Research Paper

The microRNA miR-29c-5p inhibits cell proliferation and migration by targeting TMEM98 in head and neck carcinoma

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Abbreviation: HNSCC: head and neck squamous cell carcinoma; GEO: Gene Expression Omnibus; OS: overall survival; CI: confidence intervals; DEMs: differential expressed miRNAs
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ABSTRACT

Head and neck squamous cell carcinoma (HNSCC), which occurs frequently worldwide, is characterized by high risk of metastasis. MicroRNAs (miRNAs) play crucial roles in tumorigenesis and cancer development. In this study, miR-29c-5p was identified using three high throughput microarrays. We measure miR-29c-5p expression in HNSCC tissues and cell lines. To determine the function of miR-29c-5p in HNSCC, we evaluated its effects in vitro on cell proliferation, the cell cycle, apoptosis, and cell migration. We employed a mouse tumor xenograft model to determine the effects of miR-29c-5p on tumors generated by HNSCC cell lines. The miR-29c-5p expression was lower in HNSCC tissues than in normal tissues. Upregulated miR-29c-5p expression in HNSCC cells inhibited migration and arrested cells in the G2/M phase of the cell cycle. Further, upregulated miR-29c-5p expression inhibited the proliferation of HNSCC cells *in vivo* and *in vitro*. In addition, transmembrane protein 98 (TMEM98) was identified as a direct target of miR-29c-5p by using a luciferase reporter assay. These findings provide new insights that link the regulation of miR-29c-5p expression to the malignant phenotype of HNSCC and suggest that employing miR-29c-5p may serve as a therapeutic strategy for managing patients with HNSCC.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is one of the most frequently diagnosed carcinomas in Head and Neck Surgery [1, 2]. Approximately 70% of patients with HNSCC in developed countries suffer from metastasis [3]. Despite advances in the development and implementation of screening technologies, HNSCC is frequently diagnosed, and its associated mortality and social economic burden are sharply rising worldwide [3, 4]. Therefore, it is critically important to identify high-risk factors and underlying biologic processes that regulate the malignant phenotype of HNSCC [5, 6].

Increasing evidence demonstrates that the pathogenesis of HNSCC involves numerous factors such as genetic alterations, molecular function, ethnicity, environment, and hormone levels [3, 7, 8]. Although this information contributes to earlier diagnosis, improved strategies for administering individualized treatment and accurate predictions of prognosis, there is limited availability of prognostic markers, such as molecular alterations, to manage aggressive tumor progression or to implement individualized therapeutic regimens for patients with HNSCC [6].

MicroRNAs (miRNAs) play crucial roles in cells by regulating posttranscriptional levels of gene expression in plants and animals [9, 10]. For example, miRNAs affect the pathogenesis, progression, resistance to treatment, histopathological phenotypes, and prognosis of numerous carcinomas [11–13]. Large-scale analyses of genetic alterations that affect the binding of miRNAs to their

targets has identified numerous gene regions that provide insights into the mechanisms of cancer risk [14]. For example, reliable miRNA signatures are used in a clinical model to predict prognosis and overall survival of patients with esophageal squamous cell carcinoma who undergo lymph node-positive locoregional resection [15]. Moreover, miR-142-5p significantly suppresses the proliferation, apoptosis, and migration of numerous types of cancer cells through targeting multiple apoptosis inhibitory genes and components of the NRF2/ARE pathway [16]. Despite these important advances in clinical and basic research, the regulatory role of microRNAs in HNSCC is largely unknown.

Rapid development of high-throughput technologies has made possible comprehensive bioinformatics analyses that detect differentially expressed miRNAs, which in turn, facilitate screening for therapeutic targets that are significantly associated with the pathogenesis and progression of HNSCC [17, 18]. Despite studies of genomic alterations and molecular interactions associated with HNSCC, it is difficult to identify patients at high risk to guide therapy. To address this challenge, here we analyzed three HNSCC microarray datasets acquired from the United States National Center of Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo). We screened for miRNAs that were differentially expressed (DEMs) in tumors vs adjacent normal tissues. We hypothesize that these DEMs have significant prognostic implications, and investigations of these DEMS may provide therapeutic targets for HNSCC.

RESULTS

Screening and identification of has-miR-29c-5p as a DEG in HNSCC

Analysis of three microarrays led to the identification of significant DEGs (155 probes, GSE32115; 67 probes, GSE34536; and 91 probes, GSE11163). Each dataset included three microRNAs in common: has-miR-29c, has-miR-210, and has-miR-18a (Figure 1A). We then analyzed the expression of these three microRNAs in our cohort and found the expression level of miR-29c-5p was significantly diminished in HNSCC tissues (0.483 ± 0.015) compared with normal tissues in our cohort (1.000 ± 0.023; $P<0.05$; Figure 1B).

To evaluate the potential prognostic value of miR-29c-5p in HNSCC, we performed overall survival analysis of our cohort. Decreased miR-29c-5p expression was significantly associated with unfavorable prognosis of patients with HNSCC (Figure 1C). Compared with INOE cells, the levels of miR-29c-5p were significantly reduced in FaDu and HSC-3 cells (Figure 1D), which were therefore selected for subsequent experiments.

MiR-29c-5p regulates the proliferation of FaDu and HSC-3 cells

We next analyzed miR-29c-5p expression in cells transfected with the mimic, inhibitor and found that the level of miR-29c-5p was significantly increased in cells transfected with the miR-29c-5p mimic and decreased in cells transfected with the miR-29c-5p inhibitor (FaDu and HSC-3 cells; Figure 2A).

To identify the potential function of miR-29c-5p, we used the CCK-8 assay to measure the proliferation rates of FaDu and HSC-3 cells transfected with the miR-29c-5p mimic or inhibitor. The growth rates of cells transfected with the mimic miR-29c-5p that over-expressed miR-29c-5p were significantly decreased compared with the control. Conversely, the growth rates of FaDu and HSC-3 cells transfected with the miR-29c-5p inhibitor were significantly increased compared with the negative control (Figure 2B, 2C).

Flow cytometry of transfected FaDu and HSC-3 cells revealed that downregulation of miR-29c-5p significantly increased the percentages of S-phase cells vs the control (42.2% and 40.4%, respectively), while the percentages of G2/M phase cells decreased vs the control (17.2% and 22.4%, respectively). Conversely, upregulation of miR-29c-5p in these transfectants increased the percentages of S-phase cells and decreased the percentages of G2/M phase cells ($p<0.05$) (Figure 2D–2F).

