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circ_0001006 Promotes Immune Escape in Non-small Cell Lung Cancer by Regulating the miR-320a/PD-L1 Axis

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ABSTRACT

Background: Circular RNAs are involved in the tumorigenesis of various tumors, including Non-small cell lung cancer (NSCLC).

Objective: To investigate the expression of circ_0001006 in patients with NSCLC and its role in tumorigenesis and immune escape.

Methods: A total of 115 patients with NSCLC were enrolled in the study. The expression of circ_0001006 and PD-L1 mRNA were detected using RT-qPCR. Cell proliferation activity, cell migration and invasion abilities were measured using the CCK-8 assay and Transwell chambers assay. Coculture of NSCLC cells with CD8 cytotoxic T cells was conducted to measure the levels of INF-γ, TNF-α, IL-2, and lactate dehydrogenase release in culture supernatants. Bioinformatic analysis was used to predict the target relevance among circ_0001006, miR-320a, and PD-L1.

Results: The circ_0001006 and PD-L1 mRNA levels were elevated in NSCLC tissues and cells. Patients with high levels of circ_0001006 had a shorter overall survival rate. Inhibiting circ_0001006 reduced the proliferation, migration, and invasion of NSCLC cells, while increasing PD-L1 partially counteracting the inhibitory effects of si-circ_0001006. The co-culture system of NSCLC and CD8+ T cell was found to reduce the viability of activated CD8+ T cell when circ_0001006 is present. Knocking down circ_0001006 in co-culture cells led to an increase in the expression of INF-γ, TNF-α, and IL-2. The ability of si-circ_0001006 to enhance the activation of CD8+ T cells was diminished when PD-L1 was overexpressed.

Conclusion: circ_0001006 may serve as a potential prognostic predictor and therapeutic target for NSCLC. Additionally, it offers insight into a novel regulatory mechanism of circ_0001006.

Keywords: circ_0001006, miR-320a, NSCLC, PD-L1, Progression

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INTRODUCTION

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, and poses a serious thread to people' health of (1). Currently, the the main treatment options for NSCLC include surgery, radio/chemotherapy, targeted therapy, and immunotherapy (2-4). However, most patients with NSCLC diagnosed with metastasis at diagnosis and have a high rate of recurrence after treatment, resulting in a poor prognosis (5). Currently, despite significant advancement in the diagnosis and treatment of NSCLC , the overall survival rate remains low, and the risk of recurrence is heightened by metastasis (6). This current situation has promoted further exploration into te pathogenesisof NSCLC and the search for innovative interventions, such as targeted therapy and immunotherapy.

The onset and progression of malignant tumors involve an immune response (7). Immune escape is an important charachteristic of malignant tumor cells, and the development of an immunosuppressive tumor microenvironment (TME) necessitate tumor cells to sustain high transcriptional activity. This leads to the overexpression of certain immunosuppressive molecules, such as PD-L1 (also known as CD274) (8). Immunotherapy drug approvals are used in clinical settings for treating cancers (9). Circular RNAs (circRNAs) have been confirmed to regulate PD-L1 expression and participate in immunotherapy (10), such as circBRT2.2 (11), circ-VIM (12), and circ_0000190 (13) in different cancers. Has_circ_0001006 was found to be aberrantly expressed in gastric cancer (14), breast cancer with brain metastasis (15), and cardiac hypertrophy disease (16). For instance, circ_0001006 was upregulated in breast cancer with brain metastasis and had the potential to be a biomarker (15). Studies of circ_0001006, particularly in NSCLC, are limited, and its association with immunotherapy remains unclear.

Here, we assessed the potential function of circ_0001006 in NSCLC and evaluated its interaction with the immune checkpoint PD-L1. Firstly, we measured the levels of circ_0001006 and PD-L1 mRNA in tissues from patients with NSCLC. Then, we explored its functional influence and effects on the immune system in NSCLC cells. This preliminary study evaluated the effects of circ_0001006 on antitumor immunity in patients with NSCLC, aiming to provide a potential target for NSCLC immunotherapy.

MATERIAL AND METHODS

Tissue Specimens

Tissue samples (cancer tissues and adjacent normal tissues) from 115 patients with NSCLC admitted to Zhongguancun Hospital between June 2017 and March 2020 were collected. Among the NSCLC patients (mean age 49.82±10.14 years), there were 47 females and 68 males. The patients were diagnosed with NSCLC by pathologists. Non of the participants had received anti-tumor treatments prior to tissue collection. Patients with other malignancies, systemic infections, or contraindications to surgery, as well as patients with incomplete clinical data were excluded. The patients were followed up after surgery. These experiments were conducted with approval from the Ethics Committee of Zhongguancun Hospital, and all participants or their families signed the informed consent form.

