Original Article
MiR-300 suppresses laryngeal squamous cell carcinoma proliferation and metastasis by targeting ROS1

Wensheng Ge[1], Chaodong Han[1], Jing Wang[1], Yunping Zhang[2]

Departments of [1]Otolaryngology, [2]Dermatology, Liaocheng People's Hospital and EENT Hospital, Liaocheng 252000, Shandong, China

Abstract: Laryngeal squamous cell carcinoma (LSCC) is a common aggressive head and neck cancer with high mortality and incidence. MicroRNAs (miRNAs) are short, non-coding and endogenous RNAs that posttranscriptionally inhibit gene expression. In this study, we showed that miR-300 expression was downregulated in LSCC tissues compared with adjacent no-tumor tissues. MiR-300 overexpression inhibited Hep-2 cell proliferation, as well as the expression of ki-67 and PCNA. Moreover, overexpression of miR-300 repressed the cell invasion in Hep-2 cells. We identified c-ros oncogene 1 receptor tyrosine kinase (ROS1) as a direct target gene of miR-300 in Hep-2 cell. Furthermore, ROS1 expression was upregulated in LSCC tissues compared with adjacent no-tumor tissues. Interesting, there were an inverse correlation between ROS1 and miR-300 expression in the LSCC tissues. Overexpression of ROS1 increased the Hep-2 cells proliferation and invasion. Overexpression of ROS1 abrogated miR-300 induced cell growth and invasion inhibition. Therefore, our data suggested that miR-300 acted as a tumor suppressive gene in LSCC.

Keywords: Laryngeal squamous cell carcinoma, microRNAs, miRNAs, miR-300, ROS1

Introduction

Laryngeal squamous cell carcinoma (LSCC) is a common aggressive head and neck cancer with high mortality and incidence [1-6]. The worldwide incidence of LSCC was about 2.4% every year [4, 7]. Recent treatments such as radiation therapy, chemotherapy and surgical have some effect on the patients of early stage, but are littler effective on the patients of advanced cases [6, 8-10]. The 5-year (OS) overall survival of LSCC cases is poor [11-13]. Therefore, it is important to find new biomarkers to improve diagnosis and therapy of LSCC patients.

MicroRNAs (miRNAs) are short (18-22 nucleotides), non-coding endogenous RNAs that repress gene expression through binding to 3'-UTR (3' untranslated regions) of target mRNAs [14-20]. Aberrant expression of miRNAs has been found in a number of cancers such as bladder cancer, gastric cancer, ovarian cancer, and gall-bladder and hepatocellular carcinoma [21-25]. They act as important regulators in various cell biology such as cell development, cell proliferation, apoptosis, metabolize, invasion and migration [26-29]. They are also considered as a tumor suppressors or oncogenes in tumor development [30-32].

In this study, we demonstrated that miR-300 expression was downregulated in LSCC tissues and overexpression of miR-300 suppressed the cell proliferation and invasion by targeting c-ros oncogene 1 receptor tyrosine kinase (ROS1) in LSCC cell line Hep-2.

Materials and methods

Samples cell lines and cell transfected

Human LSCC specimens (n = 30) and adjacent non-tumor samples (n = 30) were received from our department with written informed consent from each patient. All experiments were approved by the Ethics Committee of Liaocheng
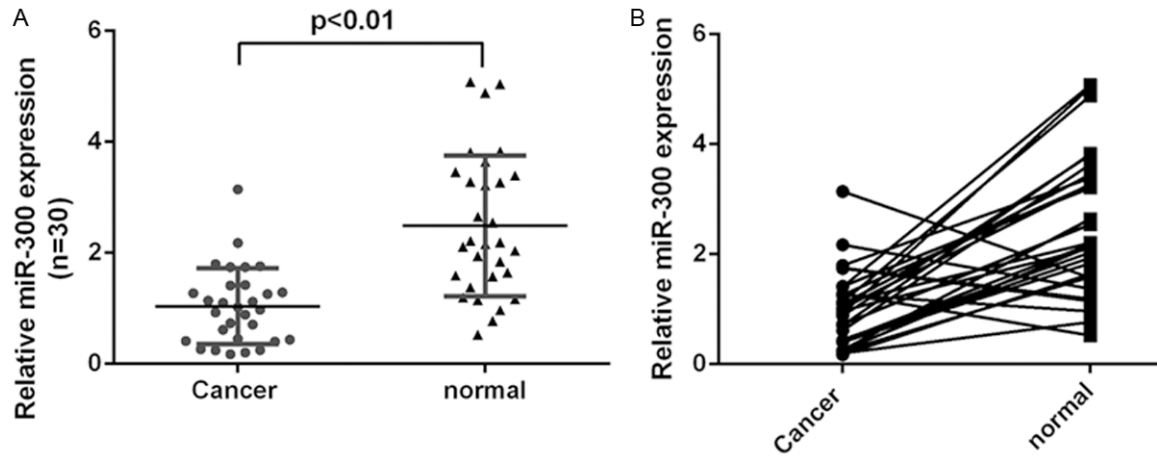
Figure 1. MiR-300 was downregulated in LSCC. A. The expression of miR-300 was measured by qRT-PCR. B. miR-300 was downregulated in 26 cases (26/30, 87%) compared with the normal adjacent tissues.

