IJMCM
Autumn 2021, Vol 10, No 4
DOI: 10.22088/IJMCM.BUMS.10.4.258

Original Article

Long Non-coding RNA snaR Promotes Proliferation in EGFR Wild Type Non-Small Cell Lung Cancer Cells

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Submmited 7 June 2021; Accepted 4 January 2022; Published 6 June 2022

Lung cancer is the second most common cancer and has high morbidity and mortality worldwide with non-small cell lung cancer (NSCLC) accounting for 85% of the cases. Over-expression of epidermal growth factor receptor (EGFR) has been clarified in different cancers, and has been shown to have a crucial role in tumor progression. In this study, we evaluated long non-coding RNA small NF90-associated RNA (snaR) expression in different EGFR-statue cell lines. Knockdown experiments were conducted to analyze snaR expression in selected cell lines. MTT and transwell assays were respectively employed to evaluate the proliferative and invasive abilities of NSCLC cells. The expression of snaR was remarkably up-regulated in SPC-A1 and A549 wild-type EGFR cell lines. Down regulation of snaR with small interfering RNA significantly inhibited cell invasion as well as proliferation of SPC-A1 and A549 cells. Our results indicate that snaR may be a potential therapeutic biomarker for NSCLC.

Key words: Long non-coding RNA, snaR, non-smallcell lung carcinoma, EGFR

Lung cancer is one of the leading causes of death and the major threats to public health among both men and women, with non-small cell lung cancer (NSCLC) being the majority of the cases (1).

Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase protein that is expressed in normal epithelial, mesenchymal, and neurogenic tissues. However, overexpression of EGFR has been reported in the pathogenesis of many human malignancies, including NSCLC (2). More studies have shown that EGFR expression in NSCLC is associated with reduced survival, frequent lymph node metastasis, and poor chemosensitivity (3,4).

EGFR belongs to the ErbB family of surface tyrosine kinases, which include ErbB1 (also known as EGFR), ErbB2 (HER2), ErbB3, and ErbB4, and is located on chromosome 7 (4).

Dysregulation of the EGFR increases intracellular pathways, via tyrosine kinase autoph-osphorylation, which leads to cell proliferation,

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[DOI: 10.22088/IJMCM.BUMS.10.4.258]

angiogenesis, invasion, and metastasis.

It has been also reported that tumors with amplified EGFR are more likely to have a significant component of solid growth, suggesting an association between EGFR amplification and a more aggressive tumor (5).

Non-coding RNAs (ncRNAs) comprise the large majority (approximately 98%) of the human transcriptome. Long ncRNAs (lncRNAs) are a large and diverse class of ncRNAs whose transcripts are longer than 200 nucleotides with no protein-coding or limited capacity (6).

LncRNAs are mostly distributed in the nucleus, and are highly similar to protein-coding RNAs regarding synthesis and processing. The similarity is attributable to the fact that RNA polymerase II also transcribes lncRNAs, which generally have fewer exons and lower expression levels than mRNAs. In addition, lncRNAs are more cell type-specific and less conserved than mRNAs (6).

Numerous studies have reported that lncRNAs participate in a variety of biological functions such as cell proliferation, immune response, stem cell differentiation, and disease pathogenesis (7,8). LncRNAs are also associated with normal physiological processes in cells based on their huge diversity. It has been suggested that lncRNAs play a significant function in many life activities, including cell cycle regulation, epigenetic regulation, and cell differentiation regulation (9-11).

Small NF90-associated RNA (snaR) is highly structured with 117 nucleotides, terminating in an oligo-(A) tract followed by an oligo-(U) tract, snaR-A was characterized as a relatively unstable RNA (half-life ~ 15 min in HeLa cells) which is transcribed by RNA polymerase III from an intragenic promoter. snaR-A is abundant in many immortal cell lines and testis and has lower levels in other parts of the body (12). Several snaR transcripts have been found in human tissues and cell lines, and are associated with ribosomes in the cytoplasm. Reports suggest that snaR transcripts are involved in tissue- and species-specific regulation of cell growth and translation (13,14).LncRNAs have been reported to act both as oncogenes or tumor suppressors in NSCLC. snaR was identified as oncogene in hormone-receptor-positive breast cancer cells, triple-negative breast cancer cells, and HER2-positive breast cancer while other studies revealed a tumor suppressive role in human colon cancer (14). In this study, we explored the expression of snaR in four different NSCLC cell lines with either wild type or mutant EGFR to demonstrate the role of snaR in the proliferation and cell invasion of cancer cells.

