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ADCY6 is a potential prognostic biomarker and suppresses OTSCC progression via Hippo signaling pathway

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Abstract

Oral tongue squamous cell carcinoma (OTSCC) is a malignant tumor. Recently, studies have found that adenylate cyclase 6 (ADCY6) plays a pivotal role in many lethal tumors formation processes. The role of ADCY6 in OTSCC remains unknown. The expression of ADCY6 in OTSCC tissue samples was detected. The clinical significance of ADCY6 in OTSCC was analyzed by statistical methods. OTSCC cell lines were selected to analyze the biological function of ADCY6. Meanwhile, the effect of ADCY6 on the growth of OTSCC in vivo was explored using subcutaneous tumorigenesis assay. WB assay was used to detect the underlying signaling pathway. Cell function recovery test used to investigate the mechanism of ADCY6-promoting OTSCC malignant biological behavior via Hippo signaling pathway. We report that ADCY6 was obviously downregulated in OTSCC tissue samples and cell lines. Importantly, lower expression of ADCY6 indicates a poorer prognosis in patients with OTSCC, and its expression is significantly correlated with TNM stage and tumor size. Functionally, forced expression of ADCY6 can significantly inhibit the proliferation, migration, invasion, and promote apoptosis of OTSCC cells. Mechanistically, we demonstrated that ADCY6 upregulation impaired Hippo signaling pathway to reduce the malignant biological behavior of OTSCC. Generally, our findings suggest that ADCY6 suppressed Hippo signaling pathway to regulate malignant biological behavior in OTSCC, which provide new cues for further exploring the mechanism of occurrence and development of OTSCC.

KEYWORDS
ADCY6, Hippo signaling pathway, OTSCC, prognostic biomarker

1 | INTRODUCTION

OTSCC is a common malignant tumor in oral and maxillofacial regions, and the prognosis of patients is poor.[1] The pathogenesis of oral cancer has not been fully clarified. Most scientists believe that it is related to environmental factors, such as heat, chronic damage, ultraviolet light, x-ray, and other radioactive elements can become carcinogenic factors.[2] Current treatments involve reducing the stimulation of external irritant elements, actively treating precancerous lesions, and

Sen Yang and Li-Juan Guo contributed equally to this study.

improving the body's disease resistance and working ability; however, the internal initiator or effector driving the development and metastasis of OTSCC remains largely unknown.[3–5] Therefore, it is still urgent to find key regulators affecting the malignant biological behavior of OTSCC.

Adenylate cyclase is a membrane integrin with both amino and carboxyl termini oriented toward the cytoplasm.[6] AC has two catalytic domains on the cytoplasmic surface of the membrane, as well as two membrane integration regions, each with six transmembrane α-helices.[7] Six adenylate cyclase isoforms have been found in mammals.[8] Because AC can convert ATP into cAMP, it initiates cellular responses. Peptides, proteins and catecholamine hormones, such as adrenaline, glucagon, insulin, adrenocorticotropin, and thyrotropin, all play roles in cellular responses through information transmission pathways involving cAMP.[9] Adenylate cyclase 6 (ADCY6) belongs to the membrane-bound adenylate cyclase family.[10,11] Increasing evidence has proven that ADCY6 is involved in pathophysiological processes of the body, such as the stability of cardiovascular system function, glucose and lipid metabolism, and even carcinogenesis.[12] In breast cancer, ADCY6 is regulated by DNA methylation of miR-27a-3p/TET1 regulatory axis, which affects malignant biological behavior of tumor cells through EMT.[13] However, the role of ADCY6 in a variety of malignant tumors is still unknown, and its role in OTSCC has not been reported.

Hippo signaling pathway is a signal pathway that inhibits cell growth, apoptosis, and metastasis.[14,15] When the receptor of Hippo signaling pathway senses the external growth inhibition signal, it phosphorylates YAP and TAZ to regulate the expression of cell proliferation- and apoptosis-related factors.[15] YAP and TAZ then interact with cytoskeletal proteins and are retained in the cytoplasm, unable to enter the nucleus to execute their transcriptional activation function.[16] In animal models, liver-specific overexpression of YAP leads to pathological enlargement of the liver and the occurrence of hepatocellular carcinoma, and the above phenomena are also observed in liver-specific knockout animal models of MST1, MST2, SAV1, and MER.[17–19] YAP/TAZ is expressed in many solid tumors and is significantly positively correlated with the poor prognosis of cancer patients.[20,21] However, the role of Hippo signaling pathway in OTSCC development is unknown, and it is not known whether ADCY6 affects the malignant progression of OTSCC through Hippo signaling pathway.

Hence, we analyzed the clinical significance of ADCY6 in OTSCC, and explored the effect of ADCY6 on the malignant biological behavior of OTSCC through in vitro and in vivo experiments. More importantly, we also preliminarily explored the mechanisms involved.

2 | MATERIALS AND METHODS

2.1 | OTSCC samples of tissues and cell lines

One-hundred twenty-nine OTSCC tissue samples were obtained from Suining Central Hospital (collected between February and August 2017) (age range, 39–73 years; gender, 69 male and 60 female). The Ethics Committee of Suining Central Hospital approved the study (approval no. 20170117). All patients gave informed written consent. The inclusion criteria were: (A) Patients with a clinically and histopathologically confirmed diagnosis of OTSCC; (B) received surgical resection as initial treatment; (C) archived tumor tissue and paired adjacent tissues that were stored; and (D) clinicopathological and follow-up data were complete and accessible. Patients were excluded if they underwent radiotherapy or chemotherapy prior to surgery.

NHOK (normal human oral keratinocyte cell lines), SCC-9 and SCC-15 (human oral squamous carcinoma cell lines), H357 (human tongue squamous cell carcinoma cell line) were procured from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. NHOK, SCC-9, SCC-15, and H357 were cultured in Dulbecco's modified Eagle's medium (Gibco) with 1% penicillin–streptomycin and 10% FBS (Gibco) at 37°C. Considering the expression of ADCY6, the cell viability and the degree of difficulty for transfection, we chose H357 and SCC-9 for further study.

2.2 | Plasmid constructs and transfection

The cells were transduced with the GV493-GFP lentivirus RNAi expression system (Shanghai GeneChem, China) expressing ADCY6-shRNA, YAP-shRNA or scrambled shRNA with Hitrans A&P (Shanghai GeneChem, China). The multiplicity of infection (MOI) was 10. The virus was transfected with serum-free medium for 10 h at 37°C. The medium was changed and replaced with complete medium and subsequent experiments were carried out after culturing for 48 h at 37°C.

