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Mechanism Study of the Effect of GSPT1 Inhibitor MRT-2359 on Immune Escape in Non-Small Cell Lung Cancer

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Abstract: *This study is dedicated to investigating the effects of the GSPT1 inhibitor MRT-2359 on the functional modulation of immune cells in the context of non-small cell lung carcinoma and to elucidate the underlying mechanisms. Accumulating evidence has highlighted the aberrant upregulation of eRF3a/GSPT1 across a spectrum of malignancies, positioning GSPT1 as a highly promising target for therapeutic intervention. Our findings demonstrate that MRT-2359 efficaciously induces the degradation of GSPT1 within NSCLC cells. Utilizing RNA sequencing (RNA-seq) and other advanced molecular techniques, we have postulated that MRT-2359 modulates the expression of CD276, thereby suppressing the tumor immune evasion phenotype of NSCLC cells. In vivo studies corroborate the tumor-suppressive effects of MRT-2359 in murine models, with immunohistochemical analyses revealing a significant reduction in GSPT1 and CD276 within tumor tissues. Concurrently, an enhanced infiltration of granzyme B (GZMB)-positive cells was observed. These collective findings provide reference value for MRT-2359 advancement towards clinical application.*

Keywords: *GSPT1, MRT-2359, CD276, Non-Small Cell Lung Cancer, Immune Escape*

1. Introduction

Lung cancer is one of the most common malignant tumors worldwide and a major cause of cancer mortality globally. Non-small cell lung cancer (NSCLC) is the most prevalent type, accounting for approximately 85% of all lung cancer cases[1] .Therefore, improving the early diagnosis rate of NSCLC, discovering new prognostic indicators, and identifying new anti-tumor therapeutic targets have become the focus of current research in NSCLC.

The occurrence and progression of tumors are associated with certain proteins in the cell cycle. Eukaryotic release factor 3a (eRF3a) is a major factor in the protein translation termination process in mammals, encoded by the G1-S phase transition protein 1 (GSPT1). Acting as a small GTPase, GSPT1 mediates the recognition of stop codons. GSPT1 forms a complex with another eukaryotic release factor, eRF1, to enhance eRF1 activity in a GTP-dependent manner and promote the release of nascent peptide chains from the ribosome[2, 3]. GSPT1 is involved in various cellular processes, such as cell cycle progression, cytoskeleton organization, and apoptosis [4, 5], It is considered a potential oncogene and therapeutic target [6].

Due to the lack of small molecule binding sites, GSPT1 has traditionally been regarded as an "undruggable" target. Recently, inducing new protein-protein interactions and degrading target proteins through the ubiquitin-proteasome system (UPS) has become an important method for addressing "undruggable" targets [7]. For example, proteolysis-targeting chimeras (PROTAC) [8] and molecular glues (MGs) [9]. MRT-2359 is a potent, selective, orally bioavailable molecular glue degrader that induces the formation of a complex between the E3 ubiquitin ligase component (CRBN) and GSPT1, leading to the targeted degradation of the GSPT1 protein [10]. MRT-2359 can inhibit the growth of drug-resistant non-small cell lung cancer and small cell lung cancer cells and is used to treat solid tumors with high expression of Myc family genes, including lung cancer and diffuse large B-cell lymphoma. The drug was approved by the FDA in September 2022 to enter Phase I/II clinical trials [11].

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Based on the aforementioned research background, this study aims to investigate the mechanisms by which the molecular glue degrader MRT-2359 affects tumor immune evasion in non-small cell lung cancer, providing a reference for the use of this drug in the treatment of non-small cell lung cancer patients.

2. Materials and methods

2.1 Cells

NSCLC cell lines A549, NCI-H292, and NCI-H520 were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. Mouse lung adenocarcinoma cell line Lewis was purchased from the ATCC cell bank.

2.2 Reagents

MRT-2359 was purchased from MCE (USA).Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (db106, Human) was purchased from Degre Biotechnology (Shanghai, China). GSPT1 antibody (10763-1-AP, Human), CD276 antibody (14453-1-AP, Human) were purchased from Proteintech (USA). PD-L1 antibody (13684S, Human) was purchased from Cell Signaling Technology (USA). CD47 antibody (AF4670, Human) was purchased from R&D Systems (USA).

