Silencing novel long non-coding RNA FKBP9P1 represses malignant progression and inhibits PI3K/AKT signaling of head and neck squamous cell carcinoma *in vitro*

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Abstract

Background: Long non-coding RNAs (lncRNAs) play key roles in human cancers. In our previous study, we demonstrated that lncRNA FKBP prolyl isomerase 9 pseudogene 1 (FKBP9P1) was highly expressed in head and neck squamous cell cancer (HNSCC) tissues. However, its functional significance remains poorly understood. In the present study, we identify the role and potential molecular biologic mechanisms of FKBP9P1 in HNSCC.

Methods: Quantitative real-time polymerase chain reaction was used to detect the expression of FKBP9P1 in HNSCC tissues, matched adjacent normal tissues, human HNSCC cells (FaDu, Cal-27, SCC4, and SCC9), and human immortalized keratinocytes cell HaCaT (normal control). Cal-27 and SCC9 cells were transfected with sh-FKBP9P1-1, sh-FKBP9P1-2, and normal control (sh-NC) lentivirus. Cell counting kit-8 assay, colony formation assay, wound healing assay, and trans-well assay were used to explore the biologic function of FKBP9P1 in HNSCC cells. Furthermore, western blotting was used to determine the mechanism of FKBP9P1 in HNSCC progression. Chi-squared test was performed to assess the clinical significance among FKBP9P1 high-expression and low-expression groups. Survival analyses were performed using the Kaplan-Meier method and assessed using the log-rank test. The comparison between two groups was analyzed by Student *t* test, and comparisons among multiple samples were performed by one-way analysis of variance and a Bonferroni *post hoc* test.

Results: FKBP9P1 expression was significantly up-regulated in HNSCC tissues (tumor *vs.* normal, 1.914 *vs.* 0.957, $t = 7.746$, $P < 0.001$) and cell lines ($P < 0.01$ in all HNSCC cell lines). Besides, the median FKBP9P1 expression of HNSCC tissues (1.677) was considered as the threshold. High FKBP9P1 level was correlated with advanced T stage ($P = 0.022$), advanced N stage ($P = 0.036$), advanced clinical stage ($P = 0.018$), and poor prognosis of HNSCC patients (overall survival, $P = 0.002$ and disease-free survival, $P < 0.001$). Knockdown of FKBP9P1 led to marked repression in proliferation, migration, and invasion of HNSCC cells *in vitro* (P all < 0.01). Mechanistically, silencing FKBP9P1 was observed to restrain the PI3K/AKT signaling pathway.

Conclusions: Silencing lncRNA FKBP9P1 represses HNSCC progression and inhibits PI3K/AKT (phosphatidylinositol 3 kinase/AKT Serine/Threonine Kinase) signaling *in vitro*. Therefore, FKBP9P1 could be a potential new target for the diagnosis and treatment of HNSCC patients.

Keywords: Head and neck squamous cell carcinoma; Long non-coding RNA; FKBP prolyl isomerase 9 pseudogene 1; PI3K/AKT signaling pathway

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide and has a poor prognosis.[1] The survival rate in HNSCC is also negatively affected by the tendency of tumor invasion and cervical lymph nodes metastasis. Although many treatments have been used for the management of HNSCC, including surgery treatment, chemotherapy, and radiotherapy, the 5-year overall survival (OS) rate is still poor, ranging from approximately 25% to 60%.[2] Novel strategies are urgently needed that change strategies from classical treatment to biomarker-guided treatment selection based on genetic differences of tumors. However, very few biomarkers are currently used in HNSCC clinical practice. Thus, it is essential to find novel molecular biomarkers of HNSCC and explore the potential molecular mechanism of HNSCC malignant progression.

Long non-coding RNAs (lncRNAs) are defined as RNA transcripts longer than 200 nucleotides without protein-coding potential. The aberrant expression of lncRNAs can

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be used as the biomarker in the diagnosis and prognosis of many human cancers.[3] FKBP prolyl isomerase 9 pseudogene 1 (FKBP9P1) is a newly identified lncRNA that is located at chromosome 7p11.2.[4,5] In our previous study, we demonstrated that FKBP9P1 (NCBI NR_027340) was highly expressed in HNSCC tissues compared with paired adjacent normal tissues by lncRNA microarrays of nine pairs patient sample.[6] However, the function and molecular biologic mechanisms of FKBP9P1 in the malignant progression of HNSCC remain unclear.