MiR-29c-5p regulates the migration of FaDu and HSC-3 cells

Upregulation of miR-29c-5p expression significantly reduced migration of the transfected FaDu and HSC-3 cells (91 ± 6 cells and 93 ± 12 cells, respectively) vs the control (201 ± 9 cells and 203 ± 15 cells, respectively), and cell numbers significantly increased after the two cell lines were transfected with the miR-29c-5p inhibitor (266 ± 11 cells and 237 ± 8 cells, respectively) compared with the negative control group (Figure 3A, 3B). After transfection of FaDu and HSC-3 cells with miR-29c-5p or the miR-29c-5p inhibitor, no significant differences were detected between apoptosis rates (Figure 3C, 3D).

MiR-29c-5p directly targets the TMEM98-3´-UTR and modulates TMEM98 expression

To identify the underlying mechanism that accounts for the effects of miR-29c-5p on cell proliferation, the cell

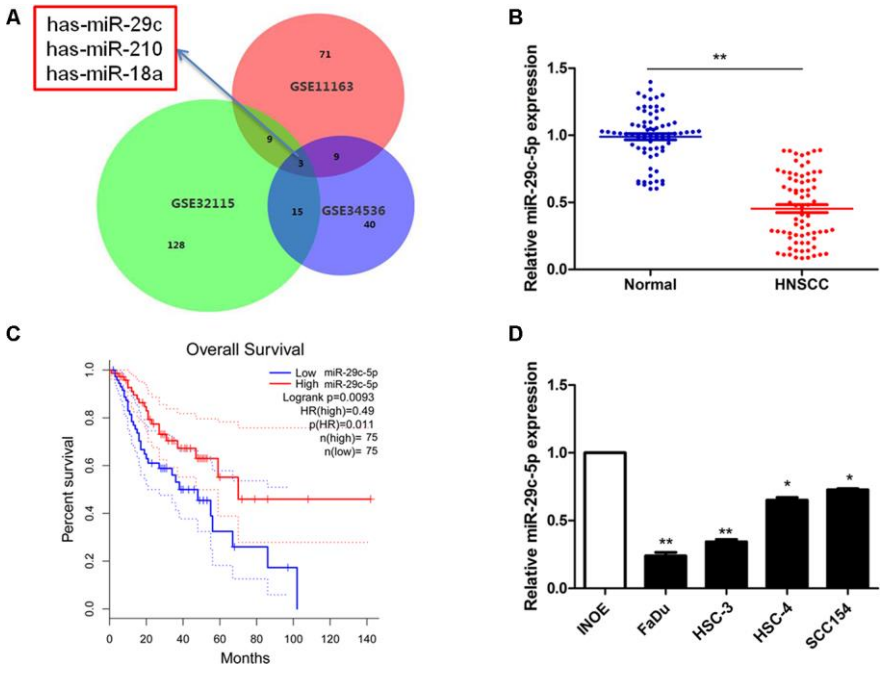

Figure 1. Detection of has-miR-29c expression in HNSCC. (**A**) The overlap among three expression datasets described in Methods included the significant miRNAs has-miR-29c, has-miR-210, has-miR-18a. (**B**) The levels of miR-29c-5p in a clinical cohort were significantly lower in HNSCC tissues compared with normal tissues (P<0.05). (**C**) Survival curves suggest that decreased miR-29c-5p expression was significantly associated with unfavorable prognosis of patients with HNSCC. (**D**) Compared with the normal control, the levels of miR-29c-5p were significantly lower in FaDu and HSC-3 cells. *p < 0.05, **p < 0.01.

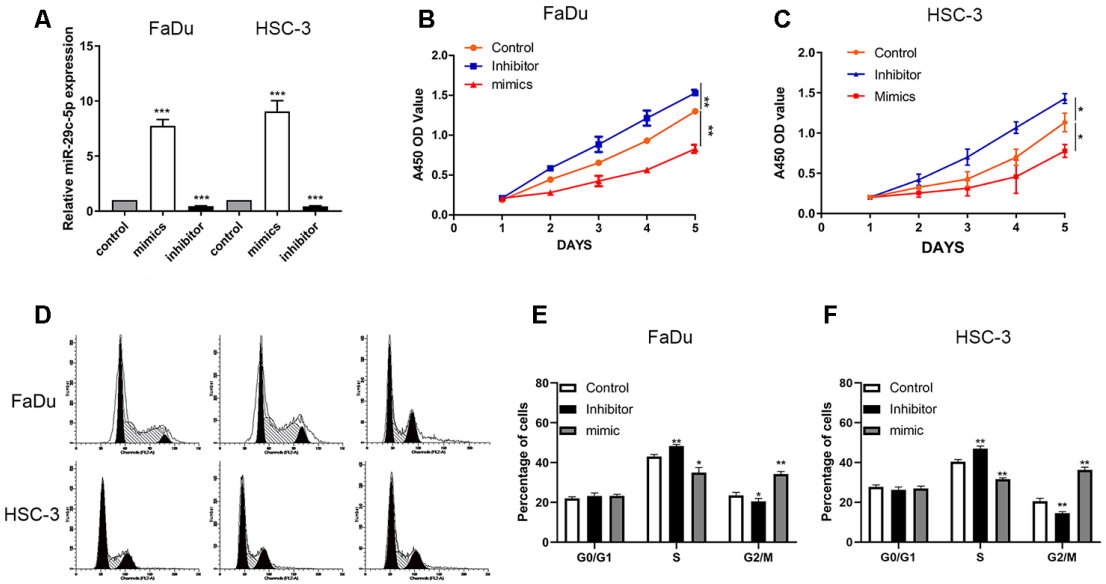

Figure 2. miR-29c-5p regulates the proliferation of FaDu and HSC-3 cells. (**A**) The levels of miR-29c-5p were significantly higher in FaDu and HSC-3 cells transfected with the miR-29c-5p mimic but lower in cells transfected with the miR-29c-5p inhibitor. (**B**, **C**) The results of CCK-8 assays suggest that upregulated miR-29c-5p expression induced by its cognate mimic significantly decreased the cell growth rate compared with the control. Conversely, the growth rates of FaDu and HSC-3 cells transfected with the miR-29c-5p inhibitor were significantly higher compared with the negative control. (**D**–**F**) Cell cycle analysis of FaDu and HSC-3 cells transfected with the miR-29c-5p inhibitor shows that the percentages of S-phase cells significantly increased while the percentages of G2/M-phase cells decreased compared with the control (p<0.05). Conversely, upregulation of miR-29c-5p expression decreased and increased the percentages of cells in S phase and G2/M phase, respectively. *p < 0.05, **p < 0.01, ***p < 0.001.

