MicroRNA-96-5p Facilitates the Viability, Migration, and Invasion and Suppresses the Apoptosis of Cervical Cancer Cells by Negatively Modulating SFRP4

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Abstract
Purpose: The current study was intended to research the functional role and regulatory mechanism of microRNA-96-5p in the progression of cervical cancer. Methods: MicroRNA-96-5p expression in cervical cancer tissues was assessed by quantitative real-time polymerase chain reaction. The association between microRNA-96-5p expression and clinicopathological features of patients with cervical cancer was analyzed. MTT, flow cytometry, wound healing, and transwell assay were performed to evaluate the viability, apoptosis, migration, and invasion of Hela and SiHa cells. Targetscan, dual-luciferase reporter gene assay, and RNA pull-down analysis were constructed to evaluate the target relationship between microRNA-96-5p and secreted frizzled-related protein 4. Results: MicroRNA-96-5p was overexpressed in cervical cancer tissues, and microRNA-96-5p expression was markedly associated with the clinical stage and lymph node metastasis of patients with cervical cancer. Overexpressed microRNA-96-5p facilitated the viability, migration, invasion, and inhibited the apoptosis of Hela and SiHa cells, whereas suppression of microRNA-96-5p exerted the opposite trend. Secreted frizzled-related protein 4 was proved to be a target of microRNA-96-5p. Silencing of secreted frizzled-related protein 4 eliminated the anti-tumor effect of microRNA-96-5p on cervical cancer cells. Conclusions: MicroRNA-96-5p facilitated the viability, migration, and invasion and inhibited the apoptosis of cervical cancer cells via negatively regulating secreted frizzled-related protein 4.

Keywords
miR-96-5p, SFRP4, cervical cancer, proliferation, migration

Abbreviations
CC, cervical cancer; LN, lymph node; miRNA, microRNA; mRNA, messenger RNA; NC, negative control; OD, optical density; qRT-PCR, quantitative real-time polymerase chain reaction; SFRP, secreted frizzled-related protein; SFRP4, secreted frizzled-related protein 4.

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Introduction
Cervical cancer (CC) is a common malignancy of the female reproductive tract globally, with an estimated 500,000 new cases and 300,000 deaths occurring each year.¹ At present, the treatment of CC mainly includes surgical treatment and radiotherapy and sometimes chemotherapy for patients with metastasis or recurrence.² However, the clinical outcomes of CC are still poor, especially in patients with advanced CC. Hence, it is necessary to study the underlying mechanism of CC and develop novel therapeutic targets for CC.

MicroRNAs (miRNAs) are a type of noncoding small RNA molecules that can modulate translational inhibition or
degradation of messenger RNA (mRNA) by binding to the 3’-UTR of the target miRNA. Current, many miRNAs are considered to be crucial regulators of multiple biological processes, such as cell proliferation, apoptosis, metabolism, as well as differentiation. More importantly, miRNAs have been reported to act as protumor or antitumor genes in cancers, including CC. For example, miR-138 has been demonstrated to act as an antitumor role in CC by repressing the proliferation of CC cells. Xu et al have concluded that miR-21 promotes the progression of CC by targeting PTEN. MicroRNA-96-5p is important in the tumor progression of several cancer types. For instance, Pei et al have found that miR-96-5p acts as an oncogenic role in progression of colorectal cancer. Li et al have illustrated that miR-96-5p restrains the proliferation of prostate cancer cells via suppressing GPC1. Nevertheless, the functional role and mechanism of miR-96-5p in CC progression remain unclear.

Secreted frizzled-related protein 4 (SFRP4), maps to the short arm of chromosome 7 (7p14.1), is a member of secreted frizzled related proteins (SFRPs). Secreted frizzled related proteins is a family of 5 secreted glycoproteins that are considered as the depressors of the Wnt signaling pathway and are believed to participate in cell proliferation, migration, embryonic development, and carcinogenesis. Secreted frizzled-related protein 4 has the function of regulating cell proliferation and as an antitumor gene in various human malignancies. For example, Kendra et al have demonstrated that SFRP4 can suppress the growth of endometrial cancer cell, suggesting its antitumor effects in endometrial cancer. Jin et al have demonstrated that SFRP4 plays an antitumor role in gastric carcinomas by suppressing Wnt target genes. However, the regulatory correlation between miR-96-5p and SFRP4 in CC is rarely researched.

