

# LncDBH-AS1 knockdown enhances proliferation of non-small cell lung cancer cells by activating the Wnt signaling pathway *via* the miR-155/AXIN1 axis

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**Abstract.** – **OBJECTIVE:** Dys-regulated long noncoding RNAs (lncRNAs) are involved in the cell growth of several malignancies and their aggressive phenotypes. LncRNA DBH-AS1 plays an important role in the advancement of various malignant tumors, but its contribution to non-small cell lung cancer (NSCLC) is still unexplored. This study intends to elucidate the role of the regulatory network of lncRNA DBH-AS1 in NSCLC progression.

**PATIENTS AND METHODS:** The LncDBH-AS1 expression in 32 paired NSCLC patients' tissue samples and NSCLC cell lines were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The role of LncDBH-AS1 in NSCLC was investigated through cell counting kit-8 (CCK-8) assay and colony formation assay *in vitro*. Besides, the interaction between LncDBH-AS1 and miR-155 was also analyzed.

**RESULTS:** The DBH-AS1 expression was significantly down-regulated in NSCLC cell lines and tissue samples. Decreased DBH-AS1 levels promoted the *in vitro* proliferation of the NSCLC cells. The mechanism was that DBH-AS1 regulated AXIN1 expression by sponging miR-155 in NSCLC cell lines. Importantly, LncDBH-AS1 might inhibit WNT/ $\beta$ -CATENIN activation in NSCLC cells.

**CONCLUSIONS:** The progression of NSCLC is facilitated by DBH-AS1 via miR-155 interaction and up-regulation of AXIN1 expression.

*Key Words:*

AXIN1, MiR-155, Metastasis, DBH-AS1, NSCLC.

## Introduction

As the most frequently occurring malignancy in humans, lung cancer has a high mortality rate around the world<sup>1</sup>. Non-small cell lung cancer (NSCLC) is an aggressive tumor with high recurrence and metastasis rates<sup>2</sup>. Given the fact of

no significant increase in the 5-year survival rate of NSCLC patients, it is essential to develop new therapeutic strategies to improve the outcomes of NSCLC patients<sup>3</sup>.

Long non-coding RNAs (lncRNAs, >200 nt) belong to the non-protein RNA family and serve as either oncogenes or tumor-suppressor genes in cancer development<sup>4</sup>. They do not code the proteins, but participate in numerous cellular processes by regulating gene expression<sup>5</sup>. LncRNA DBH-AS1 has recently been identified as an oncogene in hepatocellular carcinoma<sup>6</sup>, osteosarcoma<sup>7</sup>, and diffuse large B-cell lymphoma<sup>8</sup>. However, the potential role of DBH-AS1 in the development of NSCLC and the underlying mechanism are not fully understood. In the present study, we analyzed the expression pattern of DBH-AS1 in NSCLC samples and its influence on malignant behaviors of NSCLC cells. The results showed that DBH-AS1 mediated aggressive phenotypes of NSCLC cells by upregulating AXIN1 after it bound to miR-155. Finally, we tried to illustrate the role of the regulatory network of lncRNA DBH-AS1/miR-155/AXIN1 in NSCLC progression.

## Patients and Methods

### *Patients and Clinical Specimens*

This research was carried out as per the Declaration of Helsinki. The Research Ethics Committee of the Affiliated Hospital of Shaoxing University approved this investigation. Thirty-two paired NSCLC patients were enrolled according to NCCN Clinical Practice Guidelines in Oncology (version 8.2020). None of these patients had undergone chemotherapy, preoperative radiotherapy, or any other treatment for cancer. The sample tissues of NSCLC and paracancerous non-tu-

moral ones were immediately frozen in liquid nitrogen and maintained at  $-80^{\circ}\text{C}$  after surgical excision.

