Members of the miR-30 family inhibit the epithelial-to-mesenchymal transition of non-small-cell lung cancer cells by suppressing XB130 expression levels

KEWEI SONG[1], YINHUI JIANG[1,2], YAN ZHAO[1,2], YUAN XIE[1,2], JIANJIANG ZHOU[1,2], WENFENG YU[1,2] and QINRONG WANG[1,2]

[1]College of Sport and Health and Key Laboratory of Endemic and Ethnic Diseases of The Ministry of Education, Guizhou Medical University; [2]Key Laboratory of Medical Molecular Biology, Guizhou Medical University, Guiyang, Guizhou 550004, P.R. China

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Abstract. MicroRNAs (miRs) are associated with cancer metastasis. Aberrant expression levels of members of the miR-30 family have been observed in non-small-cell lung cancer (NSCLC). However, the effects of miR-30 family members on the epithelial-to-mesenchymal transition (EMT) of NSCLC cells and the underlying molecular mechanisms have not yet been fully elucidated. The present study investigated the effects of miR-30 family members on EMT, migration and invasion of NSCLC cells and found that overexpression of these miRs inhibited EMT via decreasing the expression levels of N-cadherin, β-catenin and SNAI1, along with weakened migration and invasion abilities. Then, XB130 was identified as a downstream target of the miR-30 family members. XB130-knockdown also inhibited EMT of NSCLC cells, whereas ectopic overexpression of XB130 partly rescued the suppressive effects of miR-30c and miR-30d on EMT. In conclusion, miR-30 family members inhibited EMT of NSCLC cells, partially via suppressing XB130 expression levels.

Introduction

Lung cancer is one of the most common types of malignant tumor worldwide, among which non-small cell lung cancer (NSCLC) accounts for 85-90% of cases (1-3). Despite progress in clinical diagnosis and treatment of NSCLC over the past several decades, the 5-year survival rate is ~15% (1,3). Therefore, it is necessary to understand the molecular mechanisms underlying NSCLC development and metastasis in order to improve diagnosis and treatment of NSCLC (4).

Cell invasion and metastasis impede the treatment of patients with NSCLC (5,6). Before acquiring these abilities, tumor cells undergo the epithelial-to-mesenchymal transition (EMT) (7). Normal EMT is a physiological cell reprogramming phenomenon during development (7). However, studies have demonstrated that deregulated EMT is associated with tumor occurrence and development (7-9). A number of molecules, such as E-cadherin, N-cadherin, β-catenin and SNAI1, are considered to be key markers of EMT (8).

MicroRNAs (miRs) regulate the expression levels of downstream target genes via binding to mRNA 3'-untranslated regions (3'-UTRs) or coding sequences (9,10). Multiple studies have shown that dysregulated miRNA expression level profiles play important roles in carcinogenesis (5,10). The expression levels of miR-30 family members (miR-30a/b/c/d/e) are repressed in a number of types of cancer, including lung cancer (7,11-14). Several miR-30 family members have critical roles in EMT, migration and invasion of NSCLC cells (7,12,13). For example, miR-30a has been shown to inhibit EMT by targeting SNAI1 and B-cell lymphoma/leukemia 11A in NSCLC (7). Low expression levels of miR-30c can promote invasion by inducing EMT in NSCLC (13). miR-30d can restrain NSCLC cell motility by targeting CCNE2 (12).

XB130, a multifunctional adaptor protein, is an oncogene that mediates cell proliferation, migration and invasion in osteosarcoma, hepatocellular and esophageal squamous cell carcinoma and pancreatic ductal adenocarcinoma, as well as prostrate, breast and gastric cancer (15-22). However, Cho *et al* (23) recently suggested that XB130 acts as a tumor suppressor in skin tumorigenesis by inhibiting inflammation, which indicates that XB130 may serve different roles in different types of tumor. Our previous study (24) demonstrated that, similar to miR-30 family members, XB130 silencing can inhibit cell migration, invasion and EMT in NSCLC. In addition, miR-30d and miR-30e are significantly upregulated in XB130 shRNA-transfected cells, suggesting that there may be a regulatory association between miR-30 family members and XB130 (25).

