

ORIGINAL ARTICLE

Overexpression of micro ribonucleic acid-591 inhibits cell proliferation and invasion of malignant pleural mesothelioma cells

Shizhao Cheng^{1,2}, Yue Xu³, Zhenliang Shi², Yongbin Lin⁴, Chuong D. Hoang³ & Xun Zhang²

1 Tianjin Medical University, Tianjin, China

2 Department of Thoracic Surgery, Tianjin Chest Hospital, Tianjin, China

3 Division of Thoracic Surgery, Department of Cardiothoracic Surgery, Stanford University School of Medicine, Stanford, California, USA

4 State Key Laboratory of Oncology in Southern China, Department of Thoracic Surgery, Sun Yat-Sen University Cancer Center, Guangzhou, China

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Correspondence

Xun Zhang, Department of Thoracic Surgery, Tianjin Chest Hospital, Tianjin 300222, China.

Tel: +86 13602036596

Fax: +86 22 8818 5406

Email: zhangxun69@163.com

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Abstract

Background: Malignant pleural mesothelioma (MPM) is an aggressive cancer refractory to current therapies. Reduced expression of micro ribonucleic acid (miR)-591 in a range of cancer types has suggested it is a potent tumor suppressor, and overexpression has been shown to inhibit tumor cell growth. The role of miR-591 in MPM is largely unknown.

Methods: miR-591 was over-expressed in vitro using micro RNA mimics in three MPM cell lines (H513, H2052, H2373), and effects on tumor cell growth, proliferation, invasion, and target gene expression were assessed.

Results: miR-591 mimic was introduced into MPM cell lines to overexpress this microRNA. The cellular growth, proliferation, and invasive capability was significantly inhibited after overexpression of miR-591. Growth inhibition caused by miR-591 correlated with upregulation of p21 and Bax. Reduced invasive capability correlated with downregulation of matrix metalloproteinase-2 and transforming growth factor- β 1.

Conclusion: miR-591 is a potent tumor suppressor in MPM. Overexpression of miR-591 may represent a novel therapeutic approach for MPM.

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor arising from the pleura, highly associated with asbestos exposure.¹ The number of MPM patients has increased worldwide, probably as a result of increased asbestos consumption in developing countries. The molecular mechanisms of MPM are poorly understood. Aside from exposure to asbestos, other cofactors, such as radiation, exposure to simian virus 40, and genetic predisposition have also been suggested.² Unfortunately, MPM is resistant to all currently available treatments and patients usually die within a year. Thus, a more comprehensive understanding of the molecular biology of MPM and development of new therapeutic strategies for MPM are urgently required.

Micro ribonucleic acids (miRNAs) are a class of short (~22 nucleotides) noncoding RNAs that regulate gene expression by binding to messenger (m)RNA, leading to mRNA degradation or the inhibition of translation.³ Increasing evidence

indicates that miRNAs are key players in cancer initiation and progression, acting as either oncogenes or tumor suppressors. In common with other cancers, the miRNA expression in MPM displays characteristic alterations.^{4–8} In a previous study, we identified and listed altered miRNAs in MPM tissues and noted a significant downregulation of miR-1 expression in tumor samples.⁸ Overexpression of miR-1 could induce apoptosis, thus making it a candidate therapeutic target. We also found significant downregulation of miR-591 expression in MPM tissues. Overexpression of miR-591 significantly repressed tumor growth in orthotopic neuroblastoma xenograft.⁹ In ovarian cancer, miR-591 resensitized paclitaxel-resistant cancer cells to paclitaxel by enhancing apoptosis and inhibiting cell migration and proliferation.^{10,11}

In the present study, we overexpressed miR-591 in MPM cells to investigate the impact of miR-591 on the cellular biological effects of MPM cells and to elucidate the cancer mechanisms involved in MPM.

Methods

Cell lines

Malignant pleural mesothelioma cell lines H513 (epithelial), H2052 (sarcomatoid), and H2373 (sarcomatoid) were obtained from the National Cancer Institute (NCI, Bethesda, MD, USA) and used in subsequent experiments.

