Long non-coding RNA B4GALT1-Antisense RNA 1/microRNA-30e/SRY-box transcription factor 9 signaling axis contributes to non-small cell lung cancer cell growth

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Abstract. Long non-coding (lnc) RNAs serve crucial functions in human cancers. However, the involvement of the lncRNA B4GALT1-antisense RNA 1 (AS1) in non-small cell lung cancer (NSCLC) has not been extensively studied. Reverse transcription-quantitative PCR was performed to detect B4GALT1-AS1 levels in NSCLC tissues and cell lines. Potential influences of B4GALT1-AS1 on biological functions of NSCLC were assessed through a series of *in vitro* experiments, and the molecular mechanism was determined via RNA immunoprecipitation (RIP) and bioinformatics analyses. The results of the present study demonstrated that knockdown of B4GALT1-AS1 significantly attenuated the proliferative ability and clonality of H1299 and A549 cells. In the present study, B4GALT1-AS1 competed as an endogenous RNA by sequestering microRNA-30e (miR-30e) leading to an enhanced expression of SRY-box transcription factor 9 (SOX9). The effects of silencing B4GALT1-AS1 on NSCLC cells proliferation could be ameliorated by inhibiting miR-30e or restoring SOX9. Hence, B4GALT1-AS1 acted as a lncRNA that drives tumor progression in NSCLC via the regulation of the miR-30e/SOX9 axis. The findings of the present study indicated that the B4GALT1-AS1/miR-30e/SOX9 axis maybe an effective target for NSCLC treatment and management.

Introduction

Lung cancer (LCa) remains one of the primary reasons of cancer mediated fatalities globally, with 5-year survival rates of <20% in 2017 (1). In the US ~234,030 new LCa cases were diagnosed and in China ~781,500 new LCa cases were diagnosed in 2018 (2). Non-small cell lung cancer (NSCLC) is the most prevalent subtype of lung cancer, and lung adenocarcinoma (LUAD) is the major subtype of NSCLC (3). In the United States, LUAD accounted for ~50% of LC cases (43 and 52% in males and females, respectively) between 1992-2013 (3). In spite of progress in NSCLC therapeutics, the molecular mechanisms underlying NSCLC progression and development are poorly understood (4,5). Further mechanistic studies to fully understand NSCLC progression are therefore urgently required.

lncRNAs (long non-coding RNAs) are a group of sequences that have been used to study several cancers, such as liver cancer, gastic cancer and colorectal carcinoma (6-8). lncRNAs are members of a family of >200 nucleotides long linear transcripts (9). They lack protein-encoding function and were hence deemed genomic 'noise' and 'junk' (10). Several recent reports have implicated lncRNAs in a range of genetic processes for gene expression modulation at epigenetic, transcriptional and post-transcriptional levels (11,12). In NSCLC, lncRNAs are frequently abnormally expressed and are involved in NSCLC occurrence and development (10-14). Hence, a detailed study of lncRNAs and their role in NSCLC may lead to unraveling potential targets to improve the prognosis and treatment of patients with NSCLC.

Recently lncRNA B4GALT1-AS1 was found as the B4GALT1 antisense counterpart, lncRNA B4GALT1-AS1 shows significant complementarity with B4GALT1 messenger RNA and enhanced colon cancer cell stemness and migration (15). Therefore, the activity of B4GALT1-AS1 in NSCLC needed to be investigated. B4GALT1-AS1 was chosen as the main focus of the present study, which aimed to study the pathogenicity of NSCLC and offer insights for its therapy.

Materials and methods

Patient details and tissue specimens. The Ethics Committee of The First Hospital of Longyan City (Longyan, China; approval no. 2019-015) gave approval for the present study which was performed according to the Declaration of Helsinki. Written informed consent was obtained from all the patients. A total of 56 patients with NSCLC, including 22 men and 34 women (mean age, 53 ± 6.2 years; age range, 32-76 years) treated by surgical resection in the First Hospital of Longyan City between January 2013 and December 2016 were recruited in the present study. The inclusion criteria were as follows: i) Histologically confirmed as NSCLC; ii) medical information was completely

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recorded and iii) informed consent was obtained prior to the study start. The exclusion criteria were as follows: i) Combined with other malignancies; ii) combined with chronic diseases and iii) therapeutic history within 3 months prior to admission. The surgically resected NSCLC and paracancerous tissues (at least 5 cm away from the tumor edge) were frozen in liquid nitrogen and stored at -80˚C until further use. The distance between the NSCLC tissue and normal adjacent tissue was more than 5 cm.