Correspondence to: Dr Qinrong Wang, College of Sport and Health and Key Laboratory of Endemic and Ethnic Diseases of The Ministry of Education, Guizhou Medical University, 9 Beijing Road, Guiyang, Guizhou 550004, P.R. China
E-mail: qin_rong_fei@163.com

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In order to understand the effects of miR-30 family members on the EMT of NSCLC cells and the related mechanisms, the present study investigated the effects of miR-30 family members overexpression and XB130 silencing on EMT in A549 and PC-9 cells. In addition, the present study explored the regulatary association between miR-30 family members and XB130, which may provide a novel theoretical basis for the diagnosis and treatment of NSCLC.

Materials and methods

Cell culture and transfection. The NSCLC cell lines A549 and PC-9 were purchased from Conservation Genetics CAS Kunming Cell Bank and FuHeng Biology Company, respectively. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS (both Gibco; Thermo Fisher Scientific, Inc.) and maintained in a humidified incubator containing 5% CO_2 at 37˚C. miR-30 family mimics or miR-30c or miR-30d inhibitors or XB130 siRNAs (Shanghai GenePharma Co., Ltd.) at a final concentration of 100 nM with or without DNA plasmids were transfected or co-transfected into cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) or Entranster™-R4000 (Engreen Biosystem Co., Ltd.) according to the manufacturer's instructions. Non-targeting sequences were used for miR mimics or inhibitors or siRNAs transfection controls. All sequences of the miRs and siRNAs used are presented in Table I. Cells used for western blotting and luciferase reporter assays were harvested 48 h after transfection. Wound healing and Matrigel invasion assays were performed 24 h after transfection.

Plasmid construction. miR-30 family binding sites in XB130 3'UTR were predicted using TargetScan (http://www.targetscan.org/vert_71/) and PicTar (https://pictar.mdc-berlin.de/) target prediction databases. In order to construct the dual-luciferase reporter plasmid, a fragment containing the miR-30 family binding sites was amplified from XB130 3'-UTR with primers WT-30-For and WT-30-Rev (Table I). Then, the fragment was cloned into psiCHECK-2 vector (Promega Corporation) and the recombinant was named WT-30. The XB130 open reading frame (ORF) was amplified from PC-9 cDNA using primers XB130-For and XB130-Rev (Table I), and then inserted to the vector pcDNA3.1(+) (Invitrogen; Thermo Fisher Scientific, Inc.), which was named pcDNA3.1-XB130 ORF. To obtain PC-9 cDNA, total RNA in PC-9 cells was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Then total RNA was reverse transcribed using a PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Biomedical Technology Co., Ltd.), with removing gDNA at 42˚C for 2 min followed by reverse transcription at 37˚C for 15 min. The synthesized cDNA was used as a template for XB130 3'-UTR and ORF amplifications. PCR reaction was performed using a PrimeSTAR® Max DNA Polymerase kit (Takara Biomedical Technology Co., Ltd.) and the reaction mixture included 25 μl 2X PrimeSTAR Max Premix, 1 μl upstream primers, 1 μl downstream primers, 1 μl cDNA template and 22 μl ddH_2O. PCR thermocycling were as follows: Denaturation at 98˚C for 10 sec, annealing at 55˚C for 15 sec and extension at 72˚C for 15 sec for 35 cycles. All primers were synthesized at the Sangon Biotech Co. Ltd. All constructs were confirmed via DNA sequencing.

Western blotting analysis. Cells were lysed in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (both Beyotime Institute of Biotechnology). The prepared protein samples were quantified using a bicinchoninic protein assay kit (Beijing Solarbio Science and Technology Co., Ltd.). Equal amounts of protein (40 μg/lane) were separated by 12% SDS-PAGE and then transferred to a PVDF membrane. The membranes were firstly blocked with 5% bovine serum albumin (Beijing Solarbio Science and Technology Co., Ltd.) at room temperature for 1 h and then incubated with primary antibodies against XB130 (1:1,000; cat. no. ab106433; Abcam), N-cadherin (1:5,000; cat. no. 22018-1-AP; ProteinTech Group, Inc.), SNAI1 (1:2,000; cat. no. 26183-1-AP; ProteinTech Group, Inc.), β-catenin (1:5,000; cat. no. 51067-2-AP; ProteinTech Group, Inc.) and GAPDH (1:5,000; cat. no. 10494-1-AP; ProteinTech Group, Inc.) overnight at 4˚C, and finally with horseradish peroxidase conjugated secondary antibodies (1:5,000; cat. no. SA00001-2; ProteinTech Group, Inc.) at room temperature for 1 h. The specific protein bands were visualized using an ECL reagent (EMD Millipore).

