

P-element Induced WImpy protein-like RNA-mediated gene silencing 2 regulates tumor cell progression, apoptosis, and metastasis in oral squamous cell carcinoma

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Abstract

Objective: P-element Induced WImpy protein-like RNA-mediated gene silencing 2 (PIWIL2) is a reported oncogene strongly associated with tumorigenesis and cancer progression. However, the potential function of PIWIL2 in oral cancer is still largely unclear.

Methods: In this study, we investigated the clinical significance of PIWIL2 expression in human oral squamous cell carcinoma (OSCC) cell lines and tissues. We also examined its function in OSCC pathogenesis by knocking down PIWIL2 expression with short hairpin RNAs, followed by phenotypic experiments focused on cell migration, invasion, proliferation, and apoptosis rates.

Results: We found that PIWIL2 was overexpressed in OSCC cell lines and tissues and significantly correlated with the malignancy stage. Furthermore, knockdown of PIWIL2 in a human OSCC cell line Tca8113 induced cell cycle arrest and apoptosis. Silencing PIWIL2 expression also significantly suppressed the migration and invasion abilities of Tca8113 cells.

Conclusions: Collectively, our results suggest a functional role of PIWIL2 in regulating OSCC pathogenesis. Our data imply that PIWIL2 could serve as a potential therapeutic target for OSCC treatment.

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Keywords

P-element Induced WImpy protein-like RNA-mediated gene silencing 2, proliferation, migration, apoptosis, disease progression, oral cancer, oncogene

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Introduction

About 405,000 new cases of oral cancer are diagnosed each year worldwide, most of them being squamous cell carcinoma (SCC) that causes significant mortality.^{1–3}

Despite advancements in clinical diagnosis and therapies for oral cancer, the survival rate is still very low.⁴ This is attributable to the lack of early stage diagnostic markers and poor sensitivity to current therapeutic strategies in advanced stage SCC. The regulatory mechanisms controlling oral squamous cell carcinoma (OSCC) tumorigenesis and development are not well understood, which limits the identification of effective diagnostic biomarkers and targeted therapies.

The current 5-year survival rates of oral cancer patients in stage I, II, III, and IV are 80%, 70%, 56.9%, and 36.8%, respectively.⁵ The advanced cancer stage is characterized by a higher occurrence of invasion to surrounding tissues, lymph nodes, and distant tissues. Metastasis is what ultimately leads to the death of the patient.⁶ Piwi (P-element Induced WImpy) proteins belong to the Argonaute family. The PIWI subfamily of genes comprise Piwi-like RNA-mediated gene silencing (PIWIL)1, PIWIL2, PIWIL3, and PIWIL4, and are upregulated in the primary tumors and metastatic tissues of epithelial ovarian cancer.⁷ PIWIL2 can reportedly regulate the invasion and metastasis of prostate cancer cells by modulating matrix metalloproteinase 9 (MMP9) expression and the epithelial–mesenchymal transition (EMT) process. Cell invasion and

migration were significantly suppressed in PC-3 cells following PIWIL2 knockdown.⁸ However, the function of PIWIL2 in OSCC tumor metastasis remains largely unknown.

In addition to metastasis, current research is also focused on inhibition of tumor proliferation and induction of apoptosis. Previous studies have indicated that high PIWIL2 expression can promote glioma cell proliferation.⁹ PIWIL2 has also been reported to promote cell proliferation by upregulating the cell cycle-related gene cyclin D1 in multiple types of cancer cells.¹⁰ Additionally, inhibiting PIWIL2 activity can induce apoptosis in a human neuroblastoma cell line.¹¹ However, whether PIWIL2 regulates OSCC cell proliferation and apoptosis remains unknown.

In the present study, we investigated the relationship between PIWIL2 expression and pathological status of OSCC. We also examined the roles of PIWIL2 in regulating cell migration, proliferation, and apoptosis in OSCC cell lines. Our results demonstrate that PIWIL2 plays a significant role in regulating OSCC progression and may serve as a therapeutic target for OSCC treatment.

