

# Inhibition of miR-18a-3p reduces proliferation of mesothelioma cells and sensitizes them to cisplatin

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**Abstract.** Malignant pleural mesothelioma is a notorious human malignancy. Despite combination chemotherapy with cisplatin and pemetrexed, the majority of patients with advanced malignant pleural mesothelioma have a poor prognosis. MicroRNAs (miRNAs/miRs) are short non-coding RNAs that regulate various biological processes by binding to the 3'-untranslated region of target gene mRNAs and suppressing their expression. Since abnormal expression patterns of miRNAs are a common feature in human malignancies, a number of them have been researched as potential therapeutic targets. Our previous study demonstrated that microRNA-18a (miR-18a) is upregulated in mesothelioma cell lines compared with in non-neoplastic mesothelial tissues, but its function remains unclear. In the present study, miRNA inhibitor was transfected into mesothelioma cell lines and then analyzed various cellular functions. Mesothelioma cells transfected with the miR-18a inhibitor exhibited lower proliferation and migration rates compared with cells transfected with a negative control inhibitor in proliferation and wound scratch assays, respectively. Additionally, the present study revealed that downregulation of miR-18a increased mesothelioma cell apoptosis. In a chemosensitivity assay, transfection of the miR-18a inhibitor significantly increased the sensitivity of mesothelioma cells to cisplatin but not to pemetrexed. Therefore, miR-18a may be a potential therapeutic target for mesothelioma resistant to cisplatin.

## Introduction

Malignant pleural mesothelioma, an aggressive tumor, is commonly caused by exposure to asbestos with a median latency of 22.8 years (1). The incidence of malignant mesothelioma is predicted to reach a peak around 2020 in Europe (2) and around 2030 in Japan (3), and has not yet declined this century in the United States (4). The incidence of malignant mesothelioma in developing countries is predicted to increase due to their heavy use of asbestos without restriction (5).

Because many cases of malignant pleural mesothelioma are detected at an advanced stage and are thus unresectable by surgery, systemic anticancer chemotherapy is the first choice of treatment. The present standard regimen of chemotherapy for advanced malignant pleural mesothelioma is a combination of pemetrexed and cisplatin; however, the median survival of patients treated with this regimen scarcely exceeds 12 months (6).

MicroRNAs (miRNAs) are short non-coding, single-strand RNAs that suppress gene expression by binding to the 3'-untranslated region of their target mRNAs (7). Recently, several studies reported different miRNAs that play important roles in the pathogenesis of various human cancers as onco-miRNAs or tumor suppressor miRNAs (8-10).

In a previous study, we investigated the expression profiles of miRNAs in mesothelioma cell lines and found some to be significantly down- or upregulated (11). In that study, microRNA-18a (miR-18a) was one of the upregulated miRNAs. Further, miR-18a has been identified as an onco-miRNA associated with many human malignancies including glioblastoma (12), esophageal cancer (13), and non-small cell lung cancer (14). The current study was performed to elucidate the biological function of miR-18a in mesothelioma.

## Materials and methods

**Mesothelioma cell lines.** Four human mesothelioma cell lines were used in this study. Two cell lines (ACC-MESO1 and ACC-MESO4) were purchased from the RIKEN BioResearch Center (Tsukuba, Japan) (15) and the other two (CRL-5915 and CRL-5946) were purchased from the American Type Culture Collection. Cells were cultured with Roswell Park Memorial

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Institute 1640 medium with GlutaMax added, containing 10% fetal bovine serum, sodium pyruvate, kanamycin, and fungizone (all purchased from Thermo Fisher Scientific). Cells were maintained in a 5% CO<sub>2</sub> incubator at 37°C.

**Transient transfection of mesothelioma cells.** The miR-18a inhibitor (mirVana; has-miR-18a-3p MH12264) and a negative control miRNA inhibitor (mirVana; negative control #1) were purchased from Thermo Fisher Scientific. Mesothelioma cells at 60-80% confluence were transfected with 50 nM of the miR-18a or negative control inhibitor using Lipofectamine RNAiMAX (Thermo Fisher Scientific) in Opti-Mem Reduced Serum Medium (Thermo Fisher Scientific) according to the manufacturer's recommended protocols.

