Upregulation of the long non-coding RNA AFAP1-AS1 affects the proliferation, invasion and survival of tongue squamous cell carcinoma via the Wnt/β-catenin signaling pathway

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Abstract

**Background:** Long non-coding RNA (lncRNA) actin filament associated protein 1 antisense RNA1 (AFAP1-AS1) is oriented in an antisense direction to the protein-coding gene AFAP1 in the opposite strand. Previous studies showed that lncRNA AFAP1-AS1 was upregulated and acted as an oncogene in a variety of tumors. However, the expression and biological functions of lncRNA AFAP1-AS1 in tongue squamous cell carcinoma (TSCC) are still unknown.

**Methods:** The expression level of AFAP1-AS1 was measured in 103 pairs of human TSCC tissues and corresponding adjacent normal tongue mucous tissues. The correlation between AFAP1-AS1 and the clinicopathological features was evaluated using the chi-square test. The effects of AFAP1-AS1 on TSCC cells were determined via a CCK-8 assay, clone formation assay, flow cytometry, wound healing assay and transwell assay. Furthermore, the effect of AFAP1-AS1 knockdown on the activation of the Wnt/β-catenin signaling pathway was investigated. Finally, CAL-27 cells with AFAP1-AS1 knockdown were subcutaneously injected into nude mice to evaluate the effect of AFAP1-AS1 on tumor growth in vivo.

**Results:** In this study, we found that lncRNA AFAP1-AS1 was increased in TSCC tissues and that patients with high AFAP1-AS1 expression had a shorter overall survival. Short hairpin RNA (shRNA)-mediated AFAP1-AS1 knockdown significantly decreased the proliferation of TSCC cells. Furthermore, AFAP1-AS1 silencing partly inhibited cell migration and invasion. Inhibition of AFAP1-AS1 decreased the activity of the Wnt/β-catenin pathway and suppressed the expression of EMT-related genes (SLUG, SNAIL1, VIM, CADN, ZEB1, ZEB2, SMAD2 and TWIST1) in TSCC cells. In addition, CAL-27 cells with AFAP1-AS1 knockdown were injected into nude mice to investigate the effect of AFAP1-AS1 on tumorigenesis in vivo. Downregulation of AFAP1-AS1 suppressed tumor growth and inhibited the expression of EMT-related genes (SLUG, SNAIL1, VIM, ZEB1, NANOG, SMAD2, NESTIN and SOX2) in vivo.

**Conclusions:** Taken together, our findings present a road map for targeting the newly identified IncRNA AFAP1-AS1 to suppress TSCC progression, and these results elucidate a novel potential therapeutic strategy for TSCC.

**Keywords:** Long non-coding RNA, AFAP1-AS1, TSCC, Proliferation, Wnt/β-catenin signaling

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**Background**

Head and neck cancer is the sixth most common malignant cancer worldwide [1–3], and epithelial tumors arising in the oral cavity are the most frequent tumors in the head and neck region. Tongue squamous cell carcinoma (TSCC) is the most common epithelial cancer identified in the oral cavity and accounts for approximately 25 to 40% of cases [1, 3]. TSCC is characterized by its high metastatic and proliferative ability and is a considerable threat to human health worldwide [3]. Over the past decades, although recent developments have been achieved in the therapeutic management of TSCC, such as surgery, chemotherapy and radiotherapy, the overall survival among TSCC patients with locally advanced disease and cervical lymph node metastasis remains dismal [4]. The five-year survival rate of TSCC remains lower than 50%, and the patient quality of life is poor [3, 4]. Therefore, it is necessary to clarify the underlying molecular mechanisms involved in TSCC invasion and metastasis and to develop novel therapeutic strategies for TSCC.

