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Original Article

NNK-mediated upregulation of DEPDC1 stimulates the progression of oral squamous cell carcinoma by inhibiting CYP27B1 expression

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Abstract: Oral squamous cell carcinoma (OSCC) is a prevalent and malignant cancer. However, the molecular mechanism of OSCC progression is not fully understood. In this study, we observed that the DEP domain containing 1 (DEPDC1) protein was overexpressed in OSCC tissues and that the increased expression of DEPDC1 was closely associated with tumor size and poor clinical outcomes in OSCC patients. The results of functional investigations demonstrated that DEPDC1 stimulates OSCC cell proliferation by inhibiting cytochrome P450 family 27 subfamily B member (CYP27B1) expression. Furthermore, we observed that upregulated DEPDC1 expression was closely associated with smoking status in OSCC patients. The results of *in vitro* experiments showed that the tobacco compound 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) stimulates DEPDC1 expression by promoting the methylation of its gene body by increasing DNMT1 expression in OSCC cells. Notably, the silencing of DEPDC1 dramatically inhibited OSCC growth by inhibiting cell proliferation and inducing apoptosis *in vivo*. These findings suggest that smoking causes DEPDC1 overexpression in OSCC through DNMT1-regulated DNA methylation and that upregulated DEPDC1 stimulates OSCC cell proliferation by inhibiting CYP27B1 expression. Our results establish a new mechanism of OSCC progression and highlight DEPDC1 as a candidate prognostic biomarker and therapeutic target in OSCC.

Keywords: OSCC, DEPDC1, CYP27B1, NNK

Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common malignant tumor worldwide [1, 2] and is estimated to have an annual incidence of 300,000 cases worldwide [3]. OSCC is also the primary branch of head and neck malignant tumors and accounts for 24% of head and neck cancers [3]. Surgical resection combined with radiotherapy and chemotherapy is the primary approach used to treat OSCC [4]. Although these clinical treatment methods have improved in recent years, the 5-year survival rate of OSCC patients remains approximately 50% [4]. Therefore, it is necessary to develop new therapeutic targets, strategies, and prognostic markers for the treatment of OSCC by gaining an in-depth understanding of the molecular mechanism of OSCC progression.

Smoking is considered to be a major risk factor for OSCC development and progression [5]. Tobacco-specific nitrosamines are a group of chemical substances formed by the nitrosation of nicotine during smoking, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [6] is a nicotine-derived nitrosamine that can induce many kinds of cancers, including OSCC, lung cancer, nasopharyngeal carcinoma, liver cancer, pancreatic cancer and cervical cancer [7]. However, the mechanism of NNK in OSCC development and progression has not been fully elu-

cidated, and no effective strategies exist to prevent smoking-induced OSCC.

DEP domain containing 1 (DEPDC1) is a highly conserved protein that is present in many species ranging from *caenorhabditis elegans* to mammals [7, 8], and studies have shown that DEPDC1 is involved in a variety of cellular functions, such as stimulating cellular proliferation and inhibiting cell apoptosis [9-11]. The aberrantly upregulated expression of DEPDC1 has expression been observed in several types of cancer, and a high levels of DEPDC1 are closely associated with cancer progression, including bladder cancer [7, 8], breast cancer [12, 13] and prostate cancer [14]. However, the expression pattern and function of DEPDC1 in OSCC remains clear. Therefore, in this study, we hypothesized that DEPDC1 is important for tumor proliferation through the inhibition of CYP27B1 expression and that NNK may enhance this process.

Materials and methods

Reagents

Fetal bovine serum (FBS) was purchased from PAN-Biotech (Aidenbach Bavaria, Germany). Cell culture medium and trypsin-EDTA (0.25%) were purchased from Gibco (Grand Island, New York, USA). 4-(Methylnitrosamino)-1-(3-pyridyl)-1- butanone (NNK) was purchased from Sigma (St. Louis, MA, USA). Anti-DEPDC1, anti-cleaved caspase-3, anti-cleaved PARP1 and anti-gamma H2AX antibodies were obtained from Abcam (Cambridge, UK). Antibodies against DNMT1, Ki-67, β-actin, and GAPDH were purchased from Proteintech (Wuhan, China). An anti-CYP27B1 antibody was obtained from Bioss (Beijing, China). TB Green™ premix Ex Taq™ II kit was purchased from Takara (Dalian, China). DNMT1, DEPDC1 and CYP27B1 shRNA overexpression plasmids were purchased from Genechem (Shanghai, China). DNMT1 shRNA was obtained from Genechem (Shanghai, China). A DNA extraction kit and TRIzol reagent were obtained from Qiagen (Dusseldorf, Germany) and Invitrogen (Carlsbad, CA, USA), respectively. A CCK-8 kit, a TUNEL cell apoptosis detection kit, cell lysis buffer, and a BCA kit were purchased from Beyotime Biotechnology (Shanghai, China). A RevertAid First Strand cDNA Synthesis kit was obtained from Thermo Scientific (Waltham, MA, USA). A kFluor555-EdU cell proliferation detection kit was obtained from Keygen Biotech (Jiangsu, China). A SureSelect Human All Exon kit was purchased from Agilent Technologies Inc. (Palo Alto, CA, USA).

Cell culture and human specimens

The cell lines CAL-27 and SCC-15 were obtained from the American Type Culture Collection (Manassas, VA, USA), while the cell lines HSC-3 and OSC-19 were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The cell line UM1 was obtained from Sichuan University (Sichuan, China). CAL-27 and UM1 cells were maintained in DMEM supplemented with 10% FBS; HSC-3 cells were maintained in MEM supplemented with 10% FBS; and SCC-15 and OSC-19 cells were maintained in DMEM/F12 supplemented with 10% FBS.

Human samples were obtained from 146 patients who were diagnosed with OSCC for the first time at Xinqiao Hospital of the Third Military Medical University. All subjects gave their informed consent for inclusion in this study before participating in the study. This study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Xinqiao Hospital of the Third Military Medical University (2019-No.108-01).

DNA methylation assay

For the DNA methylation assay, the indicated cells were treated with 5 μM NNK for 96 hours and then subjected to genomic DNA isolation. Genomic DNA was isolated using a Qiagen DNA extraction kit, and 1 μg of genomic DNA was treated with sodium bisulfite. The bisulfite-treated DNA was desalted and eluted in 40 μL of elution buffer, after which 2 μL of DNA was used for PCR amplification. Subsequently, the PCR products were ligated into the TA cloning vector and then sequenced. The primer sequences for the DEPDC1 methylation analysis are shown in Table S1.

RNA isolation and analysis

Total RNA from cells was isolated using TRIzol reagent according to the manufacturer's instructions. For RT-qPCR, total RNA was reverse transcribed into cDNA using a RevertAid First Strand cDNA Synthesis kit. RT-qPCR was performed using the TB Green™ Premix Ex Taq™ II

kit. The primers for RT-qPCR are shown in Table S1.

Transcriptome sequencing

Three paired tumor and adjacent OSCC tissue samples from patients undergoing OSCC surgery at Xinqiao Hospital were obtained and used in RNA-Seq experiments. All participants provided written informed consent. The tumor content was assessed, with an average of 60% coverage observed across samples. The exome regions were captured using an Agilent SureSelect Human All Exon kit and then sequenced on an Illumina HiSeq 2000 platform (paired end, 100 bp).

Celigo, CCK-8 and EdU assays

The indicated cells were transfected with the indicated constructs for 24 hours, after which the cells were analyzed. For the cell viability assay, cells were plated in a 96-well plate at a density of 2000 cells/well, and cell viability was determined at the indicated times after reseeding cells using a CCK-8 kit according to the manufacturer's instructions. For the Celigo assay, cells were inoculated in 96-well plates at a density of 2000 cells/well, and the plate was scanned once a day for 5 days. For the EdU assay, cells were plated in 96-well plates at a density of 5000 cells/well. After 24 hours, the assay was performed using a kFluor555-EdU cell proliferation detection kit following the manufacturer's instructions.

Apoptosis analysis

To detect apoptotic cells *in vitro*, cells were collected by trypsin digestion and low-speed centrifugation (1000 rpm for 5 min), washed with 1× PBS, and pelleted once with low-speed centrifugation. Then, the cells were resuspended in 200 μL of 1× PBS, stained with 10 μL of annexin V-APC for 15 min, and then analyzed by flow cytometry. Apoptotic cells in tissues were detected using a TUNEL cell apoptosis detection kit according to the manufacturer's instructions.