Cell culture. Three NSCLC cell lines (A549, PG49 and H1299) and a normal human lung cell line MRC-5 were acquired from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM)containing 10% heat-inactivated fetal bovine serum (FBS), 100 mg/ml streptomycin and 100 U/ml penicillin. The DMEM, FBS and antibiotics were from (Gibco; Thermo Fisher Scientific, Inc.). All the aforementioned cell lines were grown at 37˚C in an atmosphere with humidity and 5% CO_2 for the following experiments.

Cell transfection. To examine the loss-of-function, siRNA (small interfering RNA) si-B4GALT1-AS1 (against B4GALT1-AS1) and si-NC (control, non-targeting siRNA) were purchased from Guangzhou Ribobio Co., Ltd. The transfection plasmid sequences were as follows: shB4GALT1-AS1: 5'-GGTTTAGGGCTCCTCTAA-3'; sh-NC: 5'-AATTCT CACGTCACGT-3'. The antagomir-30e used for knockdown of miR-30e (forward, 5'-CCGCTCGGGAATAGGAAG GTG-3' and reverse, 5'-GCGAACCTTGGGTAGCCTCCT TGTC-3') and the negative control antagomir-NC (forward, 5'-CTTCACAGTGGCTAAGTTCCG-3' and reverse, 5'-GAA TTCCTGGTGCCAAAGCCTTGTC-3') were synthesized by Shanghai GenePharma Co., Ltd. The transfection was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in $5x10^6$ cells at a final concentration of 50 nM, according to the manufacturer's protocol. After transfection for 24 h, expression of B4GALT1-AS1 or miR-30e was validated by reverse transcription-quantitative PCR (RT-qPCR).

The synthesis of vector pcDNA3.1-SRY-box transcription factor 9 (pc-SOX9) to overexpress SOX9 and empty pcDNA3.1 vector was done by Shanghai GenePharma Co., Ltd. One day prior to transfection, NSCLC cells were seeded into 6-well plates at a density of $5x10^6$ cells until they reached 70% confluence. The transfection of the aforementioned molecular constructs was done transiently into cells using Lipofectamine 2000™ reagent from (Invitrogen; Thermo Fisher Scientific Inc.). Sox9 expression was detected via reverse-transcription quantitative (RT-q)PCR analysis 24 h post-transfection.

RT-qPCR. Total RNA was extracted from NSCLC cells and tissues and isolated using TRIzol® reagent from (Invitrogen; Thermo Fisher Scientific, Inc.), and a NanoDrop (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) was used to estimate its quality and concentration. The expression of miR-30e was done by reverse transcription using the Mir-X™ miRNA First-Strand Synthesis kit from Takara Biotechnology, Co., Ltd. and quantitative evaluation of the synthesized cDNA

was done using the Mir-X™ miRNA RT-qPCR TB Green® kit (Takara Biotechnology, Co., Ltd.). Small nuclear RNA U6 was used as the endogenous control for the normalization of the expression of miR-30e. To measure transcript levels of B4GALT1-AS1 and SOX9, the PrimeScript™ RT Reagent kit (Takara Biotechnology Co., Ltd.) was used to convert 5 µl total RNA to cDNA. Then, the amplification of cDNA products was done using the SYBR-Green PCR Master Mix (Takara Biotechnology Co., Ltd.). The level of SOX9 and B4GALT1-AS1 transcripts were normalized using GAPDH as a control. The thermocycling conditions were as follows: 94˚C for 30 sec; followed by 45 cycles of 94˚C for 5 sec, 60˚C for 15 sec and 72˚C for 15 sec; dissociation stage. The primer sequences for miR-30e were as follows: Forward, 5'-TTC ACAGAATAATTGC-3' and reverse, 5'-TTAACACTTTCA CGGGATG-3'; B4GALT1-AS1 forward, 5'-AGCTGAACG TCGAAGCGG-3' and reverse, 5'-GACACCCTGCGGCCA AAGGC-3'; U6 forward, 5'-ATTGGAACGATACAGAGA AGATT-3' and reverse, 5'-GGAACGCTTCACGAATTT G-3'; and GAPDH forward, 5'-GGTGAAGGTCGGAGTCAA CG-3' and reverse, 5-CAAAGTTGTCATGGA-3'. The $2^{-\Delta\Delta Cq}$ method was used for quantification of gene expression (16).