2.3 Cell Culture

Non-small cell lung cancer cells were cultured in DMEM supplemented with 10% FBS (Clark, Australia). Human peripheral blood mononuclear cells (PBMC) and mouse splenic lymphocytes were cultured in RPMI 1640 medium with 10% FBS at 37°C and 5% CO_2.

2.4 Co-culture of Tumor Cells and Immune Cells

A healthy male volunteer with no recent history of illness or medication use was selected. Peripheral blood was collected, and PBMC were isolated from the blood by density gradient centrifugation. The PBMC were cultured overnight in RPMI 1640 medium. The supernatant was removed, and the PBMC were activated with anti-CD3/CD28 Dyna beads™ for 24 hours according to the manufacturer's instructions. The NSCLC cells were treated with MRT-2359 for 72 hours and then transferred to a mixture of RPMI 1640 and DMEM media. Activated PBMCs were added to the wells at a ratio of 3:1 with tumor cells and co-cultured for 24 hours. The PBMCs were then collected from the supernatant, and RNA was extracted. Reverse transcription was performed, and the expression levels of IL-2 and IFN-γ secreted by T lymphocytes were detected by RT-qPCR.

2.5 Total RNA Extraction and Quantitative Real-Time PCR (RT-qPCR)

Total RNA was extracted from cells using the Trizol method, reverse transcribed into cDNA, and detected by RT-qPCR. The primers used for RT-qPCR are as follows:

Table 1 Primer sequence

Gene name	Sequence (5′→3′)
Human β-Actin	Forward: CTCCATCCTGGCCTCGCTGT
	Reverse: GCTGTCACCTTCACCGTTCC
Human CD276	Forward: AGCTGTGAGGAGGAGAATGCA
	Reverse: CTTGTCCATCATCTTCTTTGCTG
Human IFN-γ	Forward: CTGCCAGGACCCATATGTAA
	Reverse: GAGACAATTTGGCTCTGCATT
Human IL-2	Forward: ACTCACCAGGATGCTCACATT
	Reverse: AGTCCCTGGGTCTTAAGTGA

2.6 Western Blot

Cells at 80%-90% confluence were treated with carnosine. Cells were lysed in RIPA buffer with PMSF on ice for 10 min, then centrifuged at 14,000 g for 30 min at 4 ℃. Supernatants were collected and

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protein concentration determined using a BCA kit. Standard western blot protocols were followed.

2.7 Transcriptomic Analysis

NSCLC cells were treated with 3 μmol/L MRT-2359 for 3 days. Cells were then lysed with Trizol, and both treated and control samples were sent to Novogene for RNA-seq.

2.8 Mouse Xenograft Model and Intraperitoneal Treatment

Male C57BL/6 mice (5-6 weeks old) were purchased. Lewis cells in logarithmic growth were mixed 1:1 with Matrigel. Mice were randomly divided into three groups, and 4 million Lewis cells were injected into the right axilla of each mouse. After tumor formation, MRT-2359 was dissolved in DMSO and then in 20% SBE-β-CD saline. MRT-2359 was administered intraperitoneally at 10 mg/kg every 2 days for four doses.

2.9 Immunohistochemistry

After the final dose, mice were euthanized by cervical dislocation. Tumor tissues were excised and processed for fixation, dehydration, paraffin embedding, and sectioning. Sections were deparaffinized, antigen-retrieved, blocked, and stained. Slides were imaged microscopically.

2.10 Statistical Analysis

Experiments were repeated at least three times. Independent samples were analyzed using t -tests, with $P < 0.05$ considered significant. Data analysis and plotting were performed with GraphPad Prism 7.0.

3. Results

3.1 GSPT1 is highly expressed in tumor tissues, and MRT-2359 can effectively degrade GSPT1

Firstly, this study used GEPIA to compare the expression levels of GSPT1 in lung cancer tissues and other common tumors. The results showed that GSPT1 was significantly upregulated in many common tumor tissues compared with adjacent normal tissues (Figure 1A). In addition, the expression level of GSPT1 in lung cancer tissues was higher than that in adjacent tissues (Figure 1B), and patients with high GSPT1 expression had a worse prognosis (Figure 1C). After treating NSCLC cell lines (A549, H520, H292) with GSPT1 inhibitor MRT-2359 at concentrations of 0.03 μmol/L, 0.3 μmol/L, 3 μmol/L, and 30 μmol/L for 72 h, the protein expression levels of GSPT1 in lung cancer cells were detected by Western blot. It was observed that GSPT1 was significantly degraded(Figure 1D-F).

