miR-512-5p suppresses the progression of non-small cell lung cancer by targeting β-catenin

ZHEXIN WANG[1], XIAOLEI ZHU[2], TUO ZHANG[1] and FENG YAO[1]

[1]Department of Thoracic Surgery, Shanghai Chest Hospital, Shanghai Jiao Tong University,
Shanghai 200030; [2]Suzhou Institute of Systems Medicine, Centre of Systems Medicine,
Chinese Academy of Medical Sciences, Suzhou, Jiangsu 223300, P.R. China

Abstract. The oncogenic protein β-catenin is regulated by microRNAs (miRs) in non-small cell lung cancer (NSCLC). miR-512-5p is downregulated in NSCLC compared with healthy tissues and exhibits a tumour-suppressive effect. To study whether miR-512-5p acts on β-catenin to exert its anticancer effect in NSCLC, miR-512-5p mimic and inhibitor were transfected into NSCLC A549 and H1975 cells. miR-512-5p mimic inhibited the invasion of NSCLC cells and increased apoptosis, which suggested an inhibitory effect of miR-512-5p in NSCLC progression *in vitro*. By contrast, transfection with the miR-512-5p inhibitor resulted in the opposite effects. A dual-luciferase assay demonstrated that miR-512-5p complementarily bound to the 3'-untranslated region of β-catenin. miR-512-5p mimic suppressed the transcription and translation of β-catenin and reduced the expression of the downstream oncogenes cyclin D1 and matrix metalloproteinases, leading to the inhibition of Wnt/β-catenin signalling and subsequent inhibition of NSCLC tumourigenesis *in vitro*. In conclusion, miR-512-5p may function as a tumour suppressor in NSCLC by inhibiting the Wnt/β-catenin pathway.

Introduction

To date, surgical resection is the preferred treatment method for non-small cell lung cancer (NSCLC), especially among patients at early stages (I, II and certain cases of IIIA) (1). Although surgical resection is frequently used for treatment, the recurrence rate of NSCLC remains high (~30% within 5 years from surgery), which has emerged as a major contributor to the poor prognosis of patients with NSCLC (2-4). However, with the identification of molecular markers in NSCLC, targeting pathogenic genes has been used as second-line therapy as an alternative approach (5).

Activation of the Wnt/β-catenin pathway is crucial for NSCLC progression (6). Without stimulation, β-catenin mainly present in the cell membrane is maintained at a low level by the ubiquitin proteasome system (UPS) (7). Upon activation of Wnt, which is the main regulator of β-catenin, β-catenin accumulates in the cytoplasm and enters the nucleus to bind with the T-cell factor/lymphoid enhancement factor to activate the downstream oncogenes, such as cyclin D1 and matrix metalloproteinases (MMPs), leading to the proliferation and sustained aggressiveness of various cancer cells (7). β-catenin is aberrantly enhanced in NSCLC, and its downregulation has been suggested to inhibit disease development (8,9). The activity of β-catenin can be modified by several pathways, affecting its stability, cellular localization and transcriptional activity. However, the specific mechanisms that modulate β-catenin activity in NSCLC are not fully understood.

MicroRNAs (miRNAs), identified as a type of endogenous non-coding RNA molecules with sequences of 19-25 nucleotides, are known for their gene regulation and have become increasingly appreciated due to their regulatory roles in the tumourigenesis of various types of cancer. For example, miR-212 in lung cancer (10) and the miR-17-92 cluster in malignant lymphoma (11) serve as oncogenes, whereas let-7 in lung cancer (12) and miR-551a/miR-483 in colorectal cancer (13) act as tumor suppressors. miRNAs function as tumour suppressors or promoters most likely by regulating the transcription of tumour-related genes by base pairing with the 3'-untranslated region (3'-UTR) of mRNAs, resulting in the suppression of mRNAs and the subsequent degradation of gene expression (14). To date, miR-512-5p has been implicated in the tumourigenesis of a number of human malignancies, including NSCLC (15-17). β-catenin is a target of miR-3619-5p in NSCLC cells (18). However, the exact mechanism of miR-512-5p inhibition of β-catenin in the progression of NSCLC remains largely unknown.

The present study aimed to confirm the downregulation of miR-512-5p and the upregulation of β-catenin in human NSCLC. However, to the best of our knowledge, there are no reports regarding the anticancer effect of miR-512-5p in patients with NSCLC. In addition, the role of miR-512-5p in NSCLC remain elusive; therefore, the present study aimed to

---

Correspondence to: Professor Feng Yao, Department of Thoracic Surgery, Shanghai Chest Hospital, Shanghai Jiao Tong University, 241 West Huai Hai Road, Shanghai 200030, P.R. China
E-mail: yaofeng6796678@yeah.net

investigate the expression and tumour-suppressive behaviour of miR-512-5p in patients with NSCLC.

Materials and methods

Human tissue samples. Tumourous lung tissues (n=30) and adjacent non-tumourous lung tissues (n=30) were collected from patients with NSCLC at the Department of Thoracic Surgery of the Shanghai Chest Hospital (Shanghai, China) between January 2017 and December 2018. Staging for each patient was performed according to the AJCC Cancer Staging Manual, 7th edition (19), and was based on findings from physical examination and surgical resection. A total of 11 cases were stage II and 19 cases were stage III. The following information was collected at the time of diagnosis: Sex, age, pathological type and TNM stage (Table SI). All patients provided written informed consent, and the use of patient tissues and data was approved by the Ethics Committee of Shanghai Chest Hospital [approval no. KS(P)1733].

Cell culture and treatment. A549, H460, HCC827, H1975 and H157 cells were obtained from ATCC and cultured in DMEM (HyClone; GE Healthcare Life Sciences) containing 100 U/ml penicillin and 100 μg/ml streptomycin (Beijing Solarbio Science & Technology Co., Ltd.) and 10% (v/v) foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). Cells in this complete culture medium were cultured to form a monolayer at 37˚C with 5% CO_2.

