

# miR-133a suppresses cell proliferation, migration and invasion in human lung cancer by targeting MMP-14

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**Abstract.** Lung cancer is the leading cause of cancer-related mortality worldwide. Over half of lung cancer cases are diagnosed after metastasis, for which the median survival time is approximately 8 months. microRNAs (miRNAs), which are a class of single-stranded endogenous non-coding RNAs, are likely to be involved in most biological processes. miR-133 plays roles in cardiac development and disease, and recent studies showed that miR-133 is downregulated in various human malignancies, such as bladder and lung cancer. However, its detailed role in the processes of cancer remains to be determined. In the present study, we found that in the lung cancer cell lines A549 and NCI-H1299 overexpression of miR-133a suppressed cell proliferation, migration and invasion. The miR-133a-induced suppression of cell migration and invasion can be reversed by miR-133a-specific inhibitor. According to the mRNA sequence, matrix metalloproteinase (MMP)-14, which is an important regulator of metastasis, is a predicted target of miR-133a. This was confirmed by dual luciferase reporter assay. Moreover, miR-133a overexpression decreases the mRNA and protein levels of MMP-14. Collectively, these results suggest that miR-133a may inhibit lung cancer metastasis by targeting MMP-14 and may be used as an anti-metastatic therapy in lung cancer patients.

## Introduction

Metastasis, which is the spread of cancer to distant locations in the body, is a complex process. Approximately 90% of deaths associated with cancer are due to the metastasis of the original tumor cells to sites distant from the primary tumor. Metastasis occurs through a series of sequential steps including invasion of adjacent tissues, intravasation, transport of cancer cells through the circulatory system, arrest at a secondary site, extravasation and growth in a secondary organ (1). For most cancer cell types, the ability to metastasize leads to clinically incurable disease (2). However, the mechanisms underlying a cell's ability to extravasate from the primary tumor, circulate and invade new tissue remain to be established (3). The organized breakdown of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) is involved in the complex cascade of events, such as invasion and extravasation. The human MMP family is divided into secreted and membrane-type MMPs (MT-MMPs). MT-MMPs are membrane-tethered proteolytic enzymes and constitute the largest family of MMPs identified thus far (4). The type I transmembrane structural class of MT-MMPs contains MMP-14 (MT1-MMP), MMP-15 (MT2-MMP), MMP-16 (MT3-MMP) and MMP-24 (MT5-MMP). Overexpression of MT-MMPs has been observed in several types of human cancer (5). The functions of MT-MMPs are known to include roles in activation of other MMP family members, pericellular proteolysis, modulation of cellular signaling and cellular migration, the angiogenic response and regulation of cell proliferation, apoptosis and tumor metastasis (6-8). Among these members, MMP-14 is overexpressed in pancreatic ductal adenocarcinoma (PDAC) compared with normal pancreatic tissue, which is induced by TGF- $\beta$ 1 (9). The overexpression of MMP-14 is also observed in non-small cell lung carcinoma (NSCLC) (10), breast cancer (11), malignant mesothelioma (12), and supraglottic cancer (13). The activity of these proteases is tightly regulated by specific inhibitors, known as tissue inhibitors of MMPs (TIMPs) (14). A selective MMP-14 inhibitor reduces cancer cell motility and tumor growth (15). Therefore, to uncover the regulation mechanisms of MMP-14, particularly in cancer metastasis, is of great importance for understanding cancer biology and improving treatment.

microRNAs (miRNAs) are 20-23-nucleotide long single-stranded RNAs that are encoded by eukaryotic nuclear DNA

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*Abbreviations:* MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; miRNA, microRNA; PVDF, polyvinylidene difluoride membrane

