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LncRNA AC007271.3 promotes cell proliferation, invasion, migration and inhibits cell apoptosis of OSCC via the Wnt/β-catenin signaling pathway

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ABSTRACT

Aims: Long noncoding RNA (lncRNA) AC007271.3 has been identified to be dysregulated in oral squamous cell carcinoma (OSCC) in our previous study. However, the precise role of AC007271.3 in OSCC remains unclear. In this study, we investigated the potential functions and the underlying mechanisms of AC007271.3 in OSCC.
Materials and methods: The expression levels of AC007271.3 in OSCC tissues and cell lines were examined using RT-qPCR. The relationship between AC007271.3 level and clinicopathological characteristics was analyzed, and its association with patient prognosis was assessed by the Kaplan–Meier method. The biological function of AC007271.3 and its role in the development of OSCC through Wnt/β-catenin signaling pathway were studied.
Key findings: We identified that AC007271.3 was up-regulated and positively correlated with advanced clinical stage, lymph node metastasis, poor histological differentiation and unfavorable prognosis. We explored the expression, function, and molecular mechanism of AC007271.3 in OSCC cells. Overexpression of AC007271.3 remarkably promoted cell proliferation in vitro and in vivo, induced cell migration, invasion and inhibited apoptosis in vitro, while knockdown of AC007271.3 attenuated cell proliferation, migration, invasion and induced apoptosis. Mechanistically, AC007271.3 overexpression substantially increased the expression of β-catenin and the downstream target molecules CyclinD1, c-myc and Bcl-2, while silencing of AC007271.3 has the opposite effect. Rescued experiments showed that the ability to promote cell proliferation, migration, invasion and inhibiting apoptosis could be reversed when treated with the Wnt/β-catenin pathway inhibitor.
Significance: Our data indicated that AC007271.3 could promote cell proliferation, invasion and inhibit cell apoptosis of OSCC via the Wnt/β-catenin signaling pathway, which might provide a novel therapeutic approach for OSCC.

1. Introduction

Oral squamous cell carcinoma (OSCC) is one of the most frequent head and neck tumors, accounting for approximately 3% of all newly diagnosed clinical cancer cases [1]. The clinical characteristics of OSCC include invasive growth, regional metastasis and high recurrence rate [2]. Despite the improved treatment results, the average survival of OSCC cannot achieve satisfactory result at present [3]. Although some progresses have been made in the research field of OSCC, its precise underlying mechanisms are still unknown. Therefore, novel diagnostic biomarkers and therapeutic strategies are urgently needed in order to improve the prognosis of patients with OSCC.

Long noncoding RNAs (lncRNA) are > 200 base pairs in length and lack protein-coding function [4]. Recent studies have shown that lncRNAs play crucial roles in various physiological and pathological processes, such as carcinogenesis, cell proliferation, differentiation and apoptosis [5,6]. In addition, studies have identified that dysregulation of lncRNAs may be associated with cancer progression in some malignant tumors and lncRNAs can act as oncogenes and tumor suppressors [7,8].

AC007271.3, also named as ENST00000428188 and located on chromosome 2:102172621–102,182,108, is an antisense and 382 bp

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long lncRNA. We previously identified that AC007271.3 was upregulated in OSCC than in matched non-tumor samples (MNT) by microarray analysis and higher serum AC007271.3 levels of OSCC patients were significantly associated with clinical stage and poorer prognosis [9]. However, the underlying mechanism of AC007271.3 in OSCC carcinogenesis keeps unclear until now.

The Wnt/β-catenin signaling pathway, one of the canonical pathways in the process of cell signal transduction, plays a key role in human diseases, especially in cancers, probably through affecting growth, development and metabolism. The key event in the activation of Wnt/β-catenin signaling is accumulation of β-catenin signaling in the cytoplasm followed by its translation and activation in the nucleus [10]. Then, β-catenin interacts with lymphoid-enhancing factor/T-cell factor (LEF/TCF) family in the nucleus which activates target gene transcription, such as Cyclin D1, c-myc, Bcl-2 and so on, so as to affect cell proliferation, differentiation and tumorigenesis [11,12].

In the present study, we investigated the expression of AC007271.3 in OSCC and MNT. Then, we evaluated the clinical significance and biological function of AC007271.3 in OSCC. We found that AC007271.3 could promote OSCC carcinogenesis through the activation of Wnt/β-catenin signaling pathway, which provided a new approach for clinical treatment of OSCC.

2. Materials and methods

2.1. Patients and samples

From September 2014 and December 2018, OSCC samples were obtained from 97 patients of the Department of Oral and Maxillofacial Surgery, NanFang Hospital before surgery and from healthy controls. The matched normal mucosal tissues (MNTs) located at least 1.5 cm from the unaffected margins of tumors were defined as normal controls. The OSCC/MNT samples were immediately stored in RNAwait (SR0020; Solarbio, Beijing, China) and then stored at −80 °C until use. Clinicopathological data were collected. Tumor stages were determined according to the Union of International Control of Cancer (UICC) classification. All patients were informed about the aims of specimen collection and gave signed written consent in accordance with the guidelines of NanFang Hospital Ethics Committee (NFEC-2018-027).

2.2. Cell culture and transfection

Normal human oral epithelial cell line (HOK) and oral squamous cell lines (SCC9, SCC15 and SCC25) were gifted from the Institute of Antibody Engineering, Southern Medical University (Guangzhou, China). The cell lines were maintained in DEME/F12 (Invitrogen, Carlsbad, CA, USA) medium supplemented with 10% FBS (Life Technologies, Carlsbad, CA, USA) with 5% CO_2 at 37 °C.