#### **qRT-PCR**

We added the TRIzol (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) to cell/tissues to extract RNA, and a nanodrop was applied to the quantification. The qRT-PCR analysis was performed using SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II (TaKaRa, Dalian, China) and TaqMan UniversalMaster Mix II (Life Technologies Corporation, Carlsbad, CA, USA) on an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturers' instructions. The relative mRNA (DBH-AS1 and AXIN1) gene expression normalized by  $\beta$ -actin and miRNA (miR-155) gene expression normalized by U6 were calculated by the  $2^{-\Delta\Delta\text{Ct}}$  method. Primers for PCR were synthesized by RiboBio (Guangdong, Guangzhou, China) and listed below.

LncDBH-AS1 primer sequence, forward, 5'-CGTCCACTCGTCTGTTCACT-3' and reverse, 5'-TAACACCCCATCCGCTTGT-3';

AXIN1 primer sequence, forward, 5'-GGAC-AAATGCGTGGATACCT-3' and reverse, 5'-TGCTTG GAGACA ATG CTGTT-3';

$\beta$ -actin primer sequence, forward, 5'-GTGGAGATCGCAAACCT-3' and reverse, 5'-CCTATGGGCTTACTTGCAAGT-3';

miR-155 primer sequence, forward, 5'-GATCAAAGTCTTCAAATATGCCTAAAGG-3' and reverse, 5'-TGAACAAGCCAAAACCTGC-3';

U6 primer sequence, forward, 5'-TTGAGCGGGCAAATCG-3' and reverse, 5'-GCCCTATCGATGCAAGCA-3'.

#### **Cell Culture and Transfection**

NSCLC cell lines (A549 and H129) offered by KeyGen Biotech Co Ltd. (Nanjing, Jiangsu, China) were maintained in the DMEM media containing with FBS (10%) (Invitrogen, Carlsbad, CA, USA), 100  $\mu\text{g}/\text{mL}$  streptomycin and 100 UI/mL penicillin. Lentivirus-mediated shRNA or short hairpin RNA targeting DBH-AS1 (si-DBH-AS1) and the NC or shCon (negative control) vector came from Genechem (Shanghai, China). Small interfering RNA (siRNA) targeting DBH-AS1 (siDBH-AS1) and the negative control or siCon, the mimic of miR-155, miR-NC or the negative control, inhibitors of miR-155 and miRNA (miR-NC inhibitor) were prepared and provided by Genechem. Lipofectamine<sup>3000</sup> from Invitrogen was used for cell transfection.

#### **Cell Counting Kit-8 (CCK-8) Assay**

NSCLC cells were seeded ( $2 \times 10^3$  cells/well) in plates of 96 wells. After 1, 2, 3, or 4 full days, 20  $\mu\text{L}$  of 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide ( ) was added into the wells, which were then kept 20 min in DMSO (200  $\mu\text{L}$ ). The OD values at 490 nm were estimated with an analyzer for enzyme immunoassays from Bio-Rad Laboratories (Hercules, CA, USA).

#### **RNA Immunoprecipitation (RIP) Assay**

RIP was conducted using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (#17-701, Millipore, Bedford, MA, USA). Transfected NSCLC cells were lysed in protease and RIP lysis buffer containing RNase inhibitors. Subsequently, the cell lysate was induced in RIP buffer containing Ago-coated magnetic beads (Millipore, Billerica, MA, USA) for 2 h at  $4^{\circ}\text{C}$ , with IgG as the negative control (input group). Finally, co-precipitated RNAs were isolated and analyzed by qRT-PCR.

#### **Dual-Luciferase Reporter Assay**

Wild-type (WT) and mutant-type (MUT) sequences of DBH-AS1 sharing the binding site of miR-155 were synthesized by GenePharma. After co-transfection of luciferase plasmids and miR-155 mimics, the relative activity of luciferase was measured using the Dual-Luciferase reporter kit (Promega, Madison, WI, USA).

