

PGM5-AS1 Promotes Progression of Diffuse Large B-Cell Lymphoma and Immune Escape by Regulating miR-503-5p

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Purpose: Diffuse large B-cell lymphoma (DLBCL) is a prevalent malignant condition with a dismal prognosis. LncRNA PGM5 antisense RNA 1 (PGM5-AS1) appears to be intricately involved in the progression of DLBCL, yet the modulatory mechanism remains unclear. The purpose of this study was to explore the expression of lncRNA PGM5-AS1 in DLBCL and its effect on the disease progression of DLBCL, as well as to explore its mechanisms.

Patients and Methods: A total of 35 patients were included in the study. The expression levels of PGM5-AS1 and miR-503-5p in DLBCL tumor tissues and cell lines were detected by RT-qPCR. Cell proliferation was assessed using CCK8. Apoptosis rate was determined by flow cytometry. Cell invasion was examined by transwell assays. The specific interaction between PGM5-AS1 and miR-503-5p was verified through dual luciferase reporter gene assays. The immune related factors were detected by ELISA kits. The CD8⁺ T cells cytotoxicity was evaluated by LDH cytotoxicity kit.

Results: In DLBCL tumor tissues and cells, upregulated PGM5-AS1 expression, downregulated miR-503-5p expression, and elevated PD-L1 expression were observed. PGM5-AS1 functioned as a regulator in controlling DLBCL cell proliferation, apoptosis, and invasion by downregulating miR-503-5p expression. When CD8⁺ T cells were co-cultured with cells transfected with si-PGM5-AS1, the secretion of immunoregulatory factors increased, and the cytotoxicity of CD8⁺ T cells increased. These effects were mitigated by miR-503-5p inhibitors.

Conclusion: PGM5-AS1 accelerated DLBCL development and facilitated tumor immune escape through the miR-503-5p. Our discoveries offered an insight into lncRNA PGM5-AS1 serving as a prospective therapeutic target for DLBCL.

Keywords: PGM5-AS1, miR-503-5p, DLBCL, immune escape

Introduction

Diffuse large B-cell lymphoma (DLBCL) represents the most prevalent subtype of B-cell non-Hodgkin's lymphoma (NHL).¹ DLBCL is a highly aggressive form of B-cell lymphoma, and offers a curative prospect for roughly half of the patients, but about one-third of patients with refractory type or eventually relapsing, while patients with relapsed/refractory DLBCL requiring salvage therapy have a poor prognosis, only about 10% of patients eventually achieve complete cure.² It is precisely because of the high heterogeneity of DLBCL and the limitations of current treatments, especially for relapsed and refractory DLBCL, that new pathogenesis drivers, therapeutic objectives and effectiveness indicators are promptly required.

In recent years, the role of lncRNA in the emergence and progression of diffuse large B-cell lymphoma has naturally become the focus of researchers. LncRNA nuclear-enriched abundant transcript 1 (NEAT1) controlled diffuse large B-cell lymphoma through targeting the miR-495-3p/PD-L1 pathway, NEAT1 served as a biomarker for DLBCL.³ PGM5 is a phosphotransferase enzyme that catalyzes the conversion between glucose-1-phosphate and glucose-6-phosphate.⁴

LncRNA PGM5 antisense RNA 1 (PGM5-AS1) is a newly discovered lncRNA in recent years. Yuexiong Liang et al compared the expression patterns of lncRNA in early relapsed Hodgkin lymphoma with those in late relapsed Hodgkin lymphoma, and found that PGM5-AS1 expression was upregulated in late relapsed Hodgkin lymphoma.⁵

miRNAs are recognized for their role in post-transcriptional gene control and are expressed in a variety of cancerous tumors.^{6,7} miR-503-5p was detectable in a diverse range of tumors, including ovarian cancer.⁸ Nonetheless, the connection between miR-503-5p and PGM5-AS1 in DLBCL, as well as their respective contributions to DLBCL progression, remain obscure.

The protein programmed cell death-1 ligand 1 (PD-L1), also identified as B7-H1 or CD274, was originally described by Dong et al in the year 1999. It functions to modulate immune responses by inhibiting cellular immune reactions.⁹ The upregulation of PD-L1 by dendritic cells played a pivotal role in modulating the immune response against cancer.¹⁰ PD-L1 was highly expressed in a variety of cancers and cells evaded T cell immunity through PD-L1/PD-1 signaling.¹¹ There were a number of clinical studies on the use of immunosuppressive agents in tumors.¹²

This study provided the first insight into lncRNA PGM5-AS1 as a prospective therapeutic target for DLBCL and explored the molecular mechanism by which PGM5-AS1 affects DLBCL.

Materials and Methods

Patients and Tissues

A total of 35 individuals diagnosed with DLBCL by pathology at Jiangsu Cancer Hospital from June 2022 to September 2023 were chosen as research subjects. Patients ranged in age from 20 to 75 years, with a mean age of 48.53 ± 13.22 , including 19 males (54.3%) and 16 females (45.7%). DLBCL tissues were removed during surgery. Normal tissues adjacent to tumor tissues were collected as controls. None of the participants had undergone radiotherapy or chemotherapy before their surgical procedure. All samples were rapidly frozen and preserved in liquid nitrogen containers at -80°C . This work was carried out in accordance with the Declaration of Helsinki and was authorized by the Ethics Committee of Jiangsu Cancer Hospital, and all participants provided written consent.

Cell Lines and Cell Culture

All cells (human lymphoblastoid B cell GM12878 and human DLBCL cell lines OCI-Ly10, OCI-Ly7, and OCI-Ly3) acquired from the Chinese Center for Type Culture Collection (Shanghai, China). Cells were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin solution (Gibco, Grand Island, NY, USA) in a humidified environment with 5% CO_2 at 37°C .