**Cell proliferation assay.** After transfection, mesothelioma cell lines were incubated in growth medium in a 96-well plate. The proliferation rate was determined at 24, 48, and 72 h with the Cell Titer Glo 2.0 reagent (Promega KK), which measures the number of viable cells relative to their ATP level, using a GloMax Explore microplate reader (Promega) according to the manufacturer's recommended protocols.

**Colony formation assay.** Mesothelioma cell lines transfected with the miRNA inhibitor or control were seeded into collagen-coated 6-well plates at a density of 500 cells/well and incubated in growth medium for three weeks. Cellmatrix Type I-A (Nitta Gelatin) was used for collagen coating. Colonies were stained by Cell Stain (EMD Millipore) and counted.

**Wound scratch assay.** The migration ability of mesothelioma cells was analyzed by a wound scratch assay. miRNA inhibitor- and control-transfected cells were incubated in collagen-coated 24-well plates. After making wounds with 1 ml micropipette tips, floating cells were removed by washing twice with fresh growth medium. Microscopic images were obtained at 0, 12, and 24 h (ACC-MESO1 cells), or at 0, 24, and 48 h (ACC-MESO4, CRL-5915, and CRL-5946 cells). The wound area was measured using TScratch software (16).

**Cell invasion assay.** Mesothelioma cell lines were incubated with the miRNA or control inhibitor in FluoroBlok culture inserts with 8- $\mu$ m pores (BD Biosciences) and coated with Geltrex Matrigel (Thermo Fisher Scientific) according to the manufacturers' protocols. Invaded cells were measured at 24 or 48 h after incubation with the miRNA inhibitor.

**Apoptosis and necrosis assays.** Mesothelioma cells were incubated with the miRNA or control inhibitor in 96-well plates for 24 h, and the RealTime Glo Annexin V Apoptosis Assay reagent (Promega) was added to the cells after transfection. Relative levels of apoptosis and necrosis were measured by analyzing luminescence and fluorescence with a GloMax microplate reader according to the manufacturer's recommended protocol.

**Chemosensitivity to pemetrexed and cisplatin.** Mesothelioma cells transfected with the miRNA or control inhibitor were

seeded into 96-well plates containing 0 to 50  $\mu$ M cisplatin or 0-100  $\mu$ M pemetrexed (both purchased from Fujifilm Wako Pure Chemical Corporation). Viable cells were measured with the Cell Titer Glo 2.0 reagent (Promega) 72 h after the addition of chemical agents.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** RNA was extracted from cells transfected with the miRNA inhibitor or control using a Maxwell RSC simplyRNA Cells kit and analyzed on a Maxwell RSC Instrument (Promega) according to the manufacturer's instructions. The extracted RNA was reverse-transcribed with SuperScript IV VILO Master Mix (Thermo Fisher Scientific) and amplified using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) on an AriaMax Real-Time PCR System (Agilent Technologies). Relative expression levels were calculated according to the comparative CT ( $\Delta\Delta$ CT) method (17). Expression levels were normalized against the expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following primers were used: CDKN2D forward, 5'-TCACACTGCTGTGGTCAGCTTT-3', reverse, 5'-AGGATGTCCACGAGGTCCTGA-3', GAPDH forward, 5'-ACAACCTTTTGGTATCATGGAAGG-3', and reverse, 5'-GCCATCACGCCACAGTTTC-3'.

**Statistical analysis.** All experiments were conducted at least three times. Experimental data are presented as means  $\pm$  standard deviation. Statistical significance of differences between two groups was analyzed with an unpaired Student's t-test. Statistical significance was set at  $P < 0.05$ .

## Results

**Inhibition of miR-18a reduces mesothelioma cell proliferation.** Inhibition of miR-18a significantly decreased proliferation of mesothelioma cells compared to that by the negative control inhibitor in all four cell lines (Fig. 1A). After 3 days, inhibition of miR-18a significantly reduced viability by 42.3% in ACC-MESO1, 33.5% in ACC-MESO4, 32.9% in CRL-5915, and 27.5% in CRL-5946 cells. The role of miR-18a in mesothelioma cell proliferation was also evaluated by the colony formation assay (Fig. 1B and C). Inhibition of miR-18a significantly reduced the colony forming ability of all four cell lines.

