High expression of IncRNA GACAT3 inhibits invasion and metastasis of non-small cell lung cancer to enhance the effect of radiotherapy

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Abstract. – OBJECTIVE: To clarify the role of long non-coding RNA (IncRNA) GACAT3 in invasion and metastasis of non-small cell lung cancer (NSCLC) and its effect on radiotherapy.

PATIENTS AND METHODS: The expression of GACAT3 and TIMP2 in cells and tissues of NSCLC were detected by quantitative Real-time PCR (qRT-PCR). The influence of GACAT3 on cell proliferation and the capacity of colony formation were estimated by MTT test and colony forming experiment respectively. Luciferase reporting assay was used to confirm the correlation between GACAT3 and TIMP2. In addition, we observed the influence of GACAT3 on radiosensitivity of NSCLC cells.

RESULTS: Using IncRNA array analysis, we found that GACAT3 expression increased significantly. Further studies showed that overexpression of ectopic GACAT3 in A549 cells promoted cell proliferation and migration, and enhanced the sensitivity of lung cancer cells to radiotherapy. TIMP2, confirmed a direct target of GACAT3 by bioinformatics analysis and our experiments, may be involved in the GACAT3-induced upregulation of MMP10.

CONCLUSIONS: LncRNA GACAT3 may be a potential biomarker for the evaluation of curative effect and prognosis of lung cancer.

Key Words: LncRNA, GACAT3, NSCLC, Radiosensitivity, TIMP2.

Introduction

As the second most common malignant tumor, lung cancer has become the main cause of cancer-related deaths. In the United States, 226,000 new cases of lung carcinoma were found in 201212. Among all kinds of carcinoma of the lungs, NSCLC, accounting for about 85%, is the largest subset1. NSCLC is often not found until it is advanced, leading to its high mortality3,4. At present, chest radiotherapy is the main means for patients with advanced NSCLC. Nevertheless, the effectiveness of radiotherapy is still limited because of the acquired radioresistance in the process of therapy6. At the same time, the prognosis of NSCLC is not satisfied due to tumor metastasis or recurrence7. Thus, looking for new therapeutic methods to reduce radioresistance and improve the radiotherapy efficacy is imperative for ameliorating the prognosis of patients with NSCLC.

LncRNA contains more than 200 nucleotides in length and highly conserved without encoding proteins8. They play a key role in many processes of cell activities such as apoptosis, proliferation, and progression of tumor9. Recent evidence suggests that lncRNAs are often out of control in many kinds of tumors. Meanwhile, they participate in the development of cancer10,11. For example, IncRNA X inactivation-specific transcripts (XISTs) are upregulated and have been identified as oncogenes by epigenetically inhibiting the expression of KLF2 in NSCLC12. By regulating the miR-337-3p-E2F3 pathway, up-regulation of IncRNA-rich transcription factor 1 (NEAT1) enhances NSCLC cells growth, invasion and metastasis13. Additionally, upregulated tumor suppressor factor 7 (TUSC7) inhibits NSCLC cell proliferation14. LncRNA gastric cancer-associated transcript 3 (GACAT3) coexists with many known oncogenes in different solid tumors, and have oncogenic effects in lots of malignant tumors like carcinoma of lungs15, stomach16, and pancreas17. Researches18,19 have recently shown that GACAT3 level is closely related to clinical pa-
Prameters of many cancers, such as recurrence and survival rate. However, the effect of GACAT3 on NSCLC and its potential mechanism of regulating the radiosensitivity of non-small cell lung carcinoma still remain to be studied.

Patients and Materials

**Patient Characteristics and Sample Collection**
In this study, 62 pairs of NSCLC tissues (40 adenocarcinomas and 22 squamous cell carcinomas) and adjacent normal tissues were obtained from the Affiliated Hospital of Xuzhou Medical University. All the tissues were pathologically confirmed as NSCLC, and all the patients had never received preoperative chemotherapy or radiation. After surgical resection, tissues were immediately frozen in liquid nitrogen and stored at -80°C until RNA and protein analysis. To observe the changes of lncRNA expression before and after radiotherapy, 10 non-NSCLC patients confirmed by biopsy were included in this study. All patients signed an informed consent form. The study was conducted with the approval of the Affiliated Hospital of Xuzhou Medical University Ethics Review Board.

**Detection of lncRNA GACAT3 Expression in NSCLC Tissue**
Total RNA of tumor tissue was isolated using the IncrVana™ lncRNA Isolation Kit. The RNA concentration and quality were determined, and reverse transcription and qPCR were then performed. U6 was used as a standardized internal control and, then, the fold change in gene expression was calculated using the 2^(-ΔΔCt) method.

