LncRNA TGFB2-AS1 regulates lung adenocarcinoma progression via act as a sponge for miR-340-5p to target EDNRB expression

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Abstract: Long non-coding RNA TGFB2-antisense RNA1 (TGFB2-AS1) has been reported could regulate tumorigenesis. However, the roles of TGFB2-AS1 in lung adenocarcinoma (LUAD) remain largely unknown. In this work, we aimed to explore the expression levels of TGFB2-AS1 and mechanisms in regulating LUAD progression. Expression level of TGFB2-AS1 in LUAD tissues and normal tissues was analyzed at StarBase. Moreover, its expression in LUAD cells and normal cell was analyzed with quantitative real-time polymerase chain reaction method. Gain- and loss-of-function studies were conducted to analyze the biological roles of TGFB2-AS1 in LUAD. Results indicated TGFB2-AS1 was evidently downregulated in LUAD tissues and cells. Moreover, as analyzed by cell counting kit-8 assay, wound-healing and transwell invasion assays, results revealed TGFB2-AS1 overexpression could suppress proliferation, migration and invasion abilities of LUAD cells in vitro and tumor growth in vivo. In addition, LncBase V2.0 and TargetScan prediction tools showed TGFB2-AS1 and endothelin receptor type B (EDNRB) shares binding site in microRNA-340-5p (miR-340-5p). Furthermore, luciferase activity reporter assay and RT-qPCR assay validated these prediction results. Furthermore, we showed TGFB2-AS1 functions as sponge for miR-340-5p to regulate EDNRB expression. Collectively, our results indicated TGFB2-AS1/miR-340-5p/EDNRB axis plays crucial roles in regulating LUAD progression, indicating TGFB2-AS1 may be a novel therapeutic target for LUAD.

Keywords: TGFB2-AS1, miR-340-5p, EDNRB, lung adenocarcinoma

Introduction

The cases of newly diagnosed and cancer-related deaths of lung cancer each year ranked No. 1 among all cancer types in China [1]. Non-small lung cancer (NSCLC) represents 85-90% of lung cancer cases, while lung adenocarcinoma (LUAD) is main subtype of NSCLC [1, 2]. Although recently progresses in therapeutic methods for LUAD, its overall survival remains undesirable.

Long non-coding RNA (lncRNA) is a class of RNAs with limited protein coding capability [3]. Emerging evidence indicated thousands of lncRNAs were aberrantly expressed in tumors [4-6]. Some of these lncRNAs have been functional characterized and revealed to have close associations with tumor malignance behaviors, however, most of lncRNAs remained to be explored.

In recent years, multiplies studies have been performed to demonstrate the functions of lncRNAs in LUAD. LncRNA Titin-antisense RNA1 (TTN-AS1) was found highly expressed in LUAD, and correlated with late tumor stages, poorer lymph node metastasis, and poorer postoperative prognosis [7]. In addition, TTN-AS1 overexpression was revealed could promote LUAD epithelial mesenchymal transition via silencing microRNA-142-5p (miR-142-5p) and hence to promote cyclin-dependent kinase 5 expression [7]. Moreover, IncRNA MAFG antisense 1 (MAFG-AS1) was also revealed expressed at a high level in LUAD tissues, and correlated with poorer prognosis of cancer patients [8]. Functional assays showed MAFG-AS1 overexpression could promote LUAD cell proliferation and inhibit cell apoptosis via regulating miR-744-5p/MAF bZIP transcription factor G axis [8]. IncRNA TGFB2-antisense RNA1 (TGFB2-AS1) was revealed decreased expression in hepa-
cellular carcinoma, and correlated with advanced tumor stage [9]. Moreover, it was found hepatocellular carcinoma cell metastasis can be suppressed by TGFB2-AS1 [9].