Cell counting assay. Assessment of cell proliferation was done using the Cell Counting Kit-8 assay from Shanghai Haling Biotechnology, Co., Ltd (http://haling.bioon.com.cn), according to the manufacturer's protocol. After incubating the transfected cells for 24 h, they were collected after trypsinization and seeded ($2x10^3$ cells/well) into 96-well plates. CCK-8 solution (10 µl) was added per well and kept for 2 h at 37˚C. The absorbance of the mixture was estimated in a microplate reader from Bio-Rad Laboratories, Inc. at 450 nm.

Colony formation assay. A549 and H1299 cell lines infected with siNC or si-B4GALT1-AS1 ($1x10^3$/well) were seeded into a culture dish (10 cm) and cultured for 14 days at 37˚C in an atmosphere with humidity and 5% CO_2. Finally, colonies were stained with 4% paraformaldehyde for 25 min at 37˚C and stained with crystal violet for 15 min at 37˚C followed by the counting of colonies. The number of colonies (diameter >1 mm) were counted and each experiment was repeated at least 3 times.

Bioinformatics analysis. Putative B4GALT1-AS1 targets were predicted using StarBase version 3.0 (http://starbase. sysu.edu.cn/).

RNA immunoprecipitation (RIP) assay. RIP was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation kit (EMD Millipore) to assess B4GALT1-AS1 and miR-30e interaction in A549 and H1299 cells, according to the manufacturer's instructions. After treating the cells in lysis buffer, further incubation of cell lysates was performed at 4˚C overnight with magnetic beads conjugated with either human protein-argonaute 2 (AGO2) antibody (1:100; cat. no. ab32381) or IgG (negative control) (1:100; cat. no. ab109489; both purchased from Abcam). Subsequently, the immunoprecipitated RNA was isolated after collecting the magnetic beads and RT-qPCR analysis was conducted to estimate the enrichment of B4GALT1-AS1 and miR-30e.

Dual-luciferase reporter assay. Design and synthesis of B4GALT1-AS1 fragments containing binding sites for wild-type (WT) and mutant (MUT) on miR-30e was done by Shanghai GenePharma Co. Ltd. These were cloned into the target expression vector pmirGLO dual-luciferase (Promega Corporation) to get the reporter plasmids WT-B4GALT1-AS1 and MUT-B4GALT1-AS1. One night prior to transfection, seeding of cells (60-70% confluent) was done in 24-well plates using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h of transfection, luciferase activity of the cells was estimated using the Dual-Luciferase Reporter Assay system (Promega Corporation). The data normalization was done by comparison with *Renilla luciferase* activity.

Statistical analyses. All results of independent experiments conducted 3 times are presented as the mean ± SD. Two groups were compared using paired and unpaired Student's t-test based on dependence and independence of samples, respectively. Multiple comparisons were conducted using one-way ANOVA followed by the post hoc Tukey's test. Pearson correlation coefficients were used for correlation analysis between B4GALT1-AS1 and Sox9 in NSCLC. P<0.05 was considered to indicate a statistically significant difference.

Results

B4GALT1-AS1 is increased in NSCLC tissues and cells. Expression of B4GALT1-AS1 was first assessed by RT-qPCR in 56 NSCLC tissues and adjacent healthy tissues. An elevated B4GALT1-AS1 mRNA level was observed in NSCLC tissues compared with adjacent healthy tissues (Fig. 1A). In addition, RT-qPCR was performed in 3 NSCLC cell lines (PG49, A549 and H1299) and MRC-5 (human normal lung cell line) and a significantly higher B4GALT1-AS1 mRNA expression level was found in NSCLC cell lines compared with MRC-5 cells. Meanwhile, H-1299 cells had the highest level of B4GALT1-AS1 compared with the others cell lines (Fig. 1B).

In vitro B4GALT1-AS1 silencing leads to inhibition of the NSCLC cell malignant phenotype. A higher B4GALT1-AS1 mRNA expression was observed in A549 and H1299 cells compared with PG49 cells; hence, for subsequent analysis, H1299 and A549 cells were used. To examine the function of B4GALT1-AS1 in the progression of NSCLC, si-B4GALT1-AS1 to deplete B4GALT1-AS1 was transfected in A549 and H1299 cells and si-NC transfected cells were used as the control, and the level of B4GALT1-AS1 was significantly decreased in A549 and H1299 cells transfected with si-B4GALT1-AS1 (P<0.05; Fig. 2A). The proliferative ability of both A549 and H1299 cells harboring si-B4GALT1-AS1 was measured using the CCK-8 assay and demonstrated inhibition compared with control cells (P<0.05; Fig. 2B). In addition, the colony-forming capacity of A549 and H1299 cells deficient in B4GALT1-AS1 (after transfection with si-B4GALT1-AS1) was greatly inhibited (P<0.05; Fig. 2C and D). These results strongly indicated the cancer-enhancing activity of lncRNA B4GALT1-AS1 in NSCLC.

