

Original Paper

Role of MiR-3619-5p in β -Catenin-Mediated Non-Small Cell Lung Cancer Growth and Invasion

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Background/Aims: The malignancy of non-small cell lung cancer (NSCLC) is largely due to its fast growth and invasion. WNT/ β -catenin signaling plays a critical role in regulating NSCLC carcinogenesis. Hence, suppression of β -catenin signal transduction in NSCLC cells may improve the therapeutic outcome. **Methods:** We analyzed the levels of β -catenin and miR-3619-5p in NSCLC specimens, compared to paired non-tumor normal lung tissue (NT). We did Bioinformatics analyses on the binding sites of 3'-UTR of β -catenin mRNA by miR-3619-5p. We modified the levels of miR-3619-5p in NSCLC cells and examined their effects on β -catenin levels, and on the growth and invasion of NSCLC cells in an MTT assay and a transwell cell migration assay, respectively. **Results:** NSCLC specimens had significant higher levels of β -catenin, and significantly lower levels of miR-3619-5p, compared to NT. The levels of β -catenin and miR-3619-5p were inversely correlated in NSCLC specimens. Bioinformatics analyses showed that miR-3619-5p bound to 3'-UTR of β -catenin mRNA in NSCLC cells to inhibit its translation. Overexpression of miR-3619-5p decreased β -catenin protein, while depletion of miR-3619-5p increased β -catenin protein in NSCLC cells, without altering β -catenin mRNA levels. Overexpression of miR-3619-5p in NSCLC cells inhibited cell growth and invasion, while depletion of miR-3619-5p in NSCLC lines increased cell growth and invasion. **Conclusion:** Our data demonstrate a previously unappreciated role for miR-3619-5p in suppression of β -catenin-mediated cancer growth and invasion in NSCLC cells, and highlight miR-3619-5p as a novel cancer suppressor in NSCLC.

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Introduction

Non-small cell lung cancer (NSCLC) is a prevalent cancer and contains three subtypes: squamous cell carcinoma, large cell carcinoma, and adenocarcinoma [1-4]. NSCLCs are often insensitive to chemotherapy and radiotherapy, and are fast-growing and highly invasive [3-10]. Thus, understanding of the mechanisms underlying the growth and invasion of NSCLC are extremely important for its therapy.

WNT signaling pathway has been suggested to play a critical role in the carcinogenesis of NSCLC [11-13]. In murine models, activation of WNT signaling is associated with NSCLC initiation, progress and invasion [11-13]. Several WNT pathway components may be overexpressed in NSCLC, including the key factor of WNT signaling, β -catenin [14]. Aberrant activation of WNT/ β -catenin has been associated with poor prognosis clinically [14]. On the other hand, downregulation of WNT signaling inhibited NSCLC cell proliferation and xenograft growth, reduced cell motility and invasion, and induced a more differentiated phenotype [15-17].

The translation of most proteins has been found to be regulated by microRNAs (miRNAs), which target the 3'-UTR of mRNA of certain genes [4, 18-25]. Among all miRNAs, the role of miR-3619-5p in cancers has not been reported so far.

Here, we showed that NSCLC specimens had significant higher levels of β -catenin, and significantly lower levels of miR-3619-5p, compared to paired non-tumor tissue. The levels of β -catenin and miR-3619-5p are inversely correlated in NSCLC specimens. Bioinformatics analyses showed that miR-3619-5p bound to 3'-UTR of β -catenin mRNA in NSCLC cells to inhibit its translation. Overexpression of miR-3619-5p decreased β -catenin protein, while depletion of miR-3619-5p increased β -catenin protein in NSCLC cells, without affecting β -catenin mRNA levels. Overexpression of miR-3619-5p in NSCLC cells inhibited cell growth and invasion, while depletion of miR-3619-5p in NSCLC lines increased cell growth and invasion.

Materials and Methods

Patient specimen

Resected cancer specimens from 25 NSCLC patients were obtained together with the matched tumor-adjacent normal lung tissues (NLT) from 2009 to 2013 at the Forth People's Hospital of Jinan. All patients provided signed, informed consent for their tissues to be used for scientific research. Ethical approval for the current study was obtained from the Forth People's Hospital of Jinan. 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. Tissues were obtained prior to chemotherapy and radiotherapy and were immediately frozen and stored at -70°C prior to RT-qPCR and Western blot.