Cell Lines Culture and Treatment

Human NSCLC cells (H1650, A549, H1299, and HCC827) and lung epithelial BEAS-2B cells were purchased from Procell company (Wuhan, China). The cells were cultured at 37°C in RPMI 1640 medium (Invitrogen) with 10% FBS (Gibco, Life Technologies). They adapted well to this medium and condition over time and grew well.

CD8+ T cells were isolated from the whole blood of healthy donors using the EasySep Human CD8+ T cell Enrichment

kit (Stemcell). The cells were then cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) before being used in further experiments.

Small molecule interfering RNA (siRNA) targeting circ_0001006 (circ_0001006 siRNA/ si-circRNA), miR-320a mimic, inhibitor, pcDNA3.1 (oe-NC), pcDNA3.1-PD-L1 (oe-PD-L1), and respective negative controls (si-NC, miR-NC, inhibitor-NC) were synthesized by Ribobio (Guangzhou, China). Transfection was performed using Lipofectamine 2000 transfection reagent.

RT-qPCR

Total RNA was extracted from tissue specimens and cells using a TRIzol kit and then reverse-transcribed into cDNA. Subsequently, the SYBR Green Super Mix kit was used for PCR amplification. GAPDH served as the internal reference for circ_0001006 and PD-L1 mRNA, while U6 was the internal reference for miR-320a. The fold change in relative expression is calculated using the $2^{-\Delta\Delta Ct}$ method.

CCK-8 Method

The CCK-8 method was used to detect cell proliferative activity (17). After a 48 hour transfection, approximately 5000 NSCLC cells/well were seeded in 96-well plates. At the various incubation times (0, 24, 48, 72 h), the CCK-8 kit (Beyotime, Shanghai, China) was added to each well and the cell culture continued for 1 h. The optical density (OD) value of each well at 450 nm was read using an iMark microplate reader (Bio-Rad).

Migration and Invasion Assay

The cell suspension (2×10^4 cells) in serum-free medium is added to the top Transwell chamber (Corning, USA) and the medium (RPMI 1640+10% FBS) is placed in the lower chamber. The cells that migrate to the submembrane surface are stained and then counted under an inverted microscope after 24 h of incubation. In the invasion assay, cells are seeded in a Transwell chamber with Matrigel and the other steps are the same as in the migration assay (18).

Cell Viability Assay

The Transwell chamber (3 μm pore size; Corning) was utilized for the co-culture of CD8+ T cells and transfected A549 cells. Inthe control group CD8+ T cells were cultured separately, while the remaining corresponding transfected NSCLC cells were placed in the upper chamber and CD8+ T cells in the lower chamber. After 48 hours of co-culture , cells were stained with 0.4% trypan blue solution for 20 min, following which live and dead (blue) cells were counted using a light microscope. The cell viability was calculated as follows: cell viability (%)=(total cells - dead cells) / total cells×100%.

LDH Cytotoxicity Experiment

The lactate dehydrogenase (LDH) assay was used to assess cytotoxicity (19).The LDH Cytotoxicity Assay Kit (Beyotime) was used to determine cytotoxicity in lung cancer cells, using A549 cells as target cells and CD8 cytotoxic T cells as effector cells. Transfected A549 cells were co-cultured with activated CD8+ T cells at various effector:target ratios for 24 h. After centrifugation, cell culture supernatant was collected for LDH cytotoxicity assays. CD8+ T cell cytotoxicity was determined by measuring the OD value at 490 nm using a microplate reader. The cytotoxicity in cells (%)=(LDH release in mixture of target and effector cells – LDH release in effector cells)/LDH release in target cells ×100.

Cytokine Secretion Assay

After 48 h of co-culture, the culture medium was collected and the supernatant was centrifuged at 1000 g at 4°C for 10 min. The levels of human soluble PD-L1 (sPD-L1), IFN-γ, TNF-α, and IL-2 were measured in the supernatant using ELISA kits (Enzyme-linked Biotechnology Co., Ltd, Shanghai, China)

Dual-luciferase Reporter Assay

The binding sites between miR-320a and circ_0001006, as well asPD-L1 were predicted using circBank and miRDB databases. Partial sequences of circ_0001006 and PD-L1 mRNA, which contained binding sites with miR-320a, were cloned into pmirGLO vectors and named circRNA-WT and PD-L1-WT. Mutant sequences of circ_0001006 and PD-L1 mRNA were generated using site-directed mutagenesis to construct circRNA-MUT and PD-L1-MUT. The luciferase recombinant plasmids mentioned above were then co-transfected with miR-320a mimic or mimic NC into A549 cells. After 48 h of transfection, luciferase activity was detected.

Statistical Analysis

GraphPad Prism 9.0 and SPSS 26.0 software were used for data comparison (mean±SD). Pearson analysis was used to analyze the interaction between circ_0001006 and PD-L1. The difference between groups was compared by conducting a Student's t-test or one-way ANOVA. A *P*-value less than 0.05 was considered to be statistically significant.

