Tumor-endogenous PD-1 promotes cell proliferation and predicts poor survival in non-small cell lung cancer

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Background: Immunotherapy has emerged as a promising treatment strategy in malignant tumors. Inhibitors and neutralizing antibodies targeted PD-1 or PD-L1 show improved clinical outcomes in advanced non-small cell lung cancers (NSCLCs). Previous studies concluded that PD-1 was mainly expressed on activated T cells, B cells and other immune cells. Recently, two studies suggested that PD-1 could be detected in the tumor cells of melanoma and hepatocellular carcinoma. Tumor-endogenous PD-1 in NSCLCs has not been reported at present. Thus, we intended to explore the expression and prognostic relevance of tumor-endogenous PD-1 in NSCLCs.

Methods: We detected the PD-1 expression in fresh tumor samples and tumor slices by flow cytometer and immunohistochemical analysis respectively. Overall survival (OS) and dis-ease-free survival (DFS) were analyzed with Kaplan-Meier survival curve. We explored the PD-1 expression in lung cancer cell lines and investigated its effect on cell proliferation.

Results: Flow cytometry showed that tumor cells with endogenous PD-1 constituted 2.08%, 2.42%, 1.79%, and 1.21% in 4 clinical NSCLC samples respectively. Thirty-four patients (34%) in the cohort were positive for tumor-endogenous PD-1 in IHC analysis. Patients with tumor-endogenous PD-1 had significantly worse 5-year overall survival (OS) and disease-free survival (DFS). Multivariate analysis identified tumor-endogenous PD-1 as an independent risk factor (HR =3.807, 95% CI: 2.031–7.135, P<0.05). PD-1 could be observed in 4 kinds of lung cancer cell lines (A549, H1975, H1299 and HCC827). PD-1 overexpression could enhance proliferation and clone formation of these cell lines.

Conclusions: PD-1 was endogenously expressed on the tumor cells of NSCLCs, and it could serve as an independent prognostic risk factor. The molecular mechanism of inferior survival of tumor-endogenous PD-1 may attribute to its role in promoting cell proliferation and clone formation. Our results demonstrated the non-immune role of PD-1 in tumor microenvironment and may provide new thinking for the therapy of NSCLCs.

Keywords: Lung cancer; tumor-endogenous PD-1; survival analysis; immunotherapy

Introduction

Lung cancers constitute the most common type of malignancy with the highest cancer-related mortality worldwide, among which non-small cell lung cancers (NSCLCs) take 84% (1). Surgical resection for early-stage lung cancer can improve the survival of patients (2). Unfortunately, most patients are diagnosed at an advanced stage without the opportunity for surgical treatment and have to receive comprehensive therapy, including chemotherapy and radiotherapy (3). Severe side effects of chemotherapy and radiotherapy extremely affect the life quality and survival time of patients. Immunotherapy has emerged as a promising treatment modality in most malignant tumors including advanced lung cancer (4). Immunotherapy generally can be divided into active and passive strategies. Active immunotherapy is to stimulate human immune system with immune checkpoint inhibitors (ICIs), cancer vaccines or cytokines and induce *de novo* cancer-specific immunity. Passive immunotherapy is to induce human immune response through directly transferring T cells. Although these strategies are based on different mechanisms, they both aim to activate tumor-specific cytotoxic T lymphocytes (CTLs) to eliminate tumor cells (5).

It is well-accepted that the immune system could inhibit or conversely promote tumor progression at different phases of tumorigenesis (6). Under normal circumstances, immune system identifies and eliminates tumor cells, the process called immune surveillance. However, mutagenesis induced by the stress of immune surveillance can provide increased cell-to-cell variations in tumor cells. The variations appear to create a condition for selected tumor cells escaping from the immune surveillance and finally lead to immune escape. Up-regulating immune checkpoint proteins is the common way (6). Therefore, ICIs targeting the crucial checkpoint protein programmed cell death protein 1 (PD-1) and its ligand (PD-L1) have shown improved clinical outcomes in NSCLCs. In 2015, Food and Drug Administration (FDA) approved the first immune checkpoint inhibitor nivolumab for advanced NSCLC that has progressed during or after treatment with platinum-based chemotherapy (7). Recently, FDA approved immune checkpoint inhibitors pembrolizumab and Atezolizumab for the first-line treatment of selected patients with stage III NSCLC based on the KEYNOTE-042 study (8).

PD-1, a type I transmembrane protein, was discovered in the screening of apoptosis-related genes, later proved to be a co-inhibitory immune checkpoint, negatively regulating immune response (9). Previous studies have demonstrated that PD-1 was mainly expressed on the activated T cells and B cells undergoing β-selection (10,11). In the process of tumor progression, some tumor cells up-regulate the expression of PD-L1. PD-L1 binding to PD-1 inhibits the activity of CTLs and the proliferation of regulatory T-cells (Treg). Therefore, PD-1/PD-L1 axis becomes the therapeutic target of ICIs (12-14).