NSCLC cell line culture

A549 and H460 are two human NSCLC lines purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and L-glutamine in a humidified chamber with 5% CO_2 at 37°C . 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 58-year-old Caucasian male [26]. H460 cell line was deposited at the ATCC by Dr. Adi F. Gazdar [27]. They were both widely used NSCLC lines.

Modification of miR-3619-5p levels in NSCLC cell lines

MiR-3619-5p-modification-plasmids were prepared using routine methods. Briefly, the constructs for miR-3619-5p, or antisense (as)-miR-3619-5p or control null were cloned into pcDNA3.1-EGFP to generate the corresponding plasmids. The sequences were: miR-3619-5p sequence: 5'-UCAGCAGGCAGCUGGUGCAGC-3', miR-3619-5p antisense sequence (as-miR-3619-5p): 5'-GCUGCACCAGCCUGCCUGCUGA-3', control null

sequence: 5'-UUGUACUACACAAAAGUAAUG-3'. These plasmids of 2 µg were transfected into cultured NSCLC cells using Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen). The plasmids also contained a GFP reporter to allow determination of transfection efficiency, which was nearly 100% in the current study.

MTT assay

For assay of cell growth, cells were seeded at 5×10^3 per well into a 96 well-plate and subjected to a Cell Viability Kit (MTT, Roche, Indianapolis, IN, USA), according to the instruction of the manufacturer. The MTT assay is a colorimetric assay for assessing viable cell number, taking advantage that NADPH-dependent cellular oxidoreductase enzymes in viable cells reduce the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its insoluble formazan in purple readily being quantified by absorbance value (OD) at 570 nm in a microtiter plate reader (Promega, Fitchburg, WI, USA). Experiments were performed 5 times.

Quantitative PCR (RT-qPCR)

MiRNA and total RNA were extracted from lung specimen or from the cultured cells with miRNeasy mini kit or RNeasy kit (Qiagen, Hilden, Germany), respectively. For cDNA synthesis, complementary DNA (cDNA) was randomly primed from 2 µg of total RNA using the Omniscript reverse transcription kit (Qiagen). RT-qPCR was subsequently performed in triplicate with a 1:4 dilution of cDNA using the Quantitect SyBr green PCR system (Qiagen) on a Rotorgene 6000 series PCR machine. All primers were purchased from Qiagen. Data were collected and analyzed using $2^{-\Delta\Delta Ct}$ method. Values of genes were first normalized against β -actin, and then compared to the experimental controls.

Western blot

For analysis of total protein, the protein was extracted from the lung specimens or from the cultured cells, and homogenized in RIPA lysis buffer (1% NP40, 0.1% SDS, 100µg/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, in PBS) on ice. The supernatants were collected after centrifugation at 12000 × g at 4°C for 20 min. Protein concentration was determined using a BCA protein assay kit (Bio-rad, China), and whole lysates were mixed with 4×SDS loading buffer (125mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100mmol/l DTT, and 0.2% bromophenol blue) at a ratio of 1:3. Protein samples were heated at 100°C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies for Western Blot are anti- β -catenin and β -actin (all from Cell Signaling, San Jose, CA, USA). β -actin was used as protein loading controls. Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). Images shown in the figures were representative from 5 individuals. Densitometry of Western blots was quantified with NIH ImageJ software (Bethesda, MA, USA). The protein levels were first normalized to β -actin, and then normalized to experimental controls.

MicroRNA target prediction and Luciferase-reporter activity assay

MiRNAs targets were predicted as has been described before, using the algorithms TargetSan (<https://www.targetscan.org>) [28]. Luciferase-reporters were successfully constructed using molecular cloning technology. Target sequence was inserted into pGL3-Basic vector (Promega) to obtain pGL3- β -catenin-3'-UTR containing the miR-3619-5p binding sequence (β -catenin-3'-UTR sequence). MiR-3619-5p-modified NSCLC cells were seeded in 24-well plates for 24 hours, after which they were transfected with 1µg of Luciferase-reporter plasmids per well using PEI Transfection Reagent. Then luciferase activities were measured using the dual-luciferase reporter gene assay kit (Promega), according to the manufacturer's instructions.