B4GALT1-AS1 interacts directly with miR-30e in NSCLC cells and acts as a miRNA sponge. lncRNAs participate in several
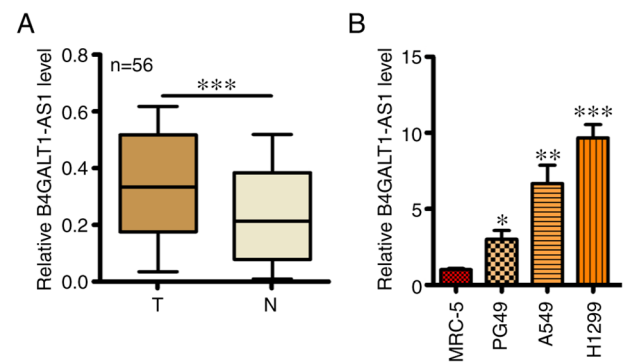

Figure 1. B4GALT1-AS1 expression is enhanced in NSCLC. (A) B4GALT1-AS1 expression was estimated by RT-qPCR in NSCLC and control adjacent healthy tissues (n=56). (B) Total RNA was extracted from 3 NSCLC cell lines (PG49, A549 and H1299) and a human healthy lung cell line (MRC-5) and was analyzed by RT-qPCR to evaluate the mRNA expression of B4GALT1-AS1. ***P<0.001, **P<0.01, and *P<0.05. T, tumor; N, normal; AS1, anti-sense RNA; RT-q, reverse-transcription quantitative; NSCLC, non-small cell lung carcinoma; OD, optical density.

biological processes by functioning as ceRNA (competing endogenous RNA) against miRNAs (9). Next, StarBase version 3.0, a publicly available algorithm was used to predict the miRNAs that interact directly with B4GALT1-AS1. A binding sites was identified between B4GALT1-AS1 and miR-30e (Fig. 3A). In addition, miR-30e has also been reported to inhibit NSCLC progression (17). An increase in miR-30e expression in A549 and H1299 cells transfected with si-B4GALT1-AS1 compared with si-NC (Fig. 3B). In addition, the luciferase activity of cells harboring WT-B4GALT1-AS1 reduced significantly following agomiR-30e transfection; however, there was no change in MUT-B4GALT1-AS1 activity when miR-30e was overexpressed (Fig. 3C). Next, the RIP assay was conducted to assess the miR-30e and B4GALT1-AS1 interaction and miR-30e and B4GALT1-AS1 were found to be enriched in immunoprecipitates containing AGO2 in comparison with the IgG control in A549 and H1299 cell lines (Fig. 3D). Subsequently, it was tested if expression of miR-30e was modulated by B4GALT1-AS1 in NSCLC cells and it was found to be upregulated in A549 and H1299 cells due to the silencing of B4GALT1-AS1 (Fig. 3E). The aforementioned findings indicated the miR-30e quenching function of B4GALT1-AS1 in NSCLC cells.