In this study, we found TGFB2-AS1 was decreased expression in LUAD tissues and cell lines. Gain- and loss-of-function experiments indicated TGFB2-AS1 could inhibit LUAD tumor growth by sponging miR-340-5p to regulate endothelin receptor type B (EDNRB) expression. Our results indicated TGFB2-AS1 functions as ceRNA in LUAD tumorigenesis and may be developed as putative therapeutic targets for LUAD.

Materials and methods

StarBase analysis

StarBase is a web server contains gene expression data obtained from 32 types of cancers which are derived from 10,882 RNA-seq and 10,546 miRNA-seq data. Expression levels of TGFB2-AS1, miR-340-5p, and EDNRB in LUAD tissues and normal tissues were analyzed at StarBase. Moreover, correlation of TGFB2-AS1 and EDNRB in LUAD tissues was analyzed at StarBase.

UCALAN analysis

Protein level of EDNRB in LUAD tissues and normal tissues was analyzed at UCALAN (http://ualcan.path.uab.edu/cgi-bin/CPTAC-Result.pl?genenam=EDNRB&ctype=LUAD).

Cell culture

LUAD cells (A549 and PC9) and human lung epithelial cell (BEAS-2B) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco’s Modified Eagle’s Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplement with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) was used to incubate these cells at a 37°C moist incubator contains 5% CO₂.

Cell transfection

To overexpress TGFB2-AS1 or EDNRB, their sequences were inserted into pcDNA3.1 to produce pTGFB2-AS1 or pEDNRB (GenScript, Nanjing, Jiangsu, China). To knockdown TGFB2-AS1, small interfering RNA (siRNA) against TGFB2-AS1 was built (RibiBio, Guangzhou, Guangdong, China) and labeled as si-TGFB2-AS1. To knockdown miR-340-5p, miR-340-5p inhibitor was purchased from Ribobio. Moreover, negative control siRNA (si-NC) or miRNA (mi-NC) were also purchased from Ribobio. For transfection, cells were seeded in 6-well plate, incubated for 24 h, and then transfected with siRNAs, miRNAs, or expression plasmids using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific).

Quantitative real-time polymerase chain reaction (qRT-PCR) assay

Total RNA was isolated using Trizol reagent (Beyotime, Haimen, Jiangsu, China) in accordance with manufacturer’s instructions. RNA was used to synthesize cDNA using Reverse Transcription System Kit (Takara, Dalian, Liaoning, China). qRT-PCR was performed using BeyoFast™ SYBR Green qPCR Mix (Beyotime) at ABI 7900 (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control to normalize expression levels of TGFB2-AS1 and EDNRB, while U6 small nuclear RNA (U6 snRNA) was used as reference for miR-340-5p. Relative gene expression levels were calculated with 2-ΔΔCt method. PCR primers were as below: TGFB2-AS1: forward, 5'-AGGGGATGTTGAATGAGG-3', reverse, 5'-GGGTGGATGAGTACATTCAAC-3'; EDNRB: forward, 5'-GGTTGCCCTGCTTCTTGGT-3', reverse, 5'-GTGGCACTGTCTTTGTG-3'; GAPDH: forward, 5'-AGGATTGTGTCGTTGGGCG-3', reverse, 5'-GGCGCGTTAAAGCAATGGAGG-3'; miR-340-5p: forward, 5'-CCCGCTTATAAATGAGG-3', reverse, 5'-GGGTGGATGAGTACATTCAAC-3'; U6 snRNA: forward, 5'-GTGGCCTTCGAGGAGGACAA-3', reverse, 5'-AACGCTTCAGAATTTCATGGC-3'. Experiments were repeated in triplicates.

Western blot

Protein sample was isolated with commercial kit purchased from Beyotime. Protein sample was separated using SDS-PAGE after concentration quantification, and then transferred to PVDF membrane. Membranes were first blocked with fat-free milk, and incubated with antibodies (anti-PCNA: ab92552; anti-E-Cadherin: ab1416; anti-N-Cadherin: ab202030; anti-Vimentin: ab92547; anti-GAPDH: ab1816-02; Abcam, Cambridge, MA, USA) at 4°C for overnight. Then, goat anti-rabbit secondary antibody (ab6721; Abcam) at 37°C for 4 h.
BeyoECL kit was used to visualize the band signals.

