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Original Article

Silencing Rab14 represses the proliferation and migration of oral squamous cell carcinoma, and enhances cisplatin sensitivity

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Abstract: Oral squamous cell carcinoma (OSCC) is a subtype of head and neck cancer with a relatively poor prognosis. The mechanisms underlying the initiation and progression of OSCC are complex and not yet fully understood; however, this information is critical for developing novel therapeutic targets and improving patient outcome. Rab14, a Ras related protein, has been implicated in multiple forms of cancer. In the present study, we confirmed that Rab14 is overexpression in human OSCC tissue, compared with normal oral mucosa samples. In addition, knockdown of Rab14 exerted potent anti-tumor effects by repressing the proliferation and migration of OSCC cell lines. Moreover, knockdown of Rab14 reduced the expression of Cyclin D1 and CXCR4, at the level of protein and mRNA, both *in vitro* and *in vivo*. Additionally, abrogation of Rab14 enhanced cisplatin sensitivity in OSCC cells *in vitro* and *in vivo*. Taken together, our data provides evidence for Rab14 as a potential therapeutic target in OSCC treatment.

Keywords: Oral squamous cell carcinoma, Rab14, proliferation, migration, chemotherapeutic sensitization

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common malignancies worldwide [1]. Over the last few decades, OSCC patients have benefited from advances in treatment options, such as radiotherapy, chemotherapy, surgery, and targeted therapy; however, overall survival remains limited [2]. In addition, disease recurrence often occurs in single-treatment strategies [3]. Therefore, a better understanding of the mechanisms responsible for this disease is critical to developing more efficacious treatments and increasing the survival rate of patients with OSCC.

A potential mechanism is the Rab family of proteins, which are involved in the regulation of membrane trafficking [4]. In recent years, the Rab family has also been implicated as key proteins in tumor progression, migration, and invasion [5-7]. Moreover, emerging evidence shows that Rab proteins play a pivotal role in the regulation of tumorigenic signaling [8, 9]. In addition, aberrant expression of Rab proteins has been shown to occur in multiple forms of cancer, including colon cancer and liver cancer [10, 11].

Of the Rab family proteins, only limited number of research studies have focused on the role of Rab14 in human cancers. In recent work, Rab14 was reported to be over-expressed in some human cancers, such as ovarian cancer and non-small cell lung cancer [12, 13]. In addition, Rab14 can promote proliferation by regulating the AKT signaling pathway, and inhibition of Rab14 has been shown to induce cell apoptosis and cell cycle arrest in gastric cancer [14]. However, the expression of Rab14 in OSCC, as well as whether it plays a role in the mechanism underlying the disease, remains unclear.

To address this question, in the present study, we performed immunohistochemistry on human OSCC tissue samples to determine Rab14 protein levels. In addition, we investigated the association between Rab14 and clinic-pathological parameters of OSCC, and examined the function of Rab14 in OSCC both *in vivo* and *in vitro*.

Material and methods

Ethics statement

All experiments of this study were approved by the Medical Ethics Committee of the People's Hospital of Zhenhai and performed in the Public Laboratory Platform of Medical School, Ningbo University.

Human OSCC samples

41 cases of human OSCC samples and 13 cases of normal oral mucosa tissues enrolled in this study were collected from the people's hospital of Zhenhai from 2014 to 2016. Consents were obtained from each patient before surgery. The histological diagnosis, pathological grade and TNM stage were estimated by pathologist according to the WHO classification guidelines.

Immunohistochemistry

Tissue samples were fixed by 4% paraformaldehyde and embedded in paraffin and made into 4 μm thick sections. Antigen retrieval was performed with citrate buffer (pH 6.0) with pressure cooker for 1.5 min. Next, sections were incubated with 0.3% hydrogen peroxide and then incubated with goat serum. SP staining kit (UltrasensitiveTM, Maixin, China) was used to perform immunohistochemical staining. Primary antibodies incubation was performed at 4°C over night. At day 2, sections were incubated with Biotinylated goat anti-rabbit or anti-mouse serum IgG, and followed incubated with HPR conjugated streptavidin-biotin. DAB kit (Maixin, China) was used for section staining. Hematoxylin was used for counterstaining.

RNA extraction and quantitative real-time PCR

Total RNA of cultured cells, nude mice tumor tissues was extracted according to the protocols of the manufacturer (TRIzol, TaKaRa, Japan). The first-strand cDNA was synthesized using 1 μg of total RNA by a Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Lithuania). Reverse transcription-polymerase chain reaction (RT-PCR) was performed by ViiA 7 Real-Time PCR System (Life Technology, USA) with the amplification conditions as follows: 1 cycle of 2 min at 95°C, then 40 cycles of 10 s at 95°C, 30 s at 60°C, and 30 s at 72°C. All experiments were performed in triplicate. The relative quantities of the RNA expression were calculated by $2^{-\Delta\Delta Ct}$ (cycle threshold) values. GAPDH was used as internal control. The primer sequences are listed in Supplementary Table.

Protein extraction and western blotting

Total protein of cultured cells and nude mice tumors was extracted by cell lysis buffer (Pierce, USA). Bradford method was used for protein quantification. 20 μg protein was added into SDS-PAGE and transferred to PVDF membrane (Millipore, USA). Incubation of primary antibodies was performed at 4°C overnight. At day 2, the membrane was incubated with HRP-coupled anti-rabbit IgG antibody (1:3000, CST, USA) at room temperature for 1 h. Protein on the membrane was visualized by ECL kit. β-actin was used as loading control.