RESULTS

circ_0001006 and PD-L1 mRNA Expression Was Measured in NSCLC

circ_0001006 levels were found to be elevated in NSCLC tissues compared to adjacent normal tissue samples (Fig. 1A). PD-L1 mRNA levels were also increased in tumor tissues (Fig. 1B). Pearson correlation analysis revealed a positive correlation between circ_0001006 expression and PD-L1 mRNA levels in NSCLC tissues (Fig. 1C). Additionally, the circ_0001006 levels in NSCLC cell lines were higher than in normal BEAS-2B cells, consistent with findings in tissue specimens (Fig. 1D).

Clinical Significance of circ_0001006 in NSCLC Patients

Based on the average level of circ_0001006 in tumor tissues, patients were divided into low-

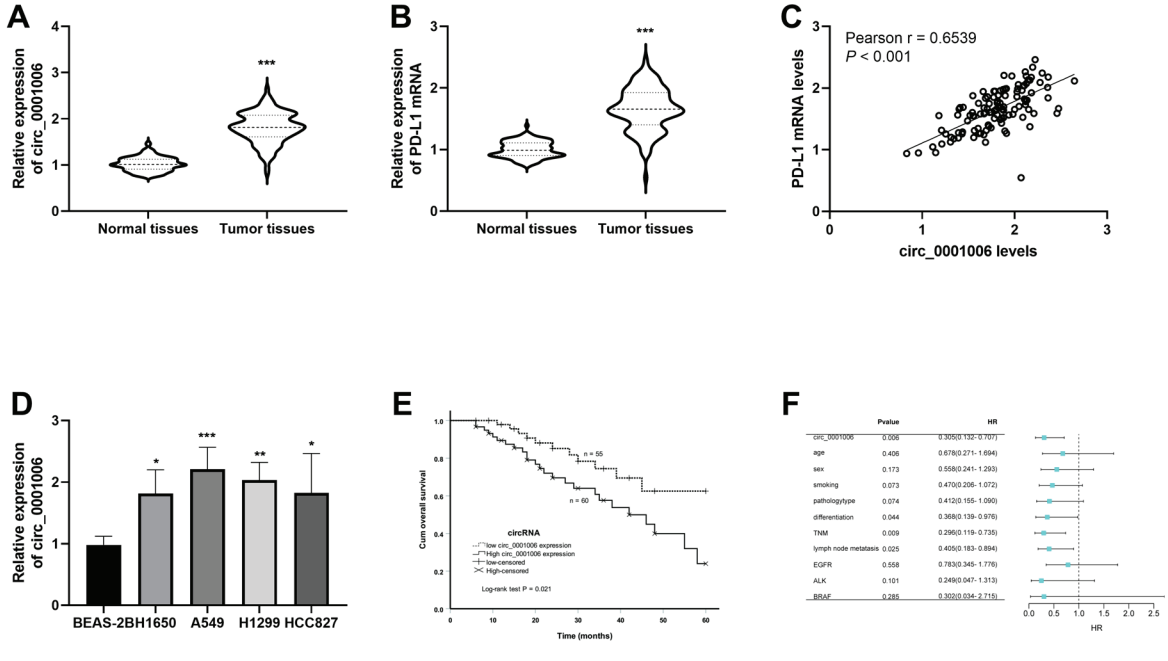

Fig. 1. circ_0001006 expression in NSCLC and its prognostic significance. A. circ_0001006 exhibited higher levels in NSCLC tissues compared to normal tissues. B. High expression levels of PD-L1 mRNA were observed in NSCLC tissues. C. A positive correlation between circ_0001006 and PD-L1 mRNA was observed in NSCLC tissues. D. circ_0001006 expression was elevated in NSCLC cells compared to normal lung BEAS-2B cells. E. The Kaplan-Meier curve indicated that high circ_0001006 expression was associated with shorter overall survival time. Log-rank test P=0.021. F. The forest plot demonstrated that circ_0001006 expression was a risk factor for survival outcome. *P<0.05, **P<0.01, ***P<0.001.

and high-expression groups. High circ_0001006 expression in tumor tissues was associated with differentiation, high TNM stage, and positive lymph node metastasis (P<0.05, Table 1).

Furthermore, the Kaplan-Meier curve showed that low circ_0001006 expression was linked to longer overall survival time (Fig. 1E). The forest plot from COX analysis indicated that circ_0001006 expression was a risk factor for survival outcome (Fig. 1F).

The Influence of circ_0001006/PD-L1 on NSCLC Cellular Behaviors

To explore the correlation between circ_0001006 and PD-L1 functions in NSCLC, transfection was performed in NSCLC cells. The transfection efficiencies are shown in Figs. 2A and 2B, as well as supplementary Fig. 1. si-circ_0001006 weakened the cell proliferative abilities of A549 and H1299 cells, while increased PD-L1 partially diminished the inhibitory role of si-circ_0001006 on proliferation (Fig. 2C). In addition, silencing of circ_0001006 weakened the cell mobility (migration and invasion) of NSCLC cells compared to the untreated cells, whereas increased expression of PD-L1 mRNA eliminated the inhibiting impacts of si-circ_0001006 (Figs. 2D and 2E).