Recent studies showed that the expression of PD-1 could be observed in tumor cells. Schatton *et al.*, reported that PD-1 was endogenously expressed in melanoma cells, and it could activate the downstream mTOR signal pathway to promote tumor progression through the PD-1/PD-L1 axis (15). Ye *et al.*, reported that liver cancer cells also express PD-1 endogenously, suggesting that the combination of PD-1 antibody and mTOR inhibitor can produce a more potent anti-tumor effect (16). These studies deepen the understanding of tumor immune relevance and provide new ICIs-related immunotherapy ideas. To our knowledge, the work about tumor cell-intrinsic PD-1 in NSCLCs has not been reported at present. Thus, we intended to explore the expression and prognostic relevance of tumor-endogenous PD-1 and its cellular effect through clinical samples and *in vitro* experiments. We present the following article in accordance with the REMARK reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-1644/rc).

Methods

Patients and tissue samples

This study's clinical samples were collected from a prospectively maintained data-base (West China Lung Cancer Database, WCLCD), including all the patients operated in the Department of Thoracic Surgery, West China Hospital. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Institutional Review Board of West China Hospital (approval number: 2016-98) and individual consent for this retrospective analysis was waived. We retrospectively collected paraffin specimens of 100 patients who underwent surgery in our department and pathologically diagnosed as NSCLC from July 2008 to June 2009. According to the 8th edition American Joint Committee on Cancer (AJCC) Classification, all cases were histologically evaluated. We collected the clinicopathologic data of these cases, including gender, age, smoke history, tumor size, tumor location, pathological type, pathological

TNM stage and adjuvant therapy. Follow-up data has been updated to January 2020. Of all these patients, 32 patients have received adjuvant chemotherapy or radiation after surgery. No patients have received immunotherapy. Additionally, we collected tumor samples from 4 patients who underwent surgery in our department and intraoperative frozen section diagnosed as adenocarcinoma in September 2018 for flow cytometry analysis.

Cell preparation and flow cytometry

We minced the collected tissue into 1–2 mm pieces using scissors, and then dispersed cells by gentle pipetting and filtered through a sterilized cell strainer for 2 times and collected the cell suspension. Centrifuge the suspension 300 g for 10 minutes at 4 ℃ and then we collected the cell pellet. We washed the pellet 3 times with cold PBS, and then resuspended with 100–200 μL volume of PBS. Finally, we added 1 μg CD45 monoclonal antibody (Innovative Research Cat# MHCD45065, RRID:AB_1475933), 1 μg PD-1 monoclonal antibody (Thermo Fisher Scientific Cat# 25-9985-82, RRID:AB_10853805) into the cell suspension, respectively. Their IgG antibodies were used for the negative control. All samples were incubated for 20 minutes at room temperature and analyzed with CytoFLEX Flow Cytometer (Beckman Coulter, Inc., Brea, CA, USA), protected from light.

Immunohistochemical (IHC)

We deparaffinized and rehydrated the tissue sections with 95%, 70% and 50% alcohols respectively for 3 minutes. We conducted antigen retrieval using 10 mM citrate buffer at 95 ℃ for 10 minutes. We then incubated and blocked the tissue sections with 3% H_2O_2 for 15 min and 10% fetal bovine serum (FBS) for 1 hour, respectively. We then incubated the tissue sections with anti-PD-1 antibody (1:200; R and D Systems Cat# AF1086, RRID:AB_354588) at 4 ℃ overnight and anti-rabbit secondary antibody (1:1,000, ZSGB-Bio Cat# PV-6001, RRID:AB_2864333) at room temperature for 30 minutes, respectively. After that, we stained the sections with DAB substrate solution and substrate-chromogen solution for 2 minutes, and hematoxylin for 1 minute, respectively. Two researchers (Chuanfen Zhang and Fanyi Gan) assessed the IHC results in a blinded way under microscopy. According to the expression of tumor-endogenous PD-1, we classified the immunostained results into positivity and negativity. Positivity was defined as tumor-endogenous PD-1 was observed in any one field by any one of the researchers during analysis. Negativity was defined as no tumor-endogenous PD-1 expression could be observed in all fields by two researchers.

