Mechanism of LncRNA FOXC2-AC1 promoting lung cancer metastasis by regulating miR-107

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Abstract. – OBJECTIVE: Previous studies have shown that long non-coding RNA (lncRNA) FOXC2-AC1 is one of cancer-promoting genes. However, the role of FOXC2-AC1 in lung cancer (LCa) has not been reported. This study aimed to investigate the expression characteristics of FOXC2-AC1 in LCa, and to further explore the mechanism by which it accelerates the metastasis of LCa.

PATIENTS AND METHODS: Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the level of FOXC2-AC1 in 62 pairs of LCa tissues and adjacent normal tissues, and the relationship between FOXC2-AC1 and LCa pathological parameters as well as the prognosis of patients were analyzed. Meanwhile, FOXC2-AC1 level was further verified in LCa cells by qRT-PCR. In addition, FOXC2-AC1 knockdown and overexpression models were constructed using lentivirus in LCa cell lines including H1299 and SPCA1, and the effect of FOXC2-AC1 on the biological function of LCa cells was analyzed by cell counting kit-8 (CCK-8) test along with transwell invasion and migration assay. Finally, the potential mechanism was explored using Western blotting assay.

RESULTS: In this study, qRT-PCR results indicated that the expression level of FOXC2-AC1 in LCa was considerably higher than that in normal tissues, with statistically significant differences. Compared with patients with low expression of FOXC2-AC1, patients with high expression of FOXC2-AC1 had higher incidence of distant metastasis and lower overall survival rate. Compared with the control group, the cell proliferation, invasion and metastasis capacities of FOXC2-AC1 overexpressing group were considerably enhanced, while opposite results were observed in the FOXC2-AC1 silencing group. In addition, miR-107 expression was found significantly reduced no matter in LCa cell lines or in tissues and showed a negative correlation with FOXC2-AC1. Subsequently, luciferase reporter gene assay demonstrated that overexpression of miR-107 significantly attenuated the luciferase activity of the wild-type FOXC2-AC1 vector without reducing the activity of the mutant vector or empty vector, further proving that FOXC2-AC1 could be targeted by miR-107 through this binding site. In addition, rescue experiment also found that FOXC2-AC1 and miR-107 have mutual regulation, which jointly affected the malignant progression of LCa.

CONCLUSIONS: These studies indicate that LncRNA FOXC2-AC1 is notably upregulated in LCa and is significantly correlated with LCa distant metastasis as well as poor prognosis. Therefore, it is suggested that lncRNA FOXC2-AC1 may promote malignant progression of LCa through the mutual regulation of miR-107.

Key Words: LncRNA FOXC2-AC1, miR-107, Lung cancer, Metastasis.

Introduction

Globally, lung cancer (LCa) is the leading malignant tumor in terms of morbidity and mortality. 1.6 million patients are diagnosed with lung cancer each year, and more than 1.4 million patients died of this cancer, among which 80% are non-small cell lung cancer (NSCLC). Currently, the conventional treatment methods for NSCLC include surgery, chemotherapy, radiotherapy, and targeted therapy, etc. However, these therapies cannot achieve the purpose of curing patients with advanced tumors; therefore, most patients still have a short lifespan after treatment with their 5-year survival rate less than 15%. Since patients with NSCLC have no specific clinical manifestations in the early stage, most of them have metastasis and local invasion at the time of diagnosis. Therefore, to explore the pathogenesis of NSCLC and early intervention in tumor cell
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Patients and Methods

Patients

Tumor and paracancerous tissue specimens were collected from 62 patients with lung cancer. All specimens were obtained from tissue specimens undergoing surgery and biopsy or bronchoscopic biopsy in oncology, thoracic surgery and respiratory medicine. In addition, the adjacent tissues of all specimens were more than 5 cm away from cancerous tissues, and no preoperative anti-tumor therapy such as radiotherapy or chemotherapy was received. The study was approved by the Ethics Committee of our hospital and all patients signed informed consent. All patients were followed up after discharge, with telephone follow-up and outpatient follow-up, including general conditions, clinical symptoms and imaging examination.