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and function in the post-transcriptional regulation of gene expression via base-pairing with complementary sequences within 3'-UTR of target mRNAs (16). Mature miRNAs, Argonaute (Ago) and several other associated proteins form the RNA-induced silencing complex (RISC) mediating gene silencing at post-transcriptional and translational levels (17). miRNAs have been shown to be involved in a wide variety of biological processes such as apoptosis, development, aging and cancer. Previous studies suggested that miRNAs contribute to the initiation and development of various types of cancer (18). There is also accumulating evidence to suggest that miRNAs are involved in tumor metastasis. miR-200 family and miR-205 can inhibit epithelial-mesenchymal transition (EMT), through which epithelial cancer cells invade and metastasize, by targeting ZEB1, ZEB1 and SIP1 (19-21). In human breast cancer, overexpression of miR-21 is significantly correlated with lymph node metastasis, advanced clinical stage and shortened survival time (22). miR-10b is also reported to initiate tumor invasion and metastasis in breast cancer (23,24). In prostate cancer progenitor cells, miR-34a acts as a potent inhibitor of metastasis by directly suppressing CD44 (25). miR-9, an MYC/MYCN-activated miRNA, is reported to enhance cell motility and invasiveness by targeting CDH1 (26). Therefore, it is reasonable to assume that more miRNAs would be discovered to govern metastasis. miR-133a has previously been reported to play roles in diabetic hearts (27), myogenic differentiation (28) and osteoblast lineage commitment program (29). Recent findings showed that miR-1 and miR-133a are frequently downregulated in various types of cancer (30). In the present study, we proved that MMP-14 is a target of miR-133a by dual luciferase reporter assay, and overexpression of miR-133a decreases the mRNA and protein levels of MMP-14 in lung cancer cell lines. In conclusion, the miR-133a-induced suppression of cell proliferation, migration and invasion addresses the anti-metastatic role of miR-133a in lung cancer.

## Materials and methods

**Cell culture and transfection.** NSCLC cell lines A549, NCI-H1299 and human HEK-293T cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (S/N:16000-044; Gibco) and antibiotics (100 U/ml penicillin and streptomycin; Invitrogen) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Cell transfection was performed using Lipofectamine™ 2000 (Invitrogen). Cells were harvested at 48 h post-transfection for protein analysis or luciferase activity assay. The related sequences are shown in Table I.

**Plasmid construction.** The full length 3'UTR of human MMP-14 was PCR-amplified from human genomic DNA and cloned into psiCHECK™-2 dual luciferase reporter plasmid (Promega, Madison, WI, USA) immediately downstream of the stop codon of the Renilla luciferase gene with *XhoI/NotI* to generate psiCHECK™-2-MMP-14-3'UTR-wt. The mutant human MMP-14 3'UTR reporter, designated as psiCHECK™-2-MMP-14-3'UTR-mut, was created within

Table I. Primers used for reverse transcription and real-time polymerase chain reaction.

Primer	Sequence
miR-133a-stem-loop RT primer	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CAG CTC-3'
RNU6-RT	5'-AAA ATA TGG AAC GCT-3'
miR-133a mimics	5'-TTT GGT CCC CTT CAA CCA GCT G-3'
Mimics control	5'-AGT GTG AGT TCT ACC ATT GCC AAA-3'
miR-133a inhibitor	5'-CAG CTG GTT GAA GGG GAC CAA A-3' (2'Ome)
miR-133a-F	5'-GTG CAT TTG GTC CCC TTC A-3'
miR-133a-R	5'-CGG GCT GTC AGT TTG TCA-3'
RNU6-F	5'-CTC GCT TCG GCA GCA CA-3'
RNU6-R	5'-AAC GCT TCA CGA ATT TGC GT-3'
GAPDH-F	5'-AGG GCA TCT TGG GCT ACA C-3'
GAPDH-R	5'-TGG TCC AGG GTT TCT TAC TCC-3'
MMP-14-F	5'-GCA GAA GTT TTA CGG CTT GCA-3'
MMP-14-R	5'-TCG AAC ATT GGC CTT GAT CTC-3'

F, forward; R, reverse; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase.

the predicted hsa-miR-133a seed sequence binding site (GGACCAA to GCTGGTAA) by site-directed PCR mutation. The construction was confirmed by DNA sequencing.

