Original Article

Silencing of *AJAP1* expression by promoter methylation activates the Wnt/β-catenin signaling pathway to promote tumor proliferation and metastasis in salivary adenoid cystic carcinoma

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Background: Salivary adenoid cystic carcinoma (SACC) is a unique malignant tumor of the salivary gland with poor prognosis, which is not effective with chemotherapy and targeted drugs. Therefore, it is important to explore the molecular mechanism underlying SACC invasion and metastasis to develop novel therapeutic strategies and targets in clinical research.

Methods: Real-time quantitative polymerase chain reaction (RT-qPCR) and western blot (WB) were performed to detect the expression of Adherens Junctions Associated Protein 1 (*AJAP1*). Methylation-specific PCR was used to evaluate the methylation of the *AJAP1* promoter. *AJAP1* was overexpressed or knocked down by lentivirus-mediated transfection. Kaplan-Meier analysis was conducted to create a survival curve and the log-rank test was used to analyze the overall survival (OS). The prognostic correlation was assessed using univariate and multivariate Cox regression analyses. Co-immunoprecipitation (Co-IP) was utilized to pull down the possible binding protein of *AJAP1* and laser scanning confocal microscopy was applied to detect the subcellular localization of *AJAP1*, E-cadherin, and β-catenin. Cell viability, colony formation, wound healing, and Transwell invasion assays were performed to evaluate the function of AJAP1 *in vitro*. A subcutaneous xenograft assay in nude mice was performed to verify the function of *AJAP1 in vivo*.

Results: *AJAP1* was downregulated in SACC tumors and was closely related to SACC lymph node/distant metastasis, which was an independent risk factor for SACC prognosis. Methylation-specific PCR confirmed that high methylation of the *AJAP1* promoter was the main cause of its silencing. Overexpression or knockdown of *AJAP1* in SACC cells could significantly inhibit or promote the proliferation, invasion, and metastasis of SACC cells, respectively, in both the *in vitro* and *in vivo* experiments. Mechanically, we found that *AJAP1* binds to E-cadherin and β-catenin to form a complex in cytomembrane, reducing the

nuclear translocation of β-catenin and blocking the Wingless/Integrated/β-catenin (Wnt/β-catenin) signaling pathway to play a suppressive role in cancer.

Conclusions: In conclusion, these results suggest that the downregulation of *AJAP1* protein expression may play a certain role in progression and metastasis of SACC. Our study indicates that *AJAP1* may be a potential prognostic molecular marker and therapeutic target for SACC.

Keywords: Salivary adenoid cystic carcinoma (SACC); *AJAP1*; methylation; β-catenin; metastasis

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Introduction

Salivary adenoid cystic carcinoma (SACC) is a unique malignant tumor of the salivary gland, accounting for approximately 10% of all salivary gland tumors and 30% of salivary gland malignancies (1). It is characterized by being highly invasive and prone to neurovascular aggression and distant metastasis (2). The 5-year survival rate of SACC is approximately 70–90% but only 20% of cases have distant invasion (3). Currently, there is a lack of effective chemotherapy and targeted drugs for patients with SACC, and surgical resection is the main treatment option (4). However, the treatment of SACC is very difficult, largely due to the particularity of the surgical location, namely, the trauma and propensity for complications as well as the high occurrence of local invasion and distant metastasis in patients. Therefore, it is important to explore the molecular mechanism underlying SACC invasion and metastasis to develop novel therapeutic strategies and targets in clinical research.

In recent years, a previous study has shown that the abnormal expression of some genes may play an important role in the malignant progression of SACC (5) and methylation of DNA promoter may be one of the main causes of abnormal gene expression. The aberrantly modified DNA methylation confers unique features on tumor cells, including sustained proliferative potential, resistance to growth-suppressive or cell death signals, augmented replicative immortality, invasion, and metastasis. As a result, DNA methylation abnormalities exhibit significant impacts on all stages of oncogenesis from its onset to progression to metastasis, which may eventually lead to tumor chemotherapy resistance. Thus, DNA methylation/demethylation pathway as a promising target for therapeutic intervention in cancer (6). Studies have shown that hypermethylation in promoters of Reversion-inducing cysteine-rich protein with kazal motifs (RECK), RAS association domain family protein1A gene (RASSF1), Suprabasin promotes the metastasis and progression of SACC, which is associated with poor prognosis of patients (7-9). This suggests that high levels of DNA methylation may play an important role in the occurrence and development of SACC. Adherens Junctions Associated Protein 1 (*AJAP1*) is a complete membrane protein with 411 amino acid residues; it includes a divisible N-terminal signal peptide (residues 1–43), extracellular domain (residues 44–282), transmembrane domain (residues 283–303), and intracellular cytoplasmic structure (residues 304–411) (10,11). In recent years, *AJAP1* has been shown to play a certain role in tumor proliferation, apoptosis, invasion, and metastasis (12-14); however, its role in the promotion of tumor progression remains

Highlight box

Key findings

- The results of our study suggest that the downregulation of AJAP1 protein expression may be closely related to the occurrence, development, and prognosis of salivary adenoid cystic carcinoma (SACC), and may play a certain role in progression and metastasis.

What is known and what is new?

- SACC is highly invasive and prone to neurovascular invasion and distant metastasis; however, its specific molecular mechanism remains unclear.
- In this study, we clarified the mechanism through which *AJAP1* regulates the progression of SACC both *in vitro* and *in vivo*. We also elucidated the causes of *AJAP1* downregulation in SACC and revealed its mechanism.

What are the implications, and what should change now?

- We believe that the results of our study will provide new ideas and targets for treating SACC and provide a new direction for the development and research of new drugs against SACC.

unclear. In glioblastoma multiforme (GBM), *AJAP1* can increase intercellular adhesion, reduce invasion and diversion, and inhibit proliferation and tumorigenicity *in vivo*, and the downregulation of *AJAP1* expression often suggests a poor prognosis (15). In liver cancer, a previous study demonstrated that Micro RNA-552 (miR-552) can promote tumor proliferation, invasion, and metastasis by downregulating *AJAP1* expression, indicating a poor prognosis (16). Moreover, Yan *et al.* observed that low expression levels of both Tet methylcytosine dioxygenase 1 (*TET1*) and *AJAP1* predict poor prognosis in urinary bladder cancer patients, and the frequently downregulated *TET1* level reduces the hydroxymethylation of the *AJAP1* promoter and subsequently activates β-catenin signaling to promote the development of urinary bladder cancer (17). Nevertheless, whether and how the mechanism of *AJAP1* inhibits the progression of SACC is currently unclear.

In this study, we clarified the mechanism through which *AJAP1* regulates the progression of SACC both *in vitro* and *in vivo*. We also elucidated the causes of *AJAP1* loss in SACC and revealed its mechanism. We present this article in accordance with the ARRIVE and MDAR reporting checklists (available at https://gs.amegroups.com/article/view/10.21037/gs-23-127/rc).

Methods

Array data

The Cancer Genome Atlas (TCGA), Gene Expression Profiling Interactive Analysis (GEPIA), GeneCards, and other databases were used to analyze the expression of *AJAP1* in various organs of the body and corresponding tumors. Genome-wide sequencing was performed on SACC tissues and paracancerous tissues to screen the differentially expressed genes and construct differentially gene expression tumor profiles. Gene Ontology (GO) and pathway enrichment were performed to analyze differentially expressed genes.

Cell culture and reagents

SACC-LM and SACC-83 were obtained from Peking University and cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS) at 37 ℃ in a humid incubator (Thermo Fisher Scientific, USA) containing 5% carbon dioxide (CO_2). The 5-Azacytidine (5-Aza) and Cell Counting Kit-8 (CCK-8 kit) were obtained from APExBIO (Houston, USA); puromycin and cell lysis buffer for western blot (WB) and IP (immunoprecipitation) were purchased from Beyotime (Shanghai, China); and the Protein Quantification Kit (BCA kit) was purchased from Thermo Fisher.

Immunohistochemistry

The SACC samples were collected by Zhejiang Provincial People's Hospital, and patients with concomitant malignancies will be excluded. Immunohistochemistry was performed as follows: the samples were fixed with formaldehyde and embedded in paraffin, before being cut into 7-μm-thick sections, dewaxed, and rehydrated to extract the antigen. The sections were then incubated with anti-AJAP1 (1:5,000; Proteintech, USA) overnight. After washing with phosphate-buffered saline (PBS), the sections were incubated with Horseradish Peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG), followed by hematoxylin and eosin (HE) staining. The slides were photographed and analyzed using a fluorescence microscope (Nikon Ni-U, Japan). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Medical Ethics Committee of Zhejiang Provincial People's Hospital (No. QT2022326) and informed consent was taken from all the patients. A protocol was prepared before the study without registration.