**Cell counting kit-8 (CCK-8) assay**

CCK-8 assay was performed to measure cell proliferation rate. Cells were seeded at the density of $3 \times 10^3$/well in 96-well plate and incubated for another 0, 24, 48 and 72 h. Optical density at 450 nm was measured after the adding of CCK-8 solution into each well and incubated for additional 4 h. Experiments were repeated in triplicates.

**Cell migration assay**

Scratch wound-healing assay was performed to analyze cell migratory ability. In brief, $2 \times 10^5$ cells were seeded at 6-well plates and then create a wound at cell surface using pipette tip. PBS was used to wash cells to remove debris. Initial distance (0 h) and distance after 48 h of scratching were captured under microscope at magnification of 200 x. Experiments were repeated in triplicates.

**Cell invasion assay**

Transwell invasion assay was used to assess cell invasive ability. $6 \times 10^3$ cells in serum-free DMEM were plated in the upper chamber of Matrigel coated chamber (BD Biosciences, Franklin Lakes, New Jersey, USA). Meanwhile, DMEM contains 10% FBS was filled into lower chamber. After incubation for 48 h, non-invaded cells were removed, while invaded cells were fixed with methanol and stained with crystal violet. Finally, numbers of invaded cells were counted under microscope. Experiments were repeated in triplicates.

**Luciferase reporter assay**

IncBase V2.0 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=incbase-sev2/index-predicted) was applied to explore miRNA target of TGFB2-AS1 and found miR-340-5p was a putative target. TargetScan (http://www.targetscan.org/vert_72/) was used to predict targets of miR-340-5p and we found EDNRB was a putative target. Sequences of TGFB2-AS1 and EDNRB containing the wild-type (wt) or mutant (mt) miR-340-5p binding sites were inserted into pGL3-Basic luciferase vector (Promega, Madison, WI, USA) to generate wt/mt-TGFB2-AS1/EDNRB. Then, luciferase activity reporter vectors were transfected into cells along with synthetic miRNAs using Lipofectamine 2000. Relative luciferase activity was measured using dual-luciferase reporter system (Promega) after 48 h of transfection. Experiments were repeated in triplicates.

**Animal experiments**

Animal experiment protocol was approved by ethic committee of the Fourth Affiliated Hospital of Guangxi Medical University. Nude mice used in this work was purchased from Shanghai Experimental Animal Center (Shanghai, China). LUAD cells with TGFB2-AS1 stable overexpression were injected into flank of nude mice. Tumor volume was calculated every 7 days by measuring tumor width and length. At 28 days after injection, nude mice were sacrificed to excise tumor tissues and weighted. Then, tissues were incubated with anti-Ki67 (ab16667). Signal was visualized using 3'-diaminobenzidine chromogen (DAB), and counterstained with hematoxylin.

**Statistical analysis**

Data collected from three independent experiments were analyzed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA) and then expressed as mean ± standard deviation. Student’s t-test and one-way ANOVA and Tukey post-hoc test were applied to analyze differences in groups. P < 0.05 was considered to indicate statistically significant.

**Results**

**TGFB2-AS1 was downregulated in LUAD tissues and cells**

StarBase analysis showed TGFB2-AS1 expression was decreased in LUAD tissues compared with normal tissues (Figure 1A). Moreover, qRT-PCR results showed TGFB2-AS1 expression level was also reduced in LUAD cells compared with normal cell line (Figure 1B).

