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Proteolytic cleavage of amyloid precursor protein by ADAM10 promotes proliferation and migration via activating MAPKs pathway in tongue squamous cell carcinoma in vitro

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ABSTRACT

APP, well-studied in the development of Alzheimer's disease, has been recently identified as the key gene correlated with TSCC. Here, we investigate the function of APP and its proteolytic cleavage by ADAM10 in the pathogenesis of TSCC. A total of 63 TSCC patients and 30 healthy controls were included and the results of IHC assay showed high expressions of ADAM10 and APP in TSCC tissues compared to paired para-carcinoma tissues. Interestingly, APP expression in TSCC patients was correlated with ADAM10 expression and their combined expression was related to the poor patients' survival. We found that APP was α-cleaved in TSCC cells to form sAPPα, and the serum level of sAPPα but not sAPPβ in TSCC patients was higher than healthy controls. Both overexpression with full-length APP and sAPPα promoted TSCC cell proliferation, migration and invasion. Downregulation of APP or ADAM10 by siRNA decreased the generation of sAPPα and inhibited the activity of ERK1/2 and p38 pathways, thereby reducing TSCC cell proliferation, migration and invasion. Treatment with ERK1/2 or p38 agonist or sAPPα overexpression reversed the effects of APP or ADAM10 knockdown. In conclusion, our data demonstrated the pathogenic roles of APP cleaved by ADAM10 to activate ERK1/2 and p38 pathways in TSCC cells. Both high expressions of ADAM10 and APP were related to poor prognosis. Targeting APP cleaved by ADAM10 might be a potential strategy in TSCC treatment.

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Introduction

Tongue squamous cell carcinoma (TSCC), the most prevalent squamous cell carcinoma of the head and neck (HNSCC), is characteristic of a high proliferation rate and high lymph node metastasis (1). Distant metastasis and postoperative relapse are the most common causes of the death of TSCC (2). Therefore, it is urgent to explore the potential molecular mechanism of the occurrence and development for TSCC to develop a new therapeutic approach.

Amyloid precursor protein (APP) is a highly conserved single type I transmembrane protein which implicated in the development of Alzheimer's disease (AD) and other non-neurogenic diseases like cancer (3). In nasopharyngeal carcinoma (NPC) and oral squamous cell carcinoma (OSCC), APP expression was identified as upregulated and it promotes cancer cell growth and migration (4, 5). Recent bioinformatics analysis indicated that there were notable differences in APP expression between TSCC tissues and the paired para-cancerous tissues (6). However, whether and how APP functioned in TSCC has not been reported.

APP was proteolytically cleaved into active fragments through two major pathways: the amyloidogenic pathway relevant to the pathogenesis of AD and the non-amyloidogenic pathway associated with carcinogenesis (7). In the amyloidogenic pathway, APP undergoes sequentially β-cleaved and γ-cleaved by secretases to form soluble N-terminal extracellular APP fragment (sAPPβ), Aβ peptide and AICD (intracellular domain of APP). In the non-amyloidogenic pathway, APP was α-cleaved (ADAM10 or ADAM17) and γ-cleaved to produce sAPPα, P3 (3 kD) and AICD (8). Aβ is extensively considered as the primary cause of AD (9). In NSCLC, AICD was involved in the modulation of the cell cycle (10). It was reported that the prevention of sAPPα generation reduced the viability of pancreatic cancer cells and improved the effectiveness of chemotherapy (11). In our study, we wished to investigate the effect and underlying molecular mechanisms of APP in the pathogenesis of TSCC.

The ADAMs family (A Disintegrin And Metalloproteinase), with a disintegrin domain and a metalloproteinase domain (12), are indispensable for ectodomain shedding of transmembrane proteins and regulate tumor metastasis by degrading the extracellular matrix (ECM) and accommodating cell mobility (13, 14). It was reported that proteolytic cleavage of APP by ADAM10 promotes the proliferation and migration of breast cancer cells (15). ADAM10 was also found to enhance the growth and migration of TSCC cells (16). Here, we investigated whether APP participated in the pathogenesis of TSCC through sAPPα gene-

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rated by ADAM cleavage in the present study.