Cell Transfection

Briefly, 3×10^5 cells were inoculated into each well of the 6-well plate one day prior to transfection and kept in serum-free medium for 2 hours prior to transfection. The negative control (si-NC) or si-PGM5-AS1 (2 $\mu\text{g}/\text{mL}$, 4390771, Thermo Fisher, CA, USA), inhibitor-NC or miR-503-5p inhibitor (50 nM, 4464084, Thermo Fisher, CA, USA) were diluted with medium, mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and added to 6-well plate. The sequence of the si-PGM5-AS1 was as follows: CGGGTAAGAGAATGTCCGAAAGA. Culture medium was changed after incubation for 6 h in the incubator, and cells were harvested after 48 hours for subsequent experiments.

Reverse Transcription-Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

The total RNA of tissues and cells was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA). The integrity of total RNA was evaluated by agarose gel electrophoresis experiment, and the purity and concentration of the extracted RNA were detected by spectrophotometer (Thermo Fisher, CA, USA), the OD260/OD280 ratio of the samples used for reverse transcription was between 1.8 and 2.0. TaqMan mRNA Reverse Transcription Kit (Biosystems, Foster City, CA, USA) was used to synthesize 1 μg of RNA to cDNA. Real-time polymerase chain reaction was carried out with Takara SYBR Green PCR reagent kit (Takara, Japan). The relative expression was calculated using the formula $2^{-\Delta\Delta\text{Ct}}$ method. The primer sequences were listed in Table 1.

Table I Real-Time PCR Primer Synthesis List

Gene		Sequences	Company	Catalog Number
PGM5-AS1	forward	5'-GACTATGTTGTGAGCCTGCG-3'	Thermo Fisher	A15629
	reverse	5'-AAAAGGGGAGGGGCAATACA-3'		
miR-503-5p	forward	5'-TCGGCAGGUAGCAGCGGGAACA-3'	Thermo Fisher	4464066
	reverse	5'-CTCAACTGGTGTCTGTGA-3'		
PD-L1	forward	5'-TGGCATTGCTGAACGCATTT-3'	Thermo Fisher	A15630
	reverse	5'-TGCAGCCAGGTCTAATTGTTTT-3'		
U6	forward	5'-CGCTTCGGCAGCACATATAC-3'	Shanghai SongonBiotech	B661602
	reverse	5'-AAATATGGAACGCTTCACGA-3'		
β-actin	forward	5'-CCTGGCACCCAGCACAAAT-3'	Shanghai SongonBiotech	B662102
	reverse	5'-GCCGATCCACACGGAGTACT-3'		

Cell Counting Kit-8 (CCK-8) Assay

The CCK-8 assay is a useful tool in cell biology and toxicology studies, as it provides a quick and reliable method for determining cell proliferation and viability. The transfected cells were enzymatically digested and inoculated into 96-well plates. The cells were incubated for 0, 24, 48, and 72 hours respectively, with 10 μL of CCK8 reagent (Invitrogen, Carlsbad, CA, USA) incorporated, followed by a 4-hour culture in a 5% CO₂ incubator at 37 °C. The absorbance at 450 nm was measured by an enzyme-linked immunosorbent assay.

Transwell Assays

The diluted Matrigel glue of 60 μL was applied to the upper chamber of transwell chamber and solidified in an incubator at 37 °C. Then 200 μL cell suspension was inoculated in the upper chamber, and 700 μL culture medium with 10% fetal bovine serum was poured into the lower chamber for a 24-hour incubation. Subsequently, the cells on the bottom surface of the chamber were carefully removed using a cotton swab, fixed in formaldehyde for 10 minutes, and stained with crystal violet for a duration of 20 minutes. Finally, it was subjected to PBS washing thrice and dried, looked under a microscope and taken pictures to count.

Flow Cytometry Detection of Cell Apoptosis

The transfected cells were digested with pancreatic enzymes and cell suspension was collected. After centrifugation, cell samples of 1×10^6 were collected and washed twice with pre-cooled PBS. Subsequently, a binding buffer solution was incorporated into the cell suspension and 5 μL Annexin V-FITC solution and 5 μL PI solution were added to mix well. The cells were cultivated in darkness at ambient conditions for 30 minutes, following which the apoptosis ratio was ascertained using flow cytometry.

Dual Luciferase Reporter Gene Assay

PGM5-AS1 and miR-503-5p were anticipated to interact directly based on the lncRNASNP2 database (lncRNASNP2-human (hust.edu.cn)). Dual luciferase reporter gene assay was performed by concurrently transfecting plasmids encoding firefly luciferase, which contained either the wild-type (WT) or mutant (MUT) sequences of PGM5-AS1. miR-503-5p mimic or mimic-NC, miR-503-5p inhibitor or inhibitor-NC were transfected into DLBCL cells via Lipofectamine 2000.

Roughly 48 hours after transfection, a dual luciferase analysis was conducted using dual luciferase reporter system (Promega, Madison, Wisconsin, USA) in accordance with the producer's guidelines.

Isolation of CD8⁺ T Cells and Co-Culture

Peripheral blood cells from healthy patients were collected and measured. CD8⁺ T cells were separated from peripheral blood mononuclear cells derived from healthy donors using Dynabeads™ CD8 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's suggested protocol. The isolated cells were propagated in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 5 µg/mL ConA.

