European Review for Medical and Pharmacological Sciences 2016; 20: 4003-4010

Regulation of Twist in the metastasis of non-small cell lung cancer by miR-92b

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Abstract. – OBJECTIVE: It is well documented that some microRNAs (miRNAs) regulates tumorigenesis and cancer metastases of non-small cell lung cancer (NSCLC). Nevertheless, a role of miR-92b in control of the metastasis of NSCLC has not been acknowledged.

MATERIALS AND METHODS: Here, we reported that miR-92b levels were significantly decreased and Twist levels were significantly increased in NSCLC specimens, compared to paired adjacent non-tumor lung tissue. Moreover, the levels of miR-92b and Twist inversely correlated.

RESULTS: Bioinformatics analyses and luciferase-reporter assay showed that miR-92b targeted the 3'-UTR of Twist mRNA to inhibit its translation. Overexpression of miR-92b inhibited Twist-mediated cell invasiveness, while depletion of miR-92b increased Twist-mediated cell invasiveness in either a transwell cell migration assay or a scratch wound healing assay.

CONCLUSIONS: Together, our data suggest that re-expression of miR-92b may inhibit Twist-mediated NSCLC metastasis.

Key Words:
Non-small cell lung cancer (NSCLC), Twist, miR-92b.

Introduction

Non-small cell lung cancer (NSCLC) is the most common lung cancer worldwide. NSCLC has 3 pathologic subgroups: squamous cell carcinoma, large cell carcinoma, and adenocarcinoma[1-3]. Some NSCLCs are susceptible to early invasion and metastasis and, thus, understanding the molecular mechanisms that underlie the regulation of NSCLC invasiveness appears to be critical to generate novel and effective therapy.

MicroRNA (miRNA) is a class of non-coding small RNA that regulate the gene expression post-transcriptionally, through its base-pairing with the 3 -untranslated region (3 -UTR) of the mRNA of the target gene[4]. Previous studies have shown strong evidence to support a critical role of miss-expressed miRNAs in the regulation of initiation and progression of the tumor, including NSCLC[5-8]. Among all miRNAs, miR-92b has only recently been reported involved in the pathogenesis of ovarian cancer, gastric cancer, cervical cancer and renal cancer[9-12]. MiR-92b appears to play a diverse role in different cancers. Hence, whether miss-expression of miR-92b may occur in NSCLC as well as its association with NSCLC tumorigenesis is largely unknown. Here, we addressed these questions.

In this study, we found that miR-92b levels were significantly decreased and Twist levels were significantly increased in NSCLC specimens, compared to paired adjacent non-tumor lung tissue. Moreover, the levels of miR-92b and Twist inversely correlated. Bioinformatics analyses and luciferase-reporter assay showed that miR-92b targeted the 3'-UTR of Twist mRNA to inhibit its translation. Overexpression of miR-92b inhibited Twist-mediated cell invasiveness, while depletion of miR-92b increased Twist-mediated cell invasiveness in either a transwell cell migration assay or a scratch wound healing assay. Together, our data suggest that re-expression of miR-92b may inhibit Twist-mediated NSCLC metastasis.

Materials and Methods

Experimental Protocol Approval

All experimental protocols were approved by the Research Bureau of Chinese PLA General Hospital. All mouse experiments were approved by the Institutional Animal Care and Use Committee at Chinese PLA General Hospital (Animal Welfare Assurance). The methods regarding animals and human specimens were carried out in "accordance" with the approved guidelines.

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Patient Specimens

Surgical specimens from 35 NSCLC patients and matched adjacent non-tumor tissues (NT) were obtained postoperatively in Chinese PLA General Hospital from 2011 to 2015. All patients gave signed, informed consent for the tissue to be used for scientific research. Ethical approval for the study was obtained from the Chinese PLA General Hospital. All diagnoses were based on pathological and/or cytological evidence. The histological features of the specimens were evaluated by senior pathologists according to the World Health Organization classification criteria.

Cell Line Culture and Transfection

A human NSCLC cell line A549 was purchased from APCC (American Type Culture Collection, Manassas, VA, USA), and have been widely used in NSCLC research. The A549 cell line was first developed in 1972 by Dr. Giard through the removal and culturing of cancerous lung tissue in the explanted tumor of a 58-year-old Caucasian male[13]. A549 cells were cultured in RPMI1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) in a humidified chamber with 5% CO_2 at 37°C.