Cell transfection. To overexpress miR-512-5p in NSCLC cells, a mimic of miR-512-5p was synthesized using the following primers: Forward, 5'-CATCACTGCCACCCAGAAGAC TG-3' and reverse, 5'-TGCCAGTGAGCTTCCCGTTCAG-3' with a 2 nt-30 overhang and a 2 nt-50 trim (Suzhou GenePharma Co., Ltd.). To downregulate miR-512-5p in NSCLC cells, an inhibitor of miR-512-5p was synthesized using the following single-stranded RNA: 5'-TAACTCGAGAACCCACTGCTT ACT-3'. A control mimic of a random miR-512-5p sequence (forward, 5'-TCGAGTCCCTCACTGTTACCCTTG-3' and reverse, 5'-TAGATGACTTAAGCCTCAGCAGCA-3') and a control inhibitor of a random miR-512-5p sequence (5'-CTA GAAGGCACACTCGAGGCTGAT-3') were used as negative controls (NCs). To study the roles of miR-512-5p in the progression of NSCLC *in vitro*, A549 and H1975 cells ($2x10^3$ cells/ml; 100 μl) were seeded into a 96-well plate and transfected with the miR-512-5p mimic inhibitor or the corresponding control using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) for 48 h, according to the manufacturer's instructions.

Flow cytometry for apoptosis detection. Following transfection for 48 h, an apoptosis assay of A549 and H1975 cells ($1x10^6$ cells) was performed using an Annexin V-FITC Apoptosis Detection kit (Sangon Biotech Co., Ltd.). A FACSCant II flow cytometry system (BD Biosciences) was used to monitor apoptosis (FlowJo v10.0.7; FlowJo LLC). Early apoptotic NSCLC cells were Annexin V (+)/propidium iodide (PI) (-) and presented in the lower right quadrant, whereas late apoptotic NSCLC cells were Annexin V (+)/PI (+) and presented in the upper right quadrant.

Transwell invasion assay. The lower side of the membrane of the Transwell inserts (Corning, Inc.) were pre-coated with a thin layer of Matrigel. Following serum starvation for 24 h, A549 and H1975 cells in 0.3 ml serum-free medium were seeded into the upper chamber ($4x10^5$ cells/well), and 0.7 ml of complete medium was added to the lower chamber. At 48 h, A549 and H1975 cells that passed through the membrane were dyed using crystal violet (Beijing Solarbio Science & Technology Co., Ltd.) and counted using a Leica DM4P microscope (magnification, x200) in three randomly selected fields.

Caspase activity assay. A549 cells were resuspended in hypotonic cell lysis buffer at a density of $1x10^6$ cells/ml and subjected to three cycles of freezing and thawing. The cell lysates were centrifuged at 16,000 x g for 20 min at 4˚C, and the supernatant fraction was collected and assayed for caspase-3 activity. Caspase activity was measured using the Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega Corporation) according to the manufacturer's instructions. Samples were analysed using a Synergy HT Plate Reader (BioTek Instruments, Inc.) at a wavelength of 594 nm.

Dual-luciferase reporter gene assay. The 3'-UTR of β-catenin targeting miR-512-5p predicted through TargetScan Release 6.2 software (http://www.targetscan.org/vert_61/) was inserted into the pGL3-Control vector (Promega Corporation). The sequences of the wild-type 3'-UTR of β-catenin were as follows: Sense, 5'-GCGGAGCTCAACCAGAAGGCCAAGTC-3' and antisense, 5'-GCGTCTAGAAAATGGACAAAGTGGGTG TGG-3'. The sequences of the mutant 3'-UTR of β-catenin were as follows: Sense, 5'-TCCCTCACTGTTACCCTT-3' and antisense, 5'-ATGACTTAAGCCTCAGCAGC-3'. A549 cells were co-transfected with 80 ng β-catenin 3'-UTR, 50 pM miR-512-5p and 5 ng pRL-TK *Renilla* plasmid (Promega Corporation) in a 96-well plate for 72 h using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) and dissolved in LBL lysis buffer (Promega Corporation). The *Renilla*/firefly luciferase activity ratio of A549 cells was assessed using a Dual-Luciferase Reporter assay system (Promega Corporation).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from lung tissues and NSCLC cell lines (A549, H460, HCC827, H1975 and H157) was extracted with TRIzol® Reagent (Sigma-Aldrich; Merck KGaA) and reverse-transcribed using a cDNA Synthesis kit (Takara Bio, Inc.). The primers used were as follows: β-catenin forward, 5'-TAACTC GAGAACCCACTGCTTACT-3' and reverse, 5'-CTAGAA GGCACACTCGAGGCTGAT-3'; miR-512-5p forward, 5'-TCG AGTCCCTCACTGTTACCCTTG-3' and reverse, 5'-TAGATG ACTTAAGCCTCAGCAGCA-3'; let-7a forward, 5'-GCCGCT GAGGTAGTAGGTTGTA-3' and reverse, 5'-GTGCAGGGT CCGAGGT-3'; APC forward, 5'-CTAGTACTGCTTCAA CTAAGTC-3' and reverse, 5'-TACCTGGAGATGTATATG ACAT-3'; cyclin D1 forward, 5'-GCCTCACACGCTTCC TCTCCAGA-3' and reverse, 5'-TGGCGCAGGCTTGACTCC AGCA-3'; MMP7 forward, 5'-ACAGGCTCAGGACTATCT CAAG-3' and reverse, 5'-ATTTCTATGACGCGGGAGTTT AA-3'; GADPH forward, 5'-CATCACTGCCACCCAGAA GACTG-3' and reverse, 5'-ATGCCAGTGAGCTTCCCGTTC AG-3'. GAPDH was used as a loading control for analysing the
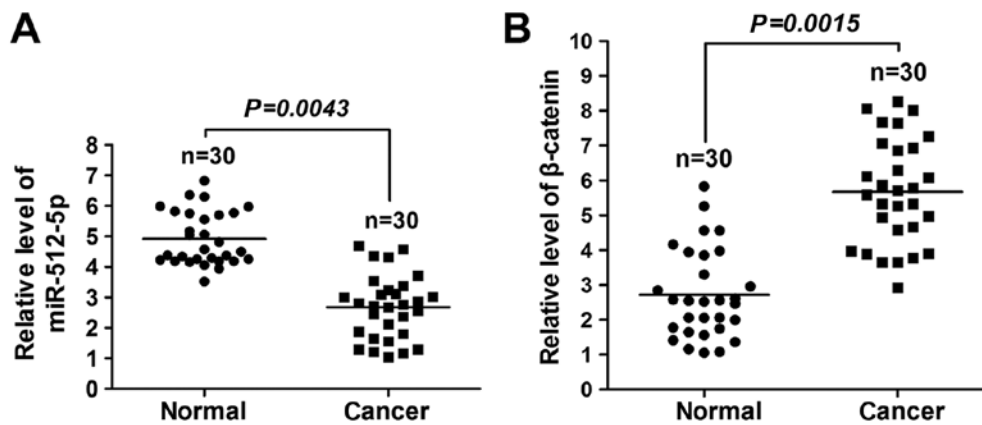
Figure 1. Expression of miR-512-5p and β-catenin in human NSCLC. Reverse transcription-quantitative PCR analysis was used to determine the levels of (A) miR-512-5p and (B) β-catenin in 30 pairs of non-tumourous and tumourous lung tissues from patients with NSCLC. NSCLC, non-small cell lung cancer; miR, microRNA.

