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Original Article

MicroRNA30a inhibits tumor invasiveness and metastasis by targeting IGF1R in non-small lung cancer

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Abstract: MicroRNAs are a number of non-coding RNAs play critical roles in the development and progression of human cancers. MiR-30a has been suggested to function as a tumor repressor in several tumors. Our previous study show that miRNA-30a is down regulated in lung cancer tissues and cell lines, in the A549 cell lines this phenomenon is most obvious, and over-express miRNA30a inhibited A549 cell proliferation by targeting IGF1R. However the role of miRNA30a in the invasion and metastasis of NSCLC is still unclear. In this study, we found that over-expression of miR-30a was able to inhibit cell invasion and migration in A549. Furthermore, silenced IGF1R expression has the similar effects in A549. These findings suggest that miR-30a inhibit cell invasion and metastasis of NSCLC cells partially through the down-regulation of IGF1R. Therefore, targeting with the miR-30a/IGF1R axis might serve as a novel therapeutic application to treat NSCLC patients.

Keywords: miR-30a, non-small cell lung cancer, invasion, migration, insulin-like growth factor 1 receptor

Introduction

MicroRNAs (miRNA) are highly conserved small non-coding RNAs about 22 nucleotides long. MiRNA could regulate gene expression post-transcriptionally by interacting with complementary sequences (usually located in the 3'-untranslated region (3'-UTR) of nucleotide) of messenger RNA (mRNA) targets. The miRNA blocking the translation of target gene or leading to degradation of it depending on the binding manners. The character of miRNA matches to the target gene open up the possibility of miRNA to regulate multiple genes [1]. The genes regulated by miRNAs were involved in various physiological processes. More over miRNAs have been shown to play a crucial role during cancer development and progression [2].

The lately Survey manifest the most commonly diagnosed cancers is lung (1.82 million), the most common causes of cancer death is lung cancer about 18% [3], and the highest size of cancer burden is for person with lung cancer about 13% [3-5]. Lung cancer is divided into two groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), 80%-85% of lung cancer cases are non-small-cell lung cancer (NSCLC) and 75% of patients with NSCLC present with locally advanced or metastatic disease [6]. Although substantial progress has been made in the past decades, including surgical treatment, radiotherapy and chemotherapy, the survival rate of NSCLS patients has changed little. It is of vital importance to search early detection method due to increased metastasis and mortality of advanced high-grade NSCLS.

MiRNA-30a is a member of the miRNA30 family. Accumulating evidences indicate that the deregulation of miR30a contributes to various malignant tumors, including breast cancer, myeloma, chronic myeloid leukemia, colorectal carcinoma, gastric cancer [7-11]. IGF1R is reported over-express in oral squamous cell carcinoma, ovarian cancer, colorectal cancer,

Table 1. Primers for plasmid construction, qRT-PCR, and oligonucleotide

Name	Sequence
miR-30a reverse-transcribed primer	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGG ATACGACCTTCCAG-3'
miR-30a forward	5'-ATCCAGTGCGTGTCGTG-3'
miR-30a reverse	5'-TGCTTGTAAACATCCTCGA-3'
U6 reverse-transcribed primer	5'-CGCTTCACGAATTTGCGTGTCAT-3'
U6 forward:	5'-GCTTCGGCAGCACATATACTAAAAT-3';
U6 reverse:	5'-CGCTTCACGAATT TGCGTGTCAT-3';
IGF1R forward:	5'-TTTCCCACAGCAGTCCACCTC-3';
IGF1R reverse:	5'-AGCATCCTAGCCTTCTCACCC-3'
β-Actin forward:	5'-TGGCACC CAGCACAATGAA-3';
β-Actin reverse:	5'-CTAAGTCATAGTCCGCCTAGAAGC A-3'.
Anti-miR-30a sequence	5'-AUCUGUGGCUUCACAGCUUCCAGU-3'.
Anti-control sequence	5'-CAGU ACUUUUGUGUAGUACAA-3'.
IGF1R siRNA sense:	5'- GAAAAGCAGUCAAUGGAUUUU-3',
Antisense:	5'- AAUCCAUUGACUGCUUUUCUU-3';
NC-siRNA sense	5'-UUCUCCGAACGUGUCACGUUU-3',
Antisense:	5'-ACGUGACACGUUCGGAGAAUU-3'.

and promote metastasis of these cancer [12-14].

