Long non-coding RNA CACNA1G-AS1 promotes cell migration, invasion and epithelial-mesenchymal transition by HNRNPA2B1 in non-small cell lung cancer

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Abstract. – OBJECTIVE: In recent years, long non-coding RNAs (lncRNAs) have been identified to participate in tumor progression. The purpose of this study was to investigate the role of CACNA1G-AS1 in non-small cell lung cancer (NSCLC).

PATIENTS AND METHODS: Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to detect the CACNA1G-AS1 expression level in 122 pairs of NSCLC and para-carcinoma normal tissue samples as well as in NSCLC cell lines. Moreover, the relationship of clinical pathological features with CACNA1G-AS1 was analyzed. Functional experiment cell lines were established using lentivirus and siRNA to study the effects of CACNA1G-AS1 on cell invasion and migration abilities. Several epithelial-mesenchymal transition (EMT) markers were measured using Western blotting. The expression level of HNRNPA2B1 was analyzed to further investigate the mechanism.

RESULTS: The expression level of CACNA1G-AS1 in NSCLC tissues was significantly higher than that in para-carcinoma normal tissues, and the expression of CACNA1G-AS1 was higher in NSCLC cell lines than that in normal BEAS-2B cells. The higher CACNA1G-AS1 level was relative to more lymph node metastasis and distant metastasis. Function experiments revealed that CACNA1G-AS1 promoted cell invasion and migration. Also, CACNA1G-AS1 over-expression increased EMT in NSCLC cells. Besides, HNRNPA2B1 was regulated by CACNA1G-AS1 in NSCLC cells.

CONCLUSIONS: CACNA1G-AS1 was identified as an oncogene in NSCLC for the first time, and could promote cell invasion, migration and EMT via increasing HNRNPA2B1 expression, providing a novel target for the biological therapy and prevention.

Key Words: LncRNA, CACNA1G-AS1, EMT, HNRNPA2B1, NSCLC.

Introduction

Lung cancer is the most common tumor in the world, and it is the primary reason for cancer deaths, among which non-small cell lung cancer (NSCLC) accounts for 85%. Despite of the gradual development in current technology, the diagnosis and treatment of NSCLC still lack rapid progression. A series of biomarkers have been found to be useful markers for early diagnosis and prognosis of NSCLC, which may provide new targets for targeted therapy for NSCLC. These biomarkers include non-coding RNAs, microRNAs, long non-coding RNAs (lncRNAs) and cirRNAs. Among them, IncRNA has more than 200 nt in length and cannot be translated to proteins. There have been already many IncRNAs found to take effect in different stages, including the development and metastasis of different tumors. For example, HOTAIR can promote cancer metastasis via reprogramming chromatin, and MALAT1 is associated with chemo resistance and can enhance poor response of colorectal cancer to chemotherapy. Also, IncRNA TSLNC8 suppresses tumor development and progression through inactivating IL-6/STAT3 signaling pathway and IncRNA FILNC1 represses renal energy metabolism and tumorigenesis. Among them, many IncRNAs are closely related to lung cancer. Pan et al proved that IncRNA FAL1 promotes NSCLC development and progression through PTEN/AKT pathway. LncRNA SNHG20 silences P21 expression to enhance lung cancer proliferation. Up-regulation of IncRNA IGFBP4-1 facilitates lung cancer development via changing energy metabolism. In addition, IncRNA 00152 and
NEAT1 can target microRNA (miRNAs) to regulate NSCLC evolution\textsuperscript{16,17}. However, the mechanism of lncRNAs in NSCLC remains unclear.

LncRNA CACNA1G-AS1 is an antisense RNA of CACNA1G, which was first found in skin keloid, and it can promote the progression of keloid and co-expressed with several genes, including CACNA1G-AS1, RABGAP1, TTC18, PDPN and TUBB6\textsuperscript{18,19}. However, no research has mentioned the relationship between CACNA1G-AS1 and tumors. The primary purpose of this study was to investigate the role of CACNA1G-AS1 in NSCLC.

Patients and Methods

Patients

NSCLC Tissue Samples

A total of 122 pairs of human NSCLC and para-carcinoma normal tissues were collected from patients receiving surgical resection from 2010 to 2016 in our hospital. Before surgery, none of patients received chemotherapy or radiotherapy. The tissues were stored in liquid nitrogen immediately after the surgical resection. The clinical pathological features, including age, gender and TNM staging, were collected based on American Joint Committee on Cancer (AJCC) standard. All patients signed the written consent and the investigation was approved by the Ethics Committee of the Second People’s Hospital of Weifang.

