MicroRNA-527 Induces Proliferation and Cell Cycle in Esophageal Squamous Cell Carcinoma Cells by Repressing PH Domain Leucine-Rich-Repeats Protein Phosphatase 2

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Abstract
Increasing evidence indicated that microRNAs served dominant roles in carcinogenesis and cancer progression by targeting potential downstream genes. In our study, we found that miR-527 was an upregulated expression in human esophageal squamous cell carcinoma (ESCC) cells and tissues. Furthermore, overexpression of miR-527 promoted cell proliferation and colony formation, enhanced anchorage-independent growth ability, and contributed to cell cycle. In addition, protein phosphatase 2 (PHLPP2) was identified as the direct downstream target gene of miR-527 and was confirmed by luciferase gene reporter assay. In summary, we concluded that miR-527 acted as an oncogenic microRNA in ESCC development by directly targeting PHLPP2 might be a novel therapeutic target for the treatment of ESCC.

Keywords
miR-527, esophageal squamous cell carcinoma, PHLPP2, cell proliferation, cell cycle

Introduction
Esophageal squamous cell carcinoma (ESCC) is the most common histological type that is often diagnosed at an advanced stage.¹ ² High rate of occurrence is the major cause of ESCC mortality.³ However, the exact molecular mechanism underlying ESCC is poorly understood. Therefore, it is urgently needed to elucidate novel biomarkers that can help early diagnosis, targeted therapy, and prognosis evaluation.

Increasing evidence suggested that microRNAs (miRNAs), known as a class of small noncoding RNAs (21-25 nucleotides), played vital regulatory roles in regulating diverse biological processes, such as cell proliferation, cell apoptosis, cell cycle, and cell metastasis.⁴-⁸ MicroRNAs serve as oncogenes or tumor suppressors that affect cancer development and progression.⁹-¹¹ MiR-30d was found to suppress the migration and invasion of ESCC by regulating enhancer of zeste homolog 2 (EZH2).¹² MiR-483-5p was reported to act as a tumor promoter and associated with poor survival of ESCC.¹³ Lu et al indicated that miR-214 suppressed invasion and migration via downregulating polypeptide-N-acetyl-galactosyltransferase 7 (GALNT7) in ESCC.¹⁴ ΔNp63 and TAp73 were reported to control the expression of miR-527 that represses the central transforming growth factor β (TGF-β) regulators.¹⁵ miR-527 was found to code region of Jun messenger RNA in malignant melanoma.¹⁶ miR-527 was reported to suppress TGF-β/drosophila mothers against decapentaplegic protein (SMAD)-induced EMT via downregulating SULF2 expression in lung cancer.¹⁷ However, the role and functional mechanism of miR-527 in ESCC had seldom been elucidated. In this study, we first found the correlation between miR-527 and ESCC by using The Cancer Genome Atlas data. We further examined miR-527 expression in ESCC tissues and cell lines. The effect of miR-527 on ESCC was detected by methyldithiazolyltetrazolium (MTT) assay.

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Received 1 February 2020; received revised 16 April 2020; accepted 30 April 2020

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tetrzolium bromide (MTT), colony formation, anchorage-independent growth, and cell cycle assay. Finally, PH domain leucine-rich-repeats protein phosphatase 2 (PHLPP2) was identified as a direct target of miR-527. Protein phosphatase 2, a novel family of Ser/Thr protein phosphatases, is down-regulated in many types of malignant tumors and played essential role in cancer progression.\textsuperscript{18-20} Taken together, miR-527 was a tumor promoter gene providing a promising prognostic biomarker and therapeutic target of ESCC.

**Materials and Methods**

**Clinical Specimens**

Eight ESCC tissues and adjacent normal tissues were obtained from patients with ESCC at Xinxiang Central Hospital (People’s Republic of China). The study was approved by the ethics committee of Xinxiang Central Hospital (Henan, People’s Republic of China). Written informed consent was obtained from all patients.