Western blot and immunohistochemistry (IHC) analyses

Cells were lysed using lysis buffer, and the protein concentration of each cell lysate was measured using a BCA kit. Equal amounts of protein samples (35 μg) were loaded onto SDS/PAGE gels and separated by electrophoresis. Subsequently, the membranes were blocked with 5% BSA for 1 hour and then incubated with the indicated primary antibodies at 4°C overnight. After being washed five times with TBST (25 min total), the membranes were incubated with secondary antibodies for 1 hour at room temperature. Then, after being washed three times with TBST (15 min total), the antibody complexes were detected using the enhanced chemiluminescence (ECL) method.

For IHC, slides were deparaffinized in xylene and rehydrated in a graded series of alcohol solutions prior to heat-induced antigen retrieval in EDTA (1 mM, pH 8.0) for 2 min. After natural cooling, a 3% hydrogen peroxide treatment for 10 min was used to block endogenous peroxidase activity. After being washed three times with PBS (15 min total), the slides were incubated with a primary antibody at 4°C overnight. The next day, the tissue sections were washed and incubated with a secondary antibody for 1 hour at room temperature. After washing, the substrate color was developed using DAB and counterstained with hematoxylin.

Animal experiments

For the subcutaneous xenograft model, fifteen 6-week-old female BALB/nude mice were divided into three groups. Then, DEPDC1-overexpressing HSC-3 cells, DEPDC1-silenced HSC-3 cells or their control cells (5×10^6 cells in 100 μL PBS) were injected into the right armpit of each mouse. Twenty-four days after cell injection, the mice were sacrificed. For orthotopic models, fifteen 6-week-old female BALB/nude mice were divided into three groups. Then, DEPDC1-overexpressing HSC-3 cells, DEPDC1-silenced HSC-3 cells or control cells (4.6×10^5 cells in 20 μL PBS) were injected into the middle part of the tongue of each mouse. The body weights of mice were measured every week, and the mice were sacrificed 21 days after cell injection. All experimental procedures involving animals were performed in accordance with the animal protocols approved by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University (AMUWEC2019483).

Statistical analyses

All data are presented as the means ± standard deviation (SD), and significant differences

between treatment groups were analyzed by Student's t-test or one-way analysis of variance (ANOVA) and Duncan's multiple range test using SAS statistical software version 6.12 (SAS Institute Inc., Cary, NC). The survival rate of patients with OSCC was calculated using Kaplan-Meier survival analysis. The significance of multiple predictors of survival was assessed using Cox regression analysis. *P* values less than 0.05 were considered significant.

Results

DEPDC1 is highly expressed in OSCC tissues and correlated with poor survival in OSCC patients

To identify genes involved in OSCC progression, we performed high-throughput sequencing using OSCC specimens and their adjacent tissues from three OSCC patients. As shown in **Figure 1A**, compared to the adjacent tissues, 404 genes were abnormally expressed in OSCC tissues. Then, we analyzed the transcriptomic data of 40 OSCC patients from the TCGA database and observed that 4359 genes were abnormally expressed in OSCC tissues compared to their adjacent tissues (**Figure 1B**). Subsequently, we observed that DEPDC1 was significantly upregulated in OSCC tissues compared to adjacent tissues in both our cohort and the TCGA dataset (**Figure 1C**). We further confirmed the difference in DEPDC1 expression between normal oral tissues and OSCC tissues at the protein level by an immunohistochemistry (IHC) assay with 146 OSCC specimens. As shown in **Figure 1D**, the DEPDC1 protein level was also significantly higher in OSCC tissues than in their adjacent tissues (**Figure 1D**). These findings suggest that DEPDC1 may be associated with OSCC progression. Thus, we investigated the correlation between DEPDC1 expression levels in OSCC primary tumors and OSCC progression. One hundred forty-six OSCC patients from our cohort (**Table 1**) were divided into DEPDC1-high and -low expression groups based on tumor section IHC results (**Figure 1E**), after which Kaplan-Meier survival analysis were performed. Our results showed that high expression levels of DEPDC1 were closely correlated with poor survival in OSCC patients (**Figure 1F**). In addition, our clinical data show that the level of DEPDC1 expression is also closely associated with tumor size in OSCC patients (**Table 1**). Importantly, multivariable analyses show that DEPDC1 is an independent predictor of OSCC patient prognosis (**Table 2**). Taken together, these findings indicate that DEPDC1 is a candidate biomarker for OSCC prognosis and that aberrantly upregulated expression of DEPDC1 may be involved in the stimulation of OSCC progression.

DEPDC1 stimulates OSCC cell proliferation and tumor growth

The clinical data showed that a high level of DEPDC1 was associated with tumor size (**Table 1**), suggesting that DEPDC1 may be involved in the regulation of cell proliferation in OSCC. Interestingly, previous studies have shown that DEPDC1 drives cancer proliferation in several types of cancer [14-16]. Therefore, to investigate whether DEPDC1 is directly involved in the regulation of OSCC cell proliferation, we measured the expression level of DEPDC1 in several OSCC cell lines, subsequently selecting the cell lines CAL-27 and HSC-3 with moderate expression level of DEPDC1 for further analysis (**Figure 2A**). Both cell lines were transfected with DEPDC1 shRNA or DEPDC1 expression constructs (**Figure 2B**) and assessed using a cell proliferation assay. Our CCK-8 assay results showed that DEPDC1 overexpression significantly stimulated cell proliferation (**Figure 2C**), whereas silencing DEPDC1 suppressed cell proliferation in both CAL-27 and HSC-3 cells (**Figure 2C**). Consistent with the CCK-8 results, Celigo imaging results also showed that DEPDC1 positively regulates OSCC cell proliferation (**Figure 2D**). Notably, flow cytometry analysis results showed that silencing DEPDC1 induced cell apoptosis in OSCC cells (**Figure 2E**). Subsequently, Western blot results demonstrated that silencing DEPDC1 stimulates pro-apoptotic proteins expression in OSCC cells, including cleaved caspase-3, cleaved PARP1 and γ-H2AX (**Figure 2F**). Furthermore, the *in vitro* results were confirmed in animal models. As shown in **Figure 3A**, the results obtained using a subcutaneous xenograft model showed that DEPDC1 overexpression significantly stimulated tumor growth and that silencing DEPDC1 dramatically suppressed tumor growth compared to that observed in the control group. In addition, IHC analysis of the cell proliferation marker protein Ki-67 showed that DEPDC1 overexpression increased the proportion of