NSCLC Cell Lines and Culture

Five NSCLC cell lines (A549, H1975, H1299, H1650 and SPCA1) and normal human bronchial epithelium cell line BEAS-2B, as normal controls, were bought from the Cell Bank of Type Culture Collection, Chinese Academy of Sciences (Shanghai, China). The six cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Corning Corporation, Corning, NY, USA) containing 10% fetal bovine serum (FBS) (Corning Corporation, Corning, NY, USA), 100 U/ml penicillin and 50 μg/mL streptomycin (Gibco, Rockville, MD, USA). After 80-90% cells covered the medium, they were digested and passed to next generation. All six cell lines were incubated at 37°C in humidified air containing 5% CO\textsubscript{2}.

Cell Transfection of Lentivirus and siRNA

For over-expressed CACNA1G-AS1, A549 cells were inoculated into a six-well plate. Lentiviral pcDNA for CACNA1G-AS1 was added into the medium using polybrene after the density was 50%. After knockdown, SPCA1 cells were platted into the six-well plate and maintained in a normal 1640 medium until the density of 60%. Using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), cells were co-cultured with appropriate amount siRNA-CACNA1G-AS1 or negative controls according to instructions. All the sequences were synthesized by GenePhama (Suzhou, China). Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was used to detect the regulation efficiency of CACNA1G-AS1 expression.

RNA Isolation and qRT-PCR

Total RNA of tissues and cell lines was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After CACNA1G-AS1 mRNA level analysis, total RNA was then reversely transcribed into cDNA using a reverse transcription kit (TaKaRa, Otsu, Shiga, Japan). Then, SYBR

Table I. Correlation between CACNA1G-AS1 level and clinicopathological features.

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LUSC: lung squamous carcinoma, LUAD: lung adenocarcinoma. The expression level of CACNA1G-AS1 was cut off by median expression level and *indicated \( p < 0.05 \).
Green Premix Kit was used to perform the qRT-PCR and GAPDH was used as control. The primers of CACNA1G-AS1 are as follows: forward: 5’-TTGTTGGCCCGAGCACTAAT-3’, reverse: 5’- TCACAGGTCAACATAGCC -3’; GAPDH: forward: 5’- CTCACCGGATGCACCAATGTT-3’, reverse: 5’-CGCGTTGCTCACAATGTTCAT-3’. QRT-PCR was performed using ABI 7900 system (ABI, Loma, Linda, CA, USA). All the relative expression RNA levels were calculated using the 2-∆∆CT method.

**Wound-Healing Assay**

Wound-healing assay was employed to study the ability of cell migration. A549 or SPCA1 cells after lentivirus or siRNA treatment were inoculated into the six-well plate until the density of 100%. After washing with phosphate-buffered saline (PBS) for 3 times, the surface of cells was scratched using a 200 μL tip. Then, cells were incubated in serum-free RPMI 1640 for 24 h and the wound-healing condition was measured under a microscope at 0 h and 24 h. Each scratch was measured by five random fields and each experiment was repeated for at least three times.

**Transwell Assay**

8 μm transwell chamber (Corning Corporation, Corning, NY, USA) were applied to further measure the cell invasion and migration ability. In cell invasion assay, a total of 3×10^4 treated cells suspended in RPMI-1640 medium containing 10% FBS were put into the upper chamber, in which matrigel (BD, Franklin Lakes, NJ, USA) was pre-plated. The lower chamber was added with 500 μL FBS-free RPMI 1640 medium. After incubation for 48 h, cells were collected and the membrane containing cells was immersed in pre-cooled methanol and stained with 0.5% crystal violet. The stained cells were calculated after photography using microscope in six random fields. In migration assay, the upper chamber was cleared before the cell plating. Other steps were the same as those in invasion assay. Each experiment was repeated for at least three times.

**Protein Extraction and Western Blotting**

To measure these protein expression levels, cells were lysed using radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) after being washed with pre-cooled PBS. The concentration of collected protein was calculated using bicinchoninic acid (BCA) protein assay kit (Beiyotime, Shanghai, China). The extracted protein was then degenerated and cooled. A total of 20 μg protein were used for 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and separated. Next, the protein was transferred onto a polyvinylidene difluoride (PVDF) membrane bought from Millipore (Billerica, MA, USA). Non-specific protein was blocked using 5% bovine serum albumin (BSA) dissolved in Tris-buffered saline and Tween 20 (TBST) at 4°C for 1 h. The membrane containing the proteins was immersed in 5% BSA at 4°C overnight, and specific primary antibodies of HNRNPA2B1 (CST, Danvers, MA, USA), E-cadherin (Abnova, Taipei, Taiwan), Vimentin (Abnova, Taipei, Taiwan), N-cadherin (Abnova, Taipei, Taiwan) and GAPDH (Abcam, Cambridge, MA, USA) were used. The membrane was washed using TBST for 10 min ×3 times. The membrane was then incubated with horseradish peroxidase (HRP)-labeled secondary antibody for 1 h. The membrane was developed using enhanced chemiluminescence (ECL) (Millipore, Billerica, MA, USA) following the instructions after being washing for three times by Tris-buffered saline and Tween 20 (TBST). Each experiment was repeated for at least three times.