Our previous study show that miRNA-30a is down regulated in lung cancer tissues and cell lines, in the A549 cell lines this phenomenon is most obvious. We demonstrated that over-expressing miR-30a suppresses A549 cell proliferation through AKT signaling pathway by targeting IGF1R [15]. In our study, the data indicated that miRNA30a inhabit the invasion and migration of non-small cell lung cancer (NSCLC)cell lines A549 by targeting IGF1R and. Therefore, over-expressing miR-30a and/or interfering with IGF1R function might be a promising NSCLC therapeutic strategy.

Materials and methods

Cell culture

The human lung cancer cell line (A549) was maintained in the Key Laboratory of Environment and Genes Related to Diseases at Xi'an Jiaotong University College of Medicine. A549 cells were cultured in RPMI-1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL, Grand Island, NY, USA) containing 2.0 mmol/L glutamine and 20 μg penicillin-streptomycin/mL at 37°C in a humidified atmosphere containing 5% CO_2.

Vectors construction, oligonucleotide synthesis and transfection

MiR-30a, anti-miR-30a, miR-control, anti-control and human IGF1R siRNA, NCsiRNA were purchased from Gene-Pharma. Lipofectamine 2000 Transfection Reagent (Invitrogen, USA) was used to transfect cells. The miR-30a expression vector (pre-miR-30a) and control vector were constructed with synthetic oligonucleotides and cloned in between the EcoRI and HindIII sites of the pcDNA6.2-GW/EmGFP vector (Invitrogen, USA). The 3'UTR of human IGF1R mRNA was constructed by synthetic oligonucleotides and cloned in between the SacI and XhoI sites of the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, USA).

Stable transfection of miR-30a

5~10×10^5 A549 cells were grown in a 6-well plate for 24 h in RPMI1640 media. The pcDNA6.2 (+)-pre-miR-30a plasmid were then transfected into cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Stable cell lines were screened with 1 mg/ml G418 (Sigma, Shanghai, China), and positive clones were identified using qRT-PCR.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from cells using TRIzol reagent according to manufacturer's protocol