Long non-coding RNAs (lncRNAs) are a type of endogenous RNA over 200 nucleotides in length that completely lack or possess limited protein-coding capacity [5–8]. Several studies have demonstrated that lncRNAs played a vital regulatory role in cellular and developmental processes [6, 7]. Aberrant expression of lncRNAs is involved in cancer biology through a variety of mechanisms ranging from transcriptional levels to post-transcriptional levels [5, 6, 9–12]. Recently, emerging evidence has indicated that lncRNAs are involved in the tumor initiation and progression of various malignant tumors, including TSCC [6, 13–21]. For example, overexpression of lncRNAs *LINC00152* and *LINC00673* promoted TSCC cell invasion and metastasis and was associated with the poor prognosis of TSCC [20, 21]. Huang et al. demonstrated that lncRNA *AKIILA* inhibits the migration and invasion of TSCC cells via suppressing epithelial-mesenchymal transition (EMT) [22]. lncRNA *MALAT1* modulated metastatic potential, inhibited apoptosis and induced EMT in TSCC cells through the regulation of small prelin rich proteins and the Wnt/β-catenin signaling pathway [23, 24]. Moreover, overexpression of lncRNA *HOTTIP* is an independent poor prognostic factor and might serve as a predictor of poor prognosis for TSCC patients [25]. *LCA1* is highly expressed in TSCC and might be correlated with cancer metastasis [26].

lncRNA actin filament associated protein 1 antisense RNA1 (*AFAP1-AS1*) is upregulated and acts as an oncogene in a variety of cancers, such as hepatocellular carcinoma, esophageal carcinoma, pancreatic ductal adenocarcinoma, colorectal cancer, cholangiocarcinoma, gallbladder cancer, and nasopharyngeal carcinoma [27–36]. However, the expression and detailed function of *AFAP1-AS1* in TSCC remains largely unknown and must be investigated. In this study, we sought to determine the expression of *AFAP1-AS1* in TSCC tissues and paired noncancerous tissues and the relationship between the expression of *AFAP1-AS1* and clinical characteristics. Further functional studies revealed that knockdown of *AFAP1-AS1* could result in the inhibition of cell proliferation and invasion in vitro and tumor growth in vivo.

**Methods**

**Human tissue samples**

Patients with TSCC who were diagnosed, treated, and followed up at the Department of Oral and Maxillofacial Surgery, The Second Xiangya Hospital, Central South University, Hunan, China, were included in the study. This study was approved by the hospital institutional review board and written informed consent was obtained from all the patients. All the protocols were reviewed by the Joint Ethics Committee of the Central South University Health Authority and performed following national guidelines. Tissue samples were collected at surgery, immediately frozen in liquid nitrogen and stored until total RNA or proteins were extracted.

**Quantitative real-time-PCR analysis**

The tissue sample was grinded in pre-chilled mortars with liquid nitrogen. TRIzol reagent (1 ml per 50–100 mg) was added when homogenizing. Then, the powders were transferred to 2-ml or 1.5-ml microcentrifuge tubes. The cultured cells were lysed directly in the dish with 0.3–0.4 ml of TRIzol reagent per 1 × 10⁵–10⁷ cells. Then, RNA was isolated from harvested cells, xenograft tumors, or human tissues with TRIzol reagent according to the manufacturer’s instructions (Invitrogen, CA, USA). Real-time PCR reactions were performed using SYBR Premix DimerEraser (Takara, Dalian, China), and human GAPDH was used as an endogenous control for mRNA detection. The expression of each gene was quantified by measuring Ct values and normalized using the 2^ΔΔct method relative to GAPDH. The gene-specific primers are shown in Table 1.

**Cells culture**

Human TSCC cell lines SCC-15, Tca8113, SCC-4, SCC-9 and CAL-27 cells were maintained in RPMI 1640 medium (Gibco, Waltham, MA, USA), supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 mg/ml streptomycin). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO2 in air.

**Vector construction and cell transfection**

The pLKO.1-puro vector used for the stable expression of shRNA against AFAP1-AS1 contained a puromycin
Table 1 The primers of the genes