**Cell Culture and Selection of Stable Cell**
Human lung cancer cell lines (A549, H157, HCC827, H838) were obtained from ATCC. All cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS) with 100 U/mL penicillin and 100 μg/mL streptomycin and, then, placed in a humidified incubator containing 5% CO2 at 37°C. A549 cells were infected with a lentiviral vector expressing pre-lncRNA GACAT3 (pLV-lncRNA GACAT3). Plasmid construction and lentivirus packaging are constructed by GeneChem Company (Shanghai, China). Three days after infection, a large number of stable green clones were sorted by BD FACS Aria III cell sorter and collected for further experiments.

**Quantitative Real-time PCR**
Total RNA was extracted from the cultured lung cancer cells using TRIzol reagent and transcribed reversely into cDNA. QRT-PCR was performed on a StepOne Plus RT-PCR System with SYBR Green Mix according to the manufacturer’s instructions.

**Western Blotting**
Cultured lung cancer cells were washed twice with phosphate-buffered saline (PBS), lysed on ice and centrifuged at 4°C for 20 min. Western blot was performed according to the routine protocol (site a paper in which this protocol was described in detail). Primary antibodies were: β-actin (AC-15; 1:2000; Sigma-Aldrich, St. Louis, MO, USA), Parp (9542; 1:1000; Cell Signaling Technology, Danvers, MA, USA), TIMP2 (5738; 1:500; Cell Signaling Technology, Danvers, MA, USA), MMP10 (Ab89638; Abcam, Cambridge, MA, USA), MMP14 (ab51074; 1:500; Abcam, Cambridge, MA, USA), MMP2 (ab92536; 1:500; Abcam, Cambridge, MA, USA).

**Construction and Transfection**
Predicted lncRNA GACAT3 luciferase reporter gene carrying the wild-type TIMP2 3’-UTR (TIMP2 3’ UTR-wt) or mutant TIMP2 3’-UTR (TIMP2 3’ UTR-mut) was synthesized and cloned into the pMiR plasmid by Sinogenomax (Beijing, China). A DNA fragment covering the coding region of TIMP2 using the Myc tag was also synthesized and cloned into pcDNA3 of Hind III and XbaI sites. All constructs were sequenced at Sangon Company (Shanghai, China). Antago-lncRNA GACAT3 and antagomir negative controls were synthesized by RiboBio (Guangzhou, China).

**MTT Assay**
Cells in logarithmic growth phase were transferred to 96-well plates with 3,000 cells, including control well of medium alone. Four hours before the end of the culture, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT 5 mg/mL) was added to each well, and the reaction was stopped by the addition of 150 μL of dimethyl sulfoxide (DMSO). The number of viable cell count was measured at a wavelength of 570 nm using a Model 680 Microplate Reader.

**Colony Formation Test**
500 cells/well were seeded into 6-well plates and irradiated with 0.2 Gy on the next day. After two weeks, the cells were fixed with 90% methanol and...
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stained with 1% crystal violet in 70% ethanol. Experiments were performed three times and colonies with diameters greater than 100 μM were counted.

**Wound Healing Assay**

A549/LV-GACAT3 and A549/LV-control cells (1 × 10^6/well) were seeded in 6-well plates in RPMI 1640 containing 10% fetal bovine serum (FBS). Then, a wound was made by dragging a pipette tip along the center of the plate after 24 h of culture. At last, we measured the distance between the cells along the wound after 6 h, 12 h or 24 h. Images were taken with a digital camera under a phase-contrast microscope.

**Transwell Assays**

Invasiveness and metastatic ability of the tumor cells were assessed using transwell chambers with 8-μm pore filters. A549/LV-lncRNA GACAT3 and A549/LV-control cells (3 × 10^4) in 100 μL of serum-free RPMI-1640 were seeded in a cell insert, and 600 μL medium containing 10% fetal bovine serum (FBS) was added to the lower chamber. After incubation at 37°C for 40 h, non-migrating cells on the upper surface of the filter were removed. Cells invading through the filter were fixed, stained, viewed and counted in three randomly chosen fields.

**Dual-luciferase Reporting Assay**

A549 cells were seeded in 24-well plates and transfected with the above plasmid (300 ng) along with the control pRL-SV40 (2 ng). Antago-lncRNA GACAT3 and antagomir-control were synthesized by RiboBio (Guangzhou, China). After 24 h of transfection, luciferase activity was detected by luciferase reporter assay system.

**Statistical Analysis**

Statistical analysis was conducted by statistical product and service solutions (SPSS 16.0, Inc., Chicago, IL, USA) software. Counting data were analyzed using χ²-test and Fisher’s test, while measurement data were assessed by bilateral t-test. p<0.05 indicated a statistically significant difference. Pearson correlation analysis was used to assess the correlation between the expression level of IncRNA GACAT3 and TIMP2 in tissue samples from lung carcinoma.