Materials and methods

Clinical study

Patients diagnosed with OSCC were recruited for this study. All patients involved in this study were informed of the details and provided written informed

consent. Included patients were in line with the pathological diagnostic criteria of oral malignant tumors¹² and never participated in chemoradiotherapy. Exclusion criteria included patients with severe primary diseases affecting the circulatory, respiratory, nervous, digestive, or blood systems and patients who could not cooperate, such as psychiatric patients. Finally, 60 OSCC patients fulfilling the above criteria were included in the study. We collected the OSCC cancer tissues and para-cancer tissues (oral epithelial dysplasia) by surgery. The progression of these patients was monitored. The present study was approved by the Ethics Committee of Heilongjiang Provincial Hospital.

Cell culture

Different oral cancer cell lines (Tca8113, SCC9, SCC25, CAL27, HN12, HSU3, and FADU) and a normal human oral keratinocyte (NHOK) cell line were obtained from American Type Culture Collection (ATCC) and were cultured in RPMI-1640 (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). Cells were cultured at 37°C in a 5% CO₂ atmosphere.

PIWIL2 knockdown

The Plko.1 vector was used for short hairpin RNA (shRNA) construction. The shRNA sequences used for silencing PIWIL2 expression: PIWIL2 shRNA-1: 5'-CTATGAGATTCTCAACTACAGAAG-3'; PIWIL2 shRNA-2: 5'-ATGAGGTT CGGCATGTTGAAGGACCATCA-3'.

The vectors were transfected into cells using X-tremeGENE HP DNA Transfection Reagent (Roche, Indianapolis, IN, USA).

Quantitative reverse transcription polymerase chain reaction (qPCR)

RNAiso plus reagent (Takara, Kusatsu, Japan) was used for total RNA isolation.

Then, RNA was reverse transcribed into cDNA with M-MLV reverse transcriptase (Takara). SYBR Premix ExTaqTM (Perfect Real Time) (Takara) was used for qPCR analysis using a LightCycler[®] 96 real-time PCR system (Roche). The relative gene expression ($2^{-\Delta\Delta C_t}$) was normalized against GAPDH expression. The primer sequences used in this study: PIWIL2: Forward 5'-TG CAGGCAGAGGCCATGTA-3', Reverse 5'-AGGCCTCGGAACATGGAGAC-3'; GAPDH: Forward 5'-CTTAGCACCCCTG GCCAAG-3', Reverse 5'-GATGTTCTGG AGAGCCCCG-3'.

Cell proliferation analysis

Cells (5×10^3 cells/well) were seeded in a 96-well plate. Following treatment, cell proliferation was measured with Cell Counting Kit-8 (CCK8) solution (Beyotime, Beijing, China) according to the manufacturer's instructions. Briefly, 10 μ L CCK-8 solution (Solarbio, Beijing, China) was added to each well and incubated for 2 hours. Relative viable cell numbers were evaluated by measuring absorbance at 450 nm using a microplate reader (Bio-Rad 680, Hercules, CA, USA).

Tumor colony formation assay

Cells (300 cells/well) were seeded in six-well plates and cultured in a 37°C 5% CO₂ incubator. The culture medium was replaced every 2 days. After 2 weeks, when colony formation became visible, the cells were washed twice with PBS and fixed with 4% paraformaldehyde (PFA) for 20 minutes, followed by staining with 0.5% crystal violet dye (Beyotime, C0121-100mL) for 15 minutes. The colonies were subsequently imaged and counted using a fluorescence inversion microscope (Olympus, Tokyo, Japan) and ImageJ software (version 1.52r).

Flow cytometry assay

For cell cycle analysis, cells were fixed with 70% alcohol at 4°C overnight. Then, RNase A (20 mg/mL final concentration) was used to degrade the RNA at 37°C for 20 minutes. Propidium iodide (PI) solution (10 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was used to stain the cellular DNA for 10 minutes in the dark. Cells were analyzed with a Cytomics FC 500 instrument (Beckman Coulter, Brea, CA, USA). For apoptosis assays, 1×10^6 cells were used for apoptotic staining using the PE-Annexin V Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA). After being washed twice with cold PBS, cells were harvested by trypsinization and incubated with 5 µL of PE Annexin V and 5 µL of PI solution for 15 minutes at room temperature. Cells were analyzed with a Cytomics FC 500 instrument (Beckman Coulter). Flow cytometry data were analyzed using FlowJo 7.6.1 software.