Cell culture

Human oral squamous cell carcinoma (OSCC) cell lines SCC4 or Tca8113 were maintained in DMEM/F12 or RPMI-1640 medium (Hyclone) with 10% FBS (fetal bovines serum, Hyclone) at 37°C in a humidified atmosphere with 5% CO_2.

Rab14-silenced OSCC cell lines establishment

Two shRNA sequences (shRNA1 and shRNA2) targeting Rab14 and negative shRNA were designed and synthesized (Genepharma, China). To construct human Rab14 shRNA plasmids, the sequences were inserted into the BamHI/EcoRI restriction sites of pGLVU6/Purolentivectors. Next, lentiviral expression vectors and packaging plasmids were co-transduced into 293 T cells. Viral particles were collected, and then infected into SCC4 or Tca8113 cells. Puromycin (2 μg/ml, R&D system, USA) was used to select the infected cells.

CCK-8 proliferation assay

Cell proliferation assay was performed by Cell Counting Kit-8 (CCK8, Dojindo, Japan). 300/well SCC4 and Tca8113 cells transfected with control shRNA or Rab14 shRNA were seeded into 96 well plates for 5 days. Then cells were incubated with CCK-8 solution for 4 h. Plates were read and measured by a reader at the wavelength of 450 nm.

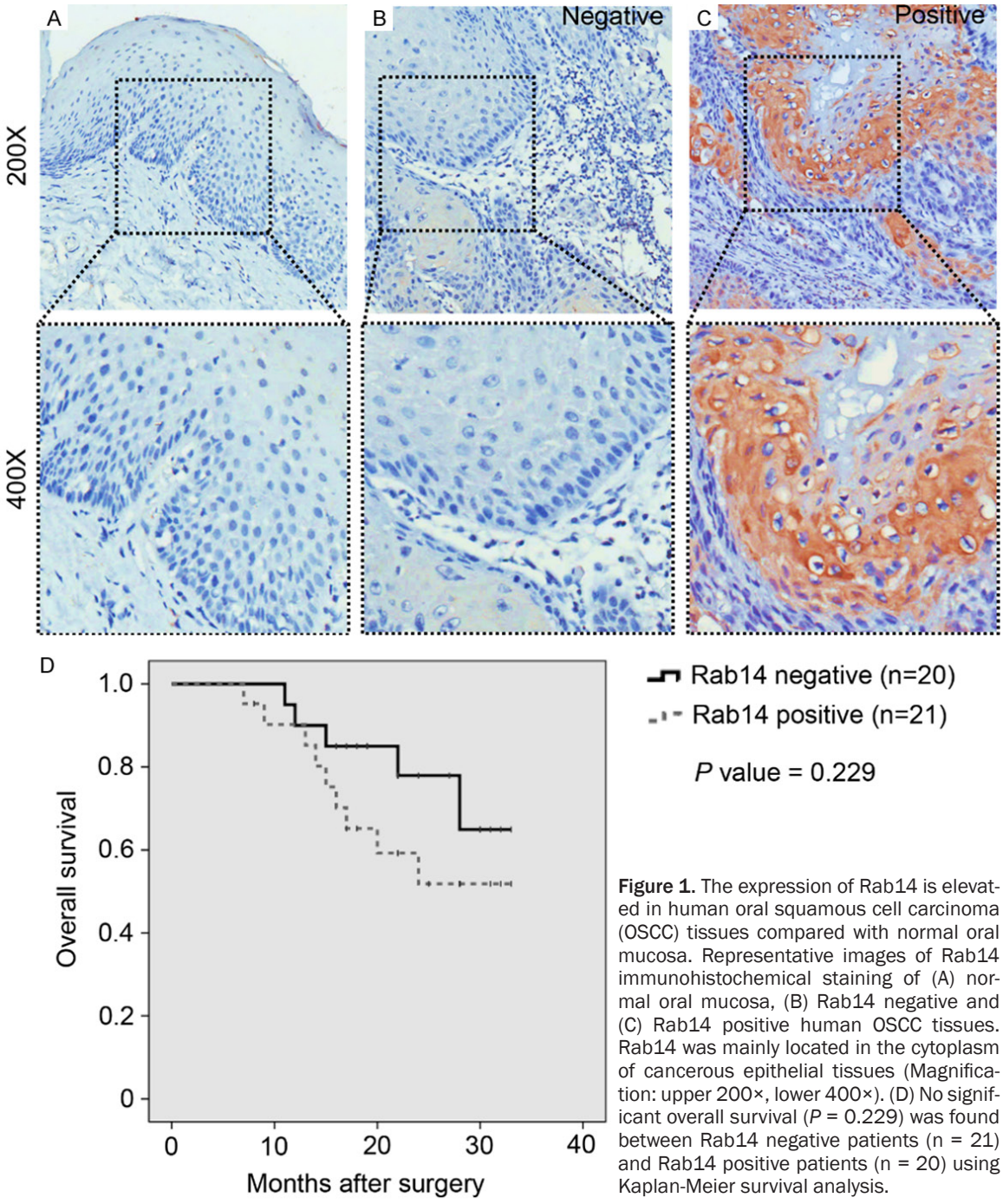

Figure 1. The expression of Rab14 is elevated in human oral squamous cell carcinoma (OSCC) tissues compared with normal oral mucosa. Representative images of Rab14 immunohistochemical staining of (A) normal oral mucosa, (B) Rab14 negative and (C) Rab14 positive human OSCC tissues. Rab14 was mainly located in the cytoplasm of cancerous epithelial tissues (Magnification: upper 200×, lower 400×). (D) No significant overall survival (P = 0.229) was found between Rab14 negative patients (n = 21) and Rab14 positive patients (n = 20) using Kaplan-Meier survival analysis.