2.3 | Reverse transcription quantitative PCR assay

Total RNA from OTSCC samples was isolated using TRIzol reagent (Invitrogen). Total RNA was reverse transcribed into cDNA using a PrimeScript RT Reagent kit (Takara Bio, Inc.). RT-qPCR was performed using SYBR Premix Ex Taq II (Takara Bio, Inc.) and a LightCycler system (Roche Diagnostics) was used for detection with the following thermocycling conditions: initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 25 s and extension at 72°C for 30 s. β-actin was used as an internal reference for ADCY6 normalization. Data analyses were performed by the $2^{-\Delta\Delta Cq}$ method. The primers used are listed in Table S1.

2.4 | Western blotting

All total protein was isolated from OTSCC samples by protein extraction buffer (Beyotime). Equal amounts of proteins (30 μg) were separated via SDS-PAGE on a 10% gel, and transferred to PVDF membranes. PVDF membranes were probed at 4°C overnight with antibodies against ADCY6 (1:3000; Abcam), YAP (1:5000; Abcam), BAX (1:5000; Abcam), BCL-2 (1:1000; Abcam), KI67 (1:5000; Abcam), and GAPDH (1:5000; Abcam). Then, PVDF membranes incubated with the secondary antibodies. Finally, protein expression was analyzed by chemiluminescence

reagents (Hyperfilm ECL). Image J (National Institutes of Health) software was used to analyze the Western blotting results.

2.5 | Immunohistochemistry

The Human Protein Atlas (HPA, https://www.proteinatlas.org) is a database based on protein, transcriptome, and system biology data, which can map tissues, cells, and organs. Immunohistochemistry analysis of ADCY6 and YAP in OTSCC were conducted with data from HPA database. The stage of OTSCC was analyzed by using hematoxylin–eosin staging.

2.6 | Public data acquisition and analysis

ADCY6 expression profile of HCC patients were downloaded from TCGA database with corresponding clinical information (https://portal.gdc.cancer.gov/repository).

2.7 | Cell apoptosis and cell cycle

For cell apoptosis, OTSCC cells (1×10^5 cells) were collected and incubated with Annexin V-FITC (Biogot Technology) and propidium

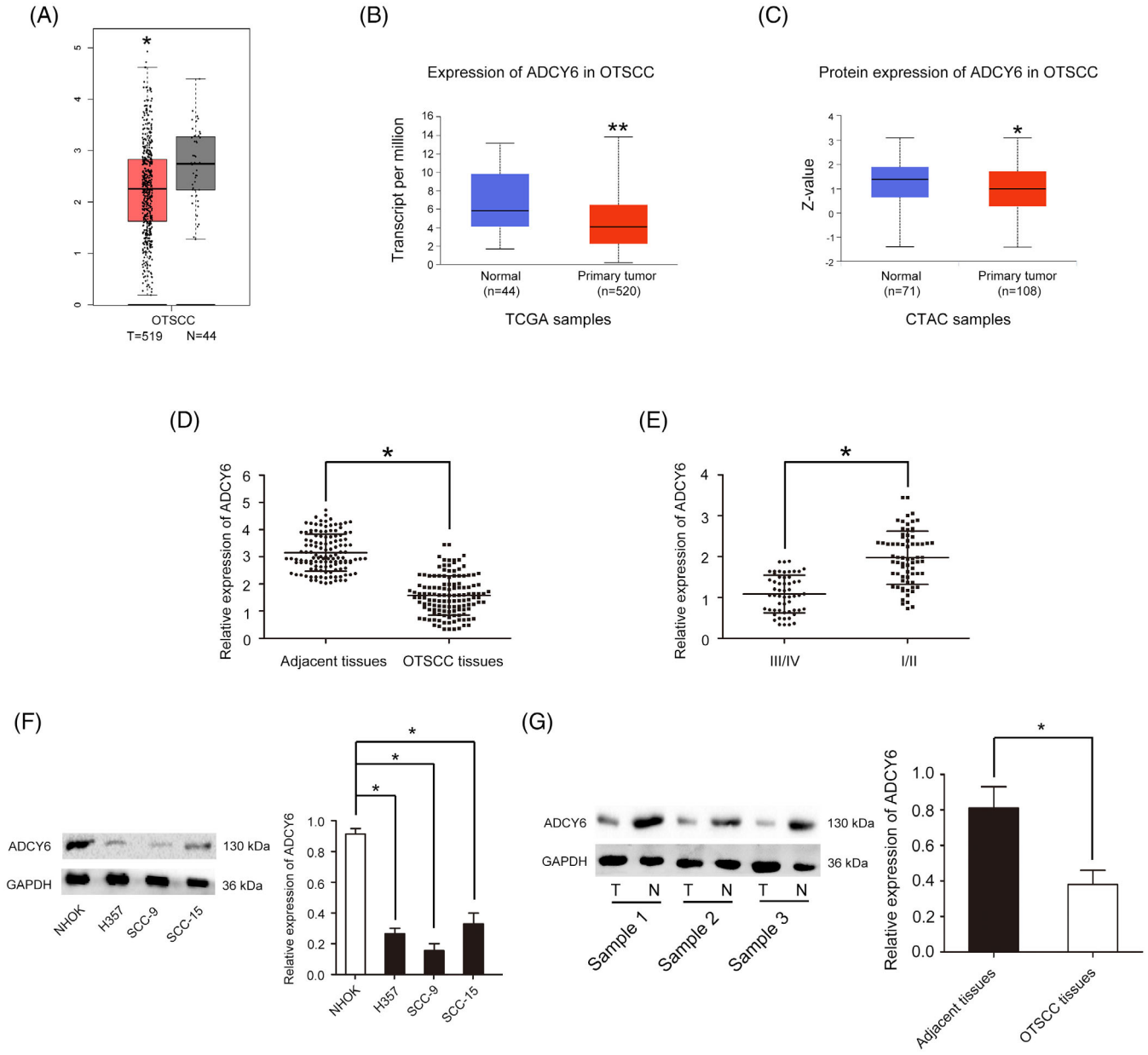


FIGURE 1 ADCY6 was downregulated in OTSCC. (A) ADCY6 expression in OTSCC in biological information database. (B) ADCY6 expression in OTSCC in TCGA database. (C) ADCY6 protein expression in OTSCC in CTAC database. (D) ADCY6 expression in OTSCC tissue samples. (E) ADCY6 expression in I/II and III/IV OTSCC tissues. (F) ADCY6 expression in NHOK and OTSCC cell lines. (G) ADCY6 expression in OTSCC tissues and paired normal adjacent tissues. *$p < 0.05$.