Western blot

RIPA buffer (Beyotime) was used to lyse cells and isolate total protein. Protein quantification was performed using a BCA Protein Assay Kit (Beyotime). Then, 10 µg protein was used for SDS-PAGE analysis. Separated proteins were transferred to a PVDF membrane (BioRad). The membrane was then incubated with primary antibodies against PIWIL2 (Proteintech, Rosemont, IL, USA, 14209-1-AP, 1:1500 dilution), GAPDH (Proteintech, 10494-1-AP, 1:1500 dilution), E-cadherin (Proteintech, 20874-1-AP, 1:1000 dilution), N-cadherin (Proteintech, 22018-1-AP, 1:1000 dilution), vimentin (Santa Cruz, Dallas, TX, USA, sc-6260, 1:1000 dilution), and MMP9 (Santa Cruz, sc-393859, 1:1000 dilution). Antibodies were also used against proliferating cell nuclear antigen (PCNA), cyclin D1, cyclin dependent kinase 2

(CDK2), p53, BCL-2, cleaved caspase-3, and cleaved caspase-9. The membrane was washed with TBST and incubated with an anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000, Proteintech, SA00001-2) at room temperature for 1 hour. ECL chemiluminescence reagent (Tanon, 180-5001, Shanghai, China) was used to detect immunoreactive proteins.

Transwell invasion assay

Cells (4×10^5) were cultured on a Matrigel-coated (BD Biosciences) 8-µm pore size membrane (Corning, Corning, NY, USA) in a transwell chamber. Then, 3% FBS RPMI medium was placed in the upper well and 10% FBS RPMI medium was placed in the lower well. After 24 hours, cells were scraped off the upper side, and cells on the lower side were fixed with 4% PFA in PBS solution for 15 minutes. Fixed cells were stained with crystal violet solution (Beyotime) and ten random fields were imaged for cell counting in each well.

Transwell migration assay

Cells (3×10^5) were cultured on an 8-µm pore size membrane (Corning) in a transwell chamber. Then, 3% FBS RPMI medium was placed in the upper well and 10% FBS RPMI medium was placed in the lower well. After 8 hours, cells were scraped off the upper side, and cells on the lower side were fixed with 4% PFA in PBS solution for 15 minutes. Fixed cells were stained with crystal violet solution (Beyotime) and ten random fields were imaged for cell counting in each well.

Wound healing test for migration assay

Cells were seeded in six-well plates and allowed to reach about 80% confluence. A scratch wound was created using a sterile 200-µL pipette tip in the central region of

each well. The cells were incubated at 37°C for 24 hours. Cell images were captured using an inverted light microscope. The migration distance was analyzed using ImageJ software. The migration rate was calculated as a ratio of wound distance at 24 hours to wound distance at 0 hours.

Statistics

A t-test, ANOVA, or chi-square test was used to determine statistical significance. Values are shown as the mean \pm standard deviation (SD). * $P < 0.05$, ** $P < 0.01$.

Results

PIWIL2 is upregulated and correlated with disease progress in OSCC patients

To determine the relationship between PIWIL2 expression and the clinical characteristics of OSCC patients, we collected OSCC cancer tissues and para-cancer tissues (para-carcinoma oral epithelial dysplasia) from 60 patients diagnosed with OSCC. Expression analysis with qPCR demonstrated that PIWIL2 mRNA levels were significantly increased in the carcinoma tissue samples (Figure 1a). From these expression results, we divided the patients into high and low expression groups using the PIWIL2 expression median value in all OSCC cancer tissues as the cutoff. The patients with high PIWIL2 expression had poor prognoses (Figure 1b). We further classified the patients into four different stages according to the TNM cancer staging system, then selected the samples from two patients with similar age, sex, and process of surgery in each of the four stages to analyze their PIWIL2 protein expression levels. With increasing malignant degree, there was a remarkable increase in PIWIL2 protein expression levels as detected by western blot analysis (Figure 1c). Therefore, the poor prognosis of those in the high