Transwell migration assay

Migration assays were performed with Costar Transwell inserts with 8 μm pore size (Coring, USA). Briefly, SCC4 and Tca8113 cells transfected with control shRNA or Rab14 shRNA were seeded into the upper chamber at a density of 20000 cells per well with 100 μl FBS-free DMEM/F12 or RPMI-1640 medium. Medium with 10% FBS was added in the bottom chamber. After incubation at 37°C for 24 h, the cells in upper chamber were removed. Cells in bottom chamber were fixed and stained with 0.1% crystal violet (Sigma, USA). Migrated cells were photographed and counted.

In vivo studies

All animalsenrolled in this study were purchased from Hunan SJA Laboratory Animal Co. Ltd.

Table 1. The overexpression of Rab14 in human oral squamous cell carcinoma

		Rab14		*P* value
		Negative	Positive	
Tissue samples				
Mucosa	13	12	1	
OSCC	41	21	20	0.008

Table 2. Association between pathological parameters and Rab14 expression

		Rab14		*P* value
		Negative	Positive	
Pathological grade				
I	9	6	3	
II+III	32	15	17	0.294
Tumor Size				
T1+T2	35	18	17	
T3	6	3	3	0.948
Lymph node involvement				
Negative	27	12	15	
Positive	14	9	5	0.228

(China). Experiments were performed according to institutional guidelines. Briefly, female athymic BALB/c nude mice (18-20 g, 5-6 weeks old) were kept in sterile laminar flow cabinets under appropriate pathogen-free conditions. SCC4 cells transfected with control shRNA or Rab14 shRNA were inoculated into the left flank of the mice. The volumes of tumors were measured by a caliper and calculated with the formula (width^2 × length)/2. At the endpoint of the experiment, the mice were euthanized for tumors harvest. For cisplatin treatment, the tumor bearing mice were treated with 10 mg/kg cisplatin by intraperitoneal injection at Day 14. The volume of Tumors from each group (Vector, Vector+Cisplatin and shRab14+cisplatin) were calculated and analyzed.

Reagents and antibodies

Cisplatin was purchased from Selleck (USA). Antibodies used in this study were listed as follows: rabbit Rab14 (Abcam, UK, 1:200 for IHC, 1:1000 for WB), rabbit Ki-67 (Abcam, UK, 1:200 for IHC), rabbit Cyclin D1 (CST, USA, 1:1000 for WB), rabbit CXCR4 (Abcam, UK, 1:1000 for WB), mouse β-actin (Proteintech, USA, 1:2000 for WB).

Statistical analysis

Collected data were analyzed by Graphpad Prism 5.0 software (Graph Pad Software Inc, USA) and expressed as mean ± SEM of repeated experiments in triplicate. The significance of differences was estimated by Student's *t* test. $P<0.05$ were defined as a statistically significant difference. Kaplan-Meier analysis and Log-rank test was performed by SPSS 20.0 (SPSS Inc., USA).

Results

Overexpression of Rab14 in human OSCC tissues

To investigate the expression pattern of Rab14 in tissue samples from patients with OSCC, immunohistochemical staining was performed on human tissue samples representing 41 OSCC cases and 13 cases of normal oral mucosae. Representative images of normal oral mucosa, Rab14-negative OSCC, and Rab14-positive OSCC are displayed in **Figure 1A-C**, respectively. Rab14 was primarily identified in the cytoplasm of cancerous epithelial cells. We found that 48.8% (20/41) of OSCC samples were positive for Rab14, and that the expression of Rab14 was significantly up-regulated in human OSCC samples compared with normal oral mucosa ($p = 0.008$; **Table 1**).

Next, the association between Rab14 expression and clinic-pathological parameters of OSCC was assessed. As shown in **Figure 1D**, a Kaplan-Meier analysis and Log-rank test were used to investigate the relationship between Rab14 expression and overall survival. We found that overall survival was not significantly different in patients that were positive for Rab14, compared to patients who were negative for Rab14 expression ($p = 0.229$). In addition, further analysis showed that expression of Rab14 was not significantly correlated with pathological tumor grade ($p = 0.294$), tumor size ($p = 0.948$), or lymph node involvement ($p = 0.228$; **Table 2**). Taken together, these data suggest that Rab14 is significantly elevated in human OSCC samples, compared with normal oral mucosa, however it is unclear what role Rab14 plays in disease progression.