Co-Culture and LDH Cytotoxicity Assay

Upon achieving a 90% growth of CD8⁺ T cells, the suspension cells and the transfected DLBCL cells were cocultured in a 3:1 ratio. Upon completion of 48 hours of coculture, the cells were collected for subsequent analysis. Subsequently, T cell cytotoxicity was determined using the LDH cytotoxicity kit (Thermo Fisher, MA, USA) in accordance with the manufacturer's guidelines.

The Secretion of Cytokines Detected by ELISA

In compliance with the guidelines, the concentrations of interferon-gamma (IFN-γ) (ab174443) and tumor necrosis factor (TNF)-α (ab309419) were detected in the supernatant of co-cultured cells utilizing ELISA kits (Abcam).

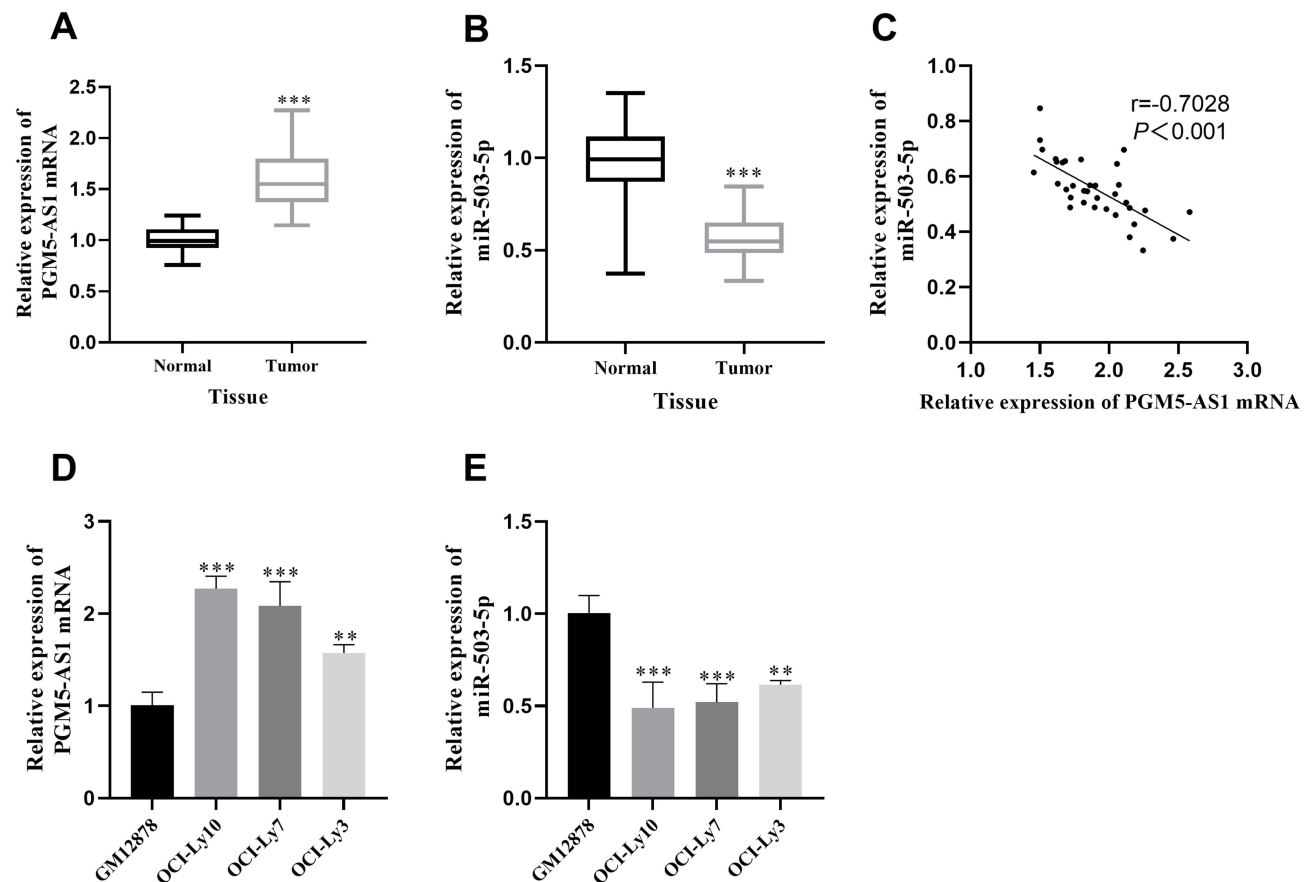


Figure 1 PGM5-AS1 and miR-503-5p were differently expressed in DLBCL tissues and cells. (**A** and **B**) The levels of PGM5-AS1 and miR-503-5p in DLBCL tumor tissues (n=35) were detected by RT-qPCR (**C**) Pearson correlation analysis between PGM5-AS1 and miR-503-5p. (**D** and **E**) The levels of PGM5-AS1 and miR-503-5p in DLBCL cell lines were detected by RT-qPCR.

Statistical Analysis

The normality of continuous variables was tested with the Kolmogorov–Smirnov test. Values are presented as the mean \pm standard deviation (SD). All data were manipulated utilizing GraphPad Prism 9.0 software (GraphPad Software Inc., San Diego, CA, USA) for statistical assessment and graphing. The *t*-test was utilized for pairwise comparisons of quantitative data, while one-way or two-way analysis of variance (ANOVA) was employed for multiple-group comparisons. The correlation between PGM5-AS1 and miR-503-5p/PD-L1 was examined through Pearson correlation analysis. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

PGM5-AS1 and miR-503-5p Were Differentially Expressed in DLBCL Tissues and Cells

The comparison between DLBCL tumor tissues and normal tissues showed that the expression of PGM5-AS1 was increased and the level of miR-503-5p was decreased in DLBCL tissues (Figure 1A and B). There was a significant negative correlation between the expression of PGM5-AS1 and miR-503-5p in DLBCL (Figure 1C). In addition, the expression of PGM5-AS1 was up-regulated and the expression of miR-503-5p was down-regulated in DLBCL cells (Figure 1D and E).