3.2 MRT-2359 treatment of NSCLC cells inhibited tumor immune escape

To further explore whether GSPT1 affects tumor immune evasion, we co-cultured MRT-2359-treated NSCLC cells (after 72 h of treatment) and control cells with PBMCs from a healthy male. The results showed that PBMCs co-cultured with MRT-2359-treated NSCLC cells expressed significantly higher levels of IFN-γand IL-2 than those co-cultured with untreated NSCLC cells. The sequences of the qPCR primers are shown in Table 1. This indicates that MRT-2359 treatment reduces the ability of NSCLC cells to suppress immune cells, thereby inhibiting tumor immune evasion (Figure 2A-F).

3.3 Transcriptomic analysis of gene expression profile changes in NSCLC cells after MRT-2359 treatment

The aforementioned results have demonstrated that MRT-2359 treatment of NSCLC cells can inhibit tumor immune evasion. To investigate the molecular mechanisms underlying this phenomenon in tumor cells, we performed transcriptomic analysis on A549, H520, and H292 cells after MRT-2359 treatment. Upon comparison of differentially expressed genes, we found that CD276 was downregulated in all three cell lines (Figure 3A-D). Subsequently, we conducted bioinformatics analysis and, through TCGA (The Cancer Genome Atlas) data analysis, we confirmed a positive correlation between the GSPT1 and CD276 genes in non-small cell lung cancer (P-Value=2.2e-13) (Figure 3E). Moreover, in lung cancer tissues, the

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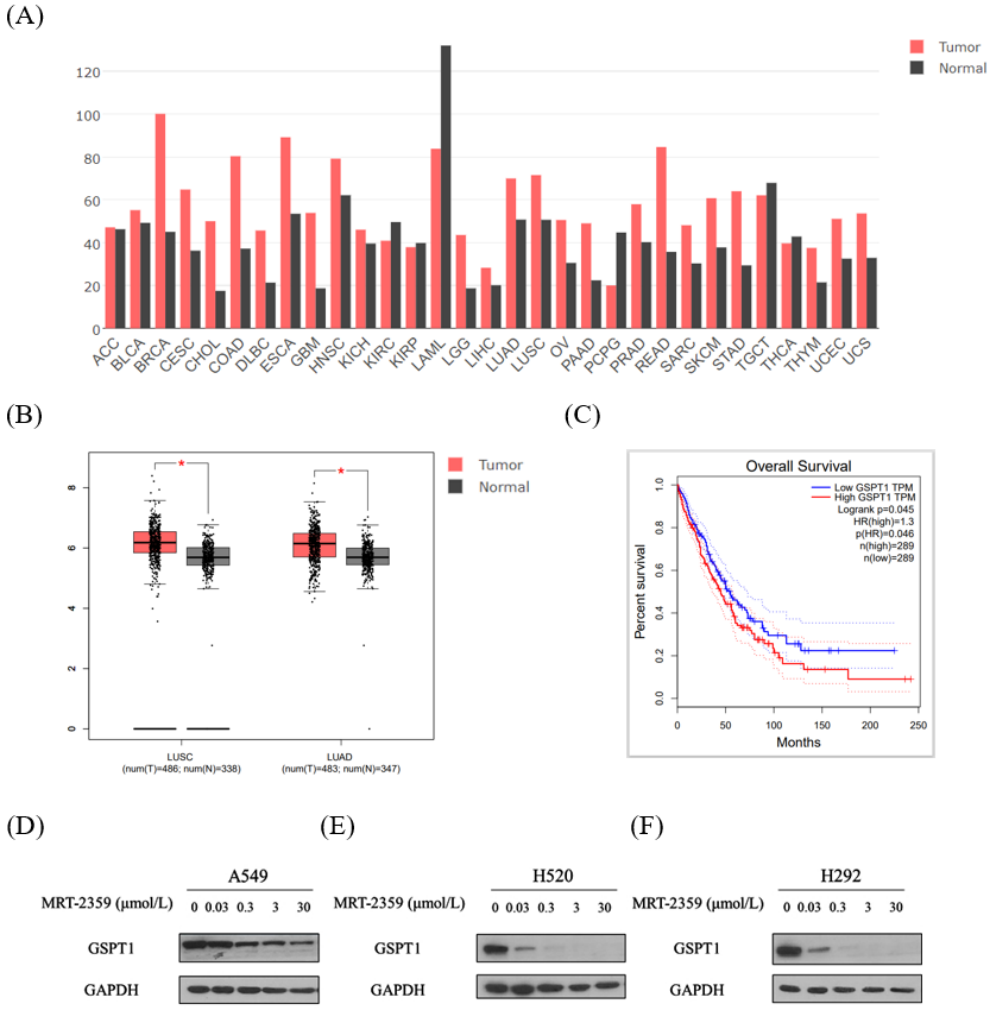
expression of CD276 was higher than that in adjacent normal tissues (Figure 3F). Additionally, survival analysis of non-small cell lung cancer patients revealed that those with high CD276 expression had a worse prognosis (Figure 3G).