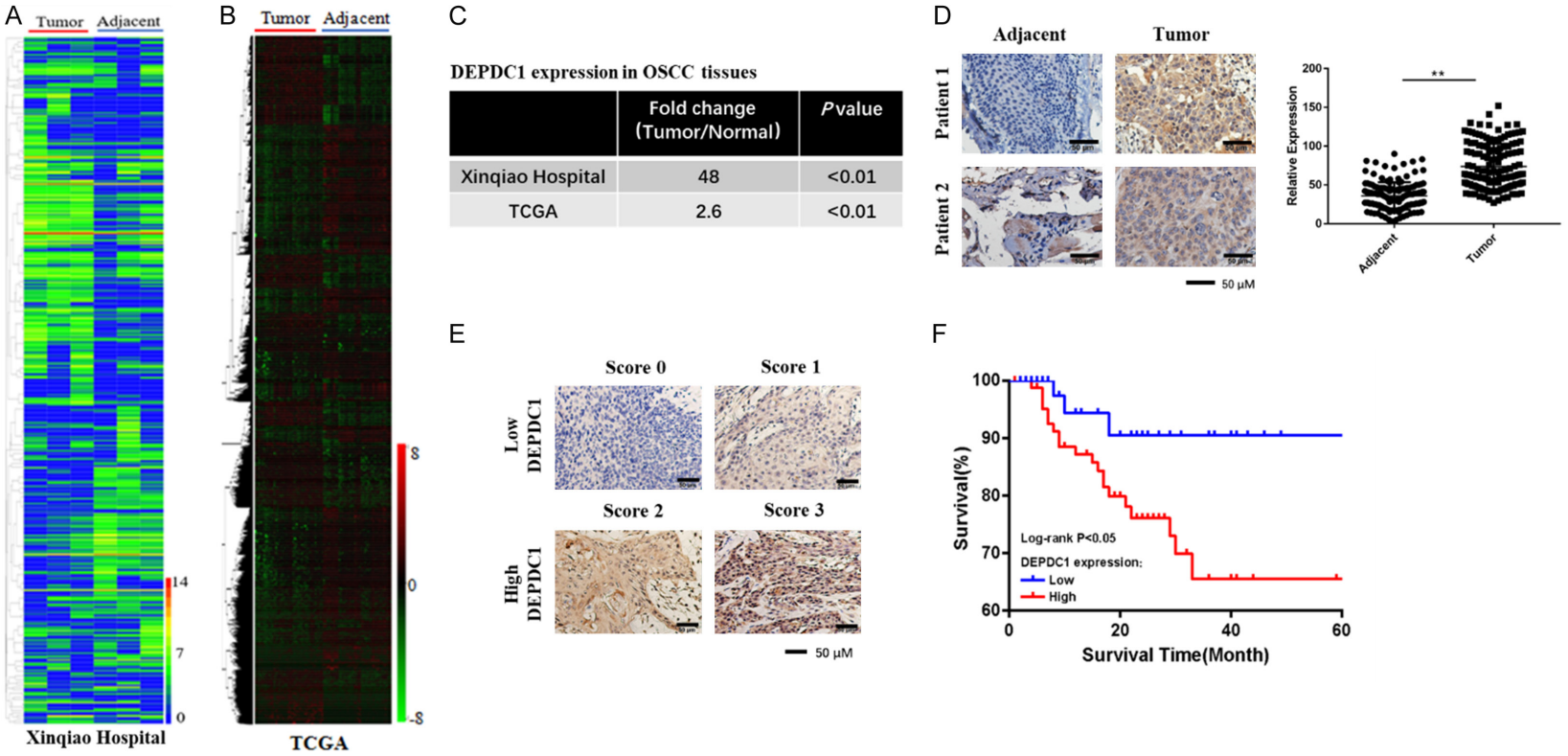


Figure 1. DEPDC1 expression is significantly increased in OSCC tissues and closely correlated with poor clinical outcomes in OSCC patients. A. The heat map of differentially expressed genes between oral squamous cell carcinoma (OSCC) and their adjacent tissues. Samples were collected from 3 patients with OSCC at Xinqiao Hospital. B. The heat map of differentially expressed genes between OSCC and their adjacent tissues. Data were obtained from the TCGA database (n=40). C. DEPDC1 was significantly upregulated in OSCC tissue compared to adjacent tissues in both our sample cohort and the TCGA dataset. D. Immunohistochemistry (IHC) assay results showed that DEPDC1 protein levels were significantly increased in OSCC tissues compared to those observed in adjacent tissues. Samples were collected from 146 OSCC patients at Xinqiao Hospital. E. DEPDC1 expression levels were measured in 146 OSCC specimens by IHC. F. Based on IHC analysis results for DEPDC1, 146 OSCC patients were divided into DEPDC1 high and low groups and then Kaplan-Meier analysis of the 5-year survival was performed. **, P<0.01.

Table 1. Characteristics of patients with OSCC

Variable	DEPDC1 expression (No of patients)		*p* value
	High (n=110)	Low (n=36)	
Sex			0.50
Male	74	22	
Female	36	14	
Smoking history			0.00
Yes	59	9	
No	51	27	
Drinking history			0.71
Yes	36	13	
No	74	23	
Pathological classification			0.54
Well	49	20	
Moderately	43	11	
Poorly	7	2	
T stage			0.01
Tx	0	1	
T1	12	9	
T2	89	18	
T3	5	1	
T4	4	0	
N stage			0.19
Nx	2	0	
N0	73	18	
N1	17	11	
N2	18	0	
Metastasis			-
No	110	29	
Yes	0	0	

Table 2. Multivariable analyses of factors that are predictive of poor overall survival in 146 patients with OSCC

Variable	HR (95% confidence interval)	*p*
Gender	0.49 (0.12-2.04)	0.33
Age	1.25 (0.43-3.60)	0.68
Smoking history	0.86 (0.19-3.98)	0.04
Drinking history	0.65 (0.15-2.80)	0.56
Pathological classification	0.66 (0.25-1.75)	0.40
T stage	1.70 (0.56-5.17)	0.00
N stage	1.93 (1.72-4.10)	0.35
DEPDC1 level	2.77 (0.77-9.98)	0.02

Ki-67-positive cells, while silencing DEPDC1 reduced the proportion of Ki-67-positive cells in OSCC tissues (**Figure 3B**). Additionally, we detected increased apoptosis in DEPDC1-silenced xenograft tumors compared to that observed in the control and DEPDC1-overexpressing tumors (**Figure 3B**). Similar results were observed in orthotopic animal models, which showed that DEPDC1 overexpression stimulated tumor growth and tumor cell proliferation (**Figure 3C** and **3D**) and that silencing DEPDC1 suppressed tumor growth (**Figure 3C**) and tumor cell proliferation and stimulated apoptosis (**Figure 3D**). In the orthotopic model, tumor formation in the tongue resulted in a decrease in body weight. Among these changes, the weight lost by the experimental mice in the DEPDC1-overexpressing group was more significant than that observed in the control group (**Figure 3E**). However, silencing DEPDC1 alleviated the weight loss caused by tumor formation (**Figure 3E**). Taken together, our data suggest that DEPDC1 plays an oncogenic role in OSCC by stimulating cell proliferation and that it is a useful therapeutic target candidate for OSCC treatment.

DEPDC1 promotes OSCC cell proliferation by inhibiting CYP27B1 expression

To investigate the regulatory mechanism of DEPDC1 in OSCC cell proliferation, we performed RNA sequencing (RNA-Seq) using HSC-3 cells with overexpressed or silenced DEPDC1. As shown in **Figure 4A**, the expression of many genes was affected by DEPDC1 overexpression or silencing in HSC-3 cells. However, only 7 genes were oppositely regulated by DEPDC1 overexpression and DEPDC1 silencing (**Figure 4B**), suggesting that these seven genes may be directly regulated by DEPDC1 in OSCC cells. Furthermore, we used RT-qPCR to confirm these RNA-Seq results and observed that MYH14 and CYP27B1 expression levels were inversely regulated in the DEPDC1-overexpressing and DEPDC1-silenced groups, and the pattern of regulation of these two genes was consistent with the RNA-Seq results (**Figures 4C** and S1). Among these two genes, CYP27B1 mRNA expression level were more significantly affected by DEPDC1 (**Figure 4C**). In addition, consistent with the observed changes in mRNA level, the Western blot analysis data showed that