expression of β-catenin, adenomatosis polyposis coli (APC), cyclin D1 and MMP7 correspondingly. A SYBR Green PCR kit (Thermo Fisher Scientific, Inc.) with an ABI 7300 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for analysis. The amplification conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 20 sec and 60°C for 45 sec. The levels of miR-512-5p were normalized to let-7a, whereas the levels of β-catenin, APC, cyclin D1 and MMP7 were normalized to that of GADPH and quantified using the $2^{-\Delta\Delta Cq}$ method (20).

Western blot analysis. A549 cells were lysed in RIPA buffer (Beyotime Institute of Biotechnology), and the extracted intracellular proteins were evaluated using a BCA protein assay kit (Sangon Biotech Co., Ltd.). Total protein (25 μg) was separated by 10% SDS-PAGE. Electrophoretically pure β-catenin, APC, cyclin D1 and MMP7 were transferred to a polyvinylidene difluoride membrane (EMD Millipore) and incubated with primary antibodies (all purchased from Abcam) against β-catenin (cat. no. ab2365; dilution, 1:1,000), APC (cat. no. ab15270; dilution, 1:500), cyclin D1 (cat. no. ab40754; dilution, 1:1,000), MMP7 (cat. no. ab5706; dilution, 1:1,000) and GAPDH (cat. no. ab16891; dilution, 1:10,000) at 4°C overnight followed by incubation with the goat-anti-rabbit secondary antibodies (cat. no. ab205718; dilution, 1:10,000) for 1 h at 25°C. The protein expression levels of β-catenin, miR-512-5p, APC, cyclin D1 and MMP7 were quantified using an enhanced chemiluminescence (ECL) system (EMD Millipore) with Bio-Rad Image Lab software version 5.1 (Bio-Rad Laboratories, Inc.) and normalized to that of GAPDH.

Immunohistochemistry. Paraffin-embedded tissue blocks were cut into 4-μm sections, dried overnight at 60°C. Sections were subsequently dewaxed in xylol, rehydrated in descending alcohol series (100, 95, 70 and 40%, 5 min each) and washed in distilled water. The sections were immersed in distilled water containing 3% hydrogen peroxidase twice to block endogenous oxidase activity. The sections were incubated with an anti-β-catenin antibody (cat. no. ab2365; dilution, 1:200) for 2 h at room temperature, followed by incubation with a goat-anti-rabbit secondary antibody (cat. no. ab205718; dilution, 1:1,000) at

room temperature for 40 min. The staining was developed by diaminobenzidine (DAB) chromogen (Bio-Rad Laboratories, Inc.). Subsequently, the tissues were rinsed in distilled water for 15 min, dehydrated in graded alcohol series and then washed in xylene. The slides were sealed by neutral gum. Five random fields of view were analysed under a light microscope with a camera (Olympus, Japan) with x100 magnification.

Statistical analysis. Data were analysed using GraphPad Prism 7.0 software (GraphPad Software, Inc.) and are presented as the mean ± standard deviation. The statistical analysis of two unpaired groups was evaluated using an unpaired Student's t-test. Statistical analysis of miR-512-5p and β-catenin expression between the tumourous lung tissues and paired non-tumourous lung tissues was evaluated using a paired Student's t-test. Any statistically significant differences between multiple groups were performed using a one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-512-5p is downregulated, whereas β-catenin is upregulated in NSCLC. The miRNA/mRNA levels of miR-512-5p and β-catenin in lung tissues of patients with NSCLC were detected. A significant reduction in miR-512-5p (Fig. 1A) and an increase in β-catenin (Fig. 1B) expression levels were observed in the tumourous lung tissues compared with the non-tumourous lung tissues, which suggested that miR-512-5p and β-catenin were involved in human NSCLC.

