

Exosomal miR-3681-3p from M2-polarized macrophages confers cisplatin resistance to gastric cancer cells by targeting MLH1

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Received August 12, 2024; Accepted January 7, 2025

DOI: 10.3892/mmr.2025.13459

Abstract. Cisplatin (DDP) is a key chemotherapeutic agent in the treatment of gastric cancer; however, its efficacy is often limited by chemoresistance, a notable challenge in clinical oncology. The present study aimed to investigate the influence of exosomes derived from M2-polarized macrophages, which promote this resistance, on the response of gastric cancer cells to DDP, examining both the effects and the underlying mechanisms. M2 macrophages, differentiated from mouse bone marrow cells with interleukin (IL)-13 and IL-4, were identified using immunofluorescence staining for CD206 and CD163. Exosomes derived from these macrophages were characterized using transmission electron microscopy and protein markers, including calnexin, tumor susceptibility gene 101 and CD9. The role of exosomal microRNA (miR)-3681-3p in DDP resistance was assessed using Cell Counting Kit-8 and apoptosis assays, while a luciferase reporter assay was used to elucidate the interaction between miR-3681-3p and MutL

protein homolog 1 (MLH1). Co-culturing gastric cancer cells with M2 macrophages enhanced DDP resistance, an effect amplified by exosomes from M2 macrophages enriched with miR-3681-3p. This microRNA directly targeted and reduced MLH1 protein expression. Overexpression of miR-3681-3p through mimic transfection, along with MLH1 silencing by small interfering RNA transfection, significantly increased DDP resistance, as evidenced by elevated IC₅₀ values in AGS cells. By contrast, the overexpression of MLH1 effectively reversed the drug resistance of AGS cells to DDP caused by miR-3681-3p mimic transfection, as evidenced by a decrease in the IC₅₀ value. In conclusion, exosomal miR-3681-3p from M2 macrophages may have a key role in conferring DDP resistance to gastric cancer by suppressing MLH1, offering a new therapeutic target for overcoming chemoresistance.

Introduction

Gastric cancer is a prevalent malignant tumor and >1,000,000 new cases were diagnosed in 2020. Notably, gastric cancer ranks as the fifth most common cancer in terms of incidence and the fourth leading cause of cancer-related mortality worldwide (1). Despite advancements in treatment modalities, the overall prognosis remains poor, highlighting the urgent need for novel therapeutic strategies (1,2). This type of cancer originates from the gastric mucosa, and exhibits diverse epidemiological patterns, risk factor profiles and molecular subtypes influenced by geographical variability (3-5). Current therapeutic options, including surgical resection, chemotherapy, targeted agents and immunotherapy, have demonstrated limited success in improving the outcome of patients, especially in cases of metastatic gastric cancer (6-9). The emergence of chemoresistance,

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Key words: gastric cancer, microRNA-3681-3p, M2 macrophages, cisplatin resistance, MLH1

particularly to platinum-based compounds such as cisplatin (DDP), severely restricts the effectiveness of standard treatments (10). Consequently, there is a need for in-depth research into the underlying mechanisms of resistance and the development of innovative therapeutic strategies. Such advancements are key for enhancing treatment response, and tailoring more efficacious and individualized interventions for patients with gastric cancer.

In the contemporary landscape of oncology, where targeted therapy and immunotherapy have become the basis of cancer treatment, research into immune cells within the tumor microenvironment (TME) is increasingly pivotal (11-13). Macrophages, as key immune effector cells, have a dual role in the TME, both influencing and being influenced by tumor dynamics (14). Macrophages are broadly classified into two phenotypes: Classically activated macrophages (M1), which enhance cytokine production and exert antitumor effects, and alternatively activated macrophages (M2), which are associated with tumor promotion and immunosuppression (15). In most solid tumors, including gastric cancer, tumor-associated macrophages (TAMs) are predominantly skewed towards the M2 phenotype (16). The density and polarization state of TAMs are intricately associated with essential oncogenic processes, such as metastasis, invasion and chemoresistance (16,17).

Exosomes, a subset of extracellular vesicles, have a key role in intercellular communication by transporting bioactive molecules, including microRNAs (miRNAs/miRs), lipids and proteins (18). Previous studies have suggested that stromal cells in the TME, particularly TAMs, can modulate tumor cell behavior and facilitate oncogenesis through exosome secretion (19,20). miRNAs, as a significant component of exosomes, are encapsulated within a bilipid layer that protects them from enzymatic degradation, ensuring their stability during transport to recipient cells (20). Upon delivery, these miRNAs can regulate gene expression by binding to target mRNAs, resulting in mRNA degradation or translational repression (19,21,22).

The present study aimed to investigate the role and underlying mechanisms of exosomes derived from M2-polarized macrophages in modulating DDP resistance in gastric cancer cells, using a well-established model of M2-polarized macrophages to mimic TAM functions in the TME (23,24). Targeting exosomal miR-3681-3p derived from M2-polarized macrophages may present a promising therapeutic strategy to overcome DDP resistance in gastric cancer, offering a potential pathway for increasing treatment efficacy.

Materials and methods

Cell culture and treatment. AGS gastric cancer cells (cat. no. CRL-1739; American Type Culture Collection) were cultured in DMEM (cat. no. 11965092, Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (cat. no. 16140071; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (cat. no. 15140122; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere with 5% CO₂. Primary mouse bone marrow cells (MBMCs; cat. no. CP-M131; Wuhan Pricella Life Technology Co. Ltd.), were maintained in DMEM/F-12 (cat. no. 11330032; Gibco; Thermo Fisher Scientific, Inc.) enriched with 10% FBS and macrophage colony-stimulating factor (cat. no. HY-P7085A;

MedChemExpress) at 37°C and 5% CO₂. For M2 polarization, MBMCs were treated with interleukin (IL)-4 (30 ng/ml; cat. no. 214-14-1MG) and IL-13 (30 ng/ml; cat. no. 210-13-1MG) (both from Gibco; Thermo Fisher Scientific, Inc.) for 48 h at 37°C. Co-culture assays were conducted using a Transwell system, wherein 5x10⁴ M2-polarized macrophages were seeded into 12-well plate inserts (upper chamber), and 1.5x10⁵ AGS cells were grown in the lower compartment at 37°C for 3 days.