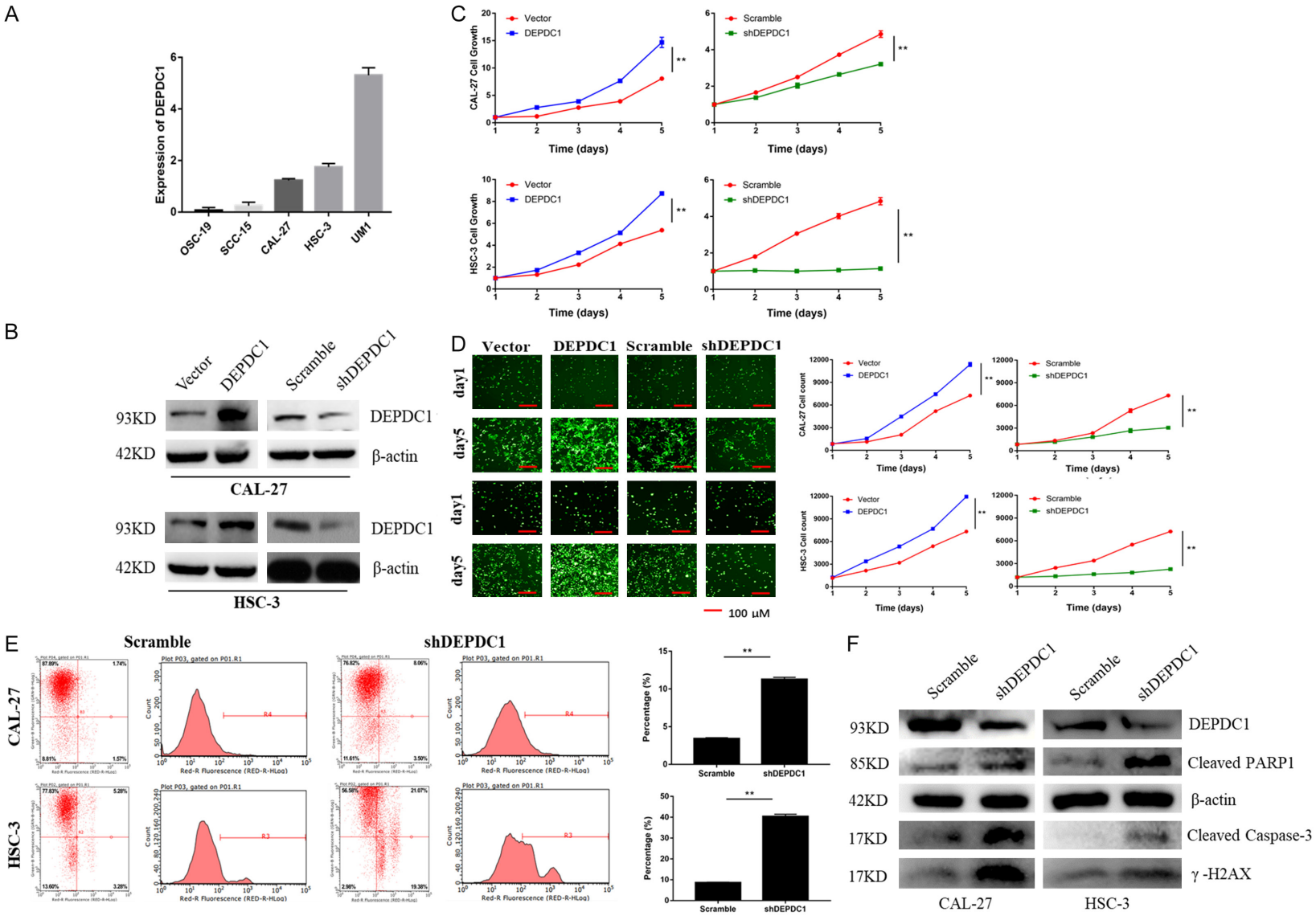


Figure 2. DEPDC1 stimulates OSCC cell proliferation. A. The level of DEPDC1 expression was measured in several OSCC cell lines using RT-qPCR. B. DEPDC1 expression in OSCC cells was measured by Western blotting. The indicated cells were transfected with the indicated constructs. After 72 hours of transfection, the cells were subjected to Western blotting. C. The effects of DEPDC1 on OSCC cell proliferation were measured by CCK-8 assays. Cells were transfected with the indicated construct. After 24 hours of transfection, the cells were subjected to CCK-8 assays. D. The effects of DEPDC1 on OSCC cell proliferation were measured with a

Celigo imaging system. E. Flow cytometry analysis results showed that silencing DEPDC1 stimulated OSCC cell apoptosis. F. Silencing DEPDC1 increased cleaved caspase-3, cleaved PARP1 and γ-H2AX expression in both CAL-27 and HSC-3 cells. The cells were transfected with the indicated constructs for 72 hours and then assayed by Western blotting. **, P<0.01.

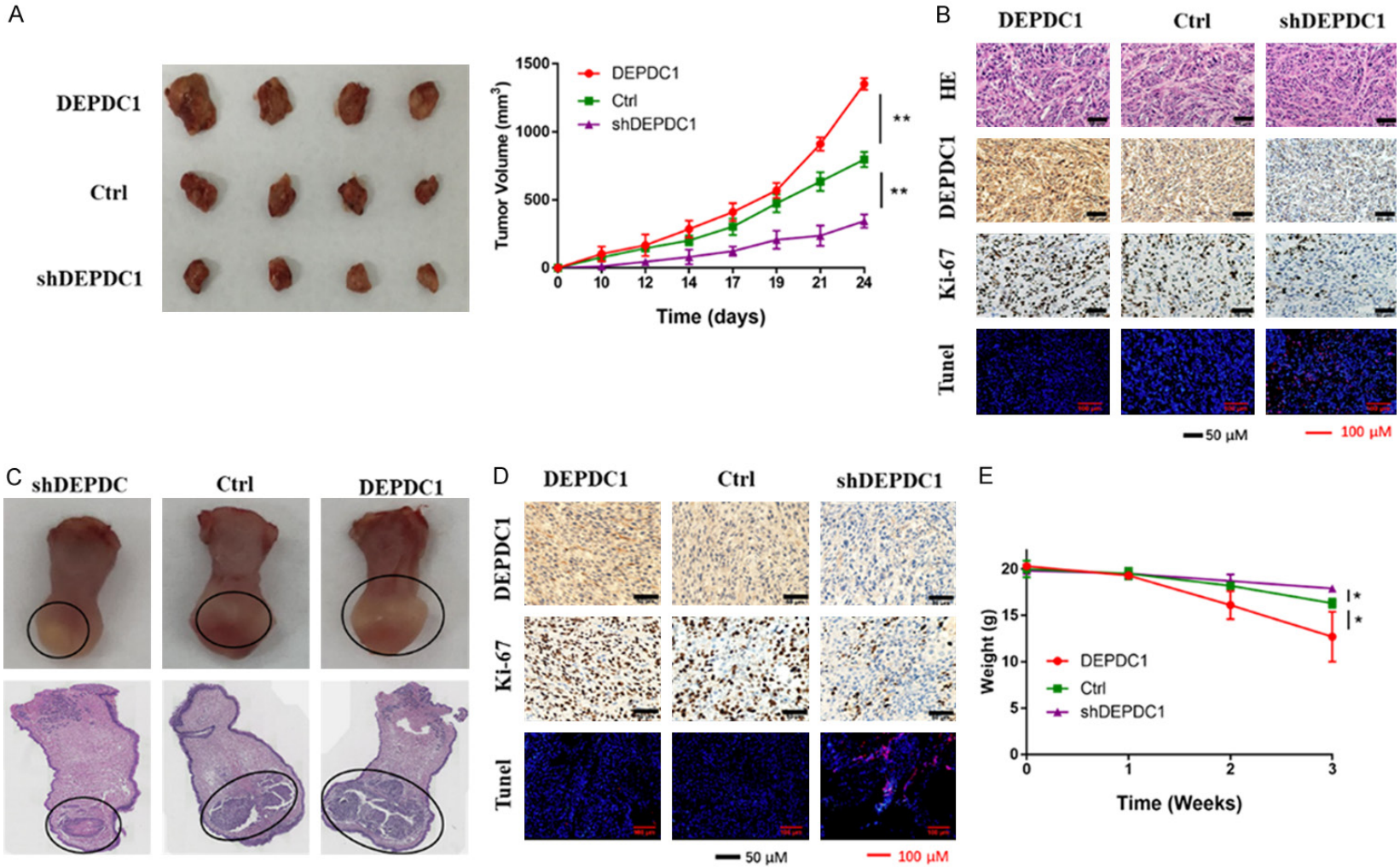


Figure 3. DEPDC1 stimulates OSCC growth *in vivo*. A. Subcutaneous xenograft model experimental results showed that DEPDC1 positively regulates OSCC growth. B. DEPDC1 positively regulates OSCC cell proliferation but negatively regulates apoptosis. DEPDC1 expression levels and Ki-67-positive cells were detected by immunohistochemistry (IHC) assays, and apoptotic cells were detected by TUNEL assays in subcutaneous xenograft tumor tissues. C. Orthotopic mouse model experi-

mental results showed that DEPDC1 positively regulates OSCC growth. D. DEPDC1 positively regulates OSCC cell proliferation but negatively regulates apoptosis. DEPDC1 expression levels and Ki-67-positive cells were detected by immunohistochemistry (IHC) assays, and apoptotic cells were detected by TUNEL assays in orthotopic model tumor tissues. E. Body weights of orthotopic model mice. Subcutaneous or orthotopic mouse models were generated by the injection of DEPDC1-overexpressing HSC-3 cells (DEPDC1) or DEPDC1-silenced HSC-3 cells (shDEPDC1) or vector and scramble cotransfected HSC-3 cells (Ctrl). *, P<0.05; **, P<0.01.