Methylation-specific polymerase chain reaction (MSP)

The DNA of SACC-LM and SACC-83 cells (SACC-LM is a subclone of SACC-83 cells, which are more capable of lung metastasis) was extracted according to the instructions of the DNA extraction kit (Servicebio, Wuhan, China), and its concentration and purity were determined by an Ultraviolet spectrophotometer (NanoDrop2000, Thermo Fisher Scientific, USA). The DNA that met the requirements was stored at –80 ℃ for use. Bisulfite modification was performed according to the instructions in the Methylation-Gold Kit (ZYMO, USA), and methylation-specific PCR (MSP) amplification was performed to analyze the Methylation state. The methylated primer design of *AJAP1* was as follows: M-*AJAP1*-F: GTTAGGTAGTAGTTGCGTTGGGC, M-*AJAP1*-R: TTCCTAAAACGAAACTAAACCACG. The unmethylated primer design was as follows: U-*AJAP1*-F:

Table 1 The primer sequence of genes

Gene	Primer sequence
AJAP1	Forward: 5'-CCTGCAGTGTTCTCACGAGT-3'
	Reverse: 5'-TCAGAAGAGGAGGGTCGGTT-3'
c-Myc	Forward: 5'-TGGAAAACCAGCCTCCCG-3'
	Reverse: 5'-TTCTCCTCCTCGTCGCAGTA-3'
CyclinD1	Forward: 5'-ATCAAGTGTGACCCGGACTG-3'
	Reverse: 5'-CCTTGGGGTCCATGTTCTGC-3'
MMP1	Forward: 5'-AGAGCAGATGTGGACCATGC-3'
	Reverse: 5'-TTGTCCCGATGATCTCCCCT-3'
GAPDH	Forward: 5'-AATGGGCAGCCGTTAGGAAA-3'
	Reverse: 5'-GCCCAATACGACCAAATCAGAG-3'

AJAP1, adherens junctions associated protein 1; *c-Myc*, transcriptional regulator Myc-like; *MMP1*, matrix metallopeptidase 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

TGGTTAGGTAGTAGTTGTGTTGGGT, U-*AJAP1*-R: ATTTCCTAAAACAAAACTAAACCACA.

The MSP products were then subjected to agarose gel electrophoresis. The homozygous methylation-positive results were defined as specific products and were mainly from the methylated primer rather than the unmethylated primer. If the unmethylated primer expanded the specific products but the methylated primer had no products, it was judged as being methylation-negative. If both primers had specific products, it was judged as being heterozygous methylation-positive. Both homozygous methylation-positive and heterozygous methylation-positive were considered methylation-positive.

Lentivirus-mediated transfection

SACC-LM and SACC-83 cells were plated in six-well plates, with 10^5 cells per well. After 24 h, lentiviruses (GENECHEM, Shanghai, China) with *AJAP1* overexpression or knockdown were added to SACC-LM or SACC-83, respectively. After 48 h, the expression rate of GFP was examined to evaluate the infection efficiency, and puromycin was used for screening. The expression of *AJAP1* was then verified using real-time quantitative polymerase chain reaction (RT-qPCR) and WB.

Real-time qPCR

Total RNA was harvested from the transfected SACC cells using TRIzol Reagent (Takara Biotechnology Co., Ltd., Kyoto, Japan) according to the manufacturer's instructions, and complementary DNA (cDNA) was obtained using a reverse transcription kit (Takara Biotechnology Co., Ltd., Kyoto, Japan). The obtained cDNA was then amplified by real-time qPCR using the following primers (*Table 1*).

WB analysis

SACC cells were lysed with Cell lysis buffer for Western and IP (WBIP) containing phenylmethanesulfonyl fluoride (PMSF). The protein concentration was determined by BCA assay. Protein samples were isolated by Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes, which were then incubated with anti-*AJAP1* (1:5,000; Proteintech), anti-E-cadherin (1:5,000; Proteintech), anti-β-catenin (1:5,000; Proteintech), anti-c-Myc (1:2,000; Proteintech), anti-CyclinD1 (1:5,000; Proteintech), anti-MMP1 (1:1,000; Proteintech), anti-actin (1:10,000; Proteintech), and anti-GAPDH (1:10,000; Proteintech) overnight. Following incubation, the membranes were washed three times with Tris-Buffered Saline and Tween 20 (TBST) and incubated with HRP-conjugated goat anti-rabbit/mouse IgG for 1 h. After washing with TBST three more times, the blots were analyzed using a gel imaging system (BIO-RAD, USA).

Cell viability and colony formation assays

For the cell viability assay, approximately 3,000 transfected SACC-LM and SACC-83 cells were plated in 96-well plates and cultured in a 37 ℃/5% CO_2 incubator for 24, 48, 72, 96, and 120 h. Following incubation, the supernatant was discarded and 100 μL of CCK8 solution was added to each well before incubating at 37 ℃ for 2 h in a 5% CO_2 incubator. Finally, the plates were read at 450 nm using a spectrometer (Bio Tek synergy LX, USA). For the colony formation assay, 1,000 cells were inoculated in each well of a six-well plate and cultured with RPMI 1640 containing 10% FBS in an incubator for 2 weeks. The colonies were stained with 0.1% crystal violet 20 min and photographed under a microscope (Nikon Ti, Japan).

Wound healing assay

SACC-LM and SACC-83 cells were plated in 12-well plates containing culture inserts (Ibidi, Germany). Next, 70 μL of cells were inoculated into each chamber of the culture insert. When the cell density reached 100%, the culture inserts were removed, and the cells were washed with PBS solution and cultured in RPMI 1640 medium without FBS. Images were taken at 0 and 24 h using a fluorescence microscope (Nikon).

Transwell invasion assay

The invasiveness of SACC-LM and SACC-83 cells was validated using a 24-well Transwell chamber (Corning Inc., USA). A layer of matrix gel was applied to the bottom of the Transwell chamber in advance, and the two SACC cells were re-suspended in serum-free 1640 medium and inoculated into the chamber. The lower layer of the chamber was inoculated with a complete medium. After 24 h, the invading cells were stained with 0.1% crystal violet 20 min and photographed under a microscope (Nikon).

Confocal microscopy

SACC-LM and SACC-83 cells were inoculated into a four-chamber glass bottom dish (Cellvis, USA), with approximately 3×10^4 cells inoculated into each well. After 24 h, the cells were fixed with methanol for 20 min and washed with PBS three times. The cells were blocked with PBS containing BSA for 20 min and washed with PBS three times before incubating with anti-flag, anti-β-catenin, and anti-E-cadherin at 4 ℃ overnight. Following incubation, the cells were washed with PBS three times and incubated with fluorescently labeled goat anti-rabbit/mouse IgG at room temperature for 1 h, before washing with PBS three times and staining with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI). After 10–15 min, the cells were subjected to laser scanning microscopy (Leica, Germany) and imaged.

Co-immunoprecipitation (Co-IP)

SACC-LM and SACC-83 cells were plated in 10 cm dishes and lysed with 500 μL cell lysis buffer for WB and IP, each containing 1 mM of PMSF to obtain proteins. The prepared protein solution was incubated with the corresponding primary antibody at 4 ℃ overnight. Next, 20 μL of fully suspended ProteinA+G Agarose (Beyotime, Shanghai, China) was added to the mixture, shaken slowly at 4 ℃ for 2 h, centrifuged at 1,000 g for 5 min, and the supernatant was discarded. Next, the precipitate was cleaned with 500 mL of PBS and centrifuged at 1,000 g for 5 min; this step was repeated five times. The supernatant was discarded for the final time, and 20 μL of 1× SDS-PAGE electrophoresis loading buffer was added to the precipitate and dried at 95 ℃ for 10 min. WB was performed using the corresponding antibodies.

Dual-luciferase reporter gene analysis

Next, to conduct dual luciferase reporter gene analysis, 2.5×10^5 SACC cells were inoculated into 24-well plates and cultured for 24 h. When the concentration reached 70–80%, the cells were transfected with β-catenin reactive double luciferin reporter plasmid Top-Flash or a negative control FOPFLASH (Merck-Millipore, USA) using the fugene6 (Invitrogen, USA) kit. After 48 h of transfection, luciferase reporter gene detection was performed according to the dual luciferase reporter gene detection system (Promega, Madison, WI, USA). Each experiment was measured in triplicate.

In vivo subcutaneous xenograft tumor model

Four-week-old nude mice were purchased from Hangzhou Ziyuan Experimental Animal Science and Technology Co., Ltd (Hangzhou, China). Animal experiments were performed under a project license (No. A2022010101) granted by Experimental Animal Welfare Ethics Committee of Zhejiang Provincial People's Hospital, in compliance with institutional guidelines for the care and use of animals.

The nude mouse transplanted tumor model was generated as follows: all nude mice were randomly divided into four groups with six mice in each group by simple randomization, and a suspension containing 10^6 SACC-LM (*AJAP1*-overexpressed and control) or SACC-83 (*AJAP1* knockout and control) cells were injected into the axilla. The tumor size and volume were measured regularly and after 30 days, the animals were executed painlessly for tumor dissection, and the samples were prepared for immunohistochemical analysis.

Statistical analysis

SPSS 18.0 statistical software (SPSS, Chicago, IL, USA) was adopted for data analysis. Counting data comparisons between groups were subjected to the χ^2 test or Fisher's exact test. P<0.05 was considered statistically significant. Survival analysis was computed by means of the Kaplan-Meier method and significance was assessed by means of the log-rank test. A multivariate analysis of the Cox regression model was used to determine prognostic factors.

