



MicroRNA-146a Inhibits Progression and Immune Evasion in Diffuse Large B-cell Lymphomas by Targeting Programmed Cell Death Ligand 1

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ABSTRACT

Background: Earlier studies have highlighted the involvement of miRNA146a in tumor suppression indicating its potential to inhibit the progression of diffuse large B-cell lymphoma (DLBCL).

Objective: To identify programmed death-ligand 1 (PD-L1) as a candidate for further research, as it plays a key role in regulating immune checkpoints in cancer and is associated with the involvement of miRNA146a in immune regulation and the response to inflammation.

Methods: The expression of miR-146a and PD-L1 in DLBCL cells was detected using qPCR analysis. Subsequently, DLBCL cells (OCI-Ly-3 and OCI-Ly-7) were treated with either the miR-146a mimic or a blank plasmid. To assess immune evasion, DLBCL cells were cocultured with peripheral blood mononuclear cells, CD8⁺ T cells, or cytokine-induced killer cells. Furthermore, the target gene of miR-146a was predicted and validated.

Results: Compared to the normal B-cell line (NCB), the level of miR-146a was significantly lower in DLBCL cells. Additionally, overexpression of miR-146a significantly reduced DLBCL viability, invasion, and immune evasion while simultaneously promoting apoptosis. Our findings also confirmed that miR-146a targeted PD-L1. Finally, the upregulation of PD-L1 notably reversed the tumor suppressive effects of miR-146a on DLBCL.

Conclusion: Our study indicates that miR-146a inhibits the progression of DLBCL by enhancing antitumor immunity through the targeting of PD-L1. The therapeutic potential of this miRNA in lymphoma is highly desirable.

Keywords: Diffuse large B-cell lymphoma; Tumor; DLBCL cells; Cytokine-induced killer; Antitumor immunity

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Cite this article as:

Li Y, Wang X, Yang K.
MicroRNA-146a Inhibits
Progression and Immune
Evasion in Diffuse Large B-cell
Lymphomas by Targeting
Programmed Cell Death Ligand 1.
Iran J Immunol. 2025; 22(1):34-46,
doi: 10.22034/iji.2025.104027.2874.

Received: 2024-09-09

Revised: 2024-12-11

Accepted: 2024-12-14

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is a common type of non-Hodgkin lymphoma (NHL) (1, 2). Reports estimate that approximately 25–35% of all NHLs are DLBCLs, with approximately 37% of tumors originating from B cells (3, 4). As a highly aggressive diffuse proliferative disease, DLBCL is very challenging to treat in the clinic (5). The first option currently considered in treating DLBCL patients is the R-CHOP regimen (6). Despite the success of the Slovak syndrome in achieving RCHOP treatment, the complex pathogenesis and lack of distinct early symptoms in DLBCL make it challenging to diagnose the disease (7) early. Consequently, this treatment program may not be effective for some patients or may result in recurrence after a specific treatment (8). Therefore, there is a significant need to identify new biomarkers that could improve the prognosis and treatment strategy for patients with DLBCL.

MiRNAs are highly conserved during evolution and play a role in posttranscriptional regulation (9-11). The altered expression of miRNAs in DLBCL has been documented in an increasing number of studies and is closely linked to the development of DLBCL. The miR-425-5p/PTEN axis can inhibit DLBCL proliferation and metastasis (12). Additionally, miR-525-5p also inhibits Myd88 transcription and impedes DLBCL progression (13). MiR-146a predominantly plays a negative regulatory role in the innate immune response process (14, 15). It can also inhibit pancreatic cancer and breast cancer by targeting SOX7 and CDKN2A, respectively (16, 17). However, whether miR-146a has any specific role in DLBCL remains to be determined.

Growing evidence highlights the central role of the immune system in cancer, indicating that immune evasion is a major mechanism through which tumor development and progression occur (18, 19). Among key immune regulatory molecules, PD-L1 plays a crucial role in this process (20).

By binding with its receptor PD-1, PD-L1 induces increased apoptosis in immune cells, thus preventing the effective elimination of tumor cells (21). PD-L1 has also been proposed to participate in various cellular processes in different types of cancers, such as hepatocellular carcinoma, melanoma, gastric cancer, and breast cancer, by silencing specific microRNAs (22-24). For example, Yu et al. (25) reported that targeting PD-L1 with miR-429 reduces immune evasion by hepatocellular carcinoma cells, leading to the inhibition of hepatocellular carcinoma progression. Based on these findings, it is reasonable to speculate that miR-146a, when interacting with PD-L1, may inhibit cell growth and immune evasion in DLBCL.

The aim of this study was to investigate the role of miR-146a/PD-L1 regulation in tumorigenesis and immune escape in DLBCL cells.

MATERIALS AND METHODS

Bioinformatic Analyses

Bioinformatic analyses were conducted using R software. The gene expression data was normalized and screened for low expression. Pathway enrichment analysis was carried out using the DAVID tool, and the emerging signaling pathways were identified through gene set enrichment analysis (GSEA). Correlation analysis was then performed in Python to generate heatmaps and visualizations of the gene expression patterns.

Cytokine Assay Stimulation

In the cytokine assay, cells were stimulated with 1 µg/ml of LPS for 24 hours. Following stimulation, the cells were allowed to produce cytokines before samples were collected. The levels of IFN-γ and TNF-α in the supernatants were then detected using ELISA.

Cell Culture

DLBCL and normal B-cell lines, including OCI-Ly-3, OCI-Ly-7, RCK-8, OCI-Ly-10,

and NCB, were obtained from the ATCC Cell Center, USA. The cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% FBS (HyClone, USA) and 80 U/ml penicillin–streptomycin at 37°C with 5% CO₂.