Cell Lines and Reagents

Five human LCa cells (A549, H1299, PC-9, H358, SPC-A1) and one normal human bronchial epithelial cell (BEAS-2B) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco, (Rockville, MD, USA). Cells were cultured in DMEM supplemented with 10% FBS and maintained at 37°C in a humidified incubator containing 5% CO₂.

Transfection

Control group (NC or anti-NC) and lentivirus (FOXC2-AC1 or anti-FOXC2-AC1) with overexpression and knockdown sequences were purchased from Shanghai Gemma Company (Shanghai, China). The cells were seeded in a 6-well plate and cultured until the cell density reached 40%. Next, they were infected with the lentivirus for 4 h after which the medium was changed. Cells were incubated for further 48 h to allow for expression and then lysed for quantitative Real-time polymerase chain reaction (qRT-PCR) analysis and cell function experiments. Controls included infection with the lentivirus generated from the empty green fluorescent protein (GFP) backbone vector.

Cell Proliferation Assay

Cells transfected for 48 h were collected and seeded into a 96-well plate with 2,000 cells per well. After the cells were cultured for 6 h, 24 h, 48 h and 72 h, the reagents of cell counting kit-8 (CCK-
8) (Dojindo Laboratories, Kumamoto, Japan) were added, and then the cell incubation continued for another 2 hours. Lastly, the optical density (OD) value of each well at 490 nm was measured in the enzyme marker and data were analyzed.

**Transwell Assay**

After transfection for 48 h, the cells were lysed by trypsin and then resuspended with serum-free medium. The density of the diluted cells was adjusted to 2.0x10^5/mL after cell counting. Transwell with or without matrix glue was placed in a 24-well plate. 200 μL of cell suspension were added in the upper chamber and 500 μL of culture medium containing 10% FBS were added in the lower chamber. The culture was maintained at 37°C. After 48 h, the chamber was taken out, and 4% paraformaldehyde was used to fix the cells for 30 minutes. After crystal violet staining for 15 minutes, the inner surface of the basement membrane of the chamber was carefully cleaned to remove the inner cells. The transmembrane cells stained in the outer layer of the basement membrane were observed under the microscope, and 5 field counts were selected randomly.

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

Total RNA was extracted from tissue samples by TRIzol (Invitrogen, Carlsbad, CA, USA). According to the instructions of AMV reverse transcription kit (Promega, Madison, WI, USA), 2 μg total RNA were added to the 20 μL system for complementary Deoxyribose Nucleic Acid (cDNA) synthesis. 2xSYBR Green PCR Master Mix was adopted for qRT-PCR, with appropriate cDNA taken as template. 3 parallel samples were set for each sample to be tested. The PCR reaction was performed on a quantitative PCR apparatus. The following primers were used: LncRNA FOXC2-AC1: forward: 5’-CCGAAACTT-TCCGCCAAGATG-3’, reverse: 5’-CCTCACT-TCCCATGGCTGAG-3’; Cally-actin: forward: 5’-CCTGGCACCCAGCACAAT-3’, reverse: 5’-TGCCGTAGGTCCCTTTG-3’; Mir-107: forward: 5’-AGCAGCAUUGUACAGGGCUAU-3’; U6: forward: 5’-CGCAAGGATGACACAAATTC-3’. ABI Step One software was used for data analysis, and 2^(-ΔΔCt) method was applied to calculate relative mRNA expression level.

**Dual Luciferase Reporter Assay**

A reporter gene plasmid was constructed to insert a specific segment of the target promoter into the ahead of the luciferase expression sequence. The transcription factor expression plasmid to be tested was co-transfected into H1299 and spc-a1 cells or other related cell lines with the reporter gene plasmid. If this transcription factor can activate the target promoter, then the luciferase gene should be expressed, and the expression of luciferase is proportional to the efficiency of transcription factor. A specific luciferase substrate was added, and the luciferase reacted with the substrate to produce fluorescence. The activity of luciferase was measured by detecting the intensity of fluorescence, so as to determine whether the transcription factor can have an effect on the target promoter fragment.