Taken together, the current study was designed to investigate whether ADAM10 functions to cleave APP to generate sAPPα, as well as the underlying molecular mechanisms of sAPPα regulate the function of TSCC cells.

Materials and Methods

Study subjects and tissue collection

A total of 63 patients were diagnosed with TSCC and treated with partial tongue resection surgery at the Jinan Stomatology Hospital from September 2019 and February 2021 were included in this study. Patients with other primary tumors were excluded from this study. Overall survival (OS) was used to evaluate the patients' prognosis. Both TSCC tissues and the para-carcinoma tissues were collected from each patient. The detailed information of 63 TSCC patients is shown in Table 1. All specimens were fixed with formalin and embedded in paraffin for immunohistochemical (IHC) analysis and histopathological diagnosis.

Table 1. Clinical characteristics 63 patient samples of TSCC.

Characteristics	Number of cases (%)
Age (years)	
≤45	21 (33.3)
>45	42(66.7)
Gender	
Male	29 (44.4)
Female	34 (55.6)
Tumor size (cm)	
≤2	37 (58.7)
>2	26 (41.3)
TNM stage	
I-II	39 (61.9)
III-IV	24 (38.1)
T classification	
T1+T2	40 (63.5)
T3+T4	23 (36.5)
N classification	
N0+N1	43 (68.3)
N2+N3	20 (31.7)
M classification	
M0	44 (69.8)
M1	19 (30.2)
Differentiation	
Well	42 (66.7)
Moderate	14 (22.2)
poor	7 (9.0)
APP expression	
Low	19 (30.2)
High	44 (69.8)
ADAM10 expression	
Low	21(33.3)
High	42 (66.7)
Relapse	
Yes	25 (39.7)
No	38 (60.3)
Death	
Yes	21 (33.3)
No	42 (66.7)

IHC staining

Paraffin-embedded specimens were cut into 4-μm slices, dewaxed with xylene and rehydrated through a series of graded ethanol. After blocking, the slices were incubated with anti-ADAM10 (CST, USA) and anti-APP N terminus (Sigma, USA) overnight at 4°C. Then the corresponding HRP-conjugated secondary antibody (Sigma, USA) was applied and incubated for 1 h. Next, the slices were visualized with diaminobenzidine (DAB, Sigma, USA), and counterstained with hematoxylin. The IHC scores were determined to conform to the percentage of positively stained tumor cells by two independent pathologists with consensus without knowledge of the clinicopathological information. The scores were classified according to the percentage of positive cells: 0 (0–5%), 1 (5–25%), 2 (25–50%), 3 (50–75%) and 4 (75–100%). A score of ≤ 2 was estimated as low expression, and scores of > 2 indicated high expression.

ELISA

The serum levels of sAPPα and sAPPβ of TSCC patients and healthy controls were evaluated by a Human sAPPα/sAPPβ ELISA Kit (Meso Scale Discovery, USA) according to the manufacturer's instruction.

Cell lines and reagents

Cell lines including HOK, H357, Cal27, SCC9 and SCC25 were purchased from the ATCC and cultured in DMEM (Sigma, USA) containing 10% FBS (Gibco, USA) and 1% P/S (Gibco, USA) at 37 °C with 5% CO_2.