| Gene name | Forward / Reverse primer(5'→3') |
|-----------|---------------------------------|
| AFAP1-AS1 | F: 5'-AATGGTGAGGGAAGGAGGA-3'  |
|           | R: 5'-CAACAGGAGGAAAGAAG-3'     |
| NANNOG    | F: 5'-GAACTCTCCACATCGTGA-3'    |
|           | R: 5'-TATCTTCGCCCATTGGTTT-3'   |
| CADN      | F: 5'-ATGCTCTACAAAGTGT-3'      |
|           | R: 5'-TGAGCTTGACTCATCAGT-3'    |
| NESTIN    | F: 5'-CCGGCTTAGAAGAATGTC-3'    |
|           | R: 5'-CTGAAAGCTGAGGAAAGG-3'    |
| SMAD22    | F: 5'-GAGGTTACCTCAAGAGG-3'     |
|           | R: 5'-CAGCAGGCTTCTTCAACAT-3'   |
| SLUG      | F: 5'-AGATCTCCGAGGAGAAG-3'     |
|           | R: 5'-GCATCGCGGAAATGTTCA-3'    |
| SNAIL1    | F: 5'-TCAAGATCCATTGAGGAGG-3'   |
|           | R: 5'-TGTCAGGAGGACATCTTG-3'    |
| SOX2      | F: 5'-TGGAAATTGTCGTGACGC-3'    |
|           | R: 5'-CACGGTGATTTATCTTGCT-3'   |
| TWIST1    | F: 5'-CAAGCGACCGTCTGGTAA-3'    |
|           | R: 5'-CCAGCGGCCTTCACCTCC-3'    |
| ZEB1      | F: 5'-GCAAAACGATGACGAGAAG-3'   |
|           | R: 5'-GCTGGAAGCTGAGGAAAGT-3'   |
| ZEB2      | F: 5'-GATGAAATAAGGGAGGTGG-3'   |
|           | R: 5'-CCTCAAATCAGTTGCAA-3'     |
| GAPDH     | F: 5'-ATCAAGATCATTGCTTCTGAG-3' |
|           | R: 5'-CTGCTTCTGCTGATACACAT-3'  |

resistance gene. The scrambled control shRNA sequence had no homology to any human genomic sequences. The cultured cells (5 × 10^5 cells/well) were seeded in 6-well culture plates and were maintained in RPMI 1640 medium with 10% FBS for 24 h before transfection. Cell transfection was performed using Lipofectamine 2000 (Invitrogen–Life Technologies, Carlsbad, CA, USA) as per the manufacturer’s instructions. For screening, puromycin (10 μg/mL) was added to the medium 72 h after transfection. The medium was replaced every 2 days for 2-3 weeks. CAL-27 and SCC-9 cells with high endogenous AFAP1-AS1 were selected for silencing. The expression of AFAP1-AS1 was confirmed by qRT-PCR. The sequence of AFAP1-AS1 shRNA and scrambled control shRNA were as follow: forward, 5'-CCGGAGACGGTCTCGACACGTGAAGATCTCGAGGAG-3' and reverse, 5'-AATTCAAAAAAGGGGTCTCAGCAGGAAATGACCTCTGAGGAGTCA TTGCTCTGAAGACCCGC T-3'; scrambled control shRNA, forward 5'-CCGGTTGTCCTCCGAAACGTGTCAC GTCTCGAGA GTGACACGTGTTTGAATTTCCTTGGT-3' and reverse, 5'-AATTCAAAAGGTCTCAGACGTTGT CACGTCTCGAGACGTGACCTCCAGGAGA-3'.

CCK-8 assay

Cell viability was determined using the CCK-8 assay. Briefly, 2000 cells/well were seeded into 96-well plates, and the absorptions of the cells were measured using a CCK-8 kit (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer’s instructions at different indicated times. Data were from three separate experiments with four replications each time.

Clone formation assay

From each group, nearly 1 × 10^4 cells were plated in each well of a 6-well culture plate. Each cell group consisted of three wells. The cells were incubated at 37 °C for 14 days with growth media being replaced every third day. Then, the cells were washed twice with PBS and stained with 0.5% crystal violet. The number of colonies containing ≥ 50 cells was counted under a microscope [plate clone formation efficiency = (number of colonies/number of cells inoculated) × 100%]. These experiments were performed in triplicate.

Cell cycle analyses by flow cytometry

Cell cycle analyses were performed using the Cell Cycle and Apoptosis Analysis Kit (Beyotime Institute of Biotechnology, Jiangsu, China) as per the manufacturer’s instructions. Cells were harvested and fixed in 70% ethanol overnight at 4 °C. Then, the cells were stained with 25 μg/ml propidium iodide containing 1 μg/ml RNase at 37 °C for 30 min. The cells were analyzed for their distribution in different phases of the cell cycle on FACScalibur flow cytometer using CellQuestPro software (Becton Dickinson, USA).