SCC9, SCC15 or HOK was used; pcDNA-AC007271.3 overexpression vector (Lv-AC007271.3) and its corresponding control vector (Lv-NC) were constructed by Obio Technology Corp., Ltd. (Shanghai, China). Specific siRNAs targeting AC007271.3 (si-AC007271.3) and scramble negative control siRNA (si-NC) were purchased from GenePharma Corp., Ltd. (Shanghai, China), for RNAi-mediated knockdown experiment. The vectors were transfected into cells using liposomes (Invitrogen, USA) following the manufacturer's protocols.

2.3. Cell viability and proliferation detected by CCK-8 and colony formation assay

SCC9 and SCC15 cells were sealed in 96-well plates, with 200 μL DMEM/F12 culture medium (approximately 2×10^3 cells per well) and set three relicated wells in each group. The Cell Counting Kit-8 (CCK-8, TransDetect, China) was used to analyze the cell viability at 0 h, 24 h, 48 h, 72 h, 96 h, respectively after RNAi-mediated knockdown treatment following the manufacturer's instruction. Results were measured according to the reference-subtracted absorbance at 450 nm with the enzyme-linked immunosorbent assay reader (Bio-Rad).

SCC9 and SCC15 cells were treated by AC007271.3 knockdown or overexpression for 72 h and sealed in 6-well plates (approximately 2000 cells per well), then the media were renewed. After culturing for another 7 days, the cells were washed twice with PBS, fixed in methanol for 30 min and then stained with Giemsa stain. We counted the number of visible colonies under the microscope. The colony formation ability was calculated as follows: (visible colonies/seeded cells) × 100%.

2.4. Cell migration and invasion assays

Cell migration was evaluated by the wound healing assay. SCC9 and SCC15 cells were plated in 6-well plates. When cells grew to confluence at approximately 80%, inserts were removed with sterile forceps to create a wound by using a 200 μL plastic pipette tip. We measure the wound-healing distance at the 0 and after 24 h to assess cell migration.

Invasiveness was measured using 24-well BioCoat cell culture inserts (Corning, NY, USA) containing a polyethylene terephthalate membrane (8-μm pores) coated with Matrigel Basement Membrane Matrix (1:12 dilution, BD Biosciences, San Jose, CA, USA). Transfected cells were plated at a density of approximately 5×10^4 cells in 100 μL serum-free F12. Cells were plated onto the upper chambers (24-well insert, pore size 8 μm; Corning, NY, USA), and the lower chambers were filled with 600 μL F12 with 20% FBS. After 36 h of incubation, the membranes were fixed with 4% methanol, stained by 0.1% crystal violet, and washed three times. At the end of the assay, the cells that did not migrate through the pores were removed with a cotton swab. Invasiveness was determined by the cells migrated to the lower side of the filter.

2.5. Flow cytometry analysis for apoptosis

The Annexin V-allophycocyanin apoptosis detection kit (TransDetect, China) was used to identify apoptotic cells. Briefly, SCC9 or SCC15 cells were seeded in 6-well plates after treated by AC007271.3 knockdown or overexpression for 48 h as described. After being trypsinized and resuspended with PBS, the cells were then stained with Annexin V–fluorescein isothiocyanate (FITC-Annexin V) and propidium iodide (PI) successively in the staining buffer at 4 °C in dark. We detected the apoptotic cells by flow cytometry with the FACSCalibur system (BD Biosciences, San Diego, CA, USA).

2.6. Immunofluorescence assay

After culture on the microscope cover glass (Thermo Fisher Scientific, Waltham, MA, USA), cells were transfected. After incubation for 48 h, SCC9 or SCC15 cells were fixed with 4% formaldehyde and blocked in 10% BSA for 30 min. Slides were then incubated with a rabbit monoclonal anti-human β-catenin (1:100; Proteintech) overnight at 4 °C, washed with PBS, and incubated with an Alexa Flour 555 conjugated anti-rabbit IgG secondary antibody (1:750; CST) for 45 min. The SCC9 or SCC15 cells were washed three times with PBS, stained with DAPI (Merck, Darmstadt, Germany), and then washed three times with PBS. We acquired the images with the fluorescence microscope (OLYMPUS FV10-MCPSU) and FV10-ASW 1.7 viewer.

2.7. RNA extraction and real-time quantitative PCR (RT-qPCR) analysis

RNA extraction from tissues and cultured cells was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), whereas total RNA in serum was performed using TRIzol LS Reagent (Ambion, Waltham, MA, USA) following the manufacturer's protocols (9). The quantitative detection of lncRNA was performed on the ABI Step one Real-Time PCR System (Applied Biosystems, San Diego, CA, USA) according to the instructions. The reactions were performed in triplicate. Gene

Table 1
The sequences of primers used in RT-qPCR.

Primers	Sequences	
	Sense (5′-3′)	Antisense (5′–3′)
AC007271.3	TACAGGCTCAACTGGTCTAACAGC	TGAAGAAGGCTTGGCTGCTC
CyclinD1	GAGACCATCCCCCTGACGGC	TCTTCCTCCTCCTCGGCGGC
β-catenin	TGCAGTTCG CCTTCACTATG	ACTAGTCGTGGAATGGCACC
c-myc	GCCCAGTGAGGATATCTGGA	ATCGCAGATGAAGCTCTGGT
GAPDH	CGCTGAGTACGTCGTGGAGTC	GCTGATGATCTTGAGGCTGTTGTC

expression was normalized to GAPDH expression, and calculated based on the $2^{-\Delta\Delta CT}$ method. The primers were designed and synthesized by Sangon Biotech (Shanghai, China). (Table 1).