#### **TOP/FOP Flash Reporter Assay**

Reporter vectors of the Wnt signaling pathway, TOP-Flash, and FOP-Flash, were provided by Promega and co-transfected to NSCLC cells with indicated transfection plasmids. Luciferase intensities were detected using the Luciferase Reporter Assay System (Promega, Madison, WI, USA).

#### **Statistical Analysis**

SPSS 17.0 (SPSS Inc. Released 2008. SPSS Statistics for Windows, Chicago, IL, USA) was used for statistical processing. Differences between groups were compared by the Student's *t*-test. A  $p < 0.05$  indicated the significance threshold.

## **Results**

#### **DBH-AS1 Was Decreased in NSCLC Cells**

GEPAI data suggested that the DBH-AS1 expression level in NSCLC tissue samples was

lower than that in normal tissues (Fold change  $>2$ ,  $p < 0.01$ , Figure 1A). Similarly, compared with that in the control tissue samples, the DBH-AS1 expression was down-regulated in NSCLC cells according to qRT-PCR data (Figure 1B). It was concluded that DBH-AS1 was down-regulated in NSCLC tissue samples.

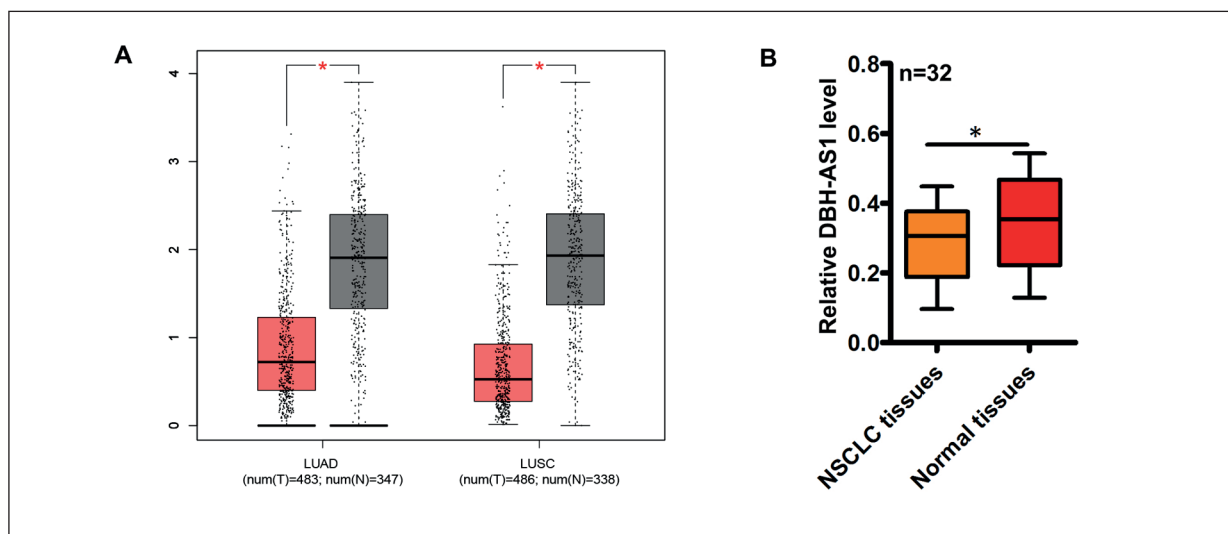
### **Knockdown of DBH-AS1 Stimulates Proliferative Capacity of NSCLC Cells**

The expression of DBH-AS1 in A549 and H1299 cells was intervened to explore its role in mediating the proliferative capacity of NSCLC cells. The effect of DBH-AS1 knockdown was first explored (Figure 2A). CCK-8 curves demonstrated that DBH-AS1 down-regulation enhanced the viability of indicated NSCLC cells (Figure 2B). Colony formation assays also achieved similar results (Figure 2C and D). Hence, DBH-AS1 was an oppressor in NSCLC cells.