Plasmids Transfection

MiR-92b-expressing and antisense (as) plasmids were prepared from a backbone plasmid containing a GFP reporter under CMV promoter (pcDNA3.1-CMV-GFP, Clontech, Mountain View, CA, USA). The miR-92b mimic, or antisense, or control null was all digested with Xhol and BamHI and then subcloned with a 2A into the pcDNA3.1-CMV-GFP plasmid. Sequencing was performed to confirm the correct orientation of the new plasmid. Transfection was done using Lipofectamine 2000 reagent (Invitrogen), according to the instructions of the manufacturer. One day after transfection, the transfected cells were purified by flow cytometry based on GFP expression.

Western Blot

The protein was extracted from the NSCLC or paired non-cancer tissue, or from cultured cells, in RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) on the ice. Protein concentration was determined using a BCA protein assay kit (Bio-Rad, China), and whole lysates were subjected to Western blot. Primary antibodies were rabbit anti-Twist and anti-α-tubulin (Cell Signaling, San Jose, CA, USA). The secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). Blotting images were representative from 5 repeats. α-tubulin was used as a protein loading control.

RT-qPCR

Total RNA was extracted from resected specimen from the patients or from cultured cells with the miRNeasy mini kit (Qiagen, Hilden, Germany) for cDNA synthesis. Complementary DNA (cDNA) was randomly primed from 2 g of total RNA using the Omniscript reverse transcription kit (Qiagen). Real-time quantitative PCR (RT-qPCR) was subsequently performed in triplicate. All primers were purchased from Qiagen. Data were collected and analyzed, using $^{2-\Delta\Delta}$Ct method for quantification of the relative mRNA expression levels. Values of genes were first normalized against α-tubulin, and then compared to experimental controls.

MicroRNA Target Prediction and 3'-UTR Luciferase-Reporter Assay

MiRNAs targets were predicted with the algorithms TargetSan. Luciferase-reporters were successfully constructed using molecular cloning technology. The Twist 3'-UTR reporter plasmid (Twist 3'-UTR) and Twist 3'-UTR reporter plasmid with a mutant at the miR-92b binding site (Twist 3'-UTR mut) were purchased from Creative Biogene (Shirley, NY, USA). A549 cells were co-transfected with Twist 3'-UTR/ Twist 3'-UTR mut and miR-92b/as-miR-92b/null by Lipofectamine 2000 (5×10^4 cells per well). Cells were collected 24 hours after transfection for assay using the dual-luciferase reporter assay system gene assay kit (Promega, Beijing, China), according to the manufacturer's instructions. The normalized control was null-transfected A549 cells with 3'-UTR of Twist mRNA (wild type).

Transwell Cell Migration Assay

Cells (10^4) were plated on the top side of polycarbonate transwell filter coated with Matrigel in the upper chamber of the BioCoatTM Invasion Chambers (Becton-Dickinson Biosciences, Bedford, MA, USA) and incubated at 37°C for 22 hours. The cells inside the upper chamber with cotton swabs were then removed. Migratory and invasive cells on the lower membrane surface were fixed, were stained with hematoxylin and

counted for 10 random 100X fields per well. Cell counts are expressed as the mean number of cells per field of view. Five independent experiments were performed, and the data are presented as mean ± standard deviation (SD).

Scratch Wound Healing Assay

Cells were seeded in 24-well plates at a density of 10^4 cells/well in complete DMEM and cultured to confluence. The cell monolayer was serum starved overnight in DMEM before initiating of the experiment. The confluent cell monolayer was then scraped with a yellow pipette tip to generate scratch wounds and washed twice with media to remove cell debris. Cells were incubated at 37 °C for 24 hours. Time-lapse images were captured after 12 hours. Images were captured from five randomly selected fields in each sample, and the wound areas are calculated by NIH ImageJ software (Bethesda, MA, USA).

Statistical Analysis

All statistical analyses were carried out using the SPSS 15.0 statistical software package (SPSS Inc., Chicago, IL, USA). Bivariate correlations were calculated by Spearman's Rank Correlation Coefficients. All values are depicted as mean ± standard deviation and are considered significant if $p < 0.05$. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher's Exact Test for comparison of two groups.

