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MiR-4513 mediates the proliferation and apoptosis of oral squamous cell carcinoma cells *via* targeting CXCL17

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Abstract. – OBJECTIVE: Emerging evidence has indicated that microRNAs (miRNAs) play crucial roles in regulating cancer carcinogenesis; however, its role in oral squamous cell carcinoma (OSCC) remains largely unknown. Our work was aimed to investigate the role of miR-4513 in regulating OSCC cells behaviors.

MATERIALS AND METHODS: MiR-4513 expression in OSCC cells was analyzed by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Cell proliferation, migration, invasion, and apoptosis were analyzed by the cell counting kit-8 (CCK-8) assay, wound-healing assay, transwell invasion assay, and flow cytometry, respectively. The connections of miR-4513 and CXC ligand 17 (CXCL17) were analyzed by luciferase reporter assay and Western blot assay.

RESULTS: MiR-4513 expression was found elevated in the OSCC cell lines. The downregulation of miR-4513 expression inhibits cell proliferation, migration, invasion and, at the same time, promotes apoptosis. Furthermore, we validated CXCL17 as a direct target of miR-4513. Knocking down the expression of CXCL17, inhibited the effects of miR-4513 on OSCC cell behaviors.

CONCLUSIONS: Our results suggested the oncogenic role of miR-4513 in OSCC, and therefore it might be used as a target for the OSCC treatment.

Key Words
MiR-4513, CXCL17, Oral squamous cell carcinoma, Cell behaviors, Oncogene.

Introduction

Lip and oral cavity cancer are highly frequent in Southern Asia and the Pacific Islands[1]. Oral squamous cell carcinoma (OSCC) accounts for about 90% of all oral cancers[2]. The survival quality of OSCC patients is poor due to the need of partial tongue removal, which will result in severe oral dysfunctions[3]. Unfortunately, we still did not fully understand the mechanisms responsible for OSCC progression. MicroRNAs (miRNAs) are non-coding RNAs to exert their functions via binding to the 3'-untranslated regions (3'-UTR) of targeted message RNA[4]. In the past decades, the importance of miRNAs in regulating biological processes including cell growth, invasion, and metastasis has been appreciated[5,6]. Multiple miRNAs have been characterized as cancer-related genes and their dysregulation was found in multiple tumors, including OSCC[7,8]. Furthermore, some studies[9] have suggested that miRNAs have dual roles in the progression of human cancers, i.e., oncogenic and tumor suppressive role[9]. Previous studies[10-13] have indicated that a large sum of miRNAs was abnormally expressed in OSCC such as miR-182-5p, miR-16, miR-21, and miR-155-5p[10-13]. MiR-4513 has been reported to be upregulated in lung adenocarcinoma and its overexpression significantly increased the drug resistance to gefitinib[14]. However, whether or not miR-4513 has a role in OSCC carcinogenesis remains unknown. CXC ligand 17 (CXCL17) is a 119 amino acid whose expression was reported to be overexpressed in colon cancer cells[15]. CXCL17 could recruit immune cells to the tumor site and be used as an indicator for poor prognosis of colon cancer patients[15]. Moreover, it was demonstrated that CXCL17 was involved in the antitumor immune response at pancreatic carcinogenesis process through triggering dendritic cells accumulation to enhance the response of tumor cells to cytolysis[16]. Moreover, low CXCL17 expression was found as a poor overall survival and recurrence-free survival predictor in hepatocellular carcinoma[17].

Here, we investigated the expression levels of miR-4513 in the OSCC cell lines and explored the

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biological functions in the development of OSCC. We also detected the potential connection between miR-4513 and CXCL17. Moreover, we investigated whether miR-4513 regulated the OSCC cell events through targeting CXCL17.

Materials and Methods

Cell Line Culture

Human normal oral epithelial keratinocytes (hNOK) and OSCC cell lines (Tca8113 and CAL-27) were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). These cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin at a 37°C humidified incubator containing 5% of CO_2.