**TGFB2-AS1 overexpression inhibits LUAD cell proliferation, migration, and invasion**

To investigate the effects of TGFB2-AS1 on LUAD cell behaviors, gain-of-function experi-
Successful transfection of pTGFB2-AS1 into LUAD cell was verified by qRT-PCR (Figure 2A). CCK-8 assay showed cell proliferation was significantly inhibited by pTGFB2-AS1 (Figure 2B). Wound-healing assay showed TGFB2-AS1 overexpression inhibits cell migration (Figure 2C). Moreover, force TGFB2-AS1 expression significantly suppressed cell invasion as indicated in the transwell invasion assay (Figure 2D). Western blot showed PCNA, N-Cadherin, and
Vimentin expression was suppressed, while E-Cadherin expression was enhanced by pTGFB2-AS1 (Figure 2E).

**TGFB2-AS1 knockdown promotes LUAD cell proliferation, migration, and invasion**

Moreover, loss-of-function experiments were conducted to further explore the biological roles of TGFB2-AS1 in LUAD. qRT-PCR showed si-TGFB2-AS1 transfection significantly decreased TGFB2-AS1 expression in LUAD cell (Figure 3A). Knockdown of TGFB2-AS1 promoted cell proliferation rate in LUAD cell (Figure 3B). Additionally, wound-healing assay and transwell invasion assay revealed si-TGFB2-AS1 transfection significantly promoted LUAD cell migration and invasion (Figure 3C and 3D). Western blot showed PCNA, N-Cadherin, and Vimentin expression was enhanced, while E-Cadherin expression was suppressed by pTGFB2-AS1 (Figure 3E).

**TGFB2-AS1 and EDNRB shared binding site for miR-340-5p**

Bioinformatic analyses showed TGFB2-AS1 and EDNRB shared the same binding sequence for miR-340-5p (Figure 4A and 4B). Moreover, luciferase activity reporter assay revealed miR-340-5p inhibitor transfection elevated relative luciferase activity of cells transfected with wt-TGFB2-AS1 or wt-EDNRB (Figure 4C and 4D). However, no obvious effects of miR-340-5p inhibitor on relative luciferase activity of cells transfected with mt-TGFB2-AS1 or mt-EDNRB was observed (Figure 4C and 4D). Moreover, we showed miR-340-5p was upregulated in tumor tissues and cells (Figure 4E and 4F). However, EDNRB was downregulated in LUAD tissues and tumor cells compared with normal tissues and normal cells (Figure 4G-I). qRT-PCR showed introduction of si-TGFB2-AS1 increased miR-340-5p expression but decreased EDNRB expression in LUAD cells (Figure 4I and 4J). Importantly, we found TGFB2-AS1 and EDNRB expression was positively correlated in LUAD patients (Figure 4K).

**TGFB2-AS1 regulates LUAD cell behaviors via regulating miR-340-5p/EDNRB axis**

Furthermore, we analyzed whether TGFB2-AS1 exerts its function in LUAD through regulating
miR-340-5p/EDNRB axis. We found pEDNRB or miR-340-5p inhibitor transfection increased EDNRB expression, while decreased cell proliferation, cell migration, and cell invasion abilities (Figure 5A-D). Importantly, we showed knockdown of miR-340-5p or overexpress of EDNRB partially reversed the effects of si-TGFB2-AS1 on EDNRB expression, cell prolifer-
TGFB2-AS1/miR-340-5p/EDNRB axis in LUAD

Figure 5. TGFB2-AS1 affects the LUAD cell behaviors via miR-340-5p/EDNRB axis. (A) EDNRB expression, (B) Cell proliferation, (C) Cell migration, and (D) Cell invasion in LUAD cell with siR-NC+pcDNA3.1, si-TGFB2-AS1+pcDNA3.1, si-TGFB2-AS1+miR-340-5p inhibitor, siR-NC+pEDNRB, siR-NC+miR-340-5p inhibitor, or si-TGFB2-AS1+pEDNRB transfection. ***P < 0.001; **P < 0.01; *P < 0.05. TGFB2-AS1: TGFB2-antisense RNA1; LUAD: lung adenocarcinoma; si-TGFB2-AS1: small interfering RNA against TGFB2-AS1; siR-NC: negative control small interfering RNA; miR-340-5p: microRNA-340-5p; EDNRB: endothelin receptor type B.