Cell migration/invasion assay

Cell migration was evaluated using a transwell assay. A total of 2 × 10^4 cells in 200 μl of serum-free medium were added to the top chamber of the transwell (8 μm pore size, BD Biosciences, New Jersey, USA) at 24 h after siRNA transfection. The bottom well contained growth medium with 20% FBS. Cells were incubated at 37 °C for 24 h. After 24 h, the cells that had migrated to the lower face of the filters were fixed with 100% methanol and stained with 0.5% crystal violet and counted. Matrigel invasion assays were performed as follows. Filters coated with Matrigel in the upper compartment were loaded with 200 μl serum-free medium containing 5 × 10^4 transfected cells, and the lower chamber was filled with 20% FBS. After 24 h, the migrated cells on the bottom surface were fixed with 100% methanol and counted after staining with 0.5% crystal violet. Numbers of invaded cells were counted in six randomly selected fields under a microscope, and the average value was calculated. Each experiment was conducted in triplicate.
Wound-healing assay
Cells were cultured until they reached 90% confluence in 6-well plates. Cell layers were scratched using a 10-μL tip to form wounded gaps, washed with PBS twice and cultured. The wounded gaps were photographed at different time points and analyzed by measuring the distance of migrating cells from five different areas for each wound.

Western blotting
For total cell lysates, cells were lysed in lysis buffer that contained 25 mM Tris (pH 7.4), 2 mM NaVO4, 10 mM NaF, 10 mM Na4P2O7, 1 mM EGTA, 1 mM EDTA, and 1% NP-40. Protease inhibitor cocktail and PhosSTOP were added fresh to the lysis buffer before each experiment. The proteins were separated on SDS-PAGE and then transferred to PVDF membrane (Merck Millipore). The membrane was blocked in Tris-buffered saline (TBS; pH 7.4) with 5% skim milk for 2 h, and then, the membranes were incubated overnight at 4 °C with diluted primary antibodies overnight. Antibodies against β-catenin (#8480), AKT (#9272), phospho-AKT (#9271), GSK3β (#12456) and phospho-GSK3β (#5558) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against AFAP1 (sc-374,655) and GAPDH (sc-32,233) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After incubation, the membranes were washed three times in TBST, followed by incubation with secondary antibody (1:5000, Santa Cruz Biotechnology, CA, USA) in TBST 10% blocking reagent for 1 h, and washed again in TBST (3 times for 20 min). Immunoblots were developed using ChemicalDocTM XRS+ (Bio-Rad, Berkeley, CA, USA). The intensity of the protein fragments was quantified using QuantityOne software (Bio-Rad, Berkeley, CA, USA).

Tumorigenicity assays in nude mice
All the animal procedures were performed in accordance with local institutional ethical guidelines. Ethical approval was obtained from the Experimental Animal Ethics Committee of the Central South University Health Authority. CAL-27 cells (1 × 10^7) with stably transfected shR-AFAP1-AS1 or shR-NC were suspended in 0.1 ml PBS and injected into the left flank of 4-week-old female BALB/c athymic nude mice. Tumor volumes were calculated using hand calipers every week after the injection using the following formula: tumor volume (mm^3) = (length × width^2)/2. At 35 days, mice were sacrificed and tumor volumes and weights were recorded.

Statistical analysis
All experiments were performed three times, and the data were analyzed with GraphPad Prism 5 (La Jolla, CA, USA). Results are presented as mean ± SD. The differences between TSCC tissues group and the adjacent normal tissues group were tested using paired Student’s t-test. Differences between groups were tested using paired and unpaired Student’s t-test, a one-way ANOVA and χ^2 tests where appropriate with the SPSS 17.0 program. A p-value of <0.05 was considered statistically significant.

Results
LncRNA AFAP1-AS1 was upregulated in TSCC tissues and associated with TSCC progression
To explore the role of lncRNA AFAP1-AS1 in TSCC, we examined the relative expression of AFAP1-AS1 in TSCC tissues (n = 103) compared with that of adjacent normal tissues (n = 103). Quantitative real-time PCR (qRT-PCR) assays showed that the expression AFAP1-AS1 was significantly increased in TSCC samples compared with the adjacent normal tissues (Student’s t-test, P < 0.001, Fig. 1a). In addition, AFAP1-AS1 expression was higher in advanced stage lesions (stage II-IV) than in low stage I lesions (P < 0.01, Fig. 1b). These changes in AFAP1-AS1 expression were further evaluated within TSCC cell lines by analyzing the expression of AFAP1-AS1 in SCC-15, Tca8113, SCC-4, SCC-9 and CAL-27. As shown in Fig. 1c, the expression level of AFAP1-AS1 was upregulated in TSCC cell lines compared to normal tissues.

