Long Noncoding RNA GAS5 Suppresses Tumorigenesis by Inhibiting miR-23a Expression in Non-Small Cell Lung Cancer

Yongcheng Mei,* Jinchun Si,† Yun Wang,‡ Zhuangshi Huang,§ Haiwen Zhu,* Shijun Feng,* Xuezhi Wu,* and Liwen Wu*

*Department of Cardiothoracic and Vascular Surgery, Zhumadian Central Hospital, Zhumadian, P.R. China
†Department of Surgery, Shangqiu Medical College, Shangqiu, P.R. China
‡Department of Respiratory, Zhumadian Central Hospital, Zhumadian, P.R. China
§Department of Thoracic Surgery, The Second Hospital Affiliated to Zhengzhou University, Zhengzhou, P.R. China

Previous studies reported that elevated expression of long noncoding RNA (lncRNA) GAS5 led to the arrest of non-small cell lung cancer (NSCLC) cell growth and a promotion of apoptosis both in vitro and in vivo. However, its underlying molecular mechanism in NSCLC is still unclear. In the present study, we noted that GAS5 was downregulated in NSCLC tissues and cells and was negatively correlated with miR-23a expression. Luciferase reporter assay and qRT-PCR analysis demonstrated that GAS5 directly interacted with miR-23a and reversely regulated its expression. miR-23a overexpression markedly promoted NSCLC cell proliferation and invasion, while GAS5 overexpression dramatically inhibited NSCLC cell proliferation and invasion and promoted apoptosis. Functional analysis indicated that miR-23a overexpression significantly abolished GAS5 overexpression-induced inhibition of proliferation and invasion, as well as promotion of apoptosis in NSCLC cells. Moreover, xenograft experiments further revealed that upregulation of GAS5 notably impaired the growth of transplanted tumors by suppressing miR-23a in nude mice. These results suggested that overexpression of lncRNA GAS5 inhibits tumorigenesis of NSCLC by inhibiting miR-23a in vitro and in vivo, providing a potential therapeutic strategy for patients with NSCLC.

Key words: Long noncoding RNA (lncRNA); GAS5; Proliferation; Invasion; Apoptosis; miR-23a; Non-small cell lung cancer (NSCLC)

INTRODUCTION

Lung cancer is currently one of the most common causes of cancer-related deaths and is expected to account for 17% of total diagnosed cancer cases1. Non-small cell lung cancer (NSCLC) comprises approximately 85% of newly found lung cancer cases and is generally diagnosed at an advanced stage because it is asymptomatic in early phases2,3. Despite tremendous advances in diagnosis and molecular-targeted therapies in the past few years, the prognosis of lung cancer is unfavorable, with an overall 5-year survival rate of only 16%.ª The main obstacles to improving the cure rate in patients with NSCLC are recurrence and early metastasis6. Thus, it is meaningful to reveal the mechanisms underlying NSCLC pathogenesis and progression in order to improve diagnosis and develop novel therapeutics for this disease.

High-throughput transcriptome analysis has discovered that transcribed human genome coding for protein accounts for only 2% of the whole genome, whereas the majority of it can be transcribed into noncoding RNAs (ncRNAs) with limited or no protein-coding capacity, including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs)7. lncRNAs are evolutionarily conserved and more than 200 nucleotides (nt) in length8. lncRNAs play crucial oncogenic and tumor-suppressive roles in various biologic processes, including cell growth, apoptosis, cell cycle progresses, and cancer metastasis9. A growing amount of evidence has demonstrated that an abnormal expression of lncRNA is involved in NSCLC tumorigenesis and development, and it has emerged as a biomarker for the diagnosis and prognosis of NSCLC10,11. Growth arrest-specific transcript 5 (GAS5) was initially identified from subtractive cDNA cloning of genes, which are preferentially expressed during growth arrest12. GAS5 is a well-known lncRNA encoded at prostate cancer-associated locus 1q25 and approximately 630 nt in length13. It is stated...
that GAS5 functions as a tumor suppressor by promoting growth arrest and apoptosis in many cell types. It is well documented that GAS5 was frequently downregulated in diverse cancers, including NSCLC, which was associated with severe clinicopathological characteristics of NSCLC. More importantly, previous studies reported that an elevated expression of GAS5 led to NSCLC cell growth arrest and a promotion of apoptosis both in vitro and in vivo. However, the underlying mechanism of GAS5 in NSCLC remains to be investigated.