Effects of circ_0001006 Knockdown on CD8+ T cell Viability and Cytotoxicity in a Co-culture System

We observed similar effects of

Table 1. Relationship between circ_0001006 expression and clinical parameters

Parameters	Cases (n)	Circ_0001006 expression		P value
	115	Low (55)	High (60)	
Age(years)				0.175
<50	64	27	37	
≥50	51	28	23	
Sex				0.338
Female	47	25	22	
Male	68	30	38	
Smoking				0.536
No	53	27	26	
Yes	62	28	34	
Pathology type				0.432
Adenocarcinoma	65	29	36	
Squamous carcinoma	50	26	24	
Differentiation				0.037
Well+Moderate	68	38	30	
Poor	47	17	30	
TNM stage				0.014
I-II	77	43	34	
III	38	12	26	
Lymph node metastasis				0.020
Negative	80	44	36	
Positive	35	11	24	
EGFR mutation				0.203
Positive	51	21	30	
Negative	64	34	30	
ALK mutation				0.343
Positive	6	4	2	
Negative	109	51	58	
BRAF mutation				0.268
Positive	4	3	1	
Negative	111	52	59	

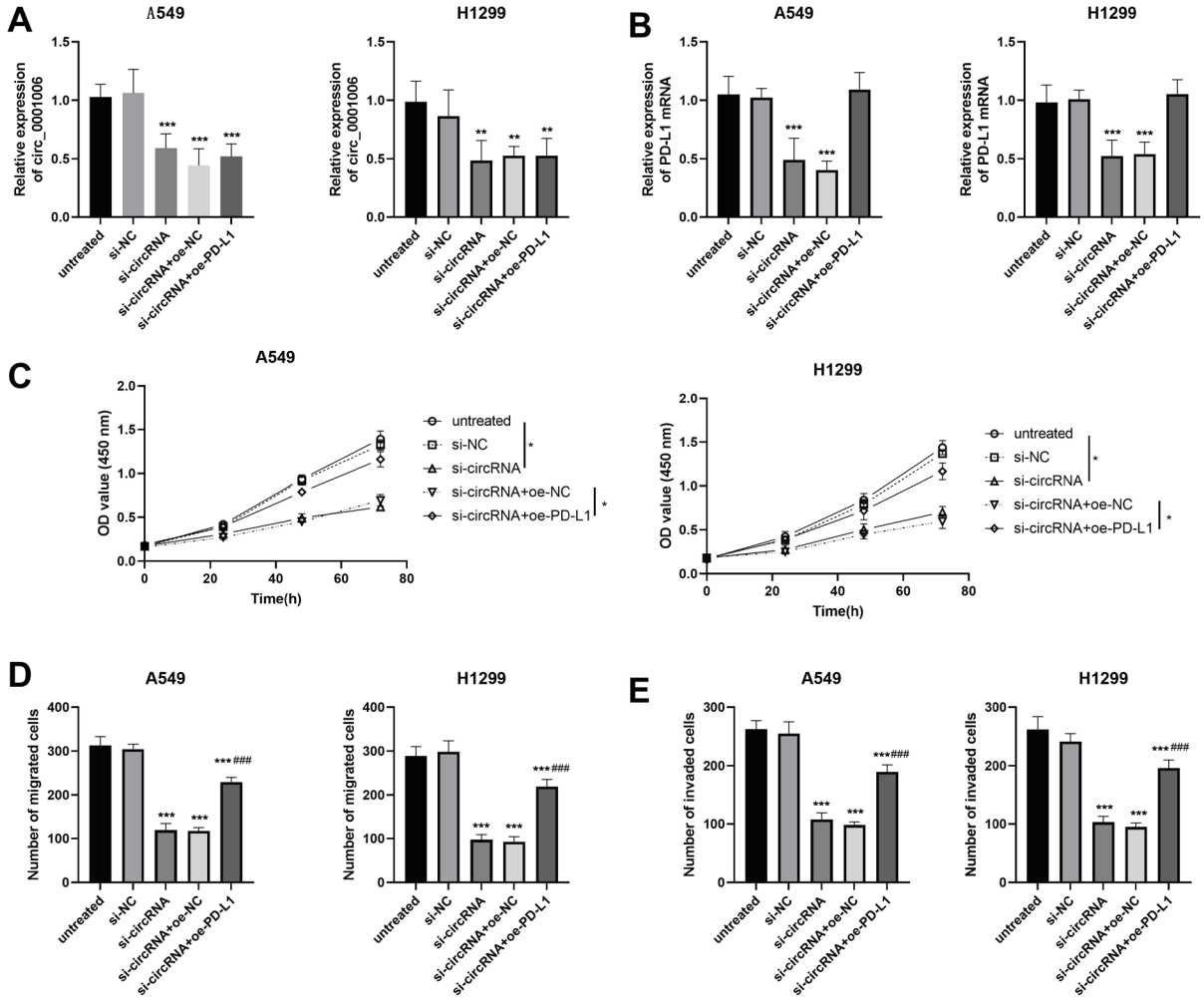

Fig. 2. The influence of circ_0001006 and PD-L1 on cellular behaviors in A549 and H1299 cells. A and B. The levels of circ_0001006 (A) and PD-L1 mRNA (B) were measured by RT-qPCR after transfection. C. The proliferative abilities of NSCLC cells were detected using the CCK-8 assay. D and E. The migration (D) and invasion (E) activities were evaluated using Transwell assays (magnification 200×). *P<0.05, **P<0.01, ***P<0.001.