miR-512-5p targets the 3'-UTR of β-catenin. The present explored whether β-catenin was a target of miR-512-5p in human NSCLC. Two putative miR-512-5p target sites were identified in β-catenin 3'-UTR using TargetScan Release 6.2 software. The results demonstrated that the 3'-UTR of β-catenin directly bound to miR-512-5p (Fig. 2A). In addition, the luciferase reporter activity of β-catenin 3'-UTR was significantly reduced by miR-512-5p compared with the NC (Fig. 2B), which suggested that miR-512-5p was a novel β-catenin-targeting miRNA.
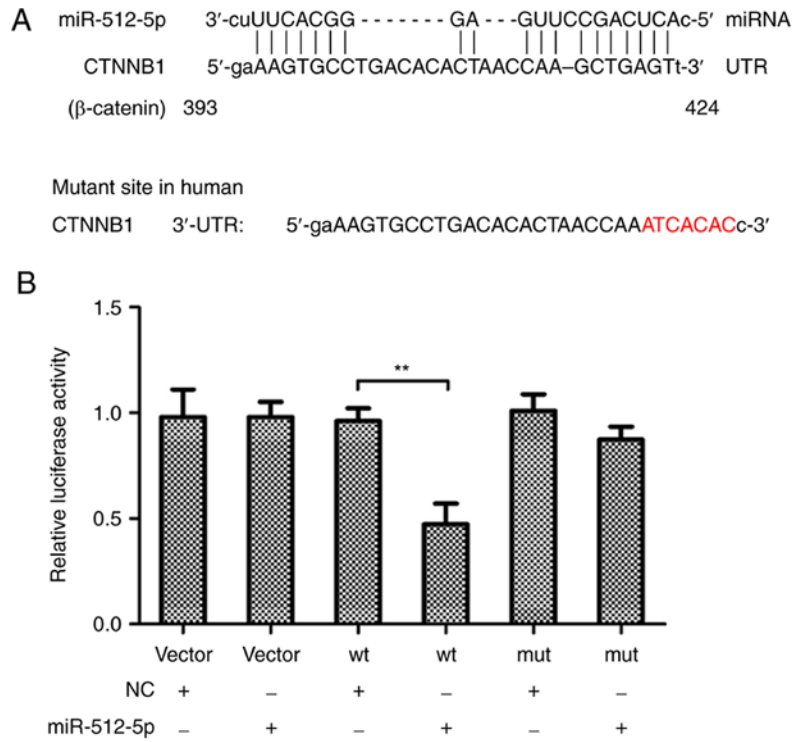
Figure 2. miR-512-5p targets β-catenin in non-small cell lung cancer cells. (A) The sequence of β-catenin 3'-UTR targeted by miR-512-5p. (B) Dual-luciferase assay in A549 cells using the WT or mutant 3'-UTR of β-catenin. **P<0.01. miR, microRNA; UTR, untranslated region; WT, wild-type; mut, mutant; CTNNB1, β-catenin.
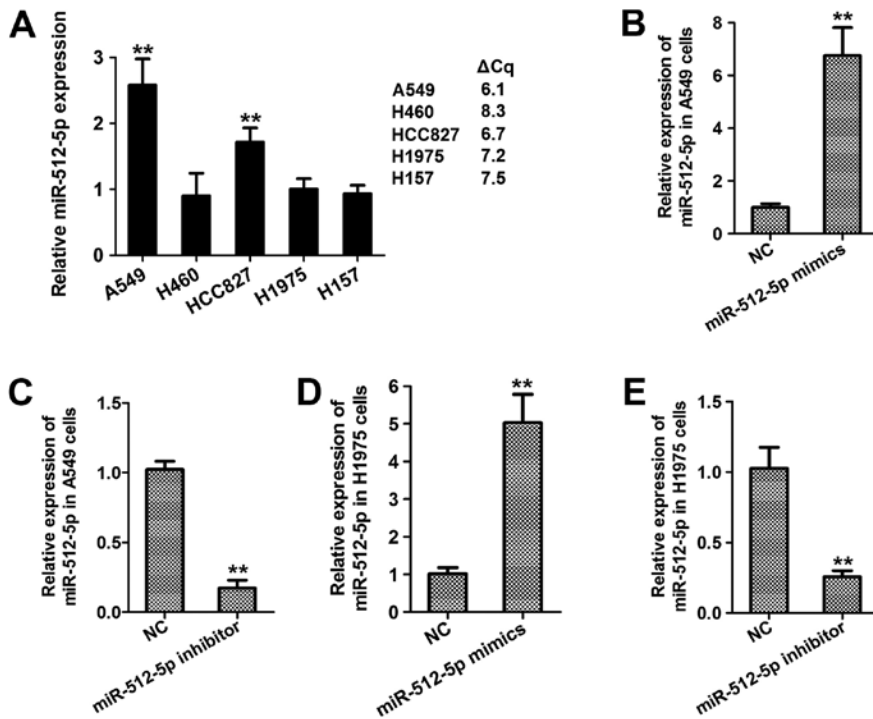

Figure 3. miR-512-5p functions in the tumourigenesis of NSCLC *in vitro*. (A) The mRNA levels of miR-512-5p in human NSCLC cell lines A549, H460, HCC827, H1975 and H157. Relative miR-512-5p expression in H1975 were set as '1'. Successful establishment of miR-512-5p (B) overexpression and (C) silencing in A549 cells analysed by RT-qPCR. Successful establishment of miR-512-5p (D) overexpression and (E) silencing in H1975 cells analysed by RT-qPCR. **P<0.01 vs. NC. NSCLC, non-small cell lung cancer; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; miR, microRNA.

miR-512-5p contributes to the progression of NSCLC in vitro. The expression levels of miR-512-5p were assessed in several NSCLC cell lines. As presented in Fig. 3A, miR-512-5p was highly expressed in A549 cells, but expressed at low levels in H460, H1975 and H157 cells. Thus, A549 and H1975 were selected for the transfection with miR-512-5p mimics
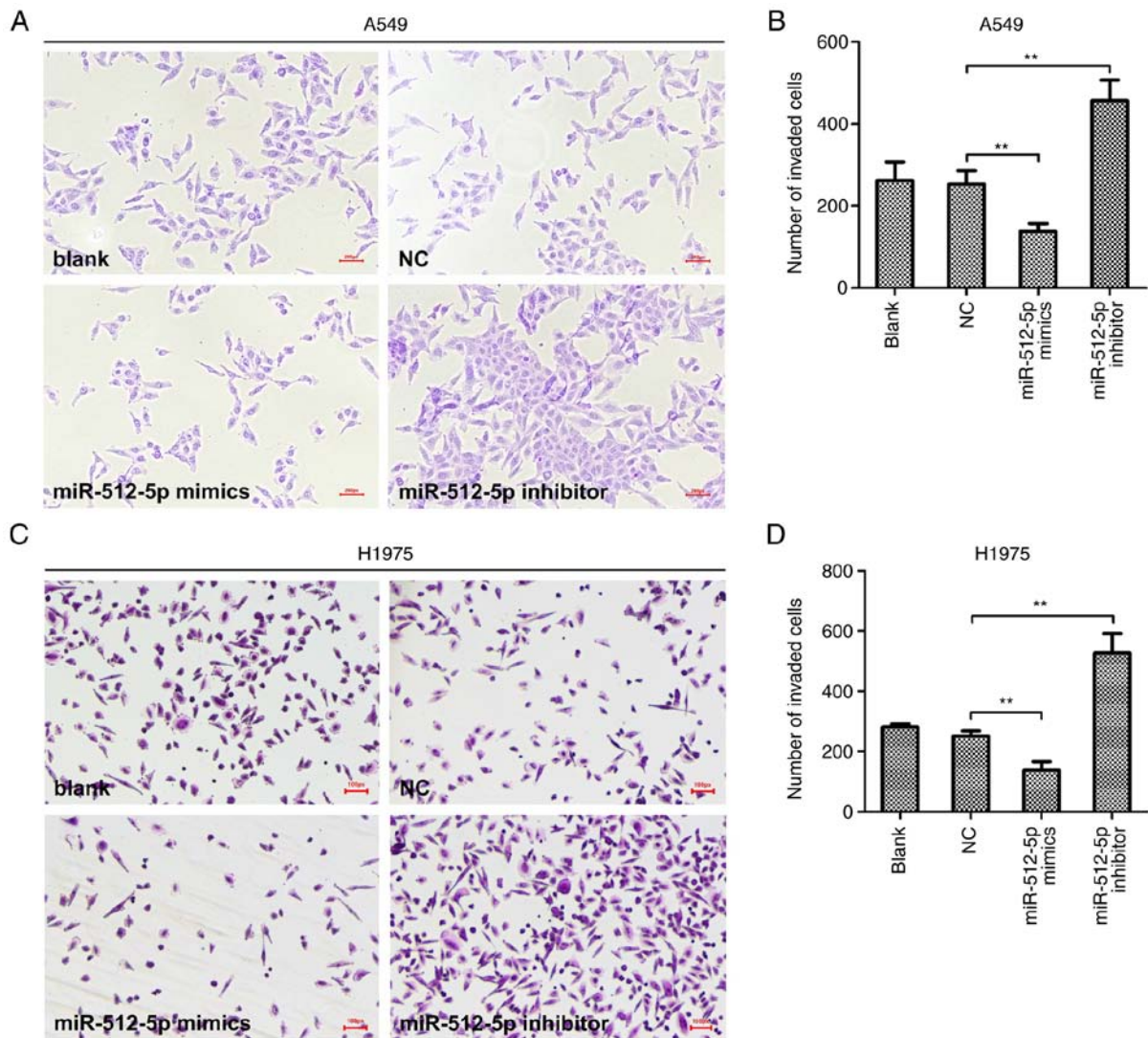
Figure 4. miR-512-5p suppresses non-small cell lung cancer cell invasion. Cell invasive ability was analysed by Transwell assays 24 h post-transfection with the inhibitor NC, miR-512-5p mimics or miR-512-5p inhibitor. Representative images of crystal violet-stained (A) A549 and (C) H1975 lung cancer cells. Quantification of invasive (B) A549 and (D) H1975 lung cancer cells. Data are presented as the mean ± standard error of the mean. **$P<0.01$. NC, negative control; miR, microRNA.