Transwell cell migration assay

The transwell cell migration assay was performed using a Fluorometric Cell Migration Assay kit with polycarbonate membrane inserts (5-µm pore size; Cell Biolabs, San Diego, CA, USA). Cells were serum-starved overnight in DMEM prior to initiation of the experiment. Cells were then incubated at 37°C for 24

hours to allow cell migration through the membrane. Migratory cells were detached from the underside of the membrane and subsequently lysed and detected by CyQuant GR dye (Invitrogen). Fluorescence measurement was performed in a FluoStar Optima fluorescence plate reader with a 485 / 520 nm filter set.

Statistics

All statistical analyses were carried out using the SPSS 18.0 statistical software package. All values in cell and animal studies are depicted as mean \pm 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' Exact Test for comparison of two groups. Bivariate correlations were calculated by Spearman's rank correlation coefficients.

Results

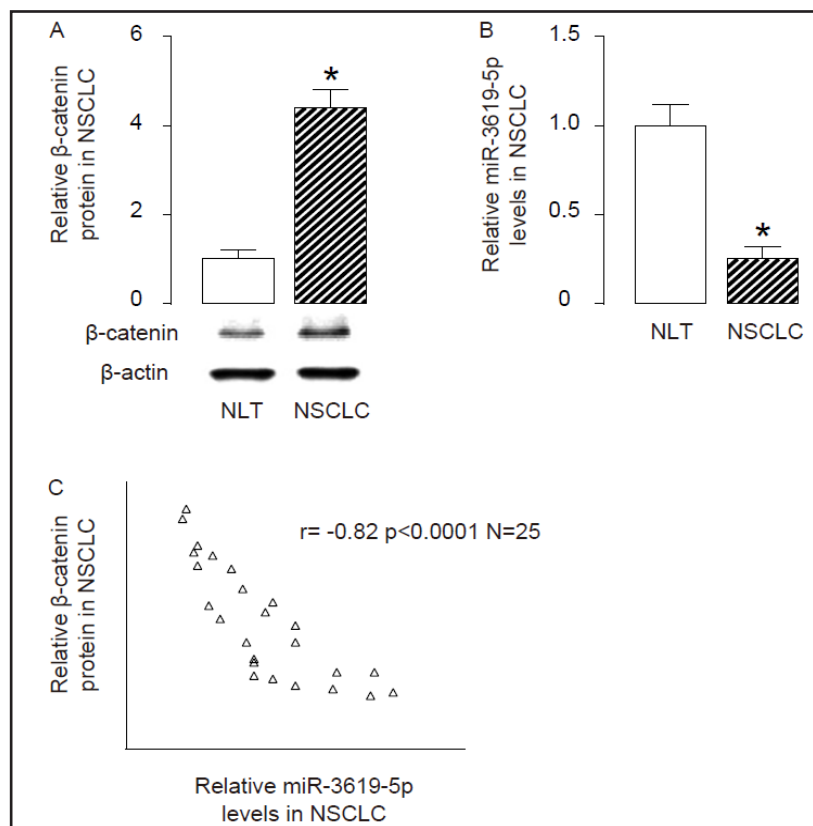
MiR-3619-5p and β -catenin levels inversely correlate in NSCLC specimens

We studied the levels of β -catenin in NSCLC specimens and detected significantly higher levels of β -catenin in NSCLC, compared to the paired adjacent non-tumor lung tissue (NLT, Fig. 1A). Moreover, we found that NSCLC expressed significantly lower levels of miR-3619-5p, compared to NLT (Fig. 1B). To figure out, whether this is a possible relationship between miR-3619-5p and β -catenin levels, we performed a correlation test in the resected NSCLC from 25 patients. A strong inverse correlation was detected between miR-3619-5p and β -catenin (Fig. 1C, $r = -0.82$; $p < 0.0001$), suggesting the presence of a regulatory relationship between miR-3619-5p and β -catenin in NSCLC cells.