Chemicals and culture medium

RPMI-1640, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Life Technologies (Foster City, CA, USA). Cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics. All cell culture wares were purchased from Corning Incorporated (Fremont, CA, USA). Unless otherwise specified, all other chemicals were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Micro ribonucleic acid (miR)-591 mimics transfection

Pre-miR miR-591 mimic and scrambled oligonucleotides were purchased from Life Technologies. Cells from each MPM cell line were seeded into six-well plates and allowed to attach overnight to achieve 60–70% confluency. miR-591 mimic or scrambled control (75 pmol) in 200 μ l of serum-free antibiotic-free medium was mixed with 7.5 μ l of Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA), dissolved in 200 μ l of the same medium, and allowed to stand at room temperature for 20 minutes. The resulting 400 μ l transfection solutions were then added to each well containing 1.6 mL of RPMI-1640 medium, supplemented with 10% FBS. Cells were grown and harvested 48 hours after transfection for additional analyses. RNA was harvested from the MPM cells at 48 hours post-transfection to evaluate miR-591 expression. Transfection efficiency was determined by quantitative real-time-polymerase chain reaction (qRT-PCR).

Crystal violet staining assay

Micro RNA-induced changes in cell growth were evaluated by crystal violet staining, as previously described.¹² In brief, cells were fixed with 1% glutaraldehyde and stained with 0.1% crystal violet. Crystal violet bound to cells, including nuclei and cytosol elements. The unbound dye was removed with water. Bound crystal violet was photographed and solubilized with 0.2% Triton-X-100 (Sigma-Aldrich, Steinheim, Germany) for quantitative analysis. Light extinction, which increases linearly with the cell number, was analyzed at

570 nm using an enzyme-linked immunosorbent assay (ELISA)-Reader (Dynex Technologies, Denkendorf, Germany).

XTT proliferation assay

Viability and proliferation of the transfected MPM cells were assessed by Cell Proliferation Kit II assay (XTT; Roche Diagnostics, Risch-Rotkreuz, Switzerland). Cells were seeded into 96-well dishes and allowed to attach overnight. At days one, three, and six post-transfection, cells were labeled with the mixture of XTT labeling reagent and electron-coupling reagent. Absorbance at 490 nm was quantitated by scanning spectrophotometer, and optical density readings from the cells of each group were recorded.

In vitro invasion assay

Transwell membranes coated with Matrigel (BD Biosciences, San Jose, CA, USA) were used to quantify in vitro MPM cell invasion. Transfected cells were plated at 5×10^4 per well in the upper chamber in medium containing 0.5% FBS. Medium containing 15% FBS was added to the lower chamber. After 24 hours of incubation, non-invasive cells were removed from the top well with a cotton swab, while the bottom cells were fixed with 3% paraformaldehyde, stained with 0.1% crystal violet, and photographed in three independent $10 \times$ fields for each well. Cell counting was performed under light microscope. Data represents mean \pm standard deviation (SD).

Quantitative real-time-polymerase chain reaction (qRT-PCR)

Ribonucleic acid isolation and qT-PCR were performed as previously described.⁸ Briefly, RNA and miRNA isolation were performed with the RNeasy Mini Kit and the miRNeasy Mini Kit, respectively (Qiagen Sciences, Valencia, CA, USA). Reverse transcription was performed using the TaqMan Reverse Transcription Kit for genes and the TaqMan miRNA Reverse Transcription Kit for miRNA (Life Technologies). qRT-PCR was performed on the ViiA 7 Real-Time PCR System with appropriate assay reagents for genes and miRNA (Life Technologies). PCR primer sequences for the genes examined in this study are shown in Table 1. Primers were tested to determine their optimal concentrations for PCR analysis, and the resulting products were run on 2% agarose gel to confirm the appropriate size and RNA integrity. Gene expression values were normalized to the endogenous control gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Similarly, miRNA expression values were normalized to the endogenous control, RNU44.¹³ All PCR reactions were performed in triplicate. Multiple, independent experiments were

Table 1 Primer sequences used in this study

Gene Name	Accession Number	Primer Sequence (5'-3')
P21	NM_078467	Forward-CAGGGGACAGCAGAGGAAGA
		Reverse-GGGCGGCCAGGGTATGTAC
BAX	NM_138763	Forward-GGCCGGGTTGTCGCCCTTTT
		Reverse-CCGCTCCCGGAGGAAGTCCA
GAPDH	NM_001256799	Forward-CCACCCATGGCAAATTCATGGCA
		Reverse-AACAAAGCTGGACAAT

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

conducted with high correlation (Pearson $r > 0.70$) among the results.