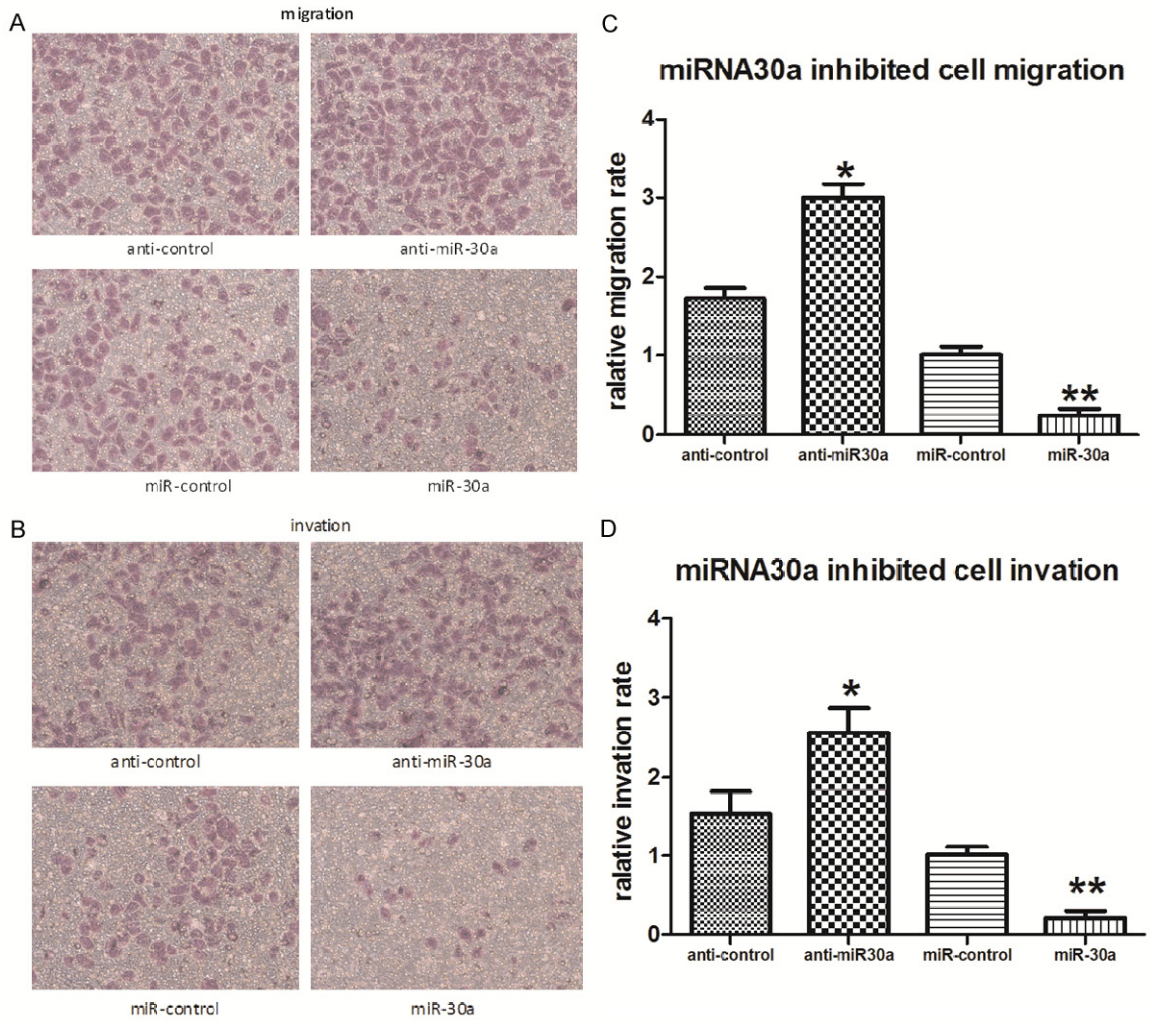

Figure 1. MiR-30a inhibited cell migration and invasion ability of A549 cells. A and B. Effects of miR-30a on migration were analyzed by Transwell migration assay. A. Representative photos. B. Quantitative analysis (*anti-miR30a compared to anti-control, miR30a compared to miR-control P<0.01). C and D. Effects of miR-30a on invasion were analyzed by Transwell invasion assay. C. Representative photos. D. Quantitative analysis. (**anti-miR30a compared to anti-control, miR30a compared to miR-control P<0.01).

(Invitrogen). RNA was reverse transcribed using SuperScript First-Strand cDNA System (Invitrogen) according to manufacturer's instructions. qRT-PCR analysis were conducted with Power SYBR Green (Takara). All protocols were performed according to the manufacturer's instructions. qRT-PCR reactions were carried out using an iQ Multicolor qRT-PCR Detection System (Bio-Rad, USA)as follow: 40 cycles of 95°C for 30 s, 95°C for 5 s, 60°C for 30 s. Fluorescence was detected at the end of each cycle. Results were normalized to the expression of U6 or β-Actin. **Table 1** listed all related DNA sequences.

Analysis of cell invasion and migration

Cell invasion and migration assays were performed using a Transwell (Millipore, Billerica, MA) method. Filters for invasion assays were coated with Matrigel (BD Biosciences, USA) in the upper compartment before cell seeding. Then, $5*10^5$ cells were seeded in the filters after 6 h cultured with no serum culture medium, and the lower compartment was filled with cell culture medium supplemented with 10% fetal bovine serum. After 24 h cultured invasive and migratory cells on the bottom surface were stained with crystal violet, then counted and measured the migrating distance.