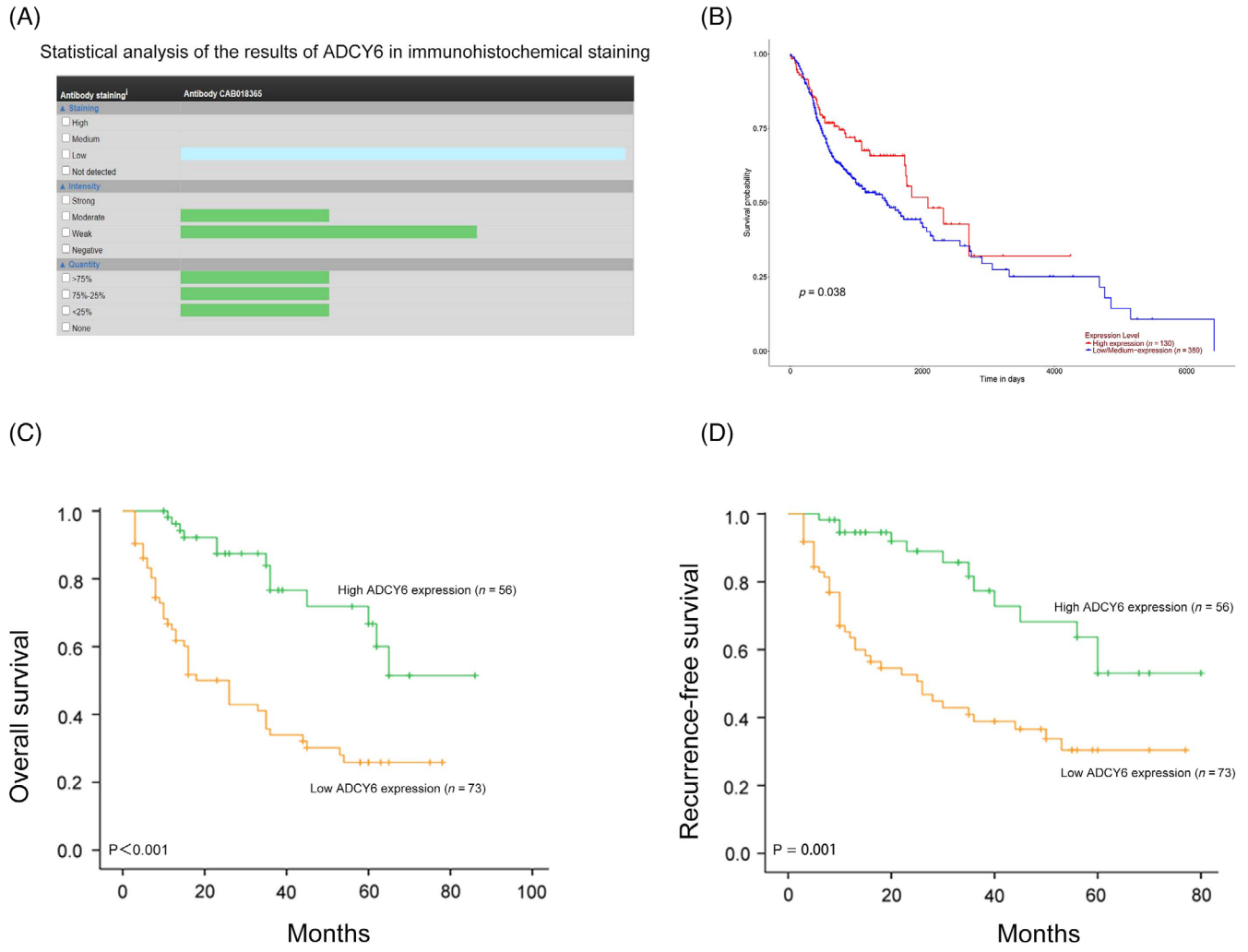


FIGURE 2 The relationship of ADCY6 expression with prognosis in OTSCC. (A) Statistical analysis (staining, intensity, quantity) of the results of ADCY6 in immunohistochemical staining in OTSCC. (B) Bioinformatics database analysis of the relationship between the expression of ADCY6 and the prognosis of patients with OTSCC. (C) Kaplan–Meier analysis of overall survival between OTSCC patients with high and low ADCY6 expression. (D) Kaplan–Meier analysis of recurrence-free survival between OTSCC patients with high and low ADCY6 expression.

iodide (PI) solution (Biogot Technology) at room temperature. Cells were subsequently suspended in binding buffer. For cell cycle, OTSCC cells were collected and fixed overnight in 75% ethanol. Then, RNase A and incubate for 20 min. Then, the cells were staining with PI and flow cytometry was performed for 5 min in the dark (Beckman).

2.8 | Cell-counting kit-8 assay

OTSCC cells were cultured in a 96-well plate (2×10^3 cells/well) for 0, 24, 48, and 72 h. CCK-8 assay (Dojindo Molecular Technologies) was used to detect cell proliferation.

2.9 | 5-Ethynyl-2′-deoxyuridine (EdU) assay

OTSCC cells were seeded in 96-well plates (1×10^5 cells/well) and cultured for 24 h. Then, the cells were immobilized with 4% polyformaldehyde, and nuclei were permeabilized with 0.5% Triton X-100 solution. EdU and DAPI staining reagents were added to 96-well plates. Image acquisition under inverted fluorescence microscope.

2.10 | Wound healing assay

OTSCC cells were grown in six-well plates (1×10^5 cells/well). After reaching the confluence, the cells with the tip of a pipette (1 mL) and washed twice with PBS. The width between scratches after 0, 24, and 48 h.

2.11 | Cell invasion assays

OTSCC cells were seeded into the upper chamber (1×10^5 cells/well). For the invasion assay, the inserts were precoated with Matrigel (1 mg/mL). Cells that migrated into the lower chamber were fixed with methanol for 20 min, and the cell outlines were stained with 0.1% crystal violet.