Cell Transfection

miR-146a mimics, a PD-L1-overexpression plasmid (OE-PD-L1), and negative controls (GenePharma, China) were obtained. DLBCL cells were transfected with the aforementioned vectors and/or oligonucleotides using Lipofectamine 3000 (Invitrogen, USA). Seventy-two hours post-transfection, 2 µg/ml puromycin was added to select stable transfectants.

CCK8 Assay

The DLBCL cell medium was changed to a gradient concentration of 2,3,5,4'-tetrahydroxyl diphenylethylene-2-O-glucoside (THSG, Sigma, USA) 24 h post-culture. Cultures were then continued for 12, 24, or 48 h, respectively. After removing the supernatant, 20 µl of CCK8 (Beyotime Biotechnology, China) was added to each well. The OD value of each well was then recorded at 450 nm using a microplate reader.

Cell Invasion Assay

After being coated with 20 µL of Matrigel at a 1:2 dilution, the inserts were treated with THSG at various concentrations for 24 hours. DLBCL cells were cultured in the upper chamber of the Matrigel-coated inserts, and 100 µL of serum-free medium was added. After 24 hours of incubation, a supplement comprising 10% FBS was added to the lower chamber. Following staining with 0.1% crystal violet, the cell morphology was observed using a microscope (Olympus, Japan).

Flow Cytometry

Apoptosis detection was conducted using flow cytometry. DLBCL cells were resuspended in 100 µL of labeling solution and incubated at 37°C for 15 minutes.

Subsequently, the fluorescence solution was incubated in the dark at 4°C for 20 minutes. Finally, flow cytometry (Millipore, USA) was utilized to detect the fluorescence of FITC at 515 nm and PI at 560 nm.

LDH Cytotoxicity Assay

A Dynabeads™ CD8 positive isolation kit (Invitrogen, USA) was used to extract CD8+ T cells from the peripheral blood of volunteers following the manufacturer's guidelines. Subsequently, the isolated CD8+ T cells were cocultured with DLBCL cells at various ratios (1:0, 2:1, 3:1, 5:1, and 0:1). After 24 hours, the cytotoxicity of CD8+ T cells toward DLBCL cells was assessed using an LDH Cytotoxicity Detection Kit (Sigma, USA) according to the manufacturer's instructions, Fig. 1.

The Cytotoxic Effects of Cytokine-induced Killer (CIK) Cells on DLBCL Cells

For the preparation of CIK cell, PBMCs were isolated from patients and stimulated with 50 ng/ml anti-CD3, 1000 U/ml recombinant human IFN-γ, or 100 U/ml recombinant human IL-1α for 24 hours. The medium was then supplemented with 300 U/ml recombinant human IL-2 for 14 days (26). The CIK cells were collected and used to coculture OCI-Ly-3 and OCI-Ly-7 cells at a 15:1 effector:target ratio in an incubator for 24 hours. Finally, cytotoxicity was assessed using the CCK-8 assay, Fig. 1.

Dual-luciferase Reporter (DLR) Assay

PD-L1-1-WT and PD-L1-MUT constructs were created using the pmirGLO vector by combining the WT and MUT miR-144a binding sites with known fragments of PD-L1-1. 293T cells were transfected with these vectors using Lipofectamine 2000 (Invitrogen, USA) for 48 hours. Luciferase activity was measured using a GloMax® 20/20 luminometer (Promega, USA) after the cells were collected.

Quantitative PCR (qPCR)