PIWIL2 expression group is likely from the cancer progressing to a more advanced stage. Furthermore, we further investigated PIWIL2 mRNA levels in different oral cancer cell lines (Tca8113, SCC9, SCC25, CAL27, HN12, HSU3, and FADU) and NHOK cells (Figure 1d). PIWIL2 mRNA expression was significantly higher in all oral cancer cell lines compared with the NHOK cells. The data of the associations between PIWIL2 expression and pathological characteristics are shown in Table 1. We found that the patients with high PIWIL2 expression were enriched for a poor differentiation status, while patients with low PIWIL2 expression were enriched for a high differentiation status (chi-square test, $P = 0.047$). Patients with high PIWIL2 expression also tended to develop more lymph node metastasis (chi-square test, $P = 0.034$) (Table 1). Collectively, our data show that PIWIL2 expression levels correlate with disease progression in OSCC patients.

PIWIL2 regulates the proliferation of Tca8113 cells

We next examined the functional role of PIWIL2 in Tca8113 OSCC cells. CCK8 assay analysis showed that TCA8113 cell proliferation was suppressed by PIWIL2 knockdown (Figure 2a-b). Colony formation assays revealed that silencing PIWIL2 inhibited the clone formation capacity of Tca8113 cells (Figure 2c). Furthermore, cell cycle progression was disrupted in cells with PIWIL2 knockdown, which showed a significant increased number of cells in the G0/G1-phase and a decreased number in the G2/M-phase (Figure 2d). We further analyzed the levels of cell cycle-related proteins by western blot analysis and found that PIWIL2 knockdown significantly decreased the protein levels of PCNA, cyclin D1, and CDK2, while p53 protein levels were upregulated (Figure 2e).

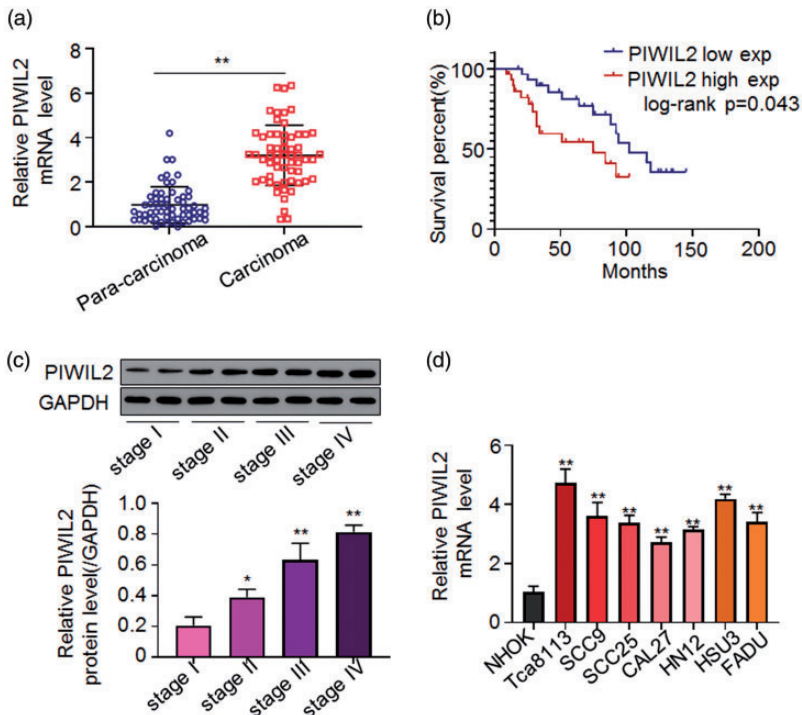


Figure 1. Piwi-like RNA-mediated gene silencing (PIWIL2) is upregulated and correlated with disease progression in oral squamous cell carcinoma (OSCC) patients. (a) qPCR detection indicated upregulated PIWIL2 mRNA in 60 OSCC cancer tissues when compared with the para-cancer tissues (oral epithelial dysplasia). (b) OSCC patients were divided into two groups according to the median level of PIWIL2 and their prognoses were followed. The Kaplan–Meier method was performed for cumulative survival rate analysis. Patients with high PIWIL2 expression levels showed poor prognosis. (c) Western blot assays showed a gradual increase of PIWIL2 protein expression levels with advancing tumor tissue malignant degree. Cancer tissues were selected from each of the four TNM malignancy groups to detect PIWIL2 protein expression. The bar graph shows the PIWIL2 protein levels normalized to GAPDH protein levels. (d) qPCR detection showed that PIWIL2 mRNA levels in different oral cancer-related cell lines (Tca8113, SCC9, SCC25, CAL27, HNI2, H5U3, and FADU) were significantly higher than in normal human oral keratinocyte (NHOK) cells. Values are presented as the mean \pm standard deviation (SD). * $P < 0.05$, ** $P < 0.01$ versus the corresponding control.