Immunofluorescence. A total of 2x10⁴ M2-polarized macrophages were cultured in 35-mm confocal dishes for 48 h, followed by fixation using 4% paraformaldehyde for 20 min at room temperature. After fixation, the cells were washed three times for 5 min with PBS and were then permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature (RT). Blocking was achieved by incubating the cells with 3% BSA (cat. no. 23225; Thermo Fisher Scientific, Inc.) in PBS for 1 h at RT. Subsequently, the cells were incubated overnight at 4°C with primary antibodies targeting CD206 (1:200 dilution; cat. no. ab64693; Abcam), CD163 (1:50 dilution; cat. no. ab316218; Abcam), CD80 (1:200 dilution; cat. no. ab315832; Abcam) and CD86 (1:100 dilution; cat. no. ab239075; Abcam). This was followed by incubation with fluorophore-conjugated secondary antibodies (1:200 dilution; cat. no. A27039; Thermo Fisher Scientific, Inc.) for 2 h at room temperature. After three additional washes with Tris-buffered saline containing 0.1% Tween-20, the nuclei were stained with DAPI (cat. no. D1306; Thermo Fisher Scientific, Inc.) for 10 min at 37°C to facilitate nuclear visualization. Imaging was carried out using a Carl Zeiss LSM 510 META confocal microscope (Zeiss GmbH) equipped with a Plan Aplanachromat 63x oil immersion objective featuring a numerical aperture of 1.4 and differential interference contrast capability.

Detection of inflammatory factors. Pro-inflammatory cytokine concentrations in the culture supernatants of M2-polarized macrophages were quantitatively evaluated using enzyme-linked immunosorbent assay (ELISA). A total of 1x10⁴ M2 macrophages were plated in 24-well plates and cultured for 48 h. Subsequently, the supernatants were collected and stored at -80°C until analysis. Levels of IL-2 (cat. no. JM-02981M1), IL-10 (cat. no. JM-02459M2), IL-6 (cat. no. JM-02446M1) and tumor necrosis factor- α (TNF- α ; cat. no. JM-02415M2) were determined using specific ELISA kits (Jiangsu Jingmei Biotechnology Co., Ltd.). All procedures were carried out according to the manufacturer's instructions.

Cell proliferation and viability assay. AGS cells were cultured in 96-well plates at a concentration of 1x10³ cells/well overnight, followed by incubation with 1 μ g/ml DDP for 1, 2, 3, 4 and 5 days at 37°C. At the end of each timepoint, 10 μ l Cell Counting Kit-8 (CCK-8) solution (cat. no. C0039; Beyotime Institute of Biotechnology) was added to each well, and the plates were incubated at 37°C for 2 h to allow the reaction to occur. Absorbance at 450 nm was measured using an Eppendorf BioPhotometer® D30 (Eppendorf SE). Cell proliferation was quantitatively determined based on the absorbance readings collected over a 5-day period.

In addition, AGS cells were cultured in 96-well plates at a concentration of 1×10^3 cells/well overnight, and were then incubated with 0.5, 1.0, 1.5 and 2.0 μM DDP for 48 h at 37°C . Then, 10 μl CCK-8 solution was added to each well, and the plates were incubated at 37°C for 2 h to allow the reaction to occur. Absorbance at 450 nm was measured using an Eppendorf BioPhotometer D30. IC_{50} values were calculated using non-linear regression in GraphPad Prism (version 8.0; Dotmatics).

Flow cytometry. The apoptosis of AGS cells was quantified using the Annexin V-FITC and PI staining kit (cat. no. G1511; Wuhan Servicebio Technology Co., Ltd.), according to the manufacturer's protocol. Analysis was carried out using a Beckman DX1800 flow cytometer (Beckman Coulter, Inc.). The identification and quantification of apoptotic cells, indicated by PI positivity, were conducted with FlowJo software (version 10.8; BD Biosciences). The apoptotic rate was calculated by summing the percentages of cells in both early and late stages of apoptosis.

Isolation of exosomes and electron microscopy. M2-polarized macrophage supernatants were collected and centrifuged at $2,000 \times g$ for 30 min at 4°C . Following centrifugation, the supernatant was filtered through a $0.22\text{-}\mu\text{m}$ syringe filter (cat. no. SLGV13SL; MilliporeSigma) and subjected to ultracentrifugation at $120,000 \times g$ overnight at 4°C using an Optima XPN-100 ultracentrifuge (cat. no. CP100MX; Hitachi, Ltd.) to precipitate exosomes. The exosomes were then resuspended in cold PBS (pH 7.4) and further purified by ultracentrifugation at $120,000 \times g$ for 90 min at 4°C . The purified exosomes were suspended in cold PBS or SDS loading buffer (cat. no. P0015F; Beyotime Institute of Biotechnology; containing 4% SDS and 100 mM Tris-HCl; pH 7.6) and stored at -80°C for further studies. For the *in vitro* experiments, exosomes were used to treat cells at a concentration of 50 μg protein/ 10^5 cells at 37°C for 48 h.

For transmission electron microscopy, freshly isolated exosome suspensions were fixed in 4% paraformaldehyde for 1 h at 4°C . Subsequently, 20 μl exosome solution was placed onto a copper grid, excess fluid was removed, and the sample was stained with 2% phosphotungstic acid for 40 sec at room temperature to enhance contrast. Visualization of the exosomes was carried out using a transmission electron microscope (model HT-7700; Hitachi, Ltd.). In addition, the exosome supernatant was denatured with 5X SDS buffer and analyzed by western blotting. Protein samples (50 μg /lane) were separated by SDS-PAGE on a 10% SDS-polyacrylamide gel and were probed with rabbit antibodies targeting calnexin (1:1,000 cat. no. ab133615; Abcam), tumor susceptibility gene 101 (TSG101; 1:1,000; cat. no. 102286-T38; Beijing Sino Technology Co. Ltd.) and CD9 (1:1,000; cat. no. ab92726; Abcam). The detailed western blotting procedures are described in the western blotting subsection.

Nanoparticle tracking analysis (NTA). M2-polarized macrophage-extracted exosomes were diluted to a total volume of 1 ml using Tris-phosphate-modified saline for subsequent analysis. To assess the size distribution of the exosomes, NTA was carried out using a NanoSight (model, N30E; NanoFCM,

Inc.) instrument. This analysis adhered to the established standard operating procedure for NTA (25), enabling precise measurement of vesicle size based on the Brownian motion of individual particles.