Results

AJAP1 expression is significantly down-regulated in SACC

In the previous experiment, we selected three pairs of SACC tissues and paracancerous tissues for whole-genome sequencing, and the results showed that 4,386 genes were upregulated and 3,119 genes were downregulated in SACC tumors. GO and pathway enrichment analyses were performed to predict the functions of the genes. The GO enrichment analysis results indicated that the enrichment degree of differentially expressed genes was the highest in Biological adhesion, followed by Regulation of cell mobility, Intrinsic component of plasma membrane, Regulation of phosphate metabolic process, Response to organic substance (P<0.01). The Pathway enrichment analysis results indicated that the enrichment degree of differentially expressed genes was the highest in Extracellular matrix organization, followed by Rheumatoid arthritis, O-linked glycosylation, Interferon alpha/beta signaling, Collagen formation (P<0.01) (*Figure 1A-1D*). As both of the different expressed genes in Biological adhesion and Extracellular matrix organization processes contain *AJAP1*, we selected *AJAP1* as the candidate research target. Furthermore, to verify the expression of *AJAP1* in SACC tissues, we detected the expression of AJAP1 protein in the cancer tissues of patients with SACC was 44.76% (47/105), while in paracancerous tissues, the positive rate of the AJAP1 protein was 86.67% (91/105), which was significantly higher than that in cancer tissues (P<0.01) (*Figure 1E-1I*).

Low AJAP1 expression was significantly related to SACC lymph node metastasis and distant metastasis

We used the chi-squared test to further explore the correlation between *AJAP1* expression and the various clinical factors of SACC. The results showed that the downregulation of *AJAP1* expression was closely related to age, lymph node metastasis/distant metastasis, and tumor nodes metastasis (TNM) stage, but was not related to sex, primary tumor site, histotype, and nerve invasion. Moreover, *AJAP1* expression was significantly downregulated in the elderly group, lymph node metastasis and distant metastasis group, and TNM stage III–IV group (P<0.05) (*Table 2*). These results suggest that *AJAP1* plays an important role in the invasion and metastasis of SACC cells.

Kaplan-Meier analysis showed that patients with lymph node/distant metastasis, TNM stage III–IV, and *AJAP1* expression loss in the SACC group all had a poorer prognosis, including both progression-free survival (PFS) and overall survival (OS). In both the TNM stage I–II and III–IV subgroups, patients with SACC with *AJAP1*-deficient expression had a poorer prognosis, including PFS and OS (*Figure 2*). Cox regression analysis further revealed that low *AJAP1* expression was an independent risk factor for SACC prognosis (P=0.035) (*Table 3*).

High methylation of the promoter cytosine-phosphoric acid-guanine (CpG) island leads to silencing of AJAP1 expression in SACC tumors

Through pan-cancer analysis, we found that *AJAP1* expression in various tumor tissues was lower than that in normal tissues (TCGA: http://ualcan.path.uab.edu/cgi-bin/Pan-cancer.pl?genenam=*AJAP1*) (GEPIA: http://gepia.cancer-pku.cn/detail.php?gene=*AJAP1*) and was highly expressed in normal salivary gland tissues (GeneCards: https://www.genecards.org/cgi-bin/carddisp.pl?gene=*AJAP1*) (*Figure 3A-3C*). To further explore the cause of downregulated *AJAP1* expression in SACC tumors, we found that the *AJAP1* promoter regions exist on numerous CpG islands through University of California, Santa Cruz (UCSC) database analysis (UCSC: http://genome.ucsc.edu/cgi-bin/hgTracks?db=hg38&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr1%3A4654609-4792534&hgsid=1404994269_Afxnfju9XzXGW6eTYUVbavPiZ2aQ) (*Figure 3D,3E*). To further verify this result, DNA was extracted from the paraffin sections of 106 groups of SACC tumors and adjacent tissues. The methylation of the *AJAP1* promoter was detected by MSP. The results showed that the methylation degree of the *AJAP1* promoter in SACC tumor tissues was significantly higher than that in adjacent normal tissues. Correlation analysis showed that *AJAP1*

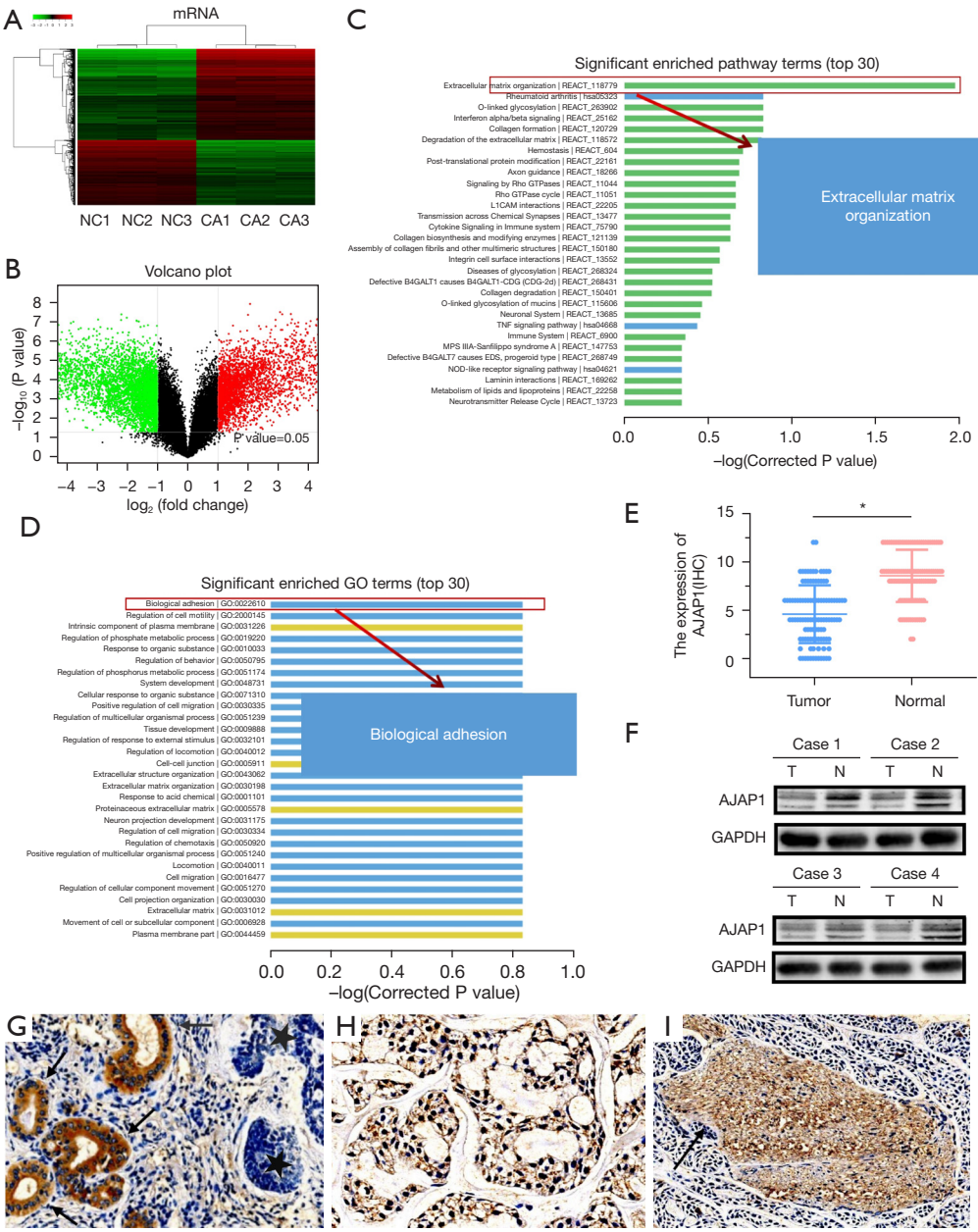

Figure 1 *AJAP1* was lowly expressed in SACC tumors. (A) Heat map of differentially expressed genes in SACC tumor tissues and paracancerous tissues; (B) Volcano map of differentially expressed genes in SACC tumor tissues and paracancerous tissues; (C) pathway enrichment analysis showed that the differentially expressed genes were mainly enriched in extracellular matrix organization; (D) GO enrichment analysis showed that the differentially expressed genes were mainly enriched in biological adhesion; (E) immunohistochemical results showed that the expression level of the *AJAP1* protein in paracancerous tumor tissues was significantly higher than that in tumor tissues; (F) Western blot results showed that the expression level of the *AJAP1* protein (45 kDa) in paracancerous tumor tissues was significantly higher than that in tumor tissues; (G) immunohistochemical showed *AJAP1* was highly expressed in the normal glandular tube (↑) but was poorly expressed in tumor tissues (×200) (★); (H) immunohistochemical showed the *AJAP1* protein was highly expressed in some tumor tissues (×200); (I) immunohistochemical showed the expression of the *AJAP1* protein was absent in tumors and highly expressed in tumor-associated nerves (↑) (×200). *, P<0.05. NC, normal control; GO, Gene Ontology; *AJAP1*, adherens junctions associated protein 1; IHC, immunohistochemistry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; T, tumor ; N, normal tissue; SACC, salivary adenoid cystic carcinoma.