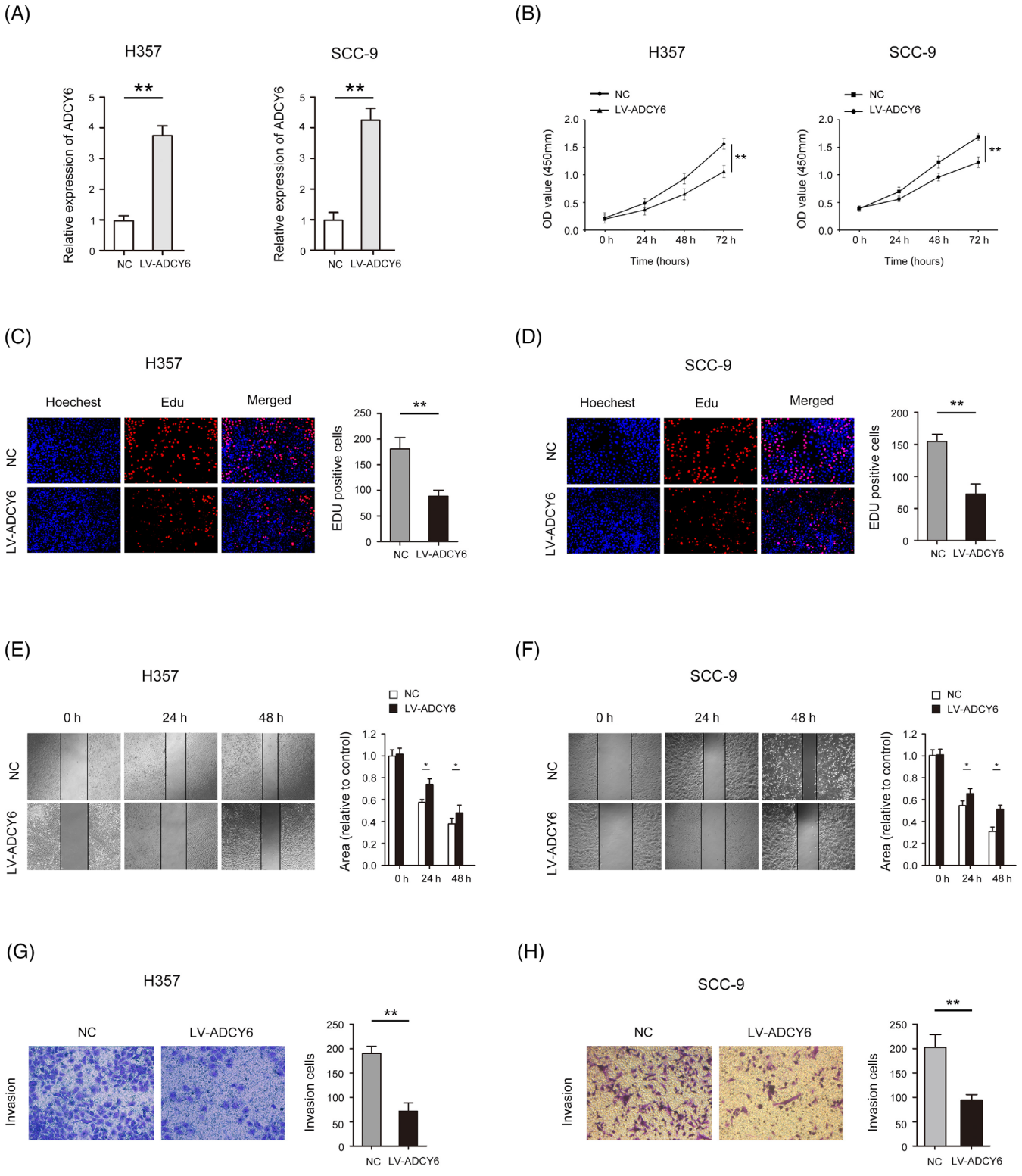


FIGURE 3 Increased ADCY6 inhibits OTSCC cell proliferation, migration and invasion. (A) Transfection efficiency validation of ADCY6 in H357 and SCC-9 cells. (B) CCK-8 assays were used to analyze the effect of ADCY6 on H357 and SCC-9 cell proliferation. (C) EdU assays were used to analyze the effect of ADCY6 on H357 cell proliferation (magnification, ×200). (D) EdU assays were used to analyze the effect of ADCY6 on SCC-9 cell proliferation (magnification, ×200). (E) Wound healing assays were performed to determine the effects of ADCY6 overexpression on H357 cell migration (magnification, ×100). (F) Wound healing assays were performed to determine the effects of ADCY6 overexpression on SCC-9 cell migration (magnification, ×100). (G) Transwell assays were performed to determine the effects of ADCY6 overexpression on H357 cell invasion (magnification, ×200). (H) Transwell assays were performed to determine the effects of ADCY6 overexpression on SCC-9 cell invasion (magnification, ×200). *$p < 0.05$, **$p < 0.01$.