miRNAs represent a type of highly conserved endogenous ncRNAs with a length of 19–22 nt that regulate gene expression posttranscriptionally by triggering either translation suppression or mRNA degradation. Numerous studies have demonstrated that miRNAs may act as oncogenes or tumor suppressors and are involved in carcinogenesis, metastasis, and invasion of lung cancer. Accordingly, miRNAs are increasingly regarded as promising tumor biomarkers or therapeutic targets for cancer progression. miR-23a is an miRNA cluster located in chromosome 19p13.12 in vertebrates and serves as an oncogene in many tumors, including NSCLC. A previous study indicated that miR-23a promoted migration and invasion by targeting IRS-1 in NSCLC and may be a potential therapeutic target in NSCLC. Recently, emerging evidence has proposed that a new regulatory circuitry between lncRNA and miRNA allows lncRNA to function as an “miRNA sponge” to negatively regulate the expression and function of miRNAs. More notably, GAS5 was reported to act as a molecular sponge to regulate miR-23a in gastric cancer. However, whether there is a correlation between GAS5 and miR-23a in NSCLC is uncertain.

In this study, we intended to analyze the interaction between GAS5 and miR-23a and the related molecular mechanism in NSCLC. The expression of GAS5 and miR-23a in NSCLC patients and cell lines, as well as their interaction, was investigated. In addition, the effect of miR-23a overexpression alone or in combination with GAS5 overexpression on the proliferation, invasion, and apoptosis of NSCLC cells was further explored. Finally, the effect of GAS5 on NSCLC cell growth in vivo was determined.

MATERIALS AND METHODS

Patients and Tissue Samples

Thirty-nine pairs of surgically resected NSCLC specimens and adjacent normal lung tissue were collected from primary NSCLC patients who underwent surgery at the Department of Cardiothoracic Surgery, Zhumadian Central Hospital, Henan Province, P.R. China, between January 2014 and December 2015. All NSCLC patients were diagnosed based on histopathological evaluation and had not received any preoperative therapy prior to surgical removal. All harvested tissue specimens were instantly frozen in liquid nitrogen for storage. This study was approved by the ethics committee of Zhumadian Central Hospital, and informed consent was obtained from each participant.

Cell Lines and Cultures

NSCLC cell lines A549, H838, H157, and HCC827 and a normal lung bronchial epithelial cell line, 16HBE, were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, P.R. China). All cells were routinely maintained in RPMI-1640 basic medium (Gibco, Grand Island, NY, USA) containing heat-inactivated 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), as well as 1% penicillin/streptomycin (Invitrogen) at 37°C in a 5% CO₂ incubator.

Cell Transfection

miR-23a mimics (miR-23a), control mimics (miR-control), siRNA against GAS5 (si-GAS5), siRNA control (si-control), pcDNA-GAS5, and pcDNA empty control (pcDNA-control) were purchased from GenePharma (Shanghai, P.R. China). Cultured A549 and H157 cells were transfected with miR-23a, si-GAS5, pcDNA-GAS5, pcDNA-GAS5+miR-23a, or their corresponding control at approximately 60%–70% confluence using Lipofectamine 2000 (Invitrogen). Transfection efficiency was checked by quantitative real-time polymerase chain reaction (qRT-PCR). Subsequent experiments were performed 48 h after transfection.

