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# MiR-373 promotes proliferation and metastasis of oral squamous cell carcinoma by targeting SPOP

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**Abstract. – OBJECTIVE: To explore the biological function of microRNA-373 (miR-373) in regulating the progression of oral squamous cell carcinoma (OSCC) and the related mechanism.**

**PATIENTS AND METHODS: 50 patients who were diagnosed as OSCC in the Department of Stomatology of the Stomatological Hospital of Chongqing Medical University were enrolled as the cancer group. 20 healthy oral mucosa specimens were obtained as the control group. The miR-373 level in both OSCC clinical samples and cell lines was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The binding relationship between speckle-type POZ protein (SPOP) and miR-373 was detected through online prediction software and luciferase reporter assay.**

**RESULTS: MiR-373 was upregulated in OSCC samples and cell lines. It could negatively regulate the protein expression of SPOP. However, it did not affect the mRNA expression of SPOP. The up-regulation of miR-373 promoted proliferation, invasion, and migration ability of the OSCC cells. However, the effects of miR-373 were abolished by the over-expression of SPOP in cells.**

**CONCLUSIONS: Up-regulated miR-373 promotes proliferation, invasion, and migration of OSCC by targeting SPOP. MiR-373/SPOP axis could be a potential therapeutic target for OSCC.**

*Key Words:*
MicroRNA-373 (miR-373), Oral squamous cell carcinoma (OSCC), Speckle-type POZ protein (SPOP).

## Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor in the head and neck. It originates from the oral mucous epithelia and has an incidence rate accounting for more than 80% of the oral and maxillofacial malignancy, ranking the 8th among total malignant tumors[1,2]. OSCC generally exhibits a rapid development. With the introduction of the multidisciplinary comprehensive and sequential therapy, some breakthroughs have been achieved to some extent. Nevertheless, the 5-year survival rate of patients after surgery remains at 50%[3]. Therefore, the deep study of the molecular mechanism of the occurrence and development of OSCC and the analysis on the effective biomarkers and therapeutic targets have important clinical significance for the prevention, control, and treatment of OSCC.

With the appearance of new approaches and techniques of bioinformatics, miRNAs have been well concerned in genetic diagnosis and treatment. Being endogenous, non-coding, and small-molecule RNAs with a length of about 22-25 nt, miRNAs can recognize specific mRNAs and promote mRNA degradation or inhibit mRNA translation to regulate the target gene expressions at transcriptional and post-transcriptional levels[4,5]. Studies have revealed that the abnormally expressed miRNAs, which have a close relationship with the incidence and devel-



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opment of tumors, are functional in the suppression or promotion of tumor growth[6]. Besides, its expression difference can serve as an important indicator for the diagnosis and prognosis of tumors[7-10], and as a new target for the treatment of tumors[11,12].

Originally, microRNA-373 (miR-373) was considered as a specific miRNA in human embryonic stem cells[13]. The subsequent findings have shown that miR-373 could directly target LATS2, a tumor-suppressor gene. MiR-373 could downregulate LATS2 level, thus inhibiting the p53 pathway. MiR-373 could also promote the cell transformation of the germ cell tumors in the testis by targeting the oncogene RAS[14]. Therefore, miR-373 is considered to be a potential oncogene. In recent years, studies on miR-373 have confirmed that it could play a promotive role in a variety of malignant tumors[15-18].

This experiment aimed to explore the role and the possible mechanism of miR-373 in the development of OSCC.

## Patients and Methods

### OSCC Clinical Samples and Cell Lines

50 patients who were diagnosed as OSCC in the Department of Stomatology of the Stomatological Hospital of Chongqing Medical University were enrolled as cancer group. All of them were primary lesions and did not receive radiotherapy and chemotherapy before surgery. 20 healthy oral mucosa specimens were obtained as the control group. Before the collection of all specimens, the informed consent from patients and the approval from the Ethics Committee of the hospital were obtained. All specimens received informed consent from the patients and were approved by the Hospital Ethics Committee. The OSCC cell line Tca8113 was purchased from Chongqing Medical University. Normal oral mucosal keratinocyte cell line HOK was bought from Chongqing Medical University. The cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator at 37°C. The cells with good condition in the logarithmic growth phase were obtained for *in vitro* study.