RT² profiler PCR array for motility genes

The Human Target RT² Profiler PCR Array from Qiagen (Cat# PAHS-128Z; Hilden, Germany) was used to analyze the expression of motility-related genes. This panel of 86 genes includes genes related with cell motility, such as matrix metalloproteinase-2 (MMP2) and transforming growth factor- β 1 (TGF- β 1). Total RNA was isolated from miR-591 transfected and control MPM cells. One microgram of total RNA was used to perform reverse transcription according to manufacturer instructions. cDNA was subsequently diluted and qRT-PCR reactions were performed on 384-well plates supplied by the manufacturer. Data were analyzed using the manufacturer's web-based software. Data analysis was based on the $\Delta\Delta$ CT method with normalization of the raw data to multiple housekeeping genes in the RT² Array from Qiagen.

Statistical analysis

Means and SDs were calculated from numerical data. In the figures, bar graphs depict the mean and SD. A student's *t*-test was performed to calculate significance. $P < 0.05$ was considered to be significant.

Results

miR-591 inhibits cell growth in malignant pleural mesothelioma (MPM) cells

We transfected three MPM cell lines (H513, H2052, and H2373) with miR-591 mimic and a scramble control and confirmed that miR-591 expression increased in all of the miR-591-transfected cells, compared with the scramble control: there was a 9.3-fold increase for H513, 8.2-fold for H2052 and 7.9-fold for H2373. To observe the impact of miR-591 overexpression on the mesothelioma phenotype, we performed crystal violet staining assay. We found that cell growth was markedly repressed in MPM cell lines with induced miR-

591 overexpression at day three (Fig 1a). We dissolved and quantitated the crystal violet dye and found a significant decrease in the crystal violet dye reading in all three miR-591 transfected MPM cell lines ($P < 0.05$; Fig 1b).

The XTT viability and proliferation experiments showed that cell proliferation in transfected cells decreased compared with the scrambled control at days one, three and six, respectively. Significant ($P < 0.05$) changes in XTT dye incorporation were quantitated at days three and six (Fig 2).

miR-591 inhibits cell invasion in MPM cells

To estimate the effect of miR-591 on invasion potential in MPM, cell invasion was examined by transwell assay. Microscopic images of the transwell assay are shown in Figure 3a. In all 3 MPM cell lines, the number of invasive cells were significantly suppressed in miR-591 transfected cells, compared with the scrambled control (232 ± 18 vs. 658 ± 58 for H513, $P < 0.01$; 17 ± 7 vs. 180 ± 20 for H2052, $P < 0.01$; 165 ± 75 vs. 452 ± 112 for H2373, $P < 0.01$; Fig 3b). These data demonstrated that tumor cell motility changed with the overexpression of miR-591.

Phenotype changes in miR-591 transfected cells is associated with alterations of apoptotic and cell cycle genes

To confirm the phenotypic changes in growth arrest, we examined genes associated with cell cycle homeostasis and pro-apoptosis using qRT-PCR. Total RNA was isolated from transfected MPM cells at 48 hours post-transfection. Gene expressions of Bax (2.5-fold increase for H513, $P < 0.01$; 3.6-fold increase for H2052, $P < 0.01$; 1.8-fold increase for H2373, $P < 0.01$) and p21 (4-fold increase for H513, $P < 0.01$; 5.8-fold increase for H2052, $P < 0.01$; 2.4-fold increase for H2373, $P < 0.01$) were both significantly increased in miR-591 overexpressed cells (Fig 4).

miR-591 affects multiple genes related with cell motility

We assessed the likely gene targets of miR-591 to gain further insight into potential mechanisms to explain why miR-591 inhibits invasion in MPM cells. RT² Profiler PCR Array analysis revealed downregulation of multiple genes, shown in Table 2. Notably, both MMP2 and TGF- β 1 were consistently downregulated in the three miR-591 overexpressed MPM cell lines.