In current research, a series of functional experiments were constructed to explore the potential role and mechanism of miR-96-5p in CC progression. First, miR-96-5p expression was detected in CC tissues. Then, the actions of miR-96-5p on the cell viability, migration, invasion, and apoptosis in CC were analyzed. Finally, we evaluated the target relationship between miR-96-5p and SFRP4 in CC cells. Our investigations suggested that miR-96-5p might function as an oncogenic gene in the development of CC, providing a possible target for the diagnosis and therapy of CC.

Materials and Methods

Clinical Specimens

Cervical cancer tissues and adjacent normal tissues were collected from 60 patients with CC who underwent surgery from June 2016 to September 2018 in our hospital. Inclusion criteria for patients recruited in this study were all the patients were diagnosed based on pathological results and none of the patients had undergone chemotherapy or radiotherapy or other treatment history. Informed consents were obtained from all patients. This study was carried out with the permission of the local ethics committee (HASFUBJY-20-009).

Cell Culture

Immortalized human epithelial cell line HaCaT were obtained from the American Type Culture Collection (ATCC), human CC cell lines (Hela, SiHa, Me180, Ms751) were purchased from China Center for Type Culture Collection (CCTCC). All cells were incubated in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum in an incubator (MCO-15 AC; SANYO) at 37 °C. Hela (epithelial cell, adenocarcinoma) and SiHa (epithelial cell, squamous cell carcinoma) cells with relatively high miR-96-5p expression were selected in the follow-up experiments.

Cell Transfection

Hela and SiHa cells (1 × 10^5 cells per well) were collected and seeded into 24-well cell culture plates. After culturing for 24 hours, cells were collected for transfection. MicroRNA-96-5p inhibitor (miR-96 inhibitor), miR-96-5p inhibitor negative control (NC-inhibitor), miR-96-5p mimic (miR-96 mimic), and miR-96-5p mimic negative control (NC-mimic; Invitrogen) were transfected into Hela and SiHa cells using Lipofectamine 3000 reagent (Invitrogen), respectively. Cells were randomly divided into 5 groups: BLANK, miR-96-5p inhibitor, NC-inhibitor, miR-96-5p mimic, NC-mimic. Hela and SiHa cells without transfection were considered as BLANK group.

Secreted frizzled-related protein 4 siRNA (si-SFRP4) and SFRP4 siRNA negative control (si-NC) were obtained from Thermo Fisher Scientific. To investigate the interaction between miR-96-5p and SFRP4 in CC cells, Hela and SiHa cells were cotransfected with si-SFRP4 or si-NC and miR-96 mimic, miR-96-5p mimic negative control (NC-mimic; Invitrogen). Cells were randomly divided into 5 groups: miR-96-5p inhibitor, NC-inhibitor, miR-96-5p mimic, NC-mimic. Hela and SiHa cells without transfection were considered as BLANK group.

Quantitative Real-Time Polymerase Chain Reaction

TRIzol Plus RNA Isolation Reagents (Invitrogen) was utilized to isolate RNA from CC tissues and cells. RNAs were reverse transcribed using the reverse transcription kit (Takara). Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using the miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems) on ABI 7500HT Fast Real-Time PCR System (Applied Biosystems). The PCR amplification conditions were 95 °C for 3 minutes, 40 cycles at 95 °C for 15 seconds, 60 °C for 30 seconds, and 72 °C for 1 minute. The mRNA expression level was calculated using the 2^-ΔΔCt method. U6 or GAPDH was used as the internal reference of miR-96-5p or SFRP4. The primer sequences are listed in Table 1.
MTT Assay

A total of $2 \times 10^4$ cells were seeded in 96-well plates. After incubated for 24, 48, 72, and 96 hours, each well was added with 20 $\mu$L of MTT (5 mg/mL; Sigma). Four hours later, 150 $\mu$L dimethyl sulphoxide (Sigma) was added to terminate the reaction. The absorbance was analyzed on a microplate reader (Bio-Rad) at 450 nm.