MiR-3619-5p targets 3'-UTR of β -catenin mRNA to inhibit its expression in NSCLC cells

We then performed bioinformatics analysis, and we found that miR-3619-5p bound to 3'-UTR of β -catenin mRNA at 723th-729th base site (Fig. 2A). Then we used 2 NSCLC lines, A549 and H460, to examine whether this binding is functional. We transfected A549

Fig. 1. MiR-3619-5p and β -catenin levels inversely correlate in NSCLC specimens. (A-B) Compared with the paired non-tumor lung tissue (NLT), NSCLC expressed significantly higher levels of β -catenin by Western blot (A), and significantly lower levels of miR-3619-5p by RT-qPCR (B). (C) A correlation test in the resected NSCLC specimens from 25 patients showing a strong inverse correlation between miR-3619-5p and β -catenin ($r = -0.82$; $p < 0.0001$). * $p < 0.05$. N = 25.



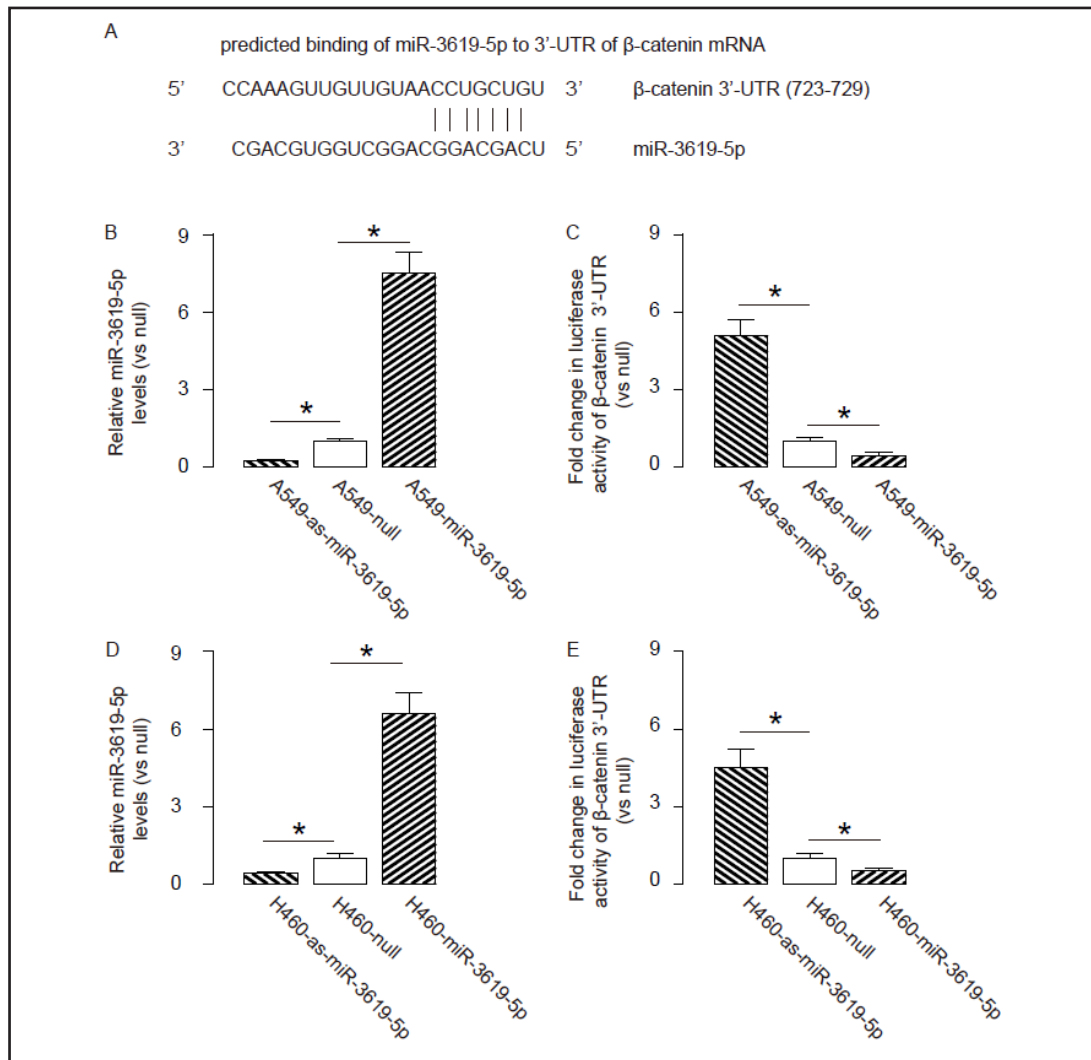


Fig. 2. MiR-3619-5p targets 3'-UTR of β -catenin mRNA to inhibit its expression in NSCLC cells. (A) Bioinformatics analysis of β -catenin targeting miRNAs, showing that miR-3619-5p binds to 3'-UTR of β -catenin mRNA at 723th-729th base site. (B) A549 cells were transfected with plasmids carrying either miR-3619-5p, or as-miR-3619-5p, or null as a control. The modification of miR-3619-5p levels in A549 cells was confirmed by RT-qPCR. (C) The miR-3619-5p-modified A549 cells were transfected with 1 μ g of β -catenin-3'-UTR Luciferase-reporter plasmid and the luciferase activities were examined. (D) H460 cells were transfected with plasmids carrying either miR-3619-5p, or as-miR-3619-5p, or null as a control. The modification of miR-3619-5p levels in H460 cells was confirmed by RT-qPCR. (E) The miR-3619-5p-modified H460 cells were transfected with 1 μ g of β -catenin-3'-UTR Luciferase-reporter plasmid and the luciferase activities were examined. *p < 0.05. N = 5.

and H460 cells with plasmids carrying either miR-3619-5p, or as-miR-3619-5p, or null as a control. The modification of miR-3619-5p levels in A549 cells was confirmed by RT-qPCR (Fig. 2B). Then, these cells were transfected with 1 μ g of β -catenin-3'-UTR Luciferase-reporter plasmid. We found that the luciferase activities in A549-as-miR-3619-5p cells were significantly higher than the control, while the luciferase activities in A549-miR-3619-5p cells were significantly lower than the control (Fig. 2C). Similarly, the modification of miR-3619-5p levels in H460 cells was confirmed by RT-qPCR (Fig. 2D). Then, these cells were transfected with 1 μ g of β -catenin-3'-UTR Luciferase-reporter plasmid. We found that the luciferase activities in H460-as-miR-3619-5p cells were significantly higher than the control,

Fig. 3. MiR-3619-5p suppresses β -catenin protein, without affecting β -catenin mRNA in NSCLC cells. (A) The mRNA levels of β -catenin in miR-3619-5p-modified A549 cells by RT-qPCR. (B) The protein levels of β -catenin in miR-3619-5p-modified A549 cells by Western blot. (C) The mRNA levels of β -catenin in miR-3619-5p-modified H460 cells by RT-qPCR. (D) The protein levels of β -catenin in miR-3619-5p-modified H460 cells by Western blot. * $p < 0.05$. NS: non-significant. N = 5.