or inhibitors. The mRNA expression of miR-512-5p was significantly enhanced by miR-512-5p mimics (Fig. 3B) and suppressed by the miR-512-5p inhibitor compared with the NC (Fig. 3C), revealing the successful establishment of miR-512-5p overexpression by the miR-512-5p mimics and miR-512-5p silencing by the miR-512-5p inhibitor in the two NSCLC cell lines.

Following transfection, the malignant behaviours of NSCLC cells were assessed. Transwell assays revealed that the invasive abilities of A549 (Fig. 4A and B) and H1975 (Fig. 4C and D) cells were reduced by miR-512-5p overexpression and increased by miR-512-5p silencing. In addition, a flow cytometry assay was performed to further determine the effects of miR-512-5p on lung cancer cell apoptosis. The results demonstrated that the group transfected with miR-512-5p mimic exhibited a significantly higher proportion of early apoptotic cells compared with the NC group in A549 (Fig. 5A and B) and H1975 (Fig. 5C and D) cells. Consistent with these findings, miR-512-5p mimic significantly increased

caspase activity in A549 cells, and the level of caspase activity was decreased in miR-512-5p inhibitor-transfected A549 cells (Fig. 5E). These results suggested an anticancer effect of miR-512-5p in NSCLC *in vitro*. By contrast, the miR-512-5p inhibitor resulted in the opposite effects, promoting NSCLC cell invasion and reducing apoptosis ($P<0.05$), indicating the promotive effect of the miR-512-5p inhibitor in the progression of NSCLC *in vitro*.

miR-512-5p prevents the activation of the Wnt/β-catenin pathway. The roles of miR-512-5p in modulating the Wnt/β-catenin pathway were investigated. In the present study, the overexpression of miR-512-5p suppressed the mRNA expression of β-catenin, APC, cyclin D1 and MMP7 compared with the NC; silencing miR-512-5p resulted in the opposite effects (Fig. 6A and B). In addition, the protein levels of β-catenin, cyclin D1 and MMP7 in A549 cells transfected with the miR-512-5p mimics or inhibitor and the corresponding NC were quantified by western blot analysis (Fig. 6C-G). The
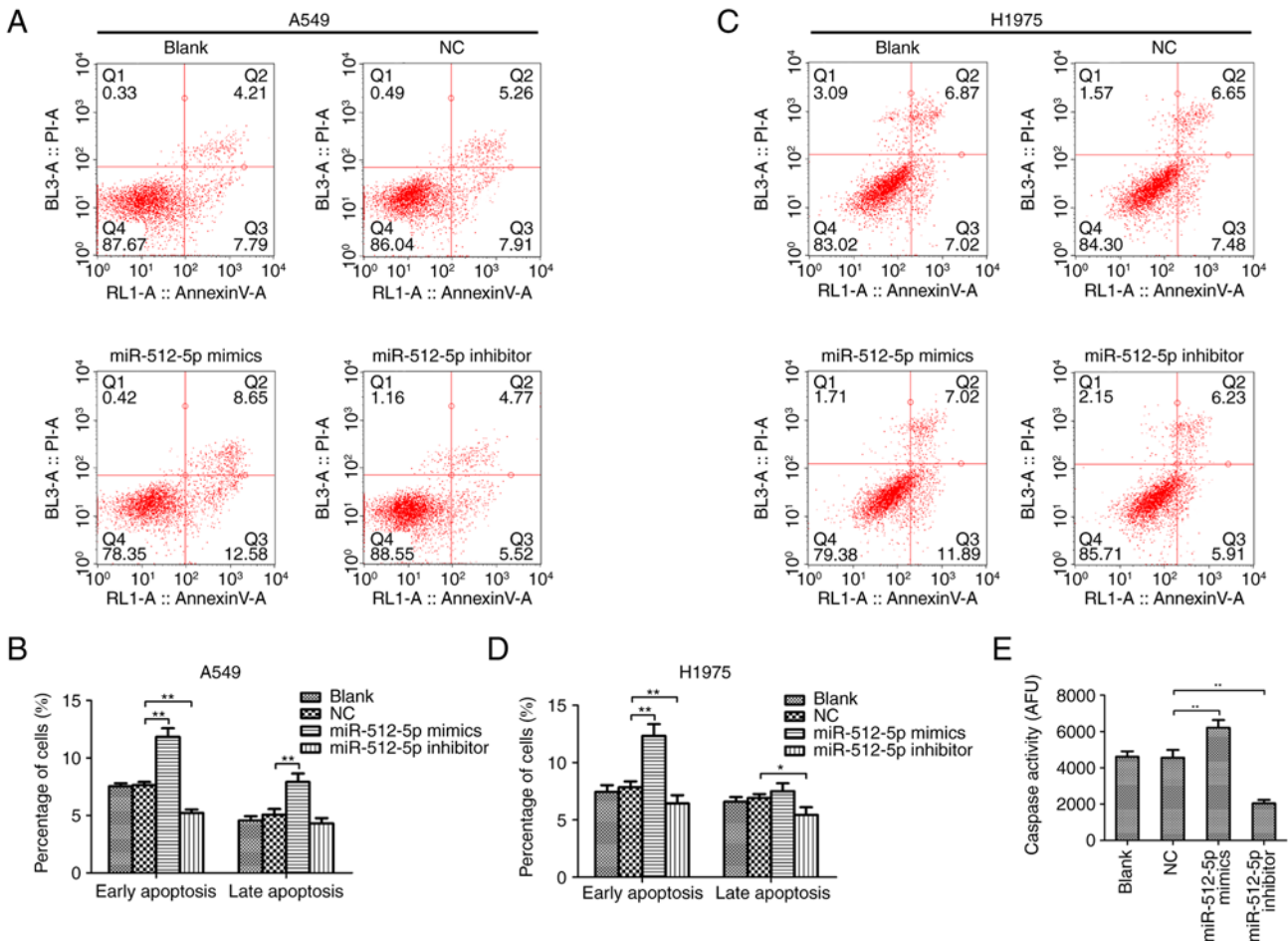
Figure 5. miR-512-5p promotes early and late stage apoptosis in lung cancer cells. Flow cytometry analyses of apoptosis in (A) A549 and (C) H1975 lung cancer cells transfected with the inhibitor NC, miR-512-5p mimics or miR-512-5p inhibitor were performed. The respective proportions of cells in the early and late stages of apoptosis in transfected (B) A549 and (D) H1975 lung cancer cells were analysed. (E) Caspase activity was measured by DEVD rhodamine fluorescence in A549 cells transfected with the inhibitor NC, miR-512-5p mimics or miR-512-5p inhibitor. Data are presented as the mean ± SEM. *P<0.05, **P<0.01. PI, propidium iodide; NC, negative control; miR, microRNA; AFU, arbitrary fluorenence units.