**miR-18a inhibition upregulates CDKN2D expression in mesothelioma cell lines.** To understand the mechanism of miR-18a in inhibiting mesothelioma cell growth, we searched for its target gene using the online miRNA target database, Target Scan Human 7.2 ([www.targetscan.org](http://www.targetscan.org)). CDKN2D was identified as a target gene of miR-18a. Furthermore, RT-qPCR analysis showed that inhibition of miR-18a significantly upregulated the expression of CDKN2D (Fig. 2).

**Inhibition of miR-18a reduces mesothelioma cell migration.** Mesothelioma cells transfected with the miR-18a inhibitor exhibited lower migration rates compared to those transfected with the negative control inhibitor in all four cell lines (Fig. 3). At 24 h, inhibition of miR-18a reduced the migration of ACC-MESO1 cells by 41.0%, and at 48 h inhibition of miR-18a reduced the migration of ACC-MESO4, CRL-5915,

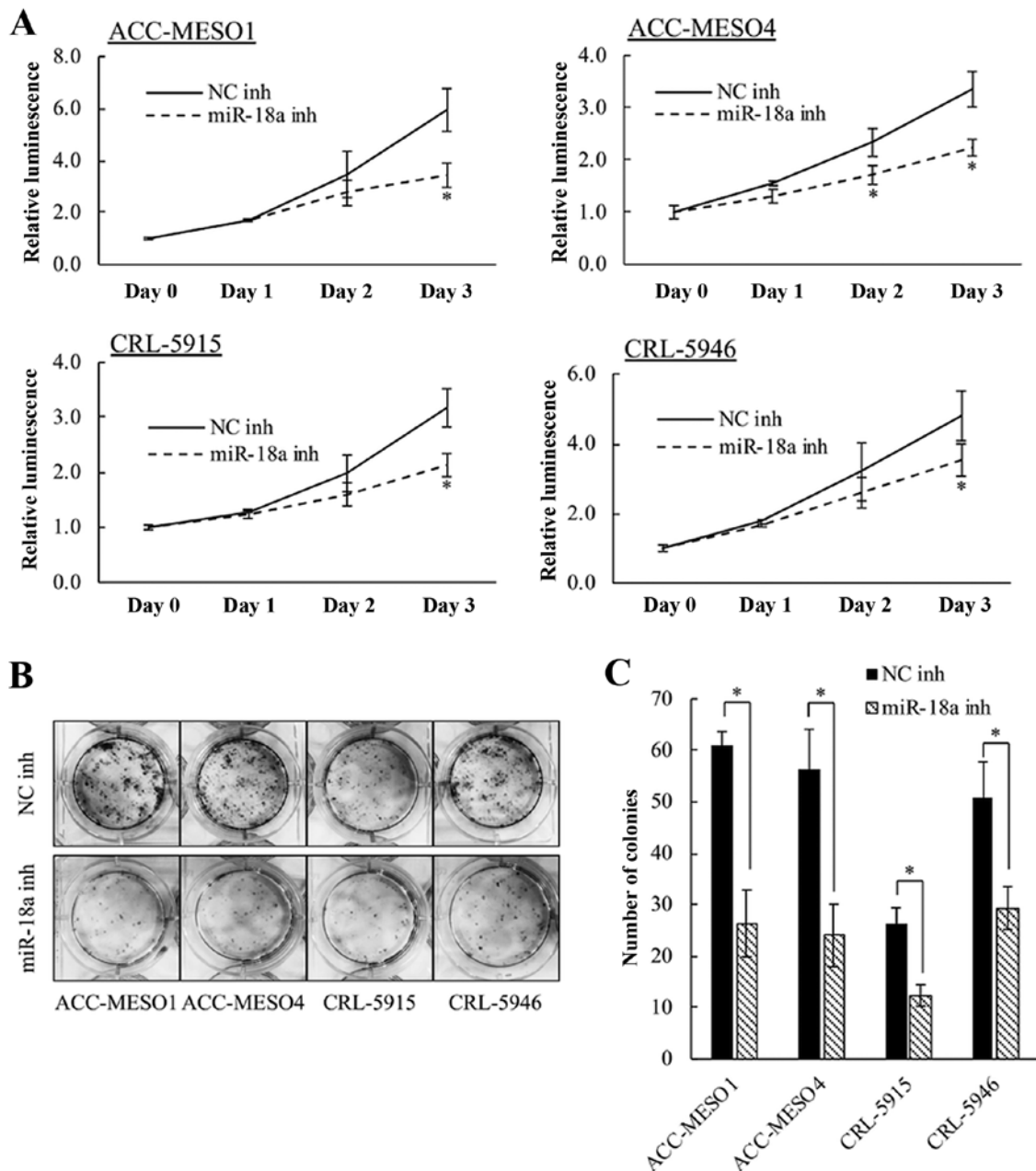


Figure 1. Cell proliferation and colony formation assay. (A) Mesothelioma cells (ACC-MESO1, ACC-MESO4, CRL-5915 and CRL-5946) transfected with the miR-18a inh exhibited lower proliferation rates compared with cells transfected with a NC inh. \* $P < 0.05$  vs. NC inh. (B) Representative