### Luciferase Reporter Assays

The predicted sequences in the SPOP 3'UTR that were matched with miR-373 mRNAs were integrated into PGL3-promoter-vector, namely PGL3-SPOP 3'UTR-WT. PGL3-SPOP 3'UTR-MUT was constructed as well. Tca8113 cells were co-transfected with miR-373/negative control (NC) and PGL3-SPOP 3'UTR-WT/PGL3-SPOP 3'UTR-MUT. After 48 h, the luciferase activity was determined according to the instructions of a dual luciferase assay kit (Promega, Madison, WI, USA).

### Transfection

MiR-373 mimics, Lv-SPOP, and miR-NC were designed, synthesized, and transfected into Tca8113 cell line in accordance with the instructions of the Lipofectamine 2000.

### Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted from tissues or cells according to the instructions of the total RNA extraction kit (TaKaRa, Otsu, Shiga, Japan). The RNA concentration was measured using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The complementary Deoxyribose Nucleic Acid (cDNA) was synthesized according to the instructions of the PrimeScript™ RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, for a total of 40 cycles. The relative expression level of the target gene was expressed by the $2^{-\Delta\Delta Ct}$ method. U6 was used as an internal reference in the quantitative analysis of miR-373 expression, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference in the quantitative analysis of the SPOP expression. The experiment was repeated for 3 times. Primer sequences used in this study were as follows: SPOP, F: 5'-GCAACGAACTAATCCTTCC-3', R: 5'-CGTATTGGGATGAACTGGCCAAGGA-3'; miR-373, F: 5'-GAACAGTGAACATCTCGCCTG-3', R: 5'-GATTGAATGTCGAAGACGCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

### Western Blot Analysis

The protein concentration was measured using the OSCCA reagent kit (Merck, Billerica, MA, USA). The cell lysates were separated on polyacrylamide gels and electroblotted onto nitrocellulose membranes. The membranes were

then blocked in blocking buffer (TBS with 0.05% Tween 20, pH 7.6 with 5% skimmed milk). After that, the membranes were incubated with anti-SPOP antibody (1:1000 dilution) or anti-GAPDH (1:1000) antibody at 4°C overnight. Thereafter, the membranes were incubated with a secondary antibody at room temperature for 2 h, washed with Tris-Buffered Saline and Tween (TBST) for 3-5 times and exposed with enhanced chemiluminescence (ECL). ImageJ (NIH, Bethesda, MD, USA) was utilized to count the gray value in each group.

### Cell Proliferation

Tca8113 cells after transfection were inoculated into a 96-well plate (100 µL/well) at $5\times10^3$ cells/well. 10 µL of cell counting kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) was added at different time points (24 h, 48 h, 72 h and 96 h) and incubated for other 2 h. A microplate reader was used to detect the optical density (OD) at 450 nm in different time points.

### Transwell Assay

After 48 h of transfection, cell migration, and invasion abilities were measured using a Transwell chamber (Corning, Corning, NY, USA) with a pore size of 8 µm, in which Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was coated at a concentration of 1:9 in the upper chamber for the detection of the invasion ability. Subsequently, the upper chamber was added with 250 µL serum-free medium while the bottom chamber was added with 700 µL medium, containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). Cells ($5\times10^4$/well) were then seeded to the upper chamber and placed in an incubator. After 24 h, the chamber was removed, and the remaining cells in the upper chamber were gently wiped off with a cotton swab. The cells in the bottom chamber were fixed with paraformaldehyde and stained with crystal violet. Finally, five fields of view were randomly selected under an inverted microscope (×100) for counting, and the average was then calculated.