Discussion

Malignant pleural mesothelioma was once an uncommon malignancy, but its incidence has increased worldwide,

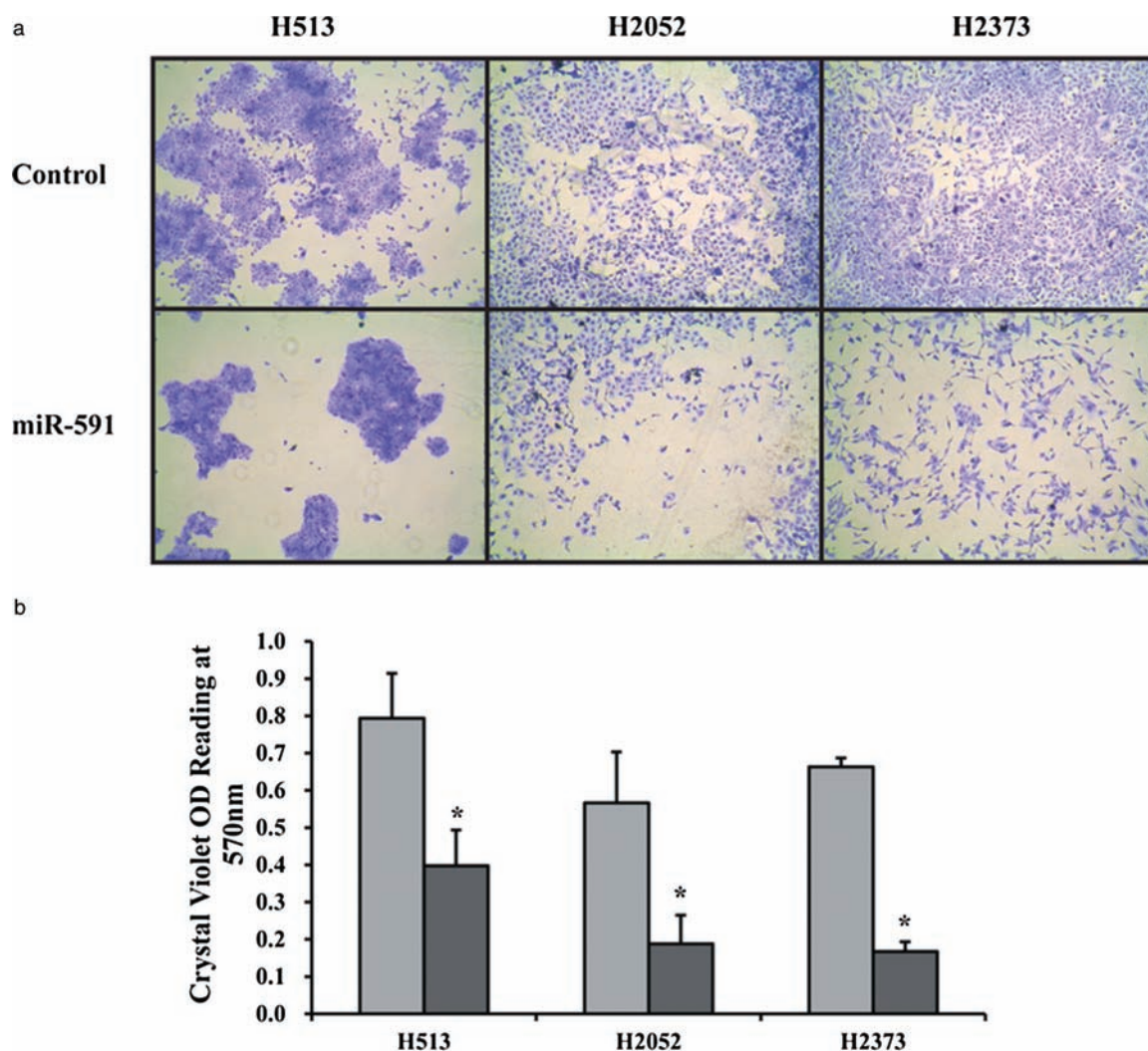


Figure 1 Growth effects of micro ribonucleic acid (miR)-591 overexpression in mesothelioma cell lines. (a) Crystal violet staining of cells on day three post-transfection of miR-591. Decreased cell growth was observed in miR-591-transfected cells (miR-591) compared with a scrambled control. Magnification, 40x. (b) Quantitative analysis of the staining assay indicated significant changes ($P < 0.05$). *Indicates significant change ($P < 0.05$). OD, optical density, measured in this assay as a correlate of a number of viable cells. ■, Control; ■, miR-591.

probably resulting from widespread exposure to asbestos. MPM is resistant to all currently available treatments and patients usually die within a year; therefore, new therapeutic strategies to treat patients with MPM are needed.