B4GALT1-AS1 sequesters miR-30e and positively modulates the expression of SOX9 in NSCLC cells. miR-30e directly targets the SOX9 gene in NSCLC cells (17). On observing that B4GALT1-AS1 sequesters miR-30e, the function of B4GALT1-AS1 in SOX9 regulation in NSCLC cells was next investigated. As shown in Fig. 4A, B4GALT1-AS1 silencing markedly reduced the expression of SOX9 at transcript in NSCLC cells. A significant decline in the level of miR-30e in antagomiR-30e harboring A549 and H1299 cells was observed compared with antagomiR-NC (Fig. 4B). In addition, the decline in the Sox9 transcript because of B4GALT1-AS1 knockdown was reversed by re-introduction of antagomiR-30e in A549 and H1299 cells (P<0.05; Fig. 4C). B4GALT1-AS1 mRNA expression was also positively correlated with SOX9 mRNA levels in NSCLC tissue (P=0.001; R=0.428; Fig. 4D).
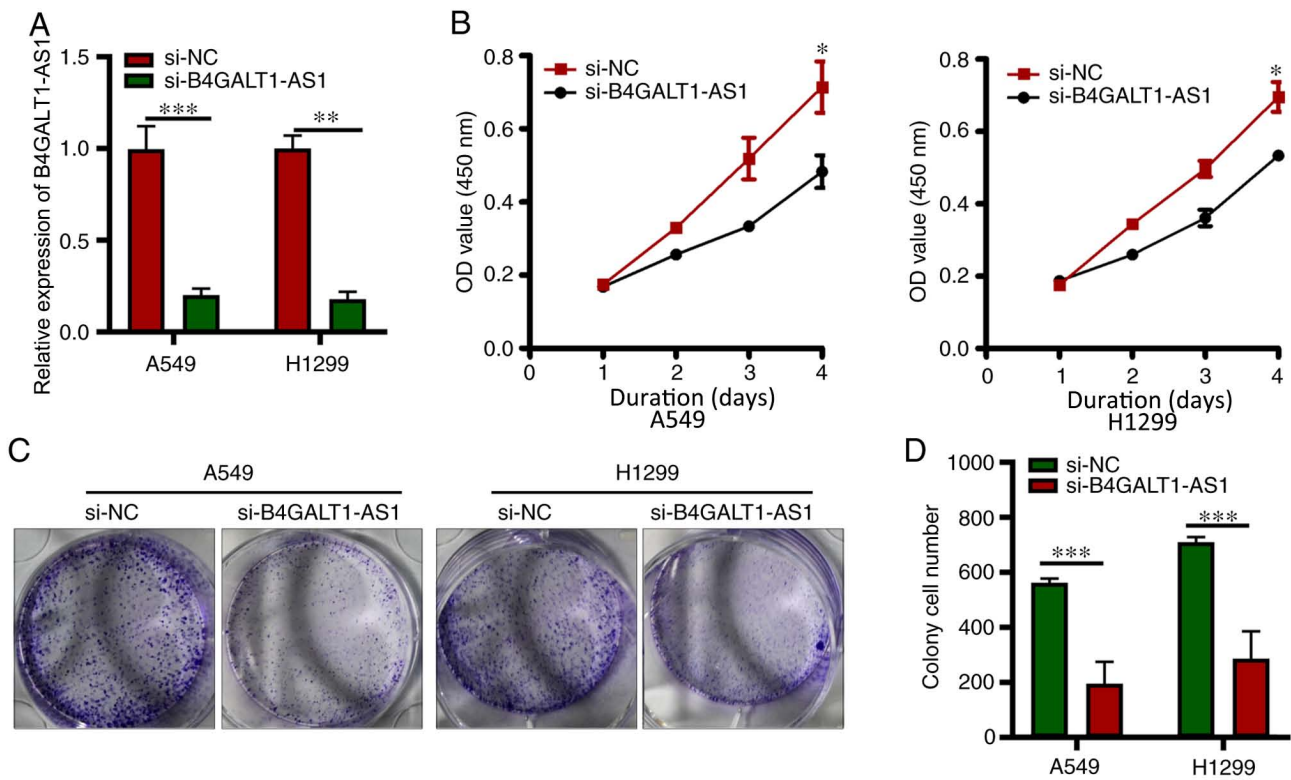
Figure 2. An *in vitro* decline in B4GALT1-AS1 level inhibited H1299 and A549 cell proliferation. (A) Expression analysis of B4GALT1-AS1 using RT-qPCR in A549 and H1299 cells post-transfection (by either si-B4GALT1-AS1 or si-NC). (B) Detection of the proliferation of H1299 and A549 cells deficient in B4GALT1-AS1 by the CCK-8 assay. (C and D) Following transfection of si-B4GALT1-AS1 or si-NC, H1299 and A549 cells were assayed for colony formation. ***P<0.001, **P<0.01, and *P<0.05. AS1, antisense RNA; RT-q, reverse-transcription quantitative; NC, negative control; si, small interfering.
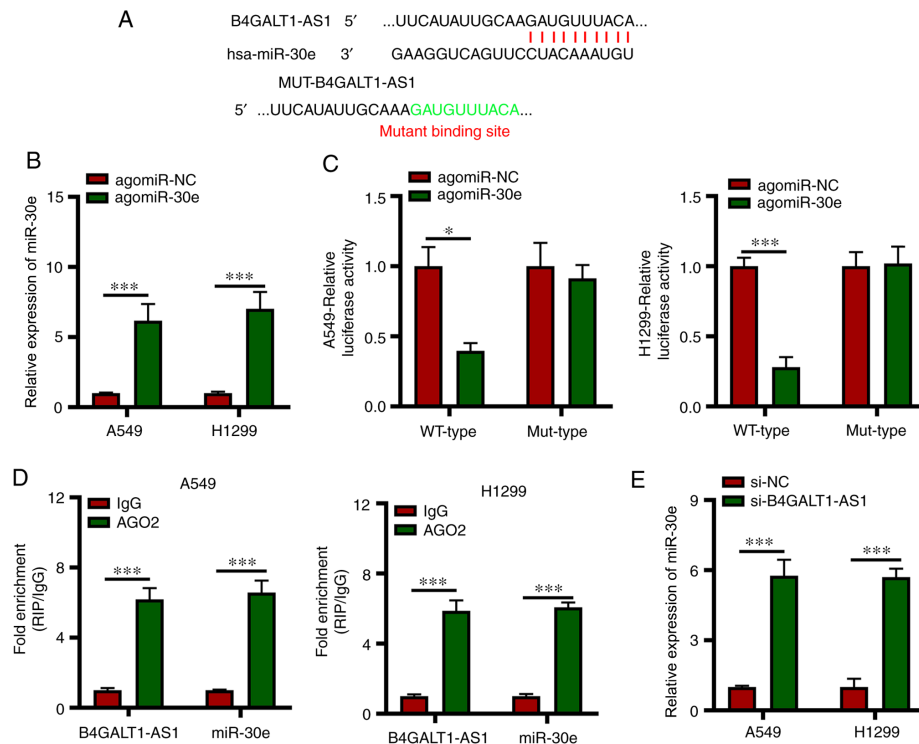