cycle, and the migration of HNSCC cells, we performed bioinformatics analysis to predict possible direct targets of miR-29c-5p. TMEM98, C20orf85, LRRC31, APN3, and SURF4 were identified as possible targets of miR-29c-5p, and their expression levels were therefore evaluated after transfection of FaDu and HSC-3 cells with the miR-29c-5p mimics (Figure 4A, 4B). We found that the levels of TMEM98 mRNA significantly decreased (Figure 4B).

Bioinformatics analysis revealed a putative binding site for miR-29c-5p in the 3´-UTR of TMEM98 mRNA (Figure 4C). Consistent with this finding, we found that the luciferase activity of FaDu cells transfected with the miR-29c-5p mimic and psiCHECK–WT was significantly decreased, whereas cotransfection with miR-29c-5p did not significantly alter the reporter activity of psiCHECK–MUT. Further, the expression of TMEM98 in FaDu and HSC-3 cells transfected with the miR-29c-5p mimic or inhibitor were downregulated or upregulated, respectively (Figure 4D, 4E). Finally, TMEM98 mRNA was expressed at higher levels in HNSCC tissues than adjacent normal tissues and was significantly associated with miR-29c-5p expression in HNSCC tissues (r^2=0.091, p<0.05; Figure 4F, 4G). Together, these results show that miR-29c-5p regulated *TMEM98* expression by directly binding to the 3´-UTR of *TMEM98* mRNA.

Down-regulation of TMEM98 represses proliferation and migration of FaDu and HSC-3 cells

To investigative the function of TMEM98 in HNSCC cells, we transfected HNSCC cells with either siRNA scramble or si-TMEM98 (Figure 5A). As shown in Figure 5B, the proliferation ability was inhibited in the si-TMEM98 treated HNSCC cells as compared with that in the scramble treated HNSCC cells. Similarly, the results from the transwell assay indicated that the migration ability in the si-TMEM98 treated HNSCC cells were lower than those in the scramble treated HNSCC cells (Figure 5C).

Overexpression of TMEM98 mRNA reverses the tumor suppressor effect of miR-29c-5p

TMEM98 mRNA and protein were detected after transfecting FaDu and HSC-3 cells with the miR-29c-5p mimic or the mimic combined with the TMEM98-overexpression plasmid. Specifically, TMEG98 expression was decreased in cells transfected with the mimic and increased in cells transfected with the

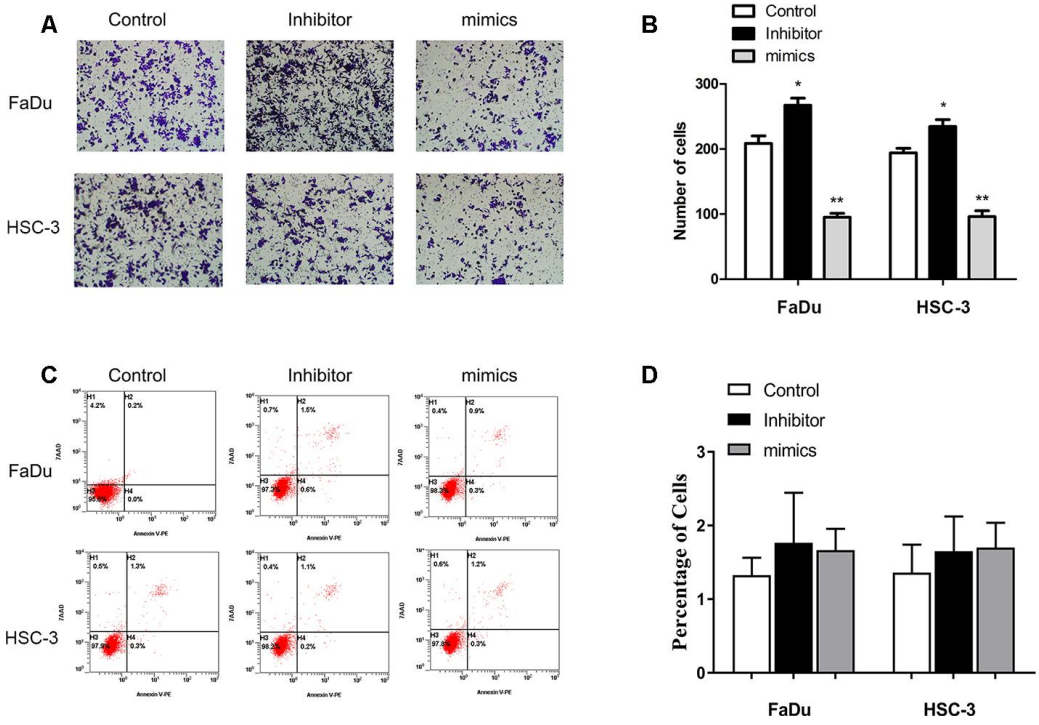

Figure 3. miR-29c-5p reduces the migration of FaDu and HSC-3 cells. (**A**, **B**) Upregulation of miR-29c-5p expression significantly reduced migration of FaDu and HSC-3 cells, while downregulation of miR-29c-5p expression using the cognate inhibitor markedly increased migration compared with the negative control. (**C**, **D**) Significant differences were not observed in the rates of apoptosis among the test and control cells. *p < 0.05, **p < 0.01.