A: The differential expression of GSPT1 in tumor tissues compared to adjacent normal tissues was detected using the GEPIA online platform.B: Analysis from the TCGA database revealed that GSPT1 expression in lung cancer tissues is significantly elevated compared to adjacent normal tissues with |log2FC|>0.45 and P<0.001.C: The correlation betweent GSPT1 expression levels and overall survival (OS) in non-small cell lung cancer patients was analyzed using the TCGA database (n=579).D-F: The protein expression level of GSPT1 in NSCLC cell lines (A549, H520, H292) after treatment with MRT-2359 for 72 hours was detected by Western Blot (, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001).*

Figure 1: Elevated GSPT1 Expression in Lung Cancer Tissue and Its Targeted Degradation by MRT-2359 in NSCLC.

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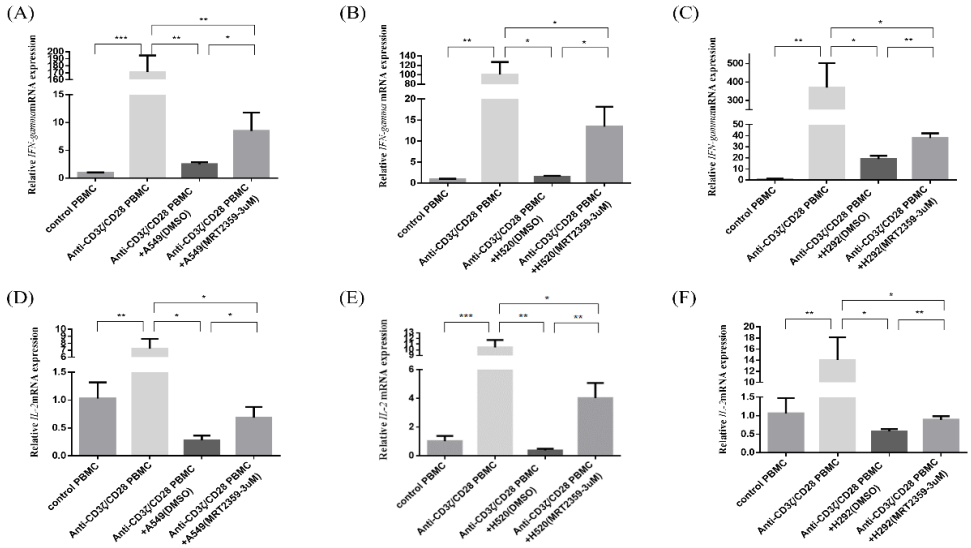
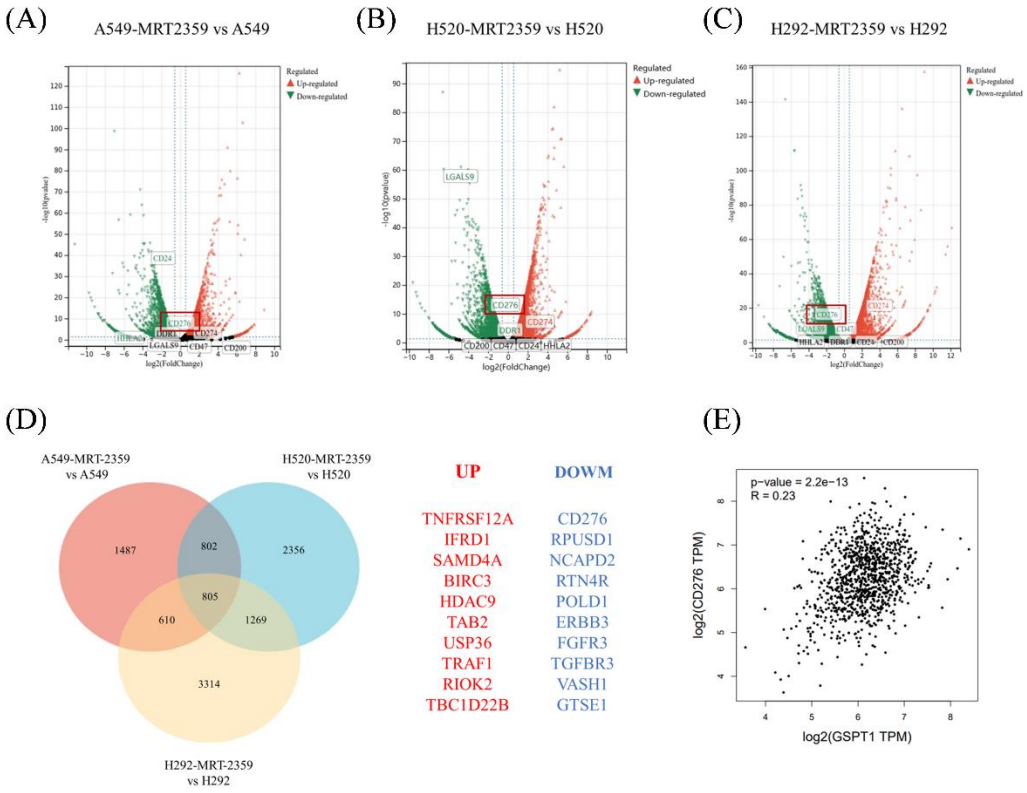