The antibodies used in this study were as follows: anti-APP N terminus (22C11) (Sigma, USA), anti-sAPPα (TECAN, Switzerland), anti-sAPPβ (Biolegend, USA), anti-ADAM10 (CST, USA), anti-p38 (CST, USA), anti-p-p38 (CST, USA), anti-ERK1/2 (CST, USA), anti-p-ERK1/2 (CST, USA), anti-GAPDH (CST, USA) and goat anti-rabbit/mouse IgG (CST, USA). The ERK1/2 agonist Pamoic acid disodium and p38 agonist 4-Hydroxylonchocarpin were purchased from MedChenExpress (MCE, USA)

The human siRNAs including negative control siRNA (si-NC), APP siRNA (si-APP) and ADAM10 siRNA (si-ADAM10) were bought from Santa Cruz Biotechnology (USA). The human plasmids of pCAX and pCAX-APP751 (full length) and pCAX-sAPPα were purchased from Addgene (USA).

Cell transfection

Cells were seeded in 12-well plates and cultured for 18 h. For knockdown experiments, cells were transfected with 12 pmol of siRNA using Lipofectamine RNAiMax (Invitrogen, USA). For overexpression experiments, cells were transfected with 1μg of plasmids using Lipofectamine 3000 (Invitrogen, USA). After 48 h of transfection, cells were harvested for further detection.

Immunoblotting

The cells were lysed with RIPA buffer (Thermo, USA)

containing protease inhibitors (Thermo, USA). The protein concentration was measured by Bradford assay (Thermo, USA). The same amounts of protein were loaded on 10% SDS-PAGE gels and transferred onto the PVDF membrane (Millipore, USA). After blocking, The membranes were incubated with the specific primary antibodies and the secondary antibody. The protein bands were detected using the ECL Plus Reagent (Thermo Fisher, USA).

EdU proliferation assay

The cell proliferation rate was assessed by the EdU-594 Cell Proliferation Assay Kit (Chemical Book, Beijing, China). Briefly, cells were seeded into 12-well plates and incubated for 48 h. Then cells were incubated with EdU solution for 3 h at 37 °C. Then the nuclei were stained with Hoechst 33258 (Thermo, USA). The images were acquired by fluorescent microscopy.

Transwell assay

Cells were digested and resuspended in serum-free DMEM. Then 0.2 mL cell suspensions were added into the upper chamber (8 μm pore size) without a coated polycarbonate membrane (Corning, USA) for migration assay while with a matrigel-coated membrane for invasion assay. The lower chamber was added with 0.5 ml DMEM with 10% FBS. After incubation for 24 h, the up chamber was stained with 0.1% crystal violet. The images were visualized and counted by a microscope.

Statistical analyses

All data from triplicate experiments were expressed as mean ± SD. Student's test and one-way ANOVA followed by Sidak post hoc test were applied to evaluate differences among two or multiple groups. Chi-square analysis was applied to measure the association between variables. The Kaplan-Meier test was used to evaluate the overall survival (OS) rates of TSCC patients. All analyses were performed with GraphPad Prism software. $P < 0.05$ was referred to be a statistically significant difference.

Results

High co-expression of ADAM10 and APP correlated with poor prognosis and APP underwent α-cleavage in TSCC

To examine the expressions of APP and ADAM10 in TSCC, IHC staining was performed in TSCC tissues and adjacent non-tumorous tissues (ANT) from 63 patients. The results indicated that the expressions of APP and ADAM10 were notably elevated in TSCC tissues in comparison with ANT (Figure 1A). It was shown that 69.8% (44/63) of the cases displayed high expression of APP, while 66.7% (42/63) of the cases displayed high expression of ADAM10 (Table 2). Then the potential correlation between the expressions of APP and ADAM10 as well as the clinical-pathological features were analyzed using the Chi-square test. As presented in Table 2, APP expression significantly correlated with TNM stage ($P = 0.02$), but not with age, gender, tumor size, T classification, N classification, M classification, differentiation, ADAM10 expression, relapse and death (all $P \geq 0.05$). Whether ADAM10 expression disturbed the prognostic value of APP expression in TSCC patients was further examined. The results determined that the cases with $APP^{hi}ADAM10^{hi}$ exhibited poor survival compared with the cases with $APP^{low}ADAM10^{low}$ (Figure 1B).