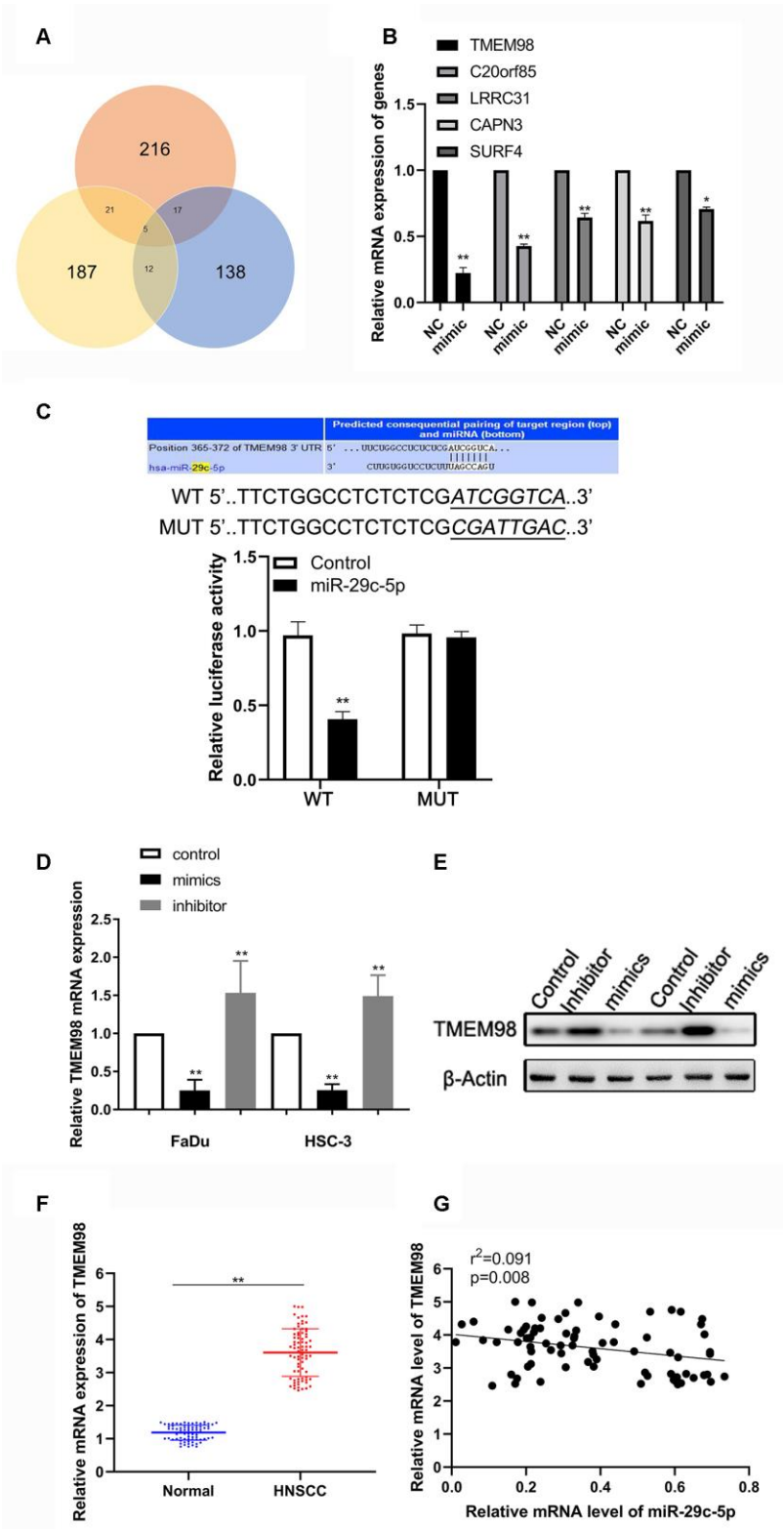

Figure 4. miR-29c-5p directly targets the 3´-UTR of *TMEM98* mRNA to modulate TMEM98 expression. (**A**) Analysis of the three bioinformatics databases showing the number of potential has-miR-29c-target genes. (**B**) Relative mRNA levels of five potential has-miR-29c-target genes in cells transfected with the has-miR-29c mimic vs the control. (**C**) The Upper is the putative binding site of miR-29c-5p in the 3´-UTR of TMEM98 mRNA. The lower is the relative luciferase activities of wild-type and mutant TMEM98 reporters in FaDu and HSC-3 cells. (**D**, **E**) mRNA and protein levels of TMEM98 in FaDu and HSC-3 cells transfected with the miR-29c-5p mimic or inhibitor. (**F**, **G**) mRNA levels of TMEM98 and their association with has-miR-29c levels in HNSCC tissues. *p < 0.05, **p < 0.01.

TMEM98-overexpression plasmid (Figure 6A, 6B). To determine whether miR-29c-5p inhibited the proliferation of FaDu and HSC-3 cells through direct targeting of *TMEM98* mRNA, we measured cell proliferation rates and the cell cycle after transfecting FaDu and HSC-3 cells with the miR-29c-5p mimic or with the mimics combined with the TMEM98-overexpression plasmid. The results indicate that cell proliferation rates and the percentages of S-phase cells were significantly decreased in cells transfected with the miR-29c-5p mimic, while these values were increased after cotransfection with TMEM98-overexpression plasmid (Figure 6C–6F). Similarly, cell migration was significantly decreased in FaDu and HSC-3 cells transfected with the miR-29c-5p mimic and increased after TMEM98 overexpression (Figure 6F, 6G).

Previous study suggested that the down-regulation of TMEM98 inhibits proliferation and migration of vascular smooth muscle cells by suppressing the AKT/GSK3β /Cyclin D1 signaling. We therefore further investigated whether miR-29c-5p-TMEM98 regulates proliferation and migration of HNSCC cells via AKT/GSK3β /Cyclin D1 signaling. Our results indicated that the level of AKT/GSK3β /Cyclin D1 signaling was markedly increased in the TMEM98 overexpression plasmid transfected HNSCC cells (Supplementary Figure 1A). Additionally, the enhanced proliferation and migration ability of TMEM98 overexpression plasmid transfected HNSCC cells could be reversed after the treatment of MK-2206 2HCL, an inhibitor of AKT/GSK3β /Cyclin D1 signaling (Supplementary Figure 1B, 1C). These results indicated that miR-29c-5p-TMEM98 might regulate HNSCC cells' proliferation and migration via AKT/GSK3β/ Cyclin D1 signaling.