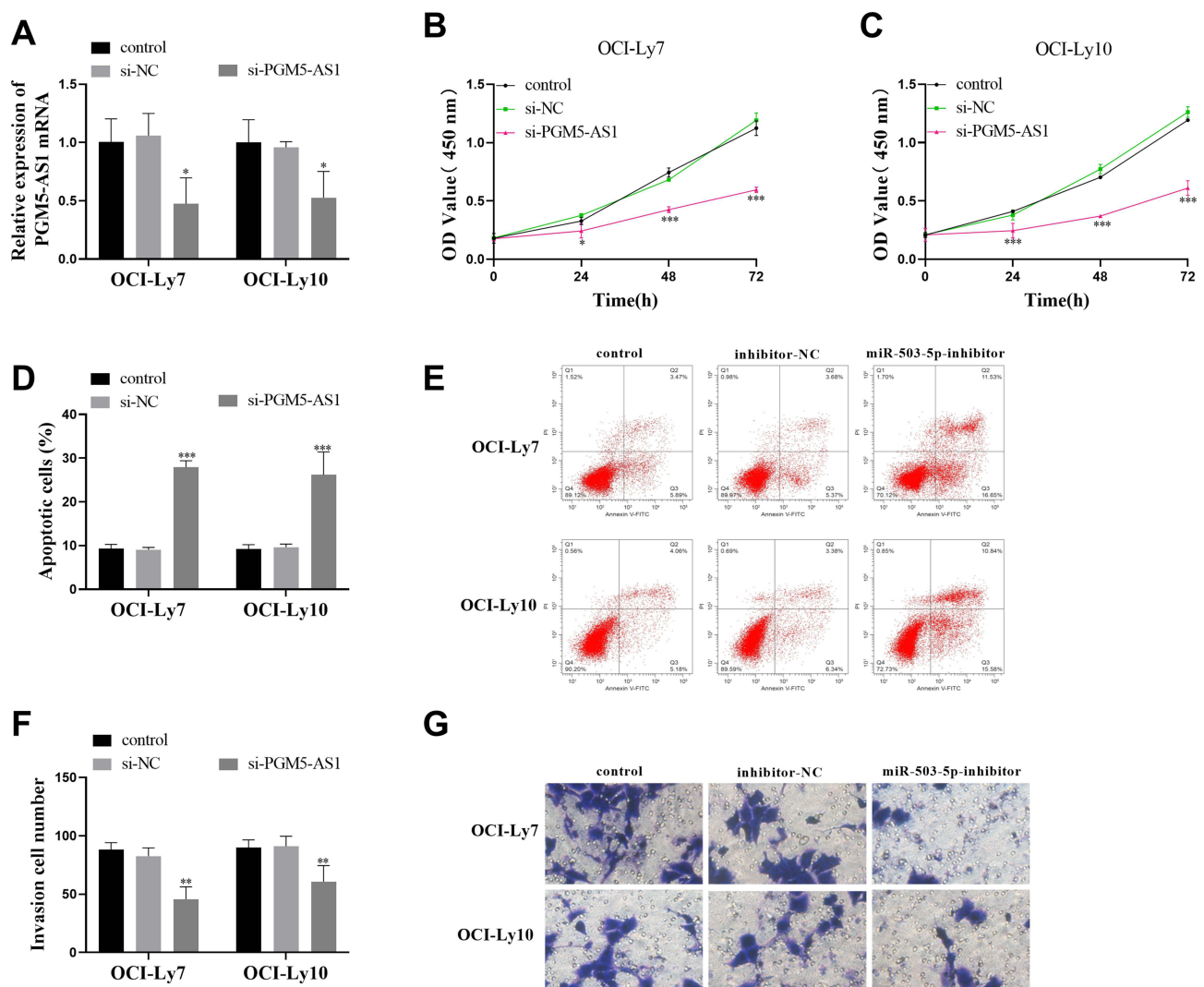


Figure 2 Downregulating PGM5-AS1 resulted in variations in cell proliferation, apoptosis, and invasion capabilities of DLBCL cells. (A) The levels of PGM5-AS1 in DLBCL cells were verified by RT-qPCR. (B and C) Viability of DLBCL cells was assessed by CCK-8. (D and E) Apoptosis in DLBCL cells was quantified by flow cytometry. (F and G) The invasion capability of DLBCL cells was assessed by the transwell assay.

PGM5-AS1 Downregulation Inhibited Cell Proliferation and Invasion, and Enhanced Apoptosis in DLBCL Cells

To inhibit PGM5-AS1 levels, OCI-Ly7 and OCI-Ly10 cells were transfected with si-PGM5-AS1 and si-NC, respectively. Subsequently, RT-qPCR was employed to assess the efficiency of PGM5-AS1 gene knockout (Figure 2A). The Results demonstrated a notable reduction in cell proliferation (Figure 2B and C). The apoptosis rate of cells treated with si-PGM5-AS1 demonstrated a significant elevation (Figure 2D and E). In addition, the number of invasive cells in DLBCL cells decreased after treatment with si-PGM5-AS1 (Figure 2F and G).

miR-503-5p Inhibition Promoted the Proliferation and Invasion, and Inhibited Apoptosis of DLBCL Cells

As illustrated in Figure 3A, following transfection with miR-503-5p inhibitors, the miR-503-5p expression was inhibited. The proliferation of DLBCL cells was enhanced after transfection with miR-503-5p inhibitor (Figure 3B and C). Moreover, the apoptosis rate decreased after miR-503-5p was inhibited (Figure 3D and E). In addition, the number of invasive cells in DLBCL cells increased (Figure 3F and G).