**Statistical Analysis**

Statistic Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) and STATA 12.0 software were used for statistical analysis. The independent-samples t-test and Spearman r² test were used for statistical analyses. These data were presented using GraphPAD prism software (La Jolla, CA, USA) and all quantitative data were presented as mean ± standard deviation. p<0.05 indicated the significant difference.

**Results**

**CACNA1G-AS1 was Upregulated in NSCLC Tissues and Cells**

CACNA1G-AS1 levels in both NSCLC and para-carcinoma normal tissues were measured via qRT-PCR, indicating that the expression of CACNA1G-AS1 is remarkably higher in NSCLC tissues than that in para-carcinoma normal tissues (Figure 1A) and the ectopic CACNA1G-AS1 expression may be involved in NSCLC progression. Next, CACNA1G-AS1 expression levels in five NSCLC cell lines and normal human bronchial epithelium cell line BEAS-2B were investigated via qRT-PCR. Results clearly showed that
CACNA1G-AS1 was expressed highly in A549, H1299, H1975, H1650 and SPCA1 compared with that in BEAS-2B cell (Figure 1B), confirming that CACNA1G-AS1 is an oncogene in NSCLC. To next identify the function of CACNA1G-AS1 in NSCLC progression, A549 cell line was chose for CACNA1G-AS1 overexpression and SPCA1 cell line after CACNA1G-AS1 knockdown. CACNA1G-AS1 expression was effectively increased in A549 cells transfected with LV-CACNA1G-AS1 or LV-NC; conversely, CACNA1G-AS1 expression was decreased in SPCA1 cells transfected with siRNA-CACNA1G-AS1 or siRNA-NC. The transfection efficiency was detected using qRT-PCR (Figure 1C-D).

**CACNA1G-AS1 was Correlated with NSCLC Clinical Pathological Features**

Next, 122 patients were divided into high CACNA1G-AS1 expression group and low CACNA1G-AS1 expression group to evaluate the correlation between several clinical pathological features and CACNA1G-AS1. As shown in Table 1, higher CACNA1G-AS1 expression level was related to more lymph node metastasis, distant metastasis and advanced TNM staging, but not related to age, gender, tumor size and histological type. These results indicate that CACNA1G-AS1 can promote NSCLC progression and indicate poor prognosis.
**Ectopic Expression of CACNA1G-AS1 Affected Cell Invasion and Migration of NSCLC**

Wound-healing assay was performed to detect the cell migration ability. After transfection with LV-CACNA1G-AS1, the wound-healing rate of A549 cells was remarkably higher than that in control group (Figure 2A-B), while SPCA1 cells treated with siRNA-CACNA1G-AS1 showed lower healing ability than that in siNC group (Figure 2C-D). Furthermore, transwell assay was employed to evaluate the cell invasion and migration. There were more invaded and migrated A549 cells after CACNA1G-AS1 over-expression than that in negative control group (Figure 3A-B). However, SPCA1 cells displayed decreased invasion and migration capacities, while CACNA1G-AS1 was downregulated. These results indicate that CACNA1G-AS1 can promote invasion and migration of NSCLC (Figure 3C-D).

**Overexpression of CACNA1G-AS1 Promoted EMT of NSCLC Cells**

As EMT is a frequent mechanism in NSCLC metastasis, several EMT markers in established experimental cell lines were detected. The ep-
Epithelial marker E-cadherin was significantly decreased after CACNA1G-AS1 up-regulation, while mesenchymal markers vimentin and N-cadherin were increased in A549 cells (Figure 4A-B). On the contrary, E-cadherin level was markedly increased but vimentin and N-cadherin levels were decreased in SPCA1 cells (Figure 4C-D). These data suggest that CACNA1G-AS1 can accelerate the EMT of NSCLC cells.