2.8. Western blot analysis

Cells were washed twice with ice-cold PBS and total proteins were lysed in RIPA buffer (Beyotime Biotechnology) supplemented with protease inhibitors. The protein concentrations were determined using BCA Protein Assay Kit (Beyotime Biotechnology). Identical quantities of proteins were separated on 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with primary anti-body overnight at 4 °C, 1 h with the secondary antibody at room temperature, followed by ECL reagent (Millipore, Bedford, MA) for chemiluminescent detection. Primary antibody of β-catenin, CyclinD1, c-myc, Bcl-2 and GAPDH (Proteintech, China) were used for WB analysis. The bands were then scanned by an Imaging System and quantified by Quantity One v4.6.2 software (Bio-Rad). Positive bands were semiquantified by densitometrical analyses using ImageJ.

2.9. RNA-fluorescence in situ hybridization (FISH)

FISH was performed according to the manufacturer's protocol (Biosearch Technology, Petaluma, California). Hybridization was performed using a digoxin-labeled (Takara Biomedical Technology, Dalian, China) AC007271.3 cDNA probe. SCC9 and SCC15 cells were observed with the Zeiss (LSM780) confocal laser scanning microscope.

2.10. In vivo tumorigenesis assay

All protocols for mouse experiments was approved by Southern Medical University Experimental Ethics Committee (L2018199). A total of 10 BALB/c nude mice (5 weeks old, 12–14 g, female) were purchased from the Animal Care Unit of Southern Medical University, and maintained under specific pathogen-free conditions. SCC-9 cells (1×10^6, 100 μL) stably infected with LV-AC007271.3 or LV-NC as aforementioned, were subcutaneously injected into right side of the armpit regions of each mice, with 5 mice per group. The mice were housed for 4 weeks, then were sacrificed. The formed tumors were excised and photographed. Xenograft volumes were evaluated.

2.11. Analysis of the activity of Wnt/β-catenin signaling pathway

TOP/FOP flash reporters (Howard Hughes Medical Institute, University of Washington, USA) were used to assess the impacts of AC007271.3 on the Wnt/β-catenin signaling pathway in SCC9 and SCC15 cells. TOP-Flash and FOP-Flash luciferase reporter vector and LV- AC007271.3 or si-AC007271.3 vector were co-transfected into OSCC cells. After incubation for 48 h, luciferase activity was measured by dual-luciferase assay kit. Fractionation of OSCC cells was carried out using the Nucleoprotein and Cytoplasmic protein Extraction Kit (P2007 400–1,683,301/800–8,283,301, Beyotime). We extracted nuclear protein and cytoprotein respectively according to the manufacturer's protocols. The expression level of β-catenin protein was measured by Western blotting. The nucleoprotein expression was normalized to H3 expression, while the cytoplasmic protein expression was normalized to GAPDH expression.

2.12. Statistical analysis

Statistical tests for data analysis included student's paired *t*-test or analysis of variance, the Wilcoxon test and χ^2 test. We used the Kaplan-Meier method to create survival curves for OSCC patients with high or low AC007271.3 expression. Data were presented as mean ± SE. Statistical analyses were performed with SPSS (Version 22, IBM, Armonk, NY, USA) and presented graphically in GraphPad Prism 6.0 (GraphPad, La Jolla, CA, USA). All tests were two-tailed and $P < .05$ was considered statistically significant.

3. Results

3.1. High expression of AC007271.3 in OSCC tissues is associated with clinicopathological characteristics and poor prognosis

Our previous study revealed that lncRNA AC007271.3 was obviously up-regulated in 30 OSCC tissues, 3 OSCC cell lines and 80 serum of OSCC patients in contrast to matched adjacent normal tissues, normal oral epithelial cell line HOK and healthy controls [9]. To further confirm the role of AC007271.3 in OSCC, we detected the AC007271.3 levels in OSCC and MNT tissues of 97 patients. Based on the average AC007271.3 expression level, we divided the 97 patients into two groups, 36 patients with low expression and 61 with high expression. The results of qPCR showed that the relative expression level of AC007271.3 was higher in OSCC tissues compared with the MNT (Fig. 1A). Furthermore, we analyzed the association between AC007271.3 expression and clinicopathological parameters of the patients. The results indicated that the expression of AC007271.3 was positively correlated with advanced clinical stage, lymph node metastasis and poor histological differentiation in OSCC patients (Table 2), and were consistent with those in serum [9]. Kaplan-Meier survival analysis revealed that patients with high AC007271.3 expression had significantly decreased overall survival (OS) compared with those with low AC007271.3 expression (Fig. 1B), which was also in agreement with that in serum [9]. Collectively, these data indicated that the up regulation of AC007271.3 might be involved in the pathogenesis of OSCC, and be a potential predictive biomarker for disease outcome and prognosis in OSCC patients.

3.2. AC007271.3 triggers OSCC cells biological properties in vitro

To further investigate the cellular function of AC007271.3 in OSCC, gain-of-function and loss-of-function assays were performed in OSCC cells.