Total RNA from DLBCL tumors and cell

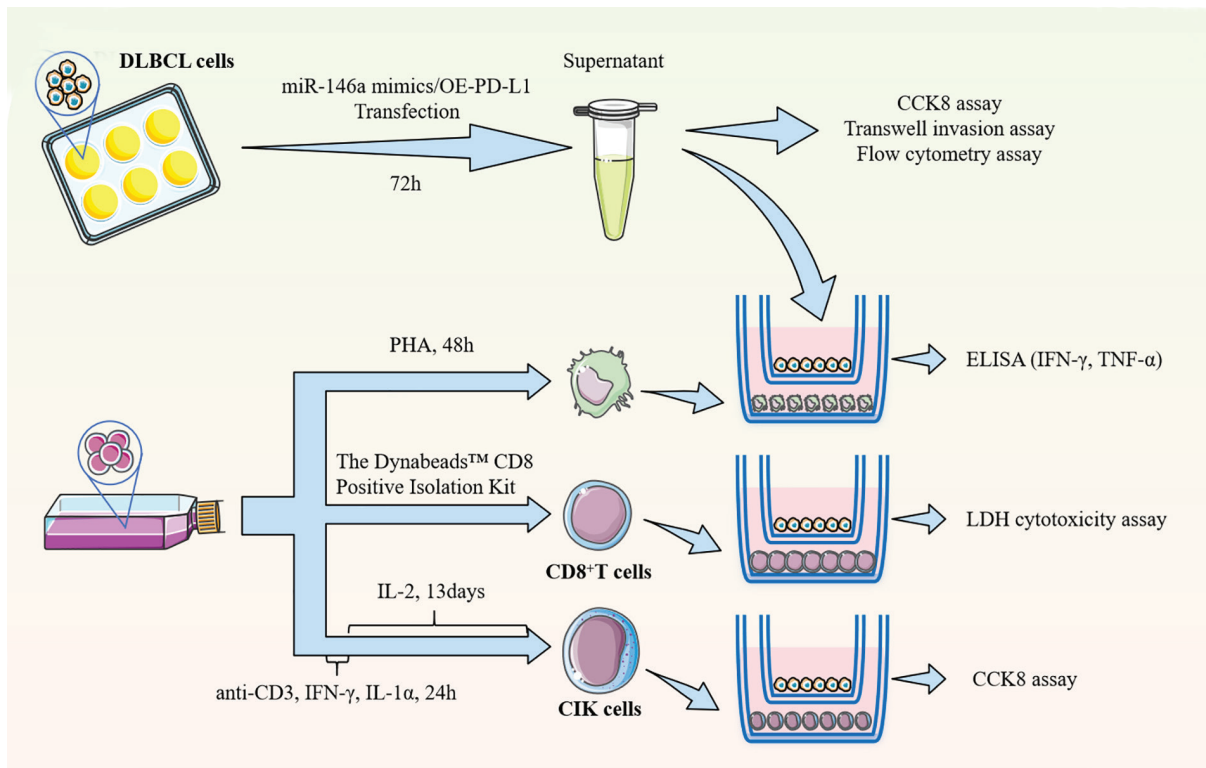


Fig. 1. Flowchart of the experimental procedure in this study

lines was extracted using TRIzol Reagent (Invitrogen, USA). A first-strand cDNA kit was used for cDNA synthesis (Tiangen, China). The SYBR Green Kit (Tiangen, China) was employed for 40 cycles of qPCR at 94°C for 5 min, followed by 20 s at 94°C, 1 min at 60°C, with signal collection at 60°C. GAPDH and U6 were used as reference genes. Expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

Western Blotting

Protein was extracted via RIPA buffer and PMSF (Shanghai Life Mode Engineering, China). The protein concentration was determined using a BCA kit (Solaibao, China). Following electrophoresis, the proteins were transferred to a PVDF membrane. The membrane was then washed and incubated with skim milk, before being diluted with the primary antibody at 4°C overnight. After gentle washing, the secondary antibody was applied for 2 hours. Prime Western Blotting Reagent (Cytiva, UK) was used to detect protein bands, and the gray values of the bands were analyzed using ImageJ.

Statistical Analysis

SPSS version 22.0 (IBM, USA) was used for analysis. The Shapiro-Wilk test was used to assess the normality of the data and determine if the variables followed a normal distribution. Based on the findings of the Shapiro-Wilk normality test, the Mann-Whitney U test was conducted for dependent variables that did not meet the normality assumption. For variables that were normally distributed, independent t-tests were used for pairwise comparisons, and one-way ANOVA with least significant difference (LSD)-T post hoc analyses was utilized for multiple group comparisons. A significance level of $P < 0.05$ was considered statistically significant.

RESULTS

miR-146a Expression in DLBCL Cell Lines

The expression of the miR-146a gene was initially analyzed in DLBCL tissues and cells using quantitative PCR. In comparison to those in the NCB group, the miR-146a levels were significantly lower in all DLBCL cell lines (Fig. 2).

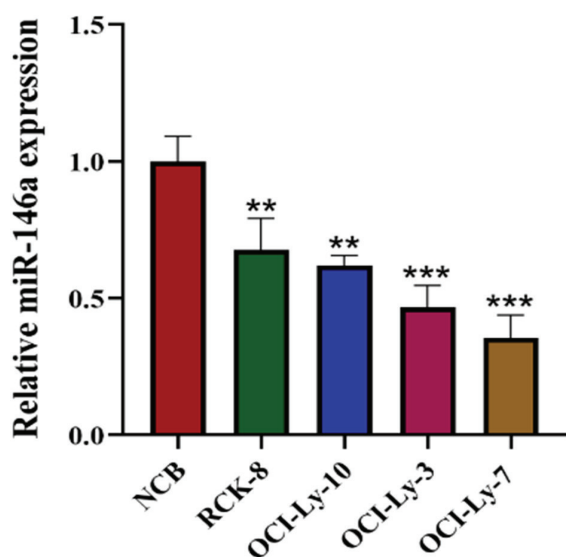


Fig. 2. The expression of miR-146a in DLBCL cell lines. ** $P < 0.01$; *** $P < 0.001$

Due to having the lowest level of miR-146a, the OCI-Ly-3 and OCI-Ly-7 cell lines were chosen for the subsequent experiments.

Upregulation of miR-146a Suppressed Viability and Invasion and Promoted Apoptosis in DLBCL Cells

The miR-146a mimic was transfected into OCI-Ly-3 and OCI-Ly-7 cells, resulted in significantly increased expression (Fig. 3A). The results of the CCK-8 assay showed that the miR-146a mimic significantly decreased the viability of DLBCL cells ($P < 0.05$) (Fig. 3B). Additionally, the Transwell invasion assay revealed that PRDX4 overexpressions significantly inhibited DLBCL cell invasiveness (Fig. 3C).