Table 2 Correlation between clinical factors and *AJAP1* expression: univariate analysis

Parameters	Cases	*AJAP1*		χ^2	P
		Negative	Positive		
Gender					
Female	63	33	30	0.520	0.417
Male	42	25	17		
Age (years)					
<51	49	21	28	5.696	0.017*
≥51	56	37	19		
Primary tumor site					
Minor salivary glands	72	41	31	0.270	0.603
Major salivary glands	33	17	16		
Histotypes					
Cribriform	58	26	32	5.731	0.057
Tubular	27	18	9		
Solid	20	14	6		
Nerve invasion					
–	51	31	20	1.234	0.276
+	54	27	27		
Lymph node/distant metastasis					
–	91	46	45	6.068	0.014*
+	14	12	2		
TNM stage					
I–II	45	14	31	18.54	0.000*
III–IV	60	44	16		

*, P<0.05. TNM, tumor nodes metastasis.

promoter methylation was closely related to low *AJAP1* expression in tumor tissues (P<0.01) (*Figure 3F*). The MSP results indicated that the target gene band (M band) of methylated primers was significantly lighter than the target gene band (U band) of unmethylated primers in the control group. However, after being treated with 5-AZA, the target gene band (U band) of unmethylated primers became lighter, and the target gene band (M band) of methylated primers became dimmer (*Figure 3G,3H*). When the methylation inhibitor 5-Aza was added to SACC cells, the messenger RNA (mRNA) and protein levels of *AJAP1* were significantly upregulated in a dose- and time-dependent manner, suggesting that the methylation of the *AJAP1* promoter in SACC tumor cells may be the main reason for its downregulation (*Figure 3I,3J*).

AJAP1 overexpression or knockdown inhibited or promoted the invasion, metastasis, and proliferation of SACC cells, respectively

To further explore the effects of changes in *AJAP1* expression on the proliferation, invasion, and metastasis of SACC cells, we detected the expression of the *AJAP1* protein in SACC-LM and SACC-83 cells under wild-type conditions. The results showed that the *AJAP1* protein level was relatively low in SACC-LM cells but was

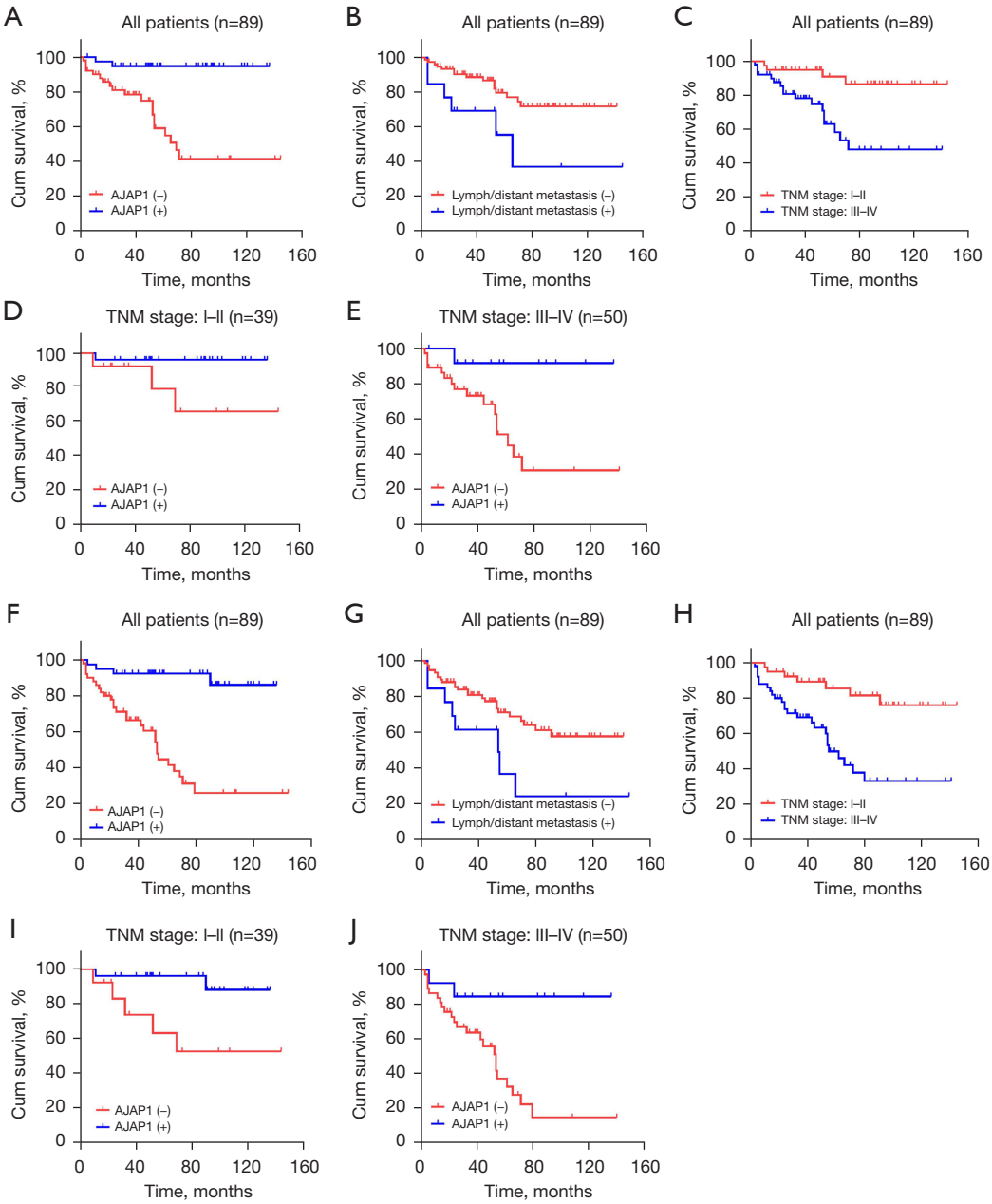

Figure 2 OS and PFS of SACC patients. (A) The OS of patients in the *AJAP1*-negative group was significantly poorer than that in the positive group; (B) the OS of patients in the lymph node/distant metastasis-positive group was significantly poorer than that in the negative group; (C) the OS in the TNM stage I–II group was significantly better than that in the stage III–IV group; (D) among patients in the TNM stage I–II group, the OS of patients in *AJAP1*-negative group was significantly poorer than that of patients in the positive group; (E) among patients in the TNM stage III–IV group, the OS of patients in the *AJAP1*-negative group was significantly poorer than that of patients in the positive group; (F) the PFS in the *AJAP1*-negative group was significantly poorer than that in the positive group; (G) the PFS in the lymph node/distant metastasis-positive group was significantly poorer than that in the negative group; (H) the PFS in the TNM stage I–II group was significantly better than that in the stage III–IV group; (I) in the TNM stage I–II group, the PFS of the *AJAP1*-negative group was significantly poorer than that of the positive group; (J) in the TNM stage III–IV group, the PFS of patients in the *AJAP1*-negative group was significantly poorer than that of patients in the positive group. TNM, tumor nodes metastasis; *AJAP1*, adherens junctions associated protein 1; OS, overall survival; PFS, progression-free survival; SACC, salivary adenoid cystic carcinoma.

Table 3 Univariate and multivariate Cox regression analysis for predicting the OS of SACC patients

Variables	Univariate analysis				Multivariate analysis			
	Exp(B)	95% CI Exp(B)		Sig.	Exp(B)	95% CI Exp(B)		Sig.
		Lower	Upper			Lower	Upper	
Gender (female *vs.* male)	0.845	0.353	2.025	0.706	–	–	–	–
Age (<51 *vs.* ≥51 years)	3.889	1.419	10.661	0.008	1.899	0.615	5.859	0.265
Primary tumor site (minor *vs.* major)	0.719	0.263	1.964	0.52	–	–	–	–
Histotypes (cribriform *vs.* tubular/solid)	3.22	1.295	8.007	0.012	1.687	0.574	4.962	0.342
Lymph node/distant metastasis (negative *vs.* positive)	2.87	1.107	7.439	0.03	1.835	0.697	4.831	0.219
Nerve invasion (negative *vs.* positive)	0.88	0.37	2.094	0.773	–	–	–	–
TNM stage (I–II *vs.* III–IV)	4.773	1.594	14.293	0.005	2.084	0.614	7.072	0.239
AJAP1 (negative *vs.* positive)	0.092	0.021	0.396	0.001	0.184	0.038	0.887	0.035

OS, overall survival; SACC, salivary adenoid cystic carcinoma; CI, confidence interval; TNM, tumor nodes metastasis.

relatively high in SACC-83 cells. Therefore, an *AJAP1* overexpression test was performed on SACC-LM cells, and an *AJAP1* knockdown test was conducted on SACC-83 cells in subsequent experiments (*Figure 4A-4D*). The CCK8 proliferation assay results showed that *AJAP1* overexpression in SACC-LM cells significantly downregulated the proliferation of tumor cells, while *AJAP1* knockdown in SACC-83 cells significantly upregulated the proliferation of tumor cells. Moreover, the colony formation assay results showed that *AJAP1* overexpression in SACC-LM cells significantly downregulated the colony formation ability of tumor cells, whereas *AJAP1* knockdown in SACC-83 cells significantly upregulated their colony formation ability (*Figure 4E-4J*). The wound healing assay results demonstrated that the migration ability of tumor cells was significantly downregulated after *AJAP1* overexpression in SACC-LM cells, and was significantly upregulated after *AJAP1* knockdown in SACC-83 cells. The Transwell assay showed that *AJAP1* overexpression in SACC-LM cells significantly downregulated the invasion ability of tumor cells, while *AJAP1* knockdown in SACC-83 cells significantly upregulated their invasion ability (*Figure 4K-4R*).