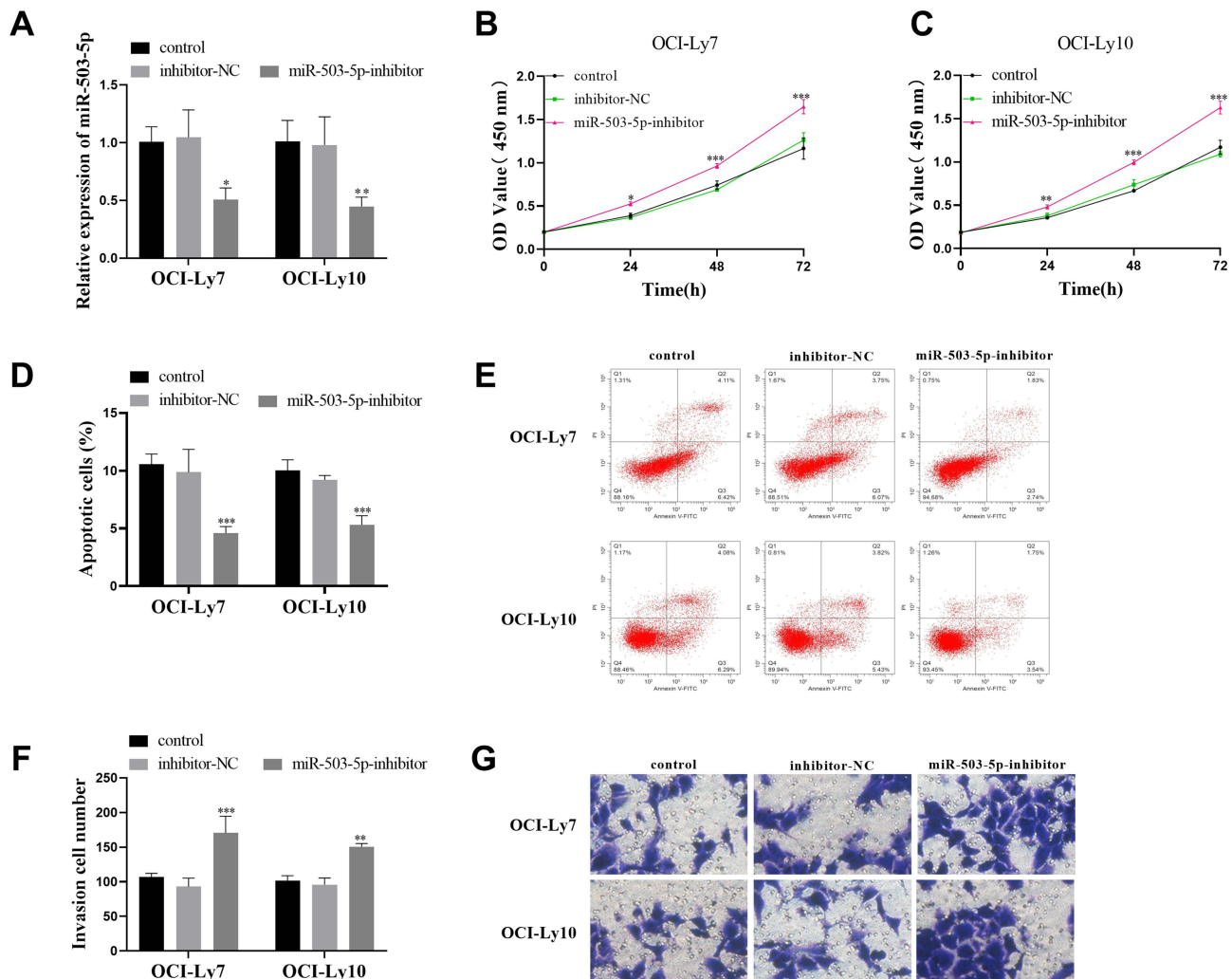


Figure 3 Inhibition of miR-503-5p resulted in variations in cell proliferation, apoptosis, and invasion capabilities of DLBCL cell lines. (A) The levels of miR-503-5p in OCI-LY-7 and OCI-LY-10 cells were verified by RT-qPCR. (B and C) Viability of DLBCL cells was assessed by CCK-8. (D and E) Apoptosis in DLBCL cells was quantified by flow cytometry. (F and G) The invasion capability of DLBCL cells was assessed by the transwell assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