People's Hospital and EENT Hospital. LSCC cell line, Hep-2, was purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were cultured in RPMI 1640 supply with 10% FBS (fetal bovine serum) at 37°C. miR-300 mimic oligonucleotide and scramble oligonucleotide was bought from GenePharma (Shanghai, China) and was transfected to cells through Lipofectamine 2000 Reagent (Invitrogen) according to manuscript's information.

Real-time PCR

RNA was extracted from cells and tissues by using Trizol (Invitrogen, CA) following to the manufacturer's explanation. MiR-300 and ROS1 expression was quantified using qRT-PCR analysis. The specific primers were used as follows: MiR-300: 5'-TATACAAGGGCAGACTCTCT-CT-3'; 5'-GTGCAGGTTCCGAGGT-3'; U6: 5'-CTC-GCTTCGGCAGCACATATACT-3', 5'-ACGCTTCACG-AATTTGCGTGTC-3'; GAPDH: 5'-AATGGGCAGC-CGTTAGGAAA-3', 5'-TGAAGGGGTCATTGATGGC-A-3'; ROS1 5'-ATGGGCTCCTGTATTGGTTG-3' and 5'-CATCAGTGCATTCTGGGAAA-3' was used as for internal control for miR-300 and GAPDH was performed to as control for ROS1.

Cell proliferation and invasion

For cell proliferation analysis, cells were cultured in 96-well plates. CCK-8 analysis (Dojindo, Japan) was performed to detect the cell proliferation and the absorbance was readied at 450 nM. For cell invasion analysis, transwell assays were done. Cells were cultured in

Matrigel matrix coated membrane (BD Biosciences) and FBS was put into the lower membrane. After 24 hours, the noninvading cells were removed and cells on the lower membrane was stained with 0.1% crystal violet and calculated.

Luciferase assay

To build a luciferase reporter vector, cDNA contained the miR-300 binding sites was amplified and cloned into the pGL3 luciferase vector. Cell was con-transfected with pGL3-ROS1 or mut pGL3-ROS1 vectors combine with miR-300 mimic or control by using Lipofectamine 2000 Reagent (Invitrogen) according to manuscript's information. The luciferase data was detected using the dual-luciferase reporter kit (Promega, USA) following to manuscript's information.

Western blot analysis

Total proteins were extracted from cell or tissues and then separated used 10% SDS-PAGE and transferred to a membrane (Bio-Rad, USA). After blocked with 5% non-fat milk for 1 hour and membrane was incubated with primary antibodies (ROS1, ki-67, PCNA, GAPDH, Sigma, USA). Enhanced chemiluminescence (ECL, USA) was performed to determine the protein concentration.