Cell Transfection

MiR-4513 inhibitor and negative control (miR-NC) were designed by GenePharma (Shanghai, China). Small interfering RNA targeting CXCL17 (si-CXCL17) and siR-NC were also designed by GenePharma (Shanghai, China). Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the supplier's instructions with the final concentration of miRNAs at 100 nmol/l, and siRNAs at 20 nmol/l.

Dual-Luciferase Activity Reporter Assay

The bioinformatical analysis showed CXCL17 was a putative target of miR-4513. The wild type 3'-untranslated region (3'-UTR) CXCL17 cloned into a pMIR vector (Promega, Madison, WI, USA) was named as pMIR-CXCL17-wt. The mutated CXCL17 3'-UTR vector was named as pMIR-CXCL17-mt. Cells were co-transfected with miR-4513 inhibitor or miR-NC and pMIR-CXCL17-wt or pMIR-CXCL17-mt using Lipofectamine 2000. After 36 h of transfection, cells were lysed to measure relative luciferase activity using the Dual-luciferase activity reporter system (Promega, Madison, WI, USA).

RNA Extraction and Quantitative Real Time PCR (qRT-PCR)

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The first strand complementary DNA was synthesized by PrimeScript miRNA cDNA synthesis kit (Takara, Dalian, China). MiR-4513 expression levels were analyzed by qRT-PCR at ABI 7500 system (Applied Biosystems, Foster City, CA, USA) using the SYBR Green Mix (TaKaRa, Otsu, Shiga, Japan) based on the manufacturer's instructions. The relative gene expression level was measured using the $2^{-\Delta\Delta Ct}$ method with U6 snRNA as internal control. The primers were as follows: miR-4513: forward, 5'-ACACTCCAGCTGGGAGACTGACGGCTGGAG-3', reverse, 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATGGGC-3'; U6 snRNA: forward, 5'-CTCGCTTCGGCAGCACA-3, reverse, 5'-ACGCTTCACGAATTTGCGT-3'.

Protein Isolation and Western Blot

Cells were lysed using Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Haimen, China) to extract total proteins according to the manufacturer's protocols. These extracted samples were separated at 10% of sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF; Millipore, Billerica, MA, USA) membranes. Then, membranes were incubated with appropriate antibodies (anti-CXCL17: MAB4207, R&D Systems, Shanghai, China; anti-GAPDH: ab8245, Abcam, Cambridge, MA, USA) for overnight at 4 °C after blocked with fat-free milk. Next, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (ab6789, Abcam) for 4 h at room temperature. The BeyoECL kit (Beyotime, Shanghai, China) was employed to visualize the protein bands.

Cell Counting Kit-8 (CCK-8) Assay

1×10^4 cells were seeded into 96-well plates and further incubated for 6 h. Subsequently, 10 μl Cell Counting Kit-8 (CCK-8) Assay (Beyotime, Shanghai, China) was added to each well and further incubated for 2 h. A microplate reader (Molecular Devices, San Jose, CA, USA) was used to measure optical density at 450 nm.

Wound-Healing Assay

5×10^5 cells were seeded to 6-well plates and incubated to about 90% confluence. A 10 μl pipette tip was used to generate a scratch at the cell surface. Cells were washed three times with phosphate-buffer saline (PBS) to remove cell debris. Cell images were captured under a microscope (Olympus, Tokyo, Japan) at 0 and 24 h.

Transwell Invasion Assay

1×10^5 cells in serum-free medium were seeded into pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) upper chamber. The lower chamber was filled with DMEM containing FBS. After incubation for 48 h, the invasion cells were fixed with methanol and stained with 0.1% of crystal violet. Invasion cell numbers were counted under a microscope.

Flow Cytometry Assay

Cells were harvested and incubated with Propidium iodide (PI) and fluorescein isothiocyanate (FITC)-labeled Annexin V mixture (Beyotime, Shanghai, China) at room temperature for 30 min. Cell apoptosis was measured at BD FACSCalibur flow cytometry (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Statistical Analysis

Data were presented as mean ± standard deviation (SD) after analyzing at SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Differences in 2 groups were analyzed using two-tailed Student's *t*-tests, whereas differences in three or above groups were analyzed with Analysis of Variance (ANOVA) and Tukey post-hoc test. *p*-value less than 0.05 was considered statistically significant.