A

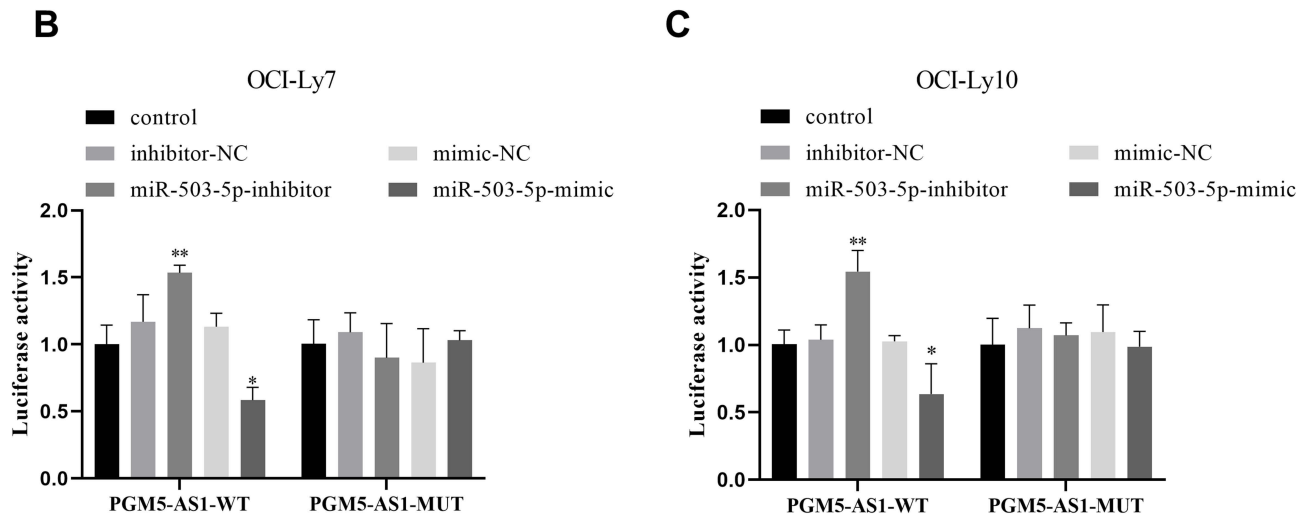


Figure 4 PGM5-AS1 functions as a miR-503-5p sponge. (A) The binding site sequence between PGM5-AS1 and miR-503-5p was forecasted utilizing the IncRNASNP2 database. (B and C) The specific interaction between PGM5-AS1 and miR-503-5p was investigated using a dual luciferase reporter gene assay.

PGM5-AS1 Acted as a miR-503-5p Sponge in DLBCL

The Figure 4A displayed the binding sites of PGM5-AS1 and miR-503-5p. The fluorescence activity was enhanced in cells co-transfected with PGM5-AS1-WT and miR-503-5p inhibitor and diminished in cells treated with PGM5-AS1-WT and miR-503-5p mimic. In contrast, no noticeable distinction was observed in cells expressing PGM5-AS1-MUT, as illustrated in Figure 4B and C.

MiR-503-5p Inhibitor Reversed the Effects on Proliferation, Apoptosis and Invasion Induced by Si-PGM5-AS1

To illustrate that PGM5-AS1 influenced the development of DLBCL by controlling miR-503-5p, we transfected si-PGM5-AS1 and miR-503-5p inhibitors into DLBCL cells. Knocking down PGM5-AS1 led to elevated miR-503-5p expression, which was counteracted by the administration of miR-503-5p inhibitors, as depicted in Figure 5A. Simultaneously, the reduction in cell viability (Figure 5B and C), the elevation in apoptosis rate (Figure 5D and E), and reduced invasion capacity triggered by PGM5-AS1 knockdown were mitigated by miR-503-5p inhibitor (Figure 5F and G).

MiR-503-5p Inhibition Reversed the Effect of PGM5-AS1 Knockdown on Cell Function

The PD-L1 expression in tumor tissues and DLBCL cell lines was up-regulated (Figure 6A and B). Moreover, the PD-L1 level in DLBCL tumor tissues showed a positive correlation with PGM5-AS1 and a negative correlation with miR-503-5p (Figure 6C and D). PGM5-AS1 silencing led to a reduction in the level of PD-L1, and the miR-503-5p inhibitor effectively restored the PD-L1 expression to a normal level (Figure 6E). To delve deeper into the impact of PGM5-AS1 on immune function, cells transfected with si-PGM5-AS1 or si-NC, si-PGM5-AS1+inhibitor-NC or si-PGM5-AS1+miR-503-5p inhibitor were co-cultured with CD8⁺ T cells. The study revealed that silencing PGM5-AS1 in DLBCL cells