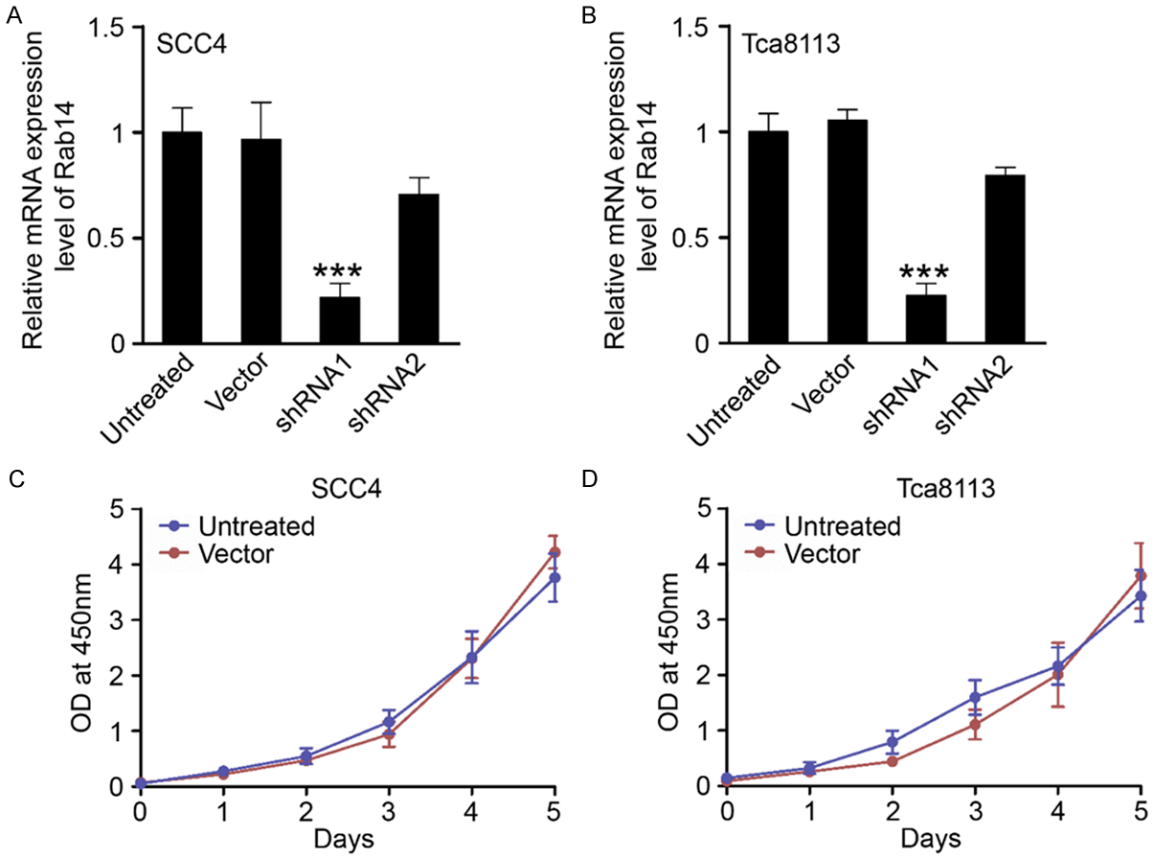

Figure 2. Establishment of Rab14-silenced OSCC cell lines. (A) SCC4 cells and (B) Tca8113 cells were transfected with control shRNA (vector) or Rab14 shRNA (shRNA1 and shRNA2). Real-time PCR indicated that shRNA1 could significantly decrease the mRNA level of Rab14 in both SCC4 and Tca8113 cells (Mean ± SEM, ***, $P<0.001$, *t* test). No extra cytotoxic effects were induced by control shRNA (vector) in (C) SCC4 cells and (D) Tca8113 cells using CCK-8 proliferation assays.

Establishment of Rab14-silenced OSCC cell lines

To develop Rab14-silenced OSCC cell lines, we transfected different cell lines with two shRNA sequences (shRNA1 and shRNA2). After the transfection of a negative control, shRNA1 and shRNA2, we found that shRNA1 could significantly inhibit levels of Rab14 mRNA in two oral OSCC cell lines: SCC4 and Tca8113 (**Figure 2A** and **2B**). Importantly, transfection in the negative control did not repress the proliferation of SCC4 and Tca8113 cells, indicating that no cytotoxic effects occurred (**Figure 2C** and **2D**).

Silencing Rab14 suppresses the proliferation and migration of OSCC cell lines

An essential oncogenic role of Rab14 has been reported in multiple studies. Therefore, we investigated the potential function(s) of Rab14 in OSCC cell lines. To this end, we performed CCK-8 cell proliferation assays to assess the role of Rab14 in cell growth. We found that cell proliferation was significantly inhibited in Rab14-silenced SCC4 and Tca8113 cells, compared to control cells (**Figure 3A** and **3B**). Furthermore, we assessed the impact of Rab14 knockdown on cell migration in OSCC cell lines, and found that silencing of Rab14 induced a significant inhibition in migration in SCC4 and Tca8113 cell lines (**Figure 3C** and **3D**). We next analyzed expression of Cyclin D1 and CXCR4 proteins in control cells and Rab14-silenced cells. Our results show that knockdown of Rab14 reduces the expression of Cyclin D1 and CXCR4 in SCC4 and Tca8113 cells (**Figure 3E**). Finally, real-time PCR assays indicated that knockdown of Rab14 decreases levels of Cyclin D1 and CXCR4 mRNA (**Figure 3F**). Taken together, these results indicate that Rab14 can regulate the proliferation and migration of OSCC cells.