Western blotting. Proteins were extracted from AGS cells using radioimmunoprecipitation assay lysis buffer (cat. no. HY-K1001; MedChemExpress), and their concentration was measured using the BCA Protein Assay Kit (cat. no. 23225; Thermo Fisher Scientific, Inc.). For SDS-PAGE, 10 μg each protein lysate was combined with 5X SDS sample buffer and separated on a 12% SDS-polyacrylamide gel. Post-electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane. To minimize non-specific interactions, the membrane was blocked with 5% non-fat milk for 2 h at RT. The membrane was then probed overnight at 4°C with the following primary antibodies: Rabbit anti-MLH1 (1:2,000; cat. no. ab92312; Abcam) and mouse anti-GAPDH (1:5,000; cat. no. ab8245; Abcam). After incubation with primary antibodies, the membrane was rinsed and exposed to HRP-conjugated secondary antibodies (1:10,000; cat. nos. ab6721 and ab205719; Abcam) for 2 h at RT. Protein bands were visualized using enhanced chemiluminescence western blot detection reagents (Thermo Fisher Scientific, Inc.). ImageJ software (version 1.53a; National Institutes of Health) was used for semi-quantification of western blot analysis.

miR-3681-3p mimic and MLH1 small interfering (si)RNA transfections. The miR-3681-3p mimic (sense: 5'-ACA CAGUGCUUCAUCCACUACU-3'; antisense: 5'-AGUAGU GGAUGAAGCACUGUGU-3') and its negative control (NC: sense: 5'-UUUGUACUACACAAAAGUACUG-3'; antisense: 5'-CAGUACUUUUGUGUAGUACAAA-3') were synthesized by Shanghai GenePharma Co., Ltd. Transfection of AGS cells with the miR-3681-3p mimic was carried out using Oligofectamine™ reagent (cat. no. 12252-011; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. This involved 24-h serum-free pre-treatment of AGS cells, followed by transfection with a final concentration of 50 nM miR-3681-3p mimic or NC for 48 h. Transfection efficiency was subsequently assessed using reverse transcription-quantitative PCR (RT-qPCR).

The MLH1 siRNA (sense: 5'-CAGCUAAUGCUAUCA AAGAGA-3'; antisense: 5'-UCUCUUUGAUAGCAUUA GUG-3') and its scrambled NC siRNA (sense: 5'-UUCUCC GAACGAGUCACGUTT-3'; antisense: 5'-ACGUGACUC GUUCGGAGAA-3') were also synthesized by Shanghai GenePharma Co., Ltd. The transfection procedure for MLH1 siRNA followed the protocol used for the miR-3681-3p mimic.

RT-qPCR. Total RNA was extracted from exosomes derived from M2 macrophages or AGS cells using the RNAiso Plus reagent (cat. no. 9109; Takara Bio, Inc.). The isolated RNA was then reverse-transcribed into cDNA using the Bestar™ qPCR RT kit (cat. no. DBI-2220; DBI Bioscience). RT followed a defined thermal profile: 5 min at 25°C , 30 min at 42°C and a final step at 85°C for 5 min. qPCR was conducted to assess the expression levels of target genes using Bestar qPCR MasterMix (cat. no. DBI-2043; DBI Bioscience) on the Agilent Mx3000P system (cat. no. Mxpro-Mx3000P; Agilent

Technologies, Inc.). The qPCR amplification used a two-step protocol: 30 sec at 95°C for initial denaturation, followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C. Relative expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method (26), with normalization of miR-3681-3p expression to U6 snRNA levels. The specific primer sequences used for amplification were as follows: miR-3681-3p forward, 5'-CGCGACACAGTGCTT CATCC-3', reverse, 5'-AGTAGTGGATGAAGCACTGT-3'; and U6 snRNA forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

Target identification and dual-luciferase reporter assay. RNAhybrid v2.2 online software (<https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid>) was used for miR-3681-3p target prediction (27). Wild-type and mutant MLH1 gene 3'-untranslated regions (3'-UTRs) were cloned into the pmirGLO dual-luciferase reporter vector (cat. no. E1910; Promega Corporation). Briefly, 3×10^5 293T cells (cat. no. CL-0005; Wuhan Pricella Life Technology Co. Ltd.) underwent co-transfection with these vectors alongside either the miR-3681-3p mimic or NC mimic using the HB-TRLF-1000 LipoFiter transfection kit (cat. no. HB-TRLF-1000; Hanbio Biotechnology Co., Ltd.). Following a 48-h incubation period at 37°C, cell lysates were collected, and luciferase activity was measured according to the instructions of the Dual-Luciferase Reporter Assay System (cat. no. E1910; Promega Corporation). Luminescence was quantified using a Lux-T020 microplate reader. The activation of the reporter gene was evaluated by calculating the normalized luminescence data, expressed as the ratio of firefly to *Renilla* luciferase activities.

Vector construction and lentiviral transduction. A lentiviral vector engineered for MLH1 overexpression was created using human MLH1 genetic sequences obtained from the NCBI GenBank (Gene Bank ID: FJ940753.1, accessible at <https://www.ncbi.nlm.nih.gov/nuccore/FJ940753.1>) by Shanghai GeneChem Co., Ltd. The coding sequence of MLH1 was inserted into the GV358 vector to yield the MLH1 overexpression vector. An empty GV358 vector was used in the NC group.

AGS cells were cultured in 6-well plates at 37°C for 36 h. Once they had reached ~70% confluence, each well was uniformly exposed to equivalent volumes (100 μ l) of MLH1-overexpressing lentiviral particles or NC (empty vector) lentiviral particles at a multiplicity of infection of 10 for 48 h at 37°C. After 48 h, the AGS cells were harvested for western blotting and the CCK-8 assay.

Statistical analysis. Quantitative data from the experiments were analyzed using GraphPad Prism software (version 8.0; Dotmatics). All experimental protocols were carried out in triplicate to ensure reproducibility and accuracy. Data are presented as the mean $\pm$ standard deviation. Statistical analysis was performed using the unpaired two-tailed Student's t-test for comparisons between two distinct groups. For multiple group comparisons, one-way ANOVA was applied, followed by Tukey's multiple comparisons test for post hoc analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Co-culturing gastric cancer cells with M2-polarized macrophages increases resistance to DDP. To investigate how M2-polarized macrophages affect the resistance of gastric cancer cells to DDP, MBMCs were treated with IL-13 and IL-4 to promote M2 polarization. These M2 macrophages were characterized by the presence of specific M2 markers, such as CD206 and CD163, and the lack of M1 markers, such as CD80 and CD86, verified through immunophenotyping (Fig. 1A). Additionally, an increase in M2-associated cytokines, including IL-2, IL-6, IL-10 and TNF- α was observed in these cells compared with in resting macrophages (Fig. 1B). In subsequent experiments, DDP treatment significantly reduced the proliferation and increased the apoptosis of AGS gastric cancer cells when compared with the untreated control. However, co-culture with M2-polarized macrophages attenuated the cytotoxic effects of DDP, suggesting that M2 macrophages may confer resistance to DDP in AGS cells (Fig. 1C and D).