Western Blot

We lysed the cultured tumor cells using RIPA lysis and extraction buffer (Solarbio, Beijing, China) with PMSF (Solarbio, Beijing, China). We measured the cell lysis buffer with the BCA protein assay kit (Solarbio, Beijing, China) and separated 50 μg protein samples in the 10% SDS-polyacrylamide gels. We then transferred the proteins onto polyvinylidene fluoride (PVDF) membrane (wet transfer, 90 minutes, 100 V). We then blocked the membrane with 5% non-fat milk at room temperature for 45 minutes. Anti-PD-1 Polyclonal IgG (1:1,000; R and D Systems Cat# AF1086, RRID:AB_354588) was used as primary antibody, and incubated at 4 ℃ overnight. An Anti-Goat IgG (1:10,000; LI-COR Biosciences Cat# 926-32214, RRID:AB_621846) was used as the secondary antibody, and incubated at room temperature for 30 minutes protected from light. Finally, we visualized the fluorescent blots with Odyssey Imager (Li-COR, NE, USA). We incubated the membrane with an anti-β-actin (1:1,000, ZSGB-Bio Cat# TA-09, RRID:AB_2636897) monoclonal antibody as the loading control.

Establishment of PD-1 overexpression cell lines

We purchased Pdcd1 overexpression lentivirus from Genechem Company, Ltd. (Shanghai, China). $2×10^5$ cells were seeded in a 6-well plate for 24 hours. Change medium and add 1 μL Pdcd1 lentivirus, and add the vector for negative control. The medium containing lentivirus was changed after 24 hours. Add puromycin (1.0 μg/mL, ThermoFisher, MA, USA, #A1113803) to select cells for about 10–14 days. Change the medium containing puromycin every 3 days and passage the cells when they reached confluency. Finally, HCC827[PD-1-OE] and H1975[PD-1-OE] stable cell lines were established.

Cell viability assay

Cells were seeded at $5×10^3$ cells per well in 96-well plates with complete medium, incubated overnight in a humidified incubator. Added 100 μL complete medium including 10 μL

of CCK-8 reagent (#HY-K0301, MedChemExpress, NJ, USA) into each well and incubated for 1 hour. The optical density (OD) value of 450 nm was analyzed on a Biotek ELx800 plate reader (BioTek Instruments, Inc., VT, USA). The results of cell viability were expressed as Relative Growth Rate [1].

$$Relative\ Growth\ Rate = (OD_t - OD_0)/OD_0 \qquad [1]$$

OD_0 is defined as the OD value 8 hours after seeded and stained with CCK-8 reagent.

OD_t is defined as the OD value 24, 48, 72, 96 hours after the first analysis with CCK-8 reagent.

Plate clone formation assays

Cells were plated $(0.5–1) \times 10^3$ cells per well in six-well plates with medium containing 10% FBS. Incubate the cells in the incubator for 2–3 weeks, and add medium containing 10% FBS every 3–4 days. Wash the cells with PBS for 2 times and fix them with 4% paraformaldehyde for 20 minutes. Then, the cells were incubated with 0.1% crystal violet at room temperature for 20 minutes. Finally, discard the crystal violet and wash the cells with PBS 3 times. Count the colonies containing >50–100 cells for analysis.

Scratch assays

Cells were plated in six-well plates with medium containing 10% FBS. Incubation for 3–4 days until the cells reached about 100% confluency. Then, remove the medium and scratches were made in the wells using the sterilized pipette tips. Wash 3 times with PBS until no obvious floating cells or cell fragments could be observed under the microscope. Add medium without FBS. Images were taken for the same labeling sites after 24 and 48 hours with a Nikon Internalight C-HGF1 fluorescence source (Nikon, Mellville, NY, USA).

Statistical analysis

SPSS 23.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Inc., La Jolla, CA, USA) were used for statistics. The relationship between tumor-endogenous PD-1 and clinicopathological variables was analyzed by the Pearson chi-square test. Survival was analyzed with Kaplan-Meier survival curve. Univariate and multivariate analyses were performed with the Cox model. The hazard ratio (HR) and a confidence interval of 95% were applied in this analysis. A two-sided P<0.05 was considered statistically significant.