**Luciferase reporter assay.** For luciferase reporter assay, HEK-293T cells (4.0x10<sup>3</sup> cells/well) were plated in a 96-well plate (Corning, USA) 24 h before transfection. Cells were co-transfected with 60 ng of either the psiCHECK™-2-MMP-14-3'UTR-wt, psiCHECK™-2-MMP-14-3'UTR-mut or psiCHECK™-2 vector and 20 nM (final concentration) of either miRNA mimics negative control or miR-133a mimics or miR-133a inhibitor, which is antisense oligonucleotides with 2'O-methyl modification (RiboBio, Guangzhou, China). Forty-eight hours after transfection, the cells were washed with PBS twice and lysed in 100 µl Passive Lysis Buffer (Promega) and the luciferase activities were measured from 20 µl lysate using the Dual-Luciferase Reporter Assay kit (Promega) following the manufacturer's instructions on a luminometer (Lumat LB 9507; Berthold, Germany). The data were obtained by averaging the results from six independent repeats. Transfection efficiency was normalized to thymidine kinase-driven Renilla luciferase activity.

**RNA isolation and real-time PCR.** Total RNA was isolated from cultured cells using TRIzol Reagent (Invitrogen). cDNA was synthesized by reverse transcription using oligo (dT) as the primer and proceeded to real-time PCR with gene-specific primers in the presence of 2X SYBR-Green Master Mix (DBI Bioscience, Shanghai, China). The relative abundance of mRNA was calculated by normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Specific stem-loop

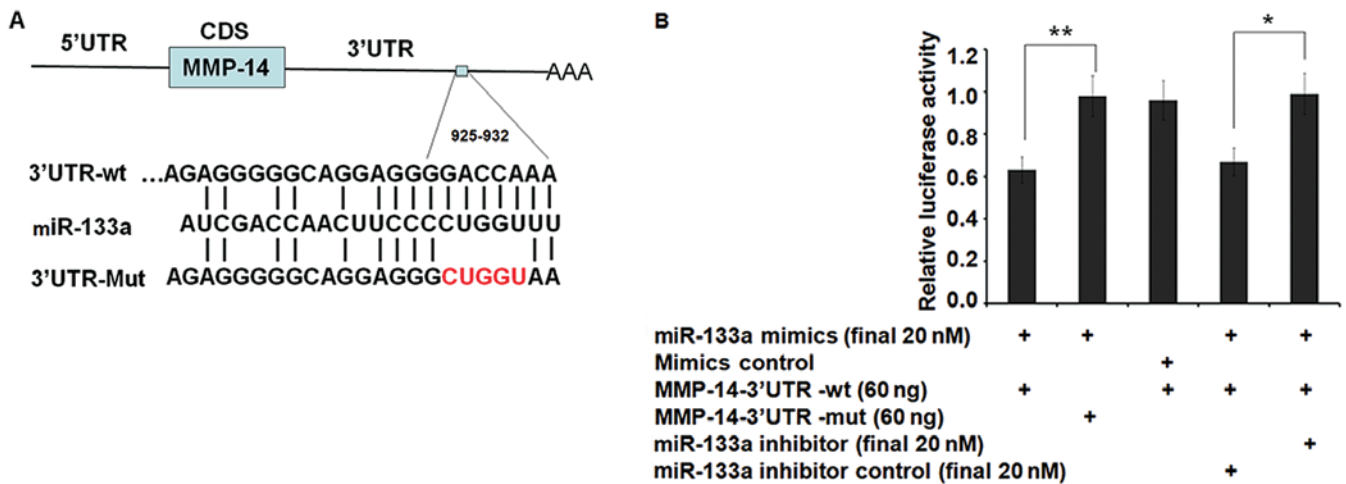


Figure 1. Matrix metalloproteinase (MMP)-14 is a target of miR-133a. (A) The highly conserved mature miR-133a sequence in mammals and the potential seed matching between miR-133a and MMP-14 3'UTR sequence are shown. The mutated bases in MMP-14-3'UTR-mut are indicated in red. (B) The transfection was performed as indicated in 293T cells, followed by dual luciferase activity assay 48 h later. Data of three independent experiments are presented as mean  $\pm$  SE. \*\* $P < 0.01$ , \* $P < 0.05$ .