Next, we detected the expressions of APP in human normal oral epithelial cell line HOK and TSCC cell lines including H357, Cal27, SCC9 and SCC25 by western blot. APP has several isoforms that are subjected to post-translational N/O-glycosylation and potential tyrosine sulfation (17). Figure 1C displayed that Cal27 and SCC9 cells had obviously high levels of APP among four TSCC cell lines. Moreover, we found soluble APP (sAPPs) secreted by TSCC cells in the culture supernatant including APP N-terminus and sAPPα, but not including sAPPβ (Figure 1C). To confirm this observation, the ELISA results determined that the serum level of sAPPα significantly increased while the level of sAPPβ had no change in TSCC patients compared with healthy controls, indicating that APP was α-cleaved to produce sAPPα in TSCC (Figure 1D).

Aberrant expression of APP affected the malignant properties of TSCC cells

To investigate the effect of APP in TSCC, APP was knocked down using its specific siRNA in Cal27 and SCC9 cells. The expression of APP was decreased in Cal27 and SCC9 cells after siRNA-APP transfection compared with siRNA-NC transfection (Figure 2A). The result of Figure 2B showed that APP knockdown significantly suppressed the proliferation of Cal27 and SCC9 cells. The migrated and invaded Cal27 and SCC9 cells were also obviously reduced by APP knockdown (Figure 2C).

To further confirm the function of APP in TSCC, either full-length APP or sAPPα were successfully transfected into Cal27 and SCC9 cells and both full-length APP and sAPPα overexpression increased the sAPPα protein level

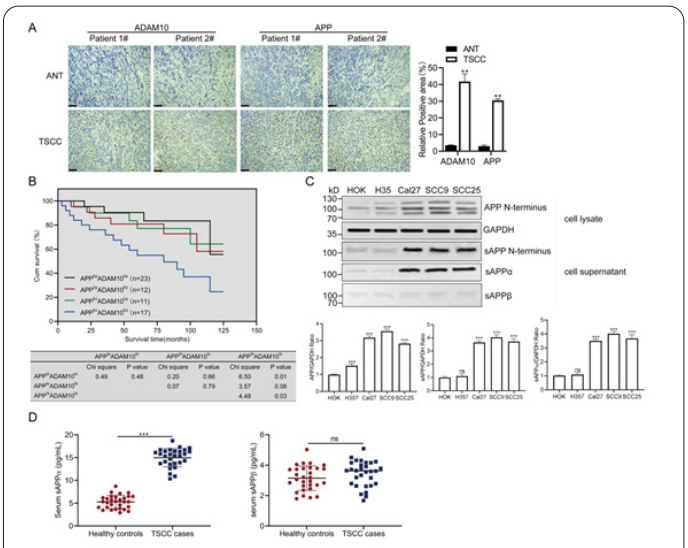

Figure 1. High co-expression of ADAM10 and APP correlates with poor prognosis of TSCC patients. (A) Immunohistochemical staining of ADAM10 and APP in two cases of representative TSCC tissues and adjacent non-tumorous tissues (ANT) (×200). Scale bar: 50μm. (B) The overall survival of TSCC patients using Kaplan-Meier analysis with combined analysis of ADAM10 and APP expression by IHC. (C) Western blot analysis using antibody against APP N-terminal (22C11), sAPPα and sAPPβ in cell lysates and culture supernatant of HOK, H357, Cal27, SCC9 and SCC25 cells (n = 3). (D) The serum levels of sAPPα and sAPPβ in TSCC patients and healthy controls were examined by ELISA (n = 30). Data were shown as mean ± SD. $^{**}P < 0.01$, and $^{***}P < 0.001$, ns no significant difference.

Table 2. Clinicopathological correlation of APP expression in TSCC.