M2-polarized macrophage-derived exosomes contribute to DDP resistance in gastric cancer cells. Electron microscopy analysis of the M2-polarized macrophage-derived supernatant revealed the presence of small vesicles consistent with exosomes, characterized by a 50-100 nm diameter (Fig. 2A and B). The exosome fraction was enriched with established markers, including the endoplasmic reticulum protein calnexin, TSG101 and CD9, as confirmed by western blotting (Fig. 2C). Notably, exosomes derived from M2 macrophages significantly promoted the proliferation and inhibited the apoptosis of DDP-treated AGS cells (Fig. 2D and E). Additionally, the expression levels of miR-3681-3p were markedly higher in exosomes from M2 macrophages than those from resting macrophages (Fig. 2F). These findings suggested that M2-polarized macrophages may mitigate the cytotoxic effects of DDP on AGS cells through the release of exosomes enriched with miR-3681-3p.

miR-3681-3p increases the DDP resistance of gastric cancer cells by downregulating MLH1. Potential target genes of miR-3681-3p were identified using the RNAhybrid v2.2 online software. Analysis indicated that miR-3681-3p may target the gene encoding MLH1 (Fig. 3A). To validate this interaction, luciferase reporter vectors were constructed containing either wild-type or mutant forms of the MLH1 3'-UTR. Transfection with the miR-3681-3p mimic resulted in a significant reduction in luciferase activity associated with the wild-type MLH1 3'-UTR compared with the miR-NC. This suppressive effect was absent in the mutated MLH1 3'-UTR construct, confirming the specificity of the interaction (Fig. 3B). To further validate whether miR-3681-3p targets and regulates the expression of MLH1, the expression of miR-3681-3p was increased in AGS cells by transfection with a miR-3681-3p mimic (Fig. 3C). Moreover, miR-3681-3p mimic transfection substantially decreased the expression levels of MLH1 protein (Fig. 3D). Additionally, knockdown of MLH1 protein expression was achieved by siRNA transfection (Fig. 3E). Functional assays demonstrated that both miR-3681-3p mimic transfection and MLH1 silencing by siRNA rescued the