This study aimed to reveal the diagnostic and prognostic values of FKBP9P1 and explore the potential molecular mechanism of FKBP9P1 in HNSCC progression.

Methods

Ethical approval

This study was approved by the Ethics Committee of Beijing Tongren Hospital, Capital Medical University. All patients were fully informed of the purpose and methods of the present study, and written informed consent was obtained from the patients. The study was undertaken in accordance with the ethical standards of the World Medical Association *Declaration of Helsinki*.

Patients and samples

One hundred and fourteen patients with HNSCC who had received surgery treatment at the Affiliated Beijing Tongren Hospital of Capital Medical University from 2011 to 2015 were enrolled for the study. Through surgical excision, tumor and adjacent normal tissues were obtained. A piece of tissue was divided into two pieces, one for quantitative real-time polymerase chain reaction (qRT-PCR) and one for histopathologic evaluation. All tumor tissues were confirmed with HNSCC and all adjacent normal tissues were confirmed with tumor-free tissue based on histopathologic examination. None of the participating patients had received chemotherapy or radiotherapy prior to surgery. For qRT-PCR, the tissue samples were snap-frozen in liquid nitrogen and stored at −80°C.

Follow-up

Follow-ups were conducted every 3 months for the first year, every 6 months for the second to the fifth years and every 12 months thereafter. Routine endoscopy examination was performed. Enhanced neck computed tomography/magnetic resonance imaging, whole-body ultrasound/bone scanning/positron emission tomography-computed tomography were conducted according to the clinical situation. OS time was defined as the time from the date of surgery to the date of death or last follow-up record. Disease-free survival (DFS) time was defined as the date of surgery to the date of metastasis or relapse. The last date of follow-up was December 2018.

Cell culture and lentivirus infection

Human HNSCC cell lines (FaDu, Cal-27, SCC4, and SCC9) and a human immortalized keratinocytes cell line HaCaT (normal control) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The Cal-27 and FaDu cells were cultured in Dulbecco modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA). SCC9 and SCC4 cells were cultured in DMEM/F12 (Gibco). HaCaT cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640, Gibco). All the medium was added with 10% fetal bovine serum (Gibco). Cells were cultured at 37°C in a humidified atmosphere containing 5% of CO_2. All cell lines were characterized by short tandem repeat profiling (ATCC) and were confirmed to be mycoplasma free. The cell lines used for experiments were all within ten passages after thawing.

For FKBP9P1 knockdown, recombinant lentiviruses expressing sh-FKBP9P1 (sh-FKBP9P1-1: forward 5′-GGTAGAAATACCCTAGGAGTG-3′; sh-FKBP9P1-2: forward 5′-GAAATACCCTAGGAGTGATGT-3′) were constructed by GeneChem Co., Ltd (Shanghai, China). The lentiviruses expressing empty vectors were regarded as normal control (sh-NC). Cal-27 and SCC9 cells were transfected with the lentivirus according to the manufacturer's instructions. To obtain stably transfected cell lines, these cells were treated with puromycin (1 μg/mL) for 14 days. After the knockdown, efficiency was confirmed by qRT-PCR. The cells were used for subsequent experiments.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from tissues or cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reversed transcribed into cDNAs using Transcriptor First Strand cDNA synthesis kit (Roche, Basel, Switzerland) according to the manufacturer's protocol. The mRNA level was measured using the SYBR Green fluorescence kit (Roche) and the ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). The expression of FKBP9P1 was normalized to housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The ratio of relative mRNA expression between the tumor (experimental) group and the normal (control) group was calculated based on the $2^{-\Delta\Delta Ct}$ method. The formula is as follows: $\Delta\Delta Ct = \Delta Ct_{experimental\ group} - \triangle Ct_{control\ group}$, $\triangle Ct = Ct_{target\ gene} - Ct_{GAPDH}$. Ct represents the amplification cycles when real-time fluorescence intensity reached threshold at reaction. The experiment was performed in triplicate. The following primers were used for qRT-PCR. FKBP9P1 forward: 5′-TCTTCTCATAGGAACACTCTCAGT-3′; FKBP9P1 reverse: 5′-CTCGCCAACG CACATCTC-3′; GAPDH forward: 5′-GATCATCAGCAATGCCTCCT-3′; GAPDH reverse: 3′-TGAGTCCT TCCACGATACCA-5′.