	APP expression (n)		*P*
	Low	High	
Age (years)			0.12
≤45	9	12	
>45	10	32	
Gender			0.21
Male	11	18	
Female	8	26	
Tumor size (cm)			0.11
≤2	14	23	
>2	5	21	
TNM stage			0.02*
I-II	16	23	
III-IV	3	21	
T classification			0.59
T1+T2	13	27	
T3+T4	6	17	
N classification			0.25
N0+N1	10	34	
N2+N3	9	10	
M classification			0.05
M0	18	26	
M1	8	11	
Differentiation			0.30
Well	10	32	
Moderate	6	8	
poor	3	4	
ADAM10 expression			0.85
Low	6	15	
High	13	29	
Relapse			0.54
Yes	6	15	
No	13	29	
Death			0.85
Yes	6	15	
No	13	29	

in the culture supernatant (Figure 2D). The proliferation and migration of Cal27 and SCC9 cells were notably elevated by both full-length APP and sAPPα overexpression (Figure 2E-F). Interestingly, the proliferation of Cal27 and SCC9 cells with full-length APP overexpression was higher than that with sAPPα overexpression, while the migration of Cal27 and SCC9 cells with full-length APP overexpression was slower than that with sAPPα overexpression.

Knockdown of APP reduced the malignant properties of TSCC cells via inhibiting ERK and p38 pathways

ERK and p38 pathways are found to be implicated in regulating TSCC cell growth and migration. To elucidate how APP functioned in TSCC, we examined whether APP knockdown could affect the activity of ERK and p38 pathways. It was found that knockdown of APP markedly reduced the levels of p-ERK1/2 and p-p38 (Figure 3A). Treatment with ERK or p38 agonist counteracted the reduced proliferation, migration and invasion of Cal27 and SCC9 cells caused by APP knockdown to a large extent (Figure 3B-C). These data demonstrated that APP functioned in TSCC cells via activating ERK1/2 and p38 pathways.

The inhibitory effects of ADAM10 knockdown could be reversed by sAPPα overexpression

ADAM10, as a α-secretase, is responsible for the α-cleavage of APP to generate sAPPα. Expectedly, higher expression of ADAM10 in Cal27 and SCC9 cells was determined by western blot (Figure 4A). Next, ADAM10 was successfully knocked down using its specific siRNA (Figure 4B). It was indicated that ADAM10 knockdown abolished the generation of sAPPα in the culture supernatant of Cal27 and SCC9 cells (Figure 4C). Consequently, the levels of p-ERK1/2 and p-p38 were decreased by ADAM10 knockdown (Figure 4D). In addition, ADAM10 knockdown remarkably reduced the proliferation, migration and invasion of Cal27 and SCC9 cells (Figure 4E-F). However, sAPPα overexpression reversed the activity of ERK1/2 and p38 and counteracted the decreased proliferation, migration and invasion of Cal27 and SCC9 cells caused by ADAM10 knockdown (Figure 4D-F).