Results

Twist and miR-92b Are Inversely Correlated in NSCLC Specimens

We detected significantly higher levels of Twist (Figure 1A), and significantly lower levels of miR-92b in NSCLC (Figure 1B), compared to the paired non-tumor lung tissues (NT) in 35 specimens. Next, we examined their correlation and detected a strong inverse correlation between miR-92b and Twist (Figure 1C, $\gamma = -0.83$, $p < 0.0001$). These data suggest the presence of a causal link between miR-92b and Twist in NSCLC.

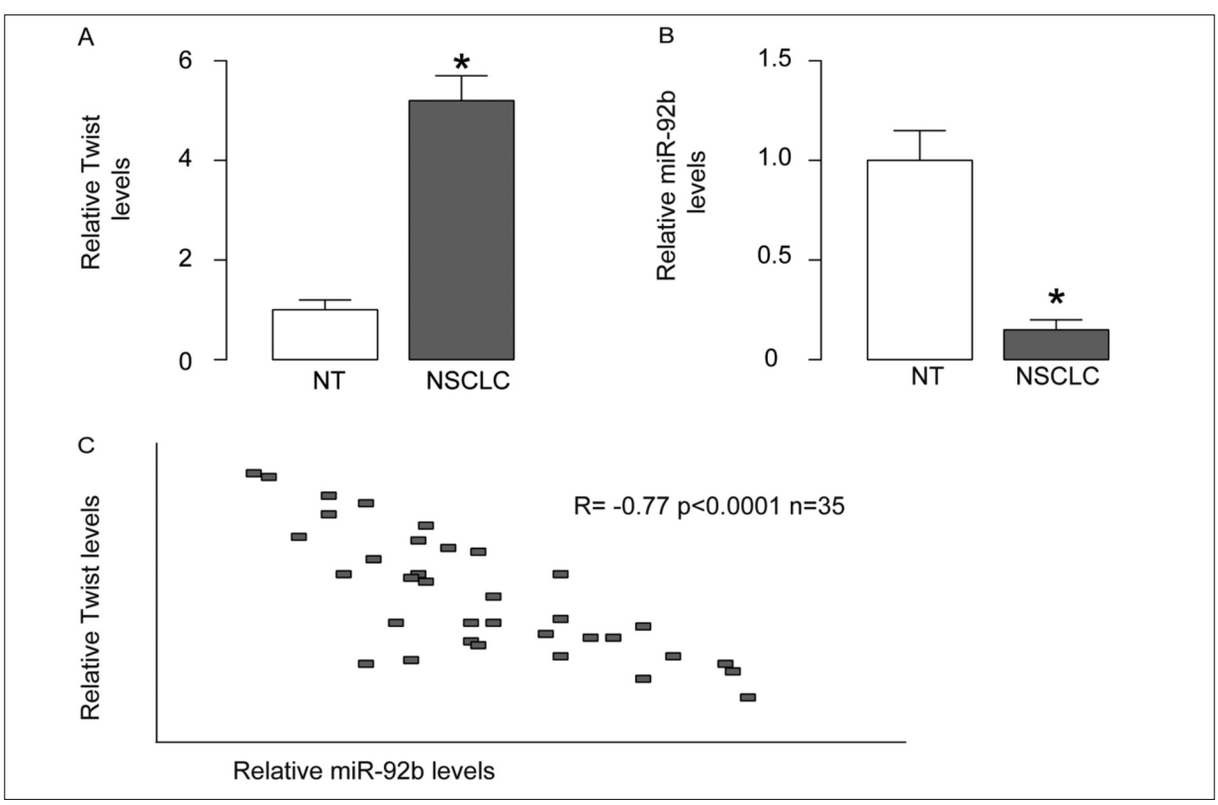

Figure 1. Twist and miR-92b are inversely correlated in NSCLC specimens. RT-qPCR on miR-92b and Western blot for Twist were performed on NSCLC and paired non-tumor lung tissues (NT) from 35 patients. ***A-B,*** Twist increased ***(A)***, and miR-92b decreased ***(B)*** in NSCLC, compared to NT. ***C,*** Correlation tests were performed between Twist and miR-92b. *$p < 0.05$. N = 35.