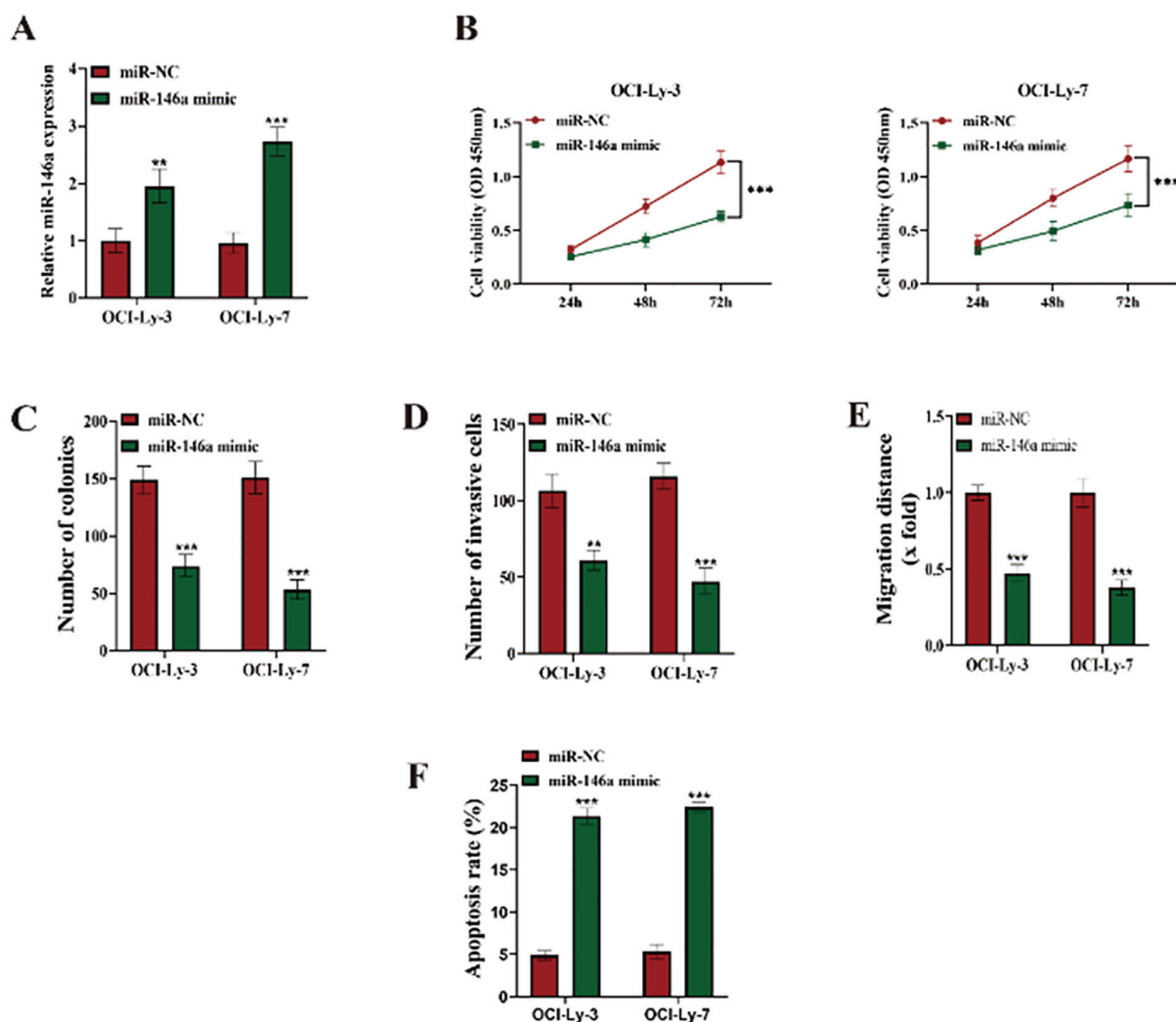


Fig. 3. Overexpression of miR-146a suppressed proliferation and metastasis, and promoted apoptosis in DLBCL cells. (A) mir-146a expression in OCI-Ly-3 and OCI-Ly-7 cells. (B) Cell viability of OCI-Ly-3 and OCI-Ly-7 in each group. (C) Cell proliferation of OCI-Ly-3 and OCI-Ly-7 cells in each group. (D) Cell invasion of OCI-Ly-3 and OCI-Ly-7 cells in each group. (E) Cell migration of OCI-Ly-3 and OCI-Ly-7 cells in each group. (F) Cell apoptosis of OCI-Ly-3 and OCI-Ly-7 cells in each group. ** $P < 0.01$; *** $P < 0.001$

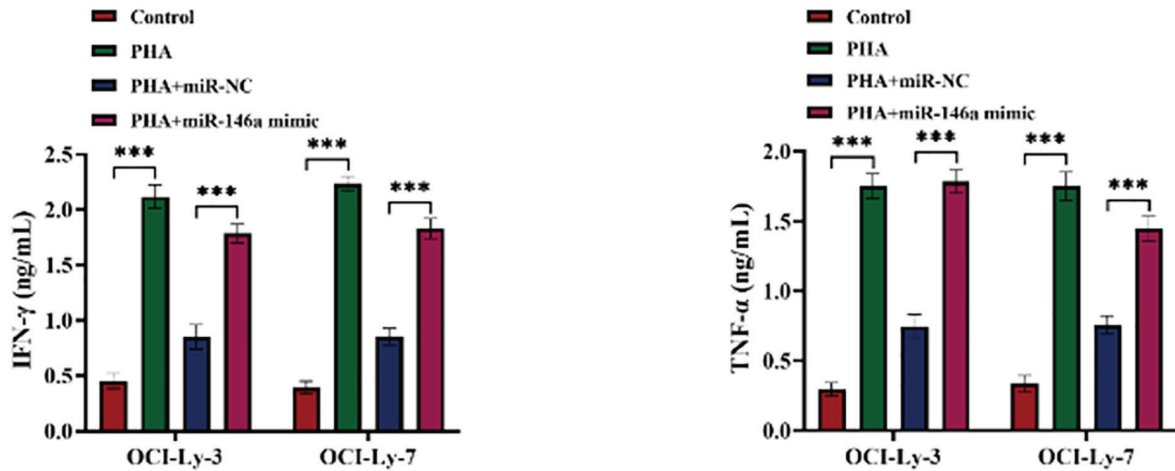
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Flow cytometry analysis demonstrated that transfection with miR-146a mimics markedly enhanced the apoptosis of DLBCL cells ($P<0.05$) (Fig. 3D).