RT primers were used for reverse transcription reaction of miR-133a. Normalization of miR-133a was performed by using RNU6 primers. All reactions were carried out in triplicate and all experiments were performed three independent times.

**Proliferation, migration and Matrigel invasion assay.** For the proliferation assay, 6 h after transfection, 3,000 cells were seeded in triplicate in 6-well plates. Cell Counting Kit-8 (CCK-8) reagent (C0038; Beyotime, China) was added at 12, 24, 48, 72 h after seeding and incubated at 37°C for half to 4 h according to the color change. The data of optical density (OD) value at 450 nm were read by a microplate reader (Synergy 2; BioTek, USA). Migration and invasion assays were performed according to the manufacturer's instructions. Cells ( $3 \times 10^4$ ) in 0.5 ml of serum-free medium were seeded onto the top of each chamber containing BD BioCoat cell culture inserts (354578) or Matrigel Invasion Chamber (354480; both from BD Biosciences), and 0.75 ml of complete growth medium containing 10% FBS was added to each well in the lower chamber. Following incubation for 48 h at 37°C, non-invasive cells were removed from the upper chamber, the cells attached to the lower chamber were fixed with methanol, stained with 0.1% crystal violet and then counted under a light microscope.

**Western blot analysis.** Fifty micrograms of total proteins were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA, USA). After blocking with 3% bovine serum albumin, the blots were incubated with antibodies against MMP-14 (Bioworld, Atlanta, GA, USA), GAPDH (Chemicon International, Temecula, CA, USA). Following incubation with red fluorescence conjugated secondary antibody, protein bands were scanned using Odyssey bands scanner (S/N ODY-2792 model: 9120) The intensity of the bands was analyzed using BandsScan Software.

**Statistical analysis.** Analysis of variance and Student's t-test were used to compare means of two or more different treatment

groups.  $P < 0.05$  was considered to indicate a statistically significant difference, unless otherwise stated. Data are expressed as mean  $\pm$  SE.

## Results

**MMP-14 is a functional target of miR-133a.** To identify potential targets of miR-133a both for experimental validation and functional studies in lung cancer, we performed *in silico* analysis of a range of miRNA target prediction databases. The target prediction of miR-133a was performed by using the following databases: TargetScan (<http://www.targetscan.org>), MicroCosm (<http://www.ebi.ac.uk/>), miRanda (<http://www.microrna.org/microrna/getGeneForm.do>) and miRGen ([www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Targets.cgi](http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Targets.cgi)). All databases analyzed presented MMP-14 as a converging target of miR-133a, which plays an essential role in the degradation of basement membranes and the expression of MMP-14 is correlated with metastasis. To validate MMP-14 as a *de novo* target of miR-133a, we constructed a luciferase reporter vector containing the entire wild-type 3'UTR of MMP-14 (psiCHECK<sup>TM</sup>-2-MMP-14-3'UTR-wt) or the mutant 3'UTR (psiCHECK<sup>TM</sup>-2-MMP-14-3'UTR-mut) with a 5-nucleotide mutation previously mentioned (Fig. 1A). HEK-293T cells, which exhibit low levels of miR-133a expression, were used for transient transfections with psiCHECK<sup>TM</sup>-2-MMP-14-3'UTR-wt or psiCHECK<sup>TM</sup>-2-MMP-14-3'UTR-mut. Co-transfection with miR-133a (a synthetic miR-133a mimic) resulted in a significant decrease (to 63%) in luciferase gene expression from the reporter vector containing the wild-type 3'UTR of MMP-14 when compared with a scrambled control (Fig. 1B), demonstrating direct targeting by miR-133a ( $P < 0.01$ ). Consistent with the data, no decrease in luciferase activity was observed when miR-133a was co-transfected with the mutant 3'UTR of MMP-14 reporter. Furthermore, miR-133a specific inhibitor, which is the antisense oligonucleotides of miR-133a can almost restore the inhibition effect ( $P < 0.05$ ) (Fig. 1B). These results indicate that MMP-14 is a direct target

