Low expression of microRNA-30c promotes invasion by inducing epithelial mesenchymal transition in non-small cell lung cancer

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Abstract. MicroRNA (miR)-30c has been identified as a tumor suppressor gene in numerous diseases. Aberrant miR-30c expression has been associated with the invasion of different types of cancer. However, the potential mechanisms underlying the association between miR-30c and invasion has been poorly elucidated in non-small-cell lung cancer (NSCLC). In the present study, quantitative polymerase chain reaction demonstrated that the expression of miR-30c was reduced in lung cancer specimens (n=85). Suppressing the expression of miR-30c promoted the invasion of A549 cells, while overexpressed miR-30c inhibited the invasion of A549 cells. Furthermore, aberrant miR-30c expression was able to control the expression levels of markers (E-cadherin, snail and vimentin) of epithelial mesenchymal transition (EMT). In conclusion, miR-30c regulated the invasion of NSCLC cells and low miR-30 levels induced EMT.

Introduction

Lung cancer is currently one of the most common types of malignant cancers (1). The incidence of lung cancer is evidently increasing in China (2). Approximately 80% of all lung cancer is non-small cell lung cancer (NSCLC) (3,4). Numerous studies have focused on improving diagnosis and therapy, but 30-40% patients with NSCLC still have a poor prognosis. The most common feature of malignancy is invasion, which is responsible for the low 5-year survival rates. Therefore, determining the mechanism underlying the association between miR-30c and invasion would facilitate the understanding of the progression of NSCLC and thus contribute to developing novel therapeutic agents.

MicroRNAs (miRNAs) had been identified as having important roles in controlling the expression of downstream target genes in various biological processes (5-7). A number of studies have suggested that miRNAs may regulate the proliferation, apoptosis, cell cycle and invasion of cancer cells (8,9). The miR-30 family (miR-30a/b/c/d/e/f) has also been reported in various diseases, including breast cancer (10), retinal pigment epithelial cell cancer (11), glioma (12) and osteoblastic cancer (13).

Epithelial-to-mesenchymal transition (EMT) has a pivotal role in the invasion of various cancer types by the transformation of polarized and adherent epithelial cells into motile and invasive mesenchymal cells (14,15). Numerous transcription factors involved in EMT, including Snail and Twist, upregulate the expression of mesenchymal markers, such as vimentin, collagen and fibronectin and downregulate the expression of epithelial markers, including E-cadherin. A breakdown of tight junctions is involved in the loss of epithelial markers and acquisition of mesenchymal makers (16-18).

The present study aimed to examine the underlying mechanism of the association between miR-30c and invasion in NSCLC, in order to provide further evidence to facilitate improvement of the therapeutic strategies for this disease.

Materials and methods

Clinical samples. A total of 85 patients with NSCLC that had undergone routine surgery at The First Affiliated Hospital of Nanjing Medical University (Nanjing, China) between May 2010 and November 2012 were selected to participate in this study. The NSCLC samples and the adjacent lung tissues obtained from the 85 patients were collected, immediately snap frozen in liquid nitrogen and stored at -80˚C until RNA extraction. The tumors were classified according to World Health Organization classification (19). The present study was approved by the Ethical Committee of The First Affiliated Hospital of Nanjing Medical University and every patient provided written informed consent.

Cell culture. The A549 cell line (American Type Culture Collection, Manassas, VA, USA) was employed for the present study and was cultured in RPMI-1640 medium with 10% fetal

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bovine serum (Invitrogen, Carlsbad, CA, USA) and penicillin (100 U/ml). The cells were cultured at 37˚C with 5% CO_2.

Isolation of total RNA and quantitative polymerase chain reaction (qPCR). Total RNA was extracted from collected tissues using TRIzol reagent (Invitrogen) and then mRNA was reverse transcribed to cDNA. The stem-loop primer for miR-30c was 5'-GTCGTATCCAGTGCAGGGTCCGAGTATTCGCACTG-GATACGACGCTGA-3'. U6 small nuclear RNA was used for normalization. The PCR reactions were performed with the following primers: Forward: 5'-GCCGCTGTAAACATCCTA-CACT-3' and reverse: 5'-GTGCAGGGTCCGAGGT-3' for hsa-miR-30c; and forward: 5'-CTCGCTTCGGCAGCACA-3' and reverse: 5'-AACGCTTCACGAATTTGCGT-3' for U6. Reaction conditions were as follows: 37˚C for 15 min and 85˚C for 5 sec. Unused reaction products were stored at 4˚C. qPCR was performed using the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA).