A-C: NSCLC cell lines (A549, H520, H292) treated with MRT-2359 were co-cultured with PBMC, and the expression changes of IFN-γ in PBMC after co-culture were measured by RT-qPCR.D-F: NSCLC cell lines (A549, H520, H292) treated with MRT-2359 were co-cultured with PBMC, and the expression changes of IL-2 in PBMC after co-culture were measured by RT-qPCR (, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001).*

Figure 2: Influence of MRT-2359 on the Immunological Function in Human Non-Small Cell Lung Cancer Cells.



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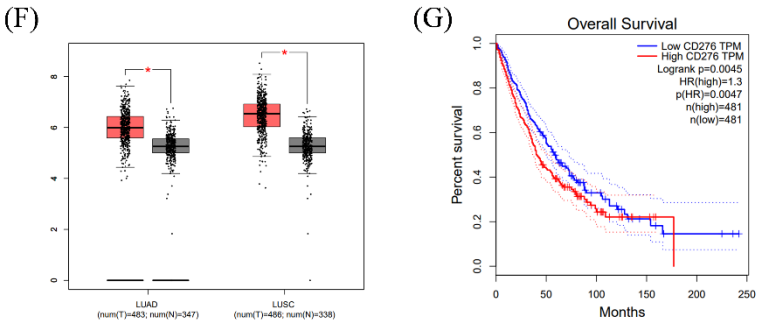


A-C: Volcano plots illustrating the distribution of differentially expressed genes after 72 hours of treatment with 3 μmol/L MRT-2359 in NSCLC cell lines (A549, H520, H292), compared to the control groups.D: Venn diagrams of the co-expressed genes between the treatment and control groups of the three lung cancer cell lines A549, H520, and H292.E: TCGA database analysis of the correlation between the GSPT1 gene and the CD276 gene in NSCLC.F: Expression of CD276 in lung cancer tissue is higher than in normal lung tissue with |log2FC| > 0.7 and P < 0.001.G: TCGA database analysis of the correlation between the expression level of CD276 and overall survival (OS) in non-small cell lung cancer patients (n=962) (, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001).*

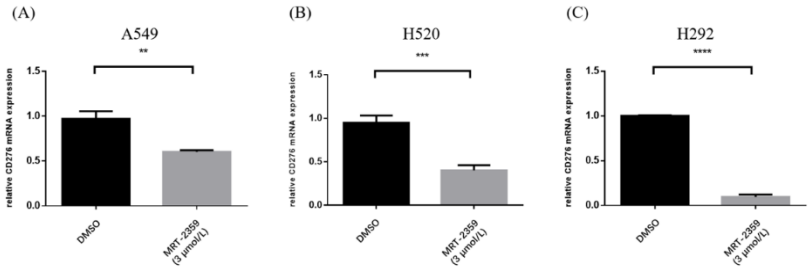
Figure 3: Gene expression profiling of human non-small cell lung cancer cells treated with MRT-2359.