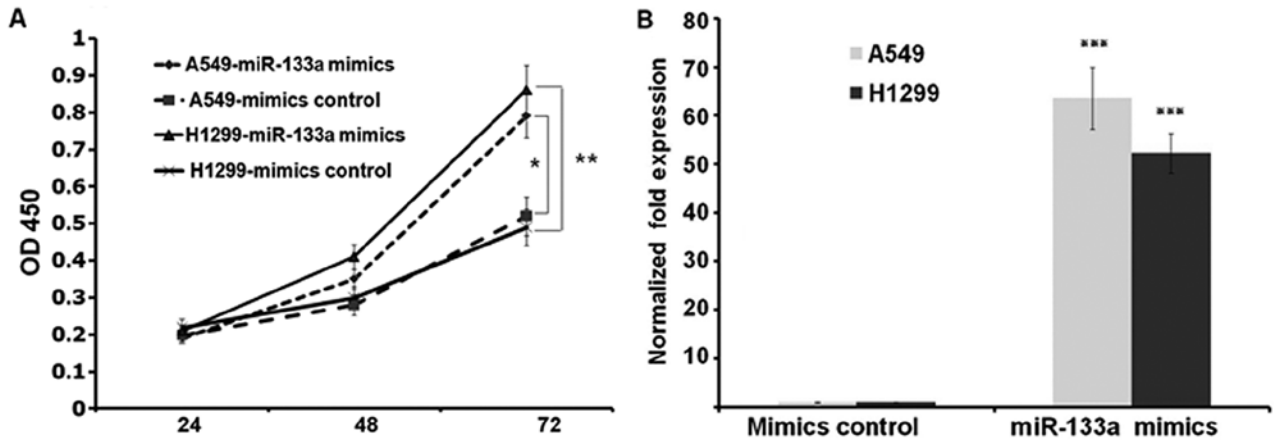


Figure 2. miR-133a suppresses cell proliferation in lung cancer cells. (A) Growth curves of A549 and NCI-H1299 cells after transfection as indicated were determined by Cell Counting Kit-8. (B) Mature miR-133a was determined using quantitative PCR with specific stem-loop reverse transcription primer after transfection.

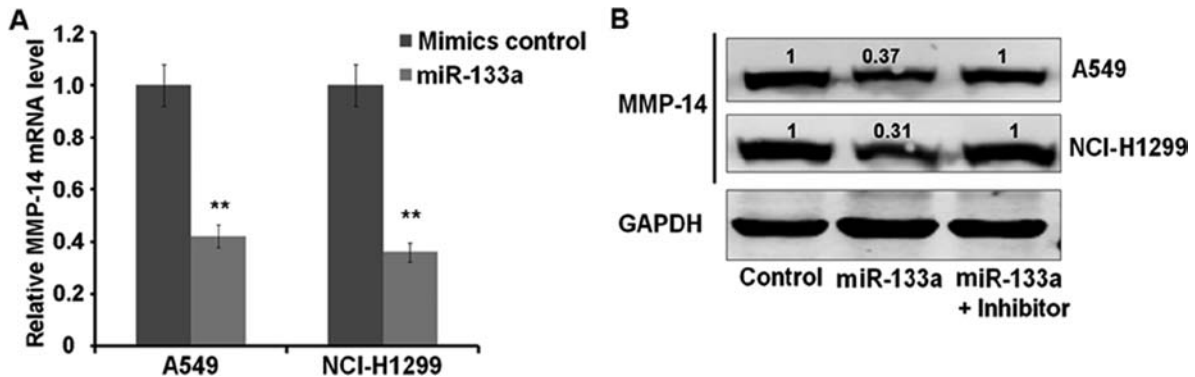


Figure 3. miR-133a decreases the mRNA and protein levels of matrix metalloproteinase (MMP)-14 (A) Total RNA was extracted from A549 and NCI-H1299 cells after transfection as indicated, and quantitative PCR was performed to determine MMP-14 expression. (B) A549 and NCI-H1299 cells were transfected as indicated. Endogenous MMP-14 and PCNA protein levels were determined by western blotting along with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. Relative protein levels were normalized by GAPDH and quantified by the ratio compared with controls.