RNA Isolation and qRT-PCR

Total RNAs of tissues and cells were extracted by TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The quantity and purity of extracted RNA were detected according to the ratio of UV absorbance at 260 and 280 nm (A260/A280 ≥ 1.8) by a NanoDrop Spectrophotometer (Thermo Scientific, Hudson, NH, USA) and gel electrophoresis. Reverse transcription into cDNA was carried out from 2 μg of total RNA using a reverse transcription kit (Takara, Dalian, P.R. China). The expressions of GAS5 and miR-23a were determined by qRT-PCR using SYBR Premix Ex Taq II (Takara) and TaqMan MicroRNA Assay System (Applied Biosystems, Foster City, CA, USA) on an ABI 7500 instrument (Applied Biosystems, respectively). Relative expressions of GAS5 and miR-23a were calculated using the 2⁻ΔΔCt method and normalized to the internal control GAPDH. The specific primers used in this study were as follows: GAS5, 5'-CTTGCCTGGACCCAGTTAAT-3' (forward) and 5'-CAAGGCAACTCTCCATACCT-3' (reverse); miR-23a, 5'-CGCGATCACATTGCCAGGG-3' (forward) and 5'-GTTAGGGTCCCGAGGTGT3' (reverse); GAPDH, 5'-GTCAACGGATTTGGTCTGATT-3' (forward) and 5'-AGTCTCGGTACCACTGGAGG-3' (reverse).
In brief, A549 and H157 cells were cultured in six-well plates for 48 h after transfection. Then the cells were trypsinized, washed, and resuspended in PBS, followed by double staining with annexin V-FITC (50 μg/ml) and propidium iodide (10 μg/ml) in the dark for 15 min at room temperature before they were subjected to flow cytometry (FACScan; BD Biosciences, San Diego, CA, USA).

**Tumor Formation Assay in a Nude Mouse Model**

Male BALB/c athymic nude mice (aged 4–5 weeks) were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. Experimental protocols of nude mice were approved by the Administrative Panel on Laboratory Animal Care of the Zhumadian Central Hospital. A549 cells (2×10⁶/ml) transfected with pcDNA-GAS5 or pcDNA-control were subcutaneously injected into either side of the posterior flank of nude mice. When the tumors were visible, tumor volume was measured every 3 days in the mice using a Vernier caliper and was calculated using the following equation: 

\[
\text{Volume} = \frac{1}{2} \times \text{length} \times \text{width}^2 \times 0.5
\]

Twenty-one days after the first measurement, mice were sacrificed, and the xenografts were resected and weighed. The expression of miR-23a in xenografts was further determined by qRT-PCR.

**Statistical Analysis**

Statistical analysis was carried out by SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The experimental data were presented as mean ± standard deviation (SD). Differences between experimental groups were determined with Student’s t-test or one-way ANOVA. The results were considered statistically significant with a value of \(p<0.05\).

**RESULTS**

**GAS5 Was Downregulated and miR-23a Was Upregulated in NSCLC Tissues**

To discover the importance of GAS5 and miR-23a in NSCLC, qRT-PCR was performed to detect GAS5 and miR-23a expression in 39 pairs of NSCLC tissues and adjacent normal lung tissues. The results indicated that GAS5 expression was significantly lower (Fig. 1A), and the miR-23a level was dramatically higher (Fig. 1B) in NSCLC tissues than those observed in adjacent normal lung tissues. Furthermore, we noticed that there was a significant negative correlation between GAS5 expression and miR-23a level in NSCLC tissues (Fig. 1C). These results suggested that GAS5 and miR-23a may be associated with NSCLC progression.

**GAS5 Was Downregulated and miR-23a Was Upregulated in NSCLC Cells**

Based on the above observations, we explored the expression levels of GAS5 and miR-23a in NSCLC cells by qRT-PCR. Similarly, the results showed that the...
expression of GAS5 was markedly decreased (Fig. 2A), and miR-23a was conspicuously elevated (Fig. 2B) in NSCLC cell lines (A549, H838, H157, and HCC827) with respect to normal lung bronchial epithelial cells (16HBE), especially in A549 and H157 cells. Therefore, ectopic expression of GAS5 mediated by the pcDNA vector was carried out in A549 and H157 cells to further explore the biological function of GAS5 in NSCLC progression. qRT-PCR analysis revealed that GAS5 expression was notably increased in pcDNA-GAS5-transfected A549 (Fig. 2C) and H157 (Fig. 2D) cells. These data verified the inverse expression between GAS5 and miR-23a in NSCLC cells and the effectiveness of pcDNA-GAS5 transfection in A549 and H157 cells.