Cell counting kit-8 assay

Cells were plated in 96-well plates (2000 cells/well). Cell proliferation was determined every 24 h for 5 days according to the manufacturer's instructions. Briefly, 10 μL of cell counting kit-8 (CCK-8) reagent (MedChemExpress, Shanghai, China) was added to each well. After incubation at 37°C for 1 h, the absorbance at 450 nm was detected. The experiment was repeated three times.

Colony formation assay

For Cal-27 cell line, 1000 cells were suspended in 2 mL complete medium and seeded in 6-well plates. For SCC9 cell line, 2000 cells were cultured in 6-well plates. After 14 days, colonies were stained by 0.1% crystal violet. Photos of colonies were taken by camera, and number of colonies were analyzed by ImageJ software. The experiment was repeated three times.

Wound healing assay

Cells were seeded into six-well plates and grown for 24 h to 85% to 90% confluence. A linear wound was generated by scratching the sub-confluent cell monolayer using a 200-μL pipette tip, and the debris was removed by washing with phosphate-buffered saline. After incubation at 37°C for 48 h, cells were photographed (Olympus, Tokyo, Japan), and wound width were recorded at 0 and 48 h. Then the relative migration rate was calculated. The experiment was repeated three times.

Trans-well migration and invasion assays

Cells were re-suspended in 200-μL serum-free medium at a density of 10^6/mL and seeded into the upper chamber of 24-well trans-well chambers (8-μm pore; Costar, LA, USA) coated without (migration) and with (invasion) Matrigel (BD Biosciences, NJ, USA). The lower chambers were filled with 500 μL of medium containing 20% fetal bovine serum. After 18 h (migration) and 24 h (invasion), cells on the lower surface of the inserts were stained with 0.1% crystal violet. The invaded or migrated cells were captured under a light microscope (Olympus). The cell numbers were counted in five random fields.

Western blotting

Total protein was extracted from cells using radioimmunoprecipitation assay buffer with cocktail (Applygen, Beijing, China). Protein lysates separated by sulfate-polyacrylamide gel electrophoresis were then transferred onto polyvinylidene difluoride membrane (Millipore, MA, USA). Anti-PI3K, anti-phosphorylation of PI3K (anti-p-PI3K), anti-AKT, anti-phosphorylation of AKT (anti-p-AKT) primary antibodies (rabbit; Abcam, Cambridge, MA, USA) were added as primary antibodies and anti-GAPDH (rabbit; Cell Signaling Technology, MA, USA) was used as an internal reference control. After incubation with secondary antibodies (rabbit; Beyotime, Shanghai, China), the protein strips were visualized and detected using a chemiluminescent reagent kit (Thermo Fisher Scientific, MA, USA).

Statistical analysis

Statistical analyses were performed using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA) was used to plot the data. The Chi-squared test was performed to assess the clinicopathologic significance among the FKBP9P1 high- and low-expression groups. Survival analyses were performed using the Kaplan-Meier method and assessed using the log-rank test. The comparison between two groups was analyzed by Student *t* test. (HNSCC tissue group and paired adjacent normal tissue group). The comparisons among multiple samples were performed by one-way analysis of variance and a Bonferroni *post hoc* test. $P < 0.05$ was considered statistically significant. Values are expressed as mean ± standard deviation.

Results

LncRNA FKBP9P1 is up-regulated in HNSCC tissues and cell lines

Initially, FKBP9P1 expression was measured in all 114 HNSCC tissue samples and paired adjacent normal tissues using qRT-PCR. As shown in Figure 1A, we found that FKBP9P1 expression was significantly higher in HNSCC tissues than in adjacent normal tissues (tumor *vs.* normal, 1.914 ± 1.430 *vs.* 0.957 ± 1.430, $t = 7.746$, $P < 0.001$). We also detected FKBP9P1 expression in cell lines [Figure 1B], and its expression in HNSCC cells (FaDu, Cal-27, SCC4, and SCC9) was significantly higher than that in normal control cell line HaCaT (all $P < 0.01$). These results indicate that FKBP9P1 may play an oncogene role in HNSCC.