Results

We prepared tumor cell suspensions from 4 patients, operated in our center and intraoperative frozen section diagnosed as adenocarcinoma. Flow cytometry analysis was performed with PD-1 and CD45. The results showed that CD45 (–)/PD-1 (+) cells constitutes 2.08%, 2.42%, 1.79%, 1.21%, respectively. CD45 (–)/PD-1 (+) cells were regarded as the tumor cells endogenously expressing PD-1 and CD45 (+)/PD-1 (+) cells were regarded as the lymphocytes expressing PD-1 (*Figure 1A,1B*). Then, to investigate the relationship between tumor-endogenous PD-1 and prognosis, we detected the expression of PD-1 by IHC in clinical samples from the WCLCD. The 100 patients of this cohort were all operated between July 2008 and June 2009 in our center and pathologically diagnosed as NSCLC. Of these patients, 34 patients (34%) were positive and 66 (66%) were negative for tumor cell-intrinsic PD-1 (*Figure 1C*). Immunohistochemical results showed TILs and a small fraction of tumor cells could be positive for PD-1 (*Figure 1D*). However, the obvious morphological differences between TILs and tumor cells make it easy to clarify the tumor cell-intrinsic PD-1 in the IHC. Correlation of tumor cell-intrinsic PD-1 expression and the clinicopathological characteristics of patients were summarized in *Table 1*. It showed that the expression of PD-1 was positively correlated with lymph nodes metastasis (OR =3.416, P=0.010), but not with gender, age, smoking history, tumor location and size, pathological type, TNM stage (I *vs.* II-IV) and adjuvant therapy. The results of PD-1 expression classified by the pathological N stage were showed in *Figure 1E*.

To explore the prognostic factor for patients with tumor cell-intrinsic PD-1, we collected the survival data from WCLCD and performed univariate and multivariate analysis (*Table 2*). No patients of the cohort have received immunotherapy ever. Patients with tumor-endogenous PD-1 had significantly worse five-year overall survival (OS) (*Figure 1F*) and disease-free survival (DFS) (*Figure 1G*). Multivariate Cox's regression analysis showed that tumor cell-intrinsic PD-1 expression was an independent prognostic risk factor (HR =3.807, 95% CI: 2.031–7.135, P<0.05*). These results together demonstrated that tumor cell-intrinsic PD-1 could be observed in lung cancer, and it was an independent prognostic risk factor.

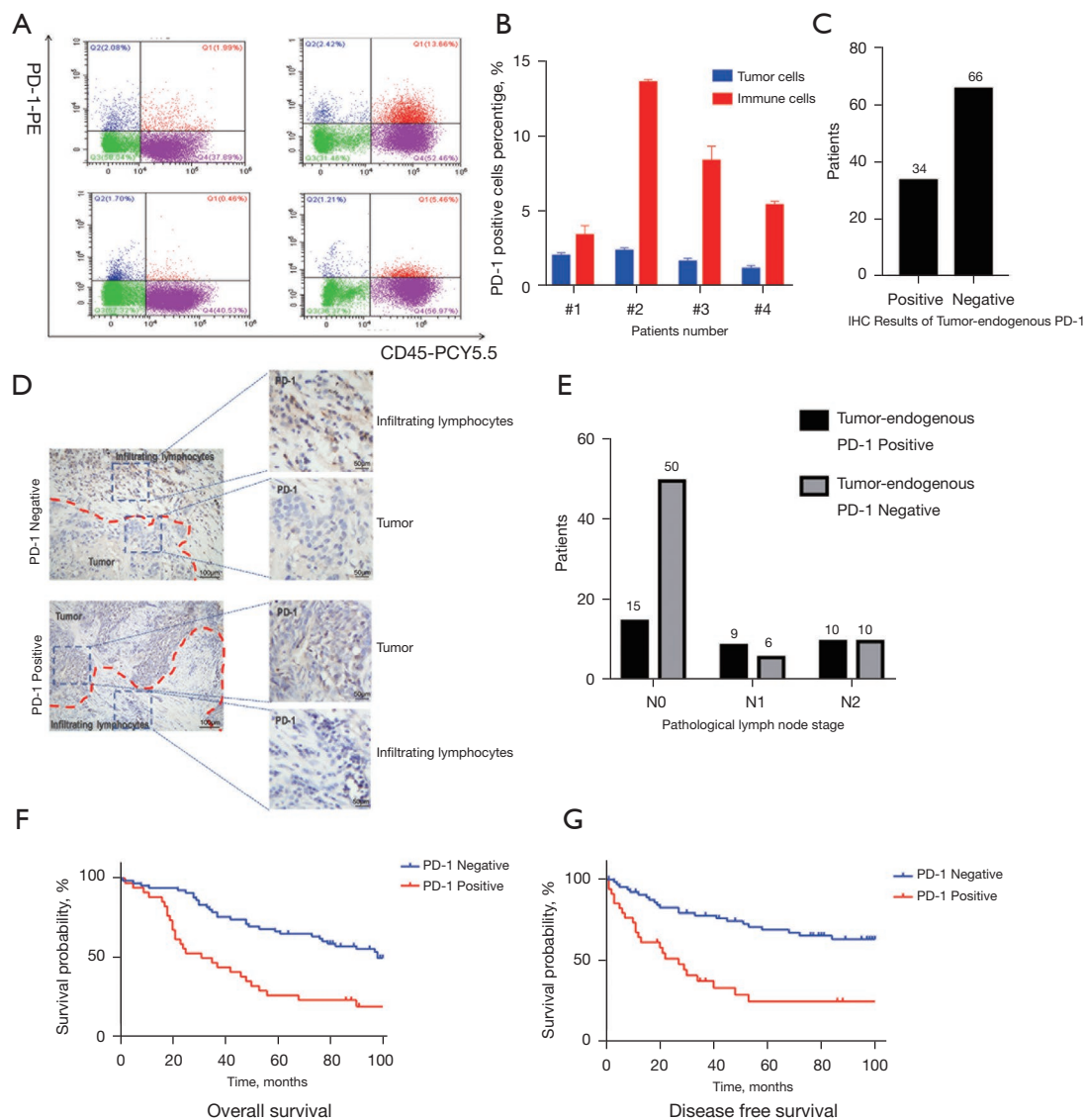To explore the expression of PD-1 in lung cancer