Flow Cytometry Analysis

Cells (1 $\times 10^5$ cells) were suspended in 500 $\mu$L binding buffer. Then, cells were stained with 5 $\mu$L Annexin V-FITC and 10 $\mu$L propidium iodide for 20 minutes at room temperature in the dark. Subsequently, cell apoptosis rate was detected via flow cytometer (BD Biosciences).

Dual-Luciferase Reporter Gene Assay

Target Scan predicted the binding site between SFRP4 and miR-96-5p. According to the predication, the fragments of the binding site and mutant fragment were cloned into PsiCHECK-2 vector (Promega), separately named SFRP4-Mut and SFRP4-Wt. Hela and SiHa cells were cotransfected with SFRP4-Mut or SFRP4-Wt and miR-96 mimic or NC-mimic (Thermo Fisher Scientific) by using Lipofectamine 3000 (Invitrogen). After transfected for 48 hours, dual-luciferase reporter gene assay system (Promega) was utilized to measure the luciferase activity.

RNA Pull-Down Assay

Biotinylated SFRP4-Wt, SFRP4-Mut, and SFRP4 NC (Bio-SFRP4-Wt, Bio-SFRP4-Mut, and Bio-NC) were purchased from GenePharma. Hela and SiHa cells were transfected with Bio-SFRP4-Wt, Bio-SFRP4-Mut, and Bio-NC, respectively. After incubation for 48 hours, cells were lysed with lysis buffer. The mixture of Biotinylated RNA and cell lysates (Hela and SiHa cells) were incubated with Dynabeads-280 (Invitrogen) at 37 $^\circ$C for 1 hour. The biotinylated RNAs were measured by qRT-PCR.

Wound Healing Assay

Cells were seeded into 6-well plates at a density of $1 \times 10^5$ cells/well. When cells reach 90% confluence, an artificial scratch was created using pipette tip. Cells were incubated for 24 hours and then observed under an inverted microscope (Olympus). The migration rate was analyzed using Image J software (v1.8.0).

Transwell Invasion Assay

The invasion of CC cells was detected by 24-well transwell inserts (8-nm pore size; Corning Inc). In brief, transwell upper inserts were precoated with Matrigel (BD Biosciences) and then added with single-cell suspension ($2 \times 10^5$ cells). A total of 600 $\mu$L of cell suspension containing 100 ng/mL Stromal cell-derived factor 1 was added to the lower inserts. Then, the transwell inserts were cultured at 37 $^\circ$C for 24 hours. Cells from the lower inserts were fixed with 100% methanol at room temperature for 15 minutes and then stained with 0.1% Crystal violet at room temperature for 30 minutes. Four random visual fields of each insert were counted under a microscope.

Statistical Analysis

All experiments were performed in triplicate. All computations were carried out using the SPSS version 22.0 statistical software (SPSS Inc). Data were expressed as mean ± standard deviation. Student $t$ test was used to compare the significant difference of 2 groups, while 1-way analysis of variance with least significant difference was applied when analyzing more than 2 groups. Differences were considered statistically when $P$ was less than .05.

Results

MicroRNA-96-5p Was Overexpressed in CC

The expression of miR-96-5p was detected in CC tissues and cells. As illustrated in Figure 1A, relative miR-96-5p expression was remarkably elevated in CC tissues than that in normal tissues ($P < .001$). MicroRNA-96-5p was markedly upregulated in CC cell lines (Hela, SiHa, Me180, and Ms751) in comparison to the immortalized human epithelial cell line HaCaT ($P < .05$; Figure 1B). In addition, Hela and SiHa cells with relatively high miR-96-5p expression were used in the follow-up experiments. As illustrated in Table 2, miR-96-5p expression was remarkably relevant to clinical stages and lymph node (LN) metastasis of patients with CC ($P < .01$). Quantitative RT-PCR revealed that relative miR-96-5p expression was increased in clinical stage II in comparison to stage I ($P < .01$; Figure 1C). Meanwhile, relative miR-96-5p expression was enhanced in LN metastasis group in comparison to non-LN metastasis group ($P < .01$; Figure 1D). These results indicated that miR-96-5p was upregulated in CC and positively associated with advanced clinical stage and LN metastasis.
MicroRNA-96-5p Facilitated the Viability and Repressed Apoptosis of CC Cells