**Statistical Analysis**

Statistical Product and Service Solutions (SPSS) 19.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for data analysis, and the expression differences of LncRNA FOXC2-AC1 in cancer tissues and normal tissues beside cancer were analyzed using variance analysis. Comparison between groups was done using One-way ANOVA test followed by post-hoc test LSD (Least Significant Difference). The expression of LncRNA FOXC2-AC1 in cancer tissues and paracancerous tissues and its relationship with various clinical and pathological parameters were analyzed using x^2-test. Kaplan-Meier method was used to analyze the relationship between LncRNA FOXC2-AC1 expression and survival time as well as prognosis, and Cox proportional risk model was used to analyze the factors influencing the prognosis of non-small cell lung cancer. Data were expressed as mean±SD, and p<0.05 was considered statistically significant.

**Results**

**FOXC2-AC1 was Highly Expressed in LCa Tissues and Cell Lines**

We detected the FOXC2 - AS1 expression in 62 pairs of LCa tissues and adjacent normal tissues as well as in LCa cell lines. The results showed that compared with adjacent normal tissues, the expression of FOXC2 - AS1 in LCa tissues decreased significantly, with statistically significant differences (Figure 1A, 1B). As for the cell lines FOXC2 - AS1 had a higher expression in LCa cell lines than in BEAS - 2 B cells (Figure 1C). Among LCa cell lines, FOXC2 - AS1 has a low level in H1299 while has a high expression in SPCA1 cel-
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Therefore, we selected these two cell lines for subsequent transfection experiments.

**FOXC2-AC1 Expression was Correlated with Distance Metastasis and Overall Survival in LCa Patients**

We divided lncRNA FOXC2-AC1 expression into high expression and low expression groups based on qRT-PCR results of 62 pairs of lncRNA FOXC2-AC1 expression in LCa tissues and adjacent tissues. $x^2$-test was used to analyze the relationship between FOXC2-AC1 expression and age, sex, clinical stage and distant metastasis of LCa patients. As shown in Table I, the expression of FOXC2-AC1 was not associated with age and gender of LCa patients, but with distant metastasis; miR-107 expression was also associated with distant metastasis. In addition, to investigate the relationship between the expression of A FOXC2-AC1 and the prognosis of patients with LCa, we collected relevant follow-up data. Kaplan-Meier survival curves showed that high expression of FOXC2-AC1 was significantly associated with poor prognosis of LCa ($p<0.05$; Figure 1D). This result suggests that FOXC2-AC1 may be a new biological indicator for predicting the prognosis of LCa.

**FOXC2-AC1 Promotes Cell Proliferation, Invasion and Migration**

To explore the effects of lncRNA FOXC2-AC1 on LCa cell proliferation and migration and invasion, we first successfully constructed the FOXC2-AC1 overexpression and knockdown model (Figure 2A). The results showed that the cell proliferation, invasion and metastasis abilities of FOXC2-AC1 overexpression group FOXC2-AC1 were considerably increased compared with control group, while in FOXC2-AC1 silencing group, opposite results occurred (Figure 2B and 2C).