Materials and methods

Cell lines and culture conditions

NSCLC cell lines including two carrying wild type EGFR (A549, SPC-A1) and two carrying mutations in EGFR (PC9 and H1975: PC9 cells carry a Glu746-Ala750 deletion mutation in exon 19 of the EGFR gene, and H1975 cells carry two-point mutations, T790M and L858R, in exons 20 and 21, respectively), and a normal human bronchial epithelial cell line (16HBE) were purchased from the Cell Bank of the Pasteur Institute of Iran (Tehran, Iran). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS), (Gibco, USA) at 37 °C in a humidified incubator with 5% CO_2, and 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, USA).

RNA extraction and quantitative RT-PCR

Total RNA was isolated from 5×10^3 cultured cells using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. RNA concentration was measured using NanoDrop ND-2000 (Thermo Fisher Scientific, USA). Total RNA (500 ng) was reverse transcribed into complementary DNA (cDNA) in a final volume of 10 µL using random

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primers under standard conditions for the PrimeScript RT reagent Kit (Takara, Japan). QPCR was performed using SYBR Select Master Mix (Takara, Japan) on an ABI 7500 system (Applied Biosystems, USA). The reaction conditions were as follows: 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Expression levels were normalized to the expression of glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) and were calculated using the comparative delta-delta CT ($2^{-\Delta\Delta Ct}$) method (11). The primer sequences were designed by using Primer3 (v. 0.4.0) software, and Primer BLAST was performed to check the specificity of both primers. All primers are listed in Table 1.

Small interfering RNA transfection

Small interfering RNAs (siRNAs) specific to snaR and scrambled negative control siRNA (si-NC) with no homology with the human genome, were synthesized by Takapuzist Gene Molecular Biotechnology Co. Ltd. (Tehran, Iran)

The sequence of si-snaR was 5′-GGGCAC GAGUUCGAGGCCA-3′ and the sequence for the negative control was 5′-CGGUACGAUCGCGGC GGGAUAUC-3′.

After overnight culture, cells were transfected with the synthesized specific snaR or control siRNA and using Lipofectamine RNAiMAX reagent (Invitrogen, USA).

MTT assay

The effect of siRNA interference for snaR on the proliferation of SPC-A1 or A549 was determined by the MTT assay (Sigma, USA) according to the manufacturer's instructions. Cells in a total volume of 200 µL were inoculated in a 96-well plate, with 5×10^3 cells in each well. At each time point, 50 µL of MTT (2 mg/mL) was added to the wells, and the cells were cultured at 37 °C for 4 h. After removal of the medium, 100 µL of dimethyl sulfoxide (DMSO) was added, and the mixture was shaken for 10 min. The optical density was then determined at 490 nm.

Cell invasion assay

The invasive properties of transfected cells were examined using transwell chambers invasion assays (8 µm; Corning Inc., USA). After 48 h of transfection, cells were added into the upper chamber of Transwell, with 3000 cells in 0.1 mL serum-free medium per well, and the lower chamber was filled up with a complete growth medium with 20% FBS. Matrigel (Millipore, USA), coated membranes were used in the invasion assay. After incubating for 22 h at 37 °C, the non-invading cells were wiped off from the upper surface of membranes, while the invaded cells were stained with 0.5% crystal violet solution. The number of invaded cells per-well was counted by a microscope ×200 magnification in random fields. All experiments were conducted at least three times.

Statistical analysis

All statistical analyses were performed using SPSS 20.0 software (IBM).

Data are presented as the mean standard deviation (SD) of three or more independent experiments. The differences in experimental results between the two groups were analyzed using Student's t-test and a statistically significant difference was defined at $P < 0.05$.

Results

Table 1. Sequence of primers used in this study

Gene	GeneID	Forward (F)	Reverse (R)	Tm (°C)	Amplicon size (bp)
snaR	100170222	5'ATTGTGGC TCAGGCCGGTT3'	5'TTTTTCCGAC CCATGTGGAC3'	F=61.6 R=60.3	320
GAPDH	100033452	5'GGGAGCCA AAAGGGTCAT3'	5'GAGTCCTTC CACGATACCAA3'	F=60.1 R=60.4	226

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Modulation of snaR expression in NSCLC cells

To investigate the functional role of snaR in NSCLC cells, we first performed qRT-PCR analysis to examine the expression of snaR in a diverse range of human NSCLC cell lines. As shown in (Figure 1), snaR expression was significantly upregulated in NSCLC cell lines (SPC-A1 and A549) in comparison with the other cell lines and the normal human bronchial epithelial cell line (16HBE). Therefore, we selected SPC-A1 and A549 which both possess wild type EGFR WT cells for further studies. Next, we used snaR siRNAs to transfect the two NSCLC cell lines. QRT-PCR analysis was performed at 48 h post-transfections.

Knockdown of snaR inhibits NSCLC cell proliferation and invasion

To assess the biological role of snaR in NSCLC, we first investigated the effect of the knockdown of snaR on cell proliferation. MTT assays showed that cell growth was significantly impaired in si- snaR -transfected SPC-A1 and A549 cells (Figure 2). Similarly, the results of cell invasion assays showed that snaR knockdown decreased invasion of SPC-A1 and A549 cells (Figure 3).