To evaluate the regulatory role of miR-96 on CC cells, miR-96-5p was silenced and overexpressed in CC cells. Quantitative RT-PCR indicated that the relative miR-96-5p expression in miR-96 inhibitor group was markedly declined, and remarkably elevated in miR-96 mimic group, in comparison to BLANK group (P < .01; Figure 2A). As presented in Figure 2B, the OD_{450} value was remarkably declined in miR-96 inhibitor group in comparison with the NC-inhibitor group, whereas remarkably enhanced in miR-96 mimic group compared to NC-mimic group (P < .05). Flow cytometry analysis illustrated that the apoptosis exerted the reverse trend in comparison with that of the cell viability (P < .01; Figure 2C). Transfection of NC-inhibitor and NC-mimic did not influence these changes in Hela and SiHa cells. These findings indicated that miR-96-5p can inhibit the proliferation and promote the apoptosis of CC cells.

MicroRNA-96-5p Promoted Migration and Invasion of CC Cells

The regulatory role of miR-96 on the migration and invasion of CC cells was further analyzed. As illustrated in Figure 3A and B, relative migration and invasion rate were remarkably declined in miR-96 inhibitor group compared to NC-inhibitor group, whereas markedly elevated in miR-96 mimic group in comparison to NC-mimic group (P < .05). Transfection of NC-inhibitor and NC-mimic did not affect the migration and

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**Table 2.** The Relationship Between MiR-96-5p Expression and Clinicopathological Features of Patients With Cervical Cancer.

| Clinicopathological features | No. of cases (N = 60) | miR-96-5p expression | P value |
|-----------------------------|-----------------------|----------------------|---------|
| Age, years                  |                       |                      |         |
| < 45                        | 41                    | 2.672 ± 0.281        | .1593   |
| ≥ 45                        | 19                    | 2.560 ± 0.286        |         |
| Clinical stages             |                       |                      |         |
| I phase                     | 36                    | 2.542 ± 0.175        | .0011^a |
| II phase                    | 24                    | 2.779 ± 0.357        |         |
| Histological classification |                       |                      |         |
| Squamous cell carcinoma     | 34                    | 2.691 ± 0.299        | .0934   |
| Adenocarcinoma              | 26                    | 2.566 ± 0.255        |         |
| Lymph node metastasis       |                       |                      |         |
| No                          | 22                    | 2.485 ± 0.201        | .0013^a |
| Yes                         | 38                    | 2.724 ± 0.292        |         |

^aP < .01.
invasion of Hela and SiHa cells. The results above indicated that miR-96-5p can inhibit the migration and invasion of CC cells.

MicroRNA-96-5p Directly Targeted SFRP4 and Negatively Modulated the Expression of SFRP4

To reveal the regulatory mechanism of miR-96-5p on CC, the downstream targets of miR-96-5p were predicted using Targetscan. A binding site between miR-96-5p and SFRP4 was shown in Figure 2A. Quantitative RT-PCR demonstrated that SFRP4 was remarkably underexpressed in CC tissues compared to normal tissues ($P < .001$; Figure 4B). Consistently, SFRP4 was markedly downregulated in Hela and SiHa cells in comparison to immortalized human epithelial cell line HaCaT ($P < .01$; Figure 4C). In addition, Spearman correlation analysis indicated a negative association between expression of miR-96-5p and SFRP4 ($P = .0070, r = -.3446$; Figure 4D). Furthermore, miR-96 mimic significantly declined the luciferase activity of SFRP4-Wt, whereas did not influence the luciferase activity of SFRP4-Mut ($P < .01$; Figure 4E). As illustrated in Figure 4F, RNA pull down analysis further validated that miR-96-5p targeted to SFRP4 directly ($P < .01$). Relative mRNA expression of SFRP4 was markedly elevated in miR-96 inhibitor group, while declined in miR-96 mimic group ($P < .01$, Figure 4G). These findings illustrated that SFRP4 is a target of miR-96-5p.

