease progression at day 264. All three are alive at the last follow-up (day 645, 539, and 461 respectively). In contrast, all three patients who had high PD-1 expression had disease progression (at day 52, 43, and
Table 1: Characteristics of 42 advanced non-small cell lung cancer patients

|                           | CD4+ PD-1 % high | CD4+ PD-1 % low | P values | Statistical Test |
|---------------------------|------------------|-----------------|----------|-----------------|
| Age (years)               | Median 65        | Median 62       | 0.371    | Mann_Whitney U  |
| Gender                    |                  |                 |          |                 |
| Male                      | 5                | 14              | 0.337    | Fisher’s        |
| Female                    | 10               | 13              | 0.337    | Fisher’s        |
| Stage                     |                  |                 |          |                 |
| IIIB                      | 2                | 2               | 0.608    | Fisher’s        |
| IV                        | 13               | 25              | 0.608    | Fisher’s        |
| Histology                 |                  |                 |          |                 |
| Adenocarcinoma            | 13               | 20              | 0.451    | Fisher’s        |
| SCC                       | 2                | 7               | 0.451    | Fisher’s        |
| Smoking status            |                  |                 |          |                 |
| Smoker                    | 11               | 24              | 0.225    | Fisher’s        |
| Non-smoker                | 4                | 3               | 0.225    | Fisher’s        |
| Treatments                |                  |                 |          |                 |
| Chemotherapy              | 11               | 16              | 0.569    | Fisher’s        |
| Immunotherapy             | 2                | 3               | 0.569    | Fisher’s        |
| Targeted therapy          | 2                | 8               | 0.569    | Fisher’s        |
| **Total**                 | **15**           | **27**          |          |                 |

Figure 1: PD-1 expression in CD4+ T cells is higher in NSCLC patients than ones in normal donors. 1 million of PBMC from patients or normal donors were used for quantification of PD-1+% in CD3+CD4+ or CD3+CD8+ T cells by flow cytometry. Statistical significance was determined by a Student t test (*, p<0.05).
120 respectively), two of whom had died at day 268 and 466 (Table 2). Although our conclusion is limited due to the small sample size, we see a trend that high PD-1 expression on CD4⁺ T cells from peripheral blood associates with poor clinical outcome after treatment with anti-PD-L1 antibody, indicating a potential value of this marker in prediction of response to PD-1 pathway inhibitors.

**DISCUSSION**

In this study, we evaluated the phenotype of peripheral blood T cells collected from NSCLC patients and determined the correlation between PD-1 expression on T cell of peripheral blood with clinical outcome. The three most important findings are: 1) PD-1 expression on CD4⁺ T cells is significantly elevated in patients with NSCLC at initial diagnosis, compared with those of

![Figure 2: No Correlation between tumor burden and PD-1 expression in CD4⁺ or CD8⁺ T cells in 42 patients with advanced NSCLC. A least-squares linear fit is shown as a thick black line. Statistical significance was determined by a Pearson Correlation (p>0.05).](image)

![Figure 3: No correlation between PD-L1 expression in lung cancer tissues and PD-1 expression in CD4⁺ or CD8⁺ T cells from patients with advanced NSCLC. Statistical significance was determined by a Student t test (p>0.05).](image)
Figure 4: High expression of PD-1 on CD4+ T cells at initial diagnosis associated with poor clinical outcome in advanced NSCLC patients. Kaplan-Meier curves of 42 patients with advanced NSCLC segregated into 2 groups A, B, according to the median value of PD-1+ CD4+ cells (with 12.27% as a threshold). PD-1+ CD4+ cells >12.27% was defined as PD-1+ high while ≤12.27% as PD-1+ low. Likewise, these 42 patients were also segregated into 2 groups C, D, according to the median value of PD-1+ CD8+ cells (with 11.75% as a threshold). PD-1+ CD8+ cells >11.75% was defined as PD-1+ high while ≤11.75% as PD-1+ low. Statistical significance was determined by a Log-rank (Mantel-Cox) Test.

Figure 5: Elevated PD-1 expression on CD4+ T cells was correlated with disease progression. 1 million of PBMC from patients with progressed disease (PD) or stable disease (SD) or partial response (PR) were used for quantification of PD-1+ in CD3+CD4+ or CD3+CD8+ T cells by flow cytometry. Statistical significance was determined by a Student t test (*, p<0.05).
healthy donors. 2) High expression of PD-1 on CD4+ T cells is strongly associated with poor clinical outcome manifested by significant shorter OS and PFS in patient with NSCLC and this is prognostic. 3) PD-1 expression on CD4+ T cells has potential predictive value in NSCLC as high PD-1 expression associates with worse clinical outcome after anti-PD-L1 treatment.