Results

MiR-4513 Expression in OSCC Cell Lines

To explore the role of miR-4513 in OSCC, we first examined miR-4513 expression in OSCC cell lines by qRT-PCR. It was found miR-4513 levels were significantly upregulated in OSCC cell lines compared with normal cell line (Figure 1).

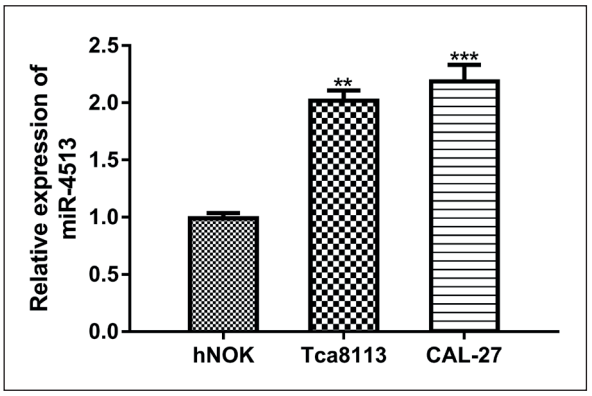

Figure 1. MiR-4513 expression was upregulated in OSCC cell lines (Tca8113 and CAL-27) compared with normal oral epithelial keratinocytes (hNOK). (**$p<0.01$) miR-4513: microRNA-4513; OSCC: oral squamous cell carcinoma.

MiR-4513 Regulates OSCC Cell Proliferation, Migration, Invasion, And Apoptosis

We then investigated the *in vitro* effects of miR-4513 expression on OSCC, miR-4513 inhibitor, and miR-NC were used to regulate miR-4513 expression level. As shown in Figure 2A, the introduction of miR-4513 inhibitor significantly reduced the levels of miR-4513 compared with miR-NC. The CCK-8 assay revealed that downregulation of miR-4513 inhibited the proliferation of Tca8113 and CAL-27 cell lines (Figure 2B). A significant inhibition on cell migration was observed in cells with miR-4513 inhibitor transfection (Figure 2C). Transwell invasion assay showed that the knockdown of miR-4513 resulted in a decrease in the cell invasion (Figure 2D). Furthermore, we analyzed the effect of miR-4513 on cell apoptosis. The inhibition of miR-4513 expression led to an increase in the cell apoptosis rate (Figure 2E). Collectively, these results indicated that the knockdown of miR-4513 inhibited OSCC cell proliferation, migration, invasion, and promoted cell apoptosis.

CXCL17 Was A Direct Target of MiR-4513

To explain how miR-4513 regulated OSCC progression, TargetScan, and miRDB databases were used to identify potential targets of miR-4513. Prediction results revealed that CXCL17 was a potential miR-4513 target (Figure 3A). Luciferase activity reporter assay revealed that miR-4513 inhibitor transfection significantly enhanced the luciferase activity of cells transfected with pMIR-CXCL17-wt but not pMIR-CXCL17-mt (Figure 3B). Besides, Western blot was conducted to analyze the CXCL17 expression in cells transfected with synthetic miRNAs. As shown in Figure 3C, the CXCL17 expression was markedly promoted by miR-4513 inhibitor. These results indicated that CXCL17 was a direct target of miR-4513.