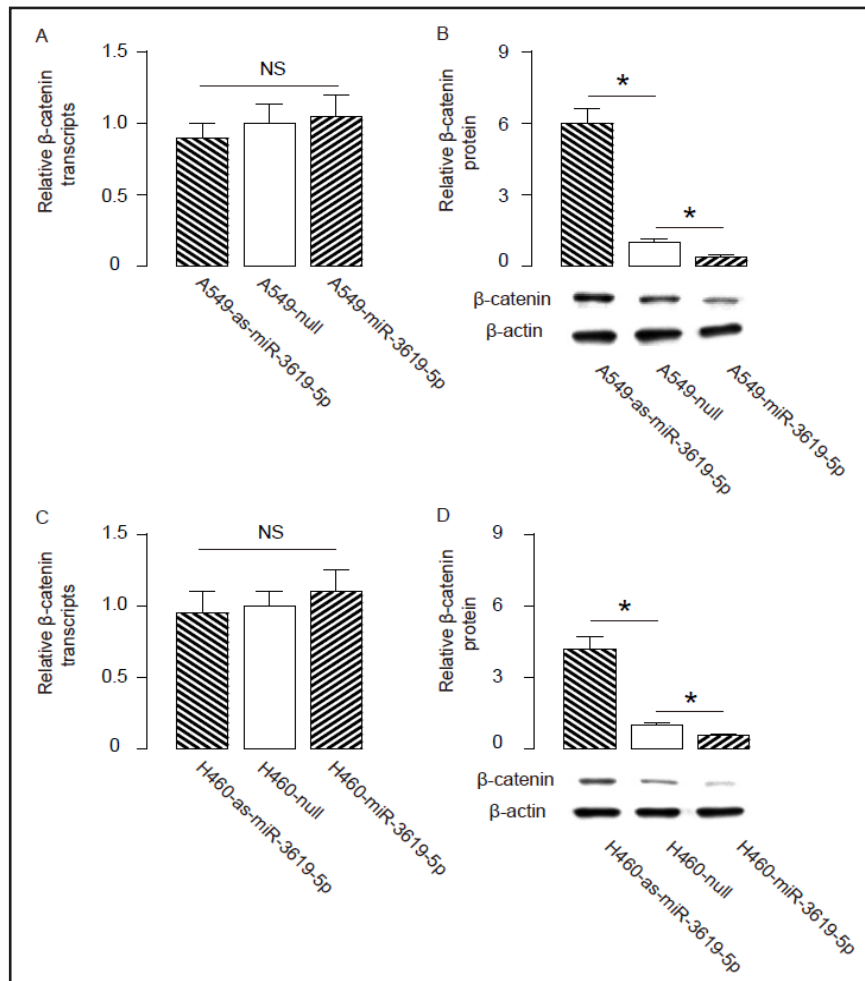
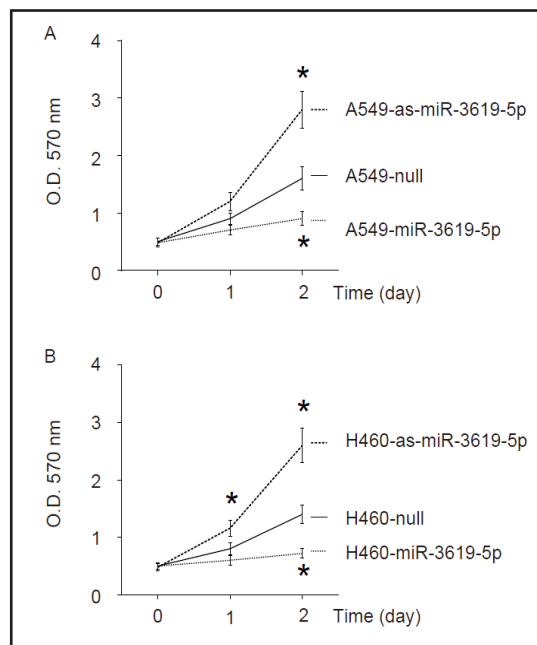