Wound healing assay. Transfected cells proliferated 12-well plates as confluent monolayers were mechanically scratched using a 200 μl pipette tip to create a straight wound. Cells were washed twice with PBS to remove the debris and then cultured with RPMI-1640 medium containing 4% FBS for 48 h to allow wound healing Images were captured at 0 and 48 h to determine cell migration using a light microscope (magnification, x100). Healing distance between the wound (%) was expressed as follows: [(Gap distance at 0 h-Gap distance at 48 h)/Gap distance at 0 h] x100%.

Matrigel invasion assay. A 24-well Matrigel transwell chamber (Costar; Corning, Inc.) was used to measure cell invasion. Briefly, transwell chamber with 8-μm pore size was precoated with 100 μl Matrigel matrix (1:8 dilution) for 1 h at 37˚C (BD Biosciences). The upper chamber was plated with $2x10^5$ cells in serum-free RPMI-1640 medium. The chambers were then inserted into a 24-well plate with 0.6 ml complete RPMI-1640 medium containing 10% FBS in each well. After incubation at 37˚C for 48 h, the cells remaining on the upper chamber were removed; cells adhering to the lower surface were fixed using methanol for 30 min and stained using crystal violet for 1 h at room temperature. Images were captured using a light microscope (magnification, x200) and counted using ImageJ software version 1.48 (National Institutes of Health).

Luciferase reporter assay. Reporter plasmids and RNA oligos were transiently co-transfected into PC-9 cells. After 48 h, the luciferase activities were measured using a Luc-Pair™ Duo-Luciferase HS Assay kit (GeneCopoeia, Inc.) on a BioTek Synergy2 Multimode Microplate Reader (BioTek Instruments, Inc.). *Firefly* luciferase was used for normalization.

Statistical analysis. Data are expressed as the mean ± SD of three independent experiments. Differences among groups were analyzed by one-way ANOVA followed by the post hoc

Table I. Sequences of primers and RNA oligos.

Name	Sequence (5'→3')
WT-30	F: CCGCTCGAGATTAAAGTGACTCTTTACT
	R: CGGGATCCGAGAATGAACATTAAACAGA
XB130	F: CTAGCTAGCATGGAGCGGTACAAAGCCCTG
	R: CCGGAATTCCTAACTTGCTCCTTTCTTCTCCCATT
hsa-miR-30a	F: UGUAAACAUCCUCGACUGGAAG
	R: CUUCCAGUCGAGGAUGUUUACA
hsa-miR-30b	F: UGUAAACAUCCUACACUCAGCU
	R: AGCUGAGUGUAGGAUGUUUACA
hsa-miR-30c	F: UGUAAACAUCCUACACUCUCAGC
	R: GCUGAGAGUGUAGGAUGUUUACA
hsa-miR-30d	F: UGUAAACAUCCCCGACUGGAAG
	R: CUUCCAGUCGGGGAUGUUUACA
hsa-miR-30e	F: UGUAAACAUCCUUGACUGGAAG
	R: CUUCCAGUCAAGGAUGUUUACA
Anti-30c	GCUGAGAGUGUAGGAUGUUUACA
Anti-30d	CUUCCAGUCGGGGAUGUUUACA
XB130 siRNA-1	F: GGAGCUAAAGGAAACCCUACU
	R: AGUAGGGUUUCCUUUAGCUCC
XB130 siRNA-2	F: GAUUCUUGACCAGGAGAAC
	R: GUUCUCCUGGUCAAGAAUC
NC siRNA/miR-cont	F: UUCUCCGAACGUGUCACGUTT
	R: ACGUGACACGUUCGGAGAATT
Anti-cont	CAGUACUUUUGUGUAGUACAA

WT, wild-type; F, forward; R, reverse; miR, microRNA; siRNA, small interfering RNA; cont, control.