Tumor xenograft model

A tumor xenograft model was established by subcutaneously injecting 4-week-old athymic nu/nu mice with FaDu cells stably transfected with the miR-29c-5p mimic. The results show that mice injected with the cells transfected with the miR-29c-5p mimic had smaller tumor volumes and lower tumor weights than the control (Figure 7A–7C). Further, Ki-67 expression in tumor tissues formed by the xenografted cells was evaluated using immunohistochemistry. Ki-67 expression decreased in cells transfected with the miR-29c-5p mimic compared with control (Figure 7D, 7E). Together, these results suggest that upregulation of miR-29c-5p expression inhibited tumor proliferation *in vivo*.

DISCUSSION

Accumulating studies show that many miRNAs contribute to the progression and recurrence of cancers via regulating prognostic genes [11, 19, 20]. Here we

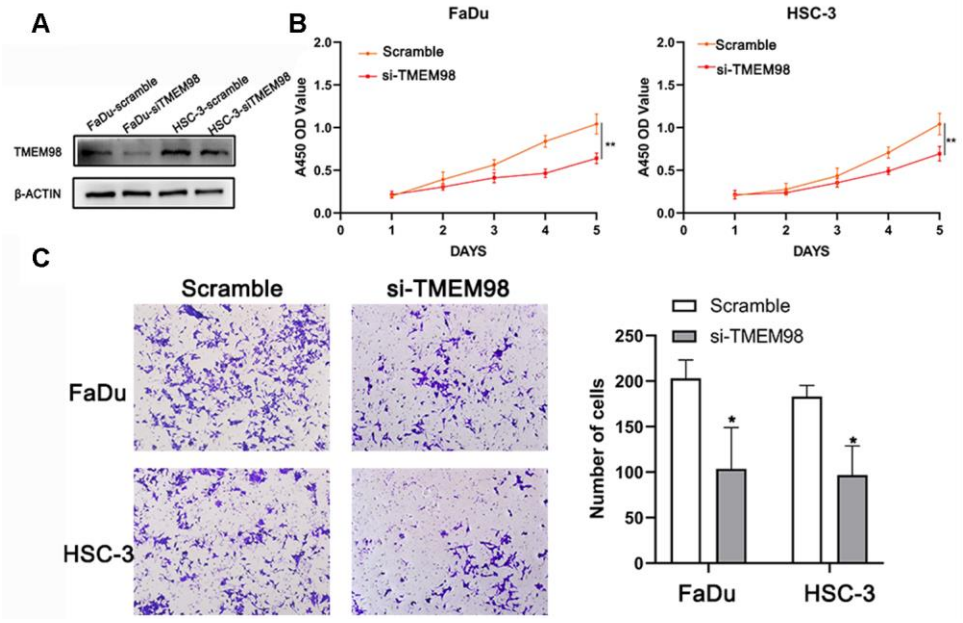

Figure 5. Down-regulation of TMEM98 represses the proliferation of FaDu and HSC-3 cells. (**A**) The expression level of TMEM98 was decreased in HNSCC cells treated with si-TMEM98 compared with cells treated with scramble. (**B**) The CCK-8 assay shows that down-regulation of TMEM98 inhibits the proliferation rate of HNSCC cells. (**C**) The migration assay indicates that down-regulation of TMEM98 represses the migration ability of HNSCC cells. *p < 0.05, **p < 0.01.

show a significant decrease in miR-29c-5p expression levels in human HNSCC tissues compared with adjacent uninvolved tissues, which significantly correlated with favorable prognosis. To identify the function of miR-29c-5p, we analyzed the proliferation, invasion, migration, cell cycle, and apoptosis of cell lines derived from HNSCCs and employed a mouse tumor xenograft model to determine the role of miR-29c-5p in HNSCC. Moreover, we manipulated the expression levels of miR-29c-5p via transfecting the HNSCC cell lines FaDu and HSC-3 with miR-29c-5p mimics or a cognate inhibitor and assessed their effects on cell proliferation and migration.

The results of luciferase reporter assays suggest that miR-29c-5p directly targets the 3´-UTR of TMEM98 mRNA to modulate TMEM98 expression. Further, TMEM98 overexpression restored the proliferation rates of FaDu and HSC-3 cells transfected with an miR-29c-5p mimic compared with the control. Finally, nude mice receiving xenografts of FaDu cells transfected with the miR-29c-5p mimic formed tumors that grew at a significantly lower rate compared with xenografted, mock-transfected cells.

TMEM98 is a predicted single-pass transmembrane protein of the endoplasmic reticulum that is expressed at