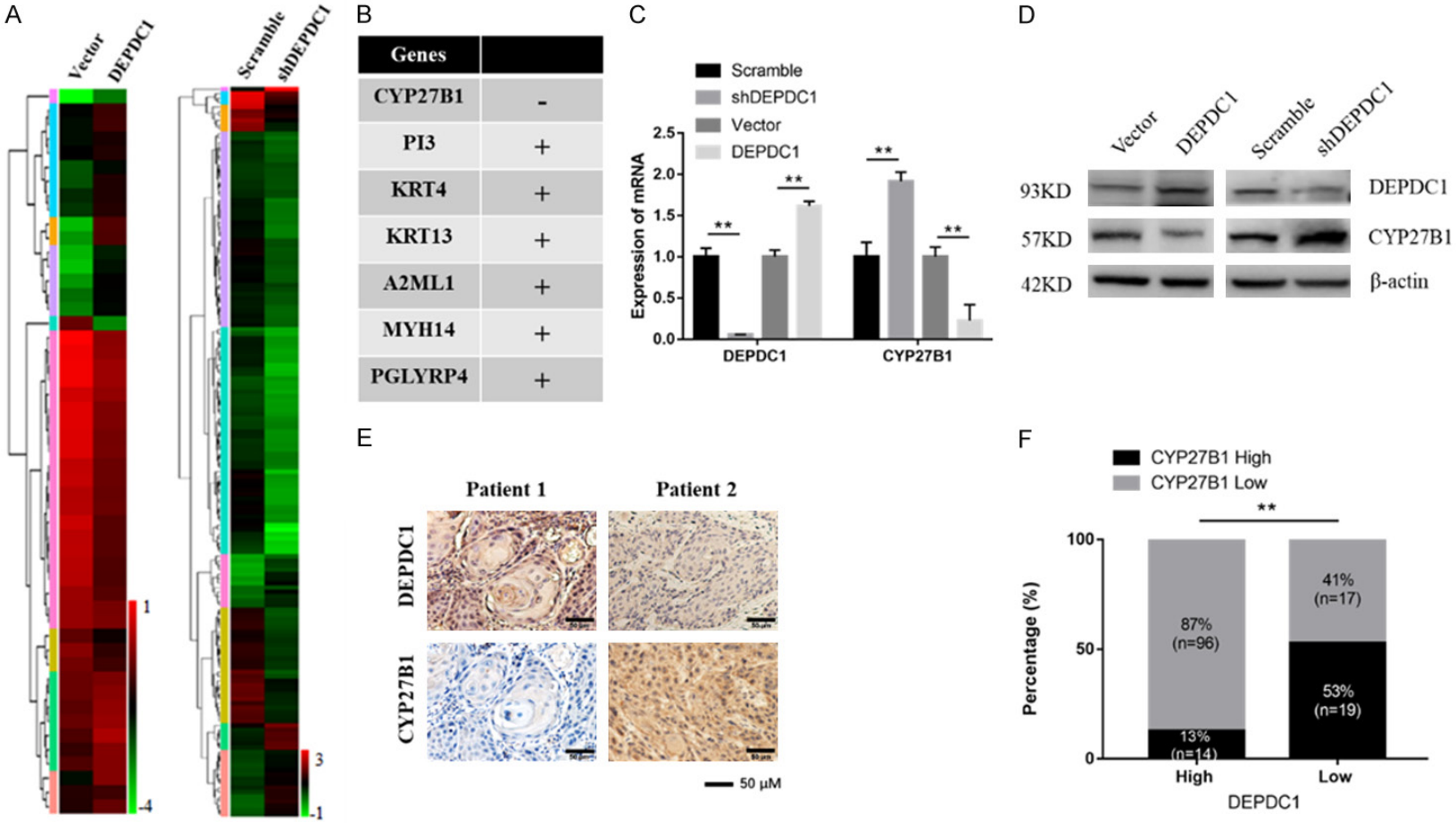


Figure 4. CYP27B1 is negatively regulated by DEPDC1 in OSCC cells. A. The heat map of genes that were differentially expressed after overexpressing or silencing DEPDC1 in HSC-3 cells. B. Inversely regulated genes between the DEPDC1-overexpressing group and the DEPDC1-silenced group in HSC-3 cells. -, negatively regulated by DEPDC1; +, positively regulated by DEPDC1. C. RT-qPCR analysis results showed that CYP27B1 mRNA levels were negatively regulated by DEPDC1 in HSC-3 cells. D. Western blot results showed that CYP27B1 protein expression was negatively regulated by DEPDC1 in HSC-3 cells. Cells were transfected with the indicated constructs for 72 hours and then subjected to RT-qPCR and Western blot analyses. E. DEPDC1 and CYP27B1 expression were measured in OSCC specimens by immunochemistry. F. DEPDC1 and CYP27B1 expression were measured by IHC in 146 OSCC specimens and analyzed for correlation. **, P<0.01.

CYP27B1 expression was also negatively regulated by DEPDC1 at the protein level in OSCC cells (**Figure 4D**). Finally, we investigated whether there was an inverse correlation between the expression levels of DEPDC1 and CYP27B1 in OSCC clinical samples. Our results showed that CYP27B1 expression was inversely correlated with DEPDC1 expression in OSCC patients (**Figure 4E** and **4F**). These findings indicate that CYP27B1 is a target of and is negatively regulated by DEPDC1 in OSCC.

Because the function of CYP27B1 in OSCC was not clear, we then investigated the effects of CYP27B1 expression on OSCC cell proliferation using CCK-8 and EdU cell proliferation assays. The CCK-8 and EdU assay results showed that overexpression of CYP27B1 (Figure S2A) dramatically suppressed OSCC cell proliferation (**Figure 5A** and **5B**) and that silencing of CYP27B1 (Figure S2B) significantly stimulated OSCC cell proliferation (**Figure 5A** and **5B**), indicating that CYP27B1 plays an anticancer role in OSCC cells. Next, we investigated whether CYP27B1 is directly involved in DEPDC1-regulated OSCC cell proliferation. Our CCK-8 and EdU assay results showed that CYP27B1 overexpression inhibited the DEPDC1 overexpression-induced stimulation of OSCC cell proliferation (Figures S2C, **5C** and **5D**). In contrast, silencing CYP27B1 attenuated the DEPDC1 silencing-induced inhibition of OSCC cell proliferation (Figures S2D, **5C** and **5D**). Taken together, our findings suggest that DEPDC1 exerts its oncogenic activity by negatively regulating the expression of the tumor suppressor gene CYP27B1 in OSCC cells.

NNK stimulates DEPDC1 expression in OSCC by upregulating DNMT1 expression

In this study, we observed that DEPDC1 is overexpressed in OSCC, but the cause of its overexpression was unclear. Interestingly, our clinical data showed that the DEPDC1 expression level is closely associated with smoking status (**Table 1** and **Figure 6A**) in OSCC patients, suggesting that smoking may be a cause of DEPDC1 overexpression in OSCC. Interestingly, our *in vitro* results showed that treatment with tobacco-specific carcinogen NNK increased the expression of DEPDC1 in OSCC cells in dose- and time-dependent manners (**Figure 6B**). Additionally, we demonstrated a negative correlation between smoking status and DEPDC1 target gene CYP27B1 expression in OSCC patients (**Figure 6A**). However, the NNK-induced reduction of CYP27B1 was inhibited by silencing DEPDC1 in OSCC cells (Figure S3), suggesting that NNK-induced inhibition of CYP27B1 occurs through upregulated DEPDC1. Next, to investigate the mechanism by which NNK upregulates DEPDC1 expression, we evaluated the effect of NNK on DNA methyltransferase 1 (DNMT1) expression and DEPDC1 methylation in OSCC cells. These assays were performed because previous studies have shown that NNK regulates gene expression partly through DNA methylation and that DNMT1 is one of the enzymes involved in DNA methylation that is affected by NNK [17, 18]. Our results showed that NNK treatment significantly induced DNA methylation of the DEPDC1 gene body but not of the promoter (**Figure 6C**). In addition, we demonstrated that NNK stimulates the expression of DNMT1 at both the mRNA and protein levels in OSCC cells (**Figure 6D**), and DNMT1 negatively regulated CYP27B1 expression in OSCC cells (**Figure 6E** and **6F**). Importantly, DNMT1 silencing blocked NNK-induced DEPDC1 upregulation in OSCC cells (**Figure 6G**). Taken together, our findings suggest that NNK stimulates DEPDC1 gene body methylation by increasing DNMT1 expression, thereby promoting DEPDC1 expression in OSCC cells.