Numerous miRNAs are dysregulated in cancers, including MPM, and a single miRNA can have multiple targets involved in different oncogenic pathways. Therefore, targeting aberrant miRNA expression to simultaneously control multiple genes within the same and/or related pathways may be more effective than any single-target agents.^{14,15} To date, a limited number of studies have evaluated the differential expression of miRNAs of MPM using different sample sources.^{4-6,8} Most recently, we compared miRNA expression in MPM tissues with miRNAs in normal parietal pleura and found significant

downregulation of miR-591 expression in MPM tissues.⁸ Several studies have investigated the effects of restoring the expression of downregulated miRNAs in MPM.^{7,16-19} The expression of miR-16 was consistently downregulated in MPM tissues and cell lines, and re-expression led to growth inhibition both in vitro and in vivo.¹⁷ The expression of miR-34b and miR-34c (miR-34b/c) was frequently downregulated by methylation in MPM cell lines and primary tumors and the forced overexpression of miR-34b/c had a significant antitumor effect that induced apoptosis in MPM cells in vitro.¹⁸ miR-34b/c could enhance the radiosensitivity of MPM cells by promoting radiation-induced apoptosis.¹⁹

In previous studies, the overexpression of miR-591 resulted in significantly decreased tumor growth and

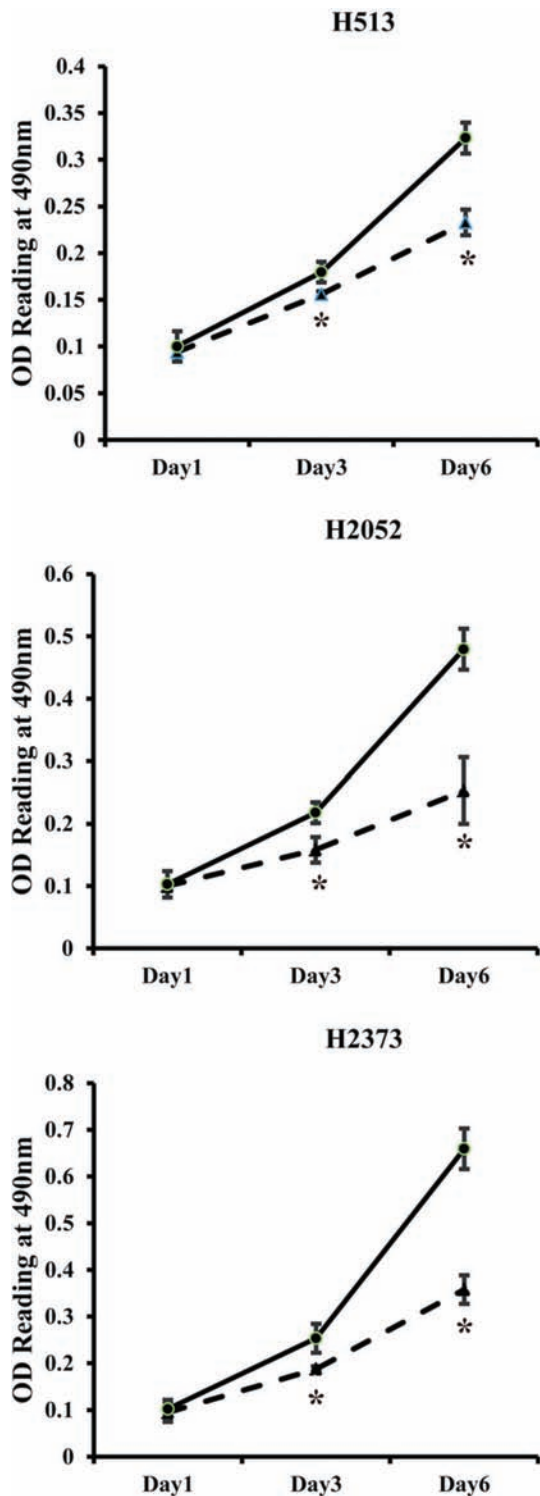


Figure 2 XTT growth assay. Significantly decreased cell proliferation was observed in micro ribonucleic acid (miR)-591-transfected cells (miR-591) compared with a scrambled control by days three and six in all three MPM cell lines. *Indicates significant change ($P < 0.05$). OD, optical density, measured in this assay as a correlate of a number of viable cells. --- , miR-591; — , Control.