Wound healing assay. The cells were plated onto 6-well plates and cultured with RPMI-1640 medium. Following 24 h, the cells were wounded with a pipette tip. Serum-free RPMI-1640 medium was added and wound closure was observed for 24 h using an XSP-4C microscope (Shanghai Changfang Optical Instrument Co. Ltd., Shanghai, China).

Transwell assay. The cell motility was measured using an 8-μm-pore polycarbonate membrane Boyden chamber insert in a Transwell apparatus (Millipore, Billerica, MA, USA). The transfected cells were treated with trypsin/EDTA solution and washed once with serum-containing RPMI-1640 medium. A total of $1x10^5$ cells in 0.2 ml serum-free RPMI-1640 medium were seeded onto a Transwell apparatus. RPMI-1640 containing 10% fetal bovine serum (600 μl) was added to the lower chamber. An invasion assay was conducted following the same procedure, with the exception that the filters of the Transwell chambers were coated with 45 μg Matrigel (BD Biosciences, San Jose, CA, USA). Following incubation of the cells for 24 h at 37˚C in a 5% CO_2 incubator, the cells on the top surface of the insert were removed by wiping with a cotton swab. The cells that invaded to the bottom surface of the insert were fixed in the 100% precooling methanol for 10 min, stained in 0.5% crystal violet for 30 min, then rinsed in phosphate-buffered saline (PBS) and subjected to microscopic inspection. The values for invasion were obtained by counting three fields per membrane and represented the average of three independent experiments.

Western blot analysis. The total proteins were prepared from the established cells, quantities using a protein assay (bicinchoninic acid method; Beyotime, Shanghai, China). The proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membrane (Millipore), blocked in 5% dry milk at room temperature for 1 h and immunostained with antibodies at 4˚C overnight using anti-E-cadherin, anti-Snail, anti-vimentin (1:1,000; Dizhao, Nanjing, China) and anti-GAPDH (1:5,000; Kangchen KangChen Bio-Tech, Shanghai, China). All of the results were visualized through a chemiluminescent detection system (Pierce ECL Substrate western blot detection system; Thermo Scientific, Pittsburgh, PA, USA) and then exposed in Molecular Imager ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA). The integrated density of the band was quantified by ImageJ software (Bio-Rad).

Table I. Expression level of miR-30c in lung cancer and corresponding adjacent tissues.

Factor	No. of samples	miR-30c low-expression (<median)	miR-30c high-expression (≥median)	P-value
Gender				0.950
Male	52	28	24	
Female	33	18	15	
Age (years)				0.629
<60	39	20	19	
≥60	46	26	20	
Smoker				0.832
No	36	19	17	
Yes	49	27	22	
Histological type				0.805
SC	47	26	21	
AC	38	20	18	
Tumor stage				0.026[a]
I-II	39	16	23	
III-IV	46	30	16	
Tumor size				0.047[a]
T1/T2	49	22	27	
T3/T4	36	24	12	
Metastasis				0.009[b]
No	31	11	20	
Yes	54	35	19	
Total	85	46	39	

[a]P<0.05 and [b]P<0.01. SC, squamous carcinoma; AC, adenocarcinoma; miR, microRNA.

Transfection. The A549 cells were plated in 6-well plates ($6x10^5$ cells/well) and 100 nm of the miR-30c mimic or 100 nm miRNA mimic control were transfected into the A549 cells, while 100 nm of the miR-30c inhibitor (anti-miR-30c) or 100 nm miRNA inhibitor control were transfected into the A549 cells, using Lipofectamine 2000 (Invitrogen Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. The miR-30c mimic, miRNA mimic control, miR-30c inhibitor and miRNA inhibitor control were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China).