images of the colony formation assay. (C) Inhibition of miR-18a reduces the colony forming ability in four mesothelioma cell lines. \* $P < 0.05$ . miR-18a inh, microRNA-18a inhibitor; NC inh, negative control inhibitor.

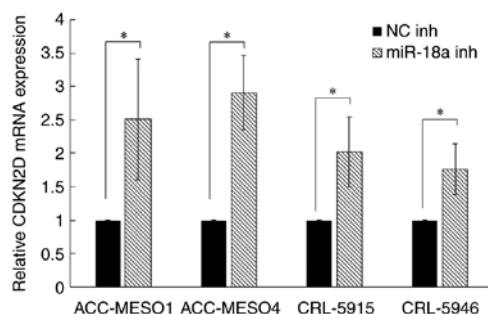


Figure 2. Reverse transcription-quantitative polymerase chain reaction analysis. CDKN2D mRNA was upregulated in mesothelioma cells transfected with miR-18a inh compared with in cells transfected with NC inh. \* $P < 0.05$ . miR-18a inh, microRNA-18a inhibitor; NC inh, negative control inhibitor.

and CRL-5946 cells by 50.5, 53.0, and 33.7%, respectively. Mesothelioma cell invasion was not significantly changed by inhibiting miR-18a (data not shown).

**Inhibition of miR-18a increases apoptosis, but not necrosis, in mesothelioma cell lines.** Transfection of the miR-18a inhibitor significantly increased the extent of apoptosis compared to that caused by the negative control inhibitor (Fig. 4A). Notably, ACC-MESO4 cells transfected with the miR-18a inhibitor exhibited over a three times increase in apoptosis compared to cells transfected with the negative control. However, no obvious change was observed in the extent of necrosis (Fig. 4B).