Relationship between AFAP1-AS1 expression and the clinicopathological features of patients with TSCC
We further assessed the association between the expression of AFAP1-AS1 and clinicopathologic characteristics of TSCC patients. For this analysis, we classified the TSCC tissues into two groups according to the relative AFAP1-AS1 expression: the low AFAP1-AS1 expression group (n = 42) in which the expression was lower than the median value and the high AFAP1-AS1 expression group (n = 61) in which the expression was higher than or equal to the median value. Patient characteristics and correlations with AFAP1-AS1 overexpression are summarized in Table 2. AFAP1-AS1 was significantly correlated with tumor differentiation (P < 0.05), T classification (P < 0.05), clinical stage (TNM, P < 0.05), depth of invasion (P < 0.05) and relapse (P < 0.01). No statistically significant correlations were obtained between AFAP1-AS1 and other clinicopathological characteristics, such as age, gender or betel-quid (BQ) chewing habit (P > 0.05, Table 2). A Kaplan-Meier analysis was used to evaluate the relationship between AFAP1-AS1 expression in TSCC and patient survival, and the results showed that high AFAP1-AS1 expression was also associated with poor survival. The survival time of patients with low AFAP1-AS1 expression (n = 42) was longer than in patients with high AFAP1-AS1 expression (n = 61) (P < 0.05,
Fig. 2a). The survival time of patients with clinical stage I \((n = 30)\) was longer than in patients with advanced stage lesions \((n = 73)\) \((P < 0.05\), Fig. 2b). \n
AFAP1-AS1 knockdown markedly suppressed the proliferation and arrested the cell cycle of TSCC cells in vitro

To examine the role of AFAP1-AS1 in TSCC cell proliferation. We knocked down the expression of AFAP1-AS1 with short hairpin RNA (shRNA) in the SCC-9 and CAL-27 cell lines. The AFAP1-AS1 expression was detected to confirm the efficiency of silencing (Fig. 3a). The results demonstrated that the shRNA could efficiently knock down AFAP1-AS1 expression by at least 50% in each of these cell lines. Next, we assessed the phenotype changes induced by AFAP1-AS1 knockdown in TSCC cell lines. As shown in Fig. 3, AFAP1-AS1 knockdown inhibited cell growth of SCC-9 and CAL-27 cell lines (Fig. 3b). Moreover, the number of colonies formed was markedly decreased in the AFAP1-AS1 knockdown cells (Fig. 3c). Further, the downregulation of AFAP1-AS1 induced G0-G1 cell cycle arrest, resulting in a considerable decrease of cell percentage in S phase and an increase of cell percentage in G0/G1-phase compared with the control group (Fig. 3d).

Silencing AFAP1-AS1 inhibited the migration and invasion of TSCC cells in vitro

Next, we studied whether AFAP1-AS1 could affect the migration and invasion of TSCC cells. A directional invasion was examined using a transwell assay with Matrigel coated in the upper compartment. The results showed that the invasion of SCC-9 (upper) and CAL-27 (lower) cells was notably decreased with AFAP1-AS1 knockdown (Fig. 4a). Furthermore, we investigated the effect of AFAP1-AS1 on cell migration by performing a transwell assay without Matrigel coating in the upper compartment and a wound-healing assay. Compared with the cells treated with control shRNA, we observed that knockdown of AFAP1-AS1 could attenuate the migratory ability of both TSCC cells (Fig. 4b and c).

Inhibition of AFAP1-AS1 decreased the activity of the Wnt/β-catenin pathway and suppressed the expression of EMT-related genes