MiR-92b Targets 3′-UTR of Twist mRNA to Inhibit its Translation

Then, we checked whether miR-92b might target Twist mRNA. Bioinformatics analyses showed that miR-92b bound to 3'-UTR of Twist mRNA at 676th to 682nd base site (Figure 2A). In order to figure out whether this binding is functional, we either overexpressed miR-92b, or inhibited miR-92b in a human NSCLC cell line, A549 cells, using transfection with a miR-92b-expressing plasmid (A549-miR-92b), or a plasmid carrying miR-92b antisense (A549-as-miR-92b), respectively. The A549 cells were also transfected with a plasmid carrying a null sequence as a control (A549-Null). The modulation of miR-92b levels in these A549 cells was first confirmed by RT-qPCR (Figure 2B). A549 cells were then transfected with 1 g plasmids of miR-92b-modified plasmids, together with plasmids carrying a luciferase reporter for 3'-UTR of Twist mRNA or a luciferase reporter for 3'-UTR of Twist mRNA with mutate at the miR-92b binding site (mut). The luciferase activities were determined in these cells, and our data showed that Twist 3'-UTR plus miR-92b had the most repression for Twist, and the 3'-UTR Twist mutant plus miR-92b had much lower repression. Moreover, the 3'-UTR in the presence of as-miR-92b restored expression of Twist (Figure 2C), suggesting that miR-92b may target 3'-UTR of Twist mRNA to inhibit its translation.

MiR-92b Inhibits Twist in NSCLC Cells

We found that modification of miR-92b levels in A549 cells did not change mRNA levels of Twist (Figure 3A). However, overexpression of miR-92b significantly decreased Twist protein in A549 cells, while suppression of miR-92b significantly increased Twist protein in A549 cells, by