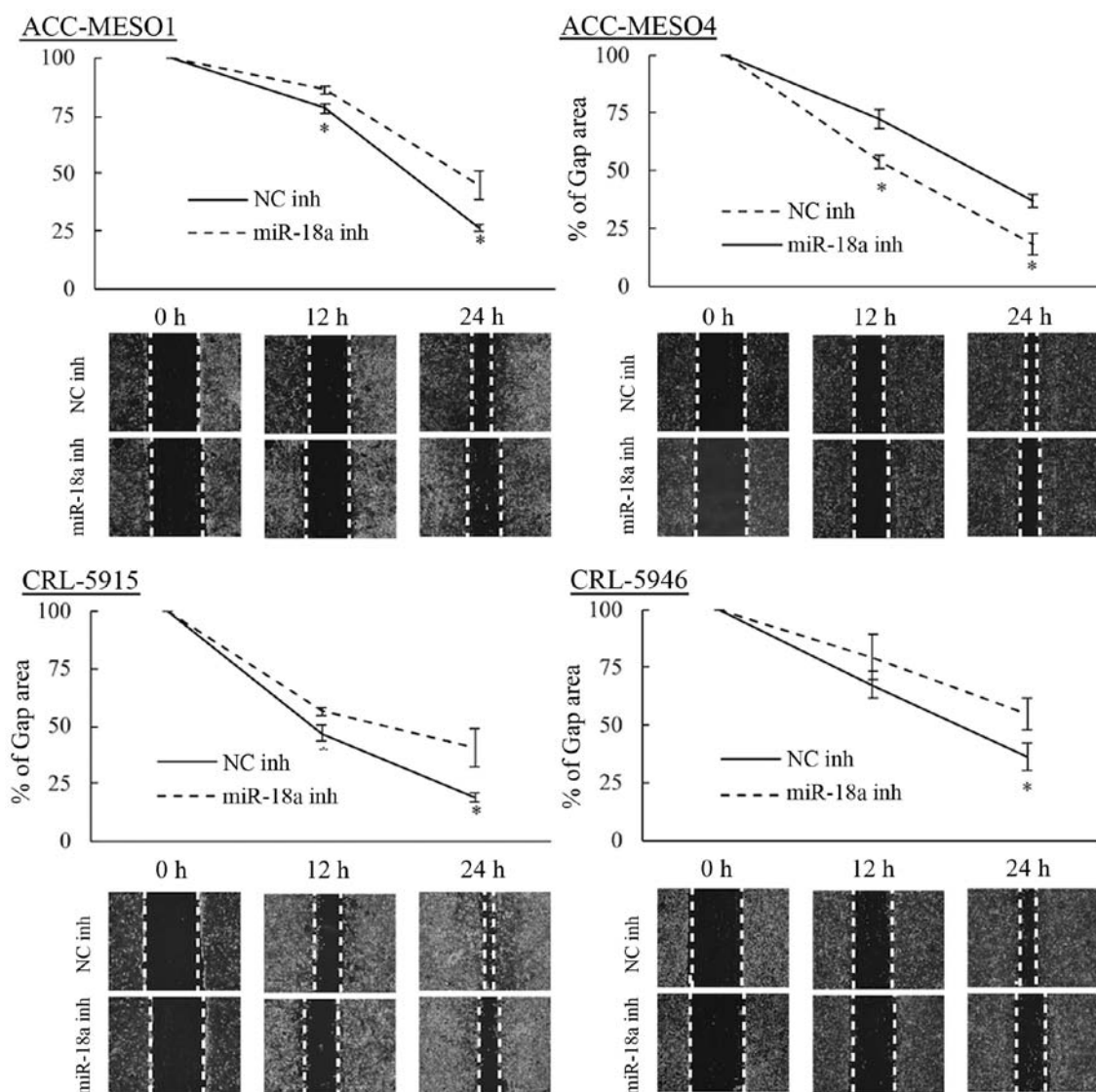


Figure 3. Wound scratch assay. Microscopy images representative of the wound gaps at 0, 12 and 24 h (ACC-MESO1 cells), or 0, 24 and 48 h (ACC-MESO4, CRL-5915 and CRL-5946 cells). Line graphs show reduced migration rates in cells transfected with miR-18a inh compared with in cells transfected with NC inh for all mesothelioma cell lines. All images were captured using an inverted microscope with a 4X objective. \* $P < 0.05$  vs. NC inh. miR-18a inh, microRNA-18a inhibitor; NC inh, negative control inhibitor.

*Inhibition of miR-18a increases the sensitivity of mesothelioma cells to cisplatin, but not pemetrexed.* In the chemosensitivity assay, CRL-5915 cells were more sensitive to both cisplatin and pemetrexed than the other three cell lines (ACC-MESO1, ACC-MESO4, and CRL-5946). We also found that transfection with the miR-18a inhibitor significantly enhanced sensitivity to cisplatin independent of the original sensitivity (Fig. 5A). At 0.5  $\mu$ M cisplatin, transfection of the miR-18a inhibitor reduced viability by 10.9, 16.0, 20.6, and 16.3% in ACC-MESO1, ACC-MESO4, CRL-5915, and CRL-5946 cells, respectively (statistically significant in ACC-MESO4, CRL-5915, and CRL-5946 cells). At 5  $\mu$ M cisplatin, transfection of the miR-18a inhibitor reduced viability by 20.5, 23.3, 20.5, and 19.7% in ACC-MESO1, ACC-MESO4, CRL-5915, and CRL-5946 cells, respectively (statistically significant in all four cell lines). In this experiment, no obvious change in pemetrexed sensitivity was observed by transfection of the miR-18a

inhibitor in cell lines relatively sensitive or resistant to this drug (Fig. 5B).

## Discussion

miRNAs are short, non-coding RNAs that perform a variety of functions through incomplete binding to the 3'-untranslated region of a target gene (18). Because many miRNAs regulate important cell functions, such as proliferation and invasion, some have been researched as therapeutic agents for various human malignancies (19).