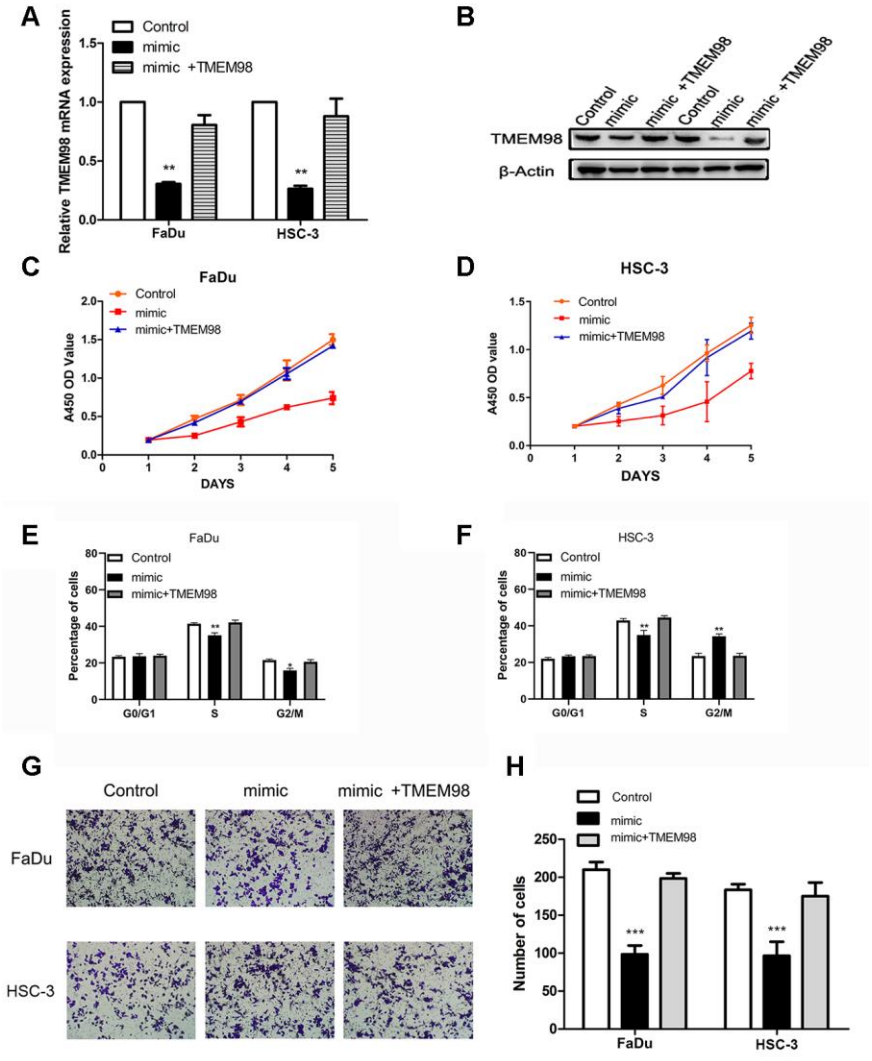

Figure 6. TMEM98 overexpression reverses the tumor suppressor effects of miR-29c-5p. (**A**, **B**) TMEM98 expression in FaDu and HSC-3 cells transfected with miR-29c-5p mimics, miR-29c-5p mimics combined with the TMEM98-overexpression plasmid, or control. (**C**, **D**) Cell proliferation was significantly lower in cells with upregulated miR-29c-5p expression, while cell numbers were restored to those of the control. (**E**, **F**) Cell cycle analysis of HNSCC cells transfected with miR-29c-5p mimics, miR-29c-5p mimics combined with the TMEM98-overexpression plasmid, or control. (**G**, **H**) Cell migration was significantly decreased in cells transfected with the miR-29c-5p mimic and increased after upregulation of TMEM98 expression. *p < 0.05, **p < 0.01, ***p < 0.001.

significantly elevated levels in adenosquamous carcinoma, suggesting its association with poor overall survival [21]. TMEM98 is a signature-specific gene in adenocarcinoma through its activation of the β-catenin pathway [21]. The binding of small interfering RNAs to TMEM98 mRNA may suppress the proliferation, invasiveness, and migration of A549 and H460 lung carcinoma cell lines [22].

Few studies report the role of miR-29c-5p in tumorigenesis and development [15, 16]. For example, a study of 186 normal breast tissue samples and 1,338 invasive breast carcinomas found that miR-29c-5p expression is significantly upregulated in luminal subtypes [23]. Interestingly, expression of TET family enzymes is significantly increased in cells in which the expression of miR-26b-5p and miR-29c-5p is silenced [24]. However, the potential prognostic value and phenotypic effects of miR-29c-5p in HNSCC remain unknown, although we show here that miR-29c-5p may serve as a novel therapeutic agent for treating HNSCC.

In conclusion, downregulation of miR-29c-5p expression was detected in human HNSCC cells and tissues, and upregulation of miR-29c-5p expression suppressed the abilities of HNSCC cell lines to proliferate and migrate, partially through direct targeting of *TMEM98* mRNA. These findings provide compelling evidence that miR-29c-5p functions as a tumor suppressor that affects HNSCC development and may therefore serve as a component of new therapeutic strategies for managing patients with HNSCC.

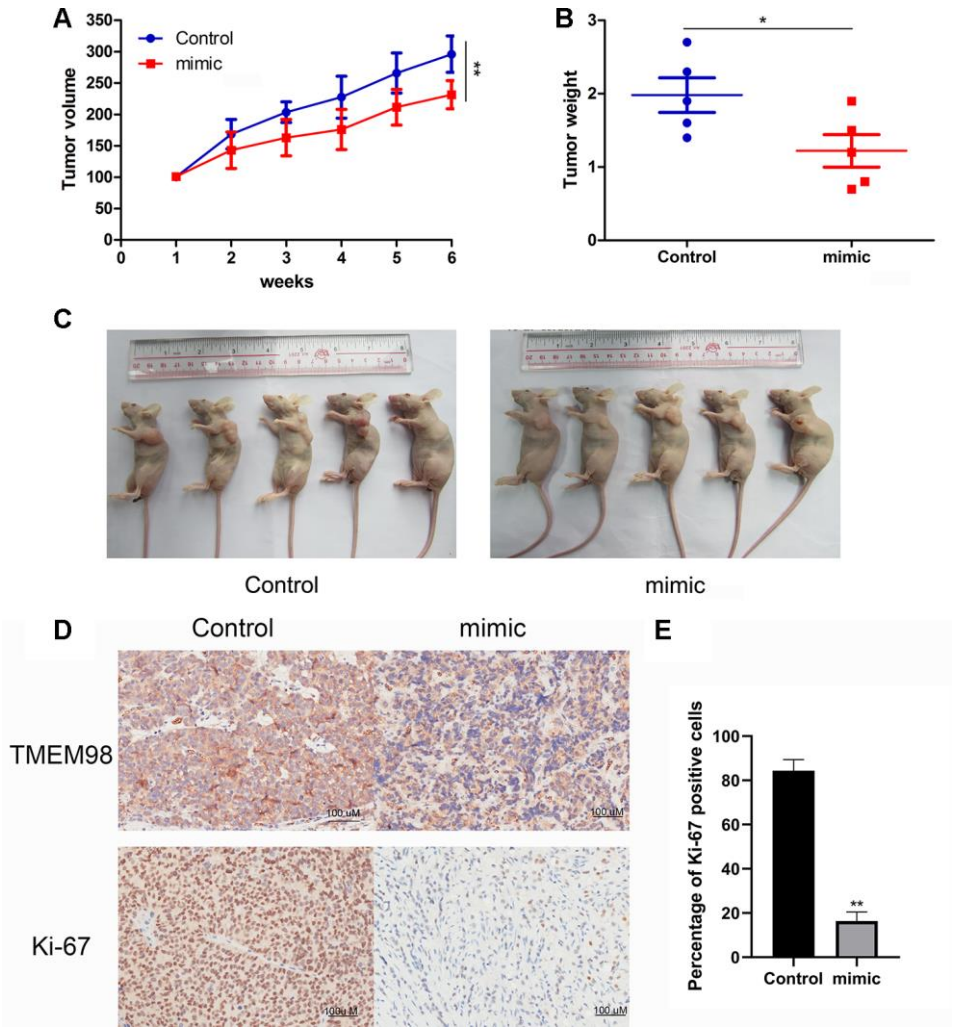

Figure 7. Analysis of a mouse tumor xenograft model of HNSCC. (**A**) Significantly lower tumor volumes were observed in mice xenografted with HNSCC cells transfected with miR-29c-5p mimic compared with control. (**B**, **C**) Tumor weights were significantly lower in mice xenografted with cells transfected with the miR-29c-5p mimic compared with control. (**D**) Ki67 and TMEM98 expression was detected in tumor tissues formed by xenografted HNSCC cells transfected with the miR-29c-5p mimics. *p < 0.05, **p < 0.01. (**E**) Bar graph indicates quantitative analysis of the percentage of Ki-67 positive cells in either miR-29c-5p mimic group or control group.