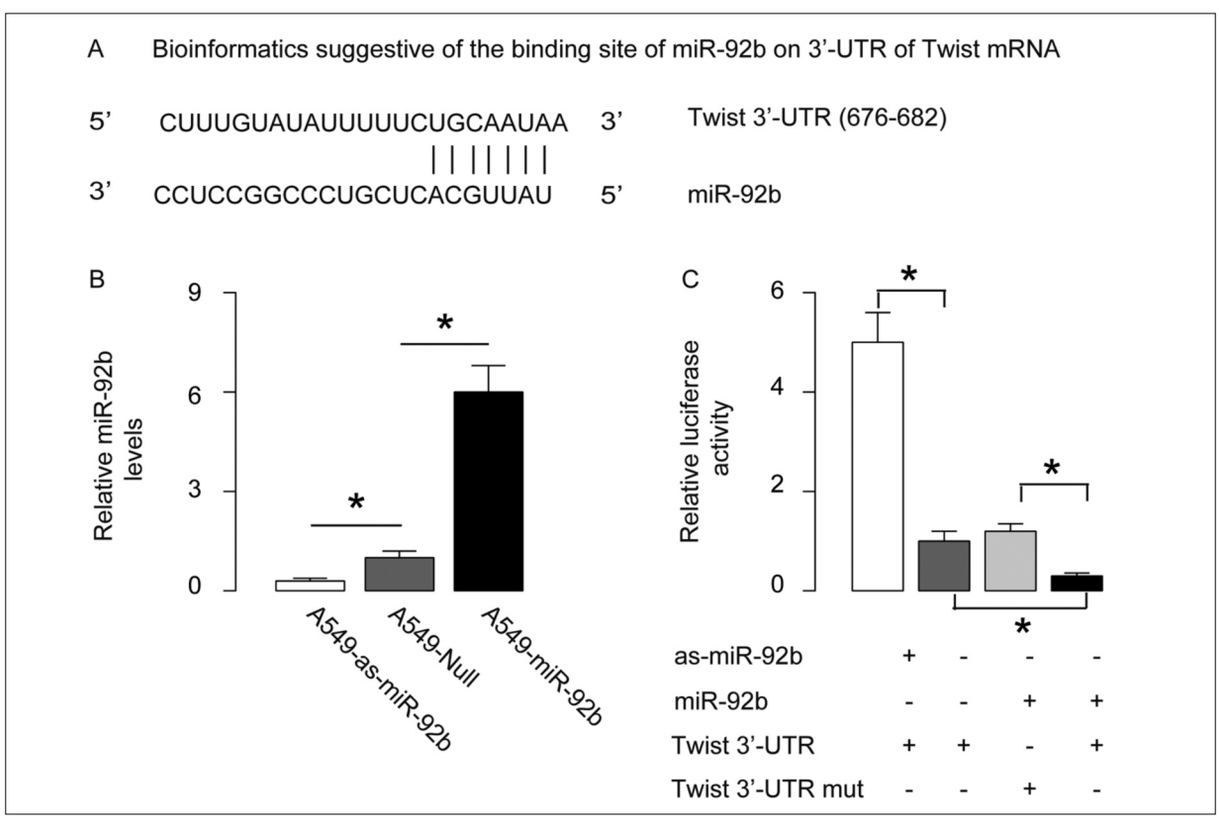

Figure 2. MiR-92b targets 3'-UTR of Twist mRNA to inhibit its expression. ***A,*** Bioinformatics analyses showing binding of miR-92b to the 3'-UTR of Twist mRNA. ***B,*** We either overexpressed miR-92b, or inhibited miR-92b in a human NSCLC cell line, A549, by transfection of the cells with a miR-92b-expressing plasmid (A549-miR-92b), or with a plasmid carrying miR-92b antisense (A549-as-miR-92b). The A549 cells were also transfected with a plasmid carrying a null sequence as a control (A549-Null). The modification of miR-92b levels in A549 cells was confirmed by RT-qPCR. ***C,*** MiR-92b-modified A549 cells were then transfected with 1μg of Twist 3'-UTR luciferase-reporter/3'-UTR of Twist mRNA with one mutate at the miR-92b binding site (mut) and miR-92b-modified plasmids. The luciferase activities were quantified. *$p < 0.05$. N = 5.

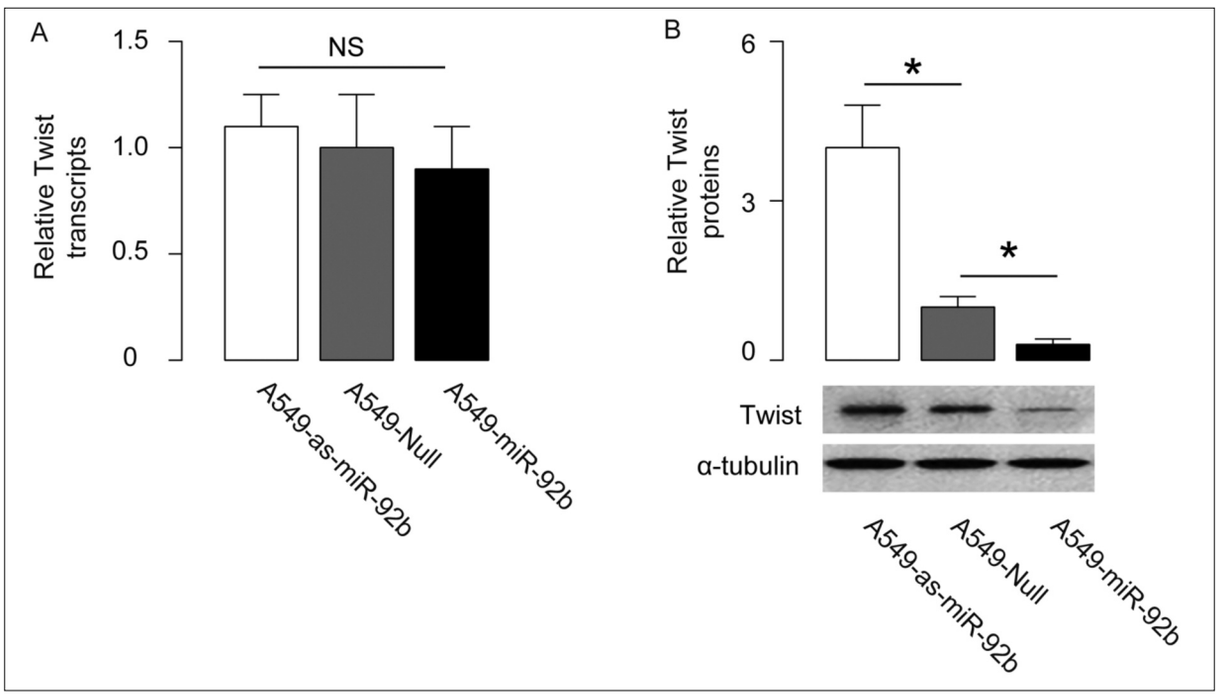

Figure 3. MiR-92b inhibits Twist in NSCLC cells. ***A-B,*** The mRNA levels ***(A)*** and protein levels ***(B)*** of Twist in miR-92b-modified A549 cells. *$p < 0.05$. NS: non-significant. N = 5.