GAS5 Negatively Regulated miR-23a Expression

It has been frequently reported that many IncRNAs function as microRNA sponges to negatively regulate specific microRNAs. Thus, the relationship between GAS5 and miR-23a in NSCLC cells was further confirmed. We used bioinformatics online software programs (Starbase v2.0 and microRNA.org-target program) to predict the potential microRNAs that interact with GAS5. As expected, we found that miR-23a contained a complementary binding site of GAS5 (Fig. 3A). To confirm this prediction, we constructed luciferase reporter plasmids containing the WT or MUT putative miR-23a binding sites of GAS5 to cotransfect with miR-control or miR-23a. Dual luciferase assay revealed that the luciferase activity of pGL3-WT-GAS5 was dramatically decreased in miR-23a-transfected A549 (Fig. 3B) and H157 (Fig. 3C) cells, whereas miR-23a overexpression had no obvious effect on the luciferase activity of pGL3-MUT-GAS5 compared with the miR-control group. To further explore the interaction between GAS5 and miR-23a, A549 and H157 were transfected with si-GAS5, pcDNA-GAS5, or matched controls. qRT-PCR results exhibited that knockdown of GAS5 strikingly increased the expression of miR-23a, while GAS5 upregulation significantly decreased miR-23a expression in A549 (Fig. 3D) and H157 (Fig. 3E) cells compared with the corresponding controls. Taken together, these results implied that GAS5 functions as a sponge to negatively regulate the expression of miR-23a in NSCLC cells.

GAS5 Overexpression Suppressed NSCLC Cell Proliferation via Inhibiting miR-23a

An MTT assay was used to verify the effect of miR-23a overexpression on the proliferation of NSCLC cells, and the results show that miR-23a overexpression triggered a significant increase in cell viability at 48 and 72 h in both A549 and H157 cells (Fig. 4A). In consideration of the regulatory effect of GAS5 on miR-23a expression in NSCLC, we supposed that GAS5 had a similar effect on miR-23a
GAS5 overexpression suppressed NSCLC cell proliferation in A549 and H157 cells, which was abolished by miR-23a upregulation (Fig. 4B). These findings uncovered the fact that enforced expression of GAS5 impaired NSCLC cell growth by inhibiting miR-23a.

**GAS5 Overexpression Repressed Cell Invasion by Inhibiting miR-23a**

A Transwell invasion assay was performed to further investigate the effect of miR-23a overexpression on NSCLC cell invasion. Compared to miR-control-transfected cells, the number of invasive cells was significantly increased in A549 and H157 cells due to miR-23a transfection (Fig. 5A). Furthermore, we found that transfection of pcDNA-GAS5 markedly reduced cell invasion capacity, whereas miR-23a overexpression strikingly recuperated this inhibitory effect (Fig. 5B). These data indicated that GAS5 overexpression suppressed NSCLC cell invasion by regulating miR-23a.

**GAS5 Overexpression Promoted Apoptosis by Inhibiting miR-23a in NSCLC Cells**

We next investigated the effect of the interaction of GAS5 and miR-23a on the apoptosis of NSCLC cells. As demonstrated by flow cytometry, pcDNA-GAS5 transfection led to a significant increase in apoptotic rates, and miR-23a overexpression conspicuously attenuated GAS5 overexpression-induced apoptosis in A549 (Fig. 6A) and H157 (Fig. 6B) cells, suggesting that GAS5 overexpression promoted apoptosis by mediating miR-23a in NSCLC cells.