results of the immunohistochemistry assay revealed that the protein expression of β-catenin was higher in lung cancer tissues compared with adjacent normal tissues, consistent with the RT-qPCR results (Fig. 7A and B). As β-catenin is a direct target gene of miR-512-5p, the results of the present study suggested that cyclin D1 and MMP7 were downstream effectors of miR-512-5p and were at least partly induced by β-catenin signalling (Fig. 7C).

Discussion

The overexpression of β-catenin is a key contributor to NSCLC development and is usually associated with the poor prognosis of this disease (8,21). The depletion of β-catenin by miRNAs impairs malignant behaviours in the progression of various types of cancer, including NSCLC, and is beneficial to the therapeutic outcome (18). The results of the present study demonstrated an increase in β-catenin expression and a decrease in miR-512-5p expression in NSCLC. Previous evidence has suggested that miR-3619-5p targets β-catenin to inhibit β-catenin expression and β-catenin-mediated malignancy of NSCLC *in vitro* (18). However, whether and how miR-512-5p regulates β-catenin in NSCLC carcinogenesis remains largely unknown.

A previous report has revealed that miR-512-5p is down-regulated in NSCLC and restricts the progression of NSCLC *in vitro* by promoting apoptosis and impairing migration; however, it exerts almost no effect on cell proliferation (16). The present study demonstrated the tumour-suppressive effect of miR-512-5p in NSCLC by suppressing cell invasion and further suggested that miR-512-5p deficiency may contribute to the malignant process of NSCLC *in vitro*. These results suggested that miR-512-5p may be a promising target for miRNA-based NSCLC treatment.