Silencing of SFRP4 Eliminated the Effects of MiR-96-5p Inhibitor on the Viability, Migration, Invasion, and Apoptosis of Hela Cells

To explore whether SFRP4 participated in the action mechanism of miR-96-5p in CC, Hela cells were cotransfected with si-SFRP4 or si-NC and miR-96 inhibitor or NC-inhibitor. Secreted frizzled-related protein 4 was lower expressed in si-SFRP4 group than that in BLANK group ($P < .01$). The OD$_{450}$ value, relative migration, and invasion rate were markedly increased in si-SFRP4 + NC-inhibitor group, in comparison to the si-NC + NC-inhibitor group. The apoptosis rate was inversed with the trend of cell viability ($P < .01$). MicroRNA-96 inhibitor partially reversed the antitumor effect of si-SFRP4 on Hela cells. Taken together, silencing of miR-96-5p inhibited the proliferation, migration, and invasion and promoted the apoptosis of Hela cells by regulating SFRP4.
Discussion

Previous researches have been confirmed that abnormal miRNAs expression is closely relevant to the occurrence and development of CC. Here, we detected that relative miR-96-5p expression was remarkably upregulated in CC tissues. MicroRNA-96 expression is dysregulated in some forms of cancers, such as hepatocellular carcinoma, non-small-cell lung cancer (NSCLC), bladder cancer, as well as colorectal cancer. Wu et al. have revealed that miR-96 is markedly upregulated in bladder cancer tissues and functions as a protumor gene in bladder cancer. Li et al. have discovered that miR-96 expression is remarkably enhanced in NSCLC tissues, and its upregulation is correlated with LN metastasis. Consistent with the previous studies, we concluded that miR-96-5p might serve as a protumor role in CC. In addition, we discovered that miR-96-5p expression was remarkably related to clinical stages and LN metastasis of patients with CC. Therefore, we considered that miR-96-5p might serve as a diagnostic biomarker for CC.

Micro-RNAs are reported to participate in numerous biological processes of CC. In our research, overexpressed miR-96-5p was detected to increase the viability, migration, invasion, and restrain the apoptosis of HeLa and SiHa cells, while downregulation of miR-96-5p exerted the opposite effects. Recent researches in diverse types of cancers were consistent with our findings. Pei et al. have illustrated that miR-96-5p expression
is elevated in colorectal tissues, and overexpression of miR-96-5p accelerates migration and invasion of SW480-7 cells. Shi et al. have revealed that miR-96-5p induces proliferation, migration, invasion, and represses apoptosis of breast cancer cells. Liu et al. have demonstrated that the expression of miR-96-5p is markedly elevated in osteosarcoma tissues and overexpressed miR-96-5p enhanced the proliferation and migration of osteosarcoma cells. Therefore, we concluded that miR-96-5p might be an oncogene in CC progression.

Micro-RNAs generally play their roles by regulating their target genes. In this study, we demonstrated that SFRP4 was targeted to miR-96-5p directly. Secreted frizzled-related protein 4 is considered to play as a vital regulator in cell self-renew, proliferation, and apoptosis. Secreted frizzled-related protein 4 is regarded as an antitumor gene in varied cancer types, such as mesothelioma, chronic lymphocytic leukemia, and esophageal adenocarcinoma. Downregulation of SFRP4 is found in these cancers, which is in line with our findings. We detected a significant decrease of SFRP4 expression in CC tissues and cell lines. Previous studies have demonstrated that SFRP4 can act as the target gene for miRNAs in diverse human diseases, including cancer. For instance, Luo et al. have illustrated that circulating miR-103b functions as a diagnostic biomarker for type 2 diabetes mellitus via regulating SFRP4. Han et al. have revealed that overexpressed miR-135b-5p facilitates the unfavorable clinical characteristics of pancreatic cancer through inhibiting SFRP4. Here, we discovered that silencing of SFRP4 eliminated the antitumor actions of miR-96-5p inhibition on CC cells. Therefore, we considered that miR-96-5p contribute to CC progression via negatively modulating SFRP4.