Tukey's test using SPSS software (version 20; SPSS, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-30 family members suppress expression levels of N-cadherin, β-catenin and SNAI1 in NSCLC cells. In order to determine the effects of miR-30 family members on the EMT of NSCLC cells, the expression levels of EMT markers, including N-cadherin, β-catenin and SNAI1, in A549 and PC-9 cells overexpressing miR-30 family members were determined. The results revealed that overexpression of miR-30 family members decreased the expression levels of EMT markers in these cells ($P<0.05$). Due to the similarity of the miR-30a-e sequences, miR-30c or miR-30d inhibitors were randomly selected. miR-30c or miR-30d inhibitors reversed the effect of miR-30c or miR-30d-overexpression on the expression levels of EMT markers (Fig. 1; $P<0.05$). These results indicated that upregulation of miR-30 family members may prevent the EMT of NSCLC cells.

miR-30 family members inhibit the migration and invasion of NSCLC cells. Since miR-30 family members suppressed the expression levels of EMT markers, the effects of miR-30 family members on cell migration and invasion were further investigated using wound healing and Matrigel Transwell assays. A549 and PC-9 cells overexpressing miR-30 family members exhibited significant decreases in invasion and migration abilities compared with cells transfected with negative control mimics (Fig. 2A and B; $P<0.05$). Moreover, the overexpression of miR-30c or miR-30d inhibitors reversed the effects of miR-30c or miR-30d mimics (Fig. 2A and B; $P<0.05$). These results indicated that increased expression levels of miR-30 family members inhibit NSCLC cell invasion and migration by impeding the EMT process.

miR-30 family members regulate XB130 expression levels in NSCLC. Next, the molecular mechanisms underlying the functions of miR-30 family members were determined. Cells transfected with miR-30 family members exhibited decreased expression levels of endogenous XB130 protein compared with the control (Fig. 3A; $P<0.05$). In order to confirm whether XB130 is a direct target of miR-30 family members, miR-30 family binding sites in XB130 3'UTR were identified using publicly available databases (TargetScan and PicTar). However, co-transfection of miR-30 family members into PC-9 cells did not inhibit the activity of *Renilla* luciferase in plasmid WT-30 compared with the control (Fig. 3B). Together, these data suggested that miR-30 family members negatively regulate the expression levels of XB130 but do not directly target its 3'UTR.

XB130 is involved in the EMT induced by miR-30 family members in NSCLC cells. In order to evaluate whether miR-30 family