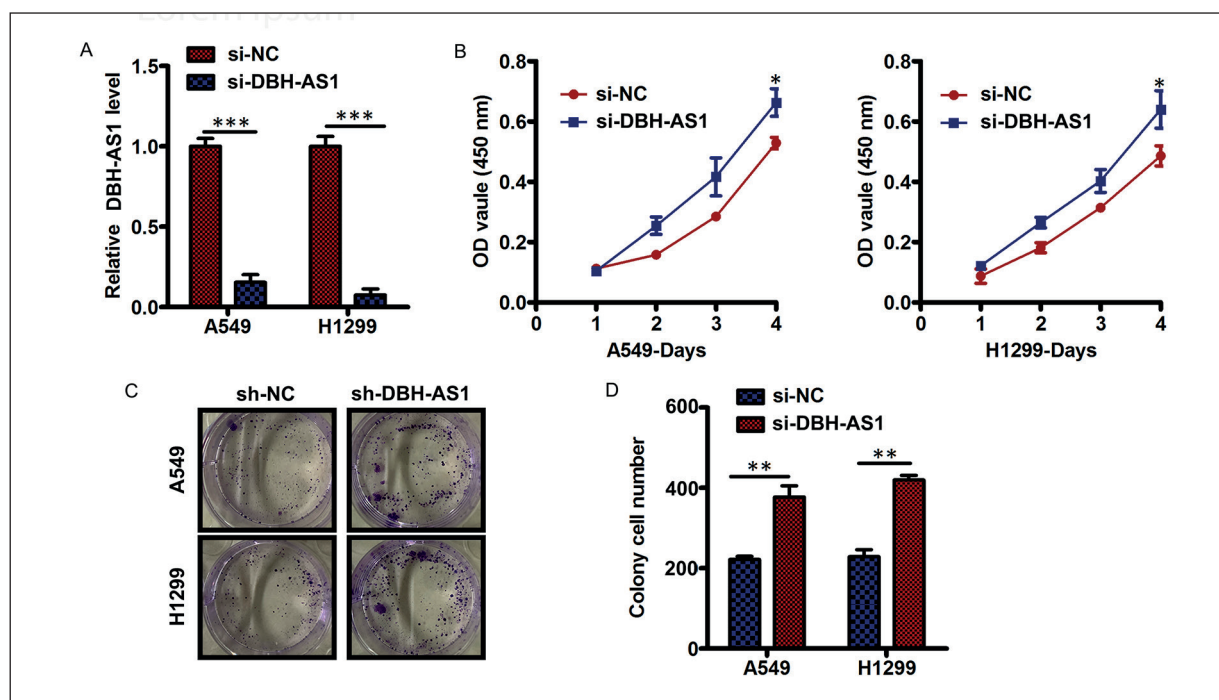
### **DBH-AS1 Sponges MiR-155 to Upregulate AXIN1 in NSCLC Cells**

To expound how DBH-AS1 mediates NSCLC progression, we used GEPIA (Gene Expression Profiling Interactive Analysis) database to predict the relationship between AXIN1 and DBH-AS1 (Figure 3A). It was found that the two were co-expressed. Interestingly, knockdown of DBH-AS1 in NSCLC cells markedly down-regulated the mRNA level of AXIN1 (Figure 3B). A growing number of evidence has substantiated