AFAP1-AS1 is the antisense RNA of AFAP1. We demonstrated that AFAP1-AS1 knockdown increased AFAP1 protein levels (Fig. 5a and b). The Wnt/β-catenin pathway and EMT play important role in tumor cell migration and invasion. We investigated the consequences of AFAP1-AS1 knockdown on the activation of the Wnt/β-
Table 2 Association of lncRNA AFAP1-AS1 expression with clinicopathologic features in TSCC patients

| Parameter                        | Total N = 103 | LncRNA AFAP1-AS1 Low n = 42(%) | High n = 61(%) | χ²  | P     |
|----------------------------------|---------------|---------------------------------|----------------|-----|-------|
| Age (years)                      |               |                                 |                |     |       |
| < 50                             | 36            | 17(47.2)                        | 19(52.7)       | 0.952 | 0.329 |
| ≥ 50                             | 67            | 25(37.3)                        | 42(62.7)       |     |       |
| Gender                           |               |                                 |                |     |       |
| Female                           | 31            | 13(41.9)                        | 18(58.1)       | 0.025 | 0.875 |
| Male                             | 72            | 29(40.3)                        | 43(59.7)       |     |       |
| Betel-quid (BQ)³ chewing habit   |               |                                 |                |     |       |
| No                               | 38            | 20(52.6)                        | 18(47.4)       | 3.504 | 0.061 |
| Yes                              | 65            | 22(33.8)                        | 43(66.2)       |     |       |
| Tumor differentiation            |               |                                 |                |     |       |
| Well/moderate                    | 69            | 33(47.8)                        | 36(52.2)       | 4.301 | 0.038 |
| Poor                             | 34            | 9(26.5)                         | 25(73.5)       |     |       |
| T classification (TNM)           |               |                                 |                |     |       |
| T1-T2                            | 61            | 30(49.2)                        | 31(50.8)       | 4.375 | 0.036 |
| T3-T4 Clinical stage             | 42            | 12(28.6)                        | 30(71.4)       |     |       |
| Depth of invasion                |               |                                 |                |     |       |
| <1 cm                            | 46            | 25(50.0)                        | 21(50.0)       | 6.339 | 0.012 |
| ≥1 cm                            | 57            | 27(47.4)                        | 40(66.7)       |     |       |
| Relapse                          |               |                                 |                |     |       |
| No                               | 63            | 32(50.8)                        | 31(49.2)       | 6.740 | 0.009 |
| Yes                              | 40            | 10(25.0)                        | 30(75.0)       |     |       |
| Status                           |               |                                 |                |     |       |
| Alive                            | 66            | 32(48.5)                        | 34(51.5)       | 4.52 | 0.033 |
| Dead                             | 37            | 10(27.0)                        | 27(73.0)       |     |       |

³Betel-quid (BQ), also called betel nut or areca nut, is one of the most commonly consumed psychoactive substances [62]. Long-term BQ chewing is strongly associated with oral precancerous conditions, including oral submucous fibrosis (OSF), and cancers of the oral cavity, pharynx, esophagus, and larynx [63–65]. Consequently, the International Agency for Research on Cancer categorized BQ as a Group 1 carcinogen in 2004 [66]. It is estimated that approximately 600 million people chew various types of BQ worldwide, predominantly in the countries of South and Southeast Asia [67]. In Mainland China, BQ chewing is mainly practiced in Hunan and Hainan provinces.

Fig. 2 Kaplan-Meier curves for overall survival in TSCC patients according to AFAP1-AS1 expression. a The survival time of patients with low AFAP1-AS1 expression (n = 42) was longer than that in patients with high AFAP1-AS1 expression (n = 61). b The survival time of patients with clinical stage I (n = 30) was longer than that in patients with clinical stage II-IV (n = 73)
catenin pathway and EMT in TSCC cells. To test the effect of AFAP1-AS1 knockdown on the Wnt/β-catenin signaling pathway, we analyzed the expression of several key proteins of this signaling by western blotting. Compared with the control group, AFAP1-AS1 silencing could decrease the phosphorylation of AKT and GSK3β and the total level of β-catenin in both SCC-9 (Fig. 5a) and CAL-27 (Fig. 5b). Total levels of AKT and GSK3-β did not show obvious differences. Additionally, to demonstrate the mechanism of AFAP1-AS1 acting as an oncogene in TSCC cells, the expression of EMT-related genes was determined by qRT-PCR. Downregulation of AFAP1-AS1 significantly downregulated EMT-related genes SLUG, SNAI1, VIM, CADN, ZEB1, ZEB2, and TWIST1 in SCC-9 cells (Fig. 5c). The expression of SLUG, VIM, CADN, ZEB1, SMAD2, and TWIST1 were downregulated in CAL-27 cells after inhibiting the expression of AFAP1-AS1 (Fig. 5d).