Figure 1 Expression of tumor-endogenous PD-1 in clinical samples. (A) PD-1 expression in fresh lung cancer tissue by flow cytometry; (B) statistical result of PD-1 expression in fresh lung cancer tissue by flow cytometry; (C) tumor-endogenous PD-1 expression in a cohort of 100 patients; (D) PD-1 expression in tumor cells and infiltrating lymphocytes in IHC; (E) tumor-endogenous PD-1 expression classified by pathological N stage; (F,G) Kaplan-Meier curves for OS and DFS of patients with tumor-endogenous PD-1. PD-1, programmed cell death protein 1; IHC, immunohistochemistry; OS, overall survival; DFS, disease-free survival.

cell lines and clarify the molecular mechanisms between PD-1 expression and prognosis, *in vitro* experiments were conducted. PD-1 expression was analyzed in 4 common lung cancer cell lines (A549, H1975, H1299 and HCC827) by western blot and flow cytometry. It showed that all of the above cell lines could be observed PD-1 expression in WB, and the PD-1 expression of H1975 and HCC827 was relatively higher than in A549 and H1299 (*Figure 2A*).

PD-1 was also detected in 4 cell lines in FCM analysis and the statistical results were showed in *Figure 2B-2F*. To sum up, the tumor-endogenous PD-1 was expressed in a small fraction of lung cancer cells, relatively lower than that in melanoma or liver cancer cells. Together with clinical samples results, we concluded that tumor cell-intrinsic PD-1 was expressed in lung cancer, and it played a crucial role in lung cancer recurrence and metastasis, which was an

Table 1 Correlation between expression of tumor-endogenous PD-1 and clinicopathological features of lung cancer

Clinicopathological variables	n	PD-1 expression		χ^2 value	P value
		Negative	Positive		
All patients	100	66	34		
Gender				0.688	0.407
Male	70	48	22		
Female	30	18	12		
Age (year)				0.186	0.666
≤60	47	30	17		
>60	53	36	17		
Smoke				0.477	0.490
No	43	30	13		
Yes	57	36	21		
Tumor location				2.350	0.125
Left	66	47	19		
Right	34	19	15		
Tumor size				0.038	0.845
≤3.0 cm	34	22	12		
>3.0 cm	66	44	22		
Pathological type				0.160	0.689
Adenocarcinoma	62	40	22		
Squamous carcinoma	38	26	12		
Lymph node metastasis				6.629	0.010
Negative	38	31	7		
Positive	62	35	27		
TNM stage				2.907	0.088
I	38	29	9		
II-IV	62	37	25		
Adjuvant chemotherapy or radiation	32	22	10	0.159	0.691

independent prognostic risk factor.

Next, the overexpression of PD-1 was produced in HCC827 and H1975 cells by lentivirus infection (*Figure 2G-2I*). We investigated the role of PD-1 in tumor proliferation, colony formation and migration. In the CCK-8 proliferation assays, HCC827PD-1-OE and H1975 PD-1-OE exhibited a more vital proliferative ability than control (*Figure 3A,3B*). Correspondingly, in the culture plate cloning assay, cell-intrinsic PD-1 promoted colony formation of HCC827 cells (P=0.0104) (*Figure 3C*). However, the difference between PD-1 overexpression and control was not statistically significant in the scratch assays, with the P value was 0.1182 and 0.2946 in HCC827 and H1975 group respectively (*Figure 3D*).