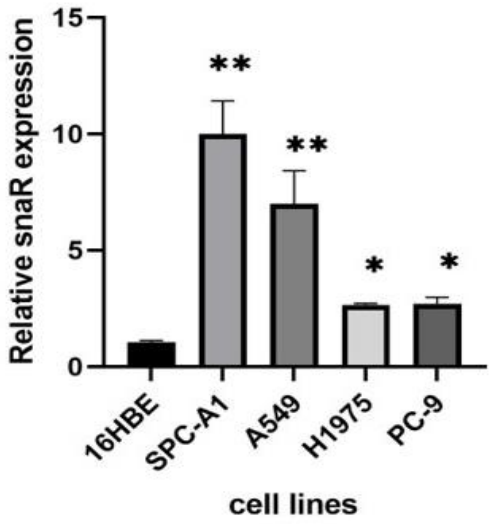

Fig. 1. snaR expression in normal human bronchial epithelial cells 16HBE and NSCLC cell lines. Data are presented as mean ± SD (*P < 0.05, **P < 0.01).

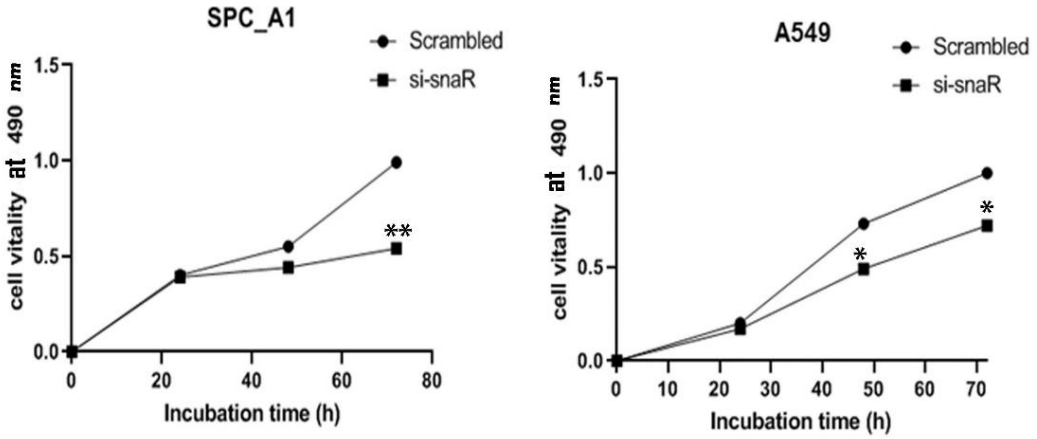

Fig. 2. Effect of si- snaR on cells viability. MTT assays were used to determine the viability of si- snaR -transfected on SPC-A1 and A549 cells. Knockdown of snaR suppressed the proliferation of SPC-A1 and A549. Data are presented as mean ± SD, compared to control treatment (*P < 0.05, **P < 0.01).

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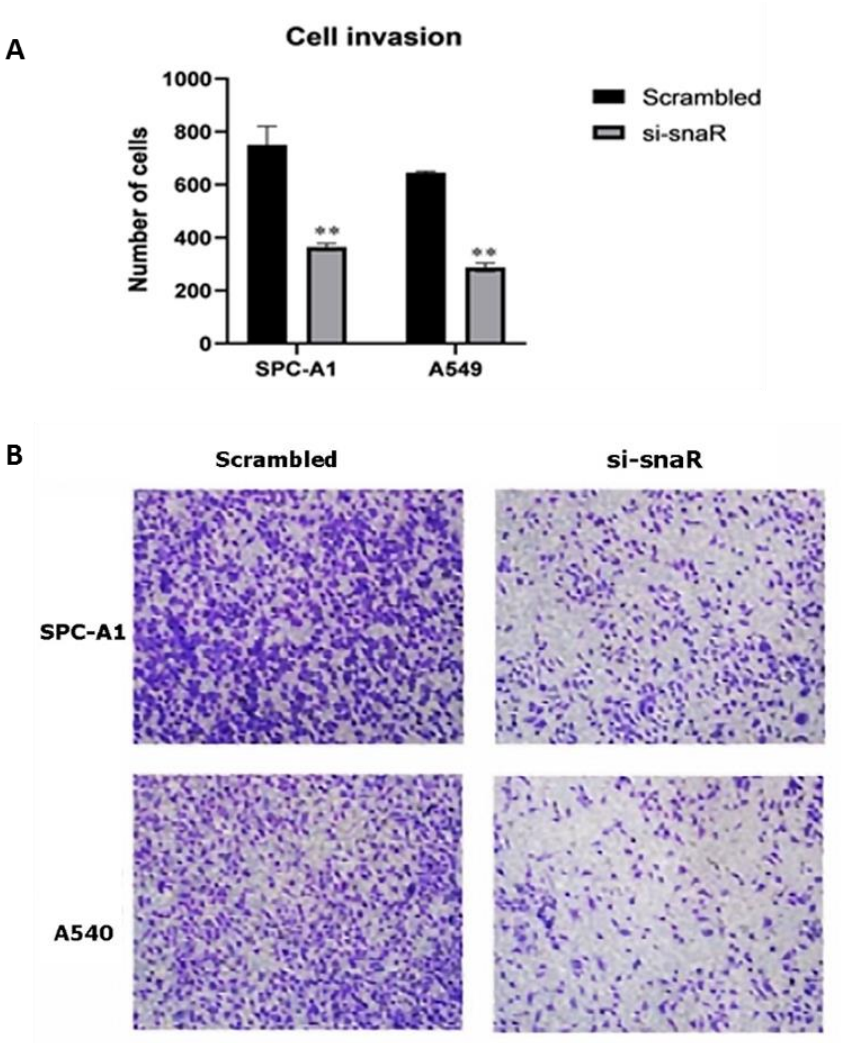