### Statistical Analysis

The statistical analysis was performed with a Student's *t*-test or One-way analysis of variance (ANOVA) followed by the post-hoc test (Least Significant Difference). All *p*-values were two-sided and $p<0.05$ were considered significant. The data were analyzed by Prism 7.01 software (La Jolla, CA, USA).

## Results

### MiR-373 Was Up-Regulated in OSCC Cells and Tissue Samples

The expression level of miR-373 in 70 clinical samples and 2 cell lines was detected by qRT-PCR, as shown in Figure 1. The expression level of miR-373 in the cancer group was significantly

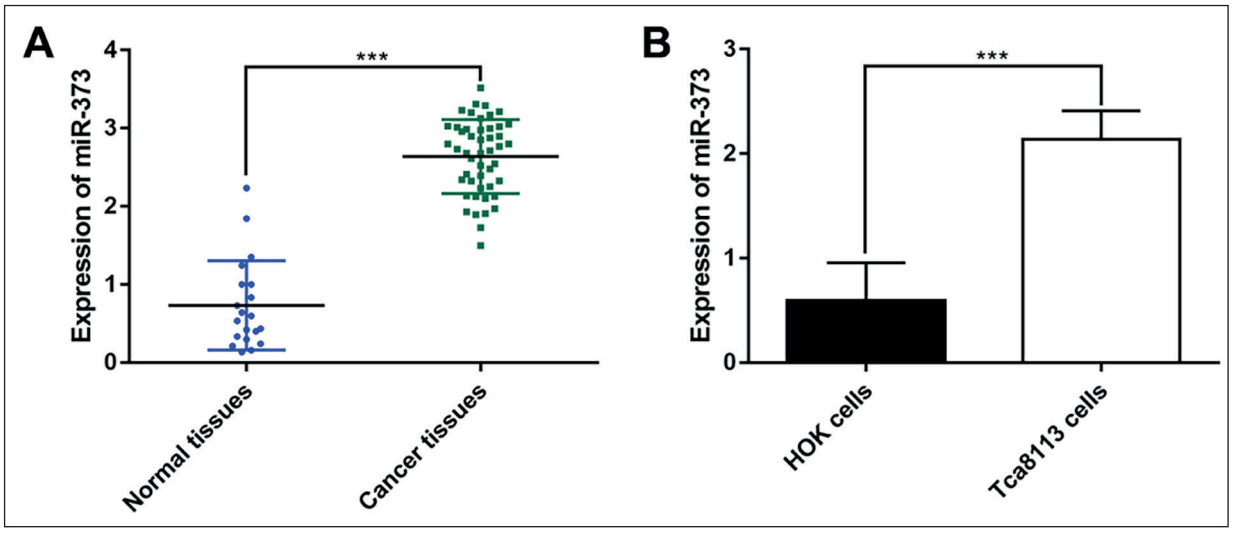


**Figure 1.** The expressions of miR-373 in oral squamous cell carcinoma (OSCC) tissue samples and cells compared with healthy oral mucosa tissues and normal esophageal epithelial cells. ***A,*** Difference in the expression of miR-373 between OSCC tissues and normal tissues. (***$p<0.001$). ***B,*** The expression of miR-373 in OSCC cells (Tca8113) and normal oral mucosal keratinocyte cells (HOK). (**$p<0.01$).

higher than that in the normal control group. Similar results were also obtained at the cellular level. These results suggested that miR-373 acted as a cancer-promoting factor in OSCC, which was consistent with other reports. Next, the transfection of miR-373 mimics can effectively increase miR-373 expression level in Tca8113 cells (Figure 2A).