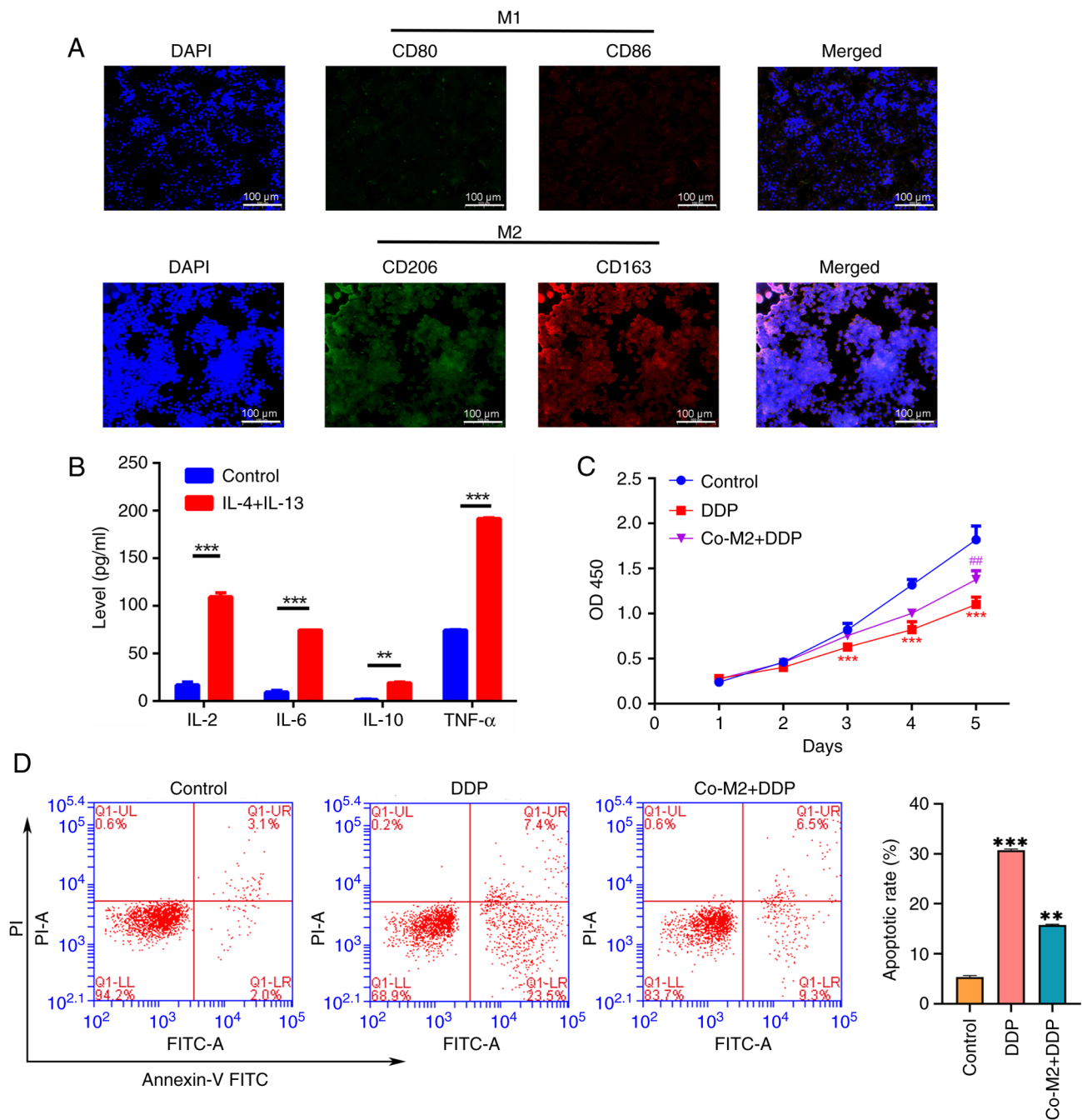


Figure 1. Co-culturing AGS gastric carcinoma cells with M2-polarized macrophages results in an increased resistance to DDP. (A) M2 macrophages, differentiated from murine bone marrow-derived precursors via stimulation with IL-13 and IL-4, were identified by immunofluorescence staining. (B) Levels of IL-2, IL-6, IL-10 and TNF- α in the culture supernatants of M2-polarized macrophages were quantified using enzyme-linked immunosorbent assay. (C) Proliferative capacity of AGS cells in co-culture with M2-polarized macrophages was assessed using the Cell Counting Kit-8 assay. (D) Apoptotic rate of AGS cells in co-culture with M2-polarized macrophages was determined through flow cytometry. Quantitative data are presented as the mean $\pm$ standard deviation (n=3). **P<0.01 and ***P<0.001 vs. control group; ##P<0.01 vs. DDP group. DDP, cisplatin; IL, interleukin; OD, optical density; TNF- α , tumor necrosis factor- α ; Co-M2, co-culture with M2-polarized macrophages.

DDP-induced suppression of AGS cell proliferation (Fig. 3F). Moreover, both miR-3681-3p mimic transfection and MLH1 silencing contributed to the drug resistance of AGS cells to DDP, as evidenced by an increase in the IC₅₀ value from 21.69 to 53.35 and 45.92 μ M, respectively (Fig. 3G). Additionally, both miR-3681-3p overexpression and MLH1 knockdown significantly reduced the apoptotic rate of AGS cells exposed to DDP treatment (Fig. 4A). By contrast, the overexpression of MLH1 significantly reversed the drug resistance of AGS cells

to DDP caused by miR-3681-3p mimic treatment, as evidenced by a decrease in the IC₅₀ value from 59.08 to 27.17 μ M (Fig. 4B and C).

Discussion

Gastric cancer, characterized by unchecked proliferation of neoplastic cells in the gastric mucosa, is a leading cause of cancer-related mortality worldwide (2,9). Its high mortality rate

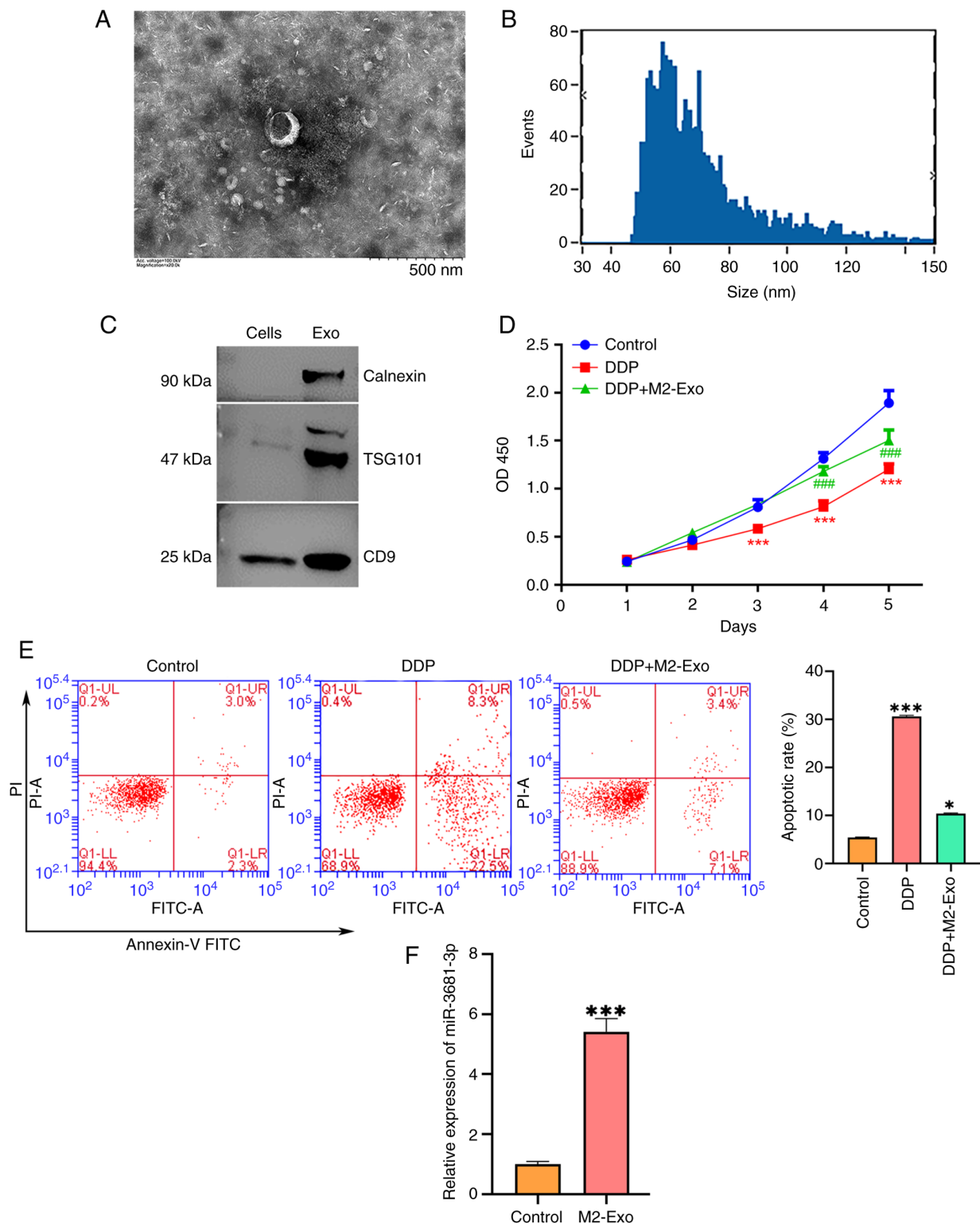


Figure 2. M2-polarized macrophage-derived exosomes increase DDP resistance in gastric cancer cells. (A) Transmission electron micrographs depicting exosomes isolated from M2-polarized macrophages. (B) NanoSight analysis was used to ascertain the size distribution of isolated exosomes in diameter. (C) Western blotting was used to detect the expression of proteins. (D) Proliferation of AGS cells treated with exosomes derived from M2-polarized macrophages was assessed using the Cell Counting Kit-8 assay. (E) Flow cytometry revealed the apoptotic rate of AGS cells treated with exosomes derived from M2-polarized macrophages. (F) Reverse transcription-quantitative PCR was used to measure the expression levels of miR-3681-3p in exosomes derived from M2-polarized macrophages. Quantitative data are presented as the mean $\pm$ standard deviation ($n=3$). * $P<0.05$ and *** $P<0.001$ vs. control group; ### $P<0.001$ vs. DDP group. DDP, cisplatin; Exo, exosomes; miR, microRNA; OD, optical density; TSG101, tumor susceptibility gene 101.