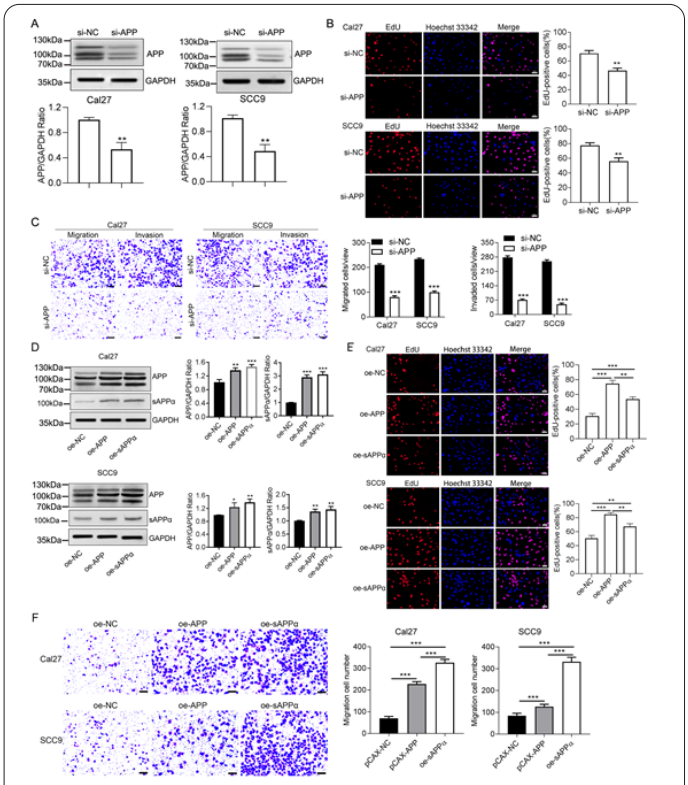

Figure 2. APP enhances the proliferation, migration and invasion of TSCC cells. (A) The protein level of APP was detected by western blotting. (B) Cell proliferation was determined by EdU assay. Scale bar: 50μm. (C) Transwell assay was used to evaluate cell migration and invasion. Scale bar: 50μm. (D) Western blotting was used to detect the expression of APP in cell lysates and the protein level of sAPPα in the culture supernatant of Cal27 and SCC9 cells. (E) Cell proliferation was determined by EdU assay. Scale bar: 50μm. (F) Transwell assay was used to detect cell migration. Scale bar: 50μm. Data were shown as mean ± SD (n=3). *$P < 0.05$, **$P < 0.01$, and *** $P < 0.001$.

Discussion

TSCC is one of the common types of HNSCC. Previous reports have indicated that APP expression was elevated in various cancer cells, which was associated with increased tumor cell differentiation, growth, migration and invasion (18-20). Zhang *et al.* proposed that APP might be a potential biomarker for TSCC (6), however, its functional roles in TSCC have not been reported yet. Herein, we indicated that APP was remarkably elevated in TSCC tissues compared with the adjacent non-tumorous tissues, and APP expression was outstandingly relevant to the TNM stage of TSCC patients. A further study presented that APP expression was also upregulated in TSCC cell lines. Knockdown of APP inhibited the growth, migration and invasion of TSCC cells, while overexpression with APP had the opposite effect. This suggested that APP upregulation was related to the development of TSCC.

The products generated during APP processing, especially sAPPα and AICD, play important roles in carcinogenesis (11, 21). It has been identified that sAPPα is highly secreted in pancreatic cancer cells and critical for chemotherapeutic tolerance and cancer cell survival and growth (22). In this study, we found that the serum level of sAPPα but not sAPPβ was notably higher in TSCC patients than that of healthy controls. Further studies indicated that APP was α-cleaved to generate sAPPα in TSCC cells. The elevated sAPPα level by transfection of full-length APP or sAPPα plasmids improved the TSCC cell proliferation, migration and invasion. These data demonstrated that sAPPα generated from proteolytical cleavage of APP participated in the development of TSCC. Notably, overexpression with full-length APP induced higher cell proliferation than sAPPα overexpression, suggesting that another cleaved fragment of APP might regulate the cell cycle in TSCC cells. In non-small cell lung cancer cells, overexpression of AICD reversed the necrotic cell death due to G0 phase arrest (10). AICD was generated by γ-cleavage of APP. The current study focused on the functional roles of sAPPα generated by α-secretase cleavage of ADAM10 in TSCC progression. Whether and how AICD or γ-secretase participates in TSCC pathogenesis is underexplored in further study.