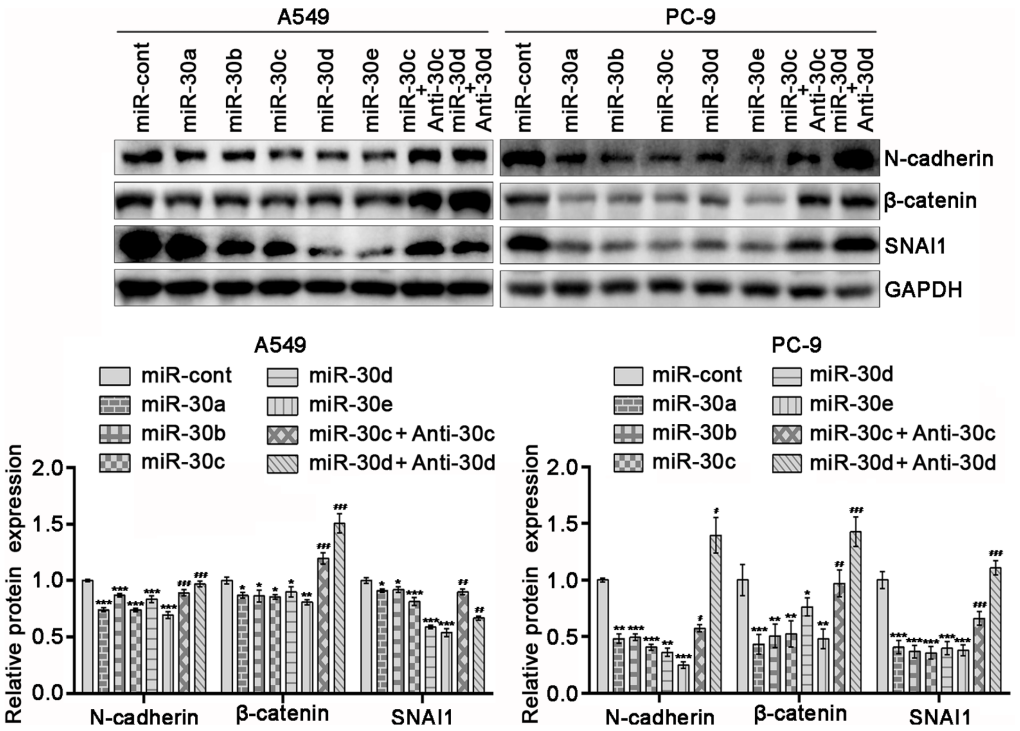

Figure 1. Overexpression of miR-30 family members represses the epithelial-to-mesenchymal transition of non-small cell lung cancer cells. A549 and PC-9 cells were co-transfected with miR mimics and inhibitors. The protein levels of N-cadherin, β-catenin and SNAI1 were determined using western blotting. GAPDH was used as a loading control. Results are representative of the mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. miR-cont; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. miR-30c or miR-30d. miR, microRNA; cont, control.

members inhibit NSCLC cell EMT partially by suppressing XB130 expression levels, siRNAs silencing XB130 were transfected into A549 and PC-9 cells. siRNA treatment led to notable decreases in the protein expression levels of XB130, N-cadherin, β-catenin and SNAI1 (Fig. 4A; P<0.05). In addition, ectopic overexpression of XB130 in A549 and PC-9 cells overexpressing miR-30c or miR-30d counteracted the inhibitory effects of miR-30c or miR-30d mimics on EMT markers (Fig. 4B; P<0.05). These observations indicated that XB30 may be involved in miR-30 family-induced EMT in NSCLC.

Discussion

EMT is a key process during tumor development and metastasis (26). miRs are involved in a number of essential biological processes, including EMT, and their dysregulation is associated with tumorigenesis (10,27). miR-30 family members are downregulated in NSCLC and are associated with the development and metastasis of NSCLC (7,11-14). The present study confirmed that overexpression of miR-30 family members in A549 and PC-9 cells reversed NSCLC EMT by inhibiting XB130 expression levels.

Previously, miR-30a and miR-30c have been demonstrated to regulate the EMT of NSCLC cells (7,13). However, the roles of other miR-30 family members in the EMT of NSCLC cells have not been fully elucidated. The present study demonstrated that overexpression of miR-30 family members significantly reversed EMT by decreasing N-cadherin, β-catenin and SNAI1 expression levels, and also attenuated migration and invasion. It was confirmed that XB130 protein expression levels were downregulated by miR-30 family overexpression. miR-30 family binding sites in *XB130* mRNA 3'UTR were identified via bioinformatics tools; however, the expression levels of *Renilla* luciferase in the reporter plasmid were not suppressed by the overexpression of miR-30 family members. It was hypothesized that miR-30 family members may suppress XB130 expression levels via other binding sites in *XB130* mRNA (28). Alternatively, the secondary structure of XB130 3'UTR transcript from the reporter plasmid or certain RNA-binding proteins binding with the XB130 3'UTR may have prevented miR-30 family members binding (29,30). However, these hypotheses require further verification. Another possible explanation is that XB130 may not be a direct target of miR-30 family members but an indirect mediator of this family, regulating EMT in NSCLC cells (25).

XB130, also known as actin filament associated protein 1-like 2, is a member of AFAP family (31). As a tumor promotor, XB130 expression levels are upregulated in numerous types of cancer tissues and can mediate cell proliferation, migration, invasion and EMT by crosslinking actin filaments, or by downstream activation of associated signaling pathways, such as PI3K/AKT (15-18,20,22,32-34). *XB130* mRNA is a good predictor of 5-year disease-free survival rate for patients with NSCLC, as well as a marker to distinguish adenocarcinoma from squamous cell carcinoma (35). To the best of our knowledge, the expression level profile of the XB130 protein in NSCLC tissues has not previously been elucidated. Shiozaki *et al* (33) demonstrated that XB130 interference decreased cell proliferation in NSCLC. Our previous study (24) confirmed that XB130 silencing inhibited NSCLC cell migration, invasion and EMT, similar to the functions of miR-30 family members. Moreover, ectopic overexpression of