**Cell Culture**

Human ESCC cell lines Eca109, Kyse180, Kyse140, Kyse410, Kyse510, Kyse520, TE-1, and Kyse30 were obtained from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China) and were cultured in Eagle’s Minimum Essential Medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Sigma). A normal human esophageal epithelial cell (Het-1A) was purchased from Cell Bank of Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences) and then cultured in RPMI-1640 medium with 10% FBS. All cells were cultured at a humidified incubator at 37 °C and 95% CO\textsubscript{2} and 5% air. MiR-527 inhibitor (miR-527-in), and negative control miRNAs were purchased from GeneCopoeia Co Ltd.

**Plasmids and Transfection**

The 3’ untranslated region (3’UTR) of PHLPP2, containing the putative miR-527-binding site, was cloned into the pGL3 luciferase assays vector (Promega). The miR-527 mimics, negative control, miR-527 inhibitor, and miR-527-mut were purchased from RiboBio Co Ltd and transfected into ESCC cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

**RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (qPCR)**

Total miRNAs were isolated from tissues and cell lines using the mirVana miRNA Isolation Kit (Ambion), and cDNA was synthesized using the Taqman miRNA reverse transcription kit (Applied Biosystems). Relative expression level of miR-527 was normalized to the expression level of RNU48 (as a control), and the relative expression levels were calculated using 2\(^{-[(\text{Ct of miR-527})-(\text{Ct of RNU48})]}\). The primers were as follows: miR-527 (HmiRQP0456, GeneCopoeia) and RNU48 (001006, ThermoFisher).

**MTT Assays and Colony Formation**

Kyse510 cells were seeded into 96-well plates in medium containing 10% FBS. At the indicated time points (1, 2, 3, 4, and 5 days), 20 μL of 5 mg/mL MTT solution (Sigma-Aldrich) was added to each well and incubated with Kyse510 cells for 4 hours at 37 °C and then replaced with 150 μL dimethyl sulfoxide (Sigma). The UV absorbance was measured in a Thermo Scientific Multiskan (Thermo Fisher Scientific).

For colony formation assay, transfected Kyse510 cells were seeded into 6-cm cell culture dishes at 500 cells per well and cultured for 2 weeks. Cells were washed with phosphate-buffered saline (PBS) and then stained with 1% crystal violet (Sigma) after fixed with 4% paraformaldehyde for 5 minutes. The number of colonies formed was counted under a light microscope (Olympus).

**Anchorage-Independent Growth Assay**

Cells were trypsinized, and 500 cells were seeded in 0.35% agar (Sigma) containing complete medium in a 6-well plate. After cultured for 2 weeks, colonies were fixed, photographed, and then counted.

**Cell Cycle Analysis**

Transfected Kyse510 cells were digested by trypsinization (Beyotime Institute of Biotechnology), washed with ice-cold PBS, fixed in 80% ice-cold ethanol in PBS at 4 °C overnight, and incubated with RNase A (final concentration 100 μg/mL; Shanghai Biotechnology Corp) and propidium iodide (Sigma-Aldrich) for 30 minutes in the dark at room temperature. Cell cycle profiles of 5 × 10\textsuperscript{4} cells were performed using a flow cytometer (BD Biosciences).

**Luciferase Activity Assay**

Cells were seeded in 24-well plates (2 × 10\textsuperscript{5}/well) 24 hours before transfection. Cotransfection of pGL3-PHLPP2-luciferase plasmid and TK-Renilla plasmid as control signals was performed with Lipofectamine 2000 from Invitrogen. Luciferase activities were measured at 48 hours after transfection using a Dual-Glo Luciferase Assay System (Promega).

**Western Blotting**

Protein lysates were extracted from ESCC cells, subjected to SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The membranes were then blocked in 5% nonfat powdered milk for 2 hours and incubated overnight at 4 °C with primary antibodies: anti-cyclin D1 (Cell Signaling Technology), anti-p21 (Cell Signaling Technology), anti-Rb (Cell Signaling Technology) and anti-p-Rb (Cell Signaling Technology). α-Tubulin was used as an internal control. And
then being incubated with antirabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz) for 2 hours at room temperature. Signals were visualized using the enhanced chemiluminescence reagents (GE).