Statistical analysis

Data was shown as mean ± SD (standard deviation). Difference between groups was mea-
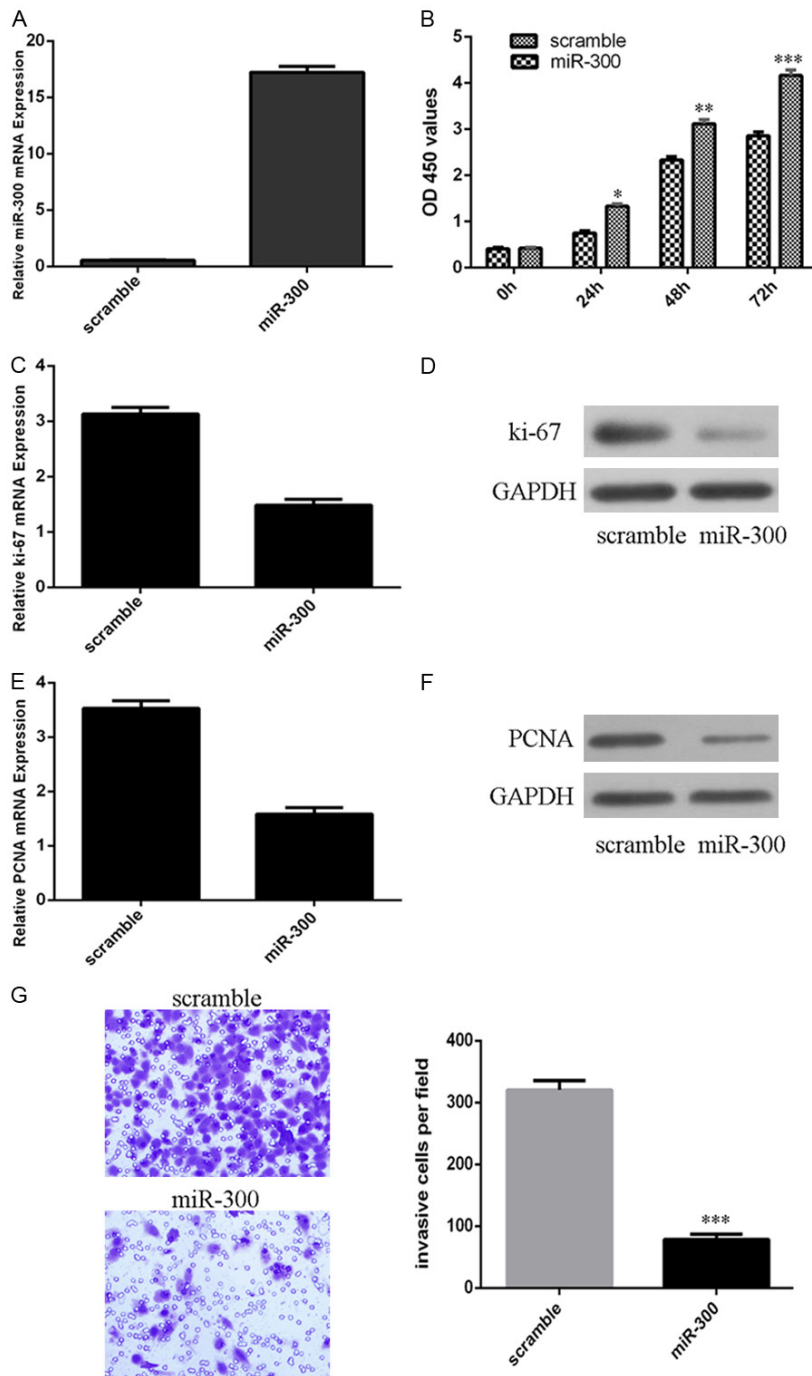
Figure 2. MiR-300 inhibited the LSCC cells proliferation and invasion. A. The expression of miR-300 was detected after treated by miR-300 mimic using qRT-PCR. B. CCK-8 analysis was performed to measure the cell proliferation. C. The mRNA expression of ki-67 was measured by qRT-PCR. D. The protein expression of ki-67 was detected by western blot. E. The mRNA expression of PCNA was measured by qRT-PCR. F. The protein expression of PCNA was detected by western blot. G. Overexpression of miR-300 inhibited the Hep-2 cell invasion. *p<0.05, **p<0.01 and ***p<0.001.

sured using SPSS V19.0 software. Student's t test was performed to compare the significance

differences of two groups and ANOVA (one-way analysis of variance) was used measure the difference between more than two groups. P<0.05 was considered to be significant.

Result

MiR-300 was downregulated in laryngeal squamous cell carcinoma

We firstly detected the miR-300 expression in the 30 pair's laryngeal squamous cell carcinoma (LSCC) tissues. qRT-PCR assay demonstrated that miR-300 expression was downregulated in LSCC tissues compared to adjacent no-tumor tissues (**Figure 1A**). Moreover, miR-300 was downregulated in 26 cases (26/30, 87%) compared with the normal adjacent tissues (**Figure 1B**).