Recent success at targeting immune inhibitory pathways has brought great deal of excitement in the field of cancer therapy. PD-1 is a key receptor expressed on T cells that can mediate immunosuppression by interacting with its ligands PD-L1 and PD-L2 on tumor or antigen presenting cells [21, 22]. Inhibiting PD-1 pathway can enhance anti-tumor activity of cytotoxic T cells. Two blocking antibodies to PD-1, nivolumab and pembrolizumab, have recently been approved by FDA for treating advanced solid tumors including NSCLC. Overall response rate of approximately 20% was achieved in patients with refractory NSCLC [23, 24]. It is important to determine the prognostic and predictive biomarkers that identify patients with high risk of disease progression and patients who will respond to PD-1 inhibitors.

Our finding of high expression of PD-1 on CD4+ T cells associates with poor clinical outcome has significant clinical implications as it provides a potential prognostic biomarker for patients with NSCLC. In our study, we used peripheral blood samples from patients with NSCLC to demonstrate a negative prognostic value of PD-1 in this population, i.e. high expression of PD-1 associates with poor PFS and OS. It is speculated that high-PD-1 expression in T cells results in immunosuppressed state and less functional for anti-tumor response leading to tumor progression and poor overall clinical outcome. Importantly, we bypassed the need for invasive tissue biopsy, which leads to treatment delays along with a potential risk of complications. Flow cytometry is a defined and well-quantitative method to determine the level of PD-1 expression on blood cells as PD-1 antibodies used in this test has been validated and widely used. Our study provided a highly feasible and effective clinical method to determine the prognosis of patients with NSCLC.

In our study, we analyzed PD-1 expression on T cells from peripheral blood of patients receiving anti-PD-L1 treatment. All patients were tested positive for PD-L1. We observed poor clinical outcome in patients with high PD-1 expression at initial diagnosis. This has a significant impact on data providing a potential predictive biomarker for NSCLC patients.

### Table 2: Characteristics of 6 advanced NSCLC patients with positive PDL-1

| Patient # | Patient #1 | Patient #2 | Patient #3 | Patient #4 | Patient #5 | Patient #6 |
|-----------|------------|------------|------------|------------|------------|------------|
| age       | 66         | 67         | 65         | 77         | 55         | 57         |
| gender    | female     | female     | female     | female     | female     | female     |
| histology | ACA        | ACA        | SCC        | ACA        | ACA        | ACA        |
| Smoking status | smoker | smoker | smoker | Non-smoker | Non-smoker | smoker     |
| PD-1% CD4+ | 6.12       | 12.18      | 9.99       | 25.89      | 15.73      | 25.53      |
| last follow-up | 645 days with | 539 days with | 264 days with | 43 days died | 120 days with | 52 days died |
|            | stable disease | stable disease | progressed disease | progressed disease | progressed disease | progressed disease |

Note: ACA is abbreviation for adenocarcinoma, SCC is abbreviation for squamous cell carcinoma.
receiving PD-1/PD-L1 targeted treatment. It should be noted that our data is limited by a small sample size.

In summary, our study demonstrates a prognostic value of PD-1 expression on CD4+ T cells from peripheral blood of patients with NSCLC. High PD-1 expression associates with poor clinical outcome. In addition, testing peripheral PD-1 expression has the potential to predict clinical response to treatment of blocking the PD-1/PD-L1 pathway.

MATERIALS AND METHODS

Patients

All 42 patients met the clinical radiological and histological criteria for advanced stage (stage IIIB or IV) NSCLC (Table 1). The patients’ age ranged from 47 to 82 years old (median 64 years old). There were 19 males and 23 females. Peripheral blood specimens from these patients were obtained, and informed consents were signed for sample collection according to a protocol approved by the Institutional Review Board of Penn State Hershey College of Medicine. Buffy coats from 25 age- and gender-matched normal donors were also obtained from the blood bank of Milton S. Hershey Medicine Center at the College of Medicine. PBMC were isolated by Ficoll-Hyphaque gradient separation as described previously [20]. Cell viability was determined by trypan blue exclusion assay with more than 95% viability in all the samples.

Immunofluorescence staining and flow cytometric analysis

One million PBMCs from patients or normal donors were used to quantify PD-1+% in CD3+CD4+ or CD3+CD8+ T cells by flow cytometry. The following antibodies were used for immunofluorescence staining: PerCP-Cy5.5 conjugated mouse anti-human CD3 (BD Pharmingen, Cat: 560835), FITC conjugated mouse anti-human CD4 (BioLegend, Cat: 317408), APC-Cy7 conjugated mouse anti-human CD8 (BioLegend, Cat: 344714) and PE conjugated mouse anti-human PD-1 (BioLegend, Cat: 329906).

Statistical analysis

Differences among two groups were statistically analyzed using a two-tailed Student's t test. The Kaplan–Meier survival curves were plotted to evaluate progression free survival (PFS) or overall survival (OS); log-rank (Mantel-Cox) test was used to analyze the statistic difference between the PD-1+% low vs. PD-1+% high patients. Correlation of PD-1 expressed T cells and tumor burden, which was measured following Recist 1.1 guideline, was assessed by Pearson Correlation analysis. A statistically significant difference was reported with p indicated where applicable. Data are reported as the mean ± SE from at least three separate experiments.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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None

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