FKBP9P1 up-regulation is associated with poor survival in HNSCC patients

We next analyzed the relationship between FKBP9P1 expression and the clinicopathologic features of HNSCC. We divided the enrolled patients into two groups (high, $n = 57$ and low, $n = 57$) based on the median expression of FKBP9P1 (1.677). A Chi-squared test was used to analyze the distribution differences of different clinical features between the two groups. As demonstrated in Table 1, high FKBP9P1 expression was correlated with advanced T stage (T3–T4 group compared with T1–T2 group, $P = 0.022$), advanced N stage (N1–N2 group compared with N0 group, $P = 0.036$), and advanced clinical stage (III–IV group compared with I–II group, $P = 0.018$). The larger the Chi-squared value, the greater the deviation between the two groups. However, there was no correlation between the level of FKBP9P1 and gender, age, alcohol consumption, smoking status, and differentiation. Additionally, survival analysis using the Kaplan-Meier method demonstrated that a higher FKBP9P1 level was associated with poor OS ($P = 0.002$) [Figure 1C] and poor DFS ($P < 0.0001$) [Figure 1D]. These results suggest that FKBP9P1 could be a potential effective prognostic biomarker for HNSCC patients.

Knockdown of FKBP9P1 decreases HNSCC cell proliferation

To investigate the effect of FKBP9P1 in HNSCC cells, we knockdown FKBP9P1 using shRNAs (sh-FKBP9P1-1, sh-FKBP9P1-2, and a normal control sh-NC) in Cal-27 and SCC9 cells, and the efficiencies of knockdown were confirmed by qRT-PCR [Figure 2A] (all $P < 0.01$). CCK-8 and colony formation assays demonstrated that FKBP9P1 knockdown significantly inhibited Cal-27 and SCC9 cell proliferation and colony formation ability [Figure 2B and 2C] (all $P < 0.01$). These results reveal that FKBP9P1 knockdown significantly decreases HNSCC cell proliferation.

Knockdown of FKBP9P1 inhibits HNSCC cell migration and invasion

To determine the effect of FKBP9P1 knockdown on the migration and invasion of HNSCC cells, wound healing