3.4 MRT-2359 inhibits the expression of CD276 in NSCLC cells

Transcriptome sequencing revealed that treatment of NSCLC cell lines (A549, H520, H292) with MRT-2359 downregulated the transcriptional expression of CD276. Therefore, RT-qPCR detection found that after treatment with MRT-2359 (3 μmol/L), the mRNA level of CD276 in NSCLC cells was reduced (Figure 4A-C). In addition, we used Western blot experiments to detect the protein expression levels of several common immune checkpoints (PD-L1, CD47, CD200, CD39, HVEM, and CD73) in NSCLC cell lines (A549, H520, H292) after 72 h of treatment with MRT-2359 (3 μmol/L). We found that only the protein expression level of CD276 was consistently decreased after MRT-2359 treatment, while the other immune checkpoints did not show a clear unified trend of change (Figure 4D-F).

3.5 MRT-2359 suppresses tumor growth in vivo

We established a mouse lung cancer xenograft model using Lewis cells. Each group comprised three mice. After tumor formation, we administered MRT-2359 via oral gavage at 10 mg/kg. The control group received a saline solution containing 10% DMSO and 20% SBE-β-CD. We dosed the mice every two days for a total of four administrations. The results showed that MRT-2359 treatment significantly inhibited tumor growth in mice compared to the control group (Fig. 5A). Immunohistochemistry revealed that MRT-2359 administration reduced GSPT1 and CD276 expression within tumors and promoted GZMB infiltration (Fig. 5B).



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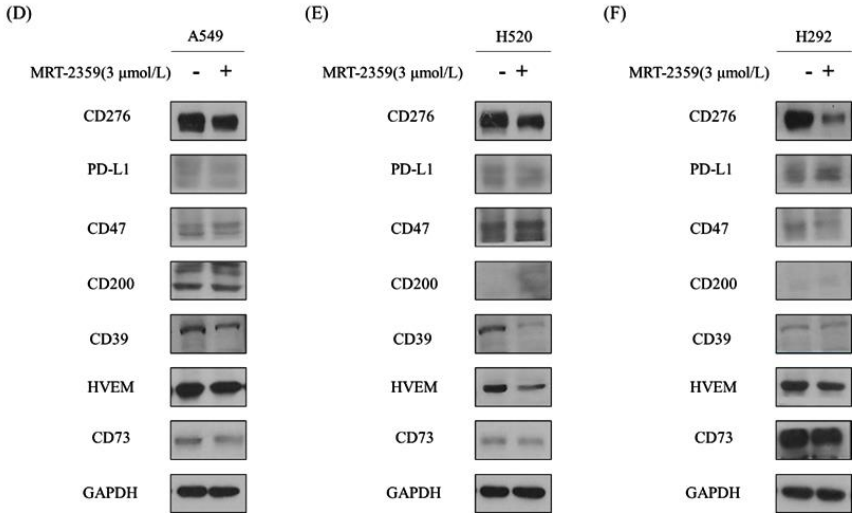
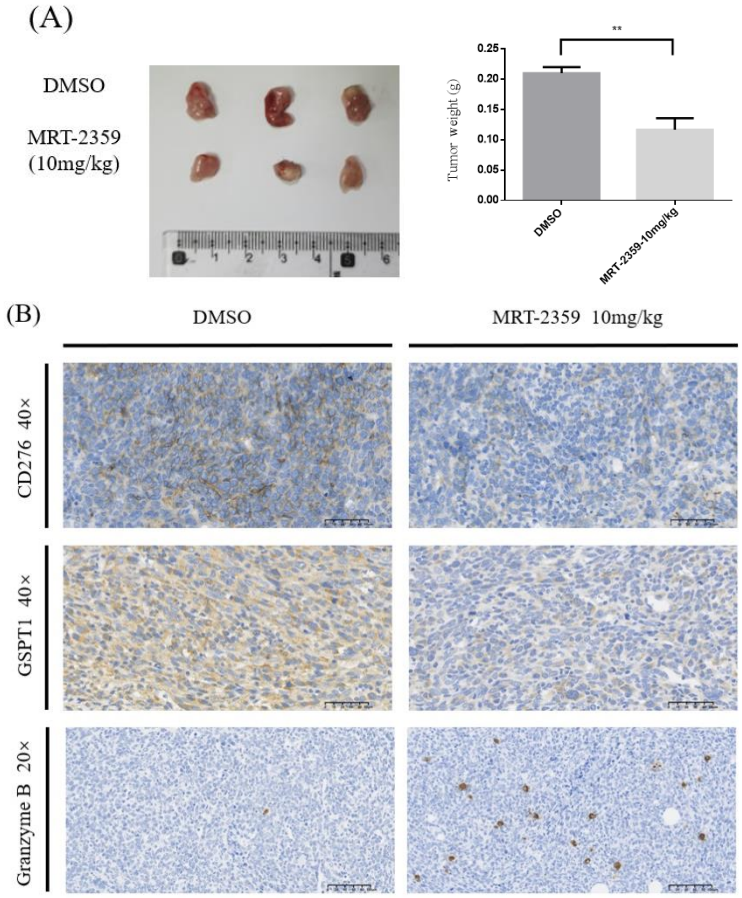