Table 2 Genes regulated by miR-591 examined by RT² array

H513	
Gene Symbol	Fold Regulation
ITGB2	-3.155
MMP2	-2.0732
MYL9	-2.3631
RHO	-2.0472
TGFB1	-2.043
H2052	
Gene Symbol	Fold Regulation
CAPN1	-2.3109
DPP4	-2.0272
MMP2	-3.1987
TGFB1	-3.1394
H2373	
Gene Symbol	Fold Regulation
CAV1	-3.8933
ENAH	-2.0907
IGF1R	-2.3054
ITGB1	-2.0777
MET	-3.625
MMP2	-5.148
MYH10	-2.7019
MYL9	-2.5385
MYLK	-2.1435
PAK1	-2.0307
PAK4	-2.7721
PIK3CA	-2.1615
PRKCA	-2.6592
PTPN1	-2.3214
RASA1	-2.3022
RHOC	-2.5315
RND3	-2.181
TGFB1	-2.6574
VIM	-2.528
WASF1	-2.6354
WIPF1	-2.9221

Gene symbol, the full names of gene symbols are available from URL: (<http://www.sabiosciences.com/genetable.php?pcatn=PAHS-128Z>). (–) value indicates fold regulation of downregulated genes. Fold regulation is the negative inverse of the fold change. Mir, micro ribonucleic acid.

migration in neuroblastoma and ovarian cancer.^{9,10} Consistent with these results, our study demonstrated that the overexpression of miR-591 inhibited proliferation and invasion in MPM cell lines.

The antiapoptotic potential of MPM makes it highly resistant to chemotherapy and radiotherapy.²⁰ Our results show that miR-591 probably induced cell cycle arrest and apoptosis by upregulating p21 and Bax. P21 is a potent negative regulator of the G1-S checkpoint, while Bax is a pro-apoptotic gene that belongs to the B cell lymphoma 2 family. Mutations and deletions of the TP53 gene are rare in MPM.²¹ The