Discussion

The investigation of the molecular mechanism of cancer progression is important for improving the clinical treatment of cancer as it allows for the identification of new therapeutic targets and prognostic markers. In particular, prognostic factors can indicate likely clinical outcomes. Thus, treatment strategies can be adjusted based on prognostic factors, which are crucial for the treatment of cancer. Unfortunately, there are no effective prognostic factors for OSCC. In this study, we analyzed clinical samples and noted that DEPDC1 expression levels were significantly upregulated in OSCC tissues compared to those observed in normal oral tissues and that high DEPDC1 levels are closely associated with tumor size and poor survival rate in OSCC patients. In addition, multivariate analysis results indicated that DEPDC1 levels are an independent prognostic factor for OSCC patient survival. Notably, silencing DEPDC1 dra-

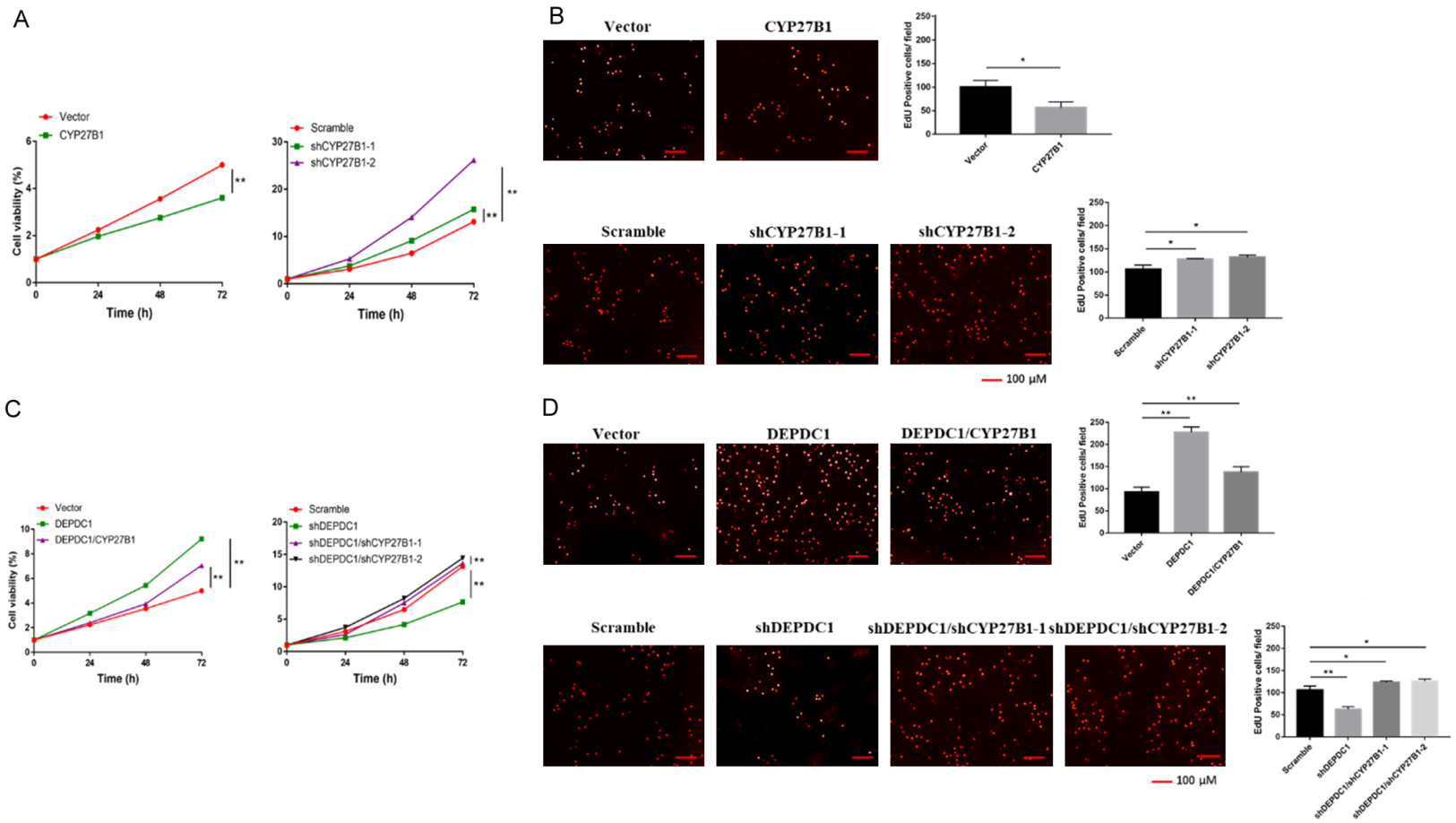
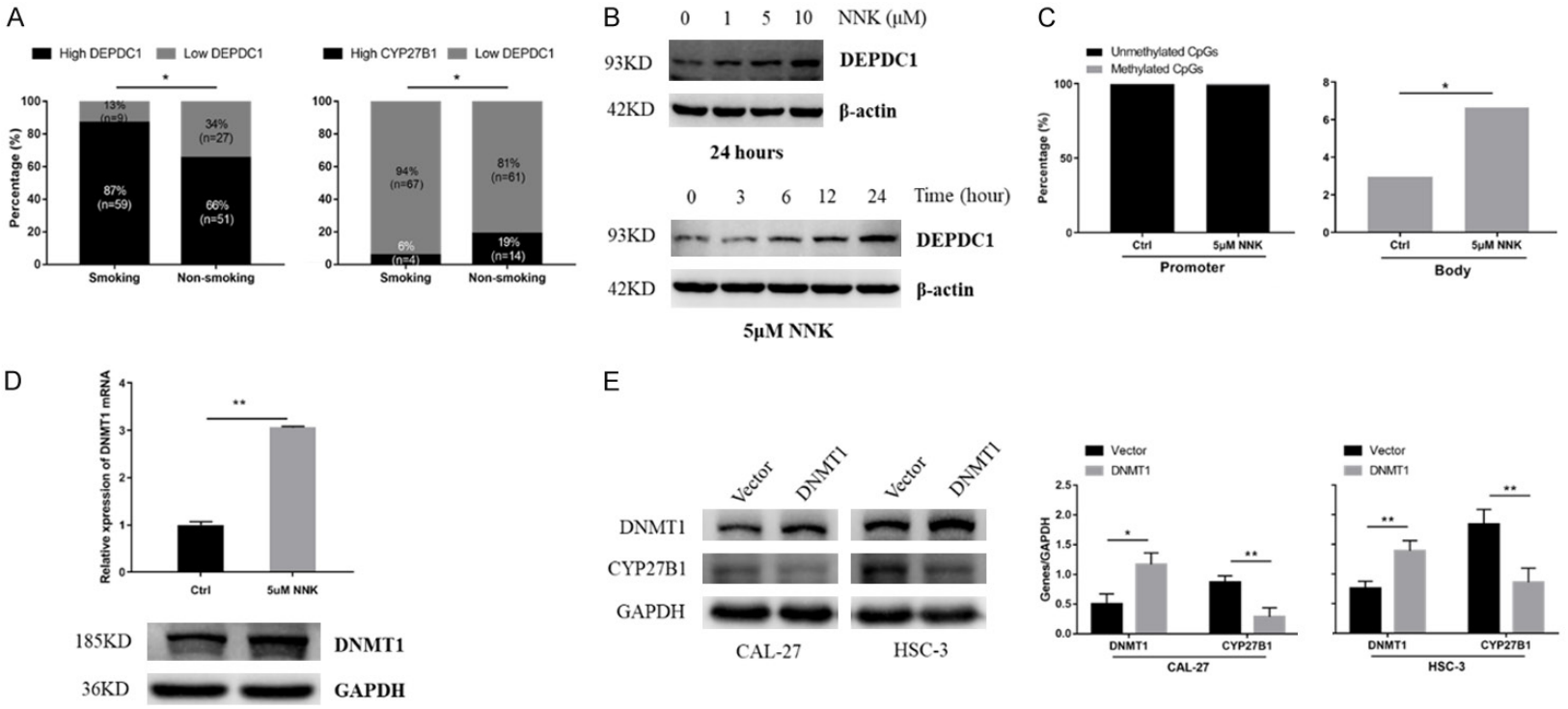