**Results**

**Relationship Between GACAT3 Expression in Tumor Tissues and Clinicopathologic Features of NSCLC**

QRT-PCR results showed that GACAT3 level in NSCLC patients was significantly higher than that in non-NSCLC patients (p<0.05) (Figure 1); we divided 62 patients into two groups based on the median of GACAT3 levels in NSCLC patients. The clinical characteristics of the patients were analyzed. As shown in Table I, high expression of GACAT3 is significantly correlated with the situation of lymph node metastasis and TNM staging (p<0.05), but not with gender, age or smoking history.

**GACAT3 Enhances NSCLC Cells Sensitivity to Radiotherapy**

To determine the change of GACAT3 level during NSCLC cell-therapy, we induced GACAT3 expression in lung cancer cell lines including A549, H157, HCC827 and H838 by lentivirus infection and found that A549 expressed a relatively lower level of GACAT3. Therefore, A549 was used for further experiments (Figure 2A). The results of qRT-PCR confirmed that A549/LV-lncRNA GACAT3 stably expressed GACAT3 (Figure 2B) and overexpression of GACAT3 could promote in vitro cell growth (Figure 2C). The result of colony formation assay indicated that GACAT3 overexpression enhanced the sensitivity of lung cancer cells to radiotherapy (2 Gy) (Figure 2D). Western blot analysis showed that upregulated GACAT3 expression significantly decreased PARP expression in A549/LV-GACAT3 cells (Figure 2E). GACAT3 accelerated the growth of tumor cells in the absence of radiotherapy, but the volume of A549/LV-GACAT3-derived cells was significantly reduced after radiotherapy, suggesting that overexpression of GACAT3 en-
enhanced NSCLC cells sensitivity to radiotherapy (Figure 2F). The above results demonstrated that GACAT3 improved the radiotherapy efficacy and promoted the apoptosis of NSCLC cells.

**GACAT3 Directly Targets TIMP2 to Regulate Invasiveness of NSCLC Cells**

Transwell assays and wound healing test were performed to clarify the influence of GACAT3 on NSCLC cell invasiveness. The former result showed that A549/LV-GACAT3 cells migrated more strongly than A549/LV-control cells (Figure 3A). Transwell migration and Matrigel invasion assays also showed the capacity of invasion and metastasis of A549/LV-GACAT3 cells were significantly enhanced compared to that of A549/LV-control cells (Figures 3B-C). The above results showed that GACAT3 could promote in vitro invasion and metastasis of NSCLC cells.

To further understand the molecular mechanism of GACAT3 in promoting tumor cell invasion, we predicted the target of GACAT3 through TargetScan and RNA-seq analysis. The results suggested that TIMP2 might be a potential target of GACAT3.

Table 1. Relationship between GACAT3 expression and clinicopathologic features in patients with NSCLC.

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<th>Clinical features</th>
<th>Group</th>
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<th>Low (n=32)</th>
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<td>≥ 60</td>
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<td>Smoking history</td>
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<td>Yes</td>
<td>24</td>
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*, p<0.05 vs. control group; †, p<0.05 vs. Lutein group; ‡, p<0.05 vs. DOX group.

To determine whether GACAT3 regulates TIMP2 expression, we examined mRNA and protein expres-

Figure 2. LncRNA GACAT3 can enhance the sensitivity of NSCLC cells to radiotherapy. **A**, GACAT3 mRNA in different lung cancer cell lines was detected by qRT-PCR. **B**, LncRNA GACAT3 expression was detected by qRT-PCR. **C**, Growth curve of A549/LV-control and A549/LV-GACAT3 cell was detected by MTT assay. **D**, The sensitivity of A549/LV-control and A549/LV-GACAT3 cells to radiotherapy (2, 4 Gy) was evaluated by colony formation assay. **E**, PARP expression level in A549/LV-control and A549/LV-GACAT3 cells was detected by Western blot. **F**, The effect of radiotherapy on NSCLC cells is shown.
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Figures 3. GACAT3 directly targets TIMP2 to regulate invasiveness of NSCLC cells. A. Cell migration was evaluated by wound-healing test. B and C, Results of in vitro migration and invasion assay of A549/LV-control and A549/LV-GACAT3 cells transfected with pcDNA3 or pcDNA3-TIMP2 are shown. B, The expression of TIMP2 mRNA was detected by qRT-PCR. E, The expression of TIMP2 protein was detected by Western blot. F, The duplex formation between human TIMP2 3'UTR and lncRNA GACAT3 was predicted.