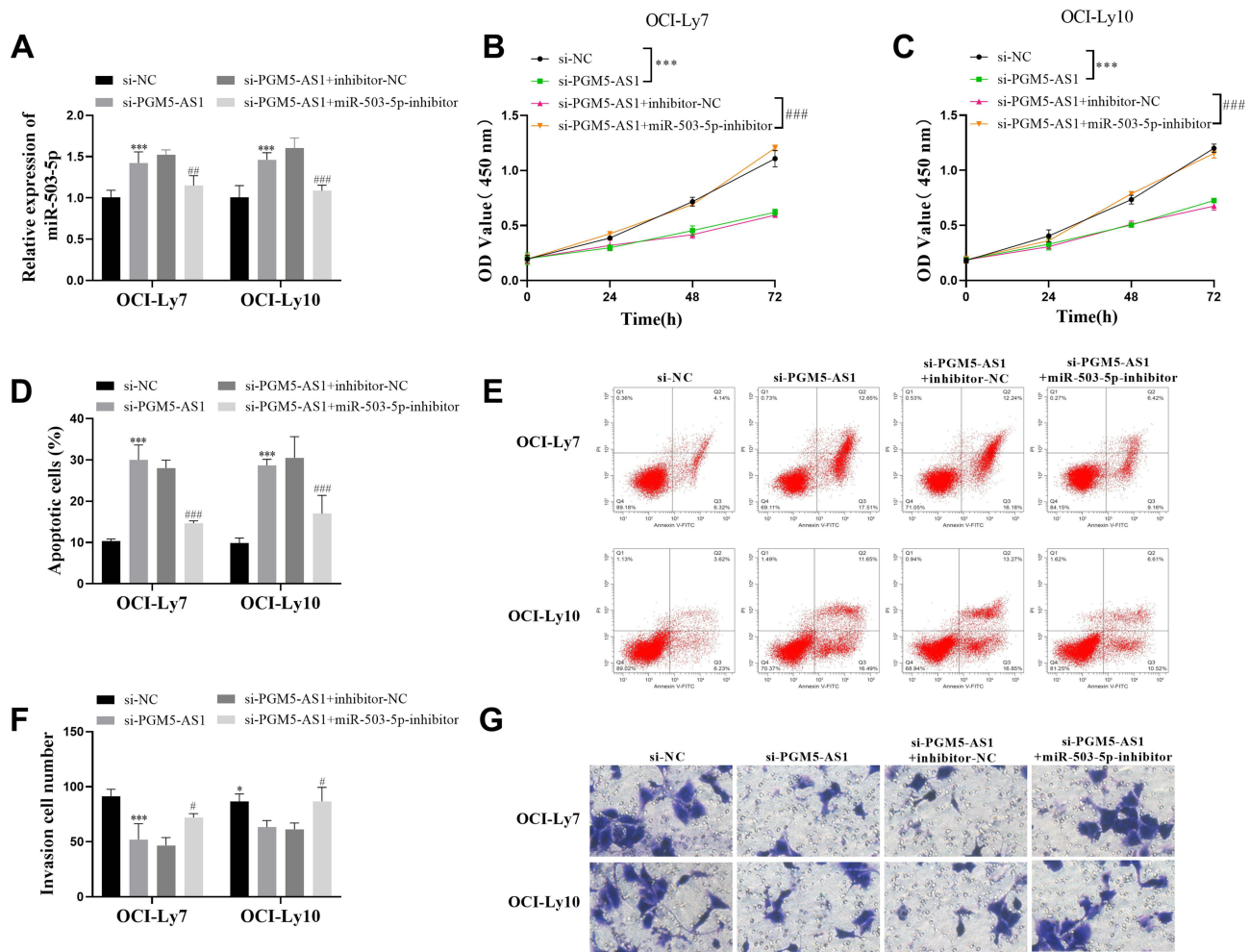


Figure 5 MiR-503-5p inhibitor reversed the effects on proliferation, apoptosis and invasion induced by si-PGM5-AS1. (A) The levels of miR-503-5p were assessed by RT-qPCR after si-PGM5-AS1 and miR-503-5p inhibitor treatment. (B and C) Cell proliferation was assessed using the CCK8 assay at intervals of 0, 24, 48, and 72 hours following si-PGM5-AS1 and miR-503-5p inhibitor administration. (D and E) Apoptosis in DLBCL cells was quantified by the Annexin V/PI assay. (F and G) The invasion capability of DLBCL cells was assessed by the transwell assay.

enhanced the level of immune related cytokines TNF- α and IFN- γ , as well as facilitated the CD8⁺ T cells cytotoxicity. This effect was mitigated by the miR-503-5p inhibitor (Figure 6F and H).

Discussion

Abnormal level of lncRNAs has been demonstrated to play a crucial role in tumorigenesis, and multiple lncRNAs have been demonstrated to control cancer cell proliferation, apoptosis, and metastasis.^{13,14} Increasing evidence suggests that lncRNAs function as crucial players in the tumorigenesis of DLBCL, opening new avenues for the detection, therapy, and prognostic assessment of this condition. lncRNA PVT1,¹⁵ lncRNA NORAD,¹⁶ and lncRNA SBF2-AS1¹⁷ have all been reported to have a close relationship with disease progression in DLBCL. Studies have shown that 9 immune-associated lncRNAs, including PGM5-AS1, have been identified in patients with anaplastic glioma.¹⁸ Overexpression of PGM5-AS1 significantly inhibited proliferation and migration of breast cancer cells and promoted apoptosis in vitro.¹⁹ In this study, increased expression of PGM5-AS1 was detected in DLBCL tissues and cells. The result was consistent with previous research.⁵ Upon PGM5-AS1 gene knockout, the proliferation, invasion capacity of DLBCL cells decreased, whereas apoptosis was elevated, indicating that PGM5-AS1 might contribute to the carcinogenic process in DLBCL progression.