MiR-300 inhibited the LSCC cells proliferation and invasion

MiR-300 was upregulated after treated with miR-300 mimics in LSCC cell line Hep-2 (**Figure 2A**). CCK-8 analysis showed that miR-300 overexpression inhibited Hep-2 cell proliferation (**Figure 2B**). We also confirmed that ectopic expression of miR-300 inhibited the mRNA expression of ki-67 (**Figure 2C**). In line with this, western blot data proved that miR-300 overexpression suppressed the ki-67 protein expression (**Figure 2D**). Overexpression of miR-300 repressed the mRNA expression of PCNA
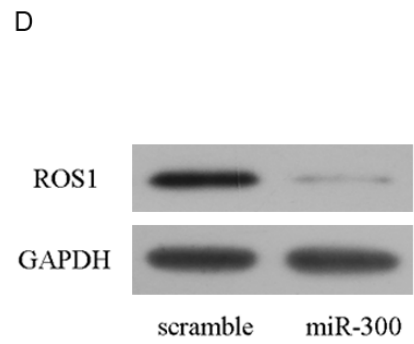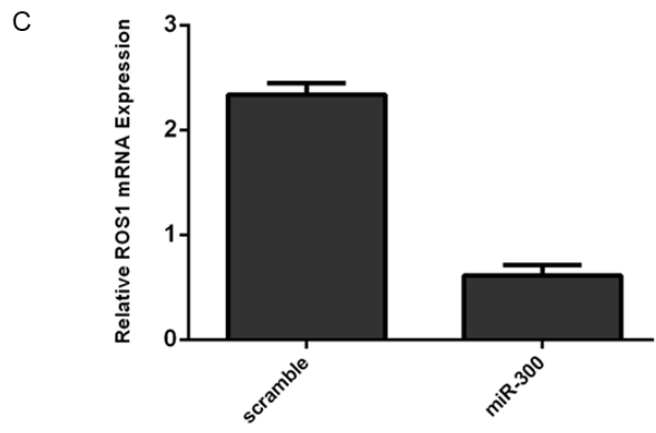
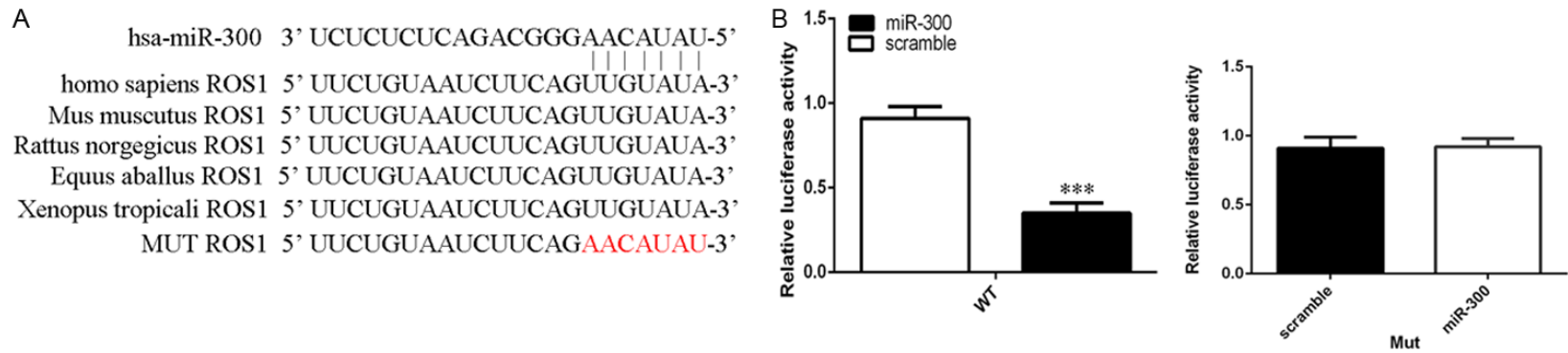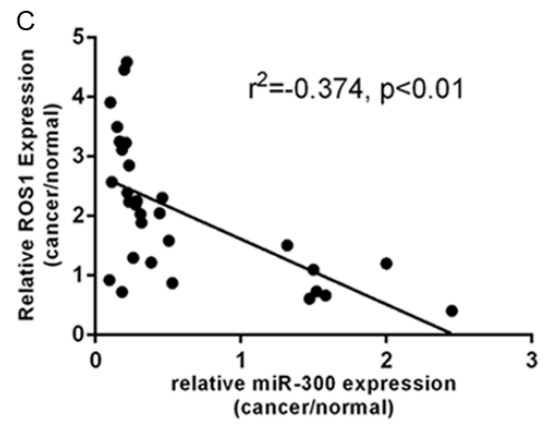**Figure 3.** Regulation of ROS1 expression by miR-300 in LSCC. A. The Targetscan data showed that there were complementary seed region in the 3'-UTR of human ROS1 complementary to miR-300. B. miR-300 overexpression decreased the luciferase activity of WT 3'-UTR of ROS1 construct, however; overexpression of miR-300 did not change the luciferase activity of MUT 3'-UTR of ROS1 construct. C. The mRNA expression of ROS1 was measured by qRT-PCR. D. The protein expression of ROS1 was detected by western blot. ***p<0.001.