Discussion

PD-1/PD-L1 axis and its related signaling pathways have

Table 2 Univariate and multivariate Cox proportional hazards analysis of prognostic factors for patients with lung cancers

Variables	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value
PD-1 expression	3.902	2.135–7.134	0.000*	3.807	2.031–7.135	0.000*
Positive *vs.* negative						
Gender	1.061	0.562–2.002	0.855			
Male *vs.* female						
Age (years)	0.905	0.500–1.639	0.742			
>60 *vs.* ≤60						
Smoke	0.81	0.448–1.463	0.485			
Yes *vs.* no						
Tumor location	1.419	0.772–2.607	0.259			
Right *vs.* left						
Tumor size (cm)	0.828	0.451–1.519	0.541	0.608		
≥3 *vs.* <3						
Lymph node metastasis	2.563	1.292–5.084	0.007*	1.166	0.216–6.306	0.858
Positive *vs.* negative						
TNM stage	2.325	1.194–4.525	0.013*	2.409	0.445–13.059	0.308
II-IV *vs.* I						

*, $P<0.05$; CI, confidence interval.

become a focus of cancer immunotherapy research over the last decade (17). PD-1 had been considered mainly expressed on the activated T cells and B cells until two studies suggested that PD-1 could be detected in melanoma and liver cancer cells, respectively (15,16). However, whether PD-1 is endogenously expressed in tumor cells of lung cancer has not been reported.

Our results showed that tumor cell-intrinsic PD-1 could be observed in clinical samples and cell lines of NSCLCs. *In vitro*, it is interesting to note that the expression of PD-1 in H1975 and HCC827 cell lines was stronger than in A549 and H1299 at the protein level. Consistently, it has been reported that the expression level of PD-L1 in H1975 and HCC827 cell lines is also relatively high (18). More than that, PD-1 functions inevitably by interacting with PD-L1. Therefore, we are prone to speculate that the PD-L1 of tumor cells may induce the expression of tumor-derived PD-1 in lung cancer. The expression level of endogenous PD-1 in lung cancer cells in this study is lower than in melanoma cells (15), which could be attributed to the unique immunologic context of different malignancies (19).

Studies have classified that tumors may be categorized based on immunologic indicators, such as immunogenicity, tumor mutation load (TMB) and inflammation-related gene expression. Next, researchers integrated these immunologic indicators and developed the "immune score" system in colorectal cancer, and found that the prediction of treatment responsiveness and prognosis was better than traditional TNM staging (20). Lung cancer and melanoma both belong to the high immunogenic tumors with high TMB and expression of inflammation-related genes. Therefore, tumor cell-intrinsic PD-1 being observed in these high immunogenic tumors could be understood. Portraying the immunologic context of different tumors or different individuals with the same tumor is critical to predicting immunotherapy's efficacy. Tumor-endogenous PD-1 may become a promising evaluation index of tumor immunologic context in the future.

In our results, the tumor-endogenous PD-1 could be observed in 34% of the included patients, and no significant correlation was observed between PD-1 expression and gender, age, smoking history, tumor location, tumor size,
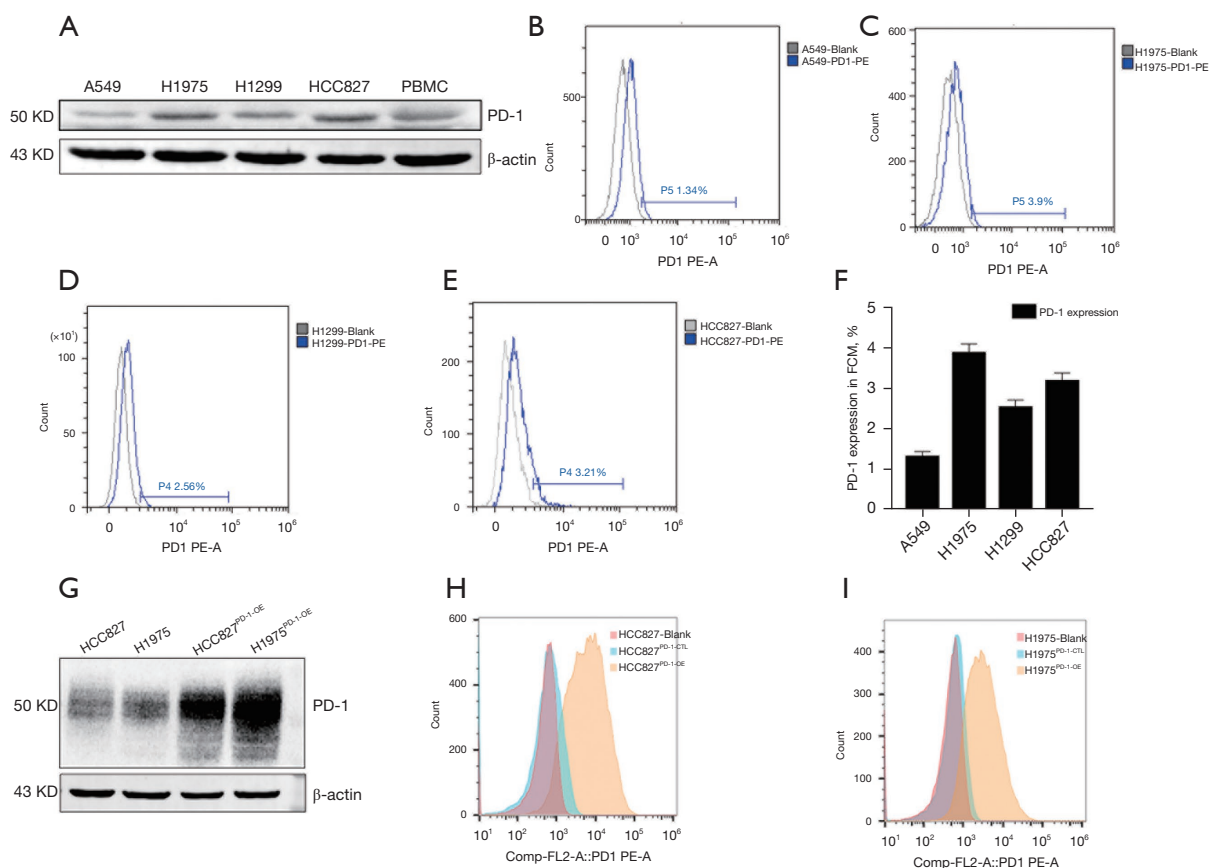
Figure 2 Expression of tumor-endogenous PD-1 in lung cancer cell lines. (A) PD-1 expression in 4 lung cancer cell lines and positive control by western blot. (B-E) PD-1 expression in A549, H1975, H1299, HCC827 cell lines by flow cytometry; (F) statistical result of PD-1 expression in 4 lung cancer cell lines by flow cytometry; (G) PD-1 expression of PD-1 overexpression cell lines of HCC827 and H1975 by western blot; (H,I) PD-1 expression of PD-1 overexpression cell lines of HCC827 and H1975 by flow cytometry. PD-1, programmed cell death protein 1.