**MiR-107 is a Direct Target of FOXC2-AC1**

To further validate the targeting of miR-107 to FOXC2-AC1, we cloned the FOXC2-AC1 sequence into the luciferase reporter plasmid--pmirGLO, and also constructed the mutant vector--pmir-
Luciferase activity of the wild-type FOXC2-AC1 vector \((p<0.05)\) without attenuating the luciferase activity containing the mutant vector \((p>0.05)\) or the empty vector \((p>0.05)\), further demonstrating that FOXC2-AC1 can be targeted by miR-107 through this binding site (Figure 3A). In addition, GLO-FOXC2-AC1-mut, and then pmirGLO-FOXC2-AS1-WT, pmirGLO-FOXC2-AC1-mut or pmirGLO and miR-107 were co-transfected into H1299 and SPCA1 cells for luciferase reporter gene experiments. The results showed that overexpression of miR-107 significantly attenuated the luciferase activity of the wild-type FOXC2-AC1 vector \((p<0.05)\) without attenuating the luciferase activity containing the mutant vector \((p>0.05)\) or the empty vector \((p>0.05)\), further demonstrating that FOXC2-AC1 can be targeted by miR-107 through this binding site (Figure 3A). In addition,
Figure 3. FOXC2-AC1 directly targets mir-170. A, The direct targeting effect of FOXC2-AC1 and mir-170 was showed by the dual-luciferase reporter gene experiment. The results of the dual-luciferase reporter gene experiment in H1299 and SPCA1 cell lines showed that overexpression of miR-170 significantly decreased the luciferase activity of the wild FOXC2-AC1 vector (\( p < 0.001 \)) without decreasing the luciferase activity of the mutant vector (\( p > 0.05 \)) or the empty vector (\( p > 0.05 \)). B, Differences in the expression of mir-170 in lung cancer tumor tissues and non-cancer adjacent tissues were detected by qRT-PCR. C, The expression level of mir-170 in lung cancer cell lines was detected by qRT-PCR. D, There was a significant negative correlation between FOXC2-AC1 and miR-170 expression in lung cancer. E, QRT-PCR verified the interference efficiency of miR-170 after transfection of FOXC2-AC1 overexpression vector in H1299 cell lines and after knockout of FOXC2-AC1 in SPCA1 cell lines. All data were mean ±SD, *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \).
qRT-PCR assay revealed that miR-107 was significantly reduced in tumor tissues compared with that in tumor-free tissues, and the difference was statistically significant (Figure 3B). And as for the cell lines, miR-107 was also significantly lower in LCa cells than in BEAS-2B, the normal human bronchial epithelial cells (Figure 3C). Therefore, we selected 16 pairs of samples in LCa tissue and detected the expression of FOXC2-AC1 and miR-107 by qRT-PCR, and the results revealed a negative correlation between mRNA expression levels of FOXC2-AC1 and mir-107 (Figure 3D); again, the above results were also demonstrated in the cell lines (Figure 3E).

**Mir-107 Modulated FOXC2-AC1 Expression in Human Lung Cells**

To further explore the pathways through which FOXC2-AC1 inhibits the malignant progression of lung cancer, a possible relationship between mir-107 and FOXC2-AC1 was found through relevant bioinformatics analysis and luciferase reporter genes. In addition, in order to further explore the interaction between FOXC2-AC1 and miR-107 in LCa cells, miR-107 was overexpressed in LCa cells overexpressing FOXC2-AC1 and silenced in the cells silencing FOXC2-AC1, and the transfection efficiency of miR-107 was tested using qRT-PCR (Figure 4A). Finally, it was found that mir-107 could counteract the effect of FOXC2-AC1 on proliferation, invasion and metastasis of LCa cells (Figure 4B and 4C).

**Discussion**

LCa is one of the common malignant tumors in the world. In recent years, the incidence and mortality of LCa in China have gradually increased, and the early diagnosis rate of LCa patients in China is still extremely low. Most of the patients have developed into the middle and late stages, so tumors in the advanced stage account for the majority1-3. Early researches3 in the diagnosis, metastasis, recurrence of LCa and adjuvant therapy after advanced LCa have become the focus of current research. Recent studies have demonstrated that IncRNA and miRNA play important roles in a variety of diseases including tumors. There are multiple abnormally expressed IncRNAs and miRNAs in LCa, which may play a crucial role in the diagnosis, treatment and prognosis of LCa21,22.
Therefore, finding the abnormal expression of lncRNA and miRNA in LCa and analyzing its correlation with clinical prognosis will contribute to improve the diagnosis and treatment of LCa, and improve the clinical prognosis of patients. Non-coding long-chain RNA was once considered to be a non-functional transcriptional gene. With the advancement of various experimental techniques, especially the development of sequencing technology, researchers have found more and more non-coding RNAs with a length exceeding 200bp play an important role in transcriptional regulation, which make them become a hot topic.6,7

At present, there are many studies on the function and mechanism of miRNA in tumors, but the research on the role of LncRNA in tumors is still in its infancy.8 It has been found that some long-chain non-coding RNAs can exert an important influence on the regulation and development of tumors. LncRNA is abnormally expressed in various malignant tumors such as liver cancer, prostate cancer and breast cancer, and plays a vital role in the progression of malignant tumors. Some lncRNAs can be even used as markers of prognosis.9 Therefore, exploring the abnormal expression of lncRNA in non-small cell lung cancer and analyzing its function will help to enhance the level of diagnosis and treatment so as to improve the prognosis.