Figure 4. ROS1 expression was upregulated in LSCC. A. The expression of ROS1 was measured by qRT-PCR. B. ROS1 was upregulated in 22 cases (22/30, 73%) compared with the normal adjacent tissues. C. There was an inverse correlation between ROS1 and miR-300 expression in the LSCC tissues.

Figure 5. Overexpression of ROS1 abrogated miR-300 induced cell growth and invasion inhibition. A. The mRNA expression of ROS1 was measured by qRT-PCR. B. The protein expression of ROS1 was detected by western blot. C. Overexpression of ROS1 increased the Hep-2 cells proliferation. D. Overexpression of ROS1 increased the Hep-2 cells invasion. E. Cell proliferation was measured by CCK-8 analysis. F. The cell invasion was measured by transwell assays.

(**Figure 2E**). Western blot data also demonstrated that miR-300 overexpression suppressed the PCNA protein expression (**Figure 2F**). Moreover, invasion analysis demonstrated that overexpression of miR-300 inhibited the Hep-2 cell invasion (**Figure 2G**).
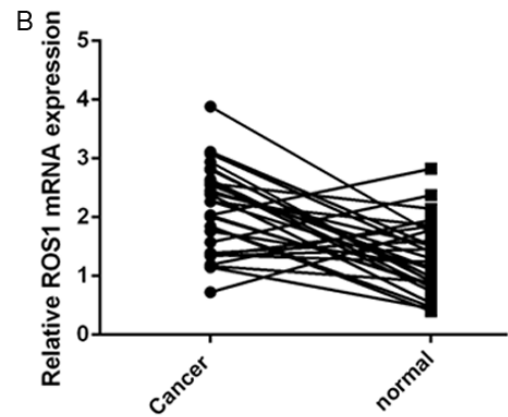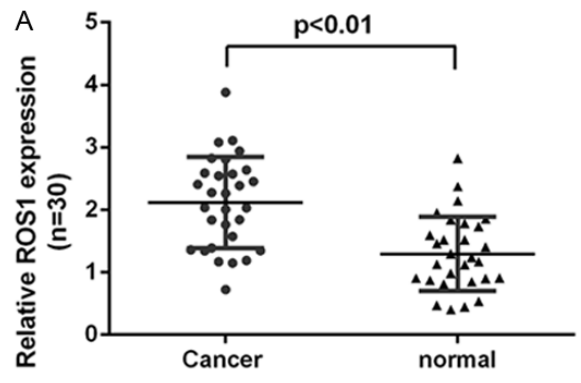
Regulation of ROS1 expression by miR-300 in LSCC

The TargetScan data showed that there were complementary seed region in the 3'-UTR of human ROS1 complementary to miR-300

(**Figure 3A**). Dual Luciferase Reporter analysis showed that miR-300 overexpression decreased the luciferase activity of WT 3'-UTR of ROS1 construct, however; overexpression of miR-300 did not change the luciferase activity of MUT 3'-UTR of ROS1 construct (**Figure 3B**). Overexpression of miR-300 inhibited the mRNA expression of ROS1 in the Hep-2 cells (**Figure 3C**). Moreover, miR-300 overexpression suppressed the ROS1 protein expression in the Hep-2 cells (**Figure 3D**).