Overall, these data show that PIWIL2 is involved in cell cycle progression in Tca8113 OSCC cells.

PIWIL2 silencing promotes apoptosis in oral cancer cells

Western blot analysis showed that the tumor suppressor protein p53 was upregulated in the cells with PIWIL2 knockdown (Figure 2e). Since p53 can promote

apoptosis, we next performed apoptosis assays to examine whether PIWIL2 knockdown could lead to cell death in Tca8113 cells. Annexin V/PI staining analyzed using flow cytometry showed that PIWIL2 knockdown caused a significant increase in the percentage of apoptotic Tca8113 cells (Figure 3a). Western blot analysis further showed that the anti-apoptotic protein BCL-2 was downregulated and the levels of cleaved caspase-3 and cleaved caspase 9

Table 1. Correlation between Piwi-like RNA-mediated gene silencing (PIWIL2) expression levels and oral squamous cell carcinoma (OSCC) clinicopathological characteristics.

Clinicopathological characteristics	Total	High expression	Low expression	χ^2	P-value
Sex					
Male	37	20	17	3.300	0.059
Female	23	10	13		
Age, years					
≤50	27	16	11	1.684	0.150
>50	33	14	19		
Differentiation					
High	20	6	14	6.109	0.047
Moderate	18	9	9		
Poor	22	15	7		
Lymph node metastasis					
Positive	26	17	9	4.344	0.034
Negative	34	13	21		
TNM stage					
I	16	3	13	11.009	0.012
II	15	7	8		
III	13	8	5		
IV	16	12	4		

were elevated after silencing of PIWIL2 (Figure 3b). Therefore, PIWIL2 silencing can promote apoptosis in oral cancer cells.

PIWIL2 modulates oral cancer cell migration

We next investigated the potential role of PIWIL2 in oral cancer cell migration. Silencing PIWIL2 suppressed the migration ability of Tca8113 cells, as detected by wound healing assays. The migration rate was significantly decreased by 50% when compared with the control group (Figure 4a). Transwell (without Matrigel) assays also revealed inhibited cell migration with PIWIL2 knockdown (Figure 4b). Furthermore, we found that the cell invasion ability was repressed in Tca8113 cells with PIWIL2 knockdown (Figure 4c). The protein expression levels of cell migration and invasion-related genes, including N-cadherin, vimentin, and MMP9, were decreased, while the epithelial marker

E-cadherin was increased (Figure 4d). Therefore, our data show that PIWIL2 supports cell migration in oral cancer cells.

Discussion

Despite the high mortality rates from oral cancer worldwide, no effective biomarkers yet exist for early diagnosis or treatment. In this study, we showed a close correlation between high PIWIL2 expression and disease progression in OSCC patients. Using an OSCC cell line, we further demonstrated the critical roles of PIWIL2 in regulating oral cancer cell proliferation, apoptosis, migration, and invasion.

PIWIL2 is one member of the P-element-Induced Wimpy testis/Argonauts (PIWI/AGO) gene subfamily¹³ and has been implicated in regulating multiple biological processes, such as germ line stem cell self-renewal activation, cell cycle progression, epigenetic modulation, and cell migration.¹⁴ Additionally, PIWIL2 is reportedly