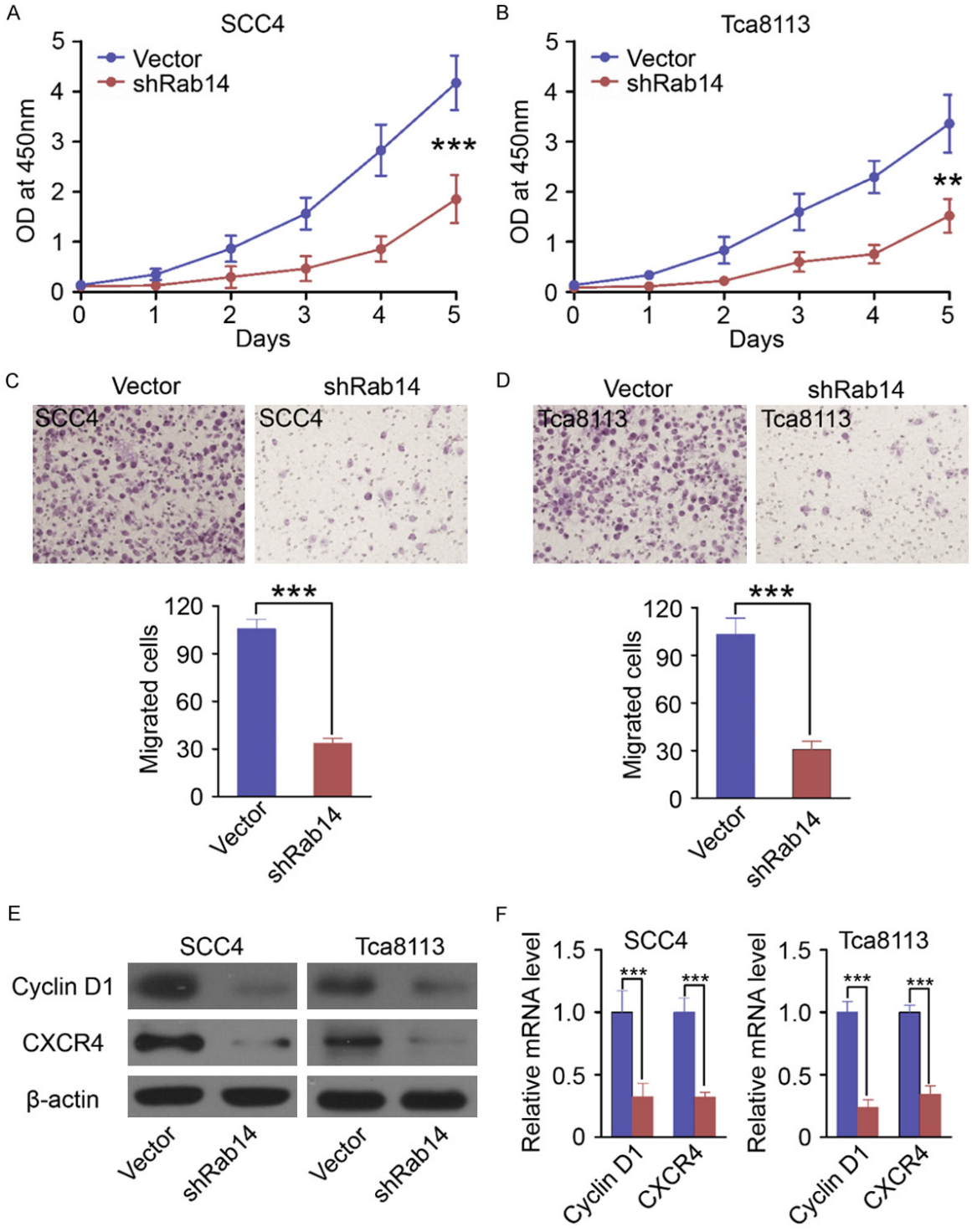

Figure 3. Silencing Rab14 suppresses the proliferation and migration of OSCC cell lines. Silencing Rab14 could induce significant repression of proliferation in (A) SCC4 and (B) Tca8113 cell lines using CCK-8 proliferation assays (Mean ± SEM, **, $P<0.01$, ***, $P<0.001$, *t* test). Transwell migration assays suggested that silencing Rab14 significantly induced inhibition of migration in (C) SCC4 and (D) Tca8113 cell lines (Mean ± SEM, ***, $P<0.001$, *t* test). (E) Western Blot showed knockdown of Rab14 downregulated the protein levels of Cyclin D1 and CXCR4 in SCC4 cells and Tca8113 cells. (F) Quantitative real-time PCR revealed that Rab14 silencing could significantly decrease the mRNA level of Cyclin D1 and CXCR4 in SCC4 cells and Tca8113 cells (Mean ± SEM, ***, $P<0.001$, t test).