pathological type, and TNM staging (I $vs.$ II-IV). However, in the TNM staging (I $vs.$ II-IV), a higher positive rate was observed in stage II-IV than stage I. Due to the limited sample size, no statistically significant difference was observed between Stage I and II-IV. Thus, we need to expand the sample size in a future study. PD-1 expression was correlated with lymph node metastasis (OR =3.416, P=0.010), suggesting that tumor-endogenous PD-1 may be involved in lung cancer progression or metastasis. Consistently, in the Kaplan-Meier survival analysis, patients with tumor-endogenous PD-1 expression showed inferior PFS and OS. Tumor-endogenous PD-1 was an independent prognostic risk factor. For clinicians, these outcomes may indicate that regular postoperative follow-up is critical for these patients with tumor-endogenous PD-1 expression,

who have a greater chance of recurrences and metastasis.

IHC results showed that tumor-endogenous PD-1 expression was mainly observed on the cell membrane, but some could be found in the cytoplasm. Studies reported that the PD-1 on the T cells is glycosylated (21). Glycosylation constitutes a common post-translational modification, including N-glycosylation and O-glycosylation, and plays a vital role in tumor proliferation, invasion and metastasis, and angiogenesis (22). Accordingly, we speculate that there are two forms of PD-1 protein in the tumor cells, membrane protein and cytosolic protein, and the PD-1 on the cell membrane has glycosylation modification. After translation, glycosylation modification of PD-1 is required for correctly locating on the cell membrane and exercise biological function. However, the unglycosylated PD-1
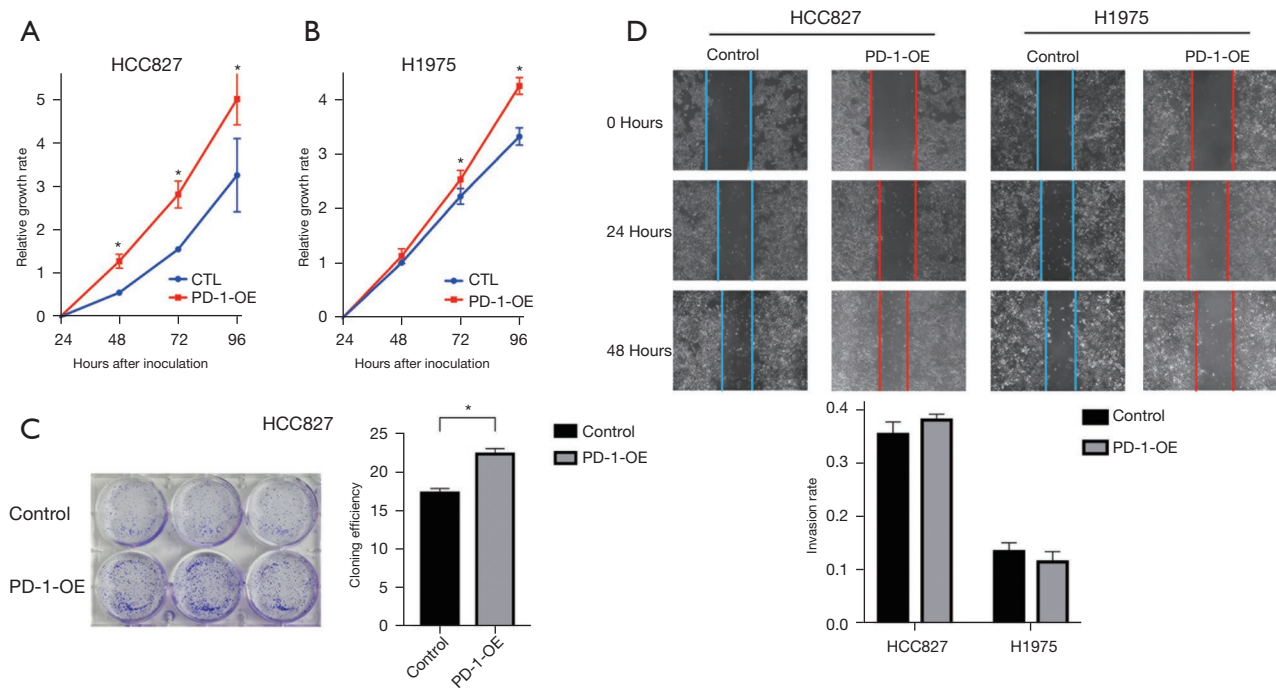
Figure 3 The effect of cell-intrinsic PD-1 on the cell proliferation, clone formation and migration of lung cancer cells. (A,B) Results of CCK-8 assays in HCC827 and H1975; (C) results of plate clone formation assays in HCC827; (D) results of scratch assays in HCC827 and H1975. PD-1, programmed cell death protein 1; CCK-8, cell counting kit-8. *, $P<0.05$.

still stays in the cytoplasm. Glycosylation modification of tumor-endogenous PD-1 is in our subsequent experiments.

The traditional view assumed that the PD-1/PD-L1 axis inhibited the immune system and induced immune escape. Moreover, blockade of the interaction between PD-1 and PD-L1 could restore or strengthen T-cell activity against tumor cells, thereby preventing cancer progressing and metastasis (12-14). Anti-PD-1 and anti-PD-L1 agents show promising efficacy in NSCLC, especially for those with high PD-L1 expression. Studies on melanoma and hepatocellular carcinoma extended our understanding of PD-1 in the tumor microenvironment and the molecular mechanisms underlying anti-PD-1 therapy (15,16). Consistently, our observations demonstrate that PD-1 was endogenously expressed on the lung cancer cells, expanding the traditional perception of PD-1 in lung cancer.