**Downregulation of AFAP1-AS1 suppressed tumor growth and inhibited the expression of EMT-related genes in vivo**

Downregulation of AFAP1-AS1 decreased Wnt/β-catenin signaling and blocks TSCC cell proliferation, invasion and migration, as verified in SCC-9 and CAL-27 cell lines. We next studied the effect of AFAP1-AS1 on tumor growth in vivo. Thus, we developed a nude mouse model with TSCC xenografts. CAL-27 cells infected with sh AFAP1-AS1 or nonspecific shRNA control were subcutaneously injected into each flank of nude mice. After 35 days, xenografted tumors were visible, and all the mice were sacrificed to harvest the xenografts (Fig. 6a). As shown in Fig. 6a and b, AFAP1-AS1 knockdown can significantly suppress the growth of TSCC xenografts in nude mice after CAL-27 cell subcutaneous inoculation. Tumor size was measured, and a smaller size was observed in the AFAP1-AS1 silencing group (Fig. 6c). The average tumor weight in the AFAP1-AS1 silencing group
was significantly lighter, compared to the control group (Fig. 6d). QRT-PCR analysis indicated that knockdown of \textit{AFAP1-AS1} led to a decrease of \textit{AFAP1-AS1} expression (Fig. 6e). In addition, the mRNA level of EMT-related genes was also detected. As shown in Fig. 6f, \textit{SLUG}, \textit{SNAIL1}, \textit{VIM}, \textit{ZEB1}, \textit{NANOG}, \textit{SMAD2}, \textit{NESTIN} and \textit{SOX2} were downregulated in the mouse model.

**Discussion**

In recent years, aberrant expression or dysfunctional activities of lncRNAs has been discovered in multiple tumor types, and substantial evidence indicates that lncRNAs participate in all steps of tumor initiation and development [6, 13, 17, 37]. Many studies have shown that lncRNAs are of great importance in the diagnosis and treatment of tumors, and that lncRNAs are useful as novel prognostic tumor biomarkers [22, 38–42]. LncRNA \textit{AFAP1-AS1} is oriented in an antisense direction to the protein-coding gene \textit{AFAP1} in the opposite strand [36, 43, 44]. High expression of \textit{AFAP1-AS1} is associated with malignancy, metastasis and poor prognosis in various cancers, such as hepatocellular carcinoma, nasopharyngeal carcinoma, esophageal carcinoma, colorectal cancer, cholangiocarcinoma, and gallbladder cancer [27–35]. \textit{AFAP1-AS1} is upregulated in hepatocellular carcinoma, and its higher expression is associated with tumor size, TNM stage, vascular invasion, and poor prognosis [45]. Furthermore, silencing \textit{AFAP1-AS1} significantly reduce cell proliferation, clonal growth, cell migration, and invasion and increase apoptosis through RhoA/Rac2 signaling [31, 45]. LncRNA \textit{AFAP1-AS1} also promote tumor growth and invasion in cholangiocarcinoma [29, 32],
esophageal squamous cell carcinoma, nasopharyngeal carcinoma and gallbladder cancer [27, 28, 34, 35]. Consistent with these studies, in the present study, IncRNA AFAP1-AS1 was also upregulated in TSCC tissues when compared with pair matched normal tissues, and the upregulation of AFAP1-AS1 was associated with clinical characteristics and poor overall survival. We also determined the effects of AFAP1-AS1 on TSCC cells. Inhibition of AFAP1-AS1 inhibited the proliferation, migration and invasion of TSCC cells in vitro and in vivo. Collectively, our results demonstrated that AFAP1-AS1, acting as an oncogene, may be a potential diagnostic and prognostic biomarker as well as a therapeutic target in TSCC.