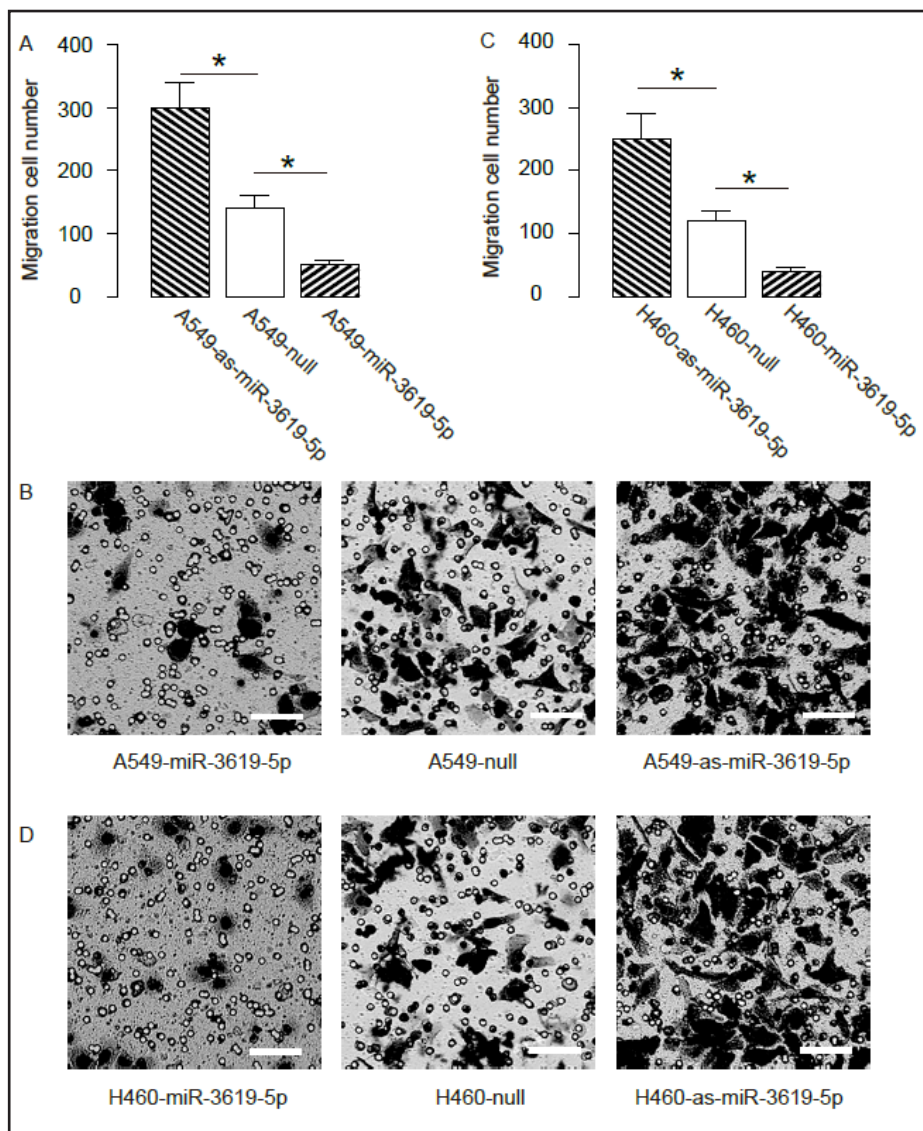