AJAP1 reduces the nuclear localization of β-catenin by forming the AJAP1/E-cadherin/β-catenin complex

To further explore the molecular mechanism through which *AJAP1* regulates the proliferation, invasion, and metastasis of SACC cells, we pulled down the possible binding protein of *AJAP1* by co-IP and found that the AJAP1 pull-down protein contained E-cadherin and β-catenin. Thus, we repeated the co-IP experiment with E-cadherin and β-catenin antibodies. Both AJAP1 and β-catenin were detected in the E-cadherin pull-down proteins, while both E-cadherin and AJAP1 were detected in the β-catenin pull-down proteins. These results suggested that AJAP1 may form a complex with E-cadherin and β-catenin in SACC cells (*Figure 5A-5C*).

We then detected the subcellular localization of *AJAP1*, E-cadherin, and β-catenin by laser scanning confocal microscopy, and the results showed co-localization in the cytomembrane (*Figure 5D*). Moreover, when *AJAP1* was knocked down or overexpressed in SACC-83/LM cells, we found that β-catenin showed increased or decreased nuclear localization, respectively. Additionally, β-catenin expression in the cytomembrane/cytoplasm and nucleus was detected by WB, which showed that when *AJAP1* was overexpressed in SACC-LM, the expression of β-catenin in the cytoplasm was increased but was downregulated in the nucleus. The knockdown of *AJAP1* expression in SACC-83 cells exhibited the opposite result (*Figure 5E*).

AJAP1 regulates the expression of β-catenin downstream genes by regulating its transcriptional activity

Previous results suggest that *AJAP1* knockdown or overexpression affects the subcellular localization of β-catenin; however, whether this directly affects the transcriptional activity of β-catenin remains unclear. To

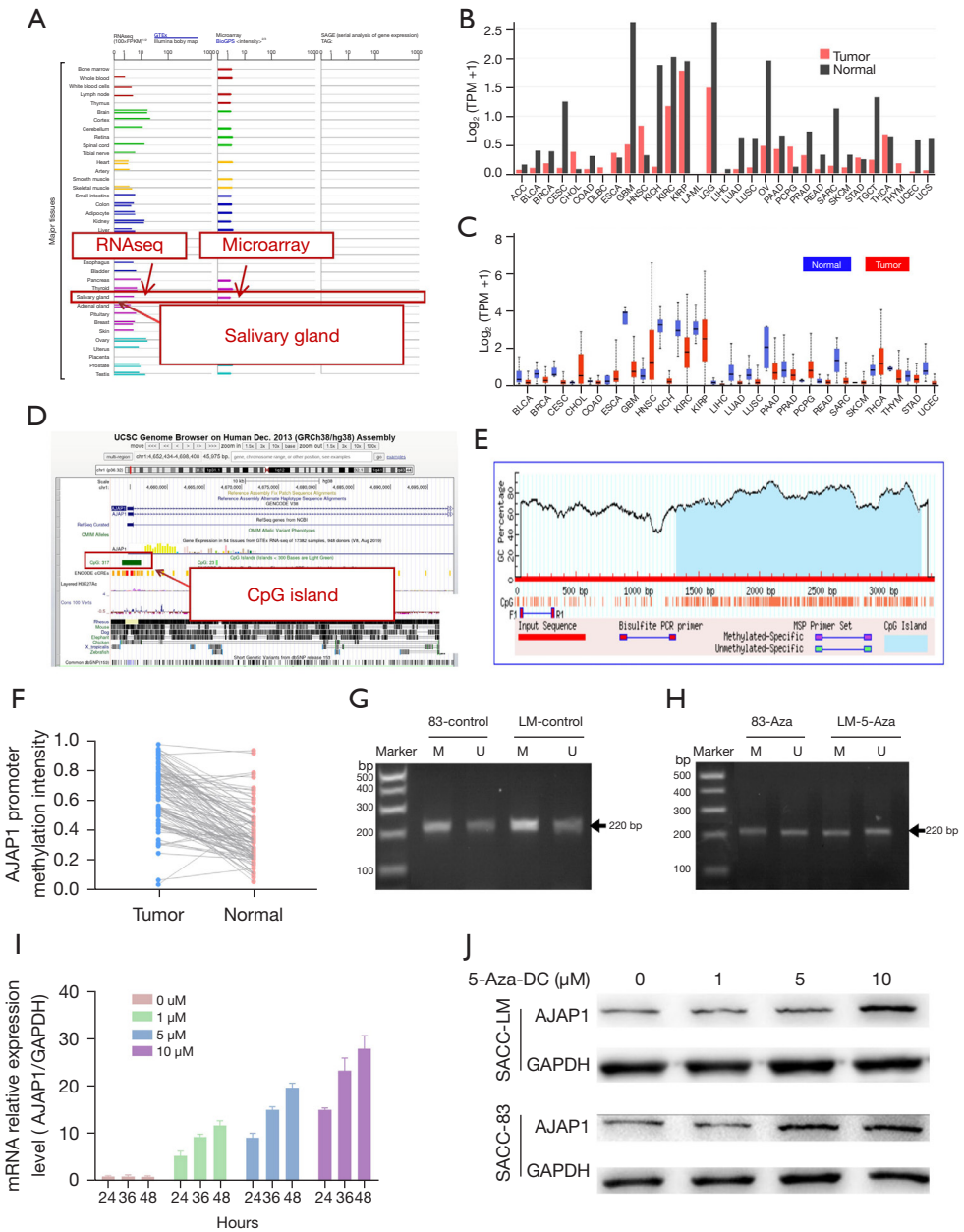

Figure 3 High methylation of the promoter CpG island leads to the silencing of *AJAP1* expression in SACC tumors. (A) The GeneCards database showed that *AJAP1* was highly expressed in normal salivary gland tissues; (B,C) the pan-cancer analysis showed that the expression of *AJAP1* in various tumor tissues was significantly lower than that in normal tissues; (D) the UCSC database showed a large number of CpG islands in the promoter region of *AJAP1* gene; (E) CpG analysis of the *AJAP1* gene promoter; (F) methylation of the *AJAP1* promoter in SACC tumor tissues was significantly higher than that in paracancerous tissues; (G) in the untreated group, methylation of the *AJAP1* gene promoter was detected by MSP; (H) promoter methylation of the *AJAP1* gene was detected by MSP in the 5-Aza treatment group; (I) the mRNA expression level of *AJAP1* increased with the increase of 5-Aza treatment concentration in SACC cells; (J) the expression of *AJAP1* protein (45 kDa) in SACC cells increased with the increase of 5-Aza treatment concentration. FPKM, Fragments Per Kilobase of exon model per Million mapped fragments; GTEx, genotype-tissue expression; TPM, transcripts per million; UCSC, University of California, Santa Cruz; *AJAP1*, adherens junctions associated protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 5-Aza, 5-Azacytidine; M, methylation; U, unmethylation; SACC, salivary adenoid cystic carcinoma; CpG, cytosine-phosphoric acid-guanine.