MATERIALS AND METHODS

Microarray data and survival analysis

Raw DNA microarray data were acquired from the GEO database and were analyzed using the Affy package of R software (version 3.3.4) [25]. The GEO-chip datasets GSE32115 (two HNSCCs and two normal samples), GSE34536 (seven each of HNSCCs and normal samples), and GSE11163 (16 HNSCCs and five normal samples) were preliminarily processed. Bioinformatics analyses of microRNA-target genes were performed using data acquired from the miRDB (http://www.mirdb.org/), StarBase (starbase.sysu.edu.cn), and TargetScan (http://www.targetscan.org/) databases.

Patients' samples

HNSCC samples (n = 76) and corresponding nontumor tissues were obtained from patients who underwent curative surgery at our institute. Total RNA was extracted from tissues and stored at −80° C. The tissue samples were used after obtaining approval from the Ethics Committee of our institute. Informed consent was obtained from all patients who participated in this study according to the committee's regulations.

Cell culture and reagents

The HNSCC-derived cell lines FaDu, HSC-3, HSC-4, SCC154, and the head and neck nontumorigenic squamous cell INOE were acquired from the American Type Culture. Cells were incubated at 37° C in RPMI-1640 medium (GIBCO, USA) supplemented with 10% fetal bovine serum and 100 μg/ml penicillin in a humidified incubator containing an atmosphere of 5% CO_2. The inhibitor of AKT signaling, MK-2206 2HCL was purchased from Selleck (Texas, U.S.).

Extraction of total RNA and quantitative real-time PCR analysis (qRT-PCR)

Total RNA was from harvested FaDu and HSC-3 cells, sample tissues were isolated using Trizol (Invitrogen, Carlsbad, CA), and qRT-PCR was performed using SYBR Premix Ex TaqTM (TaKaRa) in accordance with the sources' protocols. The primers specific for miR-29c-5p were 5′-TAGTAGTGGTTGTTTGTTTTTTT GA-3′ (forward) and 5′-CCACTCTACTAAAAAC TCCATCTCC-3′ (reverse). The primers specific for TMEM98 were 5′-CAATTGAGCTTCCACCTG-3′ (forward) and 5′-CCGACAGAGCTCTAGAGAAC-3′(reverse). The miRNA expression level of HNSCC tissues and cell lines were measured using a Hairpin-itTM microRNA qPCR Kit (GenePharma, Shanghai, China). The transcriptional levels of has-miR-29c-5p and TMEM98 were normalized to U6 or β-Actin according to the manufacturer's protocols. Relative expression levels were determined using the $2^{-\Delta\Delta Ct}$ method.

TEME98 over-expression plasmid construction and transfection

To construct a plasmid for overexpressing TMEM98, the coding sequence and 3'-UTR of TMEM98 was inserted into the p-Enter vector (Vigene Bio.) according to the manufacturer's instructions. FaDu and HSC-3 cells were transfected with miR-29c-5p mimic, an miR-29c-5p inhibitor, a negative control (RiboBio), and a TMEM98-expression vector in the presence of Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's instructions. FaDu and HSC-3 cells were harvested at least 24 h after transfection.

Cell proliferation assays

To measure cell proliferation, FaDu and HSC-3 cells (5×10^3 cells per well, each) were added to the wells of 96-well plates and cultured at 37° C for 24 h in an incubator containing an atmosphere of 5% CO_2. CCK8 solution (Keygen) was added to each well. The optical density (450 nm) of the medium was detected using a spectrophotometer.

Cell migration assays

A Transwell insert (Corning, UK) was used to measure cell migration. FaDu and HSC-3 cells (1×10^5 each) were added to serum-free medium, and the suspension was then added to the upper chamber. Medium containing 20% fetal bovine serum was added to the lower chamber. After 24 h, FaDu and HSC-3 cells on the bottom side were fixed with 4% polyoxymethylene for 15 min, stained using crystal violet, air-dried, and photographed using a microscope.

Apoptosis assay

Apoptosis was detected using Annexin V-FITC Apoptosis Detection Kit (BD, USA) in accordance with the manufacturer's procedures. Briefly, after transfection with miRNA mimics, the miRNA inhibitor, or the control, FaDu and HSC-3 cells were washed three times with phosphate-buffered saline (PBS; BD, USA) and then added to 500 μl of 1× binding buffer. Subsequently, 500 μl of cell suspension, 5 μl of Annexin V-FITC, and 5 μl of propidium iodide solution were added to each collection tube. After incubation for 15 min, apoptotic cells were detected using a FACS Analyzer (BD).

Cell cycle assay

Flow cytometry was performed to test the effects of the miR-29c-5p inhibitor and mimic on the cell cycle distribution of FaDu and HSC-3 cells compared with the negative control. The cells were transfected for 24 h, washed twice with PBS, and analyzed using a cell cycle assay kit (KeyGen) according to the manufacturer's instructions.

Luciferase reporter assay

TargetScan was utilized to identify targets of miR-29c-5p. The 3′-UTR of wild-type (WT) human TMEM98, which contains a putative miR-29c-5p DNA-binding sequence, was amplified using PCR, and the amplicon was inserted into the p-miR-reporter (Ambion, USA) to create a TMEM98-WT luciferase vector. The mutant (MUT) 3′-UTR was inserted into the p-miR-reporter to create a TMEM98-MUT luciferase vector. The WT 3′-UTR or MUT 3′-UTR of TMEM98 or the miR-29c-5p mimic was used to cotransfect FaDu cells in the presence of Lipofectamine 3000. After 48 h, luciferase activity was measured using a dual luciferase reporter assay system according to the manufacturer's instructions.