Cell migration and invasion are significant aspects of cancer progression, and the EMT is a critical biological process in tumor cell migration and invasion [5–7, 46, 47]. The Wnt/β-catenin pathway plays a critical role in cellular proliferation, survival, differentiation and EMT in cancer cells [48–50]. When Wnt ligands bind to transmembrane receptors, Wnt signaling can be initiated [49]. β-catenin accumulates in the nucleus and forms the β-catenin/TCF/LEF transcriptional complex [48]. As a result, Wnt target genes are activated and promote EMT [49]. Accumulating evidence supports that many important oncogenes or tumor suppressor genes regulate the Wnt/β-catenin signaling pathway, and thus...
modulate EMT, migration and invasion in tumor cells [51–54]. In addition, lncRNAs also participate in this regulatory process [24, 55–57]. LncRNA UCA1 enhances the Wnt/β-catenin signaling pathway to promote the EMT of breast cancer cells [55]. LncRNA PTCSC3 inhibits the proliferation and invasion of glioma cells by suppressing the Wnt/β-catenin signaling pathway [56]. In tongue cancer, MALAT1 induces EMT and inhibits apoptosis through the Wnt/β-catenin signaling pathway [24]. Moreover, overexpression of lncRNA CTD903 inhibits colorectal cancer invasion and migration by repressing Wnt/β-catenin signaling and predicts a favorable prognosis [57]. In the present study, we tested the effect of AFAP1-AS1 knockdown on the Wnt/β-catenin signaling pathway. Compared with the control group, silencing AFAP1-AS1 decreased the phosphorylation of AKT and GSK3β and the total level of β-catenin in both SCC-9 and CAL-27. Total levels of AKT and GSK3β did not show obvious differences. Additionally, the expression of EMT-related genes was determined by qRT-PCR. Downregulation of AFAP1-AS1 significantly downregulated EMT-related genes SLUG, SNAIL1, VIM, CADN, ZEB1, ZEB2, and TWIST1 in SCC-9 cells. The expression levels of SLUG, VIM, CADN, ZEB1, SMAD2, and TWIST1 were down-regulated in CAL-27 cells after inhibiting the expression of AFAP1-AS1. Additionally, we obtained similar results in the xenograft tumor model. SLUG, SNAIL1, VIM, ZEB1, NANO2, SMAD2, NESTIN, and SOX2 were down-regulated when the expression of AFAP1-AS1 was silenced in xenograft tumors. The above data indicated that AFAP1-AS1 could regulate the activity of the Wnt/β-catenin signaling pathway and affect the expression of several EMT-related genes in TSCC cells.

Previous studies have demonstrated that IncRNA activity was partly dependent on its genomic location. Antisense IncRNAs such as AFAP1-AS1 are oriented in an antisense direction with respect to a protein-coding
gene in the opposite strand and usually act as a regulator of this gene [58–60]. AFAP1-AS1 is localized in the antisense DNA strand of the AFAP1 gene [61]. We demonstrated that AFAP1-AS1 expression increased AFAP1 protein levels. However, it remains unknown whether the effects of AFAP1-AS1 on the regulation of tumor cell metastasis potential are mediated by the changed AFAP1 protein levels.

Conclusions
In conclusion, our study provided the first evidence that AFAP1-AS1 was highly expressed in TSCC tumor tissues and cell lines. The increased expression of IncRNA AFAP1-AS1 was associated with malignant behavior and poor prognosis in TSCC. Furthermore, the knockdown of AFAP1-AS1 by shRNA significantly inhibited proliferation, invasion, migration and clone formation and arrested the cell cycle in vitro. Silencing AFAP1-AS1 partially intended tumor growth in vivo. AFAP1-AS1 acted as an oncogene in TSCC through a mechanism of activating the Wnt/β-catenin signaling pathway and inhibiting the expression of EMT-related genes. Collectively, IncRNA AFAP1-AS1 is a promising biomarker and therapeutic target for human TSCC treatment.

Abbreviations
AFAP1-AS1: Actin filament associated protein 1 antisense RNA; AR: Androgen receptor; EMT: Epithelial-mesenchymal transition; IncRNA: Long Non-Coding RNA; qRT-PCR: Quantitative real-time PCR; RCC: Renal cell carcinoma; shRNA: Short hairpin RNA; TSCC: Tongue Squamous Cell Carcinoma

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Availability of data and materials
Due to our institutional policy, raw data cannot be shared.

Authors’ contributions
ZW, SZ and ZG mainly performed the experiments, analyzed the data and wrote the paper. SZ and MD helped with the experiments and analyzed the data. HP performed western blot and prepared the samples. MD helped with the protocols. SZ and HW carried out the experiment design and manuscript drafting. SZ performed construction of vectors and cells culture and transfection. All authors had edit and approved the final manuscript.

Ethics approval and consent to participate
This study was conducted at Department of Oral and Maxillofacial Surgery, The Second Xiangya Hospital, Central South University, Hunan, China. All of the protocols were reviewed and approved by the Joint Ethics Committee of the Central South University Health Authority and performed in accordance with national guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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