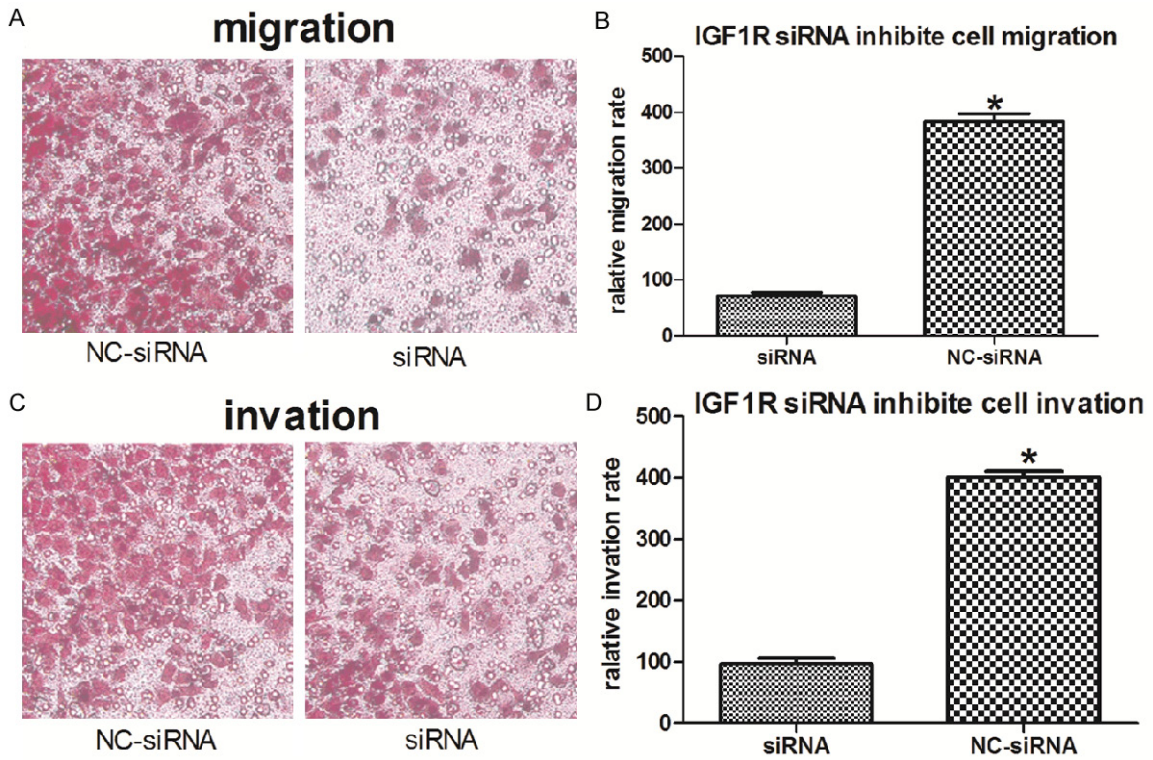

Figure 2. IGF1R promote cell migration and invasion ability of A549 cells. A and B. Effects of IGF1R on migration were analyzed by Transwell migration assay. A. Representative Transwell pictures. B. Quantitative analysis (*P<0.01). C and D. Effects of miR-30a on invasion were analyzed by Transwell invasion assay. C. Representative Transwell pictures. D. Quantitative analysis (*P<0.01).