Western blot (Figure 3B). These data suggest that miR-92b inhibits translation of the Twist mRNA in NSCLC cells.

MiR-92b Suppresses NSCLC Cell Invasiveness

Next, we examined the effects of miR-92b modification in NSCLC cells on cell invasiveness. We found that overexpression of miR-92b resulted in decreases in cell invasiveness of A549 cells in a transwell cell migration assay, by quantification (Figure 4A), and by representative images (Figure 4B). Similarly, depletion of miR-92b resulted in increases in cell invasiveness of A549 cells, by quantification (Figure 4A), and by representative images (Figure 4B). In a scratch wound healing assay, we found that overexpression of miR-92b resulted in decreases in cell invasiveness of A549 cells, by quantification (Figure 4C), and by representative images (Figure 4D). Similarly, depletion of miR-92b resulted in increases in cell invasiveness of A549 cells, by quantification (Figure 4C), and by representative images (Figure 4D). Together, these data suggest that miR-92b may inhibit NSCLC cell invasion through Twist suppression (Figure 5).

Discussion

Comprehension of the aberrant expression of miRNAs in NSCLC carcinogenesis is critical for elucidating the mechanisms underlying NSCLC progression and metastasis[14-18]. Here, by sequence matching, we found some candidate miRNAs that target Twist, a key regulator and enhancer for cancer cell invasion[19-24]. Among all these miRNAs, we specifically detected a significant decrease in miR-92b in NSCLC specimens, compared to paired non-tumor tissue. Hence, we hypothesize that miR-92b may target and regulate Twist in NSCLC cells.

The clinical data in the current work further support this hypothesis, since the low levels of miR-92b in NSCLC tissues were correlated with the levels of Twist in NSCLC specimens. Moreover, the expression levels of miR-92b and Twist were inversely correlated. The following in vitro data demonstrate that the relationship between miR-92b and Twist is not only correlative, but also regulatory, by bioinformatics data showing that 3'-UTR of Twist is a miR-92b target and by luciferase reporter assay showing that the binding of miR-92b to the 3'-UTR of Twist is functional.