Fig. 4. MiR-3619-5p inhibits NSCLC cell growth. (A-B) MTT assay for miR-3619-5p-modified A549 cells (A), and H460 cells (B). * $p < 0.05$. N = 5.



while the luciferase activities in H460-miR-3619-5p cells were significantly lower than the control (Fig. 2E). Together, these data demonstrate that miR-3619-5p targets 3'-UTR of

Fig. 5. MiR-3619-5p inhibits cell invasion in NSCLC cells. (A-B) Quantification of migrated cells in a transwell cell migration assay of miR-3619-5p-modified A549 cells by quantification (A), and by representative image (B). (C-D) Quantification of migrated cells in a transwell cell migration assay of miR-3619-5p-modified H460 cells by quantification (C), and by representative image (D). *p < 0.05. N = 5.



β -catenin to inhibit its translation in NSCLC cells, and loss of miR-3619-5p in NSCLC may result in increases in β -catenin, or augmented WNT signaling, in NSCLC cells.

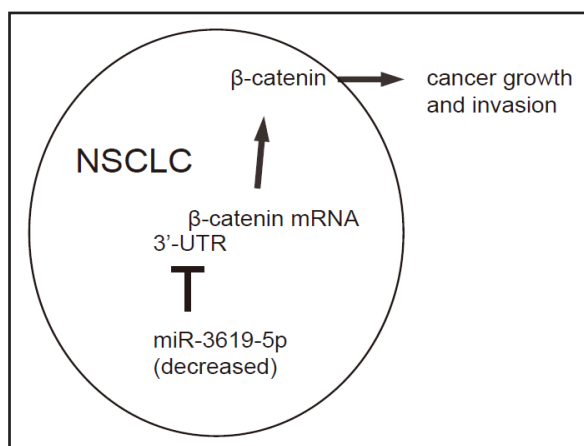
MiR-3619-5p suppresses β -catenin protein, without affecting β -catenin mRNA in NSCLC cells

Moreover, we found that although the mRNA levels of β -catenin in A549 cells were unchanged by miR-3619-5p modification (Fig. 3A), overexpression of miR-3619-5p in A549 cells decreased β -catenin protein, while depletion of miR-3619-5p in A549 cells increased β -catenin protein (Fig. 3B). Similarly, although the mRNA levels of β -catenin in H460 cells were unchanged by miR-3619-5p modification (Fig. 3C), overexpression of miR-3619-5p in H460 cells decreased β -catenin protein, while depletion of miR-3619-5p in H460 cells increased β -catenin protein (Fig. 3D). Together, these data suggest that miR-3619-5p suppresses β -catenin protein, without affecting β -catenin transcript levels in NSCLC cells.