MiR-4513 Regulates OSCC Cell Events Through Targeting CXCL17

To explore whether CXCL17 can offset the effects of miR-4513 in OSCC cell events, we co-transfected si-CXCL17 and miR-4513 inhibitor into the OSCC cell lines. Figure 4A showed that si-CXCL17 introduction markedly decreased CXCL17 levels. Figure 4B indicated that knockdown of CXCL17

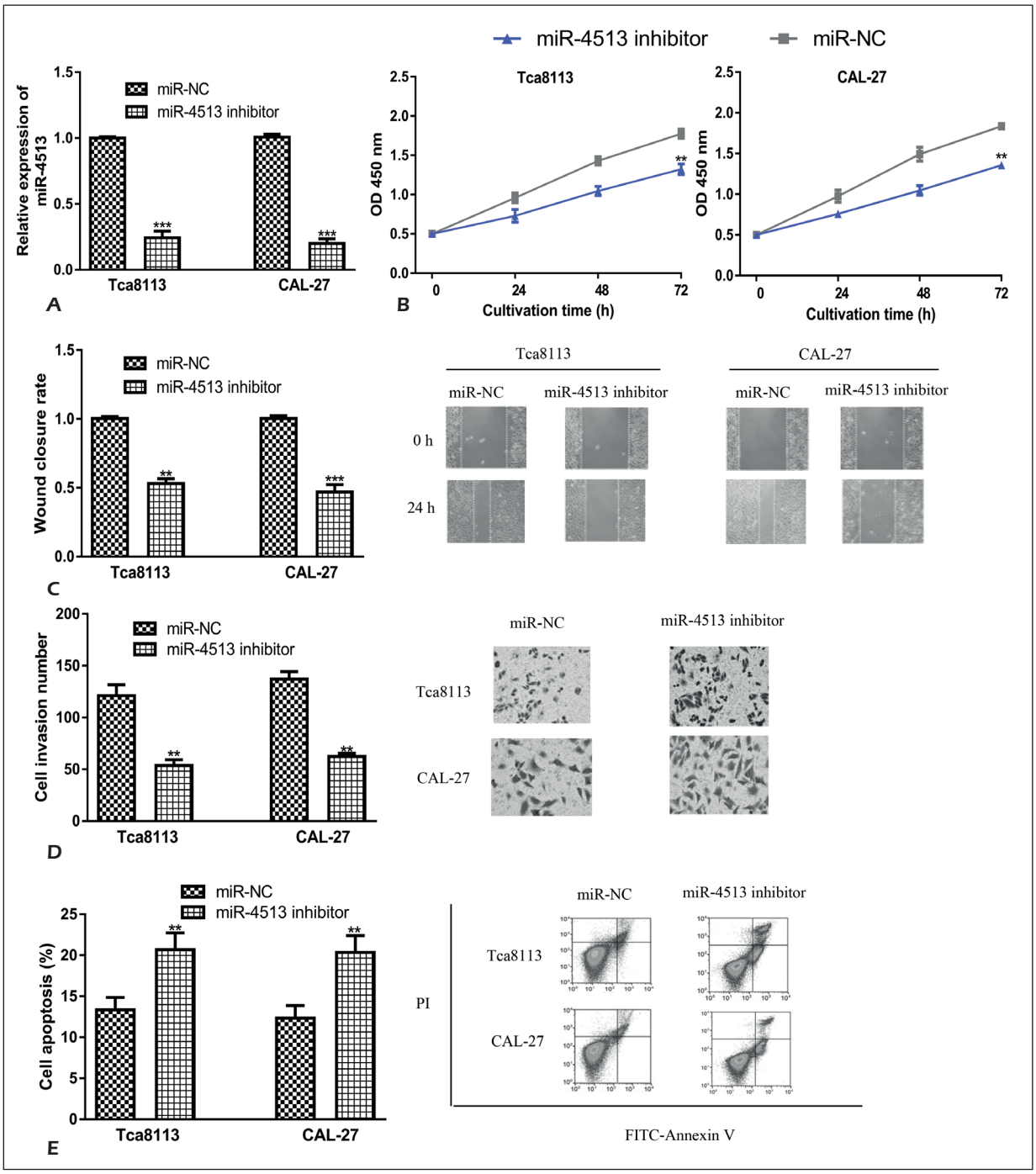

Figure 2. MiR-4513 could regulate OSCC cell proliferation, migration, invasion, and apoptosis (Magnification: 100 x). **A**, MiR-4513 expression, **B**, Cell proliferation, **C**, Cell migration, **D**, Cell invasion, and E, Cell apoptosis in OSCC cell lines transfected with miR-4513 inhibitor or miR-NC. (**p<0.01, ***p<0.001) miR-4513: microRNA-4513; OSCC: oral squamous cell carcinoma; miR-NC: negative control miRNA.