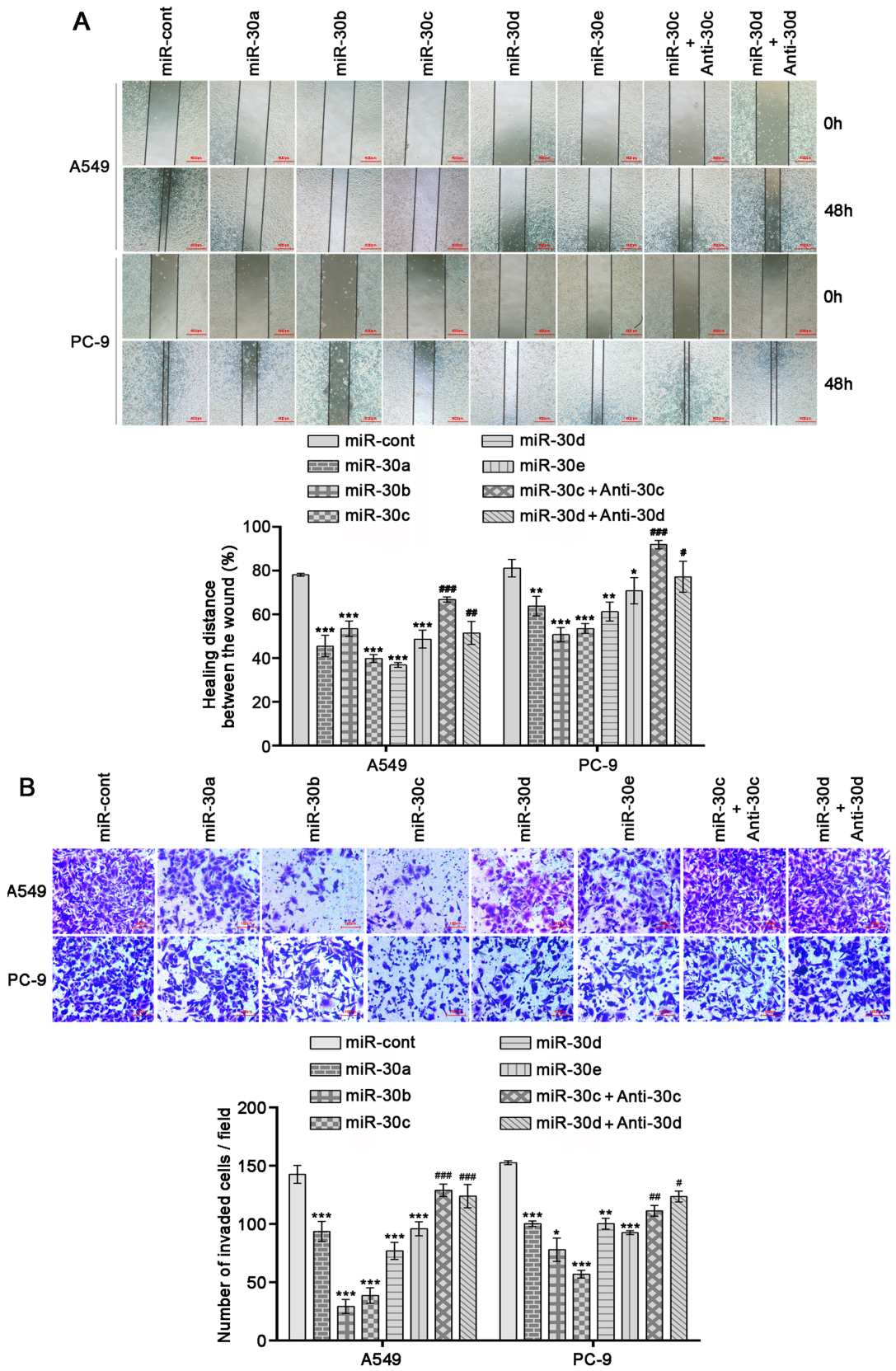

Figure 2. Overexpression of miR-30 family members inhibits the invasion of non-small cell lung cancer cells. A549 and PC-9 cells were co-transfected with miR mimics and inhibitors. (A) Cell migration was evaluated via wound healing assay. (B) Cell invasion was evaluated using a Matrigel-coated Transwell assay. Results are representative of the mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. miR-cont; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P <0.001 vs. miR-30c or miR-30d. miR, microRNA; cont, control.

XB130 in the present study reversed the effects of miR-30c or miR-30d overexpression on the levels of EMT-associated proteins, indicating that XB130 is involved in mechanism by which miR-30 family members mediate the EMT process.