### SPOP Was a Direct Target of MiR-373

The functional targets for miR-373 were searched through the target Gene Prediction Software. SPOP, a tumor-suppressor gene, was screened as our research object (Figure 2B). Further, the luciferase reporter gene assay verified the binding between miR-373 and SPOP (Figure 2C). Next, qRT-PCR and Western blot were performed to detect the expression of SPOP in these transfected cells. The transfection of miR-373 mimics could alter the protein expression of SPOP, while its mRNA levels did not change (Figure 3A-3C).

### MiR-373/SPOP Inhibited Malignant Behaviors of OSCC Cells

In the CCK-8 experiment, we evaluated the proliferative capacity of the OSCC cells. As shown in Figure 4A, the transfection of miR-373 mimics promoted the proliferation of the OSCC cells. Similarly, the transwell assay showed that miR-373 also boosted the transmembrane movement of the OSCC cells, manifesting higher migration and invasion rates in OSCC cells overexpressing miR-373 (Figure 4B). However, when we co-transfected miR-373 mimics and Lv-SPOP into OSCC cells, the protein expression of SPOP returned to the normal level (Figure 3B-3C), and the inhibitory effect of miR-373 on malignant behaviors of OSCC cells disappeared (Figure 4).

## Discussion

In recent years, the incidence of OSCC is rising and the onset age is in younger people[19]. OSCC is highly invasive and prone to early-stage cervical lymph node metastasis, seriously threatening the life and deteriorating the living quality of patients. The pathogenesis of OSCC remains unclear. According to current studies on genes, molecular levels and external factors, the OSCC might be a multigenic and multifactorial synergetic process, in which miRNAs are of great significance.

MiRNAs, non-coding and single-stranded RNA molecules with a length of about 20-25 nt, regulate about 30% of all human genes. They act as post-transcriptional regulatory factors by completely or incompletely pairing with the target gene 3'UTR to silence the target genes or hinder the translation[20]. Besides, they are very important in various biological behaviors, including cell differentiation, proliferation, apoptosis, metabolism, invasiveness, and drug resistance[5,21]. Moreover, a large number of studies have manifested that miRNAs could be used for tumor therapy, which

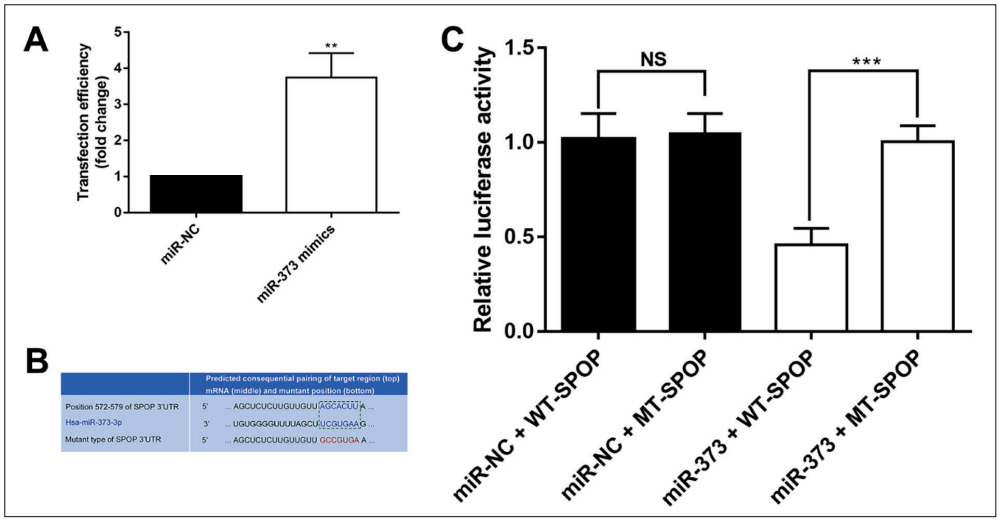


**Figure 2.** SPOP is a direct and functional target of miR-373. ***A,*** Transfection efficiency of miR-373 mimics tested by qRT-PCR (**$p<0.01$). ***B,*** Diagram of putative miR-373 binding sites of SPOP. ***C,*** Relative activities of luciferase reporters (***$p<0.001$).