is largely attributed to late-stage diagnosis and high metastatic potential (7,9). DDP, the first platinum analog approved for clinical use in 1971, is extensively used as a first-line treatment

for gastric cancer, either alone or in combination with other chemotherapeutic agents (28-30). However, DDP-based therapies face notable limitations in certain patients, primarily due to

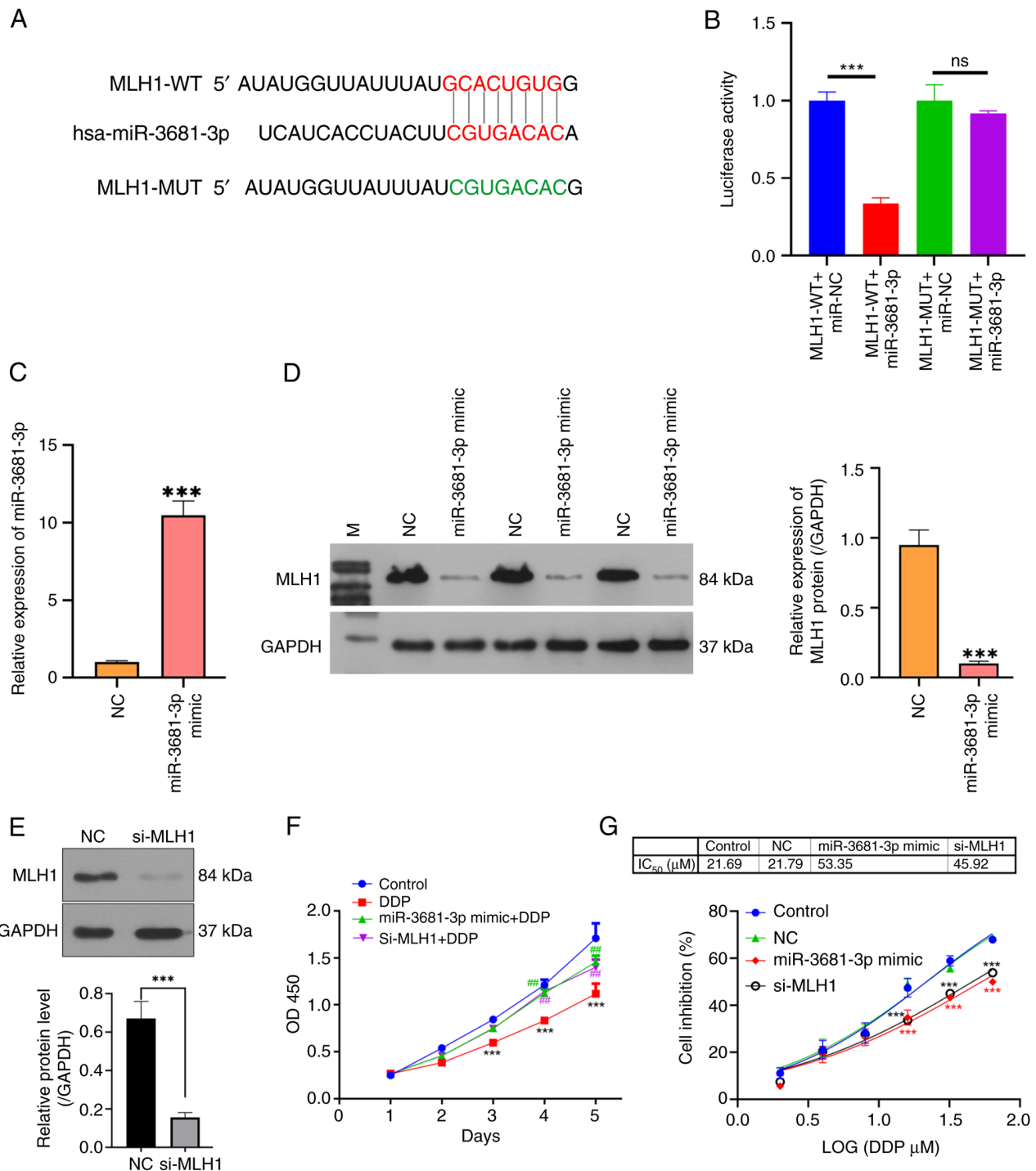


Figure 3. miR-3681-3p increases DDP resistance in gastric cancer cells by downregulating MLH1. (A) Bioinformatics analysis was utilized to predict the binding site of miR-3681-3p to MLH1. (B) Results of the dual-luciferase reporter assay assessing the interaction between miR-3681-3p and MLH1. *** $P < 0.001$. (C) Reverse transcription-quantitative PCR was used to measure the expression levels of miR-3681-3p in AGS cells after miR-3681-3p mimic transfection. (D) Western blot analysis was carried out to determine the protein levels of MLH1 in AGS cells transfected with the miR-3681-3p mimic. (E) Protein levels of MLH1 were detected by western blotting after si-MLH1 transfection. Grayscale analysis was conducted using ImageJ software. *** $P < 0.001$ vs. NC group. (F) Cell proliferation of miR-3681-3p mimic-transfected and si-MLH1-transfected AGS cells was assessed by CCK-8 assay. (G) The IC₅₀ value was calculated using GraphPad software based on CCK-8 assay results. Quantitative data are presented as the mean $\pm$ SD ($n = 3$). *** $P < 0.001$ vs. control group; ** $P < 0.01$ vs. DDP group. CCK-8, Cell Counting Kit-8; DDP, cisplatin; miR, microRNA; MLH1, MutL protein homolog 1; MUT, mutant; NC, negative control; ns, not significant; OD, optical density; si, small interfering; WT, wild-type.

adverse effects, such as nephrotoxicity, insufficient therapeutic efficacy and the development of tumor resistance (31,32).