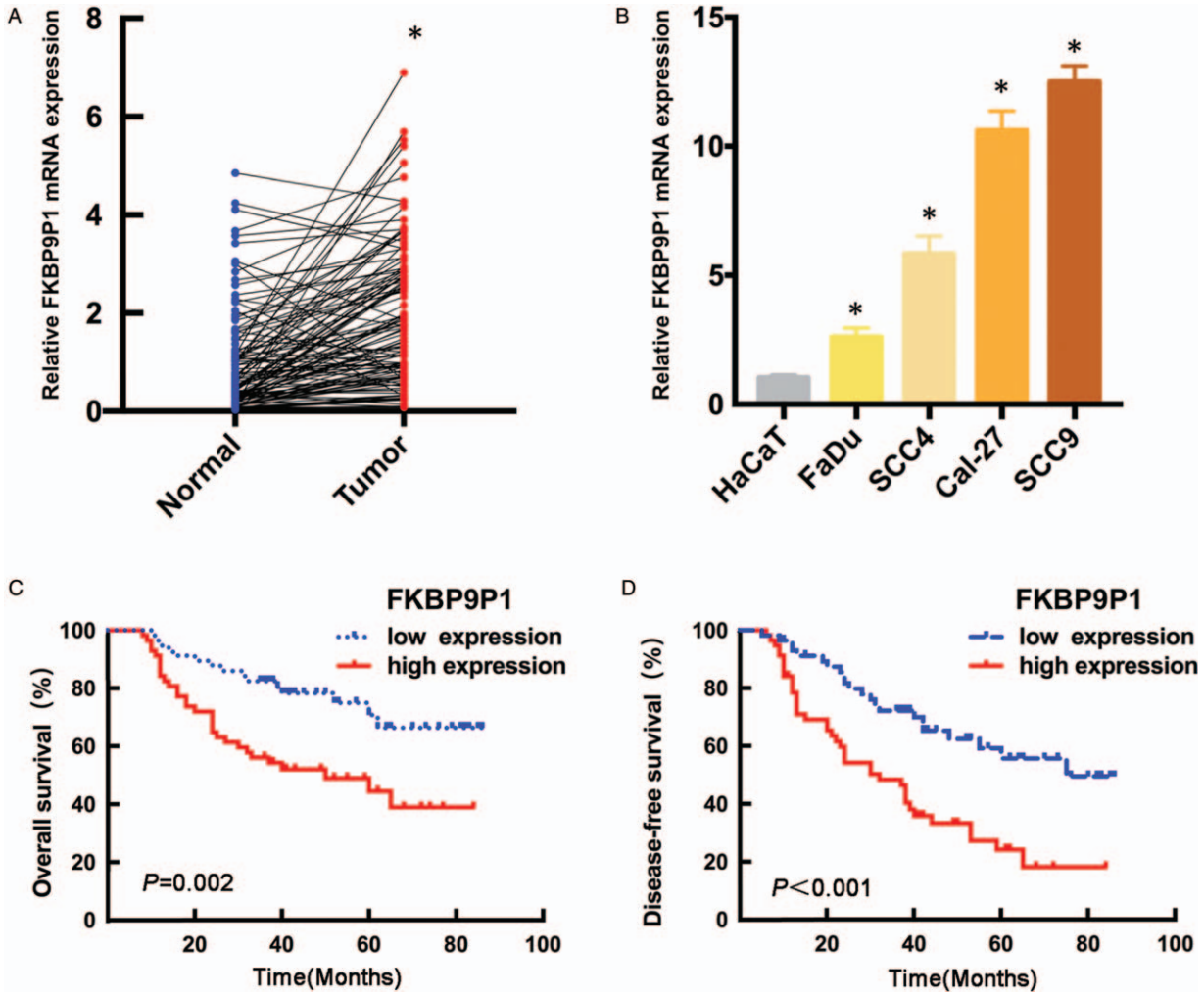

Figure 1: FKBP9P1 is upregulated in human HNSCC tissues and cell lines, and predicts a poor prognosis. (A) The mRNA expression level of FKBP9P1 in 114 HNSCC tissues (tumor group) and paired adjacent normal tissues (normal group) were detected by qRT-PCR. (B) The mRNA expression level of FKBP9P1 in four HNSCC cell lines (tumor cell groups) and HaCaT cell line (control group) were detected by qRT-PCR. (C) OS rates of patients with low (<1.667) and high levels of FKBP9P1 (≥1.667) ($P = 0.002$). (D) DFS rates of patients with low (<1.667) and high levels of FKBP9P1 (≥1.667) ($P < 0.001$). $^{*}P < 0.001$, compared with normal group, $^{**}P < 0.01$, compared with control group. FKBP9P1: FKBP prolyl isomerase 9 pseudogene 1; HNSCC: Head and neck squamous cell carcinoma; qRT-PCR: Quantitative real-time polymerase chain reaction; OS: Overall survival; DFS: Disease-free survival.

assay [Figure 2D] and trans-well assay [Figure 2E] were performed. As shown in the figures, after FKBP9P1 knockdown, the migratory, and invasion abilities of Cal-27 and SCC9 cells were both remarkably impaired (all $P < 0.01$). The results suggest that FKBP9P1 knockdown significantly inhibits HNSCC cell migration and invasion.

Knockdown of FKBP9P1 suppresses the PI3K/AKT signaling pathway in HNSCC cells

PI3K/AKT signaling pathway has been reported to contribute to various cancers, including HNSCC.[7] For elucidating the molecular mechanism underlying FKBP9P1 in HNSCC, we wonder if PI3K/AKT take part in HNSCC progression. Thus, the alteration of the PI3K/AKT signaling pathway was detected by western blotting in Cal-27 and SCC9 cells. As shown in Figure 3, FKBP9P1 knockdown suppressed the p-PI3K and p-AKT, while PI3K and AKT expression had no significant difference between FKBP9P1 knockdown cells and control cells. Our data indicate that FKBP9P1 knockdown suppresses the PI3K/AKT signaling pathway and this pathway may be the underlying mechanism of FKBP9P1 in HNSCC progression.

Discussion

HNSCC contributes to global cancer burden and covers a wide range of geographically dispersed incidences. Within the last decade, knowledge on the molecular alterations that drive HNSCC tumorigenesis and progression has rapidly been growing.[8] Less than 2% of total genome encodes protein-coding genes, suggesting that non-coding RNAs represent most of the human transcriptome.[9] LncRNA can vary in length from 200 nucleotides to 100 kilobases, and have been implicated in a diversity range of biologic processes in cancers, including HNSCC, which can be used as potential biomarkers for prognosis and therapeutic targets.[10,11] However, although thousands of lncRNAs have been identified in the past, only a small number have been functionally characterized.[12] In this

Table 1: Association between FKBP9P1 expression and clinicopathologic features of head and neck squamous cell carcinoma patients.