Some limitations also existed in this study. First, the number of included patients and samples was limited. We concluded only in a non-representative Chinese population of lung cancer patients and four lung cancer cell lines *in vitro*. Second, PD-1 was also expressed on immune cells in the tumor microenvironment, and we identified tumor-intrinsic PD-1 based on cell morphology under

a microscope. Third, WB, FCM and IHC were used to verify tumor-intrinsic PD-1 at the protein level without any experiments at the mRNA level. However, PD-1 functions at the protein level, so we think studies on the protein level prioritize. Moreover, no *in vivo* experiments verifying the role of tumor-endogenous PD-1 was carried out. Finally, a more in-depth molecular mechanism of tumor-endogenous PD-1 in lung cancer is out of this study's scope and needs further research.

Although the limitations mentioned above, our research demonstrated for the first time that PD-1 is endogenously expressed on lung cancer cells, and tumor-endogenous PD-1 could serve as an independent prognostic risk factor. Moreover, *in vitro* experiments showed that PD-1 could enhance the proliferation and clone formation of lung cancer cells. Experiments on glycosylation modification of tumor-endogenous PD-1, related downstream signaling pathways and potential therapeutic targets are needed in the future.

Conclusions

This study confirmed for the first time that PD-1 was

endogenously expressed on the cancer cell of NSCLCs. Expression of tumor-endogenous PD-1 was statistically related to lymph node metastasis, and it was an independent prognostic risk factor for patients. *In vitro* experiments demonstrated that PD-1 could enhance the proliferation and clone formation of lung cancer cell lines. These results explored the role of PD-1 in tumor immune microenvironment and may provide new thinking for immunotherapy in NSCLCs.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-1644/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-1644/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-1644/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Institutional Review Board of West China Hospital (approval number: 2016-98) and individual consent for this retrospective analysis was waived.

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