In gastric cancer, considerable knowledge gaps exist regarding DDP resistance, particularly in understanding the specific mechanisms underlying this resistance in gastric cancer

cells. Factors such as genetic mutations, epigenetic alterations and the role of extracellular vesicles remain inadequately investigated (33-35). Additionally, there is a notable absence of reliable biomarkers to predict which patients may develop resistance to DDP, impacting the implementation of personalized

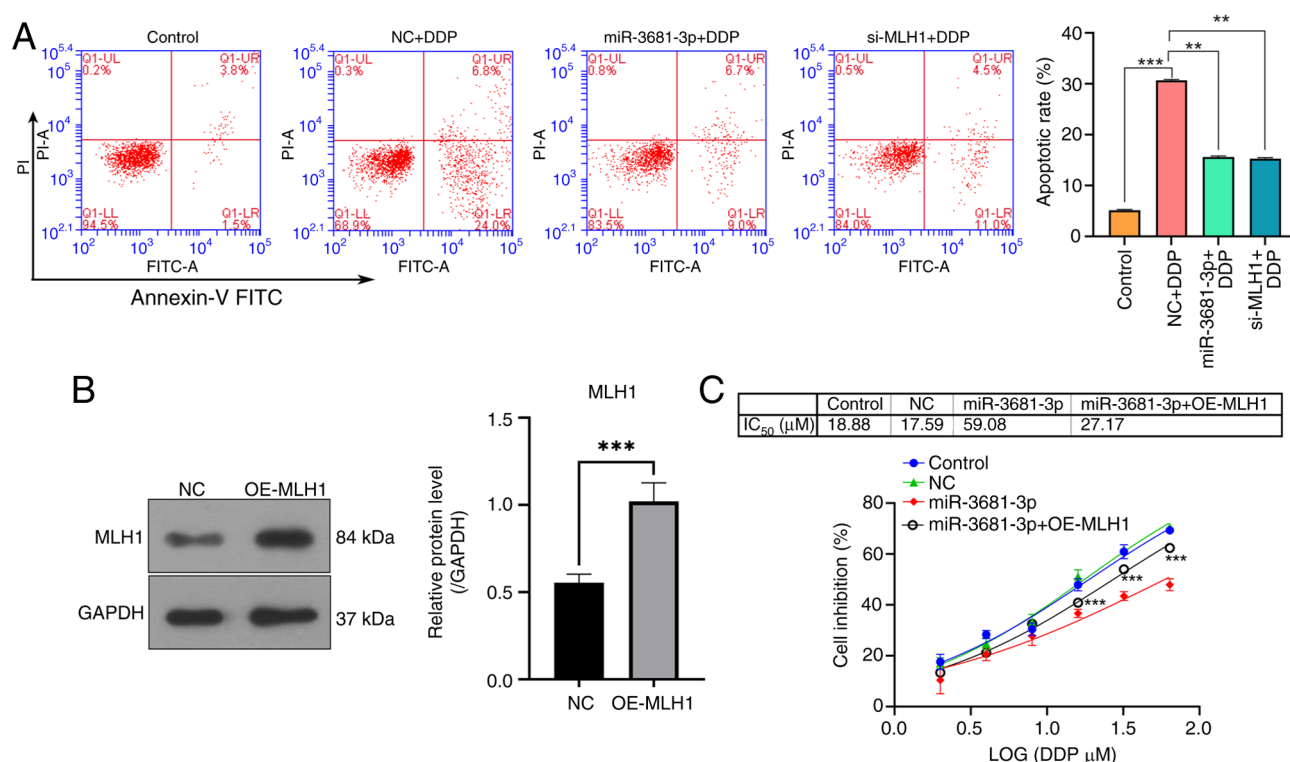


Figure 4. MLH1 OE reverses the drug resistance of AGS cells to DDP induced by a miR-3681-3p mimic. (A) Flow cytometry was used to evaluate the apoptotic rate of miR-3681-3p mimic-transfected and si-MLH1-transfected AGS cells. (B) Western blot analysis was conducted to assess the protein levels of MLH1 in AGS cells subjected to lentiviral transduction for MLH1 OE. Grayscale analysis was conducted using ImageJ software. (C) The IC₅₀ value was calculated using GraphPad software based on Cell Counting Kit-8 assay results. Quantitative data are presented as the mean $\pm$ SD (n=3). Statistical significance is indicated by **P<0.01 and ***P<0.001 vs. miR-3681-3p or as indicated. DDP; cisplatin; miR, microRNA; MLH1, MutL protein homolog 1, NC, negative control; OE, overexpression; si, small interfering.

therapy (36). While some new drugs and treatments are under investigation, effective options for managing drug-resistant gastric cancer are limited (37). Researchers are actively working to elucidate the molecular mechanisms of DDP resistance using advanced technologies, including genomics, transcriptomics and proteomics (38,39). Clinical trials are being conducted to assess new treatment combinations and alternative therapies aimed at overcoming DDP resistance (40,41). The identification of biomarkers of resistance through genomic analysis is key for developing personalized treatment plans and advancing precision medicine (42,43). In the near future, a wealth of new research findings is anticipated that will enhance the understanding of DDP resistance mechanisms, thereby laying the groundwork for innovative therapeutic strategies (44,45). Ongoing research holds promise for identifying effective biomarkers that can aid in selecting suitable patients for DDP therapy (46). Additionally, novel drugs and treatment combinations are likely to emerge in clinical practice, potentially improving outcomes for patients with DDP-resistant gastric cancer (47). Researchers are expected to strengthen interdisciplinary collaboration, merging basic and clinical research efforts to expedite the development and application of new therapies (48,49). Through these collective efforts, the aim is to effectively tackle the challenges posed by DDP resistance, and enhance treatment efficacy and survival rates for patients with gastric cancer.

Investigating the molecular mechanisms underlying DDP resistance is key for enhancing therapeutic efficacy. In the present study, the role of exosomal miR-3681-3p, derived from

M2-polarized macrophages, in fostering DDP resistance in gastric cancer cells was elucidated. This was achieved through the downregulation of MLH1 expression levels, providing novel mechanistic insights into the modulation of chemoresistance. The role of the miR-3681-3p/MLH1 axis indicates a promising therapeutic approach to overcome DDP resistance in gastric cancer.