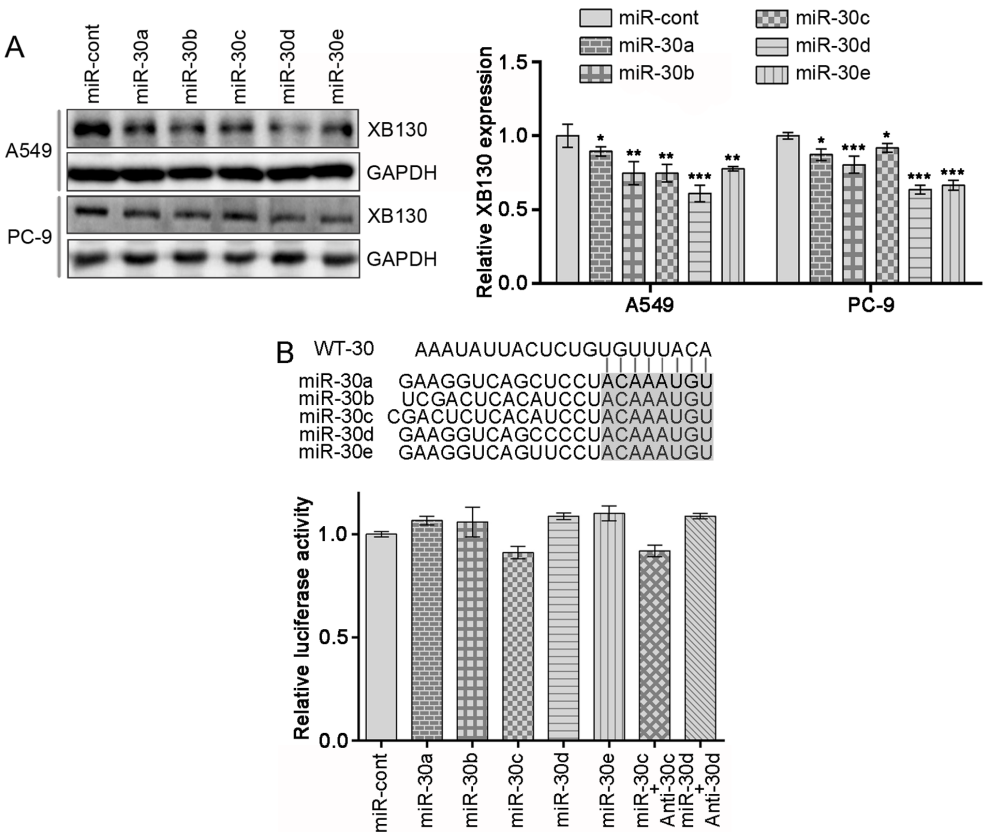

Figure 3. miR-30 family overexpression suppresses XB30 expression levels. (A) XB130 protein levels in A549 and PC-9 cells transfected with miR mimics were determined using western blotting. GAPDH was used as an internal control. (B) Potential binding sites of miR-30 family members in the XB130 3' untranslated region were predicted using TargetScan and PicTar. The luciferase reporter constructs and RNA oligos were co-transfected into PC-9 cells. Luciferase activity was determined after 48 h. Results are representative of the mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. miR-cont. miR, microRNA; cont, control.

In conclusion, the present study demonstrated that miR-30 family members decreased the EMT of NSCLC cells by suppressing XB130 expression levels. However, the molecular mechanism by which the miR-30 family inhibited XB130 expression need further investigation. For wound healing assay, culturing cells with medium containing 4% FBS to allow wound healing is a limitation of the present study. In addition, further experiments that shed light on XB130 protein expression in cancer and paracancerous tissues and perform correlations of the expression levels of the miR-30 family and XB130 in cancer tissues from patients with NSCLC are required to verify the findings of the present study. Combined with the low expression levels of miR-30 family members exhibited by patients with NSCLC (7,11-14), the present results supported the hypothesis that enhancing miR-30 family expression levels or silencing XB130 may provide improved survival benefit for patients with NSCLC (13,31,36-39).