that lncRNAs mediate biological processes as competing endogenous RNAs (ceRNAs) by sponging miRNAs. We predicted the direct target miRNA of DBH-AS1 with the online starBase 3.0. The sequences shared by MiR-155 (Figure 3C) in the promoter region were complementarily paired to those of DBH-AS1. It is reported that miR-155 targets AXIN1 in familial adenomatous polyposis<sup>9</sup>. Later, a Dual-Luciferase reporter assay was conducted to verify the relationship between miR-155 and DBH-AS1 in NSCLC cells. NSCLC cells were co-transfected with WT-DBH-AS1 or MUT-DBH-AS1 and agomiR-155 or agomiR-NC. The transfection of agomiR-155 markedly up-regulated the miR-155 level (Figure 3D). The luciferase activity in the NSCLC cells sharply decreased after co-transfection with agomiR-155 and WT-DBH-AS1, but it maintained almost unchanged after co-transfection with MUT-DBH-AS1 and agomiR-155 (Figure 3E). RIP assay was carried out to further identify the interaction between miR-155 and DBH-AS1. It was suggested that there were more DBH-AS1 and miR-155 enriched in anti-Ago2 than in IgG (Figure 3F). Furthermore, NSCLC cells were co-transfected with si-DBH and antagomiR-155 to clarify the involvement of AXIN1 in NSCLC development. The co-transfection efficacy was first examined, and antagomiR-155 played an effective role in down-regulating miR-155 (Figure 3G). Interestingly, the down-regulated AXIN1 (Figure 3H) following knockdown of DBH-AS1 was reversed in NSCLC cells co-transfected



**Figure 1.** DBH-AS1 is decreased in NSCLC cells. **A**, GEPIA data indicated a decline of DBH-AS1 in NSCLC tissues. **B**, DBH-AS1 in 32 paired NSCLC cells and ANTs were examined by real time-PCR. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



**Figure 2.** Decreased DBH-AS1 expression enhances NSCLC cell proliferation. **A**, RT-qPCR analysis of DBH-AS1 expression in indicated NSCLC cells after the transfection with si-DBH-AS1 or si-NC. **B**, The proliferation of DBH-AS1 deficient-indicated NSCLC cells was detected by the CCK-8 assay. **C**, **D**, Colony form assays were performed in indicated NSCLC cells after the transfection with si-DBH-AS1 or si-NC, magnification  $\times 1$ . \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

with antagoniR-155. Taken together, DBH-AS1 up-regulated AXIN1 in NSCLC cells by sponging miR-155.

### ***DBH-AS1 Activates the Wnt Signaling Pathway in NSCLC Cells***

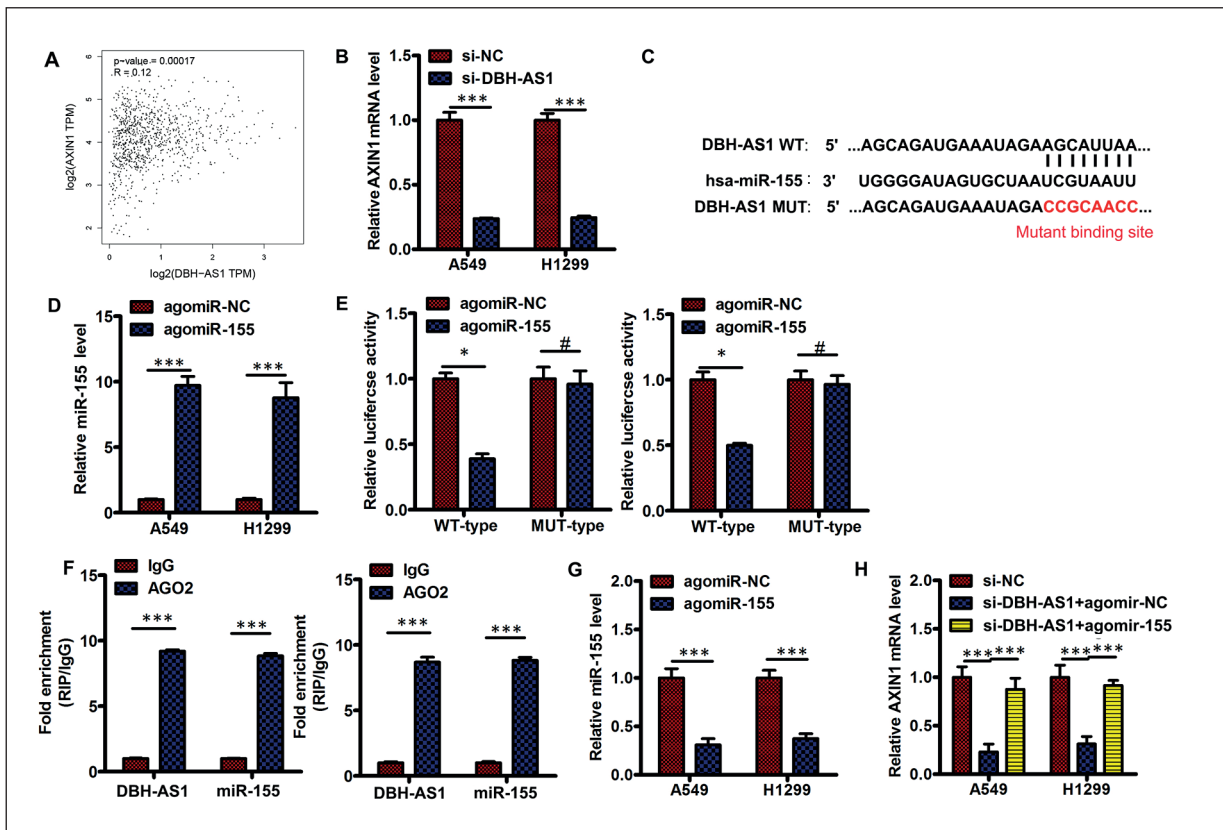
By recruiting cellular  $\beta$ -catenin and activating the Wnt signaling pathway, AXIN1 was able to mediate tumorigenesis, including NSCLC<sup>10</sup>. The above data proved the regulatory effect of DBH-AS1 on AXIN1. As for the speculation about the possibility of DBH-AS1 in mediating the Wnt signaling pathway, TOP/FOP Flash reporter assay was conducted and it was revealed that DBH-AS1 knockdown markedly accelerated the activity of the Wnt signaling pathway (Figure 4A and B).