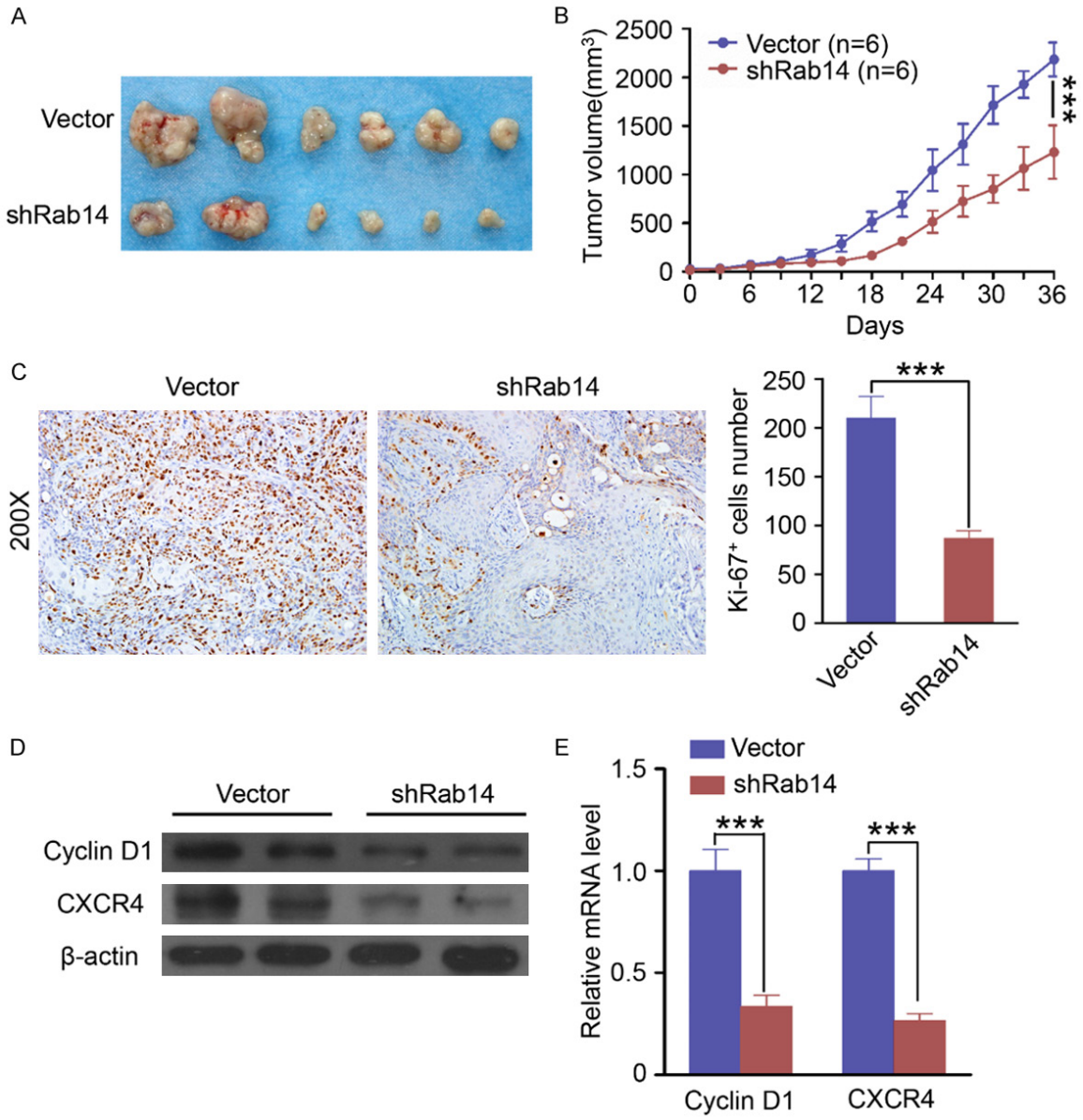

Figure 4. Rab14 silencing inhibits tumor proliferation and blunts tumor growth in vivo. A. The photos of tumors formed from SCC4 cells transfected with control shRNA (Vector, up panel) or Rab14 shRNA (down panel). B. Tumor growth curve of formed from SCC4 cells transfected with control shRNA (Vector, n = 6) or Rab14 shRNA (n = 6). Silencing Rab14 significantly repressed the tumor growth *in vivo* (Mean ± SEM, ***, $P<0.001$). C. Knockdown of Rab14 significantly decreased the Ki-67 positive tumor cells in mouse tumor tissue (Mean ± SEM, ***, $P<0.001$, *t* test, Magnification: 200×). D. Knockdown of Rab14 *in vivo* decreased the protein levels of Cyclin D1 and CXCR4 compared with control group (vector). E. Knockdown of Rab14 in vivo decreased the mRNA levels of Cyclin D1 and CXCR4 (Mean ± SEM, ***, $P<0.001$, *t* test) compared with control group (vector).

Effects of Rab14 silencing on the proliferation of OSCC cells in vivo

To explore whether Rab14 silencing effects-tumorigenesis *in vivo*, SCC4 cells were transfected with negative control shRNA or with Rab14 shRNA. Then, female nude mice (n = 6/group) were inoculated with the transfected cells. We found that tumors derived from Rab14 shRNA-transfected SCC4 cells grew significantly slower (**Figure 4A** and **4B**) than the negative controls. At the end of the experiment, tumor tissue from each group was harvested for further study.