Several studies have focused on the role of miRNAs in the progression of mesothelioma cells (20,21). Johnson *et al* (22) found that a miR-137 mimic inhibited the proliferation, invasion, and migration of mesothelioma cells, and Williams *et al* (23) found that miR-13 reduced proliferation, and increased apoptosis and necrosis of mesothelioma cells by downregulating MCL1. Further, we demonstrated previously

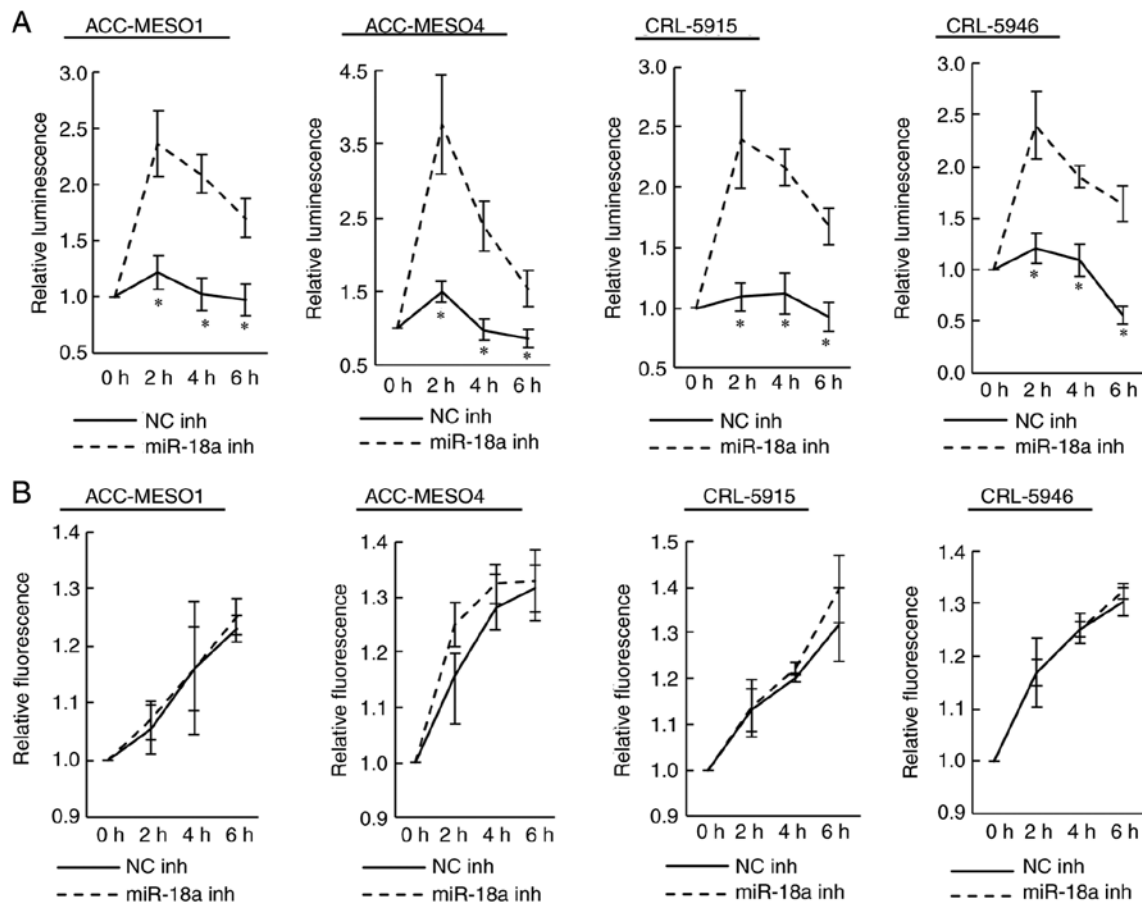


Figure 4. Apoptosis and necrosis assays. (A) Extent of apoptosis of mesothelioma cells transfected with miR-18a inh was significantly increased compared with cells transfected with NC inh. \* $P < 0.05$  vs. NC inh. (B) No significant change in necrosis was observed between mesothelioma cells transfected with miR-18a inh and NC inh. miR-18a inh, microRNA-18a inhibitor; NC inh, negative control inhibitor.

that miR-1 and miR-214 inhibited mesothelioma cell progression by suppressing PIM1 (11,24), and miR-182 and miR-183 promoted mesothelioma cell progression by suppressing FOXO1 (25).