Figure 5. CYP27B1 inhibits OSCC cell proliferation and DEPDC1 stimulates OSCC cell proliferation through CYP27B1. A. CCK-8 assay results showed that CYP27B1 negatively regulates HSC-3 cell proliferation. HSC-3 cells were transfected with the indicated construct, and after 24 hours of transfection, the cells were subjected to CCK-8 assay. B. The EdU assay results showed that CYP27B1 negatively regulates HSC-3 cell proliferation. C. CCK-8 assay results showed that DEPDC1 overexpression- or silencing-induced effects on cell proliferation were attenuated by the overexpression or silencing of CYP27B1, respectively. HSC-3 cells were transfected with the indicated construct for 24 hours and then subjected to CCK-8 assays. D. EdU assay results showed that the cell proliferation effects induced by overexpression or silencing of DEPDC1 were attenuated by the overexpression or silencing of CYP27B1, respectively. *, P<0.05; **, P<0.01.


A
High DEPDC1
Low DEPDC1
Percentage (%)
13% (n=9)
87% (n=59)
34% (n=27)
66% (n=51)
Smoking
Non-smoking
High CYP27B1
Low DEPDC1
94% (n=67)
6% (n=4)
81% (n=61)
19% (n=14)
B
0 1 5 10 NNK (μM)
93KD
DEPDC1
42KD
β-actin
24 hours
0 3 6 12 24 Time (hour)
5μM NNK
C
Unmethylated CpGs
Methylated CpGs
Ctrl
5μM NNK
Promoter
Body
D
Relative xpression of DNMT1 mRNA
185KD
DNMT1
36KD
GAPDH
E
Vector
DNMT1
CYP27B1
GAPDH
CAL-27
HSC-3
Genes/GAPDH

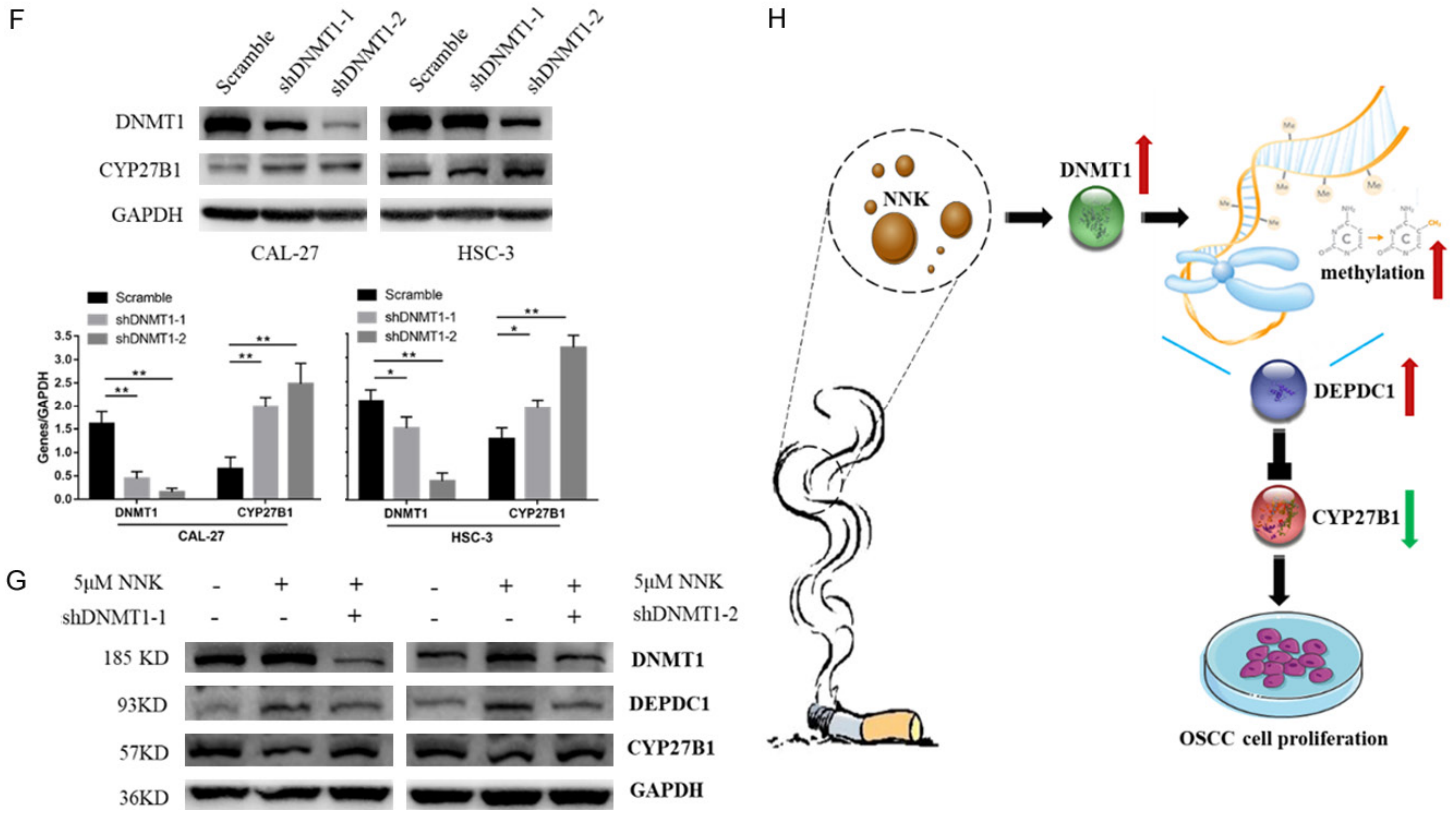


Figure 6. NNK-stimulated DEPDC1 expression through DNMT1 in OSCC cells. A. Smoking status was positively and negatively correlated with DEPDC1 and CYP27B1 expression in OSCC patients, respectively. The expression of DEPDC1 and CYP27B1 was measured by IHC in OSCC specimens from smoking and nonsmoking patients. B. NNK treatment stimulated DEPDC1 expression in HSC-3 cells. Cells were treated with the indicated concentrations of NNK for the indicated times and then subjected to Western blot analysis. C. NNK treatment increased gene body methylation of DEPDC1 in HSC-3 cells. Cells were treated with 5 μM NNK for 4 days and then subjected to a DNA methylation assay. D. NNK treatment stimulated DNMT1 expression in HSC-3 cells at both the mRNA and protein levels. Cells were treated with 5 μM NNK for 24 hours and then subjected to Western blot analysis. E. DNMT1 overexpression suppressed CYP27B1 expression in both CAL-27 and HSC-3 cells. F. DNMT1 silencing increased CYP27B1 expression in both CAL-27 and HSC-3 cells. The indicated cells were transfected with the indicated plasmid. After 72 hours of transfection, the cells were subjected to Western blot analysis. G. NNK-stimulated DEPDC1 expression was inhibited by DNMT1 silencing in HSC-3 cells. Cells were transfected with the DNMT1 shRNA expression construct. After 48 hours of transfection, cells were treated with 5 μM NNK for 24 hours, after which Western blot analyses were performed. H. Schematic diagram of NNK promoting DEPDC1 expression and DEPDC1 promoting OSCC cell proliferation. *, P<0.05; **, P<0.01.

matically suppressed OSCC growth and induced apoptosis. These findings suggest that DEPDC1 is a potential prognostic biomarker and therapeutic target for OSCC. Our findings are also supported by those from studies of other groups. Recent studies have shown that DEPDC1 is overexpressed in a variety of tumors, which is related to stimulated cell proliferation [19] and poor progression and can be used as a potential therapeutic target for breast cancer [12, 13], liver cancer [20-22] and lung cancer [23]. However, DEPDC1 needs to be further confirmed as a prognostic biomarker for OSCC in a larger cohort of OSCC clinical samples.