Analysis of Ki-67 expression with immunostaining was performed in resected tumor tissues. The number of Ki-67 positive cells in tumors

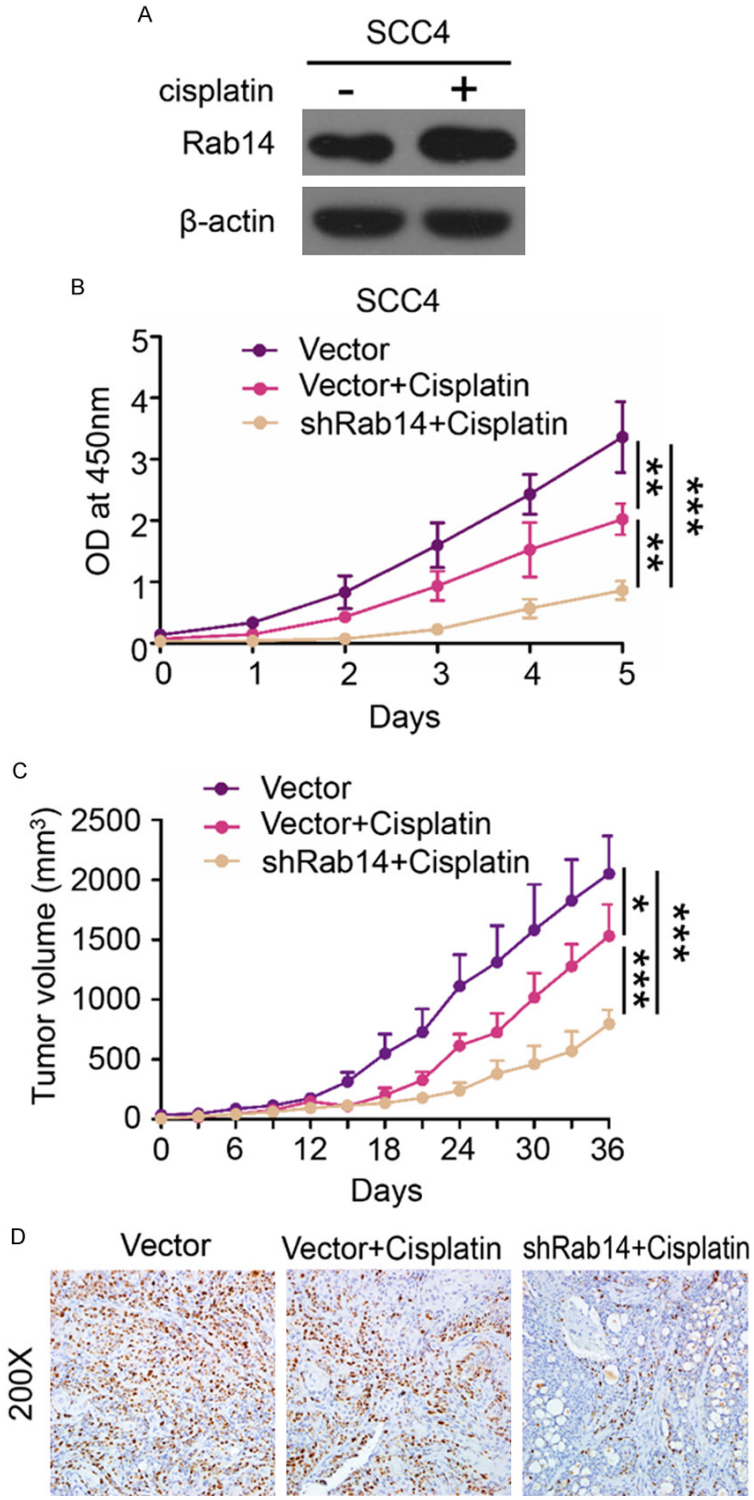

Figure 5. Rab14 silencing could enhance the cisplatin sensitivity in OSCC cells. A. Western Blot suggested ciplatin (20 μM for 24 h) treatment increased the protein level of Rab14 in SCC4 cells. B. CCK-8 proliferation assay indicated that Rab14 silencing significantly enhances the cell growth inhibition effects caused by cisplatin (Mean ± SEM, **, $P<0.01$, ***, $P<0.001$). C. The tumor growth curve of control group (Vector), Vector+Cisplatin and shRab14+Cisplatin. Rab14 silencing enhances the sensitivity of cisplatin *in vivo* (Mean ± SEM, *, $P<0.05$, ***, $P<0.001$). D. Representative images of Ki-67 immunohistochemical staining in control group (Vector), Vector+Cisplatin group and shRab14+Cisplatin group. (Magnification: 200×). ShRab14+Cisplatin mouse tumor tissue displayed less Ki-67 positive tumor cells compared with other group.

that developed from Rab14 shRNA-transfected cells was significantly lower than tumors from the negative control group (**Figure 4C**). Furthermore, we found that Rab14 silencing redu-ced the protein and mRNA levels of both Cyclin D1 and CXCR4 *in vivo* (**Figure 4D** and **4E**). These results suggest that Rab14 expression is significantly associated with the *in vivo* proliferation of OSCC cells.

Rab14 silencing enhances cisplatin sensitivity in OSCC cells

To determine if Rab14 silencing influences the sensitivity of OSCC cells to the anti-cancer drug, cisplatin, SCC4 cells were treated with 20 μM cisplatin for 24 h. We found that cisplatin treatment increased the level of Rab14 protein in SCC4 cells, suggesting a potential role of Rab14 in cisplatin resistance (**Figure 5A**). Additionally, SCC4 cells were transfected with negative control shRNA or Rab14 shRNA and treated with 20 μM cisplatin for 24 h. Then, a CCK-8 proliferation assay was performed to investigate the effect of Rab14 silencing on cisplatin sensitivity in OSCC cells. Our results revealed that cisplatin treatment was more effective on Rab14-silenced cells than on control cells (**Figure 5B**).