## **Discussion**

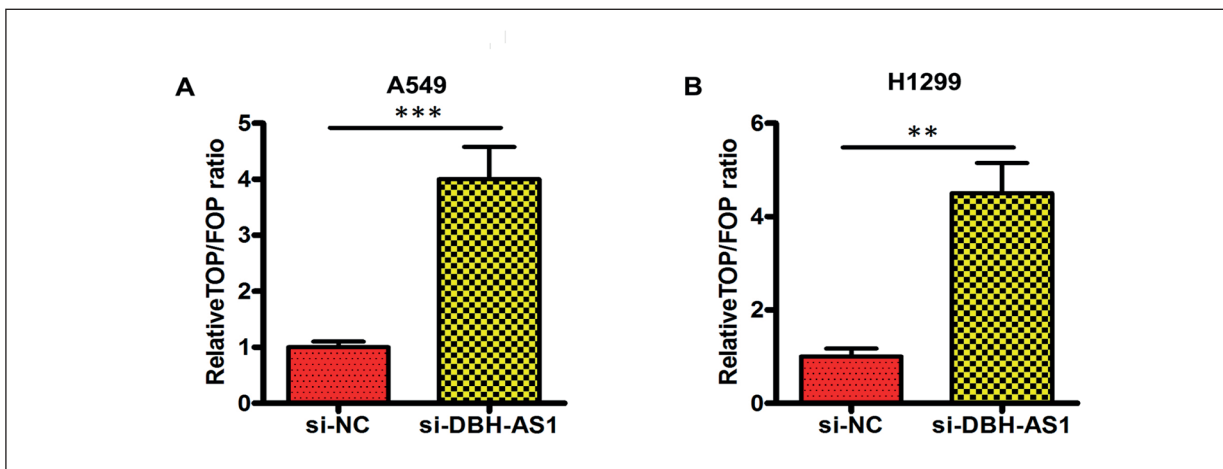
In this paper, we demonstrate that lncRNA DBH-AS1 expression is strikingly down-regulated in clinical tissues of NSCLC patients, compared with that in equivalent adjacent (normal) tissues. The silencing of DBH-AS1 enhances the proliferative capacity of NSCLC cell lines. More-

over, DBH-AS1 binds to miR-155 and regulates AXIN1 (the functional miR-155 target) expression positively. We finally prove the participation of DBH-AS1 in NSCLC progression via miR-155/AXIN1 regulation. Taken together, the DBH-AS1 acts as an oppressor in NSCLC cells.