Statistical analysis. The $2^{-\Delta\Delta Ct}$ method was used to analyze the results of qPCR in all of the experiments performed in the present study. Statistical analysis was performed using

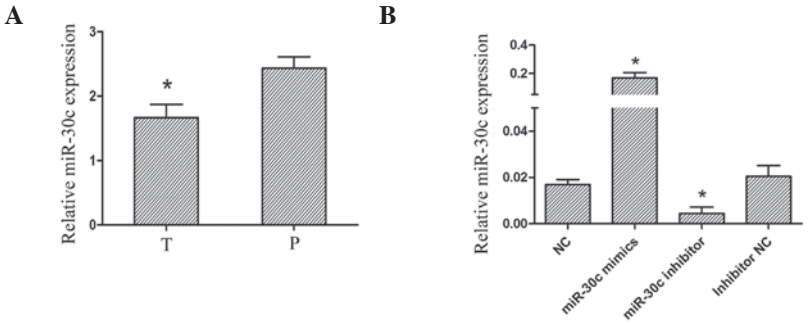

Figure 1. miR-30c is decreased in NSCLC patients. (A) The expression levels of miR-30c in the human NSCLC tissues and the corresponding adjacent tissues relative to U6 were determined by qPCR (n=85, P=0.007). *P<0.05, compared with the corresponding adjacent tissue. (B) miR-30c expression level in A549 cells transfected with miR-30c mimics, miR-30c inhibitor (anti-miR-30c), control for miR-mimics (NC) and control for anti-miR (inhibitor NC). *P<0.05, compared with the corresponding NC group. The result was validated by qPCR. The data are represented as the mean ± standard error of the mean. *P<0.05. NSCLC, non-small cell lung cancer; miR, microRNA; qPCR, quantitative polymerase chain reaction.

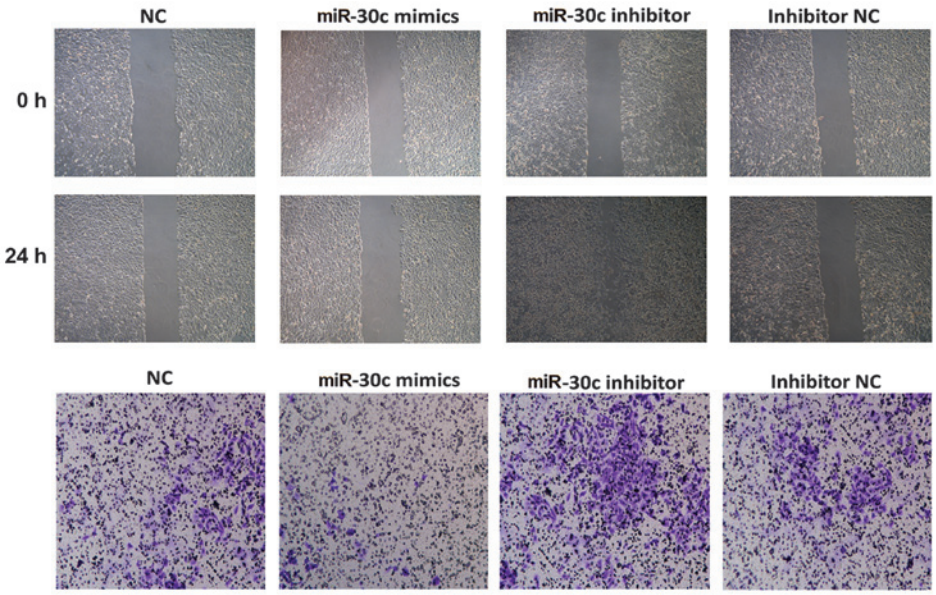

Figure 2. Effects of miR-30c expression on A549 cells invasion. The images captured at 0 h and 24 h post-wounding are revealed at magnification of x200 (upper). The Transwell assay was performed as described in Materials and methods. The cells were treated with miR-30c mimics, anti-miR-30c, control for miR-mimics (NC) and control for anti-miR (inhibitor NC) for 24 h. The representative images of invasive cells at the bottom of the membrane stained with crystal violet were visualized as demonstrated (lower). All of the experiments were performed in triplicate and the data are presented as the mean ± standard error of the mean. *P<0.05, indicates a significant difference compared with the control group. Each independent experiment was performed three times. miR, microRNA.

STATA 11, and presented with Graph Pad prism software (GraphPad Software, Inc., La Jolla, CA, USA). The results obtained from experiment *in vitro* assays are presented as the mean ± standard error of the mean from five separate experiments in triplicates per experiment, and the data was analyzed by the Wilcoxon rank-sum (Mann-Whitney) test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-30c is reduced in human lung cancer tissues. The expression of miR-30c was analyzed in lung cancer samples (n=85) and adjacent lung tissues by qPCR. The miR-30c expression was significantly lower in lung cancer tissues than paraneoplastic tissues (P=0.007; Fig. 1). There was no positive correlation with gender, age, smoking status, histological type or tumor size, however, there was an evident correla-