of miR-133a and is responsible for miR-133a targeting in the 3'UTR.

*miR-133a suppresses cell proliferation in lung cancer A549 and NCI-H1299 cell lines.* To characterize the functional impact of miR-133a on lung cancer cell behavior such as growth rate, we transfected A549 and NCI-H1299 cells with either miR-133a mimics or mimics control and transferred the cells into 96-well plates at  $3.0 \times 10^3$  cells/well post transfection. We harvested and detected the OD450 value of each well by CCK-8 kit at indicated times after seeding. These results showed that the growth rates of the two lung cancer cell lines were suppressed in miR-133a mimics transfected groups compared to control groups at 72 h ( $P < 0.01$  and  $P < 0.05$  at A549 and NCI-H1299 cell lines, respectively) (Fig. 2A). To confirm the transfection, we assessed the expressions of miR-133a at 72 h post transfection. As shown in Fig. 2B, miR-133a was upregulated to 63.6- and 52.3-fold at A549 and NCI-H1299 cells, respectively (both  $P < 0.001$ ) (Fig. 2B).

*Overexpression of miR-133a decreases the mRNA and protein levels of MMP-14.* We next assessed the effect of miR-133a

overexpression on MMP-14. Transfection of miR-133a into A549 and NCI-H1299 cells resulted in a significant decrease of MMP-14 mRNA levels compared with control-transfected cells. As measured by qRT-PCR, the MMP-14 mRNA levels were decreased to 41 and 38% in A549 and NCI-H1299, respectively (both  $P < 0.01$ ) (Fig. 3A). Subsequent western blot analysis of MMP-14 showed that miR-133a overexpression caused a reduction in MMP-14 protein levels, which can be reversed by miR-133a specific inhibitor (Fig. 3B). Measured by BandsScan Software according to grayscale of each band, MMP-14 protein is downregulated to 37 and 31% in A549 and NCI-H1299 cells, respectively. These results indicated that miR-133a can inhibit lung cancer cell proliferation, which may be mediated by downregulation of direct target MMP-14 at mRNA and protein levels.

*miR-133a inhibits lung cancer cell migration and invasion through targeting MMP-14.* MMP-14 is an MT1-MMP as a primary regulator of interstitial collagenolysis and there is evidence to suggest that MMP-14 is enhanced in metastatic PDAC lesions compared with the primary tumors and myogenic tumors (31,32). The downregulation of MMP-14 by miR-133a