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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

QW, WY and JZ designed the study. KS and YJ performed the experiments. YZ and YX analyzed the data. QW wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

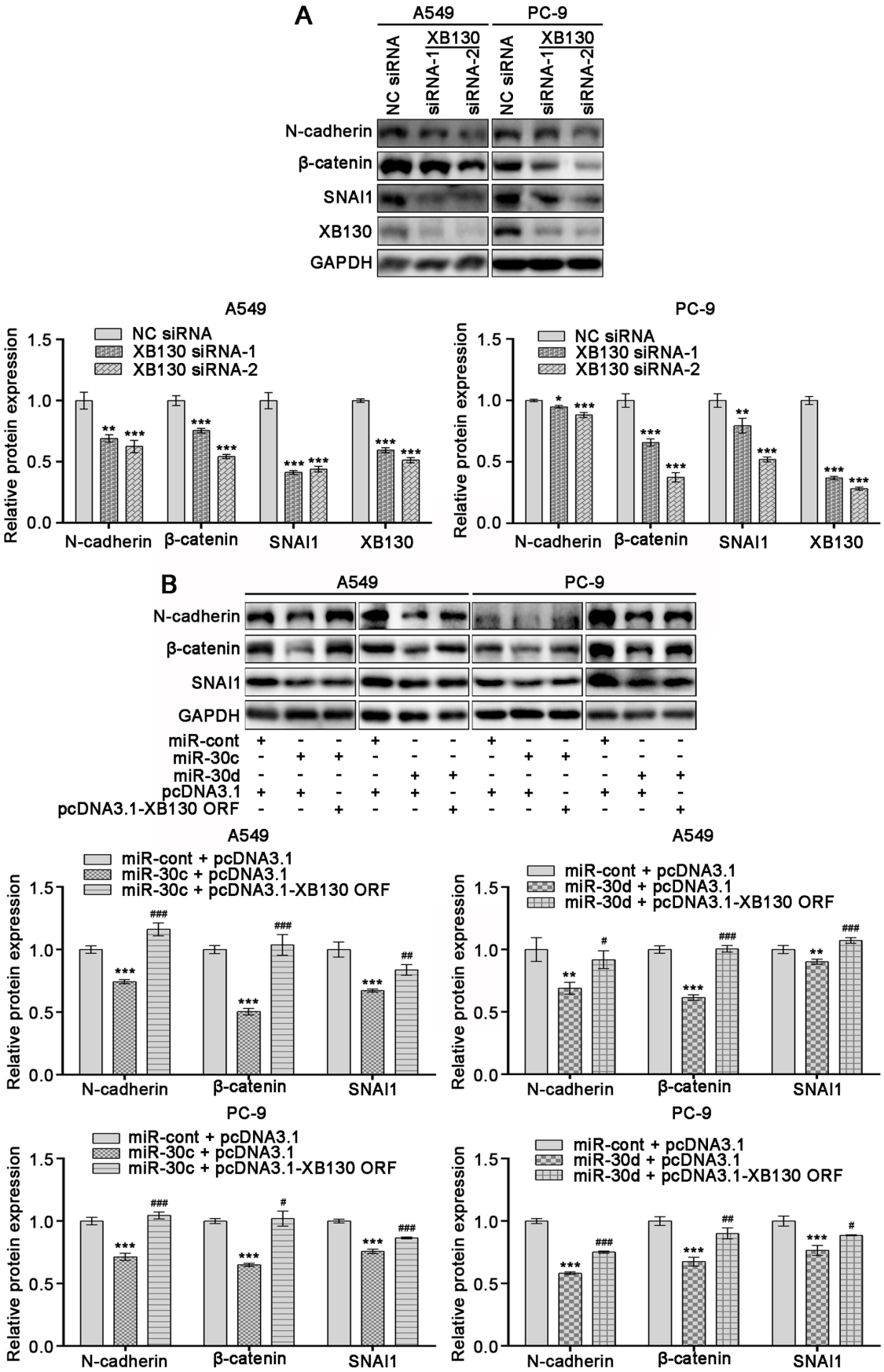

Figure 4. XB130 is involved in miR-30 family-induced epithelial-to-mesenchymal transition of non-small cell lung cancer cells. A549 and PC-9 cells were transfected with (A) XB130 siRNAs (siRNA-1 and -2) or NC siRNA or (B) co-transfected with miR mimics and DNA plasmids. After 48 h of transfection, total proteins were obtained and the expression levels of XB130, N-cadherin, β-catenin and SNAI1 were determined using western blotting. GAPDH was used as a loading control. Results are representative of the mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. NC siRNA or miR-cont and pcDNA3.1; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. miR-30c or miR-30d and pcDNA3.1. miR, microRNA; siRNA, small interfering RNA; NC, negative control; cont, control; ORF, open reading frame.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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