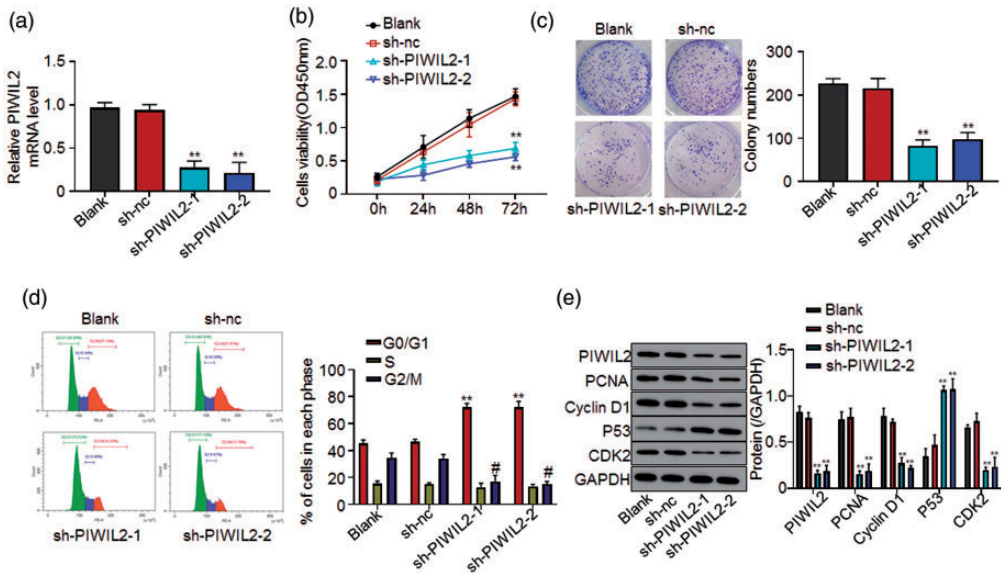


Figure 2. Piwi-like RNA-mediated gene silencing (PIWIL2) regulates the proliferation of the oral squamous cell carcinoma (OSCC) cell line TCA8113. (a) qPCR detection confirmed the effect of PIWIL2 knockdown using two PIWIL2-targeting short hairpin RNAs (shRNAs) in Tca8113 cells. (b) Cell Counting Kit-8 (CCK8) assays demonstrated repressed proliferation in Tca8113 cells with PIWIL2 knockdown. (c) Colony formation analysis showed inhibited cancer cell clone formation ability in Tca8113 cells with PIWIL2 knockdown. The statistics of the colony counts are shown in the right panel. (d) Flow cytometry analysis showed arrested cell cycle progression in Tca8113 cells following PIWIL2 knockdown. The cell population increased in the G0/G1-phase and decreased in the G2/M-phase. (e) Western blot analysis showed that PIWIL2 knockdown resulted in downregulated proliferating cell nuclear antigen (PCNA), cyclin D1, and cyclin dependent kinase 2 (CDK2) protein expression and upregulated p53 protein expression. The statistics of the protein levels are shown in right panel. Data are representative of three independent experiments. Values are presented as the mean \pm standard deviation (SD). # $P < 0.05$, ** $P < 0.01$ versus the corresponding control.

ubiquitously expressed in many tissues, including breast, colon, prostate, and gastrointestinal cancers, and plays important roles in many physiological processes.¹⁵⁻¹⁷

A potential pathological role of PIWIL2 during cancer stem cell development has been proposed in previous studies.¹⁴ The abovementioned studies suggest that PIWIL2 potentially has vital importance in the regulation of cell fitness and other pathological processes associated with the onset and progression of cancer. In our study, we found that high PIWIL2 expression correlated with disease progression in OSCC patients. PIWIL2 expression was

significantly higher in OSCC cancer tissues compared with para-cancer tissues. Moreover, higher levels of PIWIL2 in tumor tissues correlated with an advanced degree of malignancy, tendency for metastasis, and poor differentiation. Patients with low PIWIL2 expression tended to have well-differentiated carcinomas, while patients with high PIWIL2 expression were enriched for a poor differentiation status. Overall, dysregulated PIWIL2 expression is likely involved in the initiation and progression of OSCC, and can also affect the therapeutic outcome. PIWIL2 may also be used as a biomarker for earlier