Fig. 3. Effect of snaR knockdown on the invasive ability of SPC-A1 and A549 cancer cells. A) Transwell assays were performed to investigate changes in migratory abilities of SPC-A1 and A549 cells; B) knockdown of snaR inhibited migration in both cell lines with the original magnification (×100). Data are presented as mean ± SD (*P <0.05, **P <0.01).

Discussion

NSCLC is the leading cause of cancer mortality worldwide (12). NSCLC ranks among the most common and lethal malignant diseases. NSCLC accounts for approximately 80–85% of all lung cancer cases, and includes squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. The poor prognosis of early-stage NSCLC is critically linked to the beginning of tumor metastasis (1). Despite new progress in clinical treatment and diagnosis for NSCLC, the overall survival time of patients has not improved appreciably (13). In addition, the processes inducing and stimulating metastasis are complex and still not fully understood. Therefore there is an urgent need for novel therapeutic strategies due to their relative insensitivity to radiation and chemotherapy (14). EGFR is a transmembrane glycoprotein consisting of an extracellular EGF-binding domain and an intracellular tyrosine kinase domain that plays an important role in cell proliferation through various signaling pathways (4). EGFR mutations may accumulate during tumor progression, leading to the heterogeneity of EGFR status within a tumor. The presence of multiple EGFR mutations in a single tumor specimen has been reported in several studies (2, 12, 14). LncRNAs have been reported to have a diverse

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range of biological processes through various mechanisms at the transcriptional, post-transcriptional, and epigenetic regulatory levels and have been discovered to have a strong association with various malignancies (8,15). snaR is upregulated in several malignancies, and therefore, downregulation of snaR can regulate tumor proliferation, migration, or invasiveness which is related to tumor progression. Previous studies showed that the proliferation and migration of tumor cells were inhibited by the knockdown of snaR in HER2-positive breast cancer cells (10,14). Different studies have highlighted the potential therapeutic effect of targeting snaR in cancers (15-18),

For example, snaR was found to be upregulated in patients with hepatocellular carcinoma (HCC), promoting the metastasis of HCC through upregulation of TGF-β1 (19). In another study, snaR was found to be downregulated in HPV-negative CSCCs. In addition, downregulation of snaR could mediate distant recurrence of HPV-negative CSCCs through interactions with TGFβ1 (20).

The higher expression level of lncRNA in tumor cells implicates that these lncRNAs affect tumor initiation directly or indirectly. Therefore, a better understanding of their process and strategies for inhibition of specific oncogenic lncRNA would be one further step into cancer treatment.

Based on our study, snaR was significantly upregulated in various subtypes of lung cancer cell lines, especially EGFR wild-type lung cancer cells, and the fact that the downregulation of snaR can inhibit tumor proliferation and migration was proven. To investigate the functional role of snaR in the EGFR wild-type cell line, we treated the cells with the siRNA which leads to the knockdown of snaR, and confirmed the efficiency of interference by quantitative RT-PCR. The proliferation and migration of tumor cells were inhibited by the knockdown of snaR in NSCC cells (A549, SPC-A1). This means that snaR is acting as a non-coding oncogene in NSCLC tumorigenesis, and its knockdown could be helpful to develop an additional treatment agent. However, the other possible mechanisms by which snaR participate in NSCLC cell functions remain to be fully understood.

Acknowledgment

The authors wish to thank the Pasteur institute of Tehran for project support and technical help.

Conflict of Interest

Authors declare no conflict of interest.

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[DOI: 10.22088/IJMCM.BUMS.10.4.258]