OSCC cells (SCC9 and SCC15) were cultured and transfected with AC007271.3 specific siRNA (si-AC007271.3) or AC007271.3 expression vector (Lv-AC007271.3). The knockdown efficiency in SCC9 and SCC15 were approximately 84% and 68% respectively after transfection with AC007271.3 knockdown. The relative expression level of AC007271.3

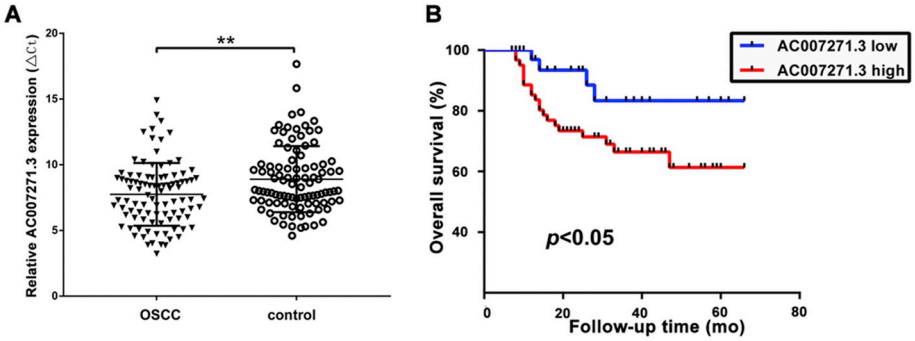

Fig. 1. A, The expression of AC007271.3 in OSCC tissues and in the MNT, measured by qPCR ($n = 97$, $P < .001$). B, Kaplan–Meier survival curve analysis indicated that OSCC patients with lower AC007271.3 expression had prolonged survival time compared with patients with high levels of AC007271.3 (log-rank test, $P < .05$).

Table 2
The correlation between AC007271.3 expression levels and clinicopathological characteristics in 97 OSCC patients.

Characteristics	No. of patients	AC007271.3 expression level		*P*-value
		Low expression $n = 36$ (%)	High expression $n = 61$ (%)	
Age, years				0.6220
< 60	40	16 (16.49)	24 (24.74)	
≥60	57	20 (20.62)	37 (38.14)	
Gender				
Male	61	23 (23.71)	38 (39.18)	0.8753
Female	36	13 (13.40)	23 (23.71)	
TNM classification				**0.0418**
I and II	41	20 (20.62)	21 (21.65)	
III and IV	56	16 (16.49)	40 (41.24)	
Lymphatic metastasis				**0.0093**
N_0	48	24 (24.74)	24 (24.74)	
N_1–N_3	49	12 (12.37)	37 (38.14)	
Differentiation				**0.0190**
Well/moderate	58	27 (27.84)	31 (31.96)	
Poor	39	9 (9.28)	30 (30.93)	

Bold type indicates that the difference was statistically significant.

in SCC9 and SCC15 was significantly up-regulated by the Lv-AC007271.3 (Fig. 2A). The CCK-8 analysis showed knockdown of AC007271.3 notably repressed the growth of OSCC cells (Fig. 2B), and significantly reduced colonies of OSCC cells were observed in colony formation assay, while opposite results were found in cells with AC007271.3 overexpression (Fig. 2C). These data suggested AC007271.3 could promote OSCC cell proliferation.

The effects of AC007271.3 on the migration and invasion of OSCC cells were evaluated by the wound-healing and Matrigel invasion assay. Results showed that AC007271.3 knockdown significantly decreased the migrating distance and had markedly weaker invading capacity as compared with negative controls (Fig. 2D-E), suggesting that AC007271.3 silencing attenuate the migration and invasion of OSCC cells. Flow cytometry showed that the rates of OSCC cell apoptosis were notably enhanced in si-AC007271.3 transfected cells and the percentage of prominent apoptotic cells was distinctly increased (Fig. 2F). In total, these results indicated that AC007271.3 induced proliferation, migration, invasion, and suppressed OSCC cell apoptosis in vitro.

3.3. AC007271.3 activates the Wnt/β-catenin pathway in OSCC cells

LncRNAs can regulate gene expression at transcriptional and post-transcriptional level. To detect the potential molecular mechanism of AC007271.3, the FISH experiment was performed to identify the distribution of AC007271.3 in SCC9 and SCC15 cells. The results suggested that AC007271.3 was localized both in the cytoplasm and nucleus (Fig. 3A), which indicated AC007271.3, similar to partial lncRNAs, might exert function at both transcriptional and post-transcriptional level.

Accumulating evidences have confirmed that lncRNAs may function in malignant tumors by cooperating with signaling transduction pathways. Wnt/β-catenin pathway is closely related with cell proliferation, invasion, migration, apoptosis, and involved in the occurrence and development of OSCC [12,13], thus it was assessed whether AC007271.3 could activate the Wnt/β-catenin pathway in OSCC cells. The results showed that the activity of luciferase was increased by the transfection of AC007271.3 overexpression vector compared with the control group, while the activity of luciferase was decreased after transfecting with AC007271.3 interference vector (*$P < .05$, **$P < .01$) (Fig. 3B). The immunofluorescence assay demonstrated that the position of β-catenin protein moved from the nucleus to cytoplasm in cells and decreased in cytoplasm when AC007271.3 was silenced, while it translocated from cytoplasm to nucleus when AC007271.3 was over expressed (Fig. 3C). Results showed that the expression level of total β-catenin protein was increased compared with the control group when AC007271.3 was overexpressed. Moreover, we extracted nuclear protein and cytoprotein respectively. We found that the trend of nucleus β-catenin expression level was consistent with that of total β-catenin protein, while opposite results were observed in OSCC cells when AC007271.3 was silenced (Fig. 3D-E). The increased expression level of total and nuclear β-catenin indicated that AC007271.3 could regulate the translocation of β-catenin and thus activate the Wnt/β-catenin signaling pathway.