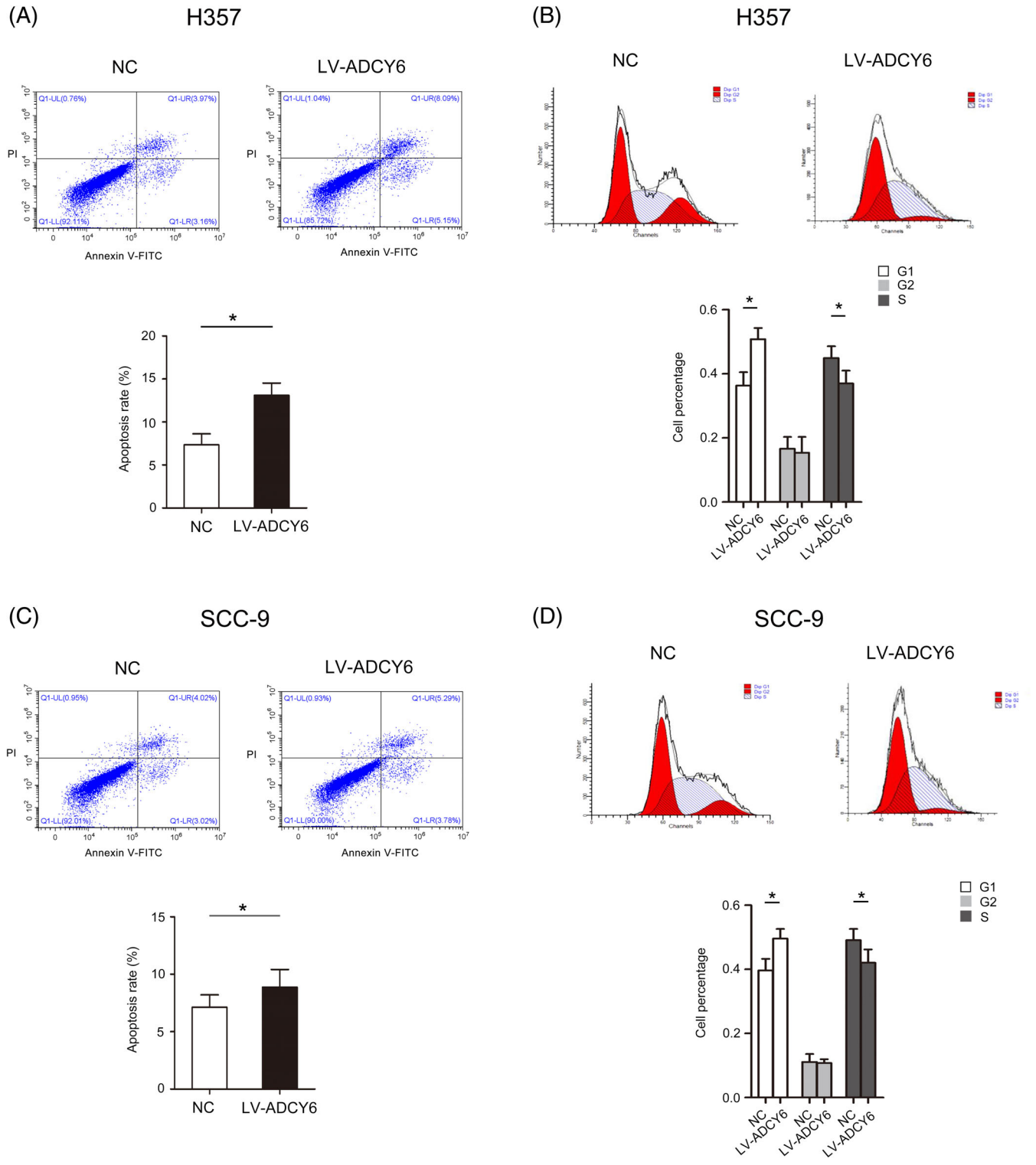


FIGURE 4 Effect of ADCY6 on the cell cycle and apoptosis of OTSCC cells. (A) The effects of ADCY6 overexpression on H357 cell apoptosis. (B) The effects of ADCY6 overexpression on the cell cycle distribution in H357 cells. (C) The effects of ADCY6 overexpression on SCC-9 cell apoptosis. (D) The effects of ADCY6 overexpression on the cell cycle distribution in SCC-9 cells. *$p < 0.05$.

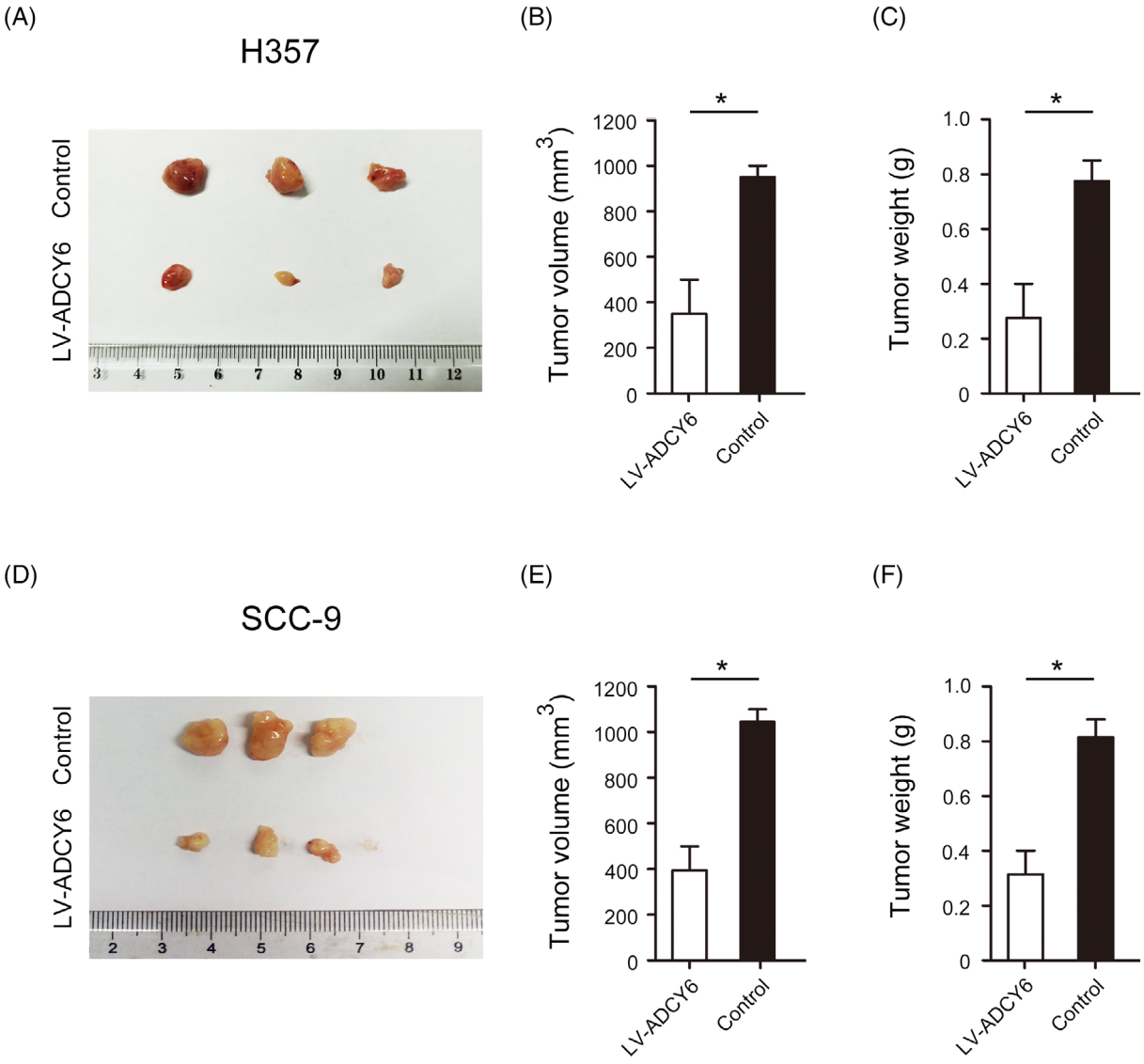


FIGURE 5 ADCY6 inhibited tumor proliferation in a mouse model of OTSCC. (A) Animal model to verify the effect of ADCY6 overexpression on tumor formation in H357 cells. (B) Statistical analysis of the volume of subcutaneous tumorigenic masses in H357 cells. (C) Statistical analysis of the weight of subcutaneous tumorigenic masses in H357 cells. (D) An animal model was used to verify the effect of ADCY6 overexpression on SCC-9 cell tumor formation. (E) Statistical analysis of the volume of subcutaneous tumorigenic masses in SCC-9 cells. (F) Statistical analysis of the weight of subcutaneous tumorigenic masses in SCC-9 cells. $^*p < 0.05$.