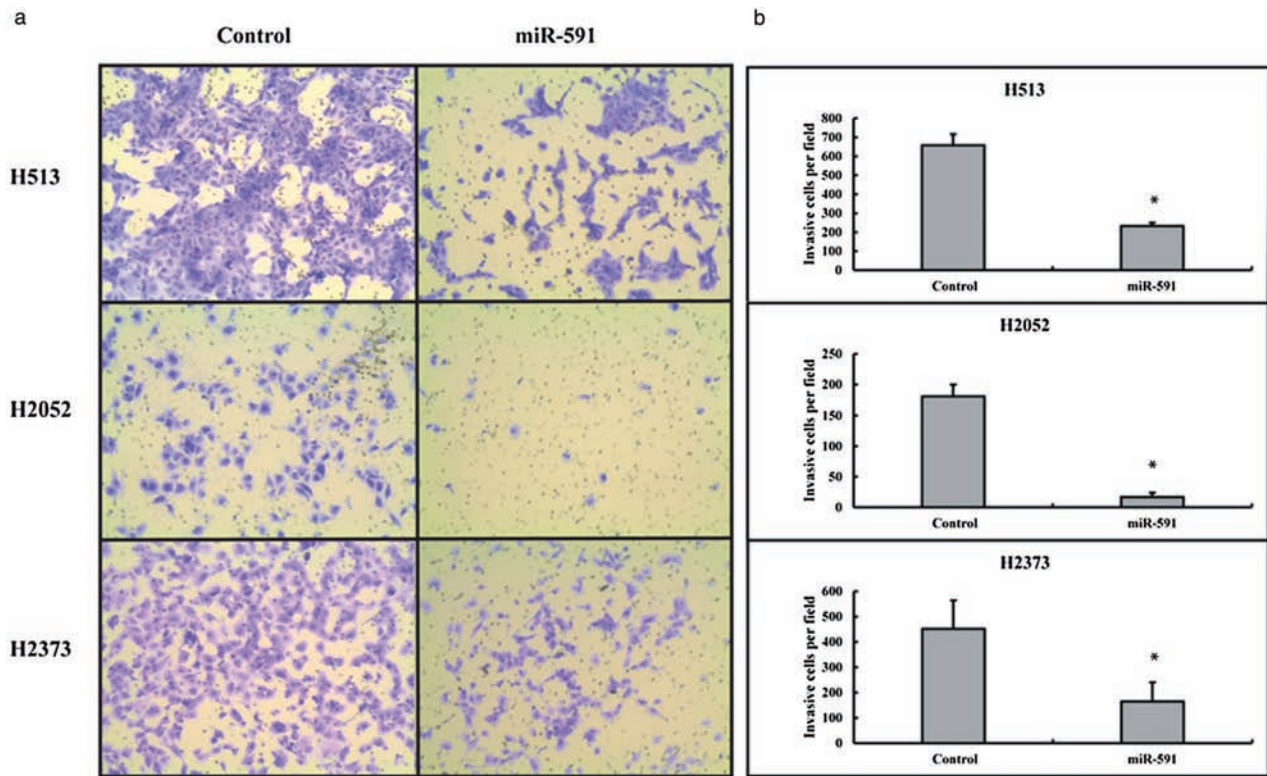


Figure 3 Transwell assay. Micro ribonucleic acid (miR)-591 suppressed tumor cell invasive capability in vitro. (a) Representative images of the transwell assay of H513, H2052, and H2373 after transfection with miR-591 or a scrambled control. Magnification, 100x. (b) Quantitative analysis of the transwell assay indicated significant changes ($P < 0.05$). *Indicates significant change ($P < 0.05$).

antiapoptotic potential of MPM has been considered to arise downstream of p53.²² Both p21 and Bax are the downstream targets of p53. Whether miR-591 directly or indirectly regulates these genes remains to be determined, as none of the predicted gene targets of miR-591 are the canonical mediators of apoptosis. miR-591 may trigger a regulatory signaling cascade and exert an indirect effect on cell cycle and apoptotic pathways.

In our study, the suppressive effects of miR-591 on invasion were confirmed in MPM cell lines by transwell assay. We found significant downregulation of MMP2 and TGF- β 1 in all three miR-591 overexpressed MPM cell lines. MMP2 plays a key role in MPM invasion and metastasis.^{23,24} TGF- β promotes tumor progression by enhancing epithelial-mesenchymal transition, metastasis, angiogenesis, etc.²⁵ TGF- β could increase the expression, secretion, and activity of MMP-2 and downregulate the expression of the protease tissue inhibitors of metalloproteinases.^{26,27} Targeting TGF- β signaling is emerging as an anti-cancer therapeutic strategy. PF-03446962 is an anti-TGF- β type I receptor monoclonal antibody. A Phase II clinical trial testing PF-03446962 in patients with advanced MPM already treated with cytotoxic chemotherapy is currently ongoing.^{28,29}

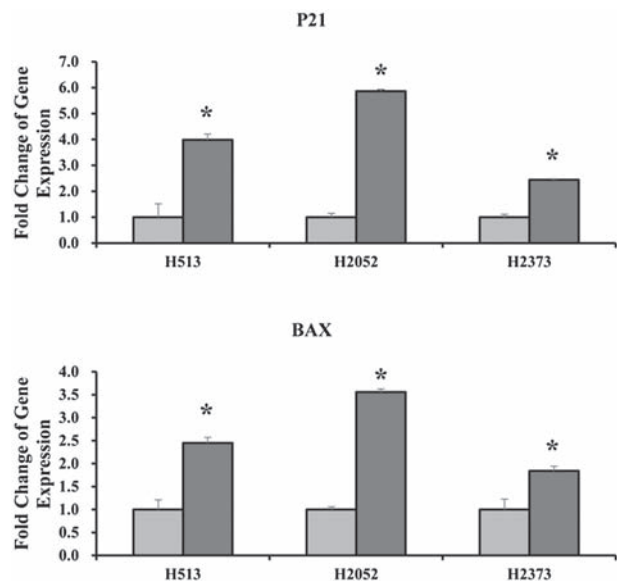


Figure 4 Gene expression changes associated with the growth arrest phenotype in micro ribonucleic acid (miR)-591 transfected mesothelioma cells. Significant increases in P21 and BAX were observed in miR-591-transfected cells compared with a scrambled control. Fold changes were calculated by the $\Delta\Delta$ Ct method. *Indicates significant change ($P < 0.05$). ■, Control; ■, miR-591.

In conclusion, our results show that overexpressing miR-591 in vitro results in significantly decreased proliferation and invasion in MPM cell lines. Our study provides new insights into the molecular pathogenesis of MPM and suggests that miR-591 could be a potential therapeutic target for MPM.

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Disclosure

No authors report any conflict of interest.

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