**Statistical Analysis**

All statistical analyses were summarized as mean ± SD, unpaired independent Student t test, and χ² test by using SPSS 17.0 software (SPSS). A P value less than 0.05 was thought to be significantly different.

**Result**

**MiR-527 Is Higher Expression in ESCC**

By analyzing the expression data downloaded from a published micro-array-based high-throughput assessment (NCBI/GEO GSE6188, including 153 ESCC tissues/104 adjacent nontumor tissues), we found that miR-527 was significantly upregulated in ESCC tissues compared with the adjacent nontumor tissues (Figure 1A). Consistently, result of real-time polymerase chain reaction (PCR) analysis indicated that miR-527 was markedly overexpressed in 8 collected ESCC samples as compared with paired adjacent nontumor tissues which were obtained from the same patient of our hospital (Figure 1B) and in all 8 ESCC cell lines (Eca109, Kyse180, Kyse140, Kyse410, Kyse510, Kyse520, TE-1, and Kyse30) analyzed compared with normal endometrial epithelial cells (NEEC1 and NEEC2; Figure 1C), implying that miR-527 may play a tumor promoting role in human ESCC development.

**MiR-527 Promoted Cell Proliferation and Cell Cycle of ESCC**

To investigate whether cell proliferation and cell cycle of ESCC were regulated by miR-527, ESCC Kyse510 cells were stably transfected with miR-527 or miR-527-in or the corresponding negative controls for further study. Relative miR-527 expression was verified using qPCR (Figures 2A and 3A). MTT assays revealed that compared to the control, miR-527 significantly increased, while miR-527-in decreased the cellular proliferation (Figures 2B and 3B), and this was further confirmed by colony formation assay (Figures 2C and 3C). Strikingly, we found that overexpression of miR-527 in Kyse510 cells drastically enhanced their anchorage-independent growth ability, while inhibition of miR-527 had the opposite effect (Figures 2D and 3D). As expected, the percentage of G0/G1 phase cells decreased and the percentage of S phase cells increased in Kyse510 cells transfected with miR-527. However, miR-527-in drastically increased the percentage of cells in the G0/G1 phase but decreased the percentage of cells in the S peak (Figures 2E and 3E).

**MiR-527 Directly Targets PHLPP2 by Binding to Its 3′-UTR**

PHLPP2 was predicted as a target of miR-527 by bioinformatics methods (Figure 4A). Western blotting analysis indicated that ectopic expression of miR-527 dramatically decreased, whereas miR-527-in increased the PHLPP2 protein expression in Kyse510 cells (Figure 4B). Furthermore, to verify whether PHLPP2 is a direct target of miR-527, PHLPP2 3′-UTR wild type was cotransfected with miR-527, miR-527-in, or miR-527-mut into Kyse510 cells, followed by measurement of luciferase activity. As shown in Figure 4C, compared with the control, miR-527 suppressed PHLPP2 luciferase activities, while miR-527-in led to a significant increase in reporter activity of PHLPP2. Moreover, miR-527-mut has no effect on the PHLPP2 luciferase activities, suggesting that miR-527 directly targets PHLPP2 (Figure 4C).

As miR-527 promoted cell growth and cell cycle of Kyse510 cells, we then explored whether the cell growth was associated with cell cycle genes (Cyclin D1, p21, pRb and Rb). Result of Western blotting assays showed that miR-527 dramatically increased, whereas miR-527-in decreased the expression of Cyclin D1 and pRb. And we found that p21 expression was downregulated in miR-527-transfected cells, while upregulated in miR-527-in-transfected cells (Figure 4D). Altogether, our
results revealed that miR-527 functionally modulated cell cycle regulators, Cyclin D1, p21, pRb, and Rb, thus relevant to cell proliferation and cell cycle.

**PHLPP2 Downregulation Counteracted the Proliferation Arrest by miR-527-in**

To further investigate the functional connection between miR-527 and PHLPP2, we transfected miR-527-in-transfected Kyse510 cells with small interfering RNA targeting PHLPP2. Western blotting analysis confirmed that PHLPP2 protein expression was reduced by siRNA targeting PHLPP2 in miR-527-in-transfected Kyse510 cells (Figure 5A). We then examined its effect on cell proliferation by colony formation and anchorage-independent growth assay, and the result indicated that downregulation of PHLPP2 in miR-527-in-transfected Kyse510 cells dramatically increased cell proliferation (Figure 5B and C). Our results revealed that inhibition of PHLPP2 by siRNA counteracted the inhibition effects of miR-527-in on proliferation.