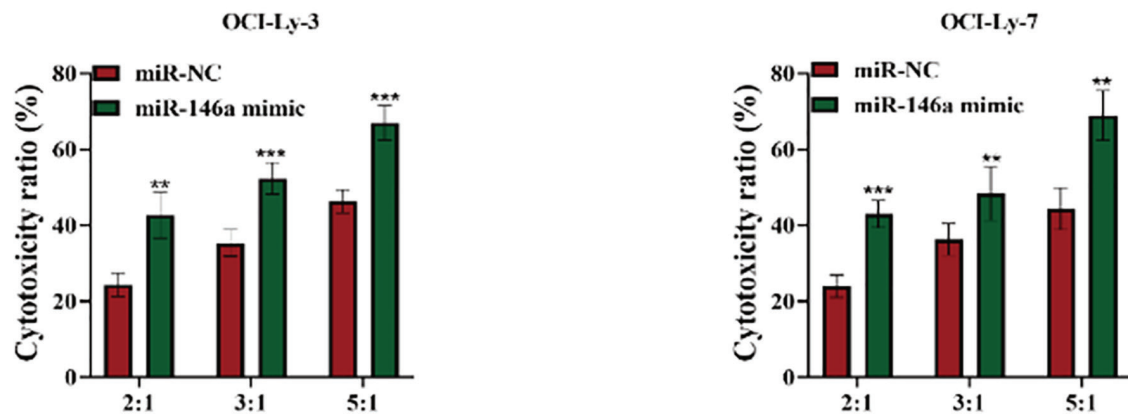
Overexpression of miR-146a Inhibited Immune Evasion in DLBCL Cells

To mimic the immune microenvironment in which tumor cells develop, PBMCs were

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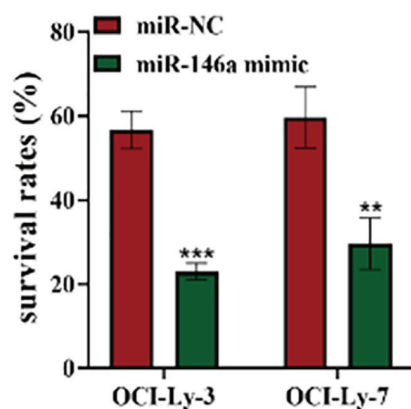


Fig. 4. Overexpression of miR-146a inhibited immune escape in DLBCL cells. (A) The levels of IFN- γ and TNF- α in the cell supernatant of each group. (B) The cytotoxicity of CD8 $^{+}$ T cells against OCI-Ly-3 and OCI-Ly-7 cells in each group. (C) The survival rates of OCI-Ly-3 and OCI-Ly-7 cells in each group. ** $P<0.01$; *** $P<0.001$.

first stimulated with phytohemagglutinin (PHA), followed by coculture with DLBCL cells. Compared to the control, PHA induced higher levels of IFN- γ and TNF- α in PBMCs (Fig. 3A). In the coculture model, the miR-146a mimic significantly increased IFN- γ and TNF- α levels compared to those in the miR-NC group ($P<0.05$) (Fig. 4A). CD8 $^{+}$ T cells play a crucial role in the direct elimination of tumor cells during antitumor immune responses. The results of the LDH cytotoxicity assay revealed the causal effect of the introduction of the miR-146a mimic on the cytotoxicity of CD8 $^{+}$ T cells against DLBCL cells ($P<0.05$) (Fig. 4B). We evaluated CIK cell-mediated cytotoxic effects on DLBCL cells, as well. The results showed that the cell survival rates were significantly lower in the miR-146a mimic group compared to the miR-NC group ($P<0.05$) (Fig. 4C). Overall, these findings suggest that miR-146a overexpression help resist immune evasion in DLBCL cells.

miR-146a Directly Targets PD-L1

A bioinformatics analysis was conducted to identify potential targets of miR-146a. Among the immune-related genes analyzed,

PD-L1 emerged as a promising candidate (Fig. 5A). Gene expression data and signaling pathway analysis have positioned PD-L1 as a compelling candidate for immune regulation and as a potential therapeutic target. The DLR assay demonstrated a decrease in relative luciferase activity in the PD-L1 WT 3'UTR with miR-146 overexpression ($P<0.05$) but no effect was observed on the PD-L1 MUT 3'UTR (Fig. 5B). The expression of PD-L1 in DLBCL cells was then detected. PD-L1 expression was significantly greater in DLBCL cells compared to NCB cells (Fig. 5C-5D). Furthermore, Western blotting showed that the expression of PD-L1 was downregulated by the miR-146a mimic ($P<0.05$) in DLBCL cells (Fig. 5E).

miR-146a Overexpression Suppressed Viability and Invasion and Promoted Apoptosis in DLBCL Cells by Targeting PD-L1

We transfected DLBCL cells to pcDNA or OE-PD-L1 into investigate the regulatory effect of miR-146a/PD-L1 on DLBCL progression. The western blotting results indicated that the expression of PD-L1 was significantly higher in the OE-PD-L1 group than in the pcDNA group ($P<0.05$) (Fig. 6A).