Western blot analysis

The A549 cells were treated by control vector, miR-30a, anti-control, anti-miR-30a, NC-siRNA or IGF1R siRNA (70 nM) for 48 h. The A549 cells were lysed in RIPA lysis buffer. Insoluble material was removed by centrifugation at 12,000 rpm for 10 min. Protein were subjected to electrophoresis using 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were blocked for 1~2 h in 5% non-fat dry milk in TBST. The membrane was incubated with primary antibodies overnight at 4°C and then incubated with secondary antibody for 1 h at room temperature. The primary monoclonal antibodies included anti-AKT (1:1,000, Santa Cruz, CA, USA), anti-p-AKT (1:1,000, Santa Cruz, CA, USA), anti-IGF1R (1:1,000, Santa Cruz, CA, USA), anti-β-Actin (1:5,000, Santa Cruz, CA, USA), anti-Ecad (1:1,000, Santa Cruz, CA, USA) and anti-N-cad (1:1,000, Santa Cruz, CA, USA). The membranes were incubated in the dark with ECL (Amersham) for chemiluminescence detection. The luminescent signal was detected by CCD camera, recorded and quantified with Syngene GBox (Syngene, UK).

Statistical analysis

Each experiment was repeated at least 3 times independently. All values are expressed as mean ± SEM. Statistical analysis was performed with SPSS 15.0 software. Student's t-tests were used to determine the statistical significance of differences between groups. Spearman's correlation was applied to identify the correlation between miR-30a and IGF1R expression. Differences with P<0.05 were considered significant.

Results

MiR-30a inhibited cell migration and invasion ability of A549 cells

To understand the impact of miR-30a on cell migration and invasion, Transwell migration and invasion and wound healing assays were performed. The results indicated that overexpression of miR-30a obviously inhibited while

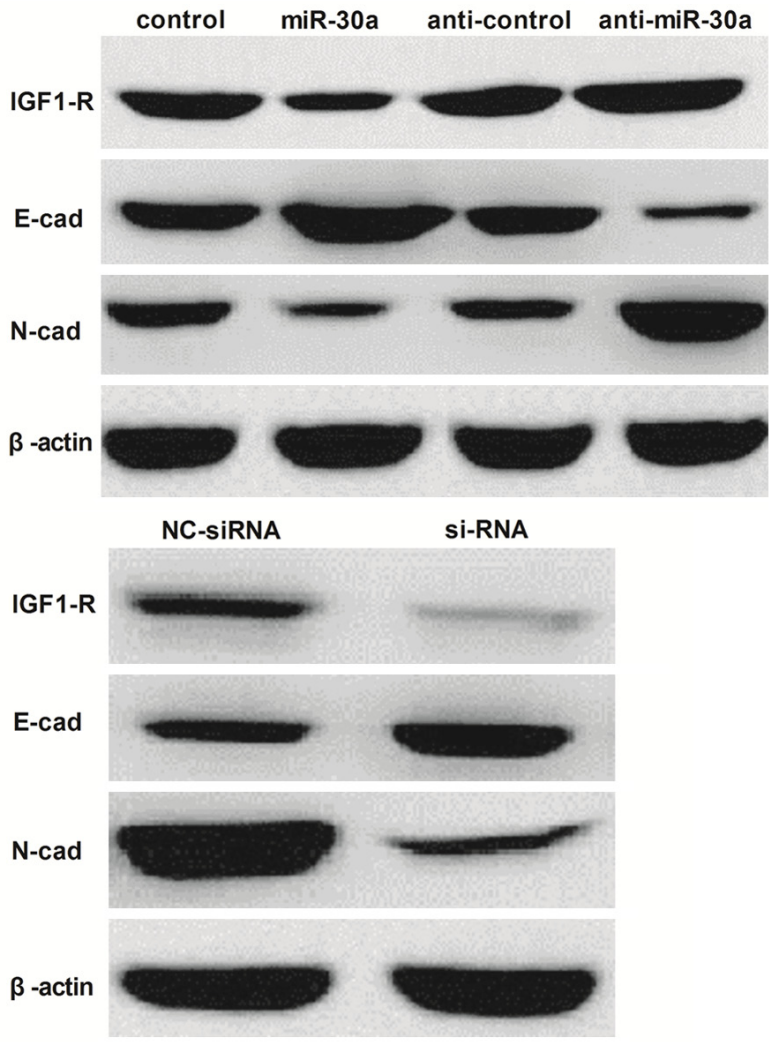

Figure 3. The expression analysis for E-cad and N-cad after miRNA30a up-regulated down-regulated (A) and IGF1R inhibited (B).

down-regulation of miR-30a promoted migration and invasion abilities of A549 cell (P<0.01) (**Figure 1A-D**).