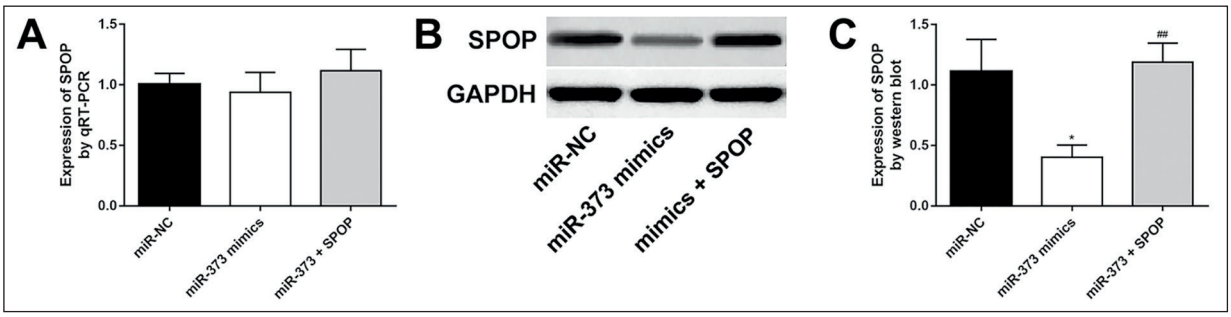


**Figure 3.** ***A,*** Expression level of SPOP by qRT-PCR analysis. ***B-C,*** Expression level of SPOP by Western blot analysis. (*$p$<0.05 *vs.* NC group; $^{\#\#}p$<0.01 *vs.* Mimics group).

provides the possibility (of applying) to apply miRNAs in early diagnosis and adjuvant treatment of OSCC. The correlations of miRNAs with OSCC have been continuously disclosed in recent years[22-25].

Speckle-type POZ protein (SPOP) is an adaptor protein of the Cul3 binding substrate protein in the ubiquitin ligase family. It consists of 374 amino acid residues with a molecular mass of 42 kDa[26]. SPOP is widely