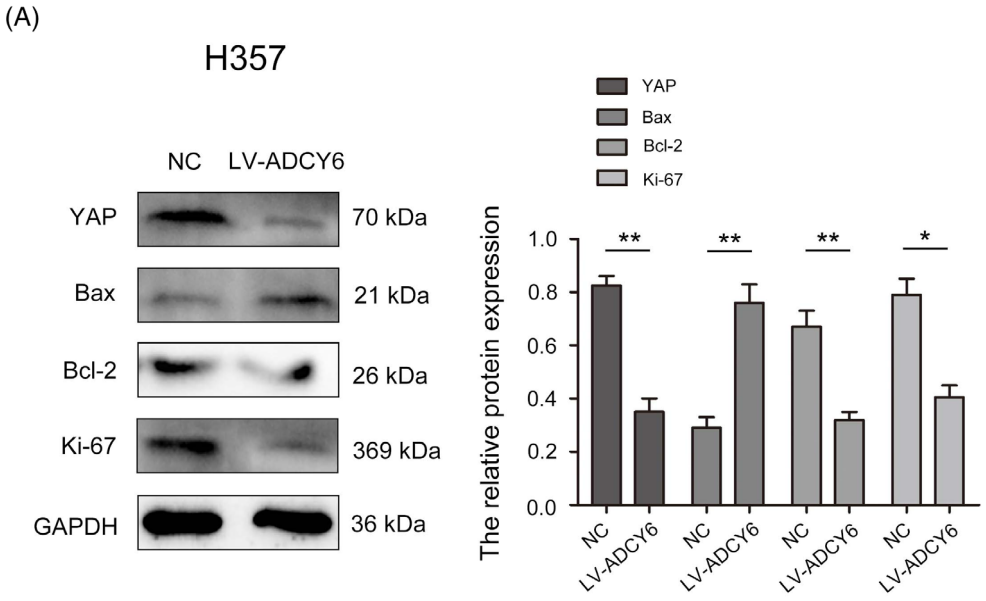
2.12 | Tumorigenesis assay

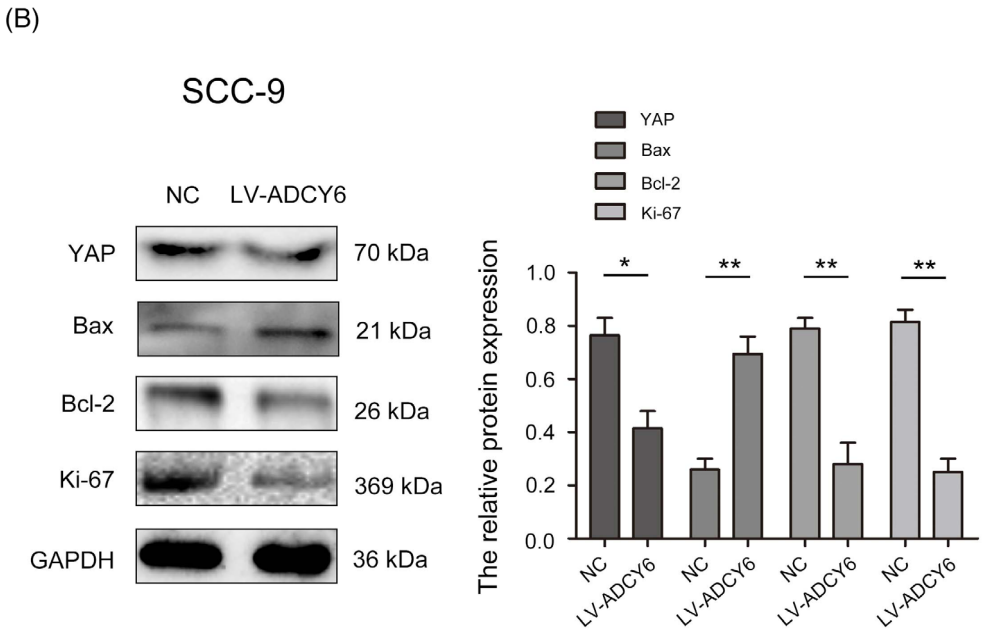
A total of 12 Male BALB/c nude mice (5-weeks-old) were purchased from the Shanghai Experimental Animal Center (Shanghai, China) and fed at the Animal Center of Suining Central Hospital (BALB/c nude mice were house with free food and water under specific-pathogen-free conditions at 21–25°C with 40%–70% humidity and 12/12-h light/dark cycle). All animal experiments were performed in accordance with the institutional guidelines and were approved by the Committee on the Ethics of Animal Experiments of Suining Central Hospital (approval no. 20210521). OTSCC cells (1×10^6 cells/ml) were subcutaneously injected into the flank of each nude mouse. Tumor volume calculation formula: volume = (length × width^2)/2. Tumor diameter was measured with Vernier calipers every 3 days after tumor emergence (13 days after inoculation) to measure tumor volume. At 30 days after injection, the mice were euthanized and tumors were collected for analysis. For this, the mice were anesthetized with 1% pentobarbital sodium (45 mg/kg, intraperitoneal) and subsequently euthanized by cervical dislocation (when the heart had stopped completely, the mouse was determined to be dead). Body weight loss >20% was considered a humane endpoint for euthanasia.

2.13 | Statistical analysis

All experiments were performed in triplicate. All data are presented as the mean ± SD. Statistical data were analyzed using SPSS version 22.0 software (IBM Corp.) and GraphPad Prism version 8.0 software (GraphPad Software, Inc.). A paired *t*-test was used to compare tumor

group and adjacent normal tissue group, and other significant differences between two groups were compared with unpaired *t*-test. One-way analysis of variance (ANOVA) was used to determine statistical significance among groups followed by Tukey's-post hoc test. Repeat the experiment three times. The *p*-value <0.05 was considered to indicate a statistically significant difference.