increased the cell proliferation. We further sought to investigate the effect of CXCL17 on cell migration and invasion. In contrast to siR-NC group, the migration and invasion ability of OSCC cell lines with si-CXCL17 transfection was promoted (Figure 4C and 4D). Lastly, we found that the knockdown of CXCL17 inhibited OSCC cell apoptosis (Figure 4E). Moreover, we showed that si-CXCL17 transfection partially reversed the effects of miR-4513 on cell events (Figure 4B-4E).

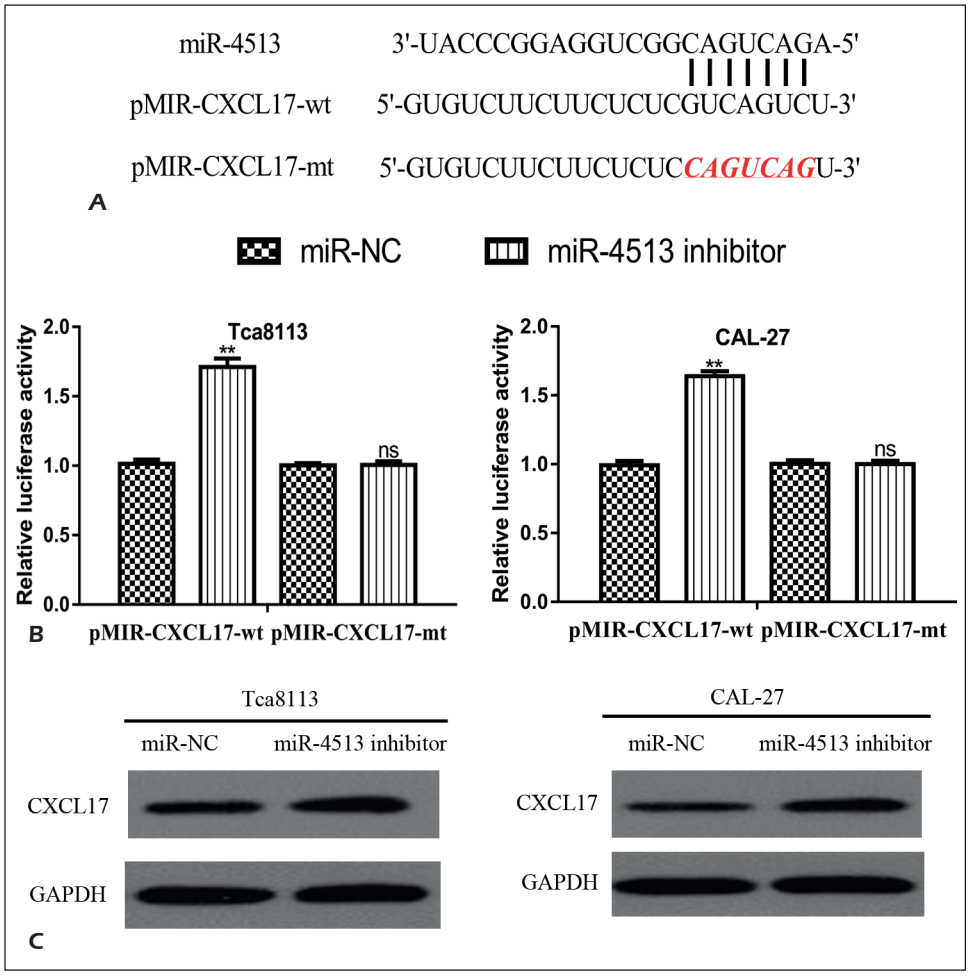

Figure 3. CXCL17 was a direct target of miR-4513. A, Binding site between miR-4513 and the 3'-UTR of CXCL17. B, Relative luciferase activity in cells transfected with pMIR-CXCL17-wt or pMIR-CXCL17-mt and miR-4513 inhibitor or miR-NC. C, CXCL17 expression in OSCC cells transfected with miR-4513 inhibitor or miR-NC. (ns not significant, $^{**}p<0.01$) miR-4513: microRNA-4513; OSCC: oral squamous cell carcinoma; miR-NC: negative control miRNA; UTR: untranslated region; wt: wild-type; mt: mutant; CXCL17: CXC ligand 17.

Discussion

Several studies[10-13,18] have illustrated that miRNA dysregulation is crucial for OSCC tumorigenesis. For instance, miR-182-5p expression was found elevated in OSCC, and the overexpression of miR-182-5p promoted cell growth and proliferation through directly targeted CAMK2N1 and inhibited the activation of AKT, ERK1/2, and NF-κB[10]. Hence, investigating the significance of miRNAs along with the possible mechanisms will help us to develop novel gene therapy methods in cancer. Even though no miRNA-based targeted therapy method exists to date due to insufficient transfection efficiency or high toxicity, researchers have made enormous efforts to explore novel delivery systems[19,20].

In this work, we found that the miR-4513 expression level was significantly elevated in OSCC cell lines by analyzing miR-4513 levels in OSCC cell lines and normal cell lines by qRT-PCR. Subsequently, we conducted a series of *in vitro* experiments to explore the biological functions of miR-4513 in the progression of OSCC. It was found that the knockdown of miR-4513 expression inhibited OSCC cell proliferation, migration, invasion and, at the same, time promoted cell apoptosis compared to miR-NC group. These results indicated that miR-4513 functions as an oncogene in the carcinogenesis of OSCC.

It has been recognized that miRNA participated mainly in the gene expression regulation by 3'-UTR binding and thus led to translational repression or mRNA degradation[21-24]. Therefore, we