IGF1R promote the migration and invasion of A549 cell

To understand the impact of IGF1R on cell migration and invasion, Transwell migration and invasion and wound healing assays were performed. The results indicated that downregulation of IGF1R obviously inhibited migration and invasion abilities of A549 cell (P<0.01) (**Figure 2A-D**).

IGF1R mediated inhibition of tumor migration and invasion of miR-30a

Based on these above evidences, we made the hypothesis that IGF1R attributed to the inhibited cell migration and invasion effect of miR-30a. To prove this untested hypothesis, we silenced IGF1R expression by siRNA method to test whether IGF1R is involved in the process of anti-tumor metastasis effects of miR-30a. The results indicated that over expression of miR-30a obviously inhibited while down-regulation of miR-30a promoted express of IGF1R and N-cad of A549 cell. Conversely the expression of E-cad is up-regulated when miR-30a is over-expressed (**Figure 3A**). Of interest, the western blot indicated that knockdown of IGF1R by siRNA could affect the expression of E-cad and N-cad which was similar to the effect of miR-30c over-expression (**Figure 3B**).

Discussion

MiRNAs are a type of non-coding RNAs which are crucial to several biological processes including proliferation, apoptosis, development and cellular differentiation [16]. A study demonstrated that more than 50% of miRNA genes are located in cancer associated genomic regions or in fragile sites [17], suggesting that miRNAs may play a more important role in the pathogenesis of a limited range of human cancers than previously thought. In this decade miRNAs were detected also have special influence in the process of cell survival, proliferation, apoptosis and migration of malignancies. MiRNA regulates the expression of the target gene by pairing with a cognate stretch in the 3'UTR of the target mRNA, because of this miRNAs can simultaneous regulation of hundreds to thousands of genes.

The role of miRNA30a in the progress of metastasis has been investigated in several cancers. It is down regulated in most reports on breast cancer [18] nasopharyngeal carcinoma [19],

and promoting or inhibiting the tumor metastasis is not coincidence in different cancers. Lung cancer is the major cause of cancer death globally; it is often diagnosed at an advanced stage and has one of the lowest survival rates of any type of cancer. Finding the key point of the cell invasion and metastasis of lung cancer is the hot spot. MiRNA30a had been reported down-regulated in NSCLC in both cell lines and tumor tissues [20], and recent studies showed it can inhibit the invasion of cell in vitro and in vivo by target different genes as BCL11A and Snai1 [21, 22].

IGF1R is often over expressed and hyperactive in cancers, and promote its metastasis [23-25, 27]. But also have converse report [26]. In NSCLC IGF1R is over expressed have been confirmed [28, 29], and is regulated by several miRNA such as miR-140, miR-486 and miR-195 [30-32]. It has been reported that IGF1R is implicated in EMT-mediated invasiveness in lung cancer cells [33].

In our study, we hypothesis that miR-30a might inversely regulate NSCLC metastasis by suppressing cell migration and invasion. Our previous studies have proved miR-30a is decreased both in clinical specimens and cell lines along with up-regulated levels of IGF1R. IGF1R is a directly functional target of miR-30a identified by luciferase assays [15]. In this study, our findings suggest that miR-30a may inhibit the metastasis of NSCLC cells through inhibiting the EMT by targeting IGF1R. MiRNA-30a and IGF1R both have an influence on the EMT of A549 cell, but IGF1R is not the only target gene of the miR-30a. MiR-30a may be used as a novel diagnostic marker and therapeutic target for NSCLC in the future.

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Disclosure of conflict of interest

None.

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