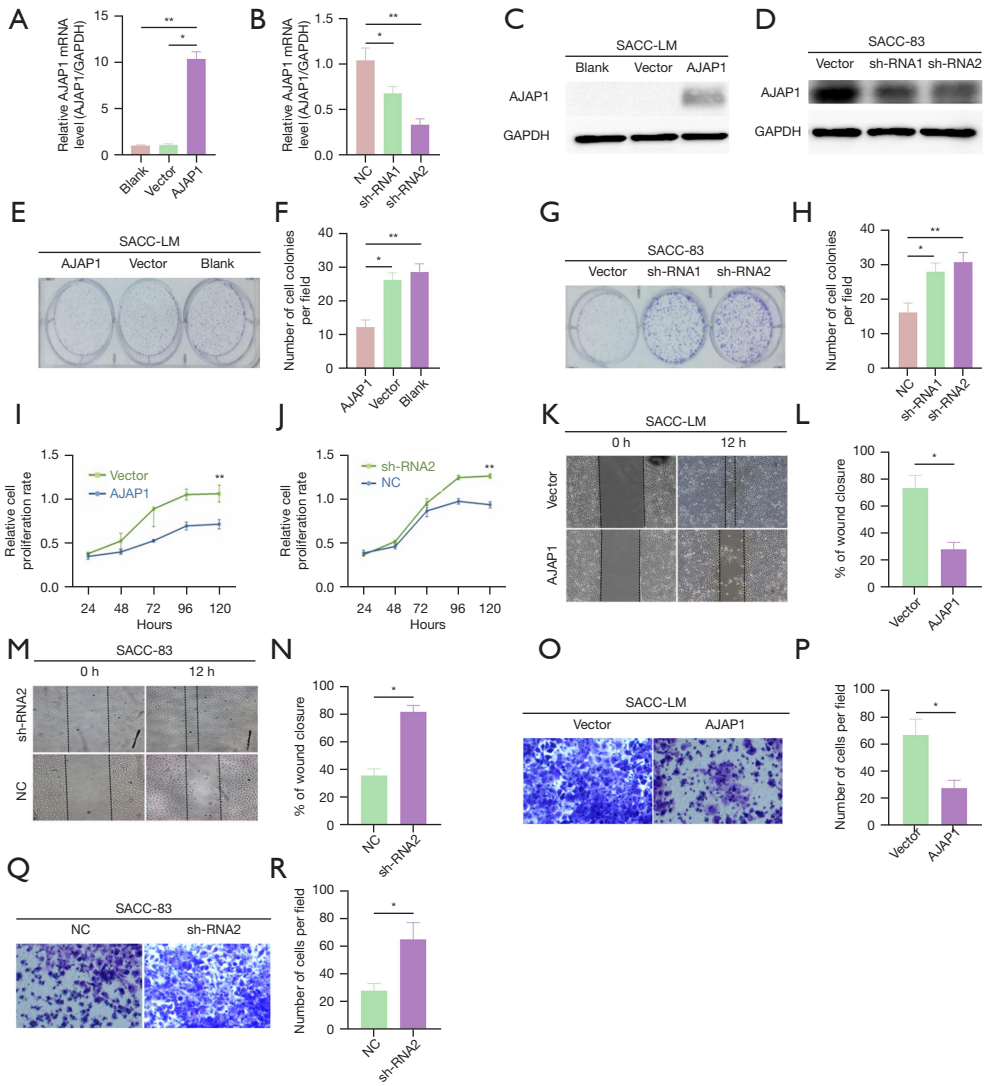

Figure 4 *AJAP1* overexpression or knockdown inhibited or promoted the invasion, metastasis, and proliferation of SACC cells. (A) The mRNA level of the *AJAP1* gene was significantly increased after the introduction of overexpressed lentivirus into SACC-LM cells; (B) the mRNA level of *AJAP1* gene was down-regulated after the introduction of knockdown lentivirus into SACC-83 cells; (C) the level of *AJAP1* protein (45 kDa) was increased after the introduction of overexpressed lentivirus into SACC-LM cells; (D) the expression of *AJAP1* protein (45 kDa) was significantly down-regulated in SACC-83 cells after the introduction of knockdown lentivirus; (E,F) the colony formation assay showed that *AJAP1* overexpression in SACC-LM cells significantly down-regulated the colony formation ability (crystal violet staining, ×4); (G,H) the colony formation assay showed that the colony formation ability was significantly up-regulated after the knockdown of *AJAP1* in SACC-83 cells (crystal violet staining, ×4); (I) the CCK8 proliferation assay showed that the overexpression of *AJAP1* significantly down-regulated the proliferation of SACC-LM cells; (J) the CCK8 proliferation assay showed that the knockdown of *AJAP1* significantly upregulated the proliferation of SACC-83 cells; (K,L) the scratch healing assay showed that the migration ability was significantly down-regulated after overexpression of *AJAP1* in SACC-LM cells (white light, high contrast resolution, ×100); (M,N) the scratch healing assay showed that the migration ability was significantly up-regulated after *AJAP1* knockdown in SSAC-83 cells (white light, high contrast resolution, ×100); (O,P) the Transwell assay showed that the invasion ability of SACC-LM cells was significantly down-regulated after the overexpression of *AJAP1* (crystal violet staining, ×200); (Q,R) the Transwell assay showed that the invasion ability of SACC-83 cells was significantly upregulated after *AJAP1* knockdown (crystal violet staining, ×200). *, P<0.05; **, P<0.01. *AJAP1*, adherens junctions associated protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SACC, salivary adenoid cystic carcinoma; NC, normal control.

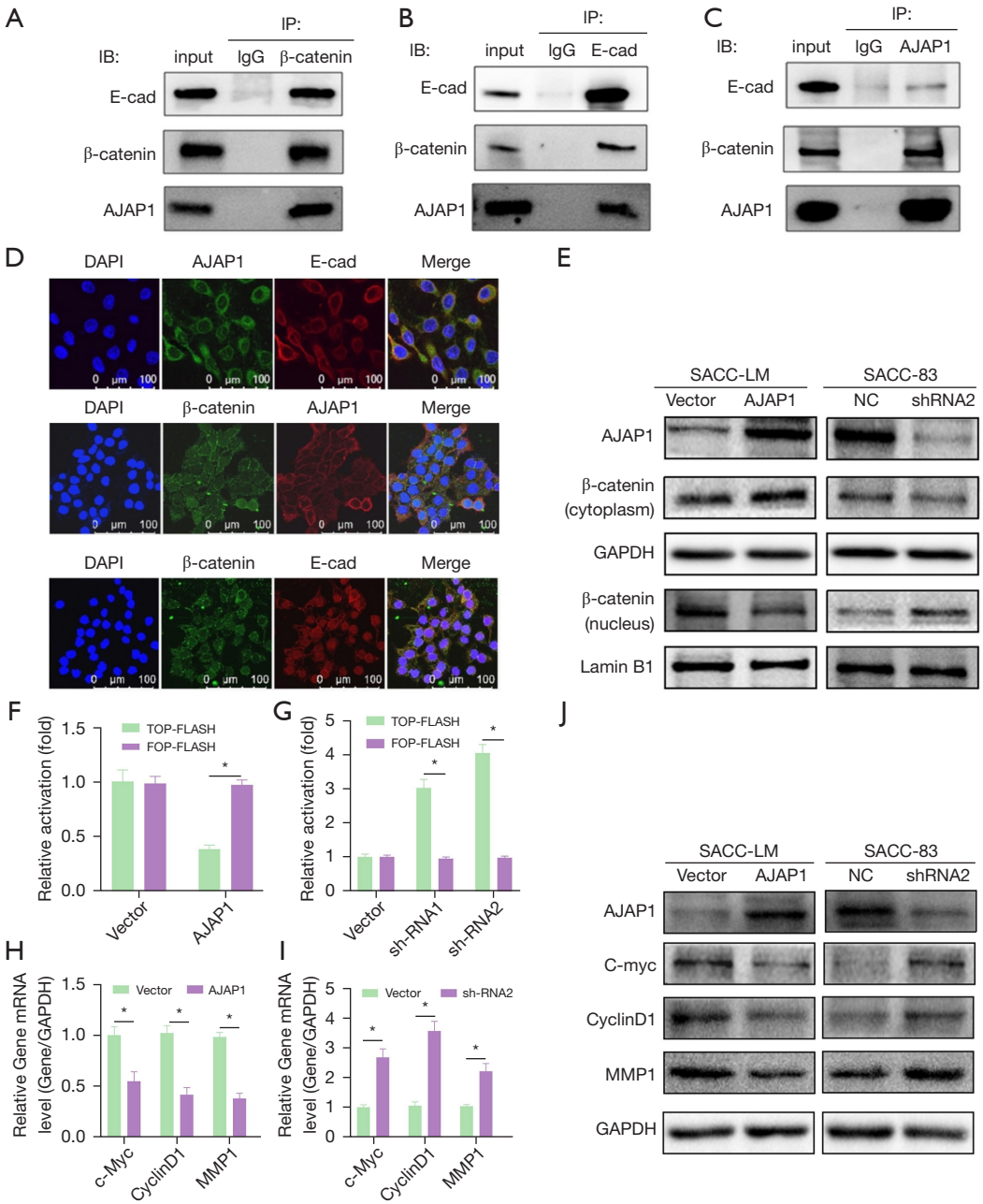

Figure 5 AJAP1 reduces the nuclear localization of β-catenin by forming the AJAP1/E-cadherin/β-catenin complex. (A-C) The co-IP assays showed that endogenous protein of *AJAP1* interacted with β-catenin and E-cadherin; (D) immunofluorescence displayed the co-localization among AJAP1 (45 kDa), E-cadherin (97 kDa), and β-catenin (85 kDa) (×200); (E) AJAP1 can bind to β-catenin in the cytoplasm, thereby reducing the content of β-catenin in the nucleus; (F) AJAP1 upregulation reduced the β-catenin/TCF/LEF-mediated transcription activity in SACC-LM cells; (G) AJAP1 depletion in SACC-83 cells increased the β-catenin/TCF/LEF-mediated transcription activity; (H-J) overexpression/knockdown of AJAP1 significantly up-regulated/down-regulated the expression of β-catenin downstream genes, respectively (AJAP1, 45 kDa; C-myc, 49 kDa; CyclinD1, 33 kDa; MMP1, 54 kDa; GAPDH, 36 kDa). *, P<0.05. IB, immunoblotting; IP, immunoprecipitation; IgG, immunoglobulin G; AJAP1, adherens junctions associated protein 1; DAPI, 4',6-diamidino-2-phenylindole; SACC, salivary adenoid cystic carcinoma; NC, normal control; co-IP, co-immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

this end, we further designed a β-catenin-responsive firefly luciferase reporter. Next, the plasmid T-cell factor/lymphoid enhancing factor (TCF/LEF) (TOP-FLASH) and non-responsive reporter plasmid (FOP-FLASH) were employed to evaluate the effect of *AJAP1* on β-catenin transcriptional activity. The results showed that the upregulation of *AJAP1* in SACC-LM cells reduced the transcriptional activity of β-catenin/TCF/LEF-dependent promoter (TOP-FLASH), but had little effect on FOP-FLASH. In contrast, *AJAP1* knockout in SACC-83 cells significantly increased the transcriptional activity of β-catenin/TCF/LEF-dependent promoters (*Figure 5F,5G*). *AJAP1* overexpression in SACC-LM cells decreased the expression of c-Myc, CyclinD1, and MMP1 at the mRNA and protein levels, while *AJAP1* knockout in SACC-83 cells upregulated the mRNA and protein expression of c-Myc, CyclinD1, and MMP1 (*Figure 5H-5J*). These results suggest that *AJAP1* deletion affects the nuclear localization of β-catenin, activates the transcriptional activity of β-catenin, and promotes the expression of its downstream genes.