Discussion

Generally, radiotherapy is regarded as one of the most effective methods for the treatment of in-
operable advanced cancer, such as advanced NSCLC\textsuperscript{20}. Nevertheless, radiation resistance induced by intrinsic mechanisms and extracellular factors such as intercellular communication are major barriers that limit the efficacy of radiotherapy for NSCLC. Also, distant metastasis and recurrence of lung cancer are major drawbacks for successful treatments\textsuperscript{21}. Hence, understanding the molecular mechanisms of NSCLC radioresistance may help to improve the therapeutic effect and prognosis of NSCLC patients.

Increasing number of evidence show that lncRNAs play an important role in the occurrence, development, clinical outcome, and therapeutic efficacy of NSCLC. Recently, it has been reported that lncRNA GACAT3 is upregulated in metastatic hepatoblastoma\textsuperscript{22}, pancreatic cancer\textsuperscript{23}, prostate tumors\textsuperscript{24}, and oxaliplatin-resistant colon cancer cells\textsuperscript{25}. Several target genes have been experimentally validated, including MZF-1, CD147, PTEN, SOX7\textsuperscript{24-27}. However, the function of lncRNA GACAT3 in NSCLC is unclear. In this study, we found that upregulation of GACAT3 can enhance NSCLC cell proliferation, in vivo xenograft tumor growth and in vitro cell migration and invasion. In all 62 NSCLC tissue specimens, GACAT3 level was found to be related to TNM staging and metastasis of lymph node, but not with age, gender, and smoking history.

It has been reported\textsuperscript{28} that GACAT3 may originate from the coding sequence of keratin19 (KRT19) gene, and activation of p53 may significantly upregulate GACAT3 expression. Previous studies\textsuperscript{28,30} have shown that KRT19 mRNA is expressed minimally in normal lymph nodes, but higher in metastatic lymph nodes. Our study showed that overexpression of GACAT3 may influence the radiosensitivity of NSCLC cells and GACAT3 overexpression in A549 cells promoted \textit{in vitro} and \textit{in vivo} cell growth, indicating that highly proliferating cells may be more sensitive to radiotherapy. We also found that GACAT3 is a potential biomarker for predicting the prognosis of NSCLC, which may provide some more effective and comprehensive therapy for NSCLC patients. However, the mechanism of how GACAT3 is involved in drug resistance or radiosensitivity of NSCLC remains unclear.

TIMP2 is a natural inhibitor of matrix metalloproteinases (MMPs). MMPs can degrade extracellular matrix (ECM) and basement membrane (BM), which is the premise of tumor metastasis\textsuperscript{31}. TIMP2 is a physiologic inhibitor of MMP2\textsuperscript{32}, MMP14\textsuperscript{33} and MMP10\textsuperscript{34}, and plays a vital role in tumorigenesis as well as cell invasion. Its role in NSCLC has been illustrated in previous studies. Vazquez-Ortiz et al\textsuperscript{35} found that MMP10 is associated with NSCLC and consistently highly expressed in NSCLC tissues. Our data indicated that the role of TIMP2 and MMP10 in NSCLC cell invasion is consistent with previous findings. TIMP-2 also controls several cell functions, such as proliferation and apoptosis\textsuperscript{36-38}. In addition, immunohistochemical results showed that tumor xenografts from A549/LV-GACAT3 cells have low TIMP2 expression compared to control tumors from A549/LV-control cells. All of these findings suggested that TIMP2 at least partially mediates the function of GACAT3 in NSCLC cell proliferation. In addition to TIMP2, there may be other factors that participate in the regulation of NSCLC cell proliferation and migration. When we overexpressed TIMP2 in lung cancer cells alone, the effect of GACAT3 on cell migration was sig-
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significant but not completely restored. However, as to whether there are other target genes of GACAT3, in addition to TIMP2, which have antiradio therapeutic effect on NSCLC, deserve further investigation.

Conclusions

We initially studied the role of GACAT3 in NSCLC cells and the regulatory mechanism of radiosensitivity. Our results showed that overexpression of GACAT3 can promote the proliferation and migration, induce apoptosis and enhance radiosensitivity of NSCLC cells by targeting TIMP2, thus, providing a new therapeutic target for improving NSCLC radiotherapy efficiency.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

21) KANG J, KIM E, KIM W, SEONG KM, YOON H, KIM JW, KIM J, YOON B. Rhamnetin and cirsiliol induce radiosensitization and inhibition of epithelial-mesenchymal transition (EMT) by miR-34a-mediated


29) HAFAANE M, GAUDIN C, HEITEL H, GRENKE C, HELLEMANN P, SCHNEIDER A, DUENST M. HPV16-E6 mRNA is superior to cytokeratin 19 mRNA as a molecular marker for the detection of disseminated tumour cells in sentinel lymph nodes of patients with cervical cancer by quantitative reverse-transcription PCR. Int J Cancer 2007; 107 (Pt 9): 2373-2379.