Figure 3. A direct interaction occurs between B4GALT1-AS1 and miR-30e to sequester the level of miR-30e expression in NSCLC cells. (A) Bioinformatics evaluation presenting the bindings sites of WT and MUT miR-30e on B4GALT1-AS1. (B) Assessment of miR-30e level in agomiR-30e or agomir-NC transfected A549 and H1299 cells. (C) WT-B4GALT1-AS1 or MUT-B4GALT1-AS1 was transfected along with agomir-NC or agomiR-30e in A549 and H1299 cells. Detection of luciferase activity 48 h following transfection. (D) B4GALT1-AS1 and miR-30e were enriched in immunoprecipitate containing AGO2 and compared with the control (IgG). (E) RT-qPCR to estimate miR-30e level in B4GALT1-AS1-silenced A549 and H1299 cells. ***P<0.001 and *P<0.05. AS1, antisense RNA; RT-q, reverse-transcription quantitative; NC, negative control; si, small interfering; NSCLC, non-small cell lung carcinoma; AGO2, protein arganaute-2; miR, micro RNA; WT, wild-type; MUT, mutant.
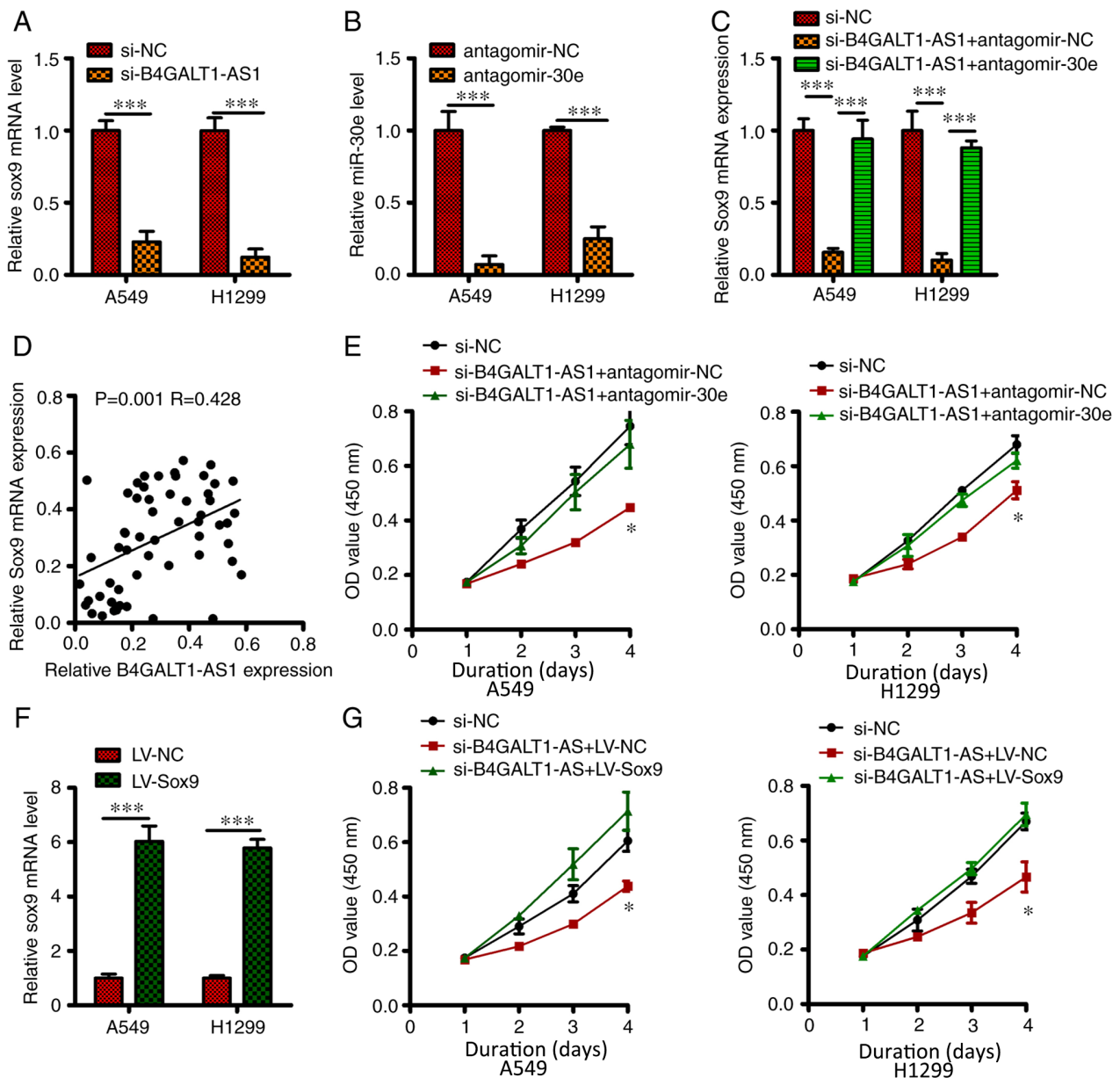
Figure 4. Role of B4GALT1-AS1 as a competing endogenous RNA which sequestered miR-30e to regulate the expression level of SOX9. (A) After transfecting si-B4GALT1-AS1 or si-NC in A549 and H1299 cells, SOX9 transcript level was quantified by RT-qPCR. (B) The level of miR-30e was estimated by RT-qPCR after the transfection of H1299 and A549 cells with antagomiR-30e or antagomir-NC. (C) Transfection of H1299 and A549 cells with antagomiR-30e or antagomir-NC in si-B4GALT1-AS1 presence. miR-30e expression was investigated via RT-qPCR assay. (D) B4GALT1-AS1 and SOX9 expression was positively correlated in NSCLC tissue. (E) Co-transfection of A549 and H1299 cells with si-B4GALT1-AS1 and either antagomiR-30e, or antagomir-NC and assessment of cell proliferation by the CCK-8 assay. (F) RT-qPCR assay to estimate SOX9 level in LV-SOX9 or LV-NC transfected A549 and H1299 cells. (G) Co-transfection of H1299 and A549 cells with si-B4GALT1-AS1 along with either pc-SOX9 or pcDNA3.1. CCK-8 assay was performed to estimate cellular proliferation. ***$P<0.001$ and *$P<0.05$. AS1, antisense RNA; RT-q, reverse-transcription quantitative; NC, negative control; si, small interfering; NSCLC, non-small cell lung carcinoma; miR, micro RNA; LV, lentiviral; OD, optical density; SOX 9, SRY-box transcription factor 9.