As a secreted protein, SFRP4 can secrete out of the cytoplasm and block the Wnt signaling pathway through binding the downstream Frizzled receptor. Bhuvanalakshmi et al. have shown that SFRP4 improves the response to commonly used chemotherapeutics in gliomas by modulating epithelial to mesenchymal transition via the Wnt/ß-catenin pathway. He et al. have found that methylation silencing of SFRP4 promotes cell growth, inhibits chemodrug-induced apoptosis, and activates Wnt pathway in mesothelioma even in the absence of

**Figure 4.** MicroRNA-96-5p directly targeted to SFRP4. A, Target scan was used to predict the binding site between miR-96-5p and SFRP4. B, Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to determine the expression of SFRP4 in cervical cancer (CC) tissues (N = 60) and normal tissues (N = 60). C, The expression of SFRP4 in CC cell lines was assessed by qRT-PCR. D, Spearman correlation analysis was used to detect the relationship between the expression of miR-96-5p and SFRP4. E, Dual-luciferase reporter gene assay was performed to verify the target correlation between miR-96-5p and SFRP4. F, RNA pull down analysis was used to analyze the interaction between miR-96-5p and SFRP4. G, The expression of SFRP4 in transfected Hela and SiHa cells was detected by qRT-PCR. BLANK, Hela, and SiHa cells without transfection; NC-inhibitor, Hela, and SiHa cells transfected with miR-96-5p inhibitor negative control; miR-96 inhibitor, Hela, and SiHa cells transfected with miR-96-5p inhibitor; NC-mimic, Hela, and SiHa cells transfected with miR-96-5p mimic negative control; miR-96 mimic, Hela, and SiHa cells transfected with miR-96-5p mimic; Bio-NC, Hela, and SiHa cells transfected with SFRP4 negative control; Bio-SFRP4-Wt, Hela, and SiHa cells transfected with Bio-SFRP4-Wt; Bio-SFRP4-Mut, Hela, and SiHa cells transfected with Bio-SFRP4-Mut. ***P < 0.001 vs normal tissues (B); **P < .01 vs HaCaT (C); ***P < .01. ***P < .001 vs NC-mimic (E); **P < .01 vs Bio-NC (F); **P < .01 vs NC-inhibitor; **P < .01 vs NC-mimic (G). NC indicates negative control; SFRP4, secreted frizzled-related protein 4.
β-catenin. It is plausible to consider that SFRP4 has a same exogenous secretion manner and similar action mechanism in CC. However, the regulatory mechanism of miR-96-5p/SFRP4 axis involving Wnt pathway is not analyzed in this study. It waits for more efforts to focus on the action mechanisms of miR-96-5p/SFRP4 in the incoming studies of CC.

In summary, miR-96-5p was upregulated in CC tissues, and miR-96-5p expression was remarkably correlated with clinical stages and LN metastasis of patients with CC. MicroRNA-96-5p enhanced the viability, migration, and invasion and suppressed apoptosis of CC cells through negatively targeting SFRP4. Consequently, we concluded that miR-96-5p might
serve as a diagnostic biomarker and a novel therapeutic target for CC treatment.

Authors’ Note
Huilang Zhang and Ruxin Chen contributed equally to this work. Huilang Zhang and Ruxin Chen made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; Jinyanshao took part in drafting the article or revising it critically for important intellectual content; all authors gave final approval of the version to be published and agree to be accountable for all aspects of the work. The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request. This study was approved by the ethics committee of Huai’an Maternity and Child Health Hospital (Approval Number HASUBJY-20-009), and all participating patients signed written informed consent.

Declaration of Conflicting Interests
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