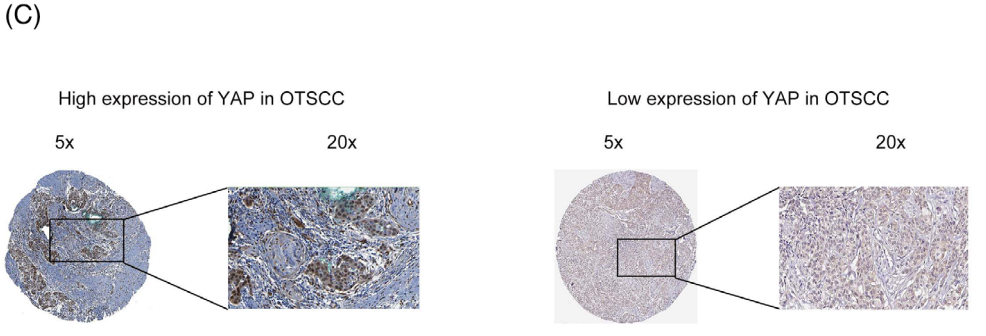


FIGURE 6 Effect of ADCY6 on Hippo signaling pathway in OTSCC. (A) Protein expression of YAP, Bax, Bcl-2, and Ki67 after overexpression of ADCY6 in H357. (B) Protein expression of YAP, Bax, Bcl-2, and Ki67 after overexpression of ADCY6 in SCC-9. (C) Immunohistochemical analysis of the expression of YAP in OTSCC. **p* < 0.05, ***p* < 0.01.

3 | RESULTS

3.1 | ADCY6 is downregulated in OTSCC tissues and cell lines

Through TCGA and GEO databases, we found that ADCY6 was significantly decreased in OTSCC cancer specimens (Figure 1A–C). RT–qPCR results showed that the ADCY6 expression was low in OTSCC tissues (Figure 1D). Moreover, ADCY6 was much lower in advanced (III/IV stage) OTSCC tissues than I/II OTSCC tissues (Figure 1E). The results of different TNM stages of OTSCC are shown in Figure S1. ADCY6 expression was also similarly decreased in OTSCC tissues and cell lines (Figure 1F,G).

3.2 | Downregulation of ADCY6 is associated with poor prognosis in OTSCC

Immunohistochemical analysis (staining, intensity, quantity) showed that the expression of ADCY6 was low in OTSCC (Figure 2A). Survival prognosis analysis showed that patients with low expression of ADCY6 had poor prognosis (Figure 2B). ADCY6 expression was positively correlated with TNM stage ($p = 0.003$) and tumor size ($p = 0.001$, Table S2). Statistical analysis of prognosis revealed that OTSCC patients with lower ADCY6 expression had significantly shorter overall survival (Figure 2C). Univariate analysis indicated that TNM stage ($p = 0.017$), tumor size ($p = 0.034$) and ADCY6 ($p = 0.007$) were