In this study, we also elucidated the mechanism by which DEPDC1 stimulates OSCC cell proliferation. CYP27B1 is the hydroxylase of vitamin D3 (VD3) and has an anticancer role in several cancers, such as prostate [24] and breast cancer [25]. Studies have shown that CYP27B1 can hydroxylate VD3 to generate calcitriol [26-28] and that calcitriol induces apoptosis [29] and inhibits cancer cell proliferation [29, 30], thereby suppressing tumor growth. In this study, we showed that CYB27B1 plays an anticancer role and is negatively regulated by DEPDC1 in OSCC cells. Clinical data also showed that the level of CYP27B1 expression was inversely correlated with DEPDC1 in OSCC patients. Importantly, CYP27B1 overexpression significantly inhibited DEPDC1 overexpression-induced oncogenic functions in OSCC cells, suggesting that DEPDC1 exerts its oncogenic activity by inhibiting the expression of tumor suppressor gene CYB27B1 in OSCC cells. However, the detailed mechanism by which DEPDC1 regulates the expression of CYP27B1 is unclear and needs further study.

Finally, we elucidated the stimulus and mechanism for the upregulation of DEPDC1 in OSCC. Smoking is considered to be one of the most important risk factors for OSCC [31, 32]. Studies have shown that the proportion of smokers with oral cancer is higher than that observed in other populations [33, 34] and that carcinogenic substances in tobacco, such as NNK, cause normal oral mucosa dysplasia and malignant tumors [35]. In this study, we used clinical sample analysis to demonstrate that smoking status is closely associated with the upregulated expression of DEPDC1 in OSCC patients. In addition, the results of *in vitro* experiments showed that NNK treatment caused the upregulation of DEPDC1 and the downregulation of its downstream protein CYP27B1 in OSCC cells, indicating that smoking is one of the causes of DEPDC1 upregulation in OSCC. Furthermore, we elucidated the mechanism by which NNK stimulates DEPDC1 expression in OSCC. According to Yang et al. [36] and Jones [37], in contrast to DNA methylation in promoters, gene body methylation is positively correlated with gene expression . As a member of the DNA methyltransferase family, DNMT1 plays a key role in maintaining DNA methylation [37] and is also involved in de novo methylation [38]. Studies have shown that the loss of DNMT1 is involved in the loss of DNA methylation at all genomic regions, including gene bodies [39], and the lack of DNMT1 expression in human cancer cells is closely associated with gene silencing [40]. In this study, our *in vitro* results showed that NNK increased DEPDC1 gene body methylation and stimulated DNMT1 expression in OSCC cells. In addition, NNK-induced overexpression of DEPDC1 was blocked by DNMT1 silencing in OSCC. These findings clearly indicate that NNK stimulates DEPDC1 expression through DNMT1-mediated regulation of DNA methylation in OSCC cells.

In summary, our findings indicate that DEPDC1 plays an oncogenic role in OSCC and is useful as a potential therapeutic target and prognostic biomarker for OSCC patients. In addition, our data clearly demonstrate that DEPDC1 exerts its oncogenic activity by inhibiting CYP27B1 protein expression and that NNK promotes the upregulation of DEPDC1 by stimulating DNMT1 expression in OSCC (**Figure 6H**).

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Disclosure of conflict of interest

None.

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Table S1. Primer sequences used in this study

Gene	Forward	Reveres
For qRT-PCR analysis		
DEPDC1	ATGCGTATGATTTCCCGAATGAG	CACAGCATAACACACATCGAGAA
CYP27B1	GGAACCCTGAACAACGTAGTC	AGTCCGAACTTGTAAAATTCCCC
PI3	CACGGGAGTTCCTGTTAAAGG	TCTTTCAAGCAGCGGTTAGGG
A2ML1	CTAGGAATGTTGGCCCTATCAC	CCAAACAAACCTTCTGAACGGA
KRT4	CGCGAACAGATCAAGCTCCT	GGGGCTCAAGGTTTTTGCTG
PGLYRP4	GGTATCAGAGGGGCTCCAGTA	CCATGCCTTGCGAGAGACC
KRT13	GACCGCCACCATTGAAAACAA	TCCAGGTCAGTCTTAGACAGAG
MYH14	TCCGGGAGCGGTACTACTC	GCTTGTACGGGTTGATGACCA
DNMT1	AGGCGGCTCAAAGATTTGGAA	GCAGAAATTCGTGCAAGAGATTC
GAPDH	TGACTTCAACAGCGACACCCA	CACCCTGTTGCTGTAGCCAAA
For DEPDC1 methylation analysis		
DEPDC1-1	AGATAGTTYGGATAGAYGGGAT	CTTTAAACCTCATTCCCAATTA
DEPDC1-2	TTAGGTTTAAGTGATTTTTTTGA	AAAAAATCATTCATCTTATTTATACC
DEPDC1-3	GGTTGGATTTTAAATAATTTYGT	CACACCCTAACTCTCCATAAA

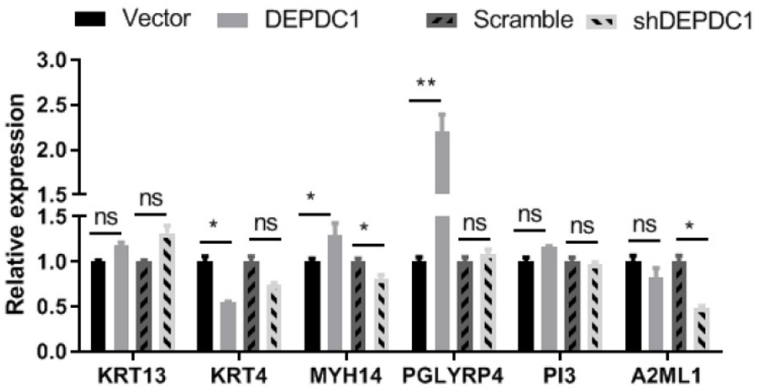

Figure S1. Indicated Gene mRNA Expression Levels were Measured by qRT-PCR. HSC-3 cells were transfected with the indicated constructs. After 72 hours of transfection, cells were subjected to qRT-PCR analysis.

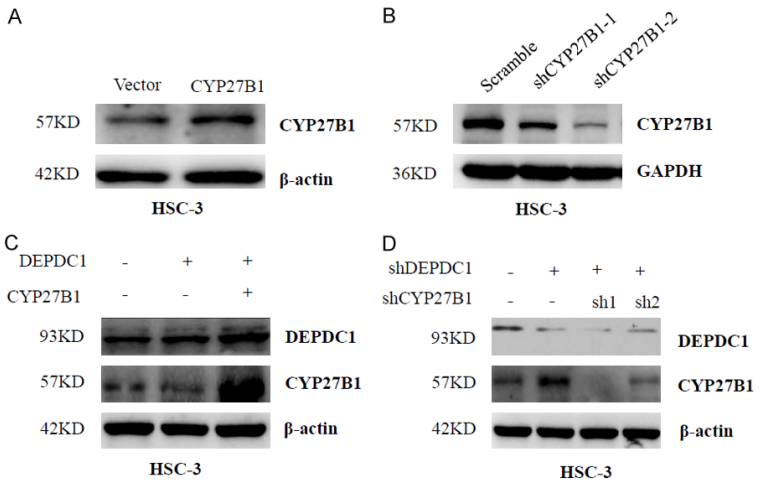

Figure S2. Indicated Protein Expression Levels were Measured by Western Blot. (A-D) HSC-3 cells were transfected with indicated plasmid. After 72 hours of transfection, cells were subjected to Western blot analysis.

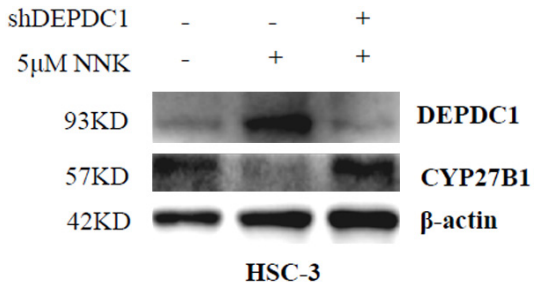

Figure S3. NNK Induced Inhibition of CYP27B1 expression was Blocked by DEPDC1 Silencing in HSC-3 Cells. HSC-3 cells were transfected with or without DEPDC1 shRNA expression construct. After 48 hours of transfection, cells were treated with 5 uM NNK for 24 hours, then subjected to Western blot.