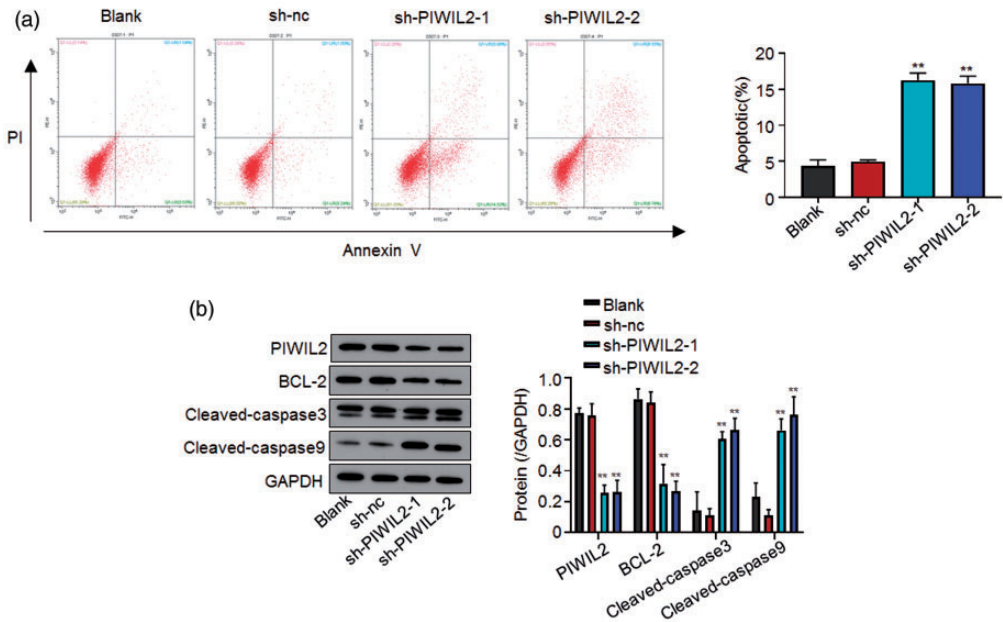


Figure 3. Piwi-like RNA-mediated gene silencing (PIWIL2) knockdown induces apoptosis in oral cancer cells. (a) Silencing PIWIL2 induced apoptosis in Tca8113 cells, as detected by Annexin V/propidium iodide (PI) staining. The statistics of the percentage of apoptotic cells are shown in the right panel. (b) The anti-apoptotic protein BCL-2 was downregulated, while cleaved caspase-3 and cleaved caspase-9 were activated, when PIWIL2 was silenced in Tca8113 cells. The statistics of the protein level quantification are shown in the right panel. Data are representative of three independent experiments. Values are presented as the mean \pm standard deviation (SD). ** $P < 0.01$ versus the corresponding control.

diagnosis of OSCC and the auxiliary reference index of cancer malignancy stage.

PIWIL2 can reportedly regulate numerous signaling pathways involved with the pathological processes of cancer. PIWIL2 can suppress the degradation of histone deacetylase 3 (HDAC3) and facilitate HDAC3 phosphorylation in cancer cells.¹⁸ PIWIL2 also promotes glioma cell proliferation and migration.⁹ PIWIL2 can promote cell cycle progression in non-small cell lung cancer cells by inducing the expression of CDK2 and cyclin A.¹⁹ Additionally, knockdown of PIWIL2 represses cell growth in liver cancer cells.²⁰ In our study, we found that PIWIL2 can regulate the proliferation, apoptosis, migration, and invasion of OSCC cells. Our data imply that PIWIL2

has an essential role in supporting proliferation and migration, as well as blocking apoptosis, in OSCC cells. These findings suggest that PIWIL2 acts as an oncogene in oral cancer tumorigenesis. Because it is involved in both proliferation and apoptosis inhibition in OSCC cells, PIWIL2 may be an important drug target for treating oral cancer. Inhibition of PIWIL2 expression or activity may be combined with existing chemotherapeutics to enhance the outcome of OSCC treatment.

Conclusions

The results of our study provide novel insights into the role of PIWIL2 dysregulation and its mechanism in OSCC