Further, rescue experiments elucidated that B4GALT1-AS1 silencing impeded proliferation of H1299 and A549 cells and inhibition of miR-30e in these cells partly neutralized the outcomes of B4GALT1-AS1 silencing ($P<0.05$; Fig. 4E). SOX9 was overexpressed in A549 and H1299 cells transfected with LV-Sox9 compared with LV-NC ($P<0.05$; Fig. 4F). Subsequently, on performing the CCK-8 assay, pc-SOX9 co-transfection was found to reverse the decline in proliferation of A549 and H1299 cells caused by the silencing of B4GALT1-AS1 ($P<0.05$; Fig. 4G). These findings indicate the oncogenic effect of B4GALT1-AS1 in NSCLC progression by acting as a ceRNA for miR-30e and consequently enhancing SOX9 expression.

Discussion

lncRNAs have attracted a lot of interest because of their vital function in the progression of cancer (18,19). It has been reported that aberrant expression of lncRNAs contributed to the progression of NSCLC (20). Hence, potential treatment targets

may be revealed by studying the roles of lncRNAs in NSCLC tumorigenesis. To the best of our knowledge, until recently the function of B4GALT1-AS1 in the malignancy of NSCLC has not been studied in detail. Hence, the present study was firstly evaluated the B4GALT1-AS1 expression level and its specific role in the malignancy of NSCLC cells and elucidated the tumor-enhancing function of B4GALT1 in NSCLC. The present study found a high expression of B4GALT1-AS1 in NSCLC tissue samples compared with adjacent normal tissues as well as the NSCLC cell lines compared with the normal lung cell line. The functional experiments conducted in the present study revealed inhibition of NSCLC cell proliferative and colony-forming capacities as a result of B4GALT1-AS1 silencing.

Notably, lncRNAs may play key role as competing endogenous RNAs (ceRNAs) to regulate miRNAs expression (21). In the present study, after revealing the tumor-enhancing role of B4GALT1-AS1 in NSCLC. Based on the results from bioinformatics analysis in the present study, it was predicted that miR-30e possesses a binding site complementary to that on B4GALT1-AS1.To further confirm the relationship between miR-30e and B4GALT1-AS1, luciferase reporter and RIP assays were conducted, which identified that miR-30e was the direct target of B4GALT1-AS1. In addition, the silencing of B4GALT1-AS1 enhanced miR-30e level and led to a decline in the expression of SOX9. In the present study, the outcomes of B4GALT1-AS1 silencing on malignant phenotypes of NSCLC could be abrogated by miR-30e inhibition or SOX9 restoration. Taken together, the findings of the present study indicate a ceRNA model that includes B4GALT1-AS1, SOX9 and miR-30e in NSCLC cells.

In multiple potential target genes, SOX9 was selected for further validation since SOX9 has been demonstrated to be involved in NSCLC carcinogenesis and progression (22). In addition, SOX9 was identified as a direct target of many diverse miRNAs in NSCLC, including miR-124 (23), miR-206 (24), and miR-32 (25). The results of the present study verified that SOX9 was a direct and functional target gene of miR-30e. In the present study, the upregulation of SOX9 targeted by miR-30e in NSCLC cells was observed and for the first time to the best of our knowledge, an upstream mechanism modulating the axis of miR-30e/SOX9 in NSCLC cells *in vitro* was identified. The present study indicated that B4GALT1-AS1 possesses a miR-30e binding site, and acts as a ceRNA and sequesters miR-30e in NSCLC cells, leading to an enhanced level of SOX9.

To the best of our knowledge, the present study identified the mode of action of B4GALT1-AS1 in enhancing NSCLC progression for the first time. B4GALT1-AS1 silencing enhanced the malignancy of NSCLC cells. B4GALT1-AS1 positively regulated SOX9 expression by sequestering miR-30e in NSCLC cells. Further studies are needed to focus on the *in vivo* function of B4GALT1-AS1 in NSCLC. Identification of the regulatory network of B4GALT1-AS1/miR-30e/SOX9 will potentially aid in fully determining the stage of NSCLC and provide possible targets for the therapy of patients with NSCLC. Future studies will focus on seeking out suitable online databases to confirm the correlation analysis between B4GALT1-AS1 and Sox9 in lung cancer. The sample size of this study was relatively small. In the future, the biological potential of B4GALT1-AS1 as a biomarker of NSCLC needs to be validated in multi-center studies with a large sample size.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

JHL and JM wrote the manuscript and contributed to the conception of the study. JHL, FNC and CXW performed the experiments and data analysis. SQH contributed to data acquisition and analysis and revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of The First Hospital of Longyan City (Longyan, China; approval no. 2019-015) and performed in accordance with the Declaration of Helsinki. All the patients provided written informed consent prior to participation in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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