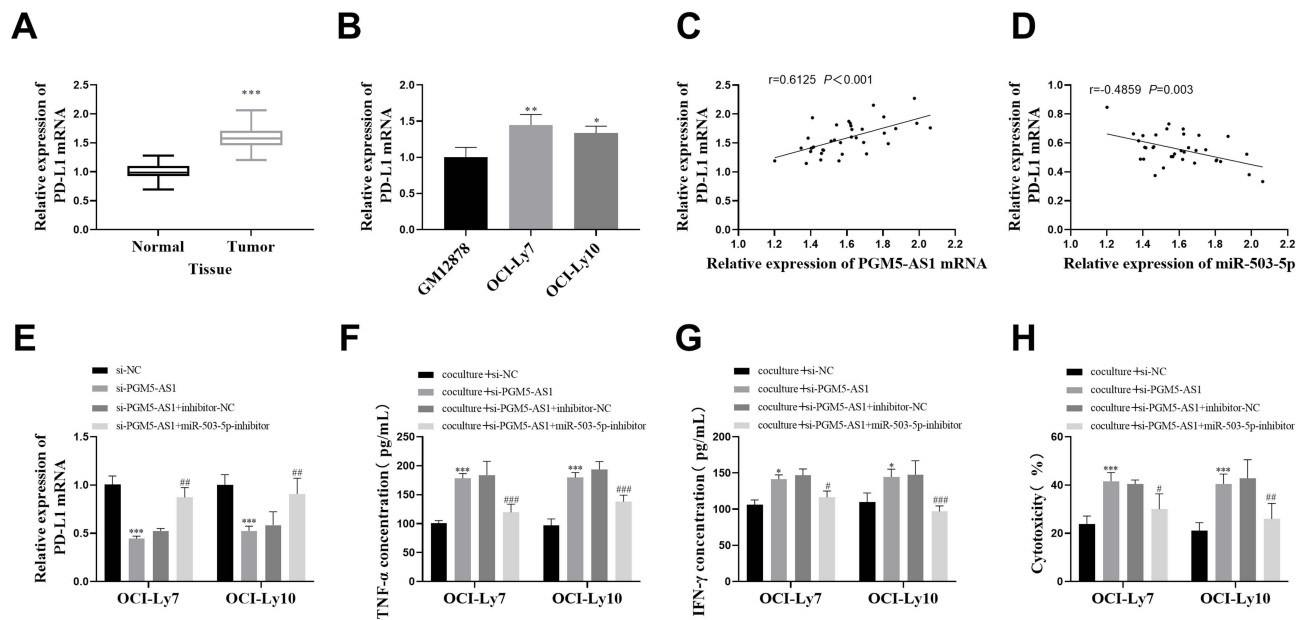


Figure 6 PGM5-AS1 exerts a regulatory influence on PD-L1 expression, IFN- γ and TNF- α secretion, and CD8⁺ T cell cytotoxicity by targeting miR-503-5p. CD8⁺ T cells were co-cultured with OCI-Ly7 and OCI-Ly10 cells with si-PGM5-AS1 and miR-503-5p inhibitor treatment. (A and B) The PD-L1 levels in DLBCL tumor tissues (n=35) and cell lines were detected by RT-qPCR. (C and D) Pearson correlation analysis between PGM5-AS1 and PD-L1, miR-503-5p and PD-L1. (E) PD-L1 levels were assessed by RT-qPCR after si-PGM5-AS1 and miR-503-5p inhibitor treatment. (F and G) Concentrations of TNF- α and IFN- γ were determined via ELISA kit. (H) The CD8⁺ T cells cytotoxicity was assessed using the LDH cytotoxicity assay kit.

LncRNAs are recognized as ceRNAs that serve as a miRNA sponge, thereby assisting in their comprehensive roles during cancer progression.^{20,21} For instance, lncRNA MALAT1 modulated the proliferation and apoptosis of ovarian cancer cells by targeting miR-503-5p.²² Overexpression of miR-503-5p promoted the apoptosis of colon cancer cells, and inhibited migration, proliferation, invasion.²³ In a prior investigation, it was observed that miR-503-5p exhibited a downregulated level in the follicular fluid of women diagnosed with Hodgkin lymphoma.²⁴ In this study, the expression of miR-503-5p was decreased in DLBCL tissues and cells. Upon miR-503-5p inhibition, DLBCL cell viability and invasion number increased, while the cell apoptosis rate was suppressed. It suggested that miR-503-5p affected the disease progression of DLBCL. In addition, this study confirmed that PGM5-AS1 may regulate the disease progression of DLBCL by regulating the expression of miR-503-5p. This provided further evidence for the involvement of PGM5-AS1 in DLBCL disease progression.

The immune system significantly contributes to the progression of malignancies. Immune checkpoint molecules are formed by ligand-receptor pairs that can either suppress or promote the immune response, including inhibitory and stimulating immune checkpoint molecules. Immune checkpoints notably contribute to the negative regulatory pathway in the immune system which maintains its own tolerance by protecting the host from autoimmunity.²⁵ Immune escape occurs when tumor cells manipulate various strategies to avoid detection and assault by the immune system, thereby enabling their unchecked growth and spread. This process serves as a crucial mechanism for promoting cancer progression.^{26,27} The activation of PD-1/PD-L1 suppressed the anticancer potency of tumor-infiltrating lymphocytes (TILs) through triggering apoptosis, suppressing granular enzymes/perforin generation, and reducing the IFN- γ /TNF- α levels.²⁸ In the investigation conducted by Parisa Lotfinejad et al on triple-negative breast cancer, suppression of PD-L1 curbed cancer cell proliferation and induced apoptosis through both intrinsic and extrinsic apoptotic pathways. By inhibiting PD-L1 in breast cancer cells, the T-cell cytotoxic activity was elevated due to an increase in the level of pro-inflammatory cytokines, including IFN- γ and TNF- α .²⁹

This research detected the PD-L1 expression in DLBCL and found that its expression was up-regulated and correlated with both PGM5-AS1 and miR-503-5p. Upon inhibition of PGM5-AS1, the PD-L1 level was suppressed. After co-culture with CD8⁺ T cells, the secretion of immune related factors IFN- γ and TNF- α was increased, and CD8⁺ T cell

cytotoxicity was enhanced, which were counteracted by miR-503-5p inhibition. These results suggested that inhibiting PGM5-AS1 may inhibit tumor immune escape and promote T cell immune activity.

This study explored the abnormal expression and partial molecular mechanism of PGM5-AS1 in DLBCL, but there were still some limitations. There may be more miRNAs server as the target of PGM5-AS1, and PGM5-AS1 may also affect the progress of DLBCL through other mechanisms, which need to be further explored. In addition, this study focused on experiments at the cellular level, which may not be complete, and we will consider tissue-level and in vivo experiments in future studies.

Conclusion

For the first time, our research delved into the efficacy of PGM5-AS1 in DLBCL, uncovering that PGM5-AS1 accelerates DLBCL development and facilitates tumor immune escape by downregulating the expression of miR-503-5p.

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Disclosure

The authors report no conflicts of interest in this work.

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