MiR-3619-5p inhibits NSCLC cell growth

In an MTT assay, we found that overexpression of miR-3619-5p in A549 cells resulted in a significant decrease in cell growth, while depletion of miR-3619-5p in A549 cells resulted in a significant increase in cell growth (Fig. 4A). Similarly, overexpression of miR-3619-5p in

Fig. 6. A schematic model. MiR-3619-5p targets 3'-UTR of β -catenin mRNA to inhibit its translation in NSCLC cells. Loss of miR-3619-5p in NSCLC promotes cancer cell growth and invasion through augmenting β -catenin.



H460 cells resulted in a significant decrease in cell growth, while depletion of miR-3619-5p in H460 cells resulted in a significant increase in cell growth (Fig. 4B). Together, these data suggest that miR-3619-5p inhibits NSCLC cell growth.

MiR-3619-5p inhibits cell invasion in NSCLC cells

Next, we evaluated the effects of miR-3619-5p on NSCLC cell invasiveness. We found that overexpression of miR-3619-5p in A549 cells resulted in a significant decrease in cell invasion, while depletion of miR-3619-5p in A549 cells resulted in a significant increase in cell invasion, in a transwell cell migration assay, by quantification (Fig. 5A), and by representative images (Fig. 5B). Similarly, overexpression of miR-3619-5p in H460 cells resulted in a significant decrease in cell invasion, while depletion of miR-3619-5p in H460 cells resulted in a significant increase in cell invasion, in a transwell cell migration assay, by quantification (Fig. 5C), and by representative images (Fig. 5D). Together, these data suggest that miR-3619-5p inhibits cell invasion in NSCLC cells. To summarize, our data demonstrate a previously unappreciated role of miR-3619-5p in suppressing β -catenin-mediated cancer vascularization, growth and invasion in NSCLC (Fig. 6).

Discussion

NSCLC is a malignant lung cancer that affects millions of people worldwide [3, 5-7]. It is one of the most common causes of cancer-associated death [3, 5-7]. The poor outcome of NSCLC largely results from its fast growth and invasion [3, 5-7]. Here, we focused on the miRNA-regulated WNT signaling in regulation of the growth and invasion of NSCLC.

Generally, the protein regulation includes regulation of protein degradation by different protein modifications, e.g. phosphorylation, SUMOylation, acetylation and ubiquitination, and regulation of protein translation by miRNAs. In this study, we focused on miRNAs. First, we compared the levels of β -catenin and some candidate miRNAs that target β -catenin in NSCLC specimens, and we specifically found that miR-3619-5p inversely correlated the levels of β -catenin in NSCLC tissue. Thus, we hypothesized that in NSCLC, β -catenin may be regulated by miR-3619-5p at post-transcriptional level. Based on bioinformatics analyses and promoter reporter assay, we found that the binding of miR-3619-5 to β -catenin mRNA is functional, which was further supported by the examination of β -catenin mRNA and protein levels in miR-3619-5p-modified NSCLC cell lines.

Furthermore, miR-3619-5p overexpression resulted in suppression of NSCLC cell growth and invasion, while miR-3619-5p depletion increased NSCLC growth and invasion. Hence, the significant reduction of miR-3619-5p levels in NSCLC specimens has significance in that it allows activation of WNT/ β -catenin signaling, which directly contributes to the cancer growth and metastases.

Future approaches may be performed to improve our understanding of the regulation of miR-3619-5p in NSCLC and to determine how miR-3619-5p is suppressed in NSCLC. On the other hand, it is possible that miR-3619-5p may have other targets that are related to either cancer cell growth or cell invasion in NSCLC cells. Elucidation of targets of miR-3619-5p other than β -catenin may improve the completeness of our understanding of the role of miR-3619-5p in the carcinogenesis of NSCLC.

Here, we have shown similar data on two NSCLC cell lines and got essentially same results. These approaches thus ruled out a possibility of our findings to be cell-line dependent. In summary, our data demonstrate a previously unappreciated role of miR-3619-5p in controlling of β -catenin-associated cancer vascularization, growth and invasion, and this regulation is conducted through modulation of β -catenin protein translation.

Disclosure Statement

The authors have declared that no competing interests exist.

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