3.4. Rescued experiments confirmed that AC007271.3 can affect the biological characteristics in OSCC through Wnt/β-catenin pathway

To further confirm that AC007271.3 promoted carcinogenesis in OSCC via Wnt/β-catenin pathway, rescued experiments was performed. SCC9 and SCC15 cells cotransfected with AC007271.3 lentiviral vectors were constructed and treated with xav939 (Selleck Chemicals, China), an inhibitor of Wnt/β-catenin signaling pathway at the concentration of 50 μM. As shown in Fig. 4A-D, the capacity of cell proliferation, migration and invasion ascended by AC007271.3 overexpression was restored when treated with xav939. Our results showed that overexpression of AC007271.3 increased the expression of the core protein β-catenin and its downstream target genes (CyclinD1, c-myc and Bcl-2) compared with the control group, while opposite results were observed in OSCC cells when silencing AC007271.3 (Fig. 3D). Nevertheless, these results were abolished when treated with xav939 (Fig. 4E). Thus, it indicated that AC007271.3 might promote OSCC proliferation, invasion, migration and suppress apoptosis via Wnt/β-catenin signaling pathway.

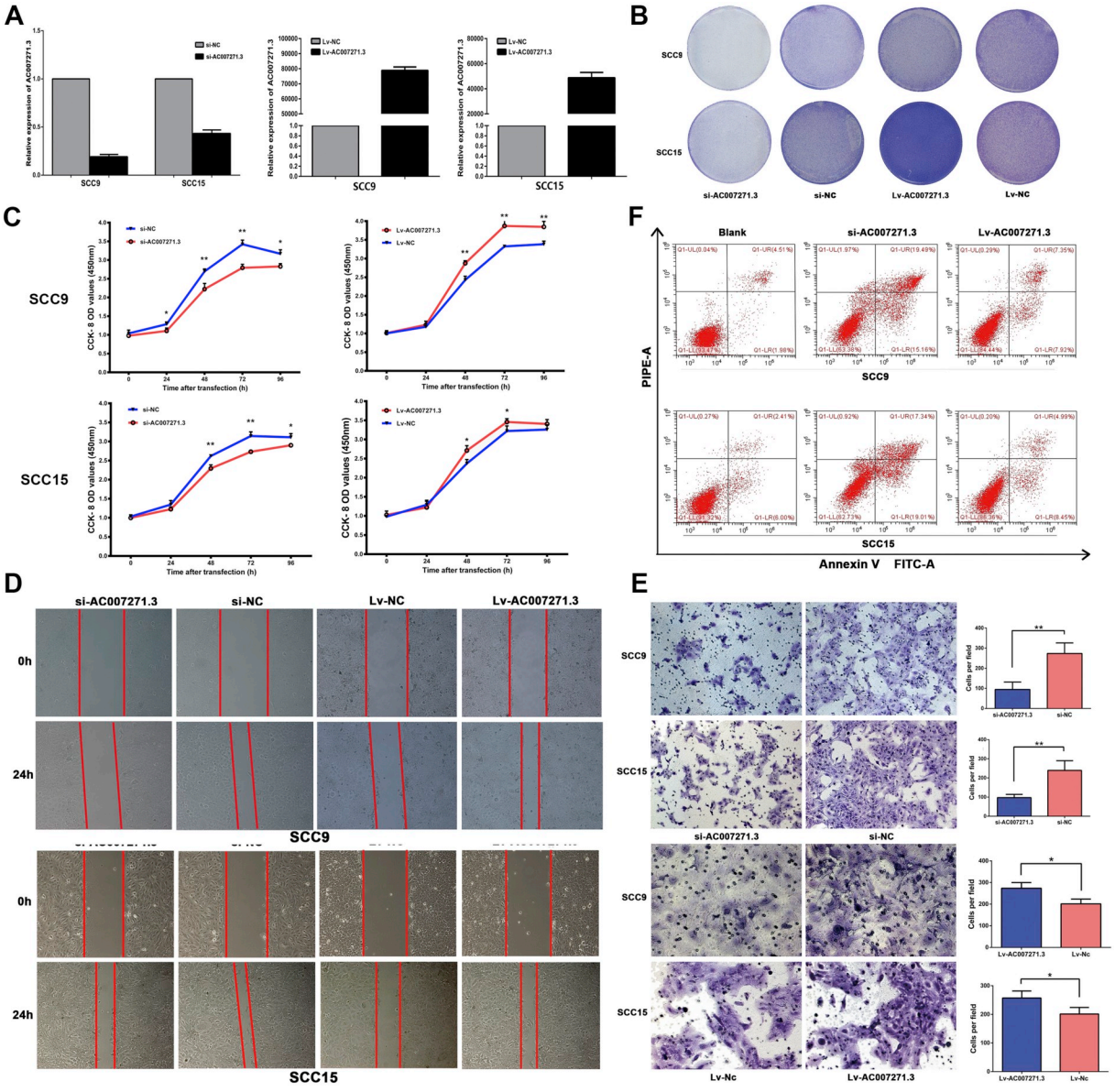

Fig. 2. Effects of down-regulation or up-regulation of AC007271.3 on cell proliferation. A, AC007271.3 expression was analyzed in OSCC cell lines by qPCR 48 h after transfection. B–C, Cell proliferation was determined by CCK-8 assay and colony formation assay. D–E, Representative images of migration and invasion of SCC9 and SCC15 cells transfected with AC007271.3 knockdown or overexpression are shown in the panels, $^{*}P < .05$, $^{**}P < .01$. F, Silencing of AC007271.3 induces apoptosis of OSCC cells.