In our previous comprehensive analysis of miRNA expression by RT-qPCR using TaqMan Low Density Array Human miRNA Cards, both miR-18a-3p and miR-18a-5p were upregulated in malignant mesothelioma cell lines (ACC-MESO1, ACC-MESO4, CRL-2081, CRL-5820, CRL-5826, CRL-5915, and CRL-5946) compared to non-neoplastic mesothelial tissues (11). He *et al* (26) found that miR-18a-5p promoted mesothelioma cell proliferation by downregulating PIAS3, but little is known about the function of miR-18a-3p in mesothelioma cells. In the present study, we showed that inhibition of miR-18a-3p reduced proliferation and migration, but increased apoptosis of mesothelioma cells. miR-16 is the most extensively investigated miRNA in malignant mesothelioma and inhibits mesothelioma cell growth and enhances sensitivity to epidermal growth factor receptor inhibition (27). A clinical phase 1 trial using TargomiR, a miR-16-based miRNA mimic that targets the epidermal growth factor receptor, has been conducted. The objective partial response rate observed was 5% and the stable disease rate 67% (28). Thus, miRNA-based therapeutic targeting of malignant mesothelioma is promising.

In this study, we found that *CDKN2D*, a target gene of miR-18a-3p in malignant mesothelioma, was upregulated. The *CDKN2D* gene (p19<sup>INK4d</sup>) is located on chromosome 19p13, and its gene product negatively regulates the cell cycle by preventing the activation of CDK4 and CDK6 (29,30). Thus, CDK4 represses the proliferation of non-small cell lung cancer (31). However, further research on the target genes is needed to understand the mechanism of miR-18a-3p in terms of migration, apoptosis, and chemosensitivity to cisplatin in malignant mesothelioma.

Recent studies have demonstrated that some miRNAs play important roles, not only in cellular proliferation and invasion, but also in chemosensitivity. For example, miR-362-5p and miR-613 suppress chemosensitivity to cisplatin in gastric cancer (32,33). Additionally, a correlation between the expression levels of miR-25, miR-145, and miR-210, and the effectiveness of pemetrexed maintenance treatment in lung adenocarcinoma, has been observed (34). Current standard chemotherapy for advanced stage malignant mesothelioma includes a combination of pemetrexed and cisplatin, but median survival remains only about 12 months, even though there is an approximately 40% response rate (6). This suggests that mesothelioma cells have chemoresistance to these anticancer agents. Moody *et al* (35) demonstrated that the loss of miR-31 enhanced sensitivity of malignant mesothelioma cells to cisplatin. Moreover,