M2 macrophages, which are involved in tissue repair and immunoregulation, significantly influence tumor progression by creating a supportive environment for cancer growth, angiogenesis and metastasis (50-52). These macrophages undergo metabolic reprogramming in the TME, supporting their survival and enhancing their immunosuppressive functions (24,53). M2 macrophages have been shown to mediate tumor resistance to various chemotherapeutic agents. For instance, M2 macrophages activate signaling pathways such as CXCR2, promoting resistance to sorafenib in hepatocellular carcinoma (54). In glioblastoma, hypoxia-driven M2 polarization facilitates cancer aggressiveness and resistance to temozolomide (55). Additionally, M2 macrophages modulate cholesterol homeostasis, suppress ferroptosis and support their own polarization, exacerbating hepatocellular carcinoma progression and contributing to drug resistance (56). In gastric adenocarcinoma, M2 macrophages have been reported to mediate resistance to 5-fluorouracil through the regulation of integrin β 3, focal adhesion kinase and cofilin expression (57). In the present study, M2 polarization of MBMCs was induced through the administration of IL-13 and IL-4. This induction resulted in the expression of M2-specific

markers (CD206 and CD163) and the upregulation of associated cytokines (IL-2, IL-6, IL-10 and TNF- α). Notably, the results of the present study indicated that co-culturing gastric cancer cells with M2-polarized macrophages increased resistance to DDP, suggesting an association between M2 polarization and DDP resistance.

M2 macrophages affect tumor cells either directly or by secreting exosomes containing specific miRNAs that modulate tumor behavior and immune responses (58-60). For example, exosomal miR-23a-3p from M2 macrophages has been shown to promote oral squamous cell carcinoma progression by inhibiting PTEN (58). Similarly, exosomes carrying miR-660-5p from M2 macrophages facilitate hepatocellular carcinoma development by downregulating KLF3 (59). Notably, exosomal miR-588 from M2-polarized macrophages has been implicated in DDP resistance in gastric cancer cells (60). These findings highlight the complex roles of M2 macrophages and their miRNA contents in driving tumor drug resistance, emphasizing the potential for therapeutic strategies targeting these mechanisms to enhance cancer treatment efficacy.

Previous studies have reported that miR-3681-3p is downregulated in lung adenocarcinoma and is associated with aggressive behaviors (61,62). However, the role and mechanism of miR-3681-3p in gastric cancer remain poorly understood. The present study revealed that exosomes derived from M2-polarized macrophages contributed to DDP resistance in gastric cancer cells, with a significant elevation of miR-3681-3p in these exosomes. Furthermore, miR-3681-3p mimic transfection significantly reduced the sensitivity of gastric cancer cells to DDP, resulting in an increased IC₅₀ value. These findings offer new insights into the key role of exosomes from M2-polarized macrophages in regulating cancer progression and highlight the novel function of miR-3681-3p in gastric cancer. The association between exosomal miR-3681-3p from M2 macrophages and its role in mediating drug resistance is an important scientific question that warrants further investigation. Understanding the mechanisms by which elevated miR-3681-3p contributes to DDP resistance in gastric cancer cells is essential for developing effective therapeutic interventions.

The MLH1 gene has a key role in maintaining genomic integrity through the mismatch repair mechanism, correcting errors during DNA replication (63). Deficiency or loss of MLH1 is associated with the initiation and progression of various types of cancer (64-66). Specifically, in colorectal carcinoma, MLH1-proficient cells were revealed to be less sensitive to 5-FU-induced cytotoxic effects (64). Moreover, MLH1 deficiency is associated with cetuximab resistance in colon cancer due to activation of the Her-2/PI3K/AKT signaling pathway (65). Notably, negative MLH1 expression was previously detected in 9.8% (28/285) of patients with gastric cancer, and was associated with chemoresistance and resulted in no improvement in survival after neoadjuvant chemotherapy (67). The present study revealed that miR-3681-3p directly targets MLH1. MLH1 downregulation significantly diminished the sensitivity of gastric cancer cells to DDP, as evidenced by an increased IC₅₀ value. By contrast, the overexpression of MLH1 effectively reversed the DDP resistance of AGS cells caused by miR-3681-3p mimic treatment, as indicated by the decrease

in the IC₅₀ value. These results confirmed that miR-3681-3p may directly target MLH1. Mechanistically, high levels of miR-3681-3p in exosomes from M2-polarized macrophages may promote DDP resistance by downregulating MLH1 expression. However, MLH1 is likely one of multiple targets of miR-3681-3p, suggesting the need for further investigation into additional regulatory factors involved in this pathway.

While the findings of the present study provided insights into the miR-3681-3p/MLH1 axis as a potential therapeutic target in gastric cancer, this study has some limitations. The interactions between miR-3681-3p and MLH1 require further validation through additional experiments. Moreover, the findings were derived exclusively from the AGS cell line, necessitating corroboration across diverse cancer cell lines to enhance their applicability. Future studies should also include animal models to comprehensively elucidate the role of the miR-3681-3p/MLH1 axis in gastric cancer treatment.

In conclusion, exosomal miR-3681-3p from M2 macrophages may contribute to DDP resistance in gastric cancer cells by suppressing MLH1 expression. The miR-3681-3p/MLH1 axis could be a promising molecular target for developing new therapeutic strategies for gastric cancer.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Scientific Research Project of High-Level Talents of Affiliated Hospital of Youjiang Medical University for Nationalities (grant no. R202011710), the Scientific Research Project of Young and Middle-Aged Key Talents of Affiliated Hospital of Youjiang Medical University for Nationalities (grant no. Y20212603), the Project of Open Subject of Guangxi Key Laboratory of Molecular Pathology of Hepatobiliary Diseases of Affiliated Hospital of Youjiang Medical University for Nationalities (grant no. GXZDSYS-009), the Project of Scientific Research and Technology Development Plan of Baise City (grant nos. Encyclopedia 20213301, Encyclopedia 20213242, Encyclopedia 20232080), Self-Funded Scientific Research Projects of Guangxi Health and Health Commission (grant nos. Z20211114 and Z20190202), Self-Funded Scientific Research Projects of the Administration of Traditional Chinese Medicine of the Guangxi Zhuang Autonomous Region (grant no. GXZYL20220304) and Scientific Research Basic Ability Enhancement Project for Young and Middle-Aged Teachers of Guangxi Colleges And Universities (grant no. 2021KY0538).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

WJW and ZYC conceptualized and designed the study. WJW, JXL, CL and RTH engaged in the acquisition, analysis and interpretation of the data. JJH, QJ and JZW contributed to the

interpretation of the data, along with manuscript drafting and finalization. QL and GDX performed the formal analysis. ZYC secured funding. WJW and ZYC confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The protocol of the present study was approved by the Medical Ethics Committee of Affiliated Hospital of Youjiang Medical University for Nationalities (approval no. YYFY-LL-2024-283).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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