Figure 6. TGFB2-AS1 overexpression inhibits LUAD tumor growth in vivo. (A) Tumor volume was decreased, (B) Tumor weight was decreased, and (C) ki-67 expression was decreased in nude mice with stable TGFB2-AS1 overexpression. ***P < 0.001; **P < 0.01; *P < 0.05. TGFB2-AS1: TGFB2-antisense RNA1; LUAD: lung adenocarcinoma.

Discussion
In this work, we investigated the biological roles of TGFB2-
AS1 in LUAD and associated mechanisms. Our work provided evidence that TGFB2-AS1 was reduced expression in both LUAD tissues and cells. In vitro, we performed gain-of and loss-of-function experiments to investigate the biological roles of TGFB2-AS1 in LUAD. We showed overexpression of TGFB2-AS1 suppresses, while knockdown of TGFB2-AS1 promotes LUAD cell proliferation, migration, and invasion. Moreover, the expression levels of proliferation marker, PCNA, and Epithelial-Mesenchymal Transition markers, E-Cadherin, N-Cadherin, and Vimentin, were also altered by changes in TGFB2-AS1 expression. In vivo, we showed nude mice with TGFB2-AS1 overexpression has smaller tumor volume, lower tumor weight, and lower ki-67 expression than those with pcDNA3.1. Investigation on the related mechanistic identified TGFB2-AS1 serves as a sponge for miR-340-5p to regulate EDNRB expression. Our work indicated that TGFB2-AS1 inhibits LUAD progression by regulating miR-340-5p/EDNRB axis.

Competitive RNA (ceRNA) theory to explain the acting mechanism of non-coding RNA was proposed by Salmena and co-authors at 2011 [10]. After that, there were more and more studies showed lncRNA mainly exert its regulatory role on target gene through ceRNA theory [11, 12]. A previous study to investigate the roles of TGFB2-AS1 in cancer failed to demonstrate whether the roles of TGFB2-AS1 were exerted through ceRNA mechanism [9]. In this case, we are interested to whether TGFB2-AS1 regulates the LUAD tumorigenesis using ceRNA theory. Here, we proposed a ceRNA regulatory triplets participated by TGFB2-AS1, miR-340-5p, and EDNRB in LUAD. Through bioinformatic prediction tools analyses, luciferase activity reporter assay, and rescue experiments, we showed effects of TGFB2-AS1 on LUAD progression were dependent on miR-340-5p/EDNRB axis. miR-340-5p was reported to have dual roles in cancers. For instance, Lim et al. revealed miR-340-5p could suppress glioblastoma multiforme aggressive cell behaviors via functioning as upstream regulate gene for Bcl-w and Sox2 [13]. On the contrary, Zhao et al. revealed miR-340-5p was overexpressed in thyroid cancer, and could promote cancer cell proliferation through targeting bone morphogenetic protein 4 [14]. Our work showed that miR-340-5p was overexpressed in LUAD, and the knockdown of miR-340-5p stimulates LUAD cell proliferation, migration, and invasion. Hence, we showed miR-340-5p may function as an oncomiR in LUAD, which is similar to its role in thyroid cancer [14]. EDNRB is a kind of G-protein-coupled receptor for endothelin-1 [15]. EDNRB was found decreased expression in hepatocellular carcinoma using immunohistochemistry analysis, and correlated with poorer prognosis of cancer patients [16]. In bladder cancer, EDNRB was found increased expression, and promoted cancer proliferation by inhibiting apoptosis [17].

Conclusion

Taken together, our work indicated TGFB2-AS1 functions as a sponge for miR-340-5p to regulate EDNRB expression in LUAD. These findings identified a novel ceRNA regulatory axis, TGFB2-AS1/miR-340-5p/EDNRB, in LUAD, which will help us to understand the mechanisms behind LUAD progression.

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Disclosure of conflict of interest

None.

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