circ_0001006/PD-L1 in two NSCLC cells, with A549 cells showing better growth. A549 cells were then used in subsequent experiments. Compared to the control group, the activity of CD8+ T cells decreased in the si-NC group. However, the activity of CD8+T cells increased in the si_circ_0001006 group (P<0.01 vs si-NC). within contrast to the si-circ_0001006+oe-NC group, the activity of CD8+ T cells was decreased in the si-circ_0001006+oe-PD-L1 group (Fig. 3A). The cytotoxicity assay showed that silencing circ_0001006 increased the cytotoxicity of CD8+ T cells , while PD-L1 overexpression diminished it (Fig. 3B).

circ_0001006 Affects Immunosuppression in A549 Cells by Regulating PD-L1

The soluble PD-L1 (sPD-L1) levels in the supernatant of the si-NC and T cells co-culture system were increased compared to the control. Knockdown of circ_0001006 partially decreased the sPD-L1 levels compared with si-NC group, while elevated PD-L1 reversed the inhibitory effect of the si-circ_0001006 (Fig. 3C). The inflammatory factors IFN-γ, TNF-α, and IL-2 levels in the co-culture si-NC group declined in contrast to the control, while these were partially increased by knockdown of circ_0001006 and decreased by PD-L1 (Figs. 3D-3F).