In this study, the level of lncRNA FOXC2-AC1 and miR-107 was firstly verified in 62 pairs of LCa tissues and paracancerous ones. The results indicated that lncRNA FOXC2-AC1 was significantly upregulated, while miR-107 was down-regulated, which was positively correlated with distant metastasis and poor prognosis. Therefore, we believe that FOXC2-AC1 plays a role in promoting cancer in LCa, while miR-107 plays the opposite role. To further explore the effects of FOXC2-AC1 and miR-107 on the biological function of LCa, we constructed a FOXC2-AC1 overexpression/knockdown model using lentivirus. CCK8, invasion and migration experiments revealed that FOXC2-AC1 indeed promoted LCa development, but its specific molecular mechanism remains elusive. In recent years, with the deepening of research on LncRNA, more and more modes of action of LncRNA have been gradually discovered, including: 1. Interfering with the expression of certain protein by transcripting in the upstream of the protein; 2. Affecting gene expression by inhibiting RNA polymerase II (RNAP II, RNAP II/PoI II), mediating chromatin remodeling or mediating histone modification; 3. Interfering with mRNA cleavage by binding to mRNA to make the shearing process of mRNA more diversified; 4. Binding to mRNA to generate siRNA under the action of Dicer enzyme, thereby regulating gene expression; 5. Binding to protein, regulating the activity of the protein; 6. Specifically forming a nucleic acid protein complex with proteins to play a role together; 7. Binding with the corresponding protein to change the action site of the protein; 8. Binding with miRNA, adsorbing miRNA and reducing the effective concentration of miRNA.10-12

Previous studies on the mechanism of miRNA expression have focused on transcriptional regulation and histone modification, but there is little recognition of post-transcriptional regulation of miRNA, that is, the clearance process of miRNA mature bodies 16-18. In recent years, a series of studies revealed a new mechanism by which the body against the post-transcription regulation of miRNA, that is the “sponges adsorption” effect of LncRNA, which means that some target genes with high affinity for lncRNA, especially some long chain non-coding RNA, can reduce the effective concentration of microRNAs so as to protect some other important physiological function from being inhibited by miRNA.23,24 This mechanism has been demonstrated in the work of tumorigenesis, muscle development, embryonic stem cell differentiation and inflammation that LncRNA is of key significance for the rapid change of miRNA abundance.25,26 MiRNA-107 is a key molecule in the lncRNA family. The results of this experiment showed that miRNA-107 had a lower expression in lung cancer tissues than in normal lung tissues and can inhibit invasion of lung cancer cells. In this study, we used bioinformatics methods to analyze the FOXC2-AC1 sequence containing a miRNA-107 binding site, and verified the direct binding of FOXC2-AC1 to downstream miRNA-107 by the dual luciferase reporter gene, and deletion of the miRNA-107 binding site in the FOXC2-AC1 vector failed to enrich miRNA-107, further validating the binding site of FOXC2-AC1 to miRNA-107. The above findings suggested that a positive feedback loop regulatory loop may exist: the “sponge adsorption” mechanism of LncRNA FOXC2-AC1 leads to a rapid decrease in miR-107 concentration, promoting the malignant progression of lung cancer.

Conclusions

We found that lncRNA FOXC2-AC1 expression was significantly elevated in LCa tissues and
cell lines and closely correlated with LCa staging, distant metastasis and poor prognosis. Therefore, we arrived a conclusion that lncRNA FOXC2-AC1 may have the ability to promote the malignant progression of lung cancer through sponge adsorption of miR-107.

Conflict of Interest
The Authors declare that they have no conflict of interest.

References

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