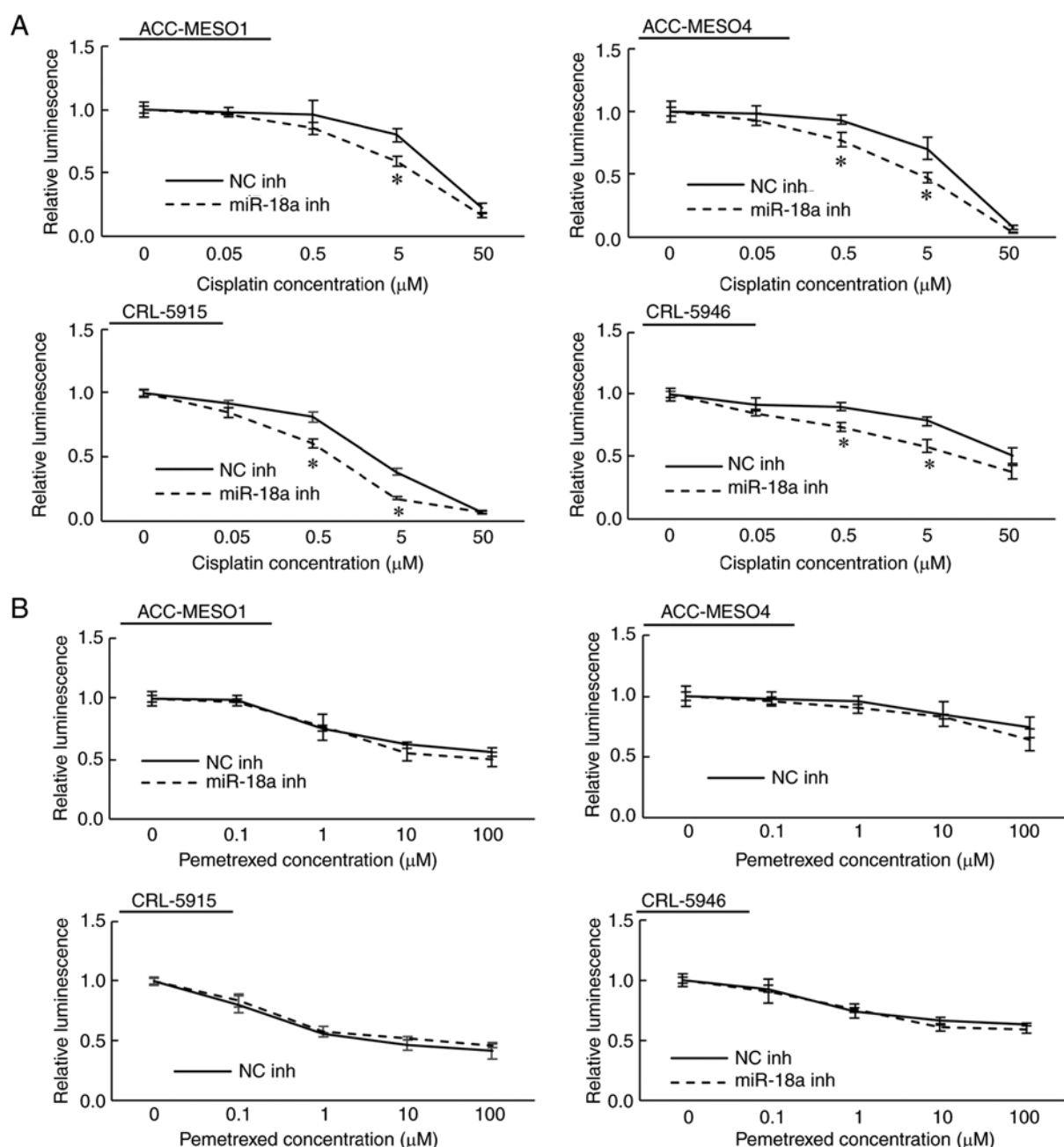


Figure 5. Chemosensitivity to cisplatin and pemetrexed. (A) Viability of mesothelioma cells transfected with miR-18a inh or NC inh 72 h after the addition of cisplatin (0-50  $\mu$ M). Transfection with miR-18a inh caused significant increases in sensitivity to 0.5 and 5  $\mu$ M cisplatin in ACC-MESO4, CRL-5915 and CRL-5946 cells, and 5  $\mu$ M cisplatin in ACC-MESO1 cells, compared with cells transfected with NC inh. \* $P$ <0.05 vs. NC inh. (B) Viability of mesothelioma cells transfected with miR-18a inh or NC inh measured 72 h after the addition of pemetrexed (0-100  $\mu$ M). Cell lines transfected with miR-18a inh exhibited no significant change in pemetrexed sensitivity compared with those transfected with NC inh. miR-18a inh, microRNA-18a inhibitor; NC inh, negative control inhibitor.

transfection of miR-145 and miR-379/411 induced chemosensitivity to pemetrexed in mesothelioma (36,37). In the current study, we found that inhibition of miR-18a enhanced chemosensitivity to cisplatin in all four cell lines tested, including ACC-MESO1, ACC-MESO4, and CRL-5946 cells that showed only a slight decrease in viability at 5  $\mu$ M. These results indicate that miR-18a targeted therapy may have benefits for patients with cisplatin-resistant mesothelioma. However, to clarify the detailed mechanism of miR-18a-3p in mesothelioma cell progression and chemosensitivity, further research is needed.

In conclusion, this study demonstrated that inhibition of miR-18a-3p significantly reduced mesothelioma progression and promoted chemosensitivity to cisplatin. Therefore, miR-18a is a potential therapeutic target for malignant mesothelioma.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

RS, VJA and YT designed the study. VJA and YT supervised and facilitated the study. RS, KK, YK, TK and YF performed the experiments. RS analyzed the data. RS and VJA interpreted the results and wrote the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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