A-C: RT-qPCR was used to measure the mRNA levels of CD276 in lung cancer cells (A549, H520, H292) after 72 hours of treatment with MRT-2359 compared to their respective control groups (, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001).D-F: Western Blot was performed to assess the changes in the expression levels of immune checkpoint proteins after 72 hours of treatment with MRT-2359 in NSCLC cell lines (A549, H520, H292).*

Figure 4: Downregulation of CD276 expression in human non-small cell lung cancer (NSCLC) cells by MRT-2359 treatment.



A: Reduction in tumor volume in mice after treatment with MRT-2359 (, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001).B: Immunohistochemical staining of tumor tissue CD276 ,GSPT1 and Granzyme B.*

Figure 5: Inhibition of Tumor Growth In Vivo by MRT-2359.

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4. Discussion

GSPT1, a translation termination factor that encodes eRF3a, recognizes stop codons and promotes the release of nascent peptide chains from ribosomes[2, 3]. It plays a key role in diseases like AML [12]and MYC-driven lung cancer, and it also promotes NSCLC cell proliferation and migration by activating nicotinic acetylcholine receptors[13]. Bioinformatics analysis via GEPIA confirmed that GSPT1 is highly expressed in lung cancer tissues and linked to poor patient prognosis.

MRT-2359 degrades GSPT1 by promoting a CRBN-GSPT1 complex [10] .After GSPT1 degradation by MRT-2359, the cell's translation termination is impaired, leading to a build-up of truncated proteins, activation of the ISR, and accumulation of ATF3/4, which triggers apoptosis[14]. Cells with high translation activity, like cancer cells, are more affected by GSPT1 degradation. Research shows MRT-2359 suppresses drug-resistant NSCLC and SCLC cell growth, with enhanced activity in MYC-driven cell lines[11]. In this study, MRT-2359 treatment led to a significant degradation of GSPT1 protein levels in NSCLC cells. Moreover, co-culture experiments revealed that IFN-γ and IL-2 expression increased when PBMC were co-cultured with MRT-2359-treated NSCLC cells, indicating reduced immune evasion.

Transcriptomic analysis showed that MRT-2359 downregulates CD276 expression in NSCLC cells. CD276 inhibits CD4+ and CD8+ T cell proliferation and reduces IL-2 and IFN-γ[15, 16]production by suppressing NF-κB and AP-1 signaling pathways. It also inhibits NK cell activity[17, 18]. Tumor stem cells use CD276 to evade immune surveillance in head and neck squamous cell carcinoma[19-20]. Additionally, CD276 has non-immune functions like promoting migration, invasion, anti-apoptosis, and EMT. Bioinformatics analysis from TCGA confirmed a positive correlation between GSPT1 and CD276. Thus, we speculate that MRT-2359 inhibits immune evasion by downregulating CD276.

5. Conclusions

Our study proves GSPT1 is highly expressed in lung cancer tissues. MRT-2359 can degrade GSPT1 in NSCLC cells. Through RNA-seq, we propose that MRT-2359 downregulates CD276 to suppress immune evasion. In vivo experiments showed MRT-2359 inhibits tumor growth in mice. Our findings offer valuable insights for treating NSCLC patients with MRT-2359.

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