Variables	Cases (*n*)	FKBP9P1 Expression		$\chi 2$	*P* values
		Low ($n = 57$)	High ($n = 57$)		
Gender					
Male	98	47	51	1.163	0.281
Female	16	10	6		
Age (years)					
<60	55	31	24	1.721	0.190
≥60	59	26	33		
Alcohol consumption					
Ever and currently	70	36	34	0.148	0.700
Never	44	21	23		
Smoking status					
Ever and currently	99	51	48	0.691	0.406
Never	15	6	9		
Differentiation					
Well + moderate	95	49	46	0.568	0.451
Poor	19	8	11		
T stage					
T1–T2	24	17	7	5.278	0.022*
T3–T4	90	40	50		
N stage					
N0	47	29	18	4.380	0.036*
N1–N2	67	28	39		
Clinical stage					
I–II	22	16	6	5.632	0.018*
III–IV	92	41	51		

* $P < 0.05$. *P* values were calculated within each variable group. FKBP9P1: FKBP prolyl isomerase 9 pseudogene 1.

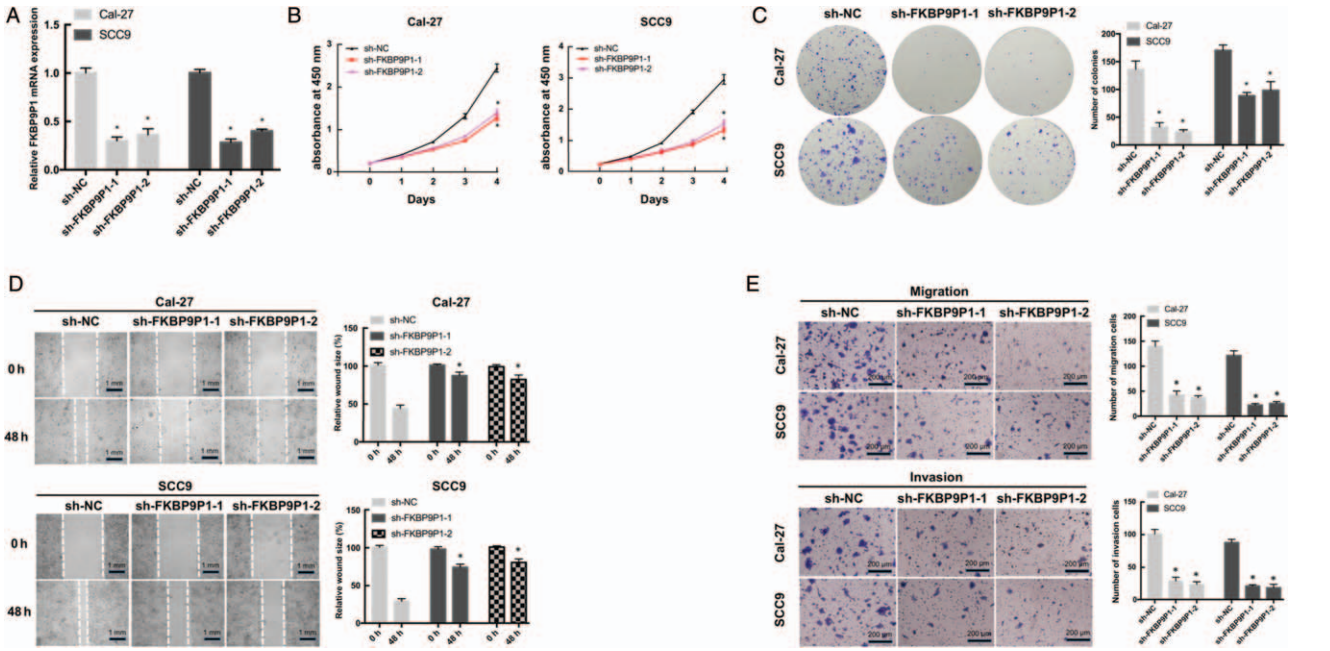

Figure 2: Knockdown of FKBP9P1 inhibits the proliferation, migration, and invasion potentials of HNSCC cells. (A) The knockdown efficiency of two different FKBP9P1 shRNA sequences (sh-FKBP9P1-1 and sh-FKBP9P1-2) compared with sh-NC was detected by qRT-PCR in the indicated cells. (B) CCK-8 assay was used to test the cell proliferation ability. (C) Colony formation assay was performed to test the cell proliferation ability. Representative images (left panel) and number of colonies (right panel) were shown. (D) Wound healing assay (bar = 1 mm) was used to reveal the migration capacity of cells. Representative images (left panel) and relative wound size (right panel) were shown. (E) Trans-well invasion and migration assays (bar = 200 μm) were performed to show the invasion and migration ability of cells. Representative images (left panel) and number of migration/invasion cells (right panel) were shown. Data showed mean ± standard deviation of three independent experiments. * $P < 0.01$ compared with sh-NC group. FKBP9P1: FKBP prolyl isomerase 9 pseudogene 1; qRT-PCR: Quantitative real-time polymerase chain reaction; HNSCC: Head and neck squamous cell carcinoma; CCK-8: Cell counting kit-8; NC: Normal control.