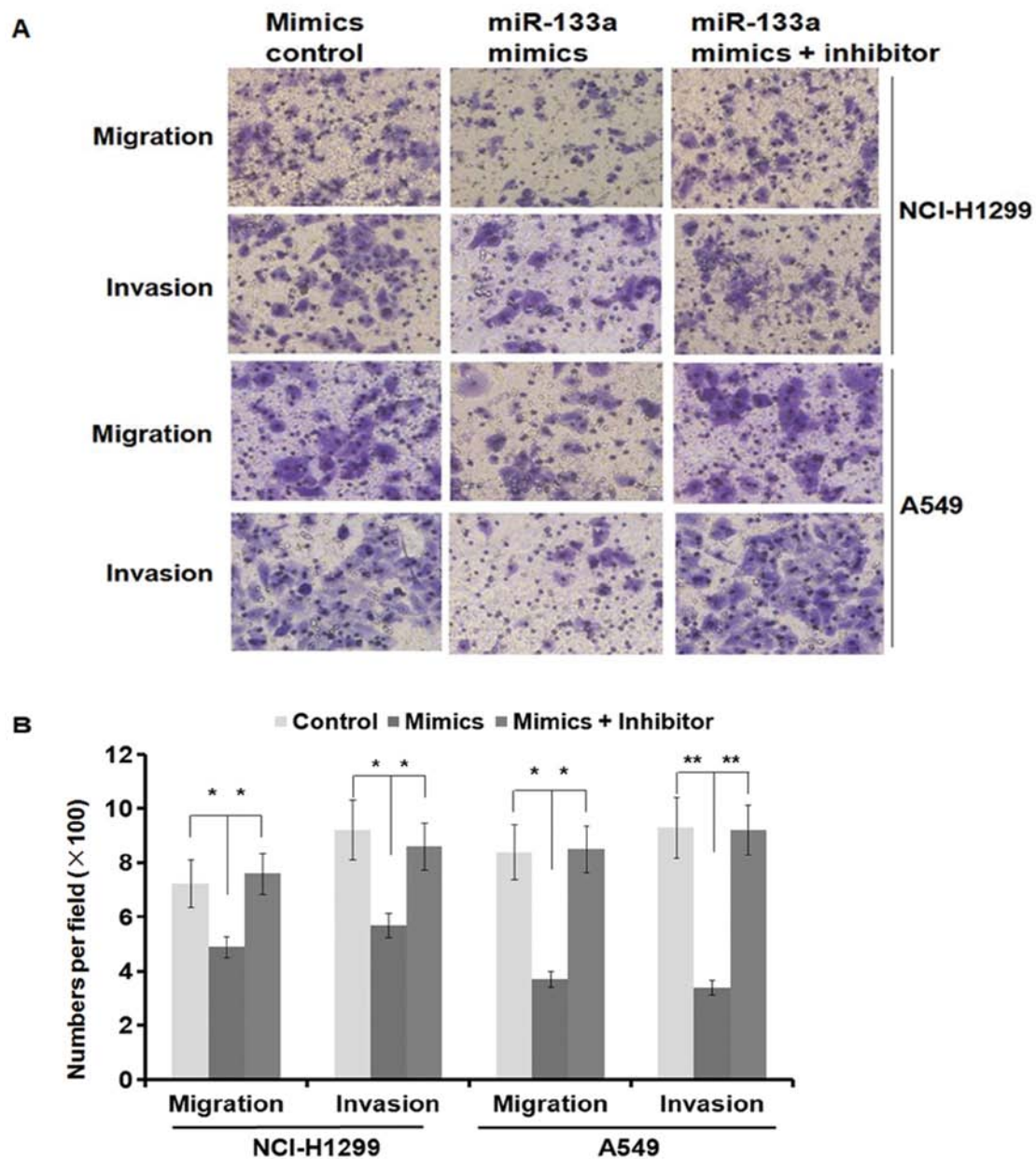


Figure 4. miR-133a inhibits cell migration and invasion in lung cancer cell lines and the miR-133a specific inhibitor reverses the effect. (A) A549 and NCI-H1299 cells were subjected to transwell migration and Matrigel invasion assays 6 h after transfection as indicated. The crystal violet dye staining images of the lower chambers are shown. (B) The data are shown as mean  $\pm$  SE of the number of cells that migrated or invaded in  $n=6$  separate experiments. \*\* $P<0.01$  and \*\*\* $P<0.001$  as compared with controls. Magnification,  $\times 100$ .

overexpression prompted us to examine whether miR-133a could modulate migration and invasion in lung cancer cell lines. We performed the transwell migration and Matrigel invasion assays in A549 and NCI-H1299 cells transfected with either miR-133a mimics or control mimics. As shown in Fig. 3A, miR-133a mimics suppressed cell migration and invasion in both A549 and NCI-H1299 cells (migration and invasion,  $P<0.05$  in the NCI-H1299 cell line and  $P<0.01$  in the A549 cell line) (Fig. 4A). The specific inhibitor of miR-133a almost restored the inhibition effect in both cell lines (Fig. 4A and B). Moreover, the MMP-14 protein levels were also restored (Fig. 3B). Collectively, these results clearly indicate that miR-133a suppresses cell migration and invasion in the A549 and NCI-H1299 cell lines by reducing MMP-14 expression.