Regulation of the Wnt/β-catenin signaling pathway could reverse the effect of AJAP1 on SACC cell function

Wnt/β-catenin agonist 2, an activator of the Wnt/β-catenin signaling pathway, was added into a culture medium of SACC-LM cells overexpressing *AJAP1*. The Transwell, wound healing, and CCK-8 assay results showed that Wnt/β-catenin agonist 2 could reverse the downregulation of invasion, migration, and proliferative ability of SACC-LM cells induced by *AJAP1* overexpression, respectively (*Figure 6*). We then added LF3, an inhibitor of the Wnt/β-catenin signaling pathway, into a culture medium containing knocked-down *AJAP1* SACC-83 cells. The Transwell, wound healing, and CCK-8 assay results showed that LF3 could reverse the upregulation of invasion, migration, and proliferative ability of SACC-83 cells induced by *AJAP1* knockdown, respectively (*Figure 6*). These results suggest that *AJAP1* may function by regulating the Wnt/β-catenin signaling pathway.

Subcutaneous xenograft assay confirmed that AJAP1 inhibited the growth and metastasis of SACC tumors

We next designed a subcutaneous xenograft assay in nude mice to verify the function of *AJAP1 in vivo*. The results showed that *AJAP1* overexpression in SACC-LM cells significantly reduced the tumorigenesis ability of SACC cells compared to the control group, suggesting that the overexpression of *AJAP1* significantly inhibited the tumorigenesis ability of SACC cells. However, when *AJAP1* was knocked down in SACC-83 cells, the tumor-forming ability was significantly stronger than that in the control group, suggesting that *AJAP1* knockdown can significantly promote the tumor-forming ability of SACC cells. Additionally, immunohistochemical detection was performed on the excised tumor tissues, and the results showed that the expression levels of the c-Myc, CyclinD1, and MMP1 downstream proteins of the β-catenin signaling pathway were significantly downregulated in the *AJAP1* overexpression group and were significantly upregulated in the *AJAP1* knockdown group (*Figure 7*).

Discussion

AJAP1 was initially identified as a novel transmembrane protein for epithelial cell adhesion, located in the 1p36 region of chromosome 1 (18). Numerous studies have identified multiple tumor suppressor-related genes in the 1p36 region, including *AJAP1*, Calcium/calmodulin dependent protein kinase II gamma (*CAMK2NA*) (19), Chromodomain helicase DNA binding protein 5 (*CHD5*) (20), Kinesin family member 1B (*KIF1B*) (21), Castor zinc finger 1 (*CASZ1*) (22), and miR-34a (23), which may be related to the occurrence and development of neural, endocrine, and reproductive tumors. In this study, high-throughput chip screening and clinicopathological analysis revealed that *AJAP1* expression deficiency may be associated with the progression and metastasis of SACC. Moreover, Cox regression analysis revealed that deficient *AJAP1* expression is an independent risk factor for SACC prognosis, and therefore, may play an important role in the progression of SACC.

At present, the mechanism underlying the upstream regulation of *AJAP1* is unclear. Qu *et al.* (16) found that *AJAP1* mRNA has a direct binding site with miR-552 in hepatocellular carcinoma (HCC) cells, and its expression may be negatively regulated by miR-552. A previous study has shown that a high degree of methylation exists in the 1p36 region in some tumors, which may explain the loss of expression of related tumor suppressor genes (24). Through database retrieval and MSP, we found that the *AJAP1* promoter region was highly methylated in SACC tumor cells and that demethylation drugs could significantly reduce the methylation level in this region and restore the expression of *AJAP1*. These results suggest that abnormal

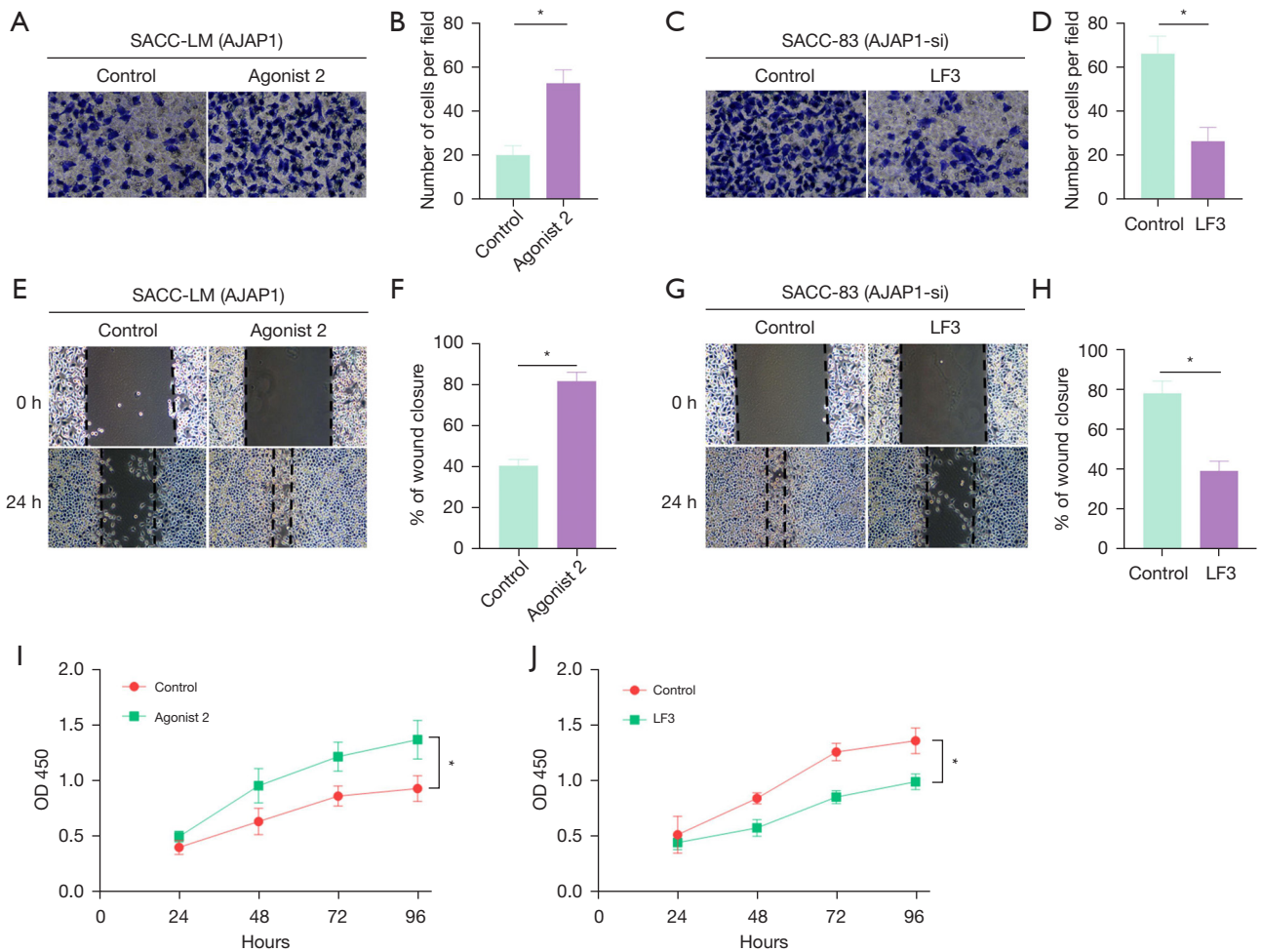

Figure 6 Regulation of Wnt/β-catenin signaling pathway could reverse the effect of *AJAP1* on the function of SACC cells. (A,B) Wnt/β-catenin agonist 2 could reverse the downregulation of invasion ability of SACC-LM cells induced by *AJAP1*overexpression (crystal violet staining, ×200); (C,D) LF3 could reverse the upregulation of invasion ability of SACC-83 cells induced by the knockdown of *AJAP1* (crystal violet staining, ×200); (E,F) Wnt/β-catenin agonist 2 could reverse the migration ability downregulation of SACC-LM cells induced by *AJAP1* overexpression (white light, high contrast resolution, ×100); (G,H) LF3 could reverse the migration ability upregulation of SACC-83 cells induced by *AJAP1* knockdown (white light, high contrast resolution, ×100); (I) Wnt/β-catenin agonist 2 could reverse the proliferative ability downregulation of SACC-LM cells induced by *AJAP1* overexpression; (J) LF3 could reverse the proliferative ability upregulation of SACC-83 cells induced by *AJAP1* knockdown. *, P<0.05. SACC, salivary adenoid cystic carcinoma; *AJAP1*, adherens junctions associated protein 1; Wnt/β-catenin signaling pathway, Wingless/Integrated/β-catenin signaling pathway; OD, optical density.

hypermethylation of the *AJAP1* promoter in SACC tumors is an important cause of the loss of *AJAP1* expression.