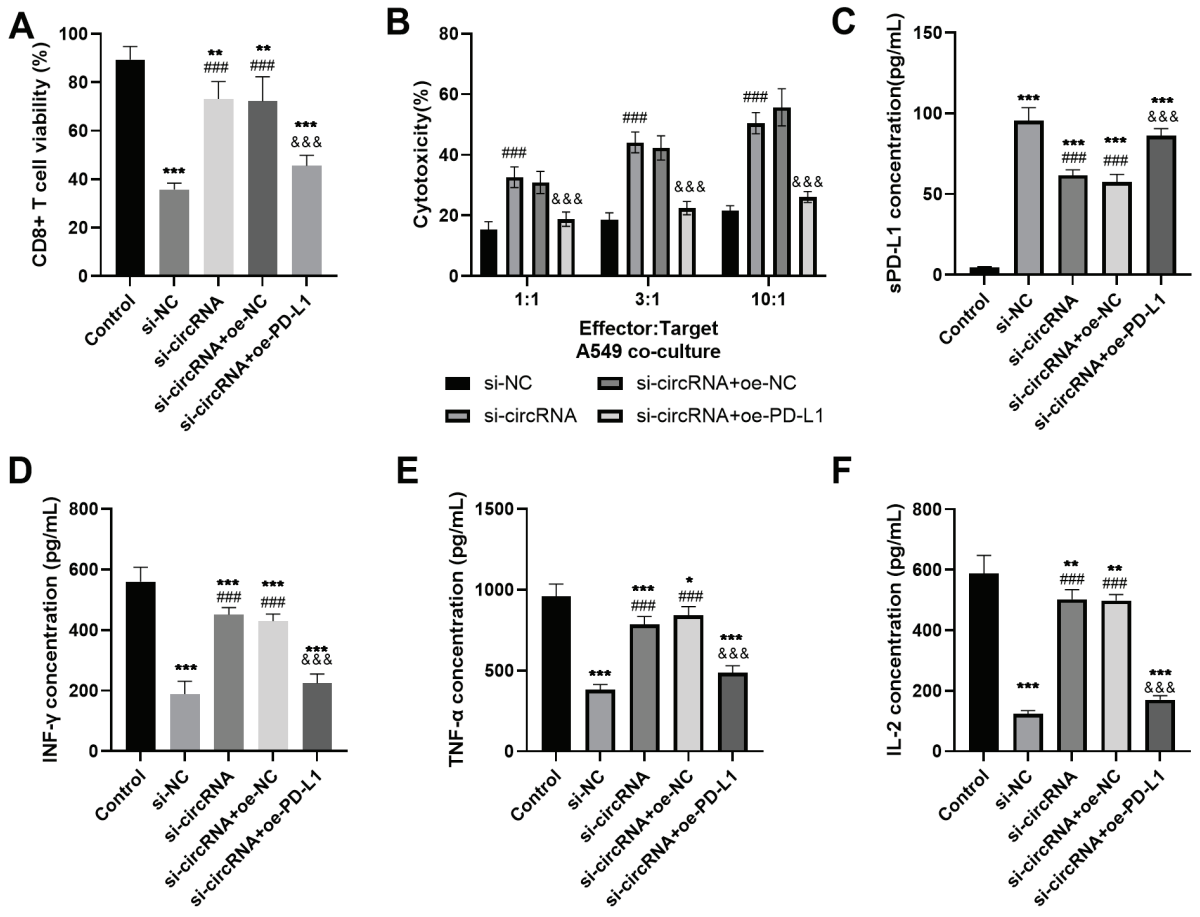

Fig. 3. Effects of circ_0001006 knockdown on CD8+ T cell viability and immune indicators in A549 cells in a co-culture system. A. Effects of circ_0001006 knockdown on CD8+ T cell viability. B. Cytotoxicity in cancer cells after coculture with CD8+ T cells was analyzed by LDH-release cytotoxicity assay. C. ELISA assay measured the sPD-L1 levels in CD8+ T cells and A549 cells co-culture system. D-F. Immune indicators, including INF-γ(D), TNF-α(E), and IL-2(F), were measured using ELISA assays. *P<0.05, **P<0.01, ***P<0.001 vs. control; ###P<0.001 vs. si-NC group; &&&P<0.001 vs. si-circRNA+oe-NC group.

circ_0001006 Acts As a Sponge for miR-320a and PD-L1 is a Direct Target of miR-320a in A549 Cells

The circBank database predicted that miR-320a was a potential target for circ_0001006 (Fig. 4A). The luciferase activity of circ_0001006-WT granulocytes containing miR-320a mimic decreased, while the luciferase activity of circRNA-MUT containing miR-320a mimic showed no significant change compared to mimic NC (Fig. 4B).

The TargetScan database predicted that the 3'UTR of PD-L1 had complementary sequences to miR-320a (Fig. 4C). Compared to the mimic NC, the luciferase activity of PD-L1-WT decreased after transfection with the miR-320a mimic, but the luciferase activity of PD-L1-MUT was not significantly changed (Fig. 4D).

In addition, the expression of miR-320a was decreased in lung cancer tissues (Fig. 4E) and was negatively correlated with circ_0001006 expression levels in tumor tissues (Fig. 4F). Although patients with low miR-320a levels showed a shorter overall survival rate compared to patients with high miR-320a levels, the difference was not statistically significant(P=0.052, Fig. 4G).

DISCUSSION

Our research indicated that the expression of tissue circ_0001006 was increased in NSCLC patients and correlated with the patients' overall survival.