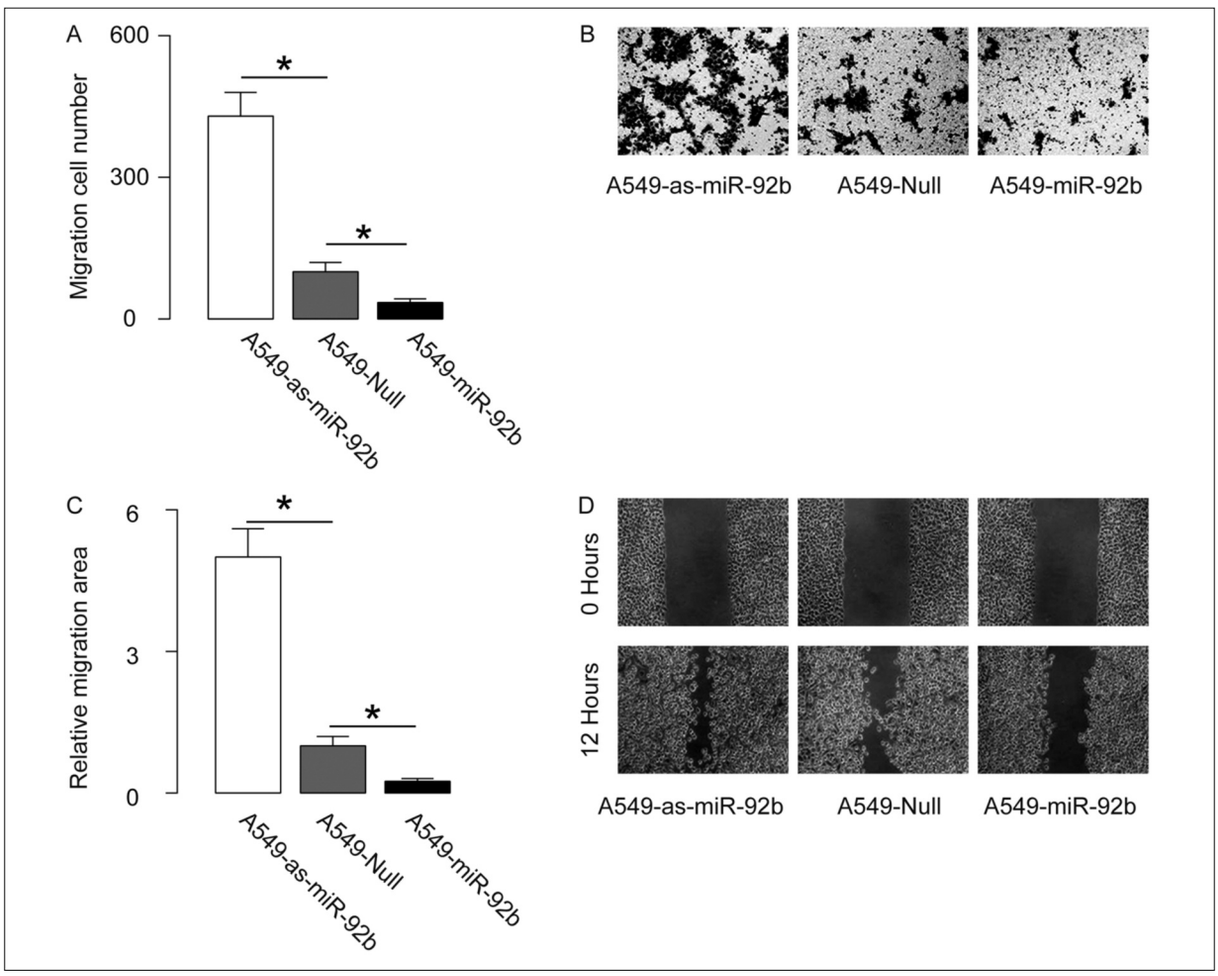

Figure 4. MiR-92b suppresses cell invasion in NSCLC cells. ***A-B,*** Cell invasiveness in a transwell cell migration assay of miR-92b-modified A549 cells, shown by quantification ***(A)***, and by representative images ***(B)***. ***C-D,*** Cell invasiveness in a scratch wound healing assay of miR-92b-modified A549 cells, shown by quantification ***(C)***, and by representative images ***(D)***. *$p < 0.05$. N = 5.

Overexpression of miR-92b inhibited cell invasion, while depletion of miR-92b increased cell invasion. We also modified miR-92b levels in NSCLC cells, and found that it did not affect Twist mRNA, but altered the protein. We have examined several other NSCLC cell lines and got essentially similar results. Thus, a possibility of the results to be cell-line-dependence could be excluded.

Conclusions

Here we propose a model of the regulation of Twist-mediated NSCLC metastasis by miR-92b. MiR-92b suppresses Twist protein translation, and subsequently decreases cell metastasis. Thus, our study suggests that miR-92b could be a promising novel target for NSCLC therapy.

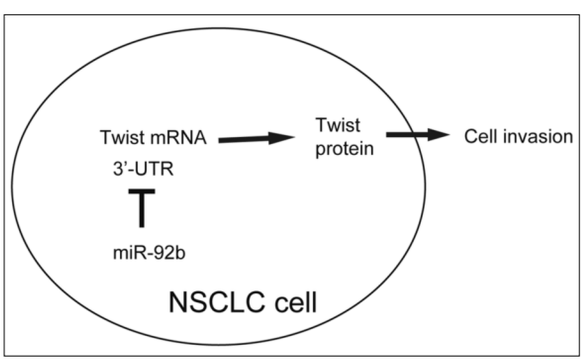

Figure 5. Schematic of the model. MiR-92b inhibits NSCLC cell invasion through suppressing Twist.

Acknowledgements

This work was supported by a grant from Beijing Natural Science Foundation (No: 7142152).

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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