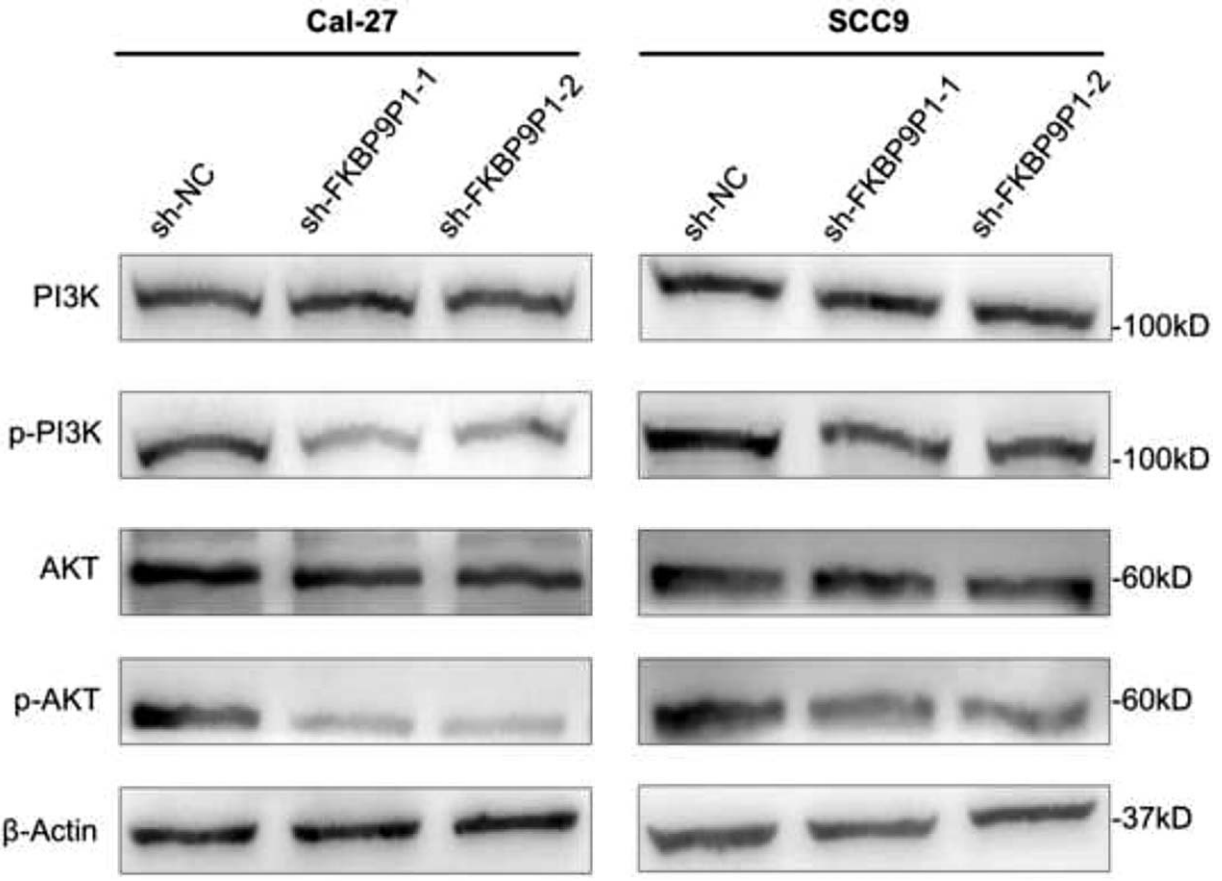

Figure 3: Knockdown of FKBP9P1 suppresses the PI3K/AKT signaling pathway in HNSCC cells. Western blotting results demonstrated that knockdown of FKBP9P1 suppresses the p-PI3K and p-AKT protein expression in HNSCC cells. FKBP9P1: FKBP prolyl isomerase 9 pseudogene 1; HNSCC: Head and neck squamous cell carcinoma; p-PI3K: Phosphorylation of PI3K; p-AKT: Phosphorylation of AKT.

study, we explored a novel lncRNA FKBP9P1 whose expression level was higher in HNSCC tissues and cell lines than normal controls and correlated with poor survival outcomes. Functional experiments demonstrated that FKBP9P1 knockdown significantly inhibited HNSCC proliferation, migration, invasion *in vitro*, and repressed the PI3K/AKT signaling pathway. Thus, our results reveal a novel mechanism of the FKBP9P1/PI3K/AKT regulatory axis involved in HNSCC progression regulation.

Currently, lncRNAs have been verified to be involved in HNSCC progression by regulating cell proliferation and metastasis. Jiang *et al* reported that lncRNA LINC00460 promoted HNSCC cell proliferation and metastasis.[13] Liang *et al* revealed that lncRNA snaR was correlated with HNSCC proliferation, migration, invasion.[14] Nishiyama *et al* demonstrated that lncRNA DLEU1 knockdown suppressed migration and invasion in oral squamous cell carcinoma cells.[15] In our study, we performed CCK-8 and colony formation assays to explore the effect of FKBP9P1 on HNSCC cells, finding that FKBP9P1 knockdown significantly suppressed proliferation and colony forming capabilities of Cal-27 and SCC9 cells. Then, we performed wound healing and trans-well assays and our results indicated that FKBP9P1 knockdown inhibited Cal-27 and SCC9 cells migration and invasion, which was consistent with clinicopathologic findings that high FKBP9P1 expression was closely related with advanced T stage, N stage, and clinical stage. Previous studies have revealed that several lncRNAs are expressed in a highly tissue- and cell type-specific manner, making them potential efficacious targets for systemic cancer treatment. LncRNA HOTAIR has been verified as an oncogene in breast cancer, lung cancer, liver cancer, pancreas cancer, and esophageal squamous cell carcinoma, and can also act as an independent predictor of survival rates.