The role of *AJAP1* in tumor progression remains controversial. In GBM, Di *et al.* (15) found that the *AJAP1* protein could increase the intercellular adhesion, reduce invasion, metastasis, proliferation, and tumor-forming ability of tumor cells, and the loss of *AJAP1* expression was frequently correlated with a poor prognosis. Chen *et al.* (25) found that AJAP1 works like a tumor suppressor in prostate cancer and hypermethylation of CpG islands of AJAP1 promoter affected AJAP1 gene expression, and low expression of AJAP1 could activate downstream JAK2/STAT3 signaling pathway, promoting prostate cancer cell migration. Moreover, Qu *et al.* (16)

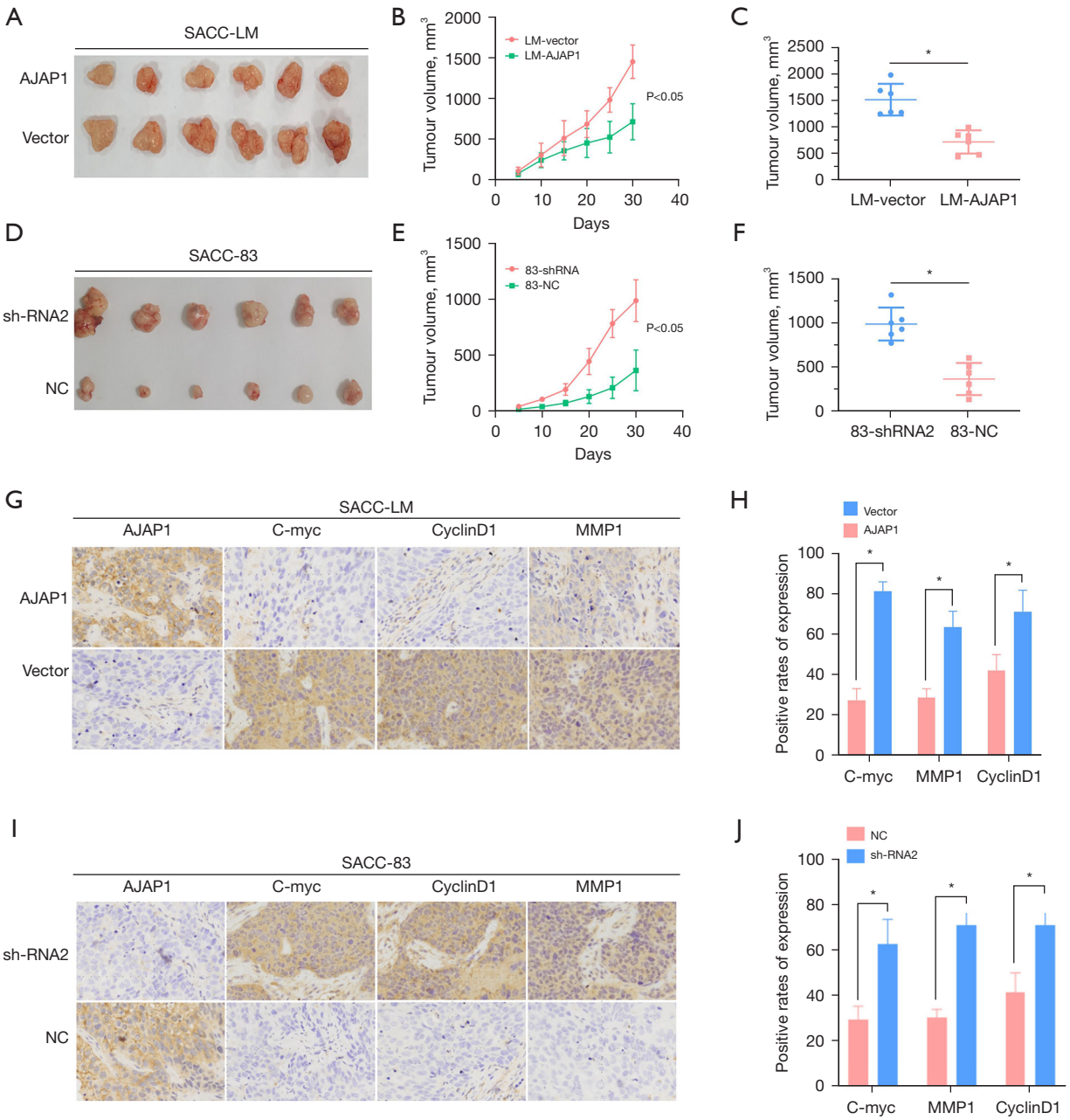

Figure 7 *AJAP1* inhibited the growth and metastasis of SACC tumors *in vivo*. (A-C) *AJAP1* overexpression in SACC-LM cells significantly reduced the subcutaneous tumorigenicity of SACC-LM cells in nude mice; (D-F) *AJAP1* knockdown in SACC-83 cells significantly increased subcutaneous tumorigenesis of SACC-83 cells in nude mice; (G,H) immunohistochemical showed *AJAP1* overexpression in SACC-LM cells significantly down-regulated the expression of β-catenin downstream genes (×200); (I,J) immunohistochemical showed *AJAP1* knockdown significantly up-regulated the expression of β-catenin downstream genes in SACC-83 cells (×200). *, P<0.05. *AJAP1*, adherens junctions associated protein 1; SACC, salivary adenoid cystic carcinoma; NC, normal control.

found that miR-552 could promote tumor proliferation, invasion, and metastasis by downregulating *AJAP1* expression in liver cancer, and showed that patients with *AJAP1* deletion had a significantly worse prognosis than those with normal expression. Furthermore, Xu *et al.* (26) demonstrated that *AJAP1* had a highly negative correlation with β-catenin nuclear expression and was a novel tumor suppressor in breast cancer, while β-catenin nuclear localization positively fed back on EGF/EGFR-attenuated *AJAP1* expression in breast cancer. In this study, by constructing *AJAP1* overexpression and knockdown cell lines, we found that *AJAP1* overexpression or knockdown could significantly inhibit or promote the proliferation, invasion, and metastasis of SACC cells, respectively, and reduce the tumorigenicity of SACC cells *in vivo*. The findings were consistent with those of Chunhui Di and Weiqing Qu, indicating that AJAP1 plays a pivotal role as a tumor suppressor gene in the progression and metastasis of SACC (15,16). Although recent studies have found that *AJAP1* plays a certain role in cell proliferation (27), apoptosis (15), invasion (13), and metastasis, the specific mechanism remains unclear. Using confocal imaging, Han *et al.* showed that *AJAP1* could stably transfect GBM cells and change the cell growth pattern, morphology, and distribution of β-tubulin and F-actin, thereby reorganizing the cytoskeletal structure, promoting intercellular adhesion, and inhibiting cell invasion and metastasis (28). *AJAP1*, which co-localizes and co-immunoprecipitates with endogenous E-cadherin, was initially described as a novel component of the adhesion of polarized epithelial cells and was suggested to bind directly to E-cadherin (29). Indeed, direct binding between E-cadherin and β-catenin has been found in a previous study (30). Therefore, in this study, the relationship between AJAP1/E-cadherin/β-catenin was detected by laser scanning confocal microscopy and immunoprecipitation assay, indicating co-localization between AJAP1/E-cadherin/β-catenin, which could directly bind to form complexes. Moreover, the loss of *AJAP1* expression could lead to the nuclear translocation of β-catenin, which reduces the content of β-catenin in the cytoplasm and capsule, but increases its content in the nucleus, thereby activating the β-catenin signaling pathway and promoting the expression of the c-Myc, CyclinD1, and MMP-1 downstream genes, thus promoting the progression and metastasis of SACC tumors. Using the activator and inhibitor of the Wnt/β-catenin signaling pathway, we further confirmed that *AJAP1* may exert its function in SACC cells by regulating the Wnt/β-catenin signaling pathway.

Wnt/β-catenin signaling pathway has been found abnormal activation in many solid cancers, including SACC. Ji *et al.* found CLDN7 inhibits cell proliferation and metastasis by inactivating the Wnt/β-catenin signaling in SACC (31). Fatty acid synthase was conformed contributes to epithelial-mesenchymal transition and invasion of SACC through PRRX1/Wnt/β-catenin pathway (32). The overactivation of Wnt/β-catenin signaling pathway was found be associated with radioresistance of cervical cancer and esophageal squamous cell carcinoma, while its role in SACC radioresistance is still unclear and more research is needed (33,34).

Meanwhile, the *in vivo* subcutaneous xenograft assay in nude mice demonstrated that overexpression or knockdown of *AJAP1* in SACC cells significantly inhibited or promoted tumorigenesis in nude mice, respectively. These results suggest that in SACC tumors, *AJAP1* binds β-catenin to the membrane and cytoplasm through the formation of an AJAP1/e-cadherin/β-catenin complex and reduces the amount of β-catenin entering the nucleus, thus blocking the Wnt/β-catenin signaling pathway and mediating its anti-cancer effects.

Conclusions

In conclusion, the high methylation of the *AJAP1* promoter leads to loss of expression, which then activates the Wnt/β-catenin signaling pathway and promotes tumor progression and metastasis. Until now, this mechanism has not been previously reported in SACC tumors. We believe that the results of our study will provide new ideas for the treatment of SACC, and direct toward the development of new targeted drugs.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://gs.amegroups.com/article/view/10.21037/gs-23-127/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Medical Ethics Committee of Zhejiang Provincial People's Hospital (No. QT2022326) and informed consent was taken from all the patients. Animal experiments were performed under a project license (No. A2022010101) granted by Experimental Animal Welfare Ethics Committee of Zhejiang Provincial People's Hospital, in compliance with institutional guidelines for the care and use of animals.

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