**Discussion**

In this study, we identified miR-527 as a novel oncogene in ESCC. We found that the expression of miR-527 was higher in ESCC cells as well as in ESCC patients’ tissues. Ectopic expression of miR-527 promoted cell proliferation and cell cycle of Kyse510 cells. Furthermore, PHLPP2 was identified as a functional target of miR-527 and was confirmed by the luciferase reporter assay. We also confirmed that the miR-527-PHLPP2 axis modulated proliferation by regulating
specific downstream tumor-associated genes (Cyclin D, p21, pRb, and Rb).

Growing evidences have indicated that miRNAs played essential roles in different types of cancer, including ESCC. MiR-202 was found to promote cell apoptosis in ESCC by targeting HSF2. Finding by Yi et al indicated that miR-193a-3p acted as an oncogene regulating cellular proliferation, migration, and apoptosis. Song et al indicated that miR-622 functioned as a tumor suppressor in ESCC by regulating E2F1. However, the role of miR-527 in ESCC has not been well understood. In our present study, we found that miR-527 expression was higher in ESCC cells and clinical tissues. Moreover, ectopic expression of miR-527 promoted cell proliferation, cell colony formation, anchorage-independent growth ability, and cell cycle of Kyse510 cells. More specifically, result of cell cycle assays indicated that miR-527 promoted cell proliferation of Kyse510 cells due to acceleration of the G1-S phase transition, then upregulation of cyclin D1 and p-Rb, and downregulation of p21 in overexpressing miR-527 Kyse510 cells.

PHLPP2 acted as a suppressive regulator of PI3K/AKT signaling pathway which was required for cell cycle progression through the G1-S phase. It has been demonstrated that PHLPP2 expression is regulated by several miRNAs in many kinds of cancers. In glioma, miR-372 regulated cell proliferation and invasion by directly targeting PHLPP2. In breast cancer, miR-32 was reported to promote cell proliferation by repressing PHLPP2 expression. In ovarian cancer, miR-760 expression was markedly upregulated and promoted cell proliferation by suppressing PHLPP2 expression. Herein, in this study, we identified the PHLPP2 was a direct target of miR-527 using bioinformatic methods and was confirmed by Luciferase assays.

Figure 3. Inhibition of miR-527 suppressed Kyse510 esophageal squamous cell carcinoma (ESCC) cell proliferation and cell cycle. A, Validation of miR-527 expression levels after transfection by polymerase chain reaction (PCR) analysis. B, MTT assays revealed that inhibition of miR-527 suppressed growth of Kyse510 ESCC cell lines. C, Representative micrographs (left) and quantification (right) of crystal violet-stained cell colonies. D, Inhibition of miR-527 impaired the anchorage-independent growth of Kyse510 ESCC cells. Representative micrographs (left) and quantification of colonies that were $>0.1$ mm (right). E, Flow cytometric analysis of the indicated Kyse510 ESCC cells transfected with NC or miR-527 inhibitor. Each bar represents the mean of 3 independent experiments. *$P < .05$. 

Xian et al
There are some limitations: miRNAs play vital role in obesity-induced cancer.\(^{29}\) The prevalence of obesity is high in Chinese population.\(^{30-32}\) Thus, obesity may have some impact on the relationship between miRNAs and ESCC.

Taken together, our study demonstrated that miR-527 repressed PHLPP2 expression and then promoted ESCC development and progression, suggesting miR-527 as a potential tumor-promoting miRNA. Our study laid theoretical foundation for promising new therapeutic targets of ESCC.

Authors’ Note

All authors designed the study together and performed the experiment together, analyzed the data and wrote the paper, and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.
Funding
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by Department of Surgical Oncology (Thoracic Tumor), Xinxian Central Hospital.

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