3.5. AC007271.3 promotes OSCC tumorigenesis via Wnt/β-catenin pathway

To investigate the role of AC007271.3 in tumorigenesis in vivo, the SCC-9 cells transfected with stably AC007271.3 overexpression were injected subcutaneously into female nude mice. All mice developed tumors at the injection sites. As the result shown, overexpression of AC007271.3 obviously increased the tumor volume and weight of the xenograft tumor in the nude mice ($P < .05$, Fig. 5A-B). Immunohistochemistry (IHC) analysis of xenograft tumors revealed that the LV-AC007271.3 group presented poorly differentiated morphology, keratinization, and abnormal nuclear divisions and the expression of Ki-67 and CD44 were significantly increased compared with the control group (Fig. 5C). Consistent with the aforementioned in vitro results, these data indicated that AC007271.3 could affect OSCC tumorigenesis in vivo.

β-Catenin and its downstream target genes including CyclinD1, c-myc and Bcl-2 play crucial roles in Wnt/β-catenin pathway. Western blot results showed up regulation of AC007271.3 promoted the expression of β-catenin compared with the control group (Fig. 5D). Therefore, these data suggested that AC007271.3 could promote the tumorigenesis of OSCC in vitro and in vivo via the Wnt/β-catenin signaling pathway.

4. Discussion

Plenty of studies have showed that lncRNAs play an important role in the genesis and development of various malignant tumors, including OSCC [13–15]. Wu et al. [16] identified that overexpression of lncRNA HOTAIR (HOX transcript antisense intergenic RNA) facilitated the growth of OSCC cells. Zhou et al. [17] showed that MALAT1 enhanced tumor growth and metastasis by triggering epithelial-mesenchymal transition (EMT) in OSCC. Consistent with these literatures, we identified that lncRNA AC007271.3 was up-regulated in OSCC tissues and cell lines in our previous study. Moreover, AC007271.3 was significantly higher in serum of OSCC patients than in controls, which was