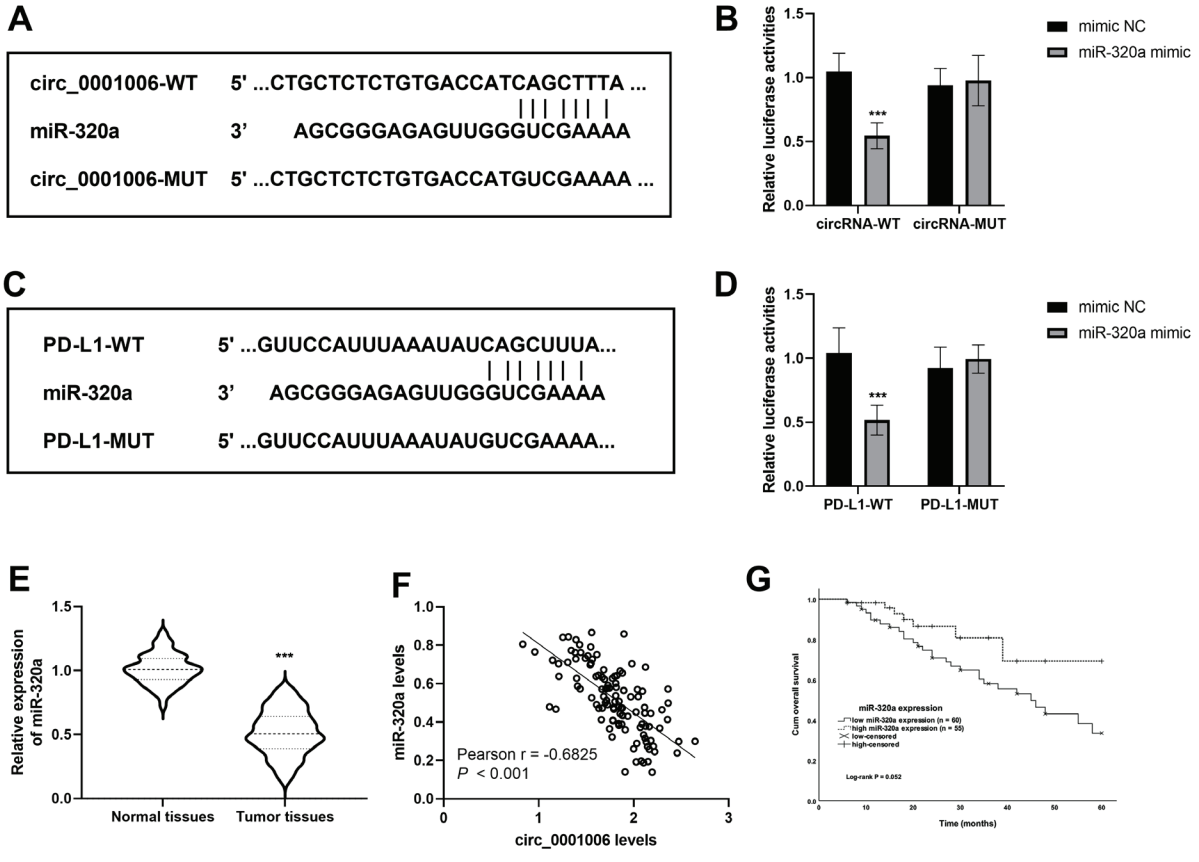

Fig. 4. miR-320a was predicted to bind to circ_0001006 and PD-L1 mRNA. A. The binding sites between circ_0001006 and miR-320a. B. Dual-luciferase reporter assay confirmed the binding relationship between circ_0001006 and miR-320a. C. The binding sites between PD-L1 and miR-320a. D. The binding relationship between miR-320a and PD-L1 was confirmed. E. Expression levels of miR-320a were measured in tissue specimens using RT-qPCR. F. Pearson correlation analysis was conducted to assess the relationship between miR-320a expression and circ_0001006 expression in NSCLC tumor tissues. G. A Kaplan-Meier curve was used to evaluate the prognostic value of miR-320a in NSCLC. ***P<0.001.

Silencing circ_0001006 expression decreased NSCLC cellular activities by regulating PD-L1 expression. Knockdown of circ_0001006 could activate CD8+ T cells in the tumor environment by downregulating the expression and secretion of PD-L1, inhibiting the immune escape of NSCLC cells through regulation of the miR-320a/PD-L1 axis.

Previous studies have shown that circ_0001006 (15) plays a role as an oncogene, while PD-L1 is an essential immune checkpoint molecule expressed at varying levels in cancers (20, 21). Additionally, miR-320a (22, 23) acts as a tumor suppressor gene in tumor progression. The expression of PD-L1 is regulated by various factors, such as transcription factors, oncogenes, tumor suppressor genes, and miRNAs, all of which can influence anti-tumor immune responses (24). Chemotherapeutic drugs that induce DNA damage and trigger the DNA damage response can also upregulate PD-L1 expression, enabling cancer cells to evade immune surveillance (25). The results of this study showed a significant increase in circ_0001006 expression in NSCLC patients and NSCLC cells. Interestingly, circ_0001006 expression was positively correlated with PD-L1 expression in tumor tissues. Knockdown of circ_0001006 could repress the proliferation, migration, and invasion of NSCLC cells, while increased PD-L1 expression partially diminished the inhibitory influence of circ_0001006 knockdown in NSCLC cells.

However, the role of circ_0001006 in immune escape from NSCLC remains to be explored.

Immunotherapies targeted against PD-L1 and PD-1 (PD-L1 receptor) could improve survival in many patients with advanced lung cancer (26, 27). Firstly, the expression pattern of circ_0001006 was measured in tissue specimens from NSCLC patients. Consistent with the trend of PD-L1 mRNA expression in NSCLC, circ_0001006 was also upregulated in tumor tissues, showing a positive correlation with PD-L1. In addition, circ_0001006 expression levels were elevated in NSCLC cells. These data suggest that circ_0001006 may play a key role in tumorigenesis and immune evasion in NSCLC.

Previous studies have indicated that circ_0001006 is a potential biomarker for cancer patients (14, 15). In this study, the clinical analysis demonstrated that the expression of circ_0001006 in tumor tissues of NSCLC patients was related to several clinical parameters that are indicators associated with survival outcomes. The patients were followed up after surgery, and high expression of circ_0001006 was correlated with a poor prognosis serving as a risk factor for shorter overall survival in patients with NSCLC. The data suggest that circ_0001006 has clinical significance for predicting prognosis in NSCLC patients.