ROS1 expression was upregulated in LSCC

ROS1 expression was upregulated in LSCC tissues compared with adjacent no-tumor tissues (**Figure 4A**). Moreover, ROS1 was upregulated in 22 cases (22/30, 73%) compared with the normal adjacent tissues (**Figure 4B**). Interesting, there was an inverse correlation between ROS1 and miR-300 expression in the LSCC tissues (**Figure 4C**).
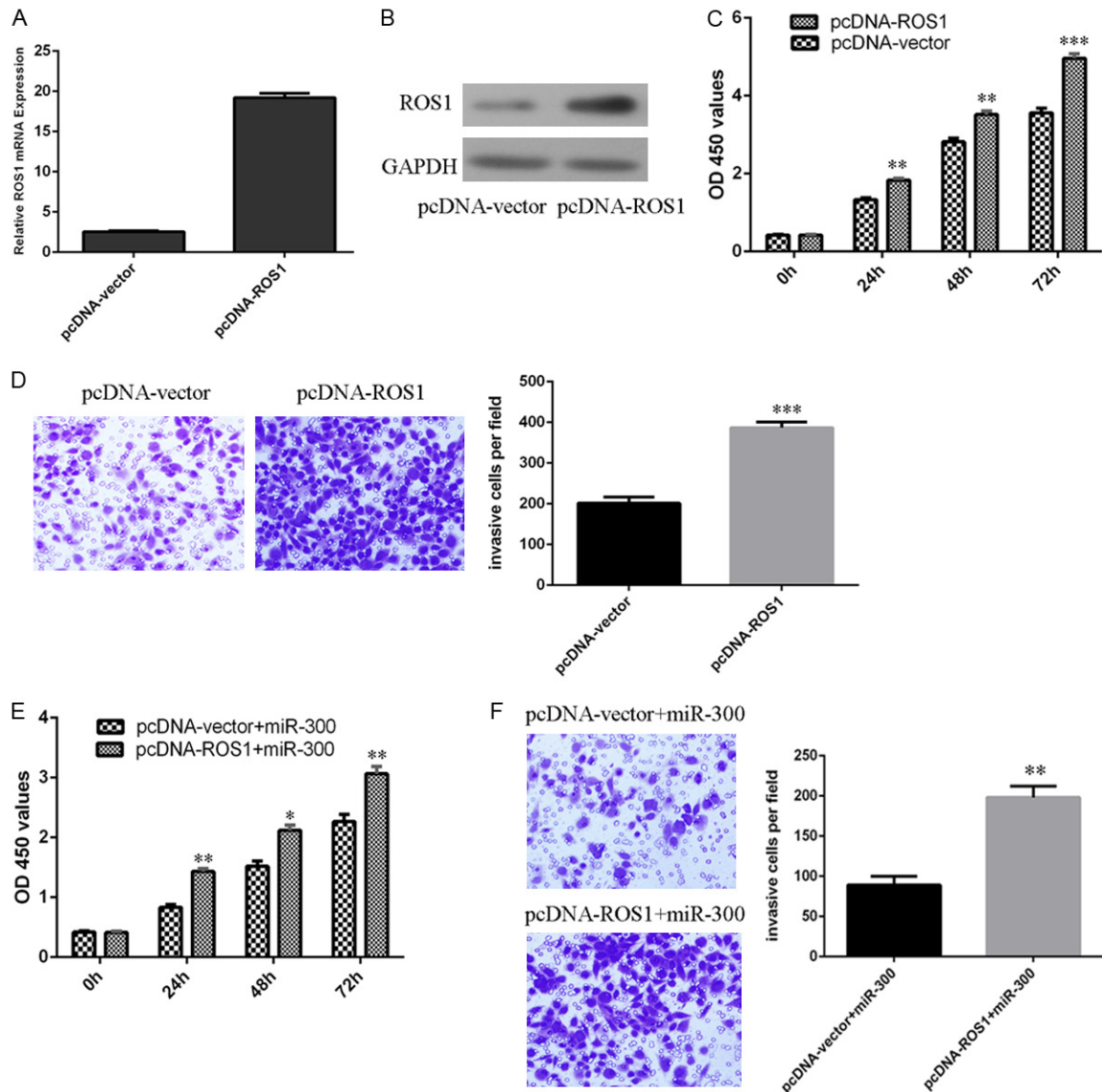
Overexpression of ROS1 abrogated miR-300 induced cell growth and invasion inhibition

The mRNA expression of ROS1 was upregulated after treated pcDNA-ROS1 in the Hep-2 cells (**Figure 5A**). Moreover, pcDNA-ROS1 also can induce the protein expression of ROS1 in the Hep-2 cells (**Figure 5B**). Overexpression of ROS1 increased the Hep-2 cells proliferation (**Figure 5C**). ROS1 overexpression promoted the Hep-2 cells invasion (**Figure 5D**). When miR-300 mimics and pcDNA-ROS1 was co-transfected into Hep-2 cells, ROS1 overexpression enhanced the ROS1-induced cell proliferation (**Figure 5E**) and invasion (**Figure 5F**) in Hep-2 cells.