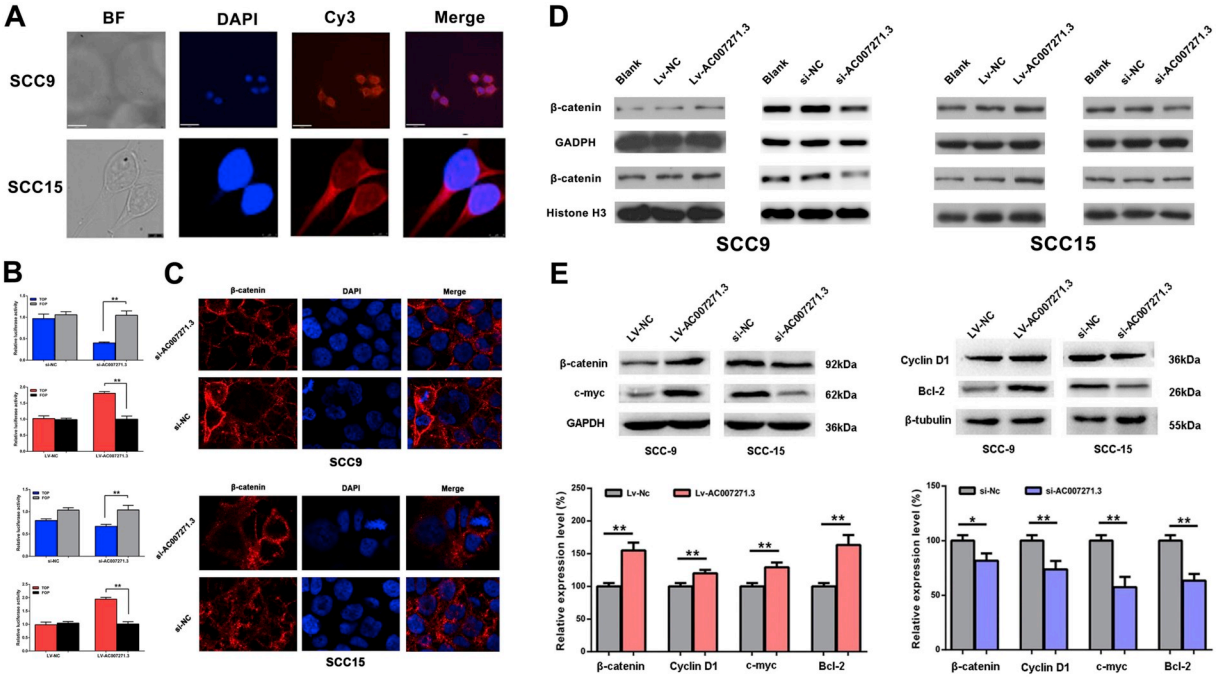

Fig. 3. AC007271.3 promotes OSCC malignant progression through Wnt/β-catenin signaling pathway. A, Location of AC007271.3 in SCC9 and SCC15. B, Luciferase reporter assay using TOP-flash vectors was carried out to detect β-catenin transcription factor/lymphoid enhancer binding factor (TCF/LEF) promoter activity; FOP-flash has mutated TCF binding sites, acting as a negative control. C, Immunofluorescence assay for β-catenin implies that the location of β-catenin in cells moves from nuclear to cytoplasmic when the expression of AC007271.3 is silenced, and that β-catenin expression decreased in the nucleus compared to si-NC. D, The expression level of total β-catenin protein, β-catenin in the cytoplasm to nucleus when AC007271.3 was overexpressed or silenced. E, Western blot analysis and measurement of the relative grey scale of β-catenin, CyclinD1, c-myc and Bcl-2 vs. GAPDH.

associated with worse prognosis, thus we established AC007271.3 as a panel of early detection for OSCC [9]. In this portion, we validated the significantly up-regulated AC007271.3 in a lager sample size of 97 OSCC tissues, and also showed that a high expression level of AC007271.3 was correlated with clinical stage, lymph node metastasis, histological differentiation, and indicated poor survival. The results indicated that AC007271.3 might perform a distinct function and be a potential prognostic factor in OSCC. However, no study has been conducted to explore the potential mechanism of AC007271.3 dysregulation in OSCC until now. In this study, our findings showed that AC007271.3 could significantly promote OSCC cell proliferation in vitro and in vivo, enhance migration, invasion and inhibited apoptosis in vitro, which suggested that AC007271.3 plays an oncogenic role in OSCC.

The Wnt/β-catenin signaling pathway, one of the classical pathways for signal transduction, plays a key role in the occurrence and progression of squamous cell carcinoma [18,19]. Emerging evidence showed that the abnormal expression of lncRNAs might be closely associated with the activation of Wnt/β-catenin signaling pathway in regulating OSCC processes [20]. For example, Liu et al. [12] reported that lncRNA MEG3 could inhibit the growth and metastasis of OSCC by negatively regulating the Wnt/β-catenin signaling pathway. Ma et al. [20] showed that silencing of lncRNA CCAT2 depressed malignancy of OSCC via Wnt/β-catenin signaling pathway. The canonical Wnt signaling pathway is activated by inhibiting GSK-3β activity, which results in the accumulation of β-catenin in cytoplasm and subsequently translocation from cytoplasm to nucleus, regulating the transcription of downstream key targeted genes like CyclinD1, c-myc and Bcl-2 [21–23]. However, the relationship between AC007271.3 and Wnt/β-catenin signaling pathway in OSCC has not been reported up till now.

In our present study, the immunofluorescence assay showed that the position of β-catenin protein moved from the nucleus to cytoplasm when silencing AC007271.3. On the contrary, it caused the accumulation of β-catenin in cytoplasm and subsequent translocation from cytoplasm to nucleus when AC007271.3 was over expressed. We also found that the trend of nucleus β-catenin expression level was consistent with that of total β-catenin protein. Thus, it indicated that AC007271.3 could regulate the translocation of β-catenin and thus activate the Wnt/β-catenin signaling pathway. It is known that CyclinD1, c-myc and Bcl-2 are the key downstream targeted molecules of this pathway, which are proved to be related with cell proliferation and apoptosis [22,23]. The western blotting analysis demonstrated that the trend of CyclinD1, c-myc and Bcl-2 protein expression was consistent with that of β-catenin when AC007271.3 was up-regulated or down-regulated. Further rescued experiments and xenograft tumor experiment in nude mice proved that AC007271.3 could promote cell proliferation, invasion, migration and reduced cell apoptosis of OSCC via the Wnt/β-catenin signaling pathway, which may provide potential pharmacological targets in OSCC.

Cumulatively, our study demonstrated that AC007271.3 is up-regulated in OSCC, and correlated with its aggressiveness and poor prognosis. AC007271.3 promoted cell proliferation, migration and invasion and inhibited cell apoptosis of OSCC via the Wnt/β-catenin signaling pathway. Our results suggest AC007271.3 be possibly used as a novel biomarker for the diagnosis and treatment of OSCC.

Declaration of competing interest

None.