circRNA is involved in the carcinogenic process in various tumors (28-30). In our study, silencing circ_0001006 resulted in decreased the cell proliferation and mobility, while increased expression of PD-L1 partially reversed the inhibitory effects of circ_0001006 knockdown on cellular behaviors. This suggests that circ_0001006 plays a promoting role in NSCLC. circRNAs have been found to regulate PD-L1/PD-1 and are involved in the immune response and immunotherapy (30). For example, circCPA4 has been shown to regulate cellular activities in lung cancer cells and deactivate CD8+ T cells by modulating the let-7/PD-L1 expression (28). To further investigate the role of circ_0001006 in NSCLC immune evasion, A549 cells and CD8+ T cells were co-cultured in a Transwell system to prevent direct cell-to-cell contact. Additionally, si-circRNA and oe-PD-L1 were cotransfected into NSCLC cells to assess the rescue effects of PD-L1 on the impact of circ_0001006. We observed that knockdown of circ_0001006 increased activated CD8+ T cell in the immune environment, while increased PD-L1 expression decreased CD8+ T cell activity in A549 cell coculture. The cytotoxicity assay revealed that silencing circ_0001006 enhanced the cytotoxicity of CD8+ T cells , wherease PD-L1 overexpression reduced it. However, the partial death rate among effector CD8 cells could introduce a certain bias in the cytotoxicity results, serving as a limitation in the present study. The data suggest that circ_0001006 expression is involved in the immune environment of NSCLC. Moreover, the secretion of sPD-L1 by A549 cells induced immune-related cytokines in the supernatant of the co-culture system, indicating that NSCLC cells deactivate CD8+ T cells by secreting sPD-L1 following the co-coincubation of A549 and CD8+ T cells. However, silencing of circ_0001006 in A549 cells reduced the secretion of PD-L1, activated CD8+ T cells, and enhanced the expression of immune-related factors IFN-γ, TNF-α, and IL-2 in the supernatant of the co-culture system. CD8+ T cells, which serve as essential effectors in immune defense, can secrete various cytokines, such as IFN-γ, TNF-α, and IL-2 (31). The inflammatory data suggest that silencing circ_0001006 blocked the immune escape of NSCLC. Additionally, circ_0001006 might sponge miR-320a and PD-L1 was identified as a direct target of miR-320a. Consistent with the decreased expression of miR-320a in NSCLC tissues in the current study, miR-320a expression is decreased and has clinical prognostic value in NSCLC (22, 32), and is downregulated in malignant mesothelioma by targeting PD-L1 (33). However, no significant prognostic value of miR-320a was observed in this study, which

may be biased by the limited sample size of participants. We speculate that circ_0001006 may participate in the tumorigenesis and immune escape of NSCLC by regulating the miR-320a/PD-L1 axis.

CONCLUSION

circ_0001006 was found to be downregulated in NSCLC patients and was associated with patients' prognosis. Silencing circ_0001006 could potentiallydecrease the expression and secretion of PD-L1, thus inhibiting the immune escape in NSCLC by regulating the miR-320a/PD-L1 axis. circ_0001006 may serve as a promising prognostic biomarker and theraputic target in NSCLC. Further in vivo experiments are needed to confirm the molecular mechanism.

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Not Applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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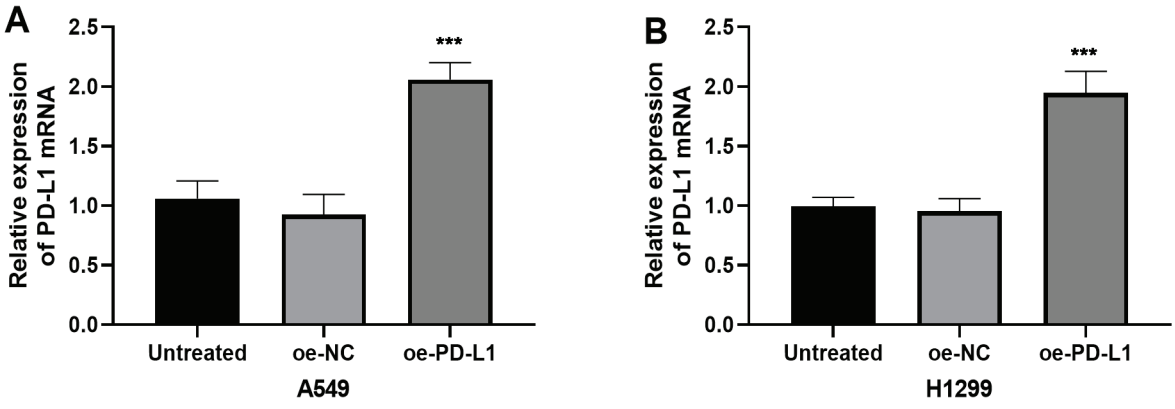

Supplementary Fig. 1. Transfection efficiency of oe-PD-L1 in A549 and H1299 cells. A. PD-L1 mRNA levels were successfully elevated by oe-PD-L1 in A549 cells. B. PD-L1 mRNA expression was upregulated by oe-PD-L1 in H1299 cells. ***P<0.001.