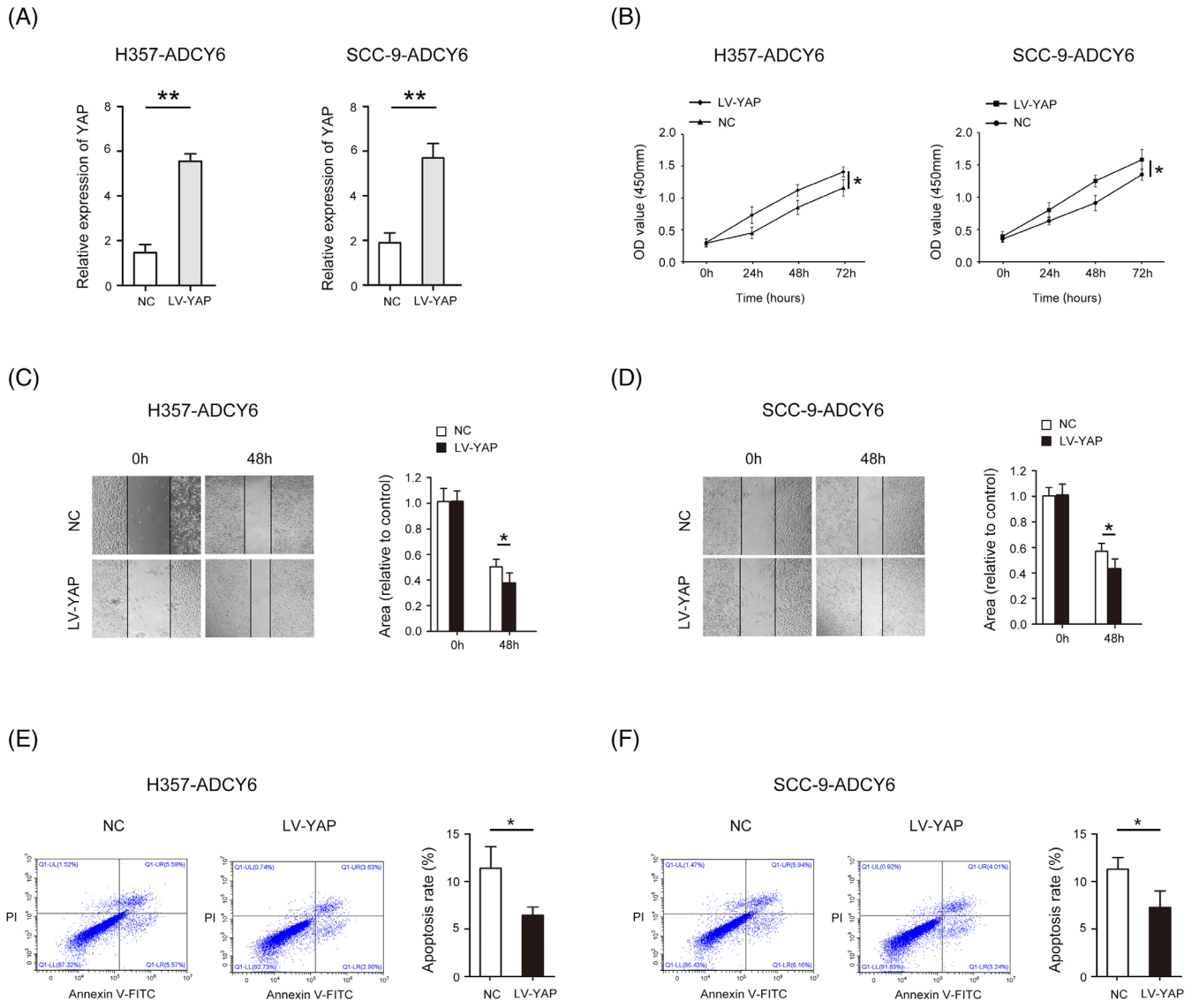


FIGURE 7 Rescue experiments are performed to confirm that YAP ameliorates the inhibitory effect of ADCY6 on OTSCC cell biological behavior. (A) The expression of YAP after increasing the expression of YAP in H357-ADCY6 and SCC-9-ADCY6 cells. (B) CCK-8 assay to detect the proliferation in H357-ADCY6 and SCC-9-ADCY6 cells that increasing the expression of YAP. (C) Wound healing assays were performed to determine the effects of YAP overexpression on H357-ADCY6 cell migration (magnification, ×100). (D) Wound healing assays were performed to determine the effects of YAP overexpression on SCC-9-ADCY6 cell migration (magnification, ×100). (E) Flow cytometry was used to detect the apoptosis in H357-ADCY6 cells that increasing the expression of YAP. (F) Flow cytometry was used to detect the apoptosis in SCC-9-ADCY6 cells that increasing the expression of YAP. *$p < 0.05$.

significantly associated with overall survival in OTSCC (Table S3). Multivariate analysis suggested that TNM stage ($p = 0.010$), tumor size ($p = 0.030$) and ADCY6 ($p = 0.011$) were independent prognostic factors for overall survival in OTSCC (Table S3). Statistical analysis of prognosis revealed that OTSCC patients with lower ADCY6 expression had significantly shorter recurrence-free survival (Figure 2D). Univariate analysis indicated that TNM stage ($p = 0.013$), tumor size ($p = 0.026$) and ADCY6 ($p = 0.015$) were significantly associated with overall survival in OTSCC (Table S4). Multivariate analysis suggested that TNM stage ($p = 0.010$), tumor size ($p = 0.020$) and ADCY6 ($p = 0.009$) were independent prognostic factors for recurrence-free survival in OTSCC (Table S4).

3.3 | Increased ADCY6 inhibits the malignant biological behavior of OTSCC cells

Gain-of-function experiments were performed in H357 and SCC-9 cell lines. ADCY6 was effectively overexpressed in H357 and SCC-9 (Figure 3A), and its overexpression significantly inhibited cell proliferation measured by CCK-8 and EdU assays (Figure 3B–D). Moreover, cell mobility and invasion ability of OTSCC cell lines with ADCY6 overexpression were obviously impaired measured by wound healing and transwell assays (Figure 3E–H).

3.3.1 | ADCY6 inhibits cell cycle and induces apoptosis of OTSCC cells

In addition to the role of ADCY6 in cell proliferation, we also detected its role in apoptosis. The results from the flow cytometry assay demonstrated that overexpression of ADCY6 elevated the cell apoptotic rate of H357 and SCC-9 (Figure 4A,C). In addition, for cells with ADCY6 overexpression, the proportion in G1 phase was increased, while the proportion in S phase was reduced (Figure 4B,D).

We further identified the role of ADCY6 in vivo. An increase in ADCY6 expression significantly inhibited tumorigenicity in H357 (Figure 5A–C). Similar results were found in SCC-9 (Figure 5D–F).

3.4 | ADCY6 had a suppressive impact on Hippo signaling pathway in OTSCC

Considering the great effect of Hippo signaling pathway on cell proliferation and organ size, we considered whether the above changes were related to Hippo signaling pathway. The increase in ADCY6 expression significantly decreased YAP, Bcl-2 and Ki-67 expression and increased Bax expression in H357 and SCC-9 (Figure 6A,B). YAP protein is an effector and key protein of Hippo signal pathway. Immunohistochemical analysis showed that the expression of YAP was high in OTSCC tissues (Figure 6C). We found that overexpression of YAP can promote the malignant biological behavior of OTSCC inhibited by ADCY6 (Figures 7 and S2).

4 | DISCUSSION

AC is widely distributed in mammalian cell membranes and is an effector in the G protein coupling system.[22,23] Increasing evidence has shown that different subtype factors of AC are inextricably linked with a variety of malignant diseases, and there are multiple nonspecific coordination or antagonistic between the effects of each subtype.[24,25] In obesity and type 2 diabetes patients, Purβ promotes the production of liver glycogen by increasing the expression of ADCY6, causes the imbalance of glucose homeostasis through cAMP/PKA/CREB axis, and promotes the disease progression of obesity and type 2 diabetes patients.[26] GPSM1 can inhibit the expression of ADCY6, regulate JNK expression through ADCY6-RAPEF3 and promote tumor growth in Ball-1 and Reh cells.[27] By analyzing the potential prognostic marker genes of breast cancer through weighted gene coexpression network, ADCY6 play a certain role in information transmission in claudin-low breast cancer and is a potential target for precise molecular therapy of breast cancer.[28] Similar to those results, low ADCY6 expression predicts the poor prognosis of OTSCC, causes the malignant proliferation and growth of OTSCC, and promotes the malignant biological behavior of OTSCC.

The Hippo signaling pathway is one of the most conserved pathways in organisms.[29,30] Hippo pathway is regulated by various signals such as mechanical environment, G protein-coupled receptor signaling, cellular energy level, oxidative stress, hypoxia.[31] At the same time, molecules of the Hippo pathway are upstream and are core molecules of tumor suppressor gene regulation; once the regulation is abnormal, it can cause uncontrolled proliferation and apoptosis inhibition.[32] The abnormal expression and dysfunction of YAP and TAZ may promote the unregulated proliferation of cells and affect the normal function of cells.[33–35] The key protein of hippo signaling pathway MST1 passes through β-catenin/DRP1 axis controls the balance of mitochondrial dynamics and affects proliferation in head and neck squamous cell carcinoma cells.[36] Our results confirm the predictive effect of ADCY6 on the poor prognosis of OTSCC patients and its impact on the malignant biology of OTSCC cells. In addition, it has also been confirmed that Hippo signaling pathway plays an important role in ADCY6 regulation of the malignant biological behavior OTSCC. However, the precise regulatory mechanism between ADCY6 and Hippo signaling, together with the underlying molecular basis in ADCY6-mediated regulation of OTSCC progression, needs further investigation. In addition, the significance of ADCY6 on the poor prognosis of OTSCC patients needs to be further confirmed by studies with larger sample size.

The present study demonstrated that ADCY6 played an anticancer role in OTSCC cells via the Hippo signaling pathway. These findings provide a scientific exploration of the role of ADCY6 in OTSCC and identify a new direction for the mechanism of OTSCC.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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