**GAS5 Overexpression Inhibited NSCLC Cell Growth In Vivo by Suppressing miR-23a Expression**

To verify the mechanism of GAS5 in NSCLC tumorigenesis in vivo, A549 cells transfected with pcDNA-GAS5 or pcDNA-control were subcutaneously injected into nude mice, and the tumor volume was measured.
every 3 days up to 21 days. An obvious growth inhibition of pcDNA-GAS5-derived tumor was observed in comparison to the pcDNA-control group (Fig. 7A). The mice were sacrificed 21 days after the first measurement, and the tumor weight was significantly lower due to GAS5 overexpression. Furthermore, the expression of miR-23a was detected in resected xenografts, and we noticed that GAS5 overexpression markedly inhibited the expression of miR-23a compared with the control group.

These findings demonstrated that GAS5 overexpression impeded NSCLC cell growth in vivo by reversely regulating miR-23a.

**DISCUSSION**

In recent years, an increasing number of lncRNAs have attracted attention, as they have been demonstrated to play a crucial role in tumorigenesis\(^2\). Accumulating evidence has indicated that IncRNAs are associated with the
occurrence and progression of NSCLC and contribute to various biological functions in human cancers, including NSCLC. In our study, we focused on lncRNA GAS5. To date, many studies have reported the biological role of GAS5 in multiple cancers and its underlying molecular mechanism. For example, Li et al. found that GAS5 was markedly decreased in ovarian cancer and promoted cell proliferation, migration, and invasion partly by regulating cyclin D1, p21, and apoptosis protease-activating factor 1 (APAF1) expression, suggesting that GAS5 may indicate a poor prognosis in ovarian cancer. Guo et al. reported that GAS5 enhanced PTEN expression to act as a tumor suppressor through inhibiting miR-103 expression in endometrial cancer. Additionally, Chang et al. showed that a low expression of GAS5 indicated a poor prognosis for hepatocellular carcinoma. Overexpression of GAS5 suppressed proliferation and invasion and promoted apoptosis by negatively regulating vimentin in hepatoma cells. Specifically, GAS5 has been shown to be downregulated and play a tumor-suppressive role in NSCLC. However, its related mechanism in NSCLC progression remains to be elucidated.

miR-23a has been demonstrated to function as an oncogene in diverse tumors. For example, miR-23a was reported to be upregulated and promote tumorigenic activity by facilitating cell cycle progress and EMT and inhibiting apoptosis through promoting IKKα expression but suppressing ST7L expression in epithelial ovarian cancer cells. In addition, miR-23a has a significantly higher expression, promotes cell proliferation and colony formation, and inhibits early apoptosis by directly targeting APAF1 in laryngeal cancer cells. A previous study has shown that miR-23a is a poor prognostic factor and played an oncogenic role in NSCLC. Consistent with the earlier study, our data further confirmed that miR-23a
overexpression significantly promoted NSCLC cell proliferation and invasion. Furthermore, functional analysis of overexpression implicated that GAS5 overexpression remarkably inhibited cell proliferation and invasion and induced apoptosis of NSCLC cells, whereas miR-23a overexpression markedly abolished these effects, suggesting that GAS5 served as a tumor suppressor by inhibiting miR-23a in NSCLC cells. In vivo xenograft experiments revealed that GAS5 overexpression dramatically suppressed tumor growth of the mice injected with...
Figure 6. Effect of miR-23a and GAS5 overexpression on apoptosis of NSCLC cells. Flow cytometry was carried out to evaluate apoptosis rate in A549 (A) and H157 (B) cells transfected with single pcDNA-GAS5 or simultaneous pcDNA-GAS5 and miR-23a. *p < 0.05 versus control group.
Moreover, the expression of miR-23a was also reduced by GAS5 overexpression in vivo, indicating that GAS5 overexpression inhibited tumor growth by inhibiting miR-23a in NSCLC.

In conclusion, our study demonstrated, for the first time, that GAS5 is negatively correlated with miR-23a expression and exerts its tumor-suppressive roles by inhibiting miR-23a in NSCLC, providing new insights into the molecular mechanism by which GAS5 functions in NSCLC.

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