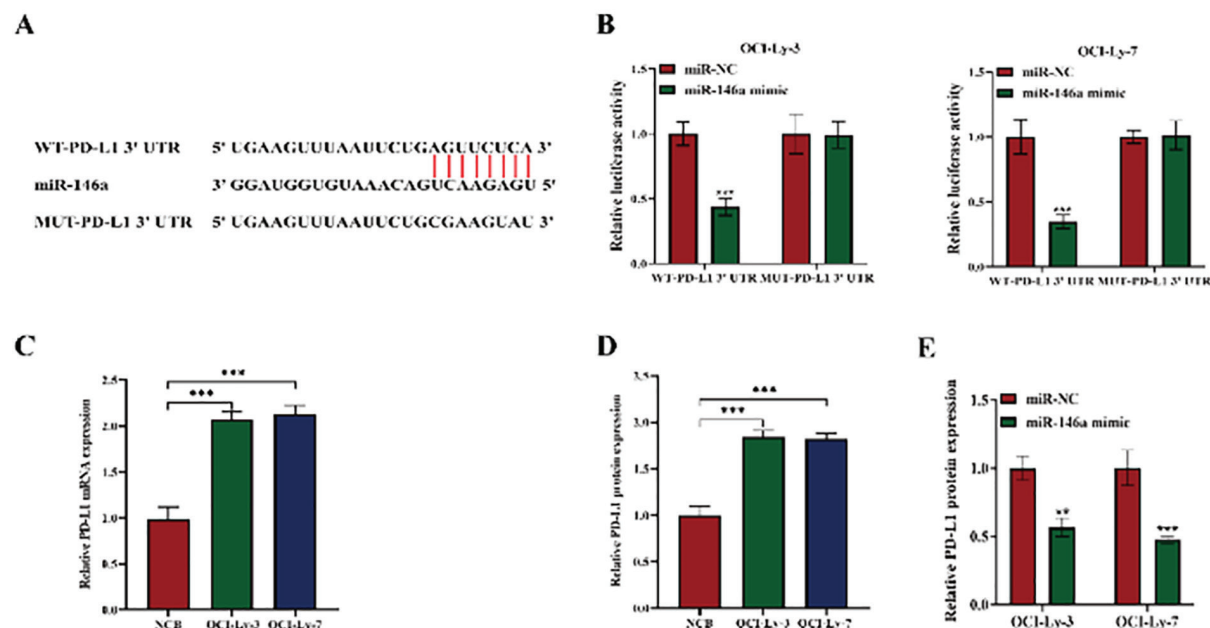


Fig. 5. MiR-146a binds to PD-L1 and suppresses its expression. (A) The binding sites between miR-146a and PD-L1. (B) Fluorescence intensity of PD-L1 3'UTR in OCI-Ly-3 and OCI-Ly-7 cells in each group. (C) The mRNA level of PD-L1 in NCB, OCI-Ly-3 and OCI-Ly-7 cells. (D) The protein level of PD-L1 in NCB, OCI-Ly-3 and OCI-Ly-7 cells. (E) The protein level of PD-L1 in OCI-Ly-3 and OCI-Ly-7 cells in each group. ** $P<0.01$; *** $P<0.001$

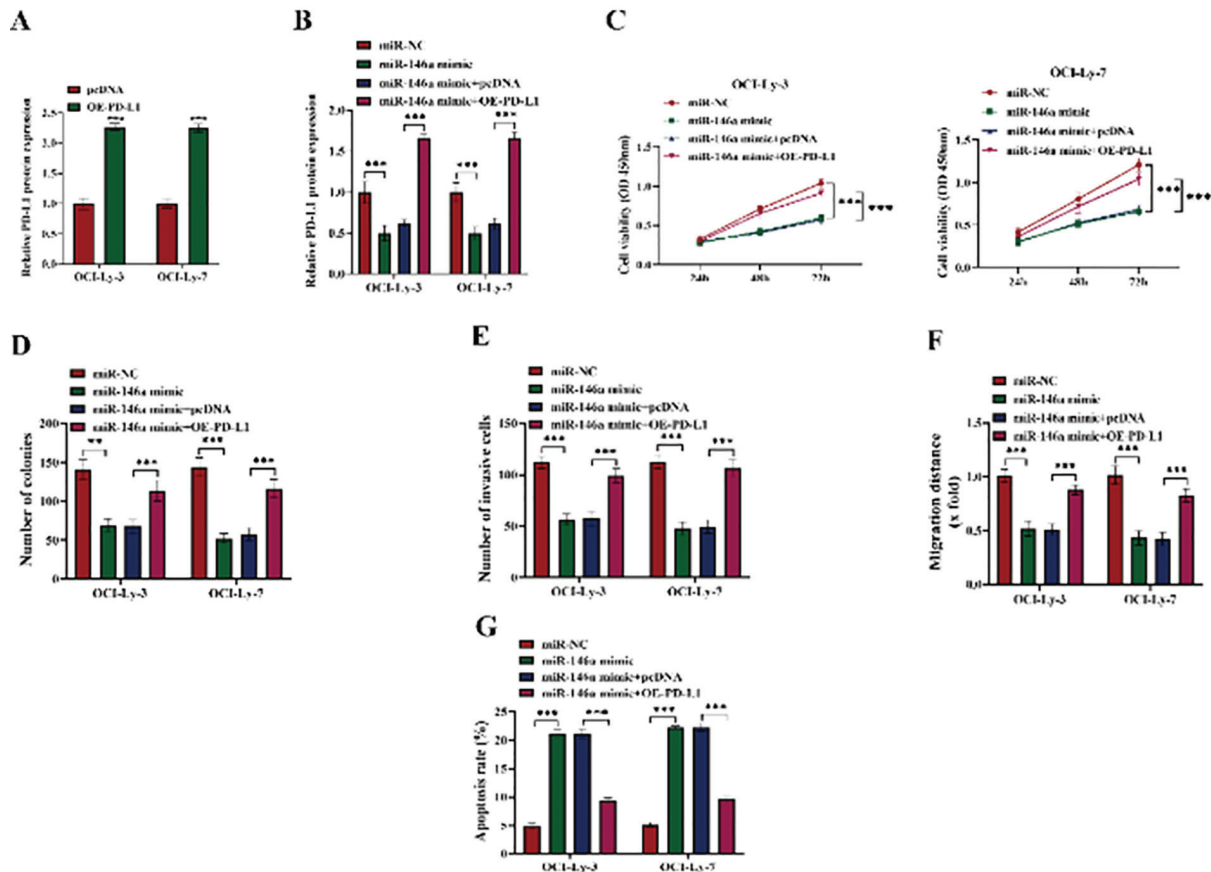


Fig. 6. MiR-146a overexpression inhibited proliferation and metastasis, and promoted apoptosis in DLBCL cells by targeting PD-L1. (A) The expression of PD-L1 in OCI-Ly-3 and OCI-Ly-7 cells in each group. (B) Cell viability of OCI-Ly-3 and OCI-Ly-7 in each group. (C) Cell proliferation of OCI-Ly-3 and OCI-Ly-7 cells in each group. (D) Cell invasion of OCI-Ly-3 and OCI-Ly-7 cells in each group. (E) Cell migration of OCI-Ly-3 and OCI-Ly-7 cells in each group. (F) Cell apoptosis of OCI-Ly-3 and OCI-Ly-7 cells in each group. ** $P < 0.01$; *** $P < 0.001$.