[16,17] LncRNA MALAT1 expression has been found to be up-regulated three- to four-fold in human breast cancers, as well as in different breast cancer cell lines. Furthermore, high level of MALAT1 has been widely considered to be poor prognostic factors and closely related to lymph node metastasis and survival rates.[18] In addition, *in vitro* and *in vivo* studies have indicated that MALAT1 therapeutic knockdown may represent an excellent option to modulate metastasis in cancer.[19] Our results provided verification that FKBP9P1 over-expression indicated advanced clinicopathologic features and poor prognosis in the 114 HNSCC patients' cohort and might be a promising prognostic and diagnostic indicator of HNSCC.

The PI3K/AKT signaling pathway is one of the most frequently activated signaling pathways in human cancer and affects many vital cellular processes including cell proliferation and metastasis.[20] Several studies have reported that lncRNAs promote the progression in several

cancers via PI3K/AKT signaling pathway.[21,22] To figure out the underlying mechanism of the FKBP9P1 in the progression of HNSCC, we performed western blotting to detect the expression of proteins related to the PI3K/AKT signaling pathway. As the results showed, FKBP9P1 knockdown could decrease the phosphorylation of PI3K and AKT. However, detailed mechanism by which FKBP9P1 modulates the PI3K/AKT signaling pathway needs to be further explored.

In conclusion, our study indicated that FKBP9P1 was up-regulated in HNSCC and that high FKBP9P1 expression was associated with tumor progression and poor survival. Silencing FKBP9P1 repressed HNSCC cell malignant progression and inhibited PI3K/AKT signaling. These findings highlight the potential of FKBP9P1 as a novel diagnostic biomarker and promising therapeutic target in HNSCC.

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Conflicts of interest

None.

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