## Discussion

miRNAs play important roles in a broad range of biological processes including development, cellular differentiation, proliferation and apoptosis (18). Increasing evidence shows that several miRNAs including the miR-200 family, miR-10b and miR-205 are involved in the lung cancer metastasis process (19-21). In the present study, we identified that miR-133a is another miRNA which can inhibit cell proliferation, migration and invasion in lung cancer cell lines by targeting MMP-14. Several lines of evidence support this idea. First, downregulation of MMP-14 by miR-133a was confirmed in A549 and NCI-H1299 cell lines at the protein and mRNA levels. Second, miR-133a repressed luciferase

reporter gene containing the MMP-14 3'UTR through its binding site and mutation of the putative binding site on MMP-14 3'UTR almost abolished the suppression effect. Third, overexpression of miR-133a in A549 and NCI-H1299 cells suppressed cell proliferation, migration and invasion through reducing MMP-14 expression. Moreover, the specific antisense miR-133a inhibitor can almost restore the inhibition effect as well.

A number of recent studies analyzed the genetic expression of MMP-14 in several types of human cancer, including tumors of the breast, colon, head, neck and oral cavity (6,8), indicating the importance of MMP-14 in cancer progression. MMP-14 is an essential molecule whose function is known to include roles in the activation of other MMP family members, pericellular proteolysis, modulation of cellular signaling and cell migration (5). It is not surprising that the expression of MMP-14 is tightly regulated at multiple levels. For example, TGF- $\beta$ 1 was reported to promote migration in an MMP-14 dependent manner in pancreatic ductal epithelial (HPDE) cells (9). In the present study, we confirmed that MMP-14 is indeed a functional target of miR-133a and found that the expression of MMP-14 is negatively regulated by miR-133a. It should be noted that mature miR-133a sequence (5'-UUUGGU CCCCUCUACACCAGCUA-3') is highly conserved among mammalian species. The potential miR-133a binding site (5'-UUUGGUC-3') is also presented in the known MMP-14 3'UTR of rat, dog, horse and cattle, suggesting that miR-133a may regulate MMP-14 expression of these species in a similar manner. Collectively, it is reasonable to hypothesize that post-transcriptional regulation of MMP-14 by miRNAs may be a major determinant of MMP-14 expression in lung cancer growth and metastasis.

The functions of mammalian miR-133a and its targets have been reported in several publications. Ectopic expression of miR-133a significantly inhibited the invasion capacity of various human cancer cell lines (27,28). It is reported that miR-133a targets FSCN1 in MCF-7 and MDA-MB-231 breast cancer cell lines. In breast cancer, miR-133a expression was found reduced by a microarray-based analysis (27), which indicated the tumor suppressive function of miR-133a. As is known, miRNAs can target hundreds of targets (16), miRNAs may play different roles in different cell lines or under different signals. We cannot exclude the possibility that a single miRNA can exert multiple functions by targeting multiple targets. In the present study, ectopic expression of miR-133a was more than 50-fold compared with endogenous expression, but MMP-14 is only downregulated to approximately 30%, indicating that the endogenous expression of miR-133a is extremely low and that MMP-14 may not be the only target in this condition. The specific inhibitor of miR-133a can almost restore the suppression of cell migration and invasion. These results suggest the function of miR-133a in lung cancer is prominently inhibition of tumor metastasis rather than tumor growth.

In summary, in the present study we described for the first time that miR-133a plays an anti-metastatic role in lung cancer and miR-133a may be a suitable tumor marker for lung cancer metastasis. We also proved that MMP-14 is a new functional target of miR-133a. In further studies, we will uncover the complex functions of miR-133a in modulating lung cancer progression.

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