Previous research has demonstrated that the translation of β-catenin is regulated by miRNAs that target the 3'-UTR of β-catenin mRNA in NSCLC cells, such as miR-3619-5p (18). The present study first demonstrated that β-catenin was also a target of miR-512-5p. miR-512-5p inhibited the activity of β-catenin by base pairing with the 3'-UTR of β-catenin, resulting in a reduction in β-catenin expression and inactivation of the Wnt/β-catenin pathway, which demonstrated the involvement of the Wnt/β-catenin pathway in the anticancer effect of miR-512-5p in NSCLC. However, an interventional study using a β-catenin activator needs to be performed to further confirm whether miR-512-5p directly acts through the Wnt/β-catenin pathway to regulate the progression of NSCLC *in vitro*. In
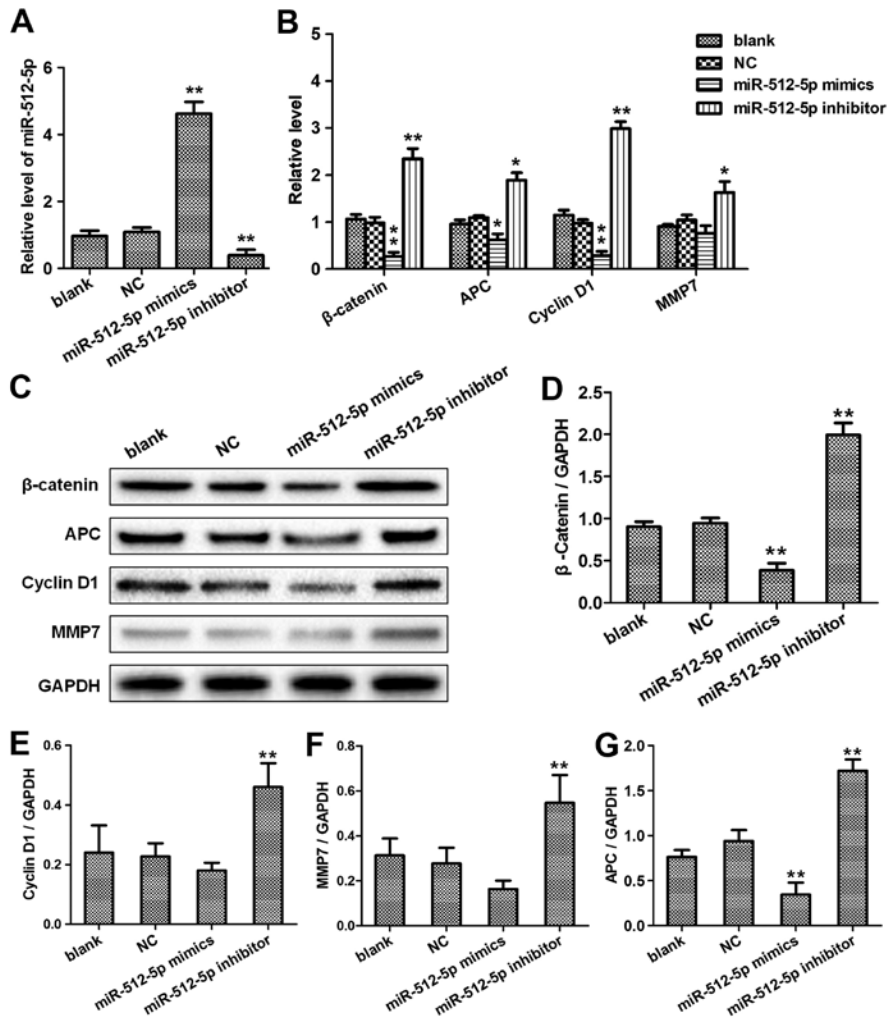
Figure 6. miR-512-5p downregulates β-catenin, APC, cyclin D1 and MMP7 expression in the A549 cell line. At 48 h post-transfection with the NC, miR-512-5p mimic or the miR-512-5p inhibitor, the expression levels of (A) miR-512-5p and (B) β-catenin, APC, cyclin D1 and MMP7 in A549 cells was analysed by reverse transcription-quantitative PCR. (C) Western blot analyses were performed to evaluate the protein expression levels of β-catenin, APC, cyclin D1 and MMP7 in A549 cells transfected with the NC, miR-512-5p mimic or miR-512-5p inhibitor. Quantitative analysis of protein levels of (D) β-catenin, (E) cyclin D1, (F) MMP7 and (G) APC. $^*P<0.05$, $^{**}P<0.01$. miR, microRNA; NC, negative control; APC, adenomatosis polyposis coli; MMP7, matrix metallopeptidase 7.
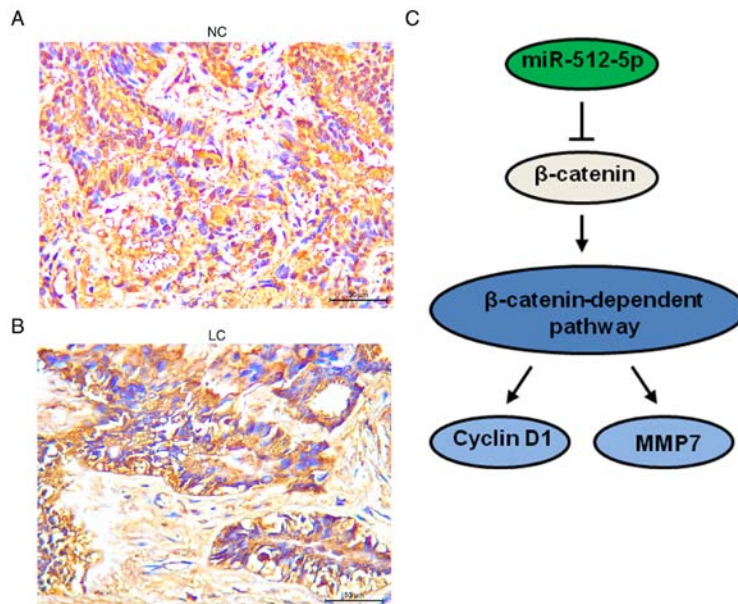

Figure 7. Roles of miR-512-5p in the Wnt/β-catenin pathway. Immunohistochemical analysis of β-catenin expression in (A) adjacent normal lung tissues and (B) lung cancer tissues. (C) A model of miR-512-5p functions in lung cancer cells. NC, normal control; LC, lung cancer; miR, microRNA; MMP7, matrix metallopeptidase 7.

addition, cytoplasmic β-catenin is broken down through the UPS pathway by a multiprotein destruction complex containing APC and Axin, as well as kinases casein kinase 1α/ε and glycogen synthase kinase 3 β (22). APC, a tumour suppressor protein, is a core constitute of canonical Wnt signalling (7). The downregulation of APC triggers the activation of Wnt/β-catenin signalling by accumulating β-catenin in the nucleus. Cyclin D1 and MMP7 are two important targets of β-catenin and are associated with NSCLC cell proliferation and invasion (23,24). The results of the present study indicated an inhibitory effect of miR-512-5p on APC and β-catenin, which may offset the enhanced β-catenin expression caused by the downregulation of APC. In addition, APC can be regulated by miRNA-129-5p and miR-582-5p in cancer progression (25,26); however, whether and how miR-512-5p influences APC expression need to be investigated in further research.

The Wnt/β-catenin signalling pathway is an evolutionarily highly conserved signalling pathway (7). When an external signal stimulates the activation of the Wnt/β-catenin signalling pathway, it activates the cytoplasmic dishevelled protein, and dephosphorylate β-catenin (27). After accumulating to a certain extent in the cytoplasm, β-catenin begins to translocate to the nucleus and activates the downstream target genes c-myc, cyclin Dl, survivin and MMP7, the promoters of which are exposed and activated, resulting in abnormal cell proliferation (28). The results of the present study indicated that overexpression of miR-512-5p negatively affected the expression of β-catenin, cyclin D1 and MMP7 in A549 cells. miR-512-5p does not contain a binding site for cyclin D1 or MMP7; therefore, miR-512-5p may bind to and inhibit β-catenin to downregulate its downstream target genes Cyclin D1 and MMP7.

In conclusion, miR-512-5p is a tumour-suppressive regulator of tumour progression of NSCLC *in vitro*. miR-512-5p may directly target β-catenin and inhibit the Wnt/β-catenin pathway and the progression of NSCLC *in vitro*. To confirm these results, a future *in vivo* study using a xenograft model will be performed.

Acknowledgements

Availability of data and materials

The datasets used and/or analysed during the present study are available from the first author on reasonable request.

Authors' contributions

ZW and XZ performed all the experiments and wrote the manuscript. TZ performed immunohistochemistry experiments and relevant specimen collection. ZW collected and analyzed the data and assisted with the writing of the manuscript. FY conceived the design and supervised the entire project. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients gave their full consent to participate in the present study, and a written consent form was obtained from each patient. All animal experiments were approved by the Research Ethics Committee of Shanghai Chest Hospital (Shanghai, China) and carried out in conformity with the Chinese governing law on the use of medical laboratory animal (authorization No. 55,1998, by ministry of health).

Patient consent for publication

A written consent form was obtained from each patient.

Competing interests

The authors declare that they have no competing interests.