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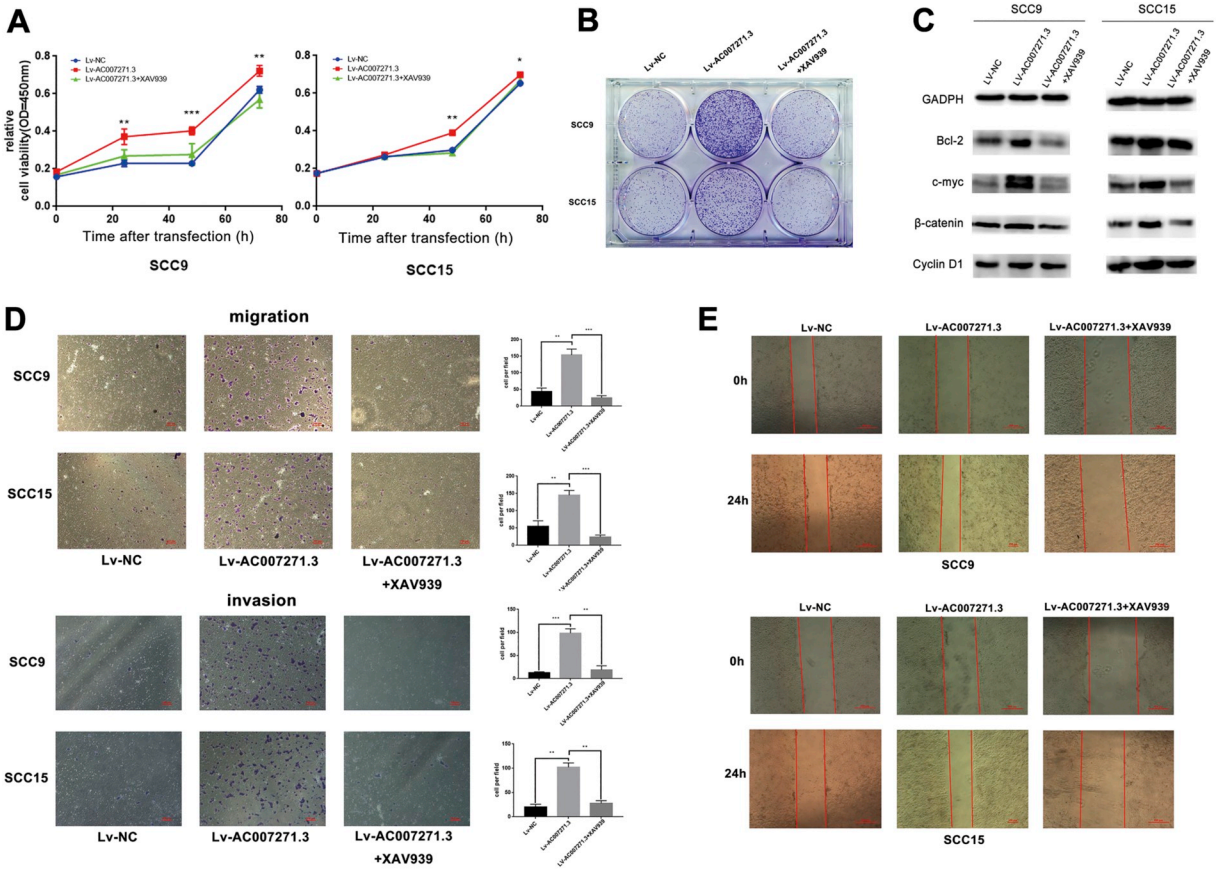

Fig. 4. The functions of AC007271.3 in OSCC cells was involved in Wnt/β-catenin pathway. The capacity of OSCC cell proliferation determined by CCK-8 assay (A) and colony formation assay (B), migration and invasion (C) ascended by AC007271.3 overexpression was restored when treated with xav939. D, The increased expression of β-catenin and downstream target genes (CyclinD1, c-myc and Bcl-2) of Wnt/β-catenin signaling pathway was abolished when treated with the pathway inhibitor xav939.

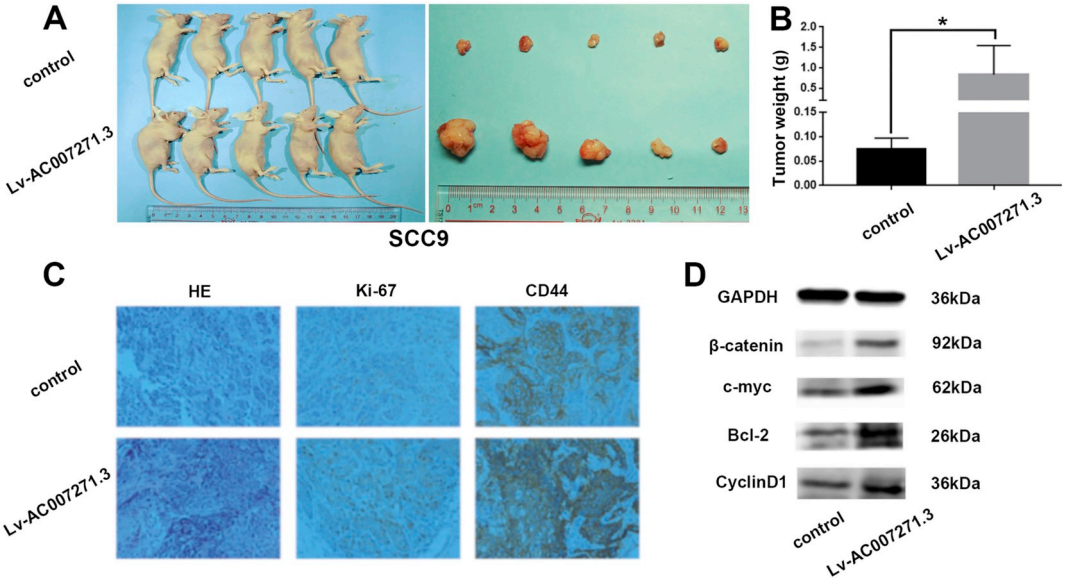

Fig. 5. Tumor volumes were quantified for 28 days in SCC9 xenograft mouse model transfected with Lv-NC and Lv-AC007271.3. A, Representative tumor images for SCC9 xenografts. B, Xenograft tumors were resected and weighted. C, Immunohistochemical Ki-67 and CD44 staining for SCC9 tumor sections are shown, $*P < .05$. D, Western blotting of β-catenin and downstream proteins in SCC9 tumor sections are shown, $*P < .05$.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.117087.

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