**CACNA1G-AS1 Increased HNRNPA2B1 Expression in NSCLC Cells**

Furthermore, to investigate the underlying mechanism of CACNA1G-AS1 in NSCLC, several
eral databases were searched and HNRNPA2B1 was found to be a potential target gene for CACNA1G-AS1. Next, the HNRNPA2B1 expression in A549 and SPCA1 cells was detected. Up-regulation of CACNA1G-AS1 significantly improved HNRNPA2B1 protein level in A549 cells (Figure 5A-B), while down-regulation of CACNA1G-AS1 reduced HNRNPA2B1 expression in SPCA1 cells. These data indicate that HNRNPA2B1 is a target for CACNA1G-AS1 in NSCLC cells (Figure 5C-D).

**Discussion**

In this work, the relationship between lncRNA CACNA1G-AS1 and NSCLC was demonstrated, and the correlation of CACNA1G-AS1 with clinical pathological features was elucidated. Furthermore, *in vitro* researches showed that CACNA1G-AS1 overexpression promoted cell invasion and migration of NSCLC. In addition, several EMT markers were detected, and it was verified that CACNA1G-AS1 accelerated the progression of EMT. At last, HNRNPA2B1 was found to be a downstream molecular of CACNA1G-AS1. As far as we know, the effect of CACNA1G-AS1 on NSCLC was elucidated for the first time.

LncRNAs have been identified to participate in several processes of NSCLC, especially in tumor metastasis, which leads to a poor prognosis of NSCLC. For example, lncRNA FAL1 promotes NSCLC metastasis via PTEN/AKT pathway, while lncRNA NKILA can inhibit metastasis via NF-kB/Snail pathway. In this study, ectopic CACNA1G-AS1 expression was correlated with more lymph node metastasis, distant metastasis and advanced TNM staging because CACNA1G-AS1...
over-expression facilitates cell invasion and migration.

EMT has been studied for decades and it has been proposed as a key mechanism in cancer progression, especially in tumor metastasis. EMT is tightly regulated by multiple molecules, that coordinate the transformation from epithelial-like phenotypes to mesenchymal phenotypes, and rely on a delicate balance between these two stages. Several studies have shown that EMT in tumors can be mediated by lncRNAs. For example, lncRNA H19 can regulate EMT and MET of breast cancer cells by differentially sponging miR-200b/c and let-7b. In cervical cancer, MALAT1 knockdown inhibits EMT to decrease cell invasion and migration. In NSCLC, linc00673 can sponge miR150 to regulate NSCLC development, lncRNA-LET suppresses cancer cell proliferation via EMT, and MEG3 contributes to the epigenetic regulation of NSCLC cell lines. In this research, it was found that CACNA1G-AS1 promoted EMT and CACNA1G-AS1 was an oncogene in NSCLC. Next, it was found through several databases that HNRNPA2B1 was a potential target of CACNA1G-AS1, and Western blotting showed that HNRNPA2B1 expression could be regulated by CACNA1G-AS1. HNRNPA2B1 was a mediator to control mRNA translation. In pancreatic cancer, HNRNPA2B1 can interact with KRAS and regulate cancer progression, regulating β-catenin protein expression in prostate cancer cells. Moreover, in NSCLC, it can be a biomarker in tumor tissue and blood for diagnosis. Also, HNRNPA2B1 is an EMT regulator in pancreatic cancer via the ERK/snail pathway. We found that CACNA1G-AS1 could promote HNRNPA2B1 to accelerate EMT; then, cell invasion and migration abilities were increased.

All these data in this study partially demonstrate that CACNA1G-AS1 can promote NSCLC

Figure 5. HNRNPA2B1 expression is affected by CACNA1G-AS1. A-B, HNRNPA2B1 is significantly up-regulated in A549 cells transfected with LV-CACNA1G-AS1 compared with LV-NC; C-D, HNRNPA2B1 is significantly down-regulated in SPCA1 cells transfected with siRNA-CACNA1G-AS1 compared with siRNA-NC. Data are presented as mean ± standard deviation in three independent experiments; *p<0.05, **p<0.01.
cell invasion, migration and EMT via HNRNPA2B1, so CACNA1G-AS1 is identified as an oncogene in NSCLC. However, more experiments, especially in vivo assays, are still needed to further study the mechanism of CACNA1G-AS1 in NSCLC.

Conclusions

We demonstrated for the first time that CACNA1G-AS1 was a tumor-promoting factor on NSCLC progression. Also, CACNA1G-AS1 could promote the EMT progression via HNRNPA2B1. These findings suggest that CACNA1G-AS1 may serve as a novel and prospective target for NSCLC therapy and prognosis prediction.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References