References

1. Zhang H, Liu C, Tan Z and Zhang T: Segmentectomy versus Wedge resection for stage I non-small cell lung cancer: A Meta-analysis. J Surg Res 243: 371-379, 2019.
2. Liu H, Yin Q, Yang G and Qie P: Including 3564 patients prognostic impact of tumor spread through air spaces in non-small cell lung cancers: A meta-analysis. Pathol Oncol Res 2019 (Epub ahead of print).
3. Im Y, Park HY, Shin S, Shin SH, Lee H, Ahn JH, Sohn I, Cho JH, Kim HK, Zo JI, *et al*: Prevalence of and risk factors for pulmonary complications after curative resection in otherwise healthy elderly patients with early stage lung cancer. Respir Res 20: 136, 2019.
4. Hung JJ, Jeng WJ, Hsu WH, Chou TY, Huang BS and Wu YC: Predictors of death, local recurrence, and distant metastasis in completely resected pathological stage-I non-small-cell lung cancer. J Thorac Oncol 7: 1115-1123, 2012.
5. Peng B, Zhang J, Woods JS and Peng W: Molecular markers and pathogenically targeted therapy in non-small cell lung cancer. Front Med China 3: 245-255, 2009.
6. Stewart DJ: Wnt signaling pathway in non-small cell lung cancer. J Natl Cancer Inst 106: djt356, 2014.
7. Shang S, Hua F and Hu ZW: The regulation of β-catenin activity and function in cancer: Therapeutic opportunities. Oncotarget 8: 33972-33989, 2017.
8. Xu X, Sun PL, Li JZ, Jheon S, Lee CT and Chung JH: Aberrant Wnt1/β-catenin expression is an independent poor prognostic marker of non-small cell lung cancer after surgery. J Thorac Oncol 6: 716-724, 2011.
9. Paul S and Dey A: Wnt signaling and cancer development: Therapeutic implication. Neoplasma 55: 165-176, 2008.
10. Chen W, Huang Y, Zhang S, Zheng X, Xie S, Mao J, Cai Y, Lu X, Hu L, Shen J, *et al*: MicroRNA-212 suppresses nonsmall lung cancer invasion and migration by regulating ubiquitin-specific protease-9. J Cell Biochem 120: 6482-6489, 2019.
11. Jiang C, Bi C, Jiang X, Tian T, Huang X, Wang C, Fernandez MR, Iqbal J, Chan WC, McKeithan TW, *et al*: The miR-17~92 cluster activates mTORC1 in mantle cell lymphoma by targeting multiple regulators in the STK11/AMPK/TSC/mTOR pathway. Br J Haematol 185: 616-620, 2019.
12. Li JP, Liao XH, Xiang Y, Yao A, Song RH, Zhang ZJ, Huang F, Dai ZT and Zhang TC: Hyperoside and let-7a-5p synergistically inhibits lung cancer cell proliferation via inducing G1/S phase arrest. Gene 679: 232-240, 2018.
13. Loo JM, Scherl A, Nguyen A, Man FY, Weinberg E, Zeng Z, Saltz L, Paty PB and Tavazoie SF: Extracellular metabolic energetics can promote cancer progression. Cell 160: 393-406, 2015.
14. John K, Wu J, Lee BW and Farah CS: MicroRNAs in head and neck cancer. Int J Dent 2013: 650218, 2013.
15. Dinami R, Buemi V, Sestito R, Zappone A, Ciani Y, Mano M, Petti E, Sacconi A, Blandino G, Giacca M, *et al*: Epigenetic silencing of miR-296 and miR-512 ensures hTERT dependent apoptosis protection and telomere maintenance in basal-type breast cancer cells. Oncotarget 8: 95674-95691, 2017.

16. Chu K, Gao G, Yang X, Ren S, Li Y, Wu H, Huang Y and Zhou C: miR-512-5p induces apoptosis and inhibits glycolysis by targeting p21 in non-small cell lung cancer cells. Int J Oncol 48: 577-586, 2016.
17. Li J, Lei H, Xu Y and Tao ZZ: miR-512-5p suppresses tumor growth by targeting hTERT in telomerase positive head and neck squamous cell carcinoma in vitro and in vivo. PLoS One 10: e0135265, 2015.
18. Niu X, Liu S, Jia L and Chen J: Role of miR-3619-5p in β-catenin-mediated non-small cell lung cancer growth and invasion. Cell Physiol Biochem 37: 1527-1536, 2015.
19. Edge SB and Compton CC: The American Joint Committee on Cancer: The 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol 17: 1471-1474, 2010.
20. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
21. Xu H, Lin D, Wang L, Liu N and Wang E: Expression and mutation of β-catenin in non-small cell lung cancer. Zhongguo Fei Ai Za Zhi 7: 409-413, 2004 (In Chinese).
22. Vargas DA, Sun M, Sadykov K, Kukuruzinska MA and Zaman MH: The integrated role of Wnt/β-catenin, N-glycosylation, and E-cadherin-mediated adhesion in network dynamics. PLoS Comput Biol 12: e1005007, 2016.
23. Xie C, Jiang G, Fan C, Zhang X, Zhang Y, Miao Y, Lin X, Wu J, Wang L, Liu Y, et al: ARMC8α promotes proliferation and invasion of non-small cell lung cancer cells by activating the canonical Wnt signaling pathway. Tumour Biol 35: 8903-8911, 2014.
24. Schneikert J and Behrens J: The canonical Wnt signalling pathway and its APC partner in colon cancer development. Gut 56: 417-425, 2007.
25. Shu Z, Chen L and Ding D: miR-582-5P induces colorectal cancer cell proliferation by targeting adenomatous polyposis coli. World J Surg Oncol 14: 239, 2016.
26. Li M, Tian L, Wang L, Yao H, Zhang J, Lu J, Sun Y, Gao X, Xiao H and Liu M: Down-regulation of miR-129-5p inhibits growth and induces apoptosis in laryngeal squamous cell carcinoma by targeting APC. PLoS One 8: e77829, 2013.
27. Wang S, Dong Y, Zhang Y, Wang X, Xu L, Yang S, Li X, Dong H, Xu L, Su L, et al: DACT2 is a functional tumor suppressor through inhibiting Wnt/β-catenin pathway and associated with poor survival in colon cancer. Oncogene 34: 2575-2585, 2015.
28. Yang Y, Yang JJ, Tao H and Jin WS: New perspectives on β-catenin control of cell fate and proliferation in colon cancer. Food Chem Toxicol 740: 14-19, 2014.