OE-PD-L1 decreased PD-L1 expression in response to the miR-146a mimic (Fig. 6B). The results of the CCK-8 assay revealed that the inhibition of cell viability by the miR-146a mimic was reversed by the overexpression of PD-L1 ($P < 0.05$) (Fig. 6C). Additionally, PD-L1 overexpression counteracted the inhibitory effect of the miR-146a mimic on the invasive ability of DLBCL cells ($P < 0.05$) (Fig. 6D). Moreover, PD-L1 overexpression reversed the promotion of apoptosis induced by the miR-146a mimic in DLBCL cells ($P < 0.05$) (Fig. 6E).

MiR-146a Overexpression Inhibited Immune Evasion in DLBCL Cells by Targeting PD-L1

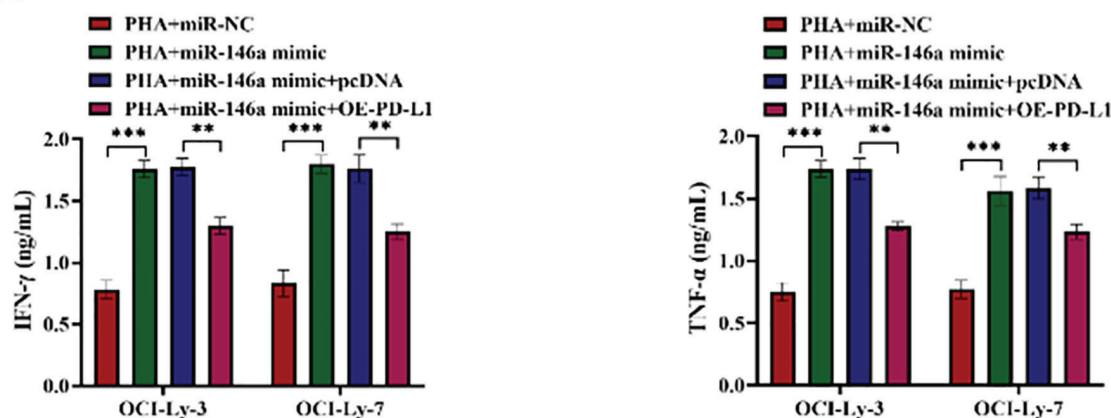
These findings indicate that the level of PD-L1 overexpression resulting from the induction of IFN-gamma and TNF-alpha is

reversed by the miR-146a mimic ($P < 0.05$) (Fig. 7A). In addition, cotransfection of the miR-146a mimic and OE-PDL1 decreased the cytotoxicity of CD8⁺ T cells to DLBCL cells compared to that in the miR-146a mimic+pcDNA group ($P < 0.05$) (Fig. 7B). Moreover, PD-L1 overexpression reversed the reduction in survival of DLBCL cells mediated by the miR-146a mimic ($P < 0.05$) (Fig. 7C).

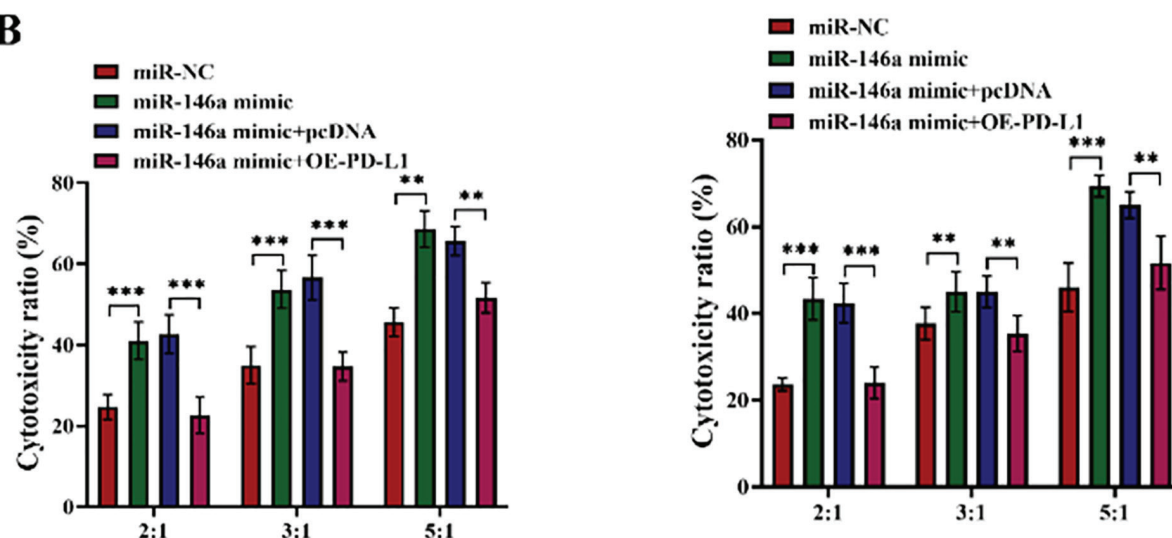
DISCUSSION

DLBCL is the most common NHL and its incidence is increasing annually (27). While treatment is often successful in achieving remission, many patients still have tumors that continue to progress (28).