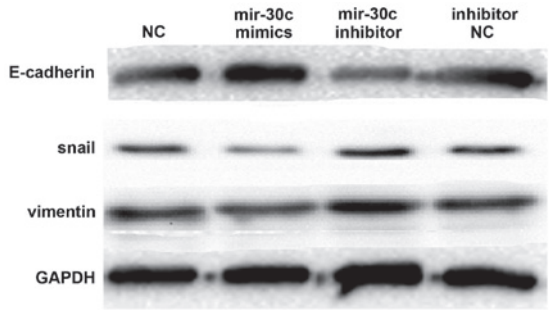

Figure 3. miR-30c expression controlled the expression levels of epithelial mesenchymal transition markers. E-cadherin, Snail and vimentin protein expression levels in A549 cells transfected with miR-30c mimics, anti-miR-30c, control for miR-mimics (NC) and control for anti-miR (inhibitor NC) were analyzed by western blotting. GAPDH was used as a loading control. Average values of integrated optical density(IOD) were assessed by analyzing five fields per slide. The data are represented as the mean ± standard error of the mean.*P<0.05. miR, microRNA.

tion with tumor stage (P=0.026) and metastasis (P=0.009; Table I). The aberrant expression level of miR-30c suggested that miR-30c may have an important role in lung cancer progression and development. Therefore, based on this expression pattern, the A549 cell line was selected to verify the effect of miR-30c.

miR-30c regulated the invasion of A549 cells in vitro. To examine the mechanism underlying the effect of miR-30c on the invasion in lung cancer, the A549 cells were transfected with miR-30c mimics, NC mimics and miR-30c inhibitor (anti-miR-30c) and inhibitor NC respectively. The transfection efficiency was validated by qPCR (Fig. 1). The wound healing assay demonstrated that the overexpression of miR-30c was able to suppress A549 cell healing, while suppression of miR-30c increased cell healing (Fig. 2). Furthermore, the Matrigel invasion assay demonstrated that overexpression of miR-30c attenuated A549 cell invasion, whereas the suppression of miR-30c reversed its effect (Fig. 2). The results suggested that miR-30c inhibited invasion of the A549 cell line *in vitro*.

Down regulated expression of miR-30c induces EMT. The A549 cells were transfected with miR-30c mimics, NC mimics and miR-30c inhibitor (anti-miR-30c) and inhibitor NC to examine whether miR-30c was involved in EMT. The epithelial marker (E-cadherin) and mesenchymal markers (vimentin and Snail) were investigated by western blot analysis. At a protein level, upregulated miR-30c expression by miR-30c mimics resulted in elevated E-cadherin expression and decreased vimentin and Snail expression. In addition, suppression of miR-30c expression by the miR-30c inhibitor resulted in decreased E-cadherin expression and increased vimentin and Snail expression (Fig. 3). Therefore, it was concluded that miR-30c contributed to regulating EMT marker expression in lung cancer cell lines.

Discussion

The present results indicated that the expression of miR-30c was decreased in lung cancer tissues (n=85), as compared with the corresponding adjacent tissues. Aberrant expression of miR-30c controlled the invasion of lung cancer cell lines *in vitro*. Furthermore, it was also identified that the overexpression of miR-30c led to elevated E-cadherin expression and decreased vimentin and Snail expression. The downregulation of miR-30c had the reverse effect. E-cadherin is an epithelial marker, while vimentin and Snail are mesenchymal markers. These results suggested that downregulation of miR-30c may promote lung cancer invasion by inducing EMT.

Decreased E-cadherin and elevated vimentin and Snail expression is a hallmark of EMT, which is a key process in cancer invasion (19). Previously, EMT has been identified to be associated with tumor invasiveness, metastasis and prognosis (20,21). Numerous studies established functional associations between non-coding microRNAs and key effectors of EMT occurring in the context of carcinogenesis and embryonic development, including microRNA-200 (22,23), microRNA-10b (24) and microRNA-21 (25,26). In addition to cancer progression, EMT contributes to chronic epithelial injury (27), leading to tissue fibrosis and organ failure (28,29).

In conclusion, compared with the adjacent tissues, the mRNA expression level of miR-30c was decreased in lung cancer. It was demonstrated that low expression of miR-30c promoted invasion via inducing EMT in lung cancer. Furthermore, the miR-30c-EMT pathway that was investigated may be exploited in a therapeutic approach for the treatment of lung cancer in the future.

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