The signaling pathways that were downstream of sAPPα were further studied. APP has been reported to promote the migration of cancer cells and via activating the MAPK pathway (23, 24). Of note, sAPPα could activate the ERK1/2, p38 and JNK MAPK signaling pathways in microglia (25). The bioinformatics analysis also determined that the activity of the MAPKs pathway was significantly increased in TSCC tissues (26). Further studies demonstrated that the p38 and ERK1/2 pathways were involved in the regulation of cancer growth and metastasis in TSCC (27). Here, we found the ERK and p38 signaling

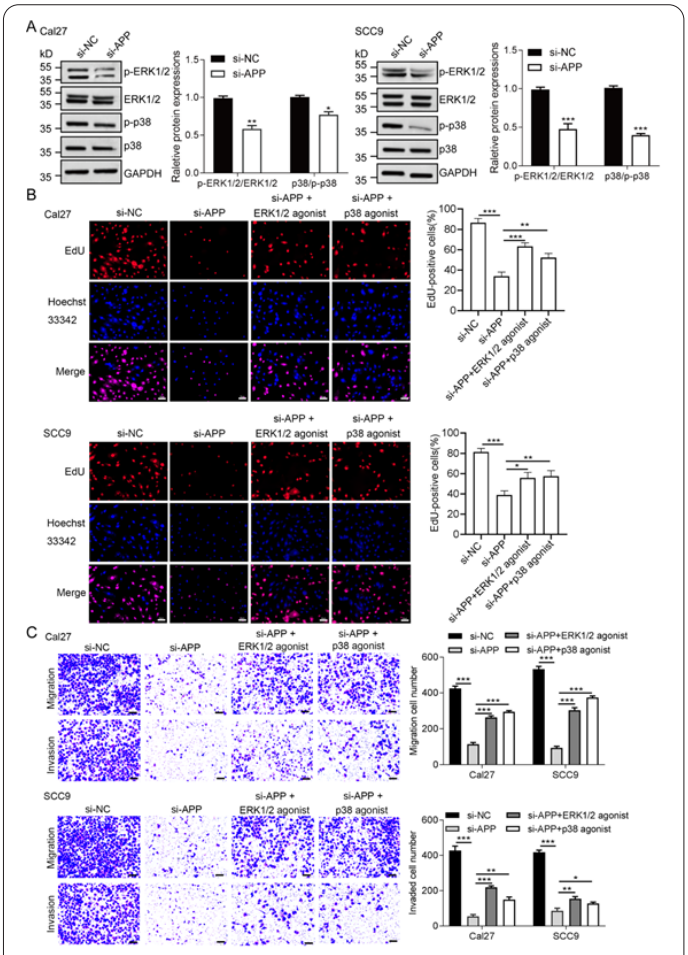

Figure 3. Knockdown of APP reduced the malignant properties of TSCC cells via inhibiting ERK and p38 pathways. (A) The protein levels of p-ERK1/2, ERK1/2, p-p38 and p38 were detected using western blot analysis. (B) Cell proliferation was determined by EdU assay. Scale bar: 50μm. (C) Transwell assay was used to detect cell migration and invasion. Scale bar: 50μm. Data were shown as mean ± SD (n=3). *$P < 0.05$, **$P < 0.01$, and *** $P < 0.001$.