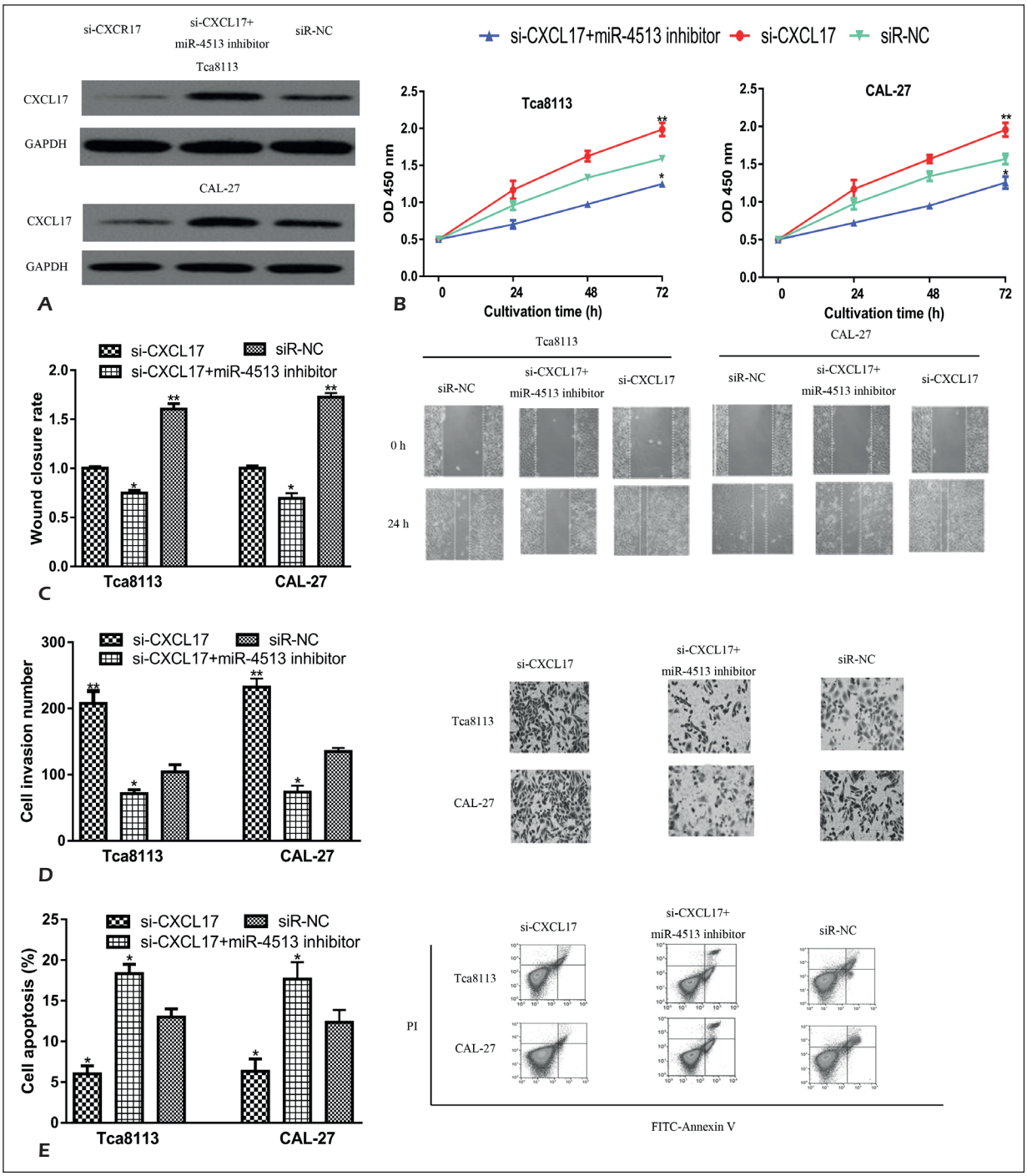

Figure 4. MiR-4513 regulates OSCC cell proliferation, migration, invasion, and apoptosis through targeting CXCL17 (Magnification: 200 x). **A**, CXCL17 expression, **B**, Cell proliferation, **C**, Cell migration, **D**, Cell invasion, and **E**, Cell apoptosis in OSCC cell lines transfected with si-CXCL17, siR-NC, or si-CXCL17 and miR-4513 inhibitor. (*p<0.05, **p<0.01) miR-4513: microRNA-4513; OSCC: oral squamous cell carcinoma; CXCL17: CXC ligand 17; si-CXCL17: small interfering RNA targeting CXCL17; siR-NC: negative control siRNA.

suspected that miR-4513 also affected OSCC cell proliferation, migration, invasion and apoptosis through targeting downstream genes expression. The putative targets of miR-4513 were predicted in TargetScan and miRDB databases. These analysis results demonstrated that CXCL17 is a potential miR-4513 target. Luciferase activity reporter assay and Western blot assay were conducted to

verify this prediction. To further observe whether CXCL17 represented a credible downstream mediator of miR-4513 in regulating OSCC cell proliferation, migration, invasion, and apoptosis, we further co-transferred si-CXCL17 and miR-4513 inhibitor into the OSCC cell lines. Rescue investigations revealed that si-CXCL17 transfection partially reversed the effects of the miR-4513 inhibitor on CXCL17 expression, cell proliferation, cell migration, cell invasion, and cell apoptosis. These results indicated that miR-4513 regulates OSCC cell events through targeting the expression of CXCL17.

Conclusions

Taken together, we discovered a new target for miR-4513 to regulate OSCC cell behaviors. The knockdown of miR-4513 inhibits OSCC cell proliferation, migration, invasion, and apoptosis. Importantly, we found that the knockdown of CXCL17 partially reversed the effects of the miR-4513 inhibitor on OSCC cell events. Therefore, the reduction of miR-4513 expression or the increase in CXCL17 expression may be possible treatment methods for OSCC.

Conflict of Interests

The authors declared that they have no conflicts of interests.

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