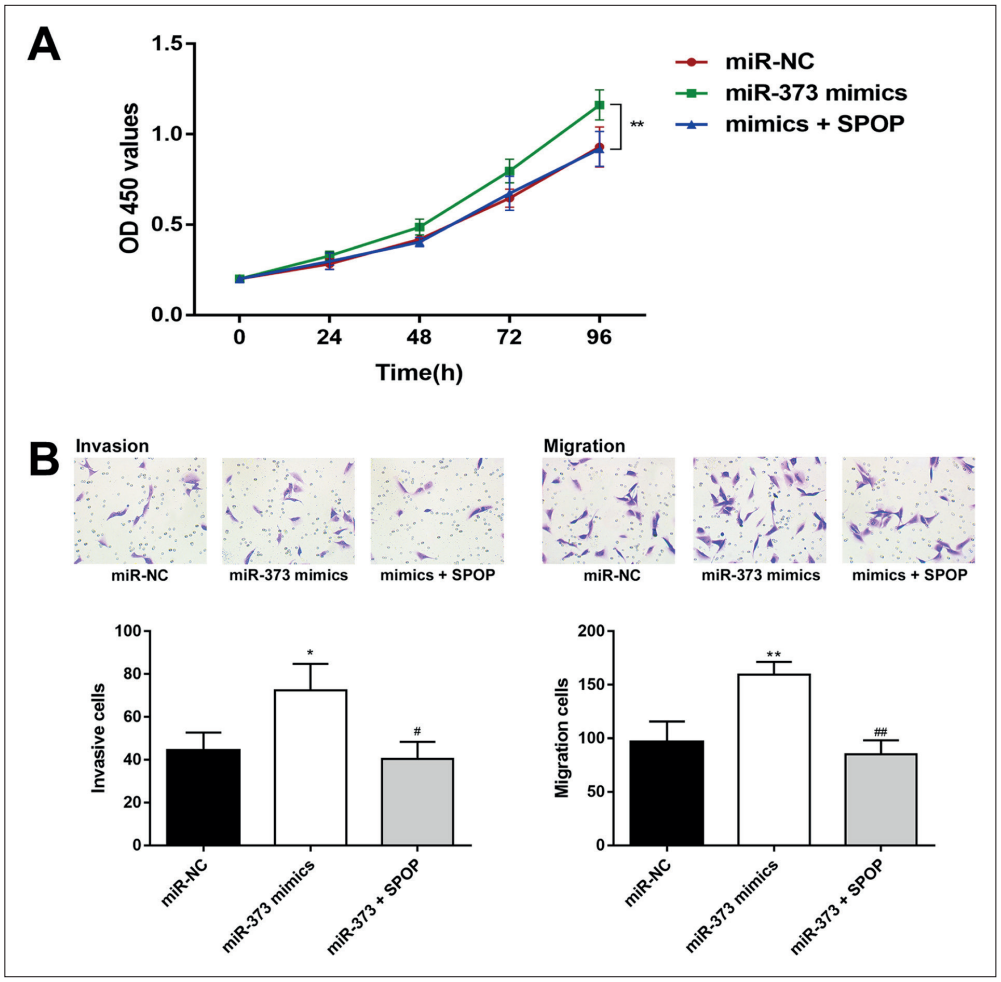


**Figure 4.** ***A,*** MiR-373 promoted the proliferation of OSCC cells. (**$p$<0.01). ***B,*** MiR-373 promoted the invasion and migration of OSCC cell (magnification × 100). (*$p$<0.05, **$p$<0.01 *vs.* NC group; $^{\#}p$<0.05, $^{\#\#}p$<0.01 *vs.* Mimics group).

distributed in mammals. Over ten proteins are known as the substrate receptors of SPOP. SPOP could exert several functions by binding to and affecting these proteins[27], such as the regulation of cell apoptosis[28], the translational process of the development and differentiation of pancreas[29], bone development and homeostasis[30], and phosphorylation processes of PI3K and MAPK signaling pathways[31]. For the past few years, studies on SPOP and cancer are very popular. By inhibiting the phosphorylation of SP1 and down regulating the expression of MMP2 in CRC cells via Sp1/PI3K/Akt signaling pathway, the overexpression of SPOP could inhibit the progression and metastasis of colon cancer[32]. Among patients with ovarian cancer, over 50% of them are detected with SPOP gene deletion. SPOP is related to histological subtype and tumor stage. This is more commonly observed in patients with advanced ovarian cancer, suggesting that SPOP could be used as a tumor-suppressor gene in ovarian cancer[33]. In breast cancer, another cancer closely related to hormone levels, SPOP decomposes the estrogen receptor by mediating ubiquitination and degradation to regulate the level of progesterone in breast cells, and thereby it inhibits the progression of breast cancer[34]. Among various SPOP-related malignant tumors known so far, SPOP acts as a tumor suppressor in most cases, while it promotes the occurrence and development of renal cancer. Cytoplasmic SPOP has an opposite function to that of the nuclear one, indeed, instead of inducing the apoptosis, it promotes cell proliferation, thus leading to the occurrence of renal clear cell carcinoma[31].

In our study, miR-373 overexpression attenuated biological functions such as proliferation, invasion, and migration of OSCC cells. However, the co-transfection of miR-373 mimics and SPOP neutralized the regulation function of miR-373 on cells. The above results indicated that the inhibition effects of miR-373 on OSCC cells were exerted by targeting SPOP.

## Conclusions

This work preliminarily revealed the molecular mechanism of miR-373 in OSCC, providing a theoretical basis for the study of the pathogenesis of OSCC and a reference value for the further developments of the biological targeted gene therapy for OSCC.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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