Western blotting

FaDu and HSC-3 cells were lysed using RIPA lysis buffer (TaKaRa, Beijing, China), and protein concentrations were determined using a bicinchoninic acid protein assay kit (Keygen, Nanjing, China). Proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrophoretically transferred to a methanol-activated polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with skimmed milk for 1 h at room temperature and then incubated with an anti-TMEM98 antibody (diluted 1:1000; Proteintech) at 4° C overnight. The next day, the membrane was rinsed three times with TBST, incubated with the secondary antibody for 1 h at room temperature, and immunocomplexes were visualized using ECL plus western blotting detection reagents (Keygen, Nanjing, China).

Tumor xenograft model

FaDu cells (100 μl, 1×10^7 cells/ml) were transfected with RV-miR-29-mimic or RV-control and then injected into the right flank of 4-week-old BALB/c-Nude mice. Tumor volume was measured using a caliper and calculated as follows: volume = (length × width)/2. Four weeks after injection, mice were killed, tumors were removed and weighed, and tumor nodules were fixed in 10% buffered formalin. Ki-67 expression in tumor and normal tissues was evaluated using immunohistochemistry according to the manufacturer's instructions. The Institutional Animal Care and Use Committee of our institute approved the experimental protocol.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad) and the R package (V. 3.3.4). All data represent the mean ± standard deviation (SD). The significance of differences in data between groups was analyzed using a two-tailed Student's *t* test or one-way ANOVA. Survival was analyzed using the Kaplan–Meier method with 95% confidence intervals and the log-rank test. $p < 0.05$ indicates a significant difference.

AUTHOR CONTRIBUTIONS

Wang Y. designed the study strategy. Li J. and Chen W. designed experiment, performed the study and analyzed data. Luo L. and Liao L. contributed to research and reviewed the manuscript. Deng X. analyzed clinical data. All authors reviewed the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

FUNDING

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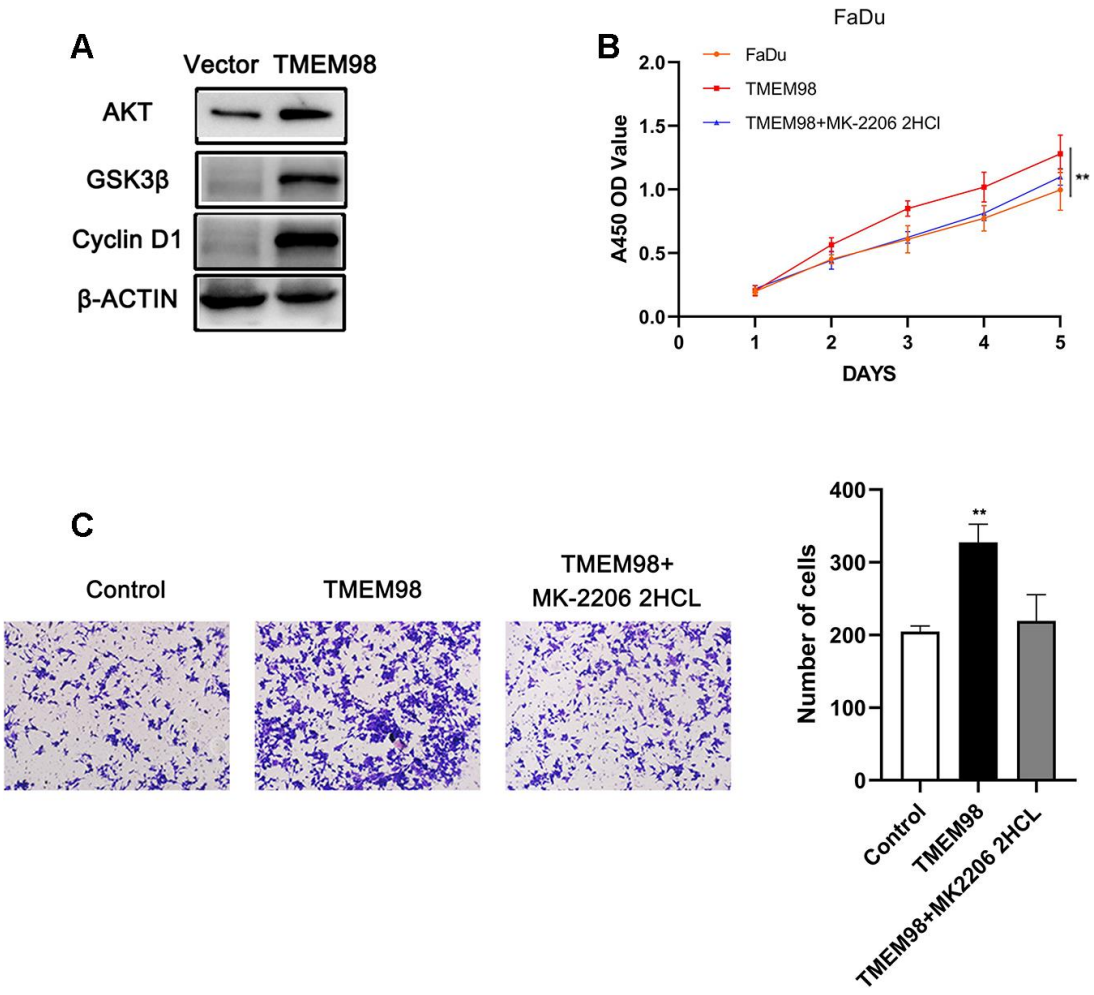
SUPPLEMENTARY MATERIALS

Supplementary Figure



Supplementary Figure 1. MiR-29c-5p-TMEM98 might regulate HNSCC cells' proliferation and migration via AKT/GSK3β /Cyclin D1 signaling. (**A**) The expression level of AKT/GSK3β /Cyclin D1 was significantly elevated in HNSCC cells transfected with TMEM98 over-expression plasmid. (**B**) The inhibitor of AKT signaling, MK-2206 2HCL, reversed the enhanced effect of TMEM98 on proliferation ability in HNSCC cells. (**C**) The enhanced migration ability of TEME98 over-expression HNSCC cells was reversed after the treatment of MK-2206 2HCL. *p < 0.05, **p < 0.01.