To investigate cisplatin sensitivity *in vivo*, female nude mice (n = 6/group) were inoculated with SCC4 cells transfected wi-

th either negative control shRNA and Rab14 shRNA. On day 14, mice received 10 mg/kg cisplatin treatment by intraperitoneal injection. As shown in **Figure 5C**, Rab silencing significantly enhanced the sensitivity of cisplatin *in vivo*. Furthermore, immunostaining of Ki-67 in the tumors from each group indicated that Rab14 knockdown significantly repressed the proliferation of SCC4 cells *in vivo* (**Figure 5D**). Taken together, our results demonstrate that Rab14 silencing enhances cisplatin sensitivity in OSCC cells both *in vitro* and *in vivo*.

Discussion

Recent studies have provided evidence for a close relationship between the Rab family of proteins and tumorigenesis in human cancers [15, 16]. Importantly, Rab proteins may exert diverse functions in different cancers [17, 18], and dysregulated expression of Rab proteins was reported in multiple forms of cancer [19, 20]. Therefore, determining the function of Rab proteins and whether they play a role in cancer progression is critical for the development of novel therapeutic strategies.

We chose to focus on Rab14, as a recent study reported high levels of Rab14 mRNA in gastric cancer tissues [14], and elevated levels of Rab14 protein have been observed in human ovarian cancer [12]. In this study, using immunohistochemistry we determined that Rab14 expression is up-regulated in human OSCC samples. In ovarian cancer, overexpression of Rab14 has been associated with the International Federation of Gynecology and Obstetrics (FIGO) stage, indicating a potential function of Rab14 in tumor progression [12]. In addition, Rab14 overexpression has been correlated with poor outcome in breast cancer [21]. However, in our study, we found that expression of Rab14 was not significantly related with prognosis, pathological grade, tumor size, or involvement of the lymph nodes. This discrepancy may be due to a small sample size and short follow-up period. Therefore, a future study should be conducted with a larger number of samples and a longer follow-up, to resolve this question.

Next, we carried out functional assays to evaluate the function of Rab14 in OSCC cell lines. Our *in vitro* study revealed that knockdown of Rab14 inhibits the proliferation and migration of OSCC and downregulates Cyclin D1 and CXCR4 protein. This may have important implications in disease progression, as Cyclin D1 plays a vital role in uncontrolled proliferation [22], and high expression of CXCR4 promotes tumor cell metastasis [23, 24]. The results of our *in vivo* study confirmed a function of Rab14 in proliferation, as we found that knockdown of Rab14 suppressed tumor growth and decreased Ki-67 expression. These results are consistent with previous studies, which have shown that Rab14 can regulate proliferation and migration in multiple forms of cancer. For example, in ovarian cancer, depletion of Rab14 inhibits its cell proliferation by decreasing Cyclin E and upregulating p21, through a Wnt signaling-dependent mechanism [12]. Moreover, in gastric cancer, Rab14 promotes cell proliferation by activating the Ser473 site of Akt [14]. In non-small cell lung carcinoma, ectopic miR-338-3p expression has been shown to regulate proliferation partially through downregulation of Rab-14 [13]. Finally, miR-320a has been suggested to modulate cell proliferation, migration, and invasion by targeting Rab14 [21].

Resistance to chemotherapeutic drugs reduces the efficacy of chemotherapy, resulting in unsuccessful treatment [25, 26]. In recent years, several of the mechanisms underlying drug resistance have been identified [27-29]. Here, we investigated a role of Rab14 in the resistance of OSCC to the chemotherapeutic drug, cisplatin. We found that levels of Rab14 protein were increased after cisplatin treatment. Moreover, the results of our CCK-8 assay and *in vivo* study showed that Rab14 knockdown enhances the cytotoxic effects of cisplatin. Interestingly, in renal cancer, miR-148a has been shown to enhance cisplatin sensitivity by negatively regulating Rab14 [30] and, in nasopharyngeal carcinoma, miR-451 inhibits Rab14 expression and increases sensitivity to radiotherapy [31]. Taken together with the literature, our results indicate that Rab14 may play a crucial role during chemotherapy or radiotherapy.

In summary, our data showed overexpression of Rab14 in human OSCC tissue, compared with normal oral mucosa samples. The association between Rab14 and clinic-pathological parameters was also analyzed. Both our *in vitro* and *in vivo* studies showed that knockdown

of Rab14 can suppress the proliferation and migration of OSCC cell lines. However, interestingly, silencing Rab14 enhances sensitivity to cisplatin. Together, our data provides evidence for a role of Rab14 in OSCC, and highlights Rab14 as a potential target for OSCC treatment.

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Disclosure of conflict of interest

None.

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Supplemental Table. Primer sequences in this study

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
Rab14	CATGGCAACTGCACCATACAAC	GCAAGATTTTCCTACTCCCATGTC
Cyclin D1	GCTGCGAAGTGGAAACCATC	CCTCCTTCTGCACACATTTGAA
CXCR4	AGACCACAGTCATCCTCATCCT	GTTCTCAAACTCACACCCTTGC