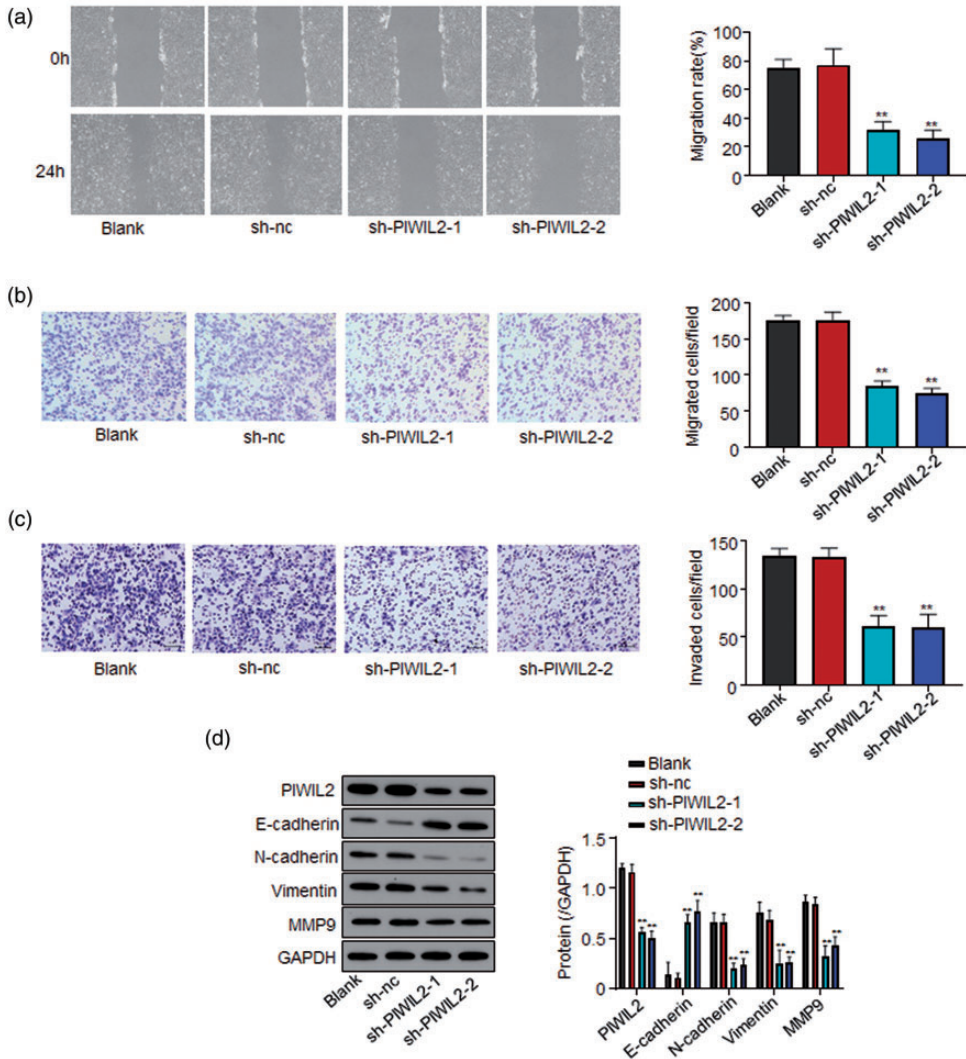


Figure 4. Piwi-like RNA-mediated gene silencing (PIWIL2) modulates oral cancer cell migration. (a) Wound healing assays showed that knockdown of PIWIL2 inhibited Tca8113 cell migration. The statistics of the migration rate experiments are shown in the right panel. (b) Transwell migration assays demonstrated inhibited migration of Tca8113 cells when PIWIL2 was silenced. The statistics of migrated cells per microscopical field of vision are shown in the right panel. (c) Transwell invasion assays revealed impaired invasion in Tca8113 cells with PIWIL2 knockdown. The statistics of invaded cells per microscopical field are shown in the right panel. (d) Western blot analyses showed that the protein expression levels of cell migration and invasion-related genes N-cadherin, vimentin, and matrix metalloproteinase 9 (MMP9) were downregulated, while epithelial gene E-cadherin was upregulated, following PIWIL2 knockdown. The bar graph shows the protein levels normalized to GAPDH protein levels. Data are representative of three independent experiments. Values are presented as the mean \pm standard deviation (SD). ** $P < 0.01$ versus the corresponding control.

pathogenesis. We showed the functional role of PIWIL2 in supporting oral cancer cell growth and migration, while silencing PIWIL2 could induce apoptosis in these cells. Our analysis of OSCC patient samples also revealed a close relationship between PIWIL2 expression levels and disease progression. Future work will focus on using animal models of oral cancer to validate the functional requirement of PIWIL2 in OSCC tumorigenesis.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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