Discussion

In our study, we showed that miR-300 expression was downregulated in laryngeal squamous cell carcinoma (LSCC) tissues compared with adjacent no-tumor tissues. MiR-300 overexpression inhibited Hep-2 cell proliferation and inhibited the expression of ki-67 and proliferating cell nuclear antigen (PCNA). Moreover, overexpression of miR-300 repressed the Hep-2 cell invasion. We identified c-ros oncogene 1 receptor tyrosine kinase (ROS1) as a direct target gene of miR-300 in Hep-2 cell. Furthermore, ROS1 expression was upregulated in LSCC tissues compared with adjacent no-tumor tis-

sues. Interesting, there were an inverse correlation between ROS1 and miR-300 expression in LSCC tissues. Overexpression of ROS1 increased the Hep-2 cells proliferation and invasion. Overexpression of ROS1 abrogated miR-300 induced cell growth and invasion inhibition in LSCC. Therefore, our data suggested that miR-300 acted as a tumor suppressive gene in LSCC.

Previous studies demonstrated that miR-300 played important roles in the tumor development. For example, Yu et al. showed that the expression of miR-300 was decreased in the head and neck squamous cell carcinoma (HNSCC) cells and breast cancer cell [33]. miR-300 overexpression blocked TGF-beta-induced epithelial-to-mesenchymal transition (EMT) and reversed the EMT phenotype in MDA-MB-231 and HN-12 cells. Xue et al. demonstrated that miR-300 expression was increased in osteosarcoma cells and tissues and miR-300 overexpression increased cell invasion, proliferation and EMT through regulating bromodomain-containing protein 7 (BRD7) expression [34]. Xu et al. showed that the expression of miR-300 was increased in breast cancer cell lines and tissues [35]. MiR-300 overexpression enhanced cell cycle progression and proliferation by inhibiting p53 expression. However, the role of miR-300 was still uncovered in LSCC. In this study, we demonstrated that miR-300 expression was downregulated in LSCC tissues compared with adjacent no-tumor tissues. MiR-300 was downregulated in 26 cases (26/30, 87%) compared with the normal adjacent tissues. Moreover, miR-300 overexpression inhibited Hep-2 cell proliferation and invasion and inhibited the expression of ki-67 and PCNA.

In our study, we identified ROS1 as a direct target gene of miR-300 Hep-2 cell. ROS1 is an oncogene that could activate various pathways, such as the SHP-1 and SHP-2, ERK1/2, phosphatidylinositol 3-kinase (PI3K), IRS-1 (isiulin receptor substrate 1), STAT3, VAV3 and protein kinase B signaling pathways [36-39]. ROS1 expression was detected in central nervous system, kidney, liver, stomach and colon [38, 40-42]. ROS1 gene rearrangement was also found in the glioblastoma multiforme, NSCLC (nonsmall cell lung cancer), colon cancer and gastric cancer [43-47]. Recently, Zhang et al. showed that miR-33a inhibited cell metastasis

and proliferation through targeting ROS1 in breast cancer [48]. In our study, we identified ROS1 as a direct target gene of miR-300 in Hep-2 cell. Furthermore, ROS1 expression was upregulated in LSCC tissues compared with adjacent no-tumor tissues. Interesting, there were an inverse correlation between ROS1 and miR-300 expression in the LSCC tissues. Overexpression of ROS1 increased the Hep-2 cells proliferation and invasion. Overexpression of ROS1 abrogated miR-300 induced cell growth and invasion inhibition.

In conclusion, we showed that miR-300 expression was downregulated in LSCC tissues and overexpression of miR-300 suppressed the LSCC cell line Hep-2 cell proliferation and invasion by targeting ROS1 expression. These data suggested that miR-300 acted as a tumor suppressive gene in LSCC.

Address correspondence to: Chaodong Han, Department of Otolaryngology, Liaocheng People's Hospital and EENT Hospital, Liaocheng 252000, Shandong, China. E-mail: hongxia1l@sina.com

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