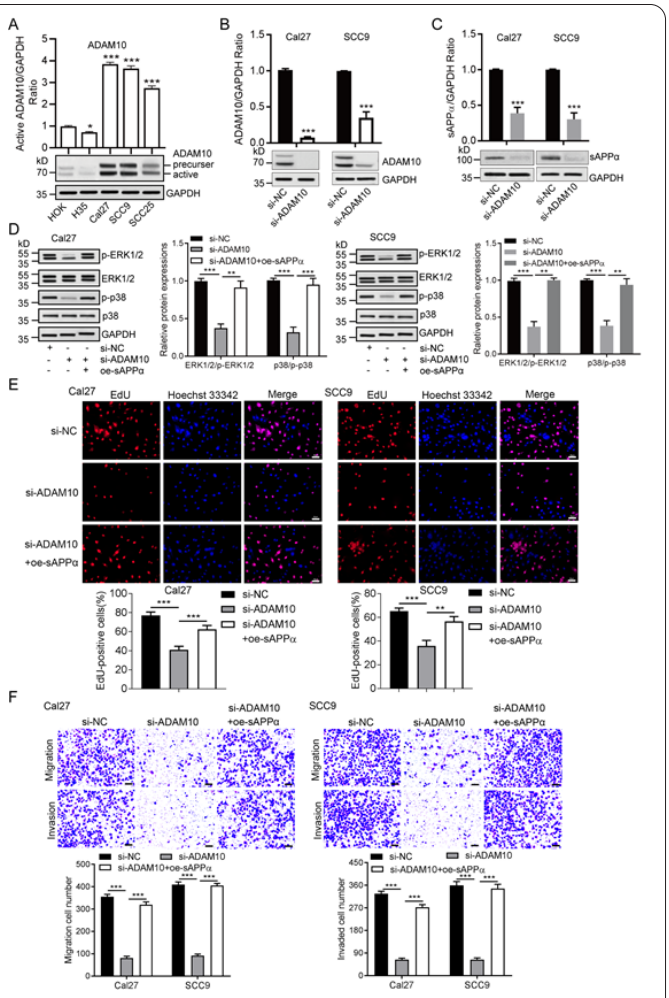

Figure 4. The inhibitory effects of ADAM10 knockdown could be reversed by sAPPα overexpression. (A) Western blotting was used to evaluate the expression of ADAM10. (B) Western blotting was used to detect the expression of ADAM10. (C) The protein level of sAPPα in the culture supernatant was detected using western blot analysis. (D) The protein levels of p-ERK1/2, ERK1/2, p-p38 and p38 were detected using western blotting. (E) Cell proliferation was determined by EdU assay. Scale bar: 50μm. (F) Transwell assay was used to detect cell migration and invasion. Scale bar: 50μm. Data were shown as mean ± SD (n=3). *$P < 0.05$, **$P < 0.01$, and *** $P < 0.001$.

pathways functioned downstream of sAPPα. Knockdown of APP blocked the p38 and ERK pathways, and activation of the p38 and ERK pathways using their agonist reversed the reduced TSCC cell proliferation, migration and invasion by APP knockdown. These data suggested that sAPPα promoted the function of TSCC cells via activating the p38 and ERK pathways.

Several studies have shown that the ADAM family is mainly performed as α-secretase, especially ADAM 9, 10, 17 and 19 (28, 29). Among them, ADAM10 is mainly responsible for the α-cleavage of APP (30). Tsang *et al.* demonstrated that in breast cancer, ADAM10 but not ADAM17 proteolytically cleaved APP to regulate cell proliferation and invasion (15). A previous study indicated that ADAM10 is implicated in the growth, invasion and migration of TSCC cells (16). In this finding, we indicated that ADAM10 was upregulated in TSCC tissues and also as an α-secretase processed APP to generate sAPPα, in consistent with the previous studies. Knockdown of ADAM10 abolished the formation of sAPPα, consequently inhibiting the p38 and ERK1/2 pathway and suppressing the proliferation, migration and invasion of TSCC cells. Overexpression of sAPPα counteracted these effects. We found that co-expression of both high ADAM10 and APP correlates with the poor prognosis of TSCC patients for the first time.

Taken together, our study demonstrated that high co-expression of ADAM10 and APP was associated with poor survival in TSCC patients. ADAM10 was responsible for the proteolytic cleavage of APP to form sAPPα, which resulted in the activation of ERK1/2 and p38 pathways to enhance TSCC cell proliferation, migration and invasion in vitro. Our study provided evidence for the application of APP as a potential therapeutic target of TSCC.

Ethics statement

The study was approved by the Ethics Committee of Jinan Stomatological Hospital. All experiments were conducted complied with the Declaration of Helsinki principles. Written informed consent was received from all participants included in the study.

Conflict of interest

The authors declare no conflict of interest.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

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