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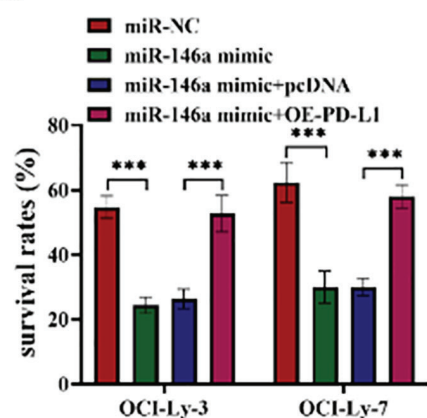


Fig. 7. MiR-146a overexpression suppressed immune evasion in DLBCL cells by targeting PD-L1. (A) The levels of IFN- γ and TNF- α in the cell supernatant of each group. (B) The cytotoxicity of CD8⁺ T cells against OCI-Ly-3 and OCI-Ly-7 cells in each group. (C) The survival rates of OCI-Ly-3 and OCI-Ly-7 cells in each group. **P<0.01; ***P<0.001

Accumulating evidence suggest a significant correlation between the misexpression of miRNAs and the initiation and progression of tumors (29, 30). Therefore, investigating miRNAs and their roles in DLBCL is crucial, hence offering great promise in the development of new and effective miRNA-based therapeutic strategies.

In this study, we have demonstrated that the expression of miR-146a is significantly reduced in DLBCL cells. MiR-146a is located on human chromosome 5q33 and has been the subject of numerous studies suggesting that it plays a role in regulating essential biological processes such as proliferation, differentiation, and apoptosis (31). Several studies have indicated that its expression is increased in various types of cancer, which is strongly linked to their development and progression (32-34). One example of these mechanisms includes targeting CCND1 and CCND2 in non-small cell lung cancer, which restrains cell proliferation and interrupts the Wnt-mediated cell cycle (35). Cui et al. (36) reported that miR-146a enhances radiosensitivity by targeting SOD2, inhibiting the proliferation of epithelial ovarian cancer cells. Moreover, miR-146a targets c-met and inhibits cell metastasis in colorectal cancer (37). Although similar findings have been reported in various cancers, the specific mechanism by which miR-146a functions in DLBCL cells progressing toward tumors remains unclear. To clarify its role, we regulated the miR-146a level in DLBCL cells. Interestingly, the expression of miR-146a inhibited cell viability and invasion and promoted the apoptosis of DLBCL cells.

PBMCs produce inflammatory regulatory factors upon tumor cell stimulation (38). The antitumor activities of IFN- γ and TNF- α have been documented (39). CD8⁺ T cells are the main effector cells in an organism's immune repertoire and play a crucial role in directly eradicating tumors. These cells respond to neoantigens presented by tumor cells, becoming activated and differentiating into cytotoxic CD8⁺ T cells that can efficiently kill

cancer cells at an early stage (40). CIK cells are gaining attention as potential candidates for cancer immunotherapy due to their unique ability to eliminate tumor cells and release various cytokines that enhance systemic antitumor immunity (41, 42). In this study, DL-BCL was cocultured independently with PHA-stimulated PBMCs, CD8⁺ T cells, and CIK cells. Further experimental results indicated that miR-146a inhibits tumor escape in DLBCL through the secretion of IFN- γ and TNF- α and increases the cytotoxicity of CD8⁺ T cells and CIK cells towards DLBCL cells.

Bioinformatics was used to predict potential targets of miR-146a in order to uncover the mechanisms by which its overexpression inhibits the progression of DLBCL. In this context, PD-L1 has been identified as a potential gene candidate through immune gene analysis (Fig. 4A). This analysis, supported by expression data and pathway analysis, emphasized the significance of PD-L1 in immune regulation and suggested it as a possible therapeutic target. The DLR assay also validated PD-L1 as a target that directly interacts with miR-146a. PD-L1 is an essential costimulatory molecule in the immune response, and its aberrant expression is a hallmark for the early diagnosis of several malignant tumors (43). The binding of PD-1/PD-L1 induces T-cell apoptosis and tumor immune evasion (44). Research has demonstrated that PD-1 knockout in human T lymphocytes can release IFN- γ , significantly amplifying T-cell immunity (45). Further investigations have revealed a close relationship between the PD-1/PD-L1 interaction and the progression of gastric cancer (23). Additionally, several studies have highlighted the ability of mRNAs to impede tumor progression by targeting PD-L1. For instance, miR-199a-5p was found to be an inhibitor of cell proliferation and metastasis in follicular thyroid carcinoma by targeting PD-L1 (46). Similarly, miR-34a-5p has been shown to sensitize ovarian cancer cells to cisplatin by targeting PD-L1, thereby inhibiting ovarian cancer progression (47).

Our findings indicate that PD-L1 expression is unregulated in DLBCL. PD-L1 overexpression in DLBCL counteracts the tumor suppressive effects of miR-146a and is association with enhanced antitumor immunity.

In summary, the investigation revealed that deregulation of miR-146a occurs in DLBCL cells. More importantly, miR-146a inhibited DLBCL growth by preventing immune escape through binding to PD-L1. Therefore, miR-146a could be a novel target for immune therapy in DLBCL.

CONCLUSION

In summary, this study demonstrated that miR-146a is downregulated in DLBCL cells and its overexpression inhibits tumor progression and immune escape through targeting of PD-L1. Further studies should be conducted to clarify the mechanistic pathways of miR-146a' effects of and assess its clinical utility as a biomarker or therapeutic tool in immune-related cancers.

AUTHORS' CONTRIBUTION

YL is responsible for the guarantor of integrity of the entire study, study concepts & design, definition of intellectual content, clinical studies, experimental studies, manuscript review; XW is responsible for the statistical analysis, manuscript preparation & editing; KNY is responsible for the literature research, data acquisition & analysis. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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