lncRNAs are capable of mediating gene expression by interacting with mRNAs, proteins or miRNAs<sup>11,12</sup>. Serving as ceRNAs, lncRNAs play their role in cancer development by binding to corresponding miRNAs<sup>13-15</sup>. In colorectal carcinoma (CRC), KCNQ10T1 up-regulates ATG4B through sponging miR-34, thus triggering chemoresistance of cancer cells<sup>16</sup>. Besides, MALAT1 mediates cell stemness of CRC through the miR-20b-5p/Oct4 axis<sup>17</sup>. Through binding to miR-346, DGCR5 slows the progress of HCC by up-regulating KLF1<sup>18,19</sup>. Based on the literature review, we held that miR-155 and AXIN1 could be involved in DBH-AS1-mediated development of NSCLC. First, a shared binding site predicted in the promoter region was paired to DBH-AS1 3'-UTR, and their binding relationship was further verified by a Dual-Luciferase reporter and RIP assays. Second, knockdown of DBH-AS1 up-regulated miR-155 and thus down-regulated AXIN1. We



**Figure 3.** DBH-AS1 directly interacts with miR-155 in NSCLC cells as a molecular miRNA sponge to regulate the AXIN1 level. **A**, GEPIA tool indicated a strong positive correlation between the expression of DBH-AS1 and AXIN1 in these NSCLC tissue samples. **B**, AXIN1 mRNA expression in indicated NSCLC cells with DBH-AS1 knocked down. **C**, Bioinformatics analysis revealed the bindings sites of WT and MUT miR-155 within DBH-AS1. **D**, miR-155 expression in indicated NSCLC cells transfected with agomiR-155 or agomiR-NC. **E**, WT-DBH-AS1 or MUT-DBH-AS1 was co-transfected with agomiR-155 or agomiR-NC into indicated NSCLC cells. Luciferase activity was detected 48 h after co-transfection. **F**, There were more DBH-AS1 and miR-155 enriched in Ago2-containing immunoprecipitated samples than in the IgG controls. **G**, miR-155 expression was detected in indicated NSCLC cells transfected with antagomiR-155 or antagomir-NC using qRT-PCR. **H**, Indicated NSCLC cells were transfected with antagomiR-155 or antagomir-NC in the presence of si-DBH-AS1. After transfection, the qRT-PCR assay was performed to detect EGFR mRNA and protein expression. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; # $p > 0.05$ .



**Figure 4.** DBH-AS1 activates the Wnt- $\beta$ -catenin signaling pathway in NSCLC cells. **A**, and **B**, TOP/FOP Flash reporter activity assay for the detection of relative Wnt signaling activity in A549 and H1299 cells. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

have identified an axis involving DBH-AS1, miR-155, and AXIN1 as a ceRNA network for mediating NSCLC development. This study had some limitations. First, it ignored the analysis of migration, invasion, EMT, and apoptosis of cell lines. Second, a study in vivo (xenograft transplantation in nude mice) was lacking.

## Conclusions

We found that lncRNA DBH-AS1 is down-regulated in human NSCLC cells. Malignant behaviors of NSCLC cells can be inhibited by DBH-AS1 via miR-155/AXIN1 axis. Our findings imply that lncRNA DBH-AS1/miR-155/AXIN axis acts as a novel target for the treatment of NSCLC.

## Conflict of Interest

The Authors declare that they have no conflict of interests.

## Author contribution

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