Long non-coding RNA RP11-284F21.9 functions as a ceRNA regulating PPWD1 by competitively binding to miR-769-3p in cervical carcinoma

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Abbreviations

IncRNA: Long non-coding RNA

PPWD1: Peptidylprolyl isomerase domain and WD repeat-containing protein1

RIP: RNA immunoprecipitation

DMEM: Dulbecco’s modified Eagle medium

FBS: fetal bovine serum
Abstract

Cervical carcinoma is the most common gynaecological cancer in women worldwide. Emerging evidence has shown that long non-coding RNAs (lncRNAs) participate in multiple biological processes of cervical carcinoma tumorigenesis. We aimed to investigate the function of a novel lncRNA RP11-284F21.9 in cervical carcinoma. We found that RP11-284F21.9 was downregulated in cervical carcinoma tissues and cell lines. Overexpression of RP11-284F21.9 inhibits proliferation, invasion and migration of cervical carcinoma cells in vitro. Further, we identified that RP11-284F21.9 directly interacted with miR-769-3p and functioned as the miR-769-3p sponge. Mechanistically, we showed that miR-769-3p regulated PPWD1 expression by targeting PPWD1 3’-UTR. Furthermore, xenograft tumor model revealed that Overexpression of RP11-284F21.9 inhibited tumor growth of cervical carcinoma in vivo. Taken together, our results demonstrate that lncRNA RP11-284F21.9 functions as tumor suppressor and regulates PPWD1 expression through competitively binding to miR-769-3p in cervical carcinoma, suggesting that RP11-284F21.9/miR-769-3p/PPWD1 axis could serve as a promising prognostic biomarker and therapeutic target for cervical carcinoma.

Key words: RP11-284F21.9; miR-769-3p; PPWD1; cervical carcinoma.
1. Introduction

Cervical carcinoma is one of the most common aggressive gynaecological cancers and ranks the 4th leading cause of cancer-related deaths in women worldwide (1). Significant advances in diagnosis and therapeutic technologies greatly improve the cervical carcinoma treatment (2). However, due to the high invasive and metastatic ability of cervical carcinoma, the prognosis remains poor in patients at advanced cancer stages (3, 4). Thus, it is critical to understand the mechanisms underlying the tumorigenesis and metastasis of cervical carcinoma and develop new therapeutic strategies to treat the disease.

LncRNAs are a class of long non-coding RNAs with > 200 nucleotides involved in multiple biological processes including cancer development and metastasis (5-7). Emerging evidence has shown that various LncRNAs are involved in the regulation of cervical carcinoma tumorigenesis and functions as oncogenes or tumor suppressor (8). For instance, LncRNA AB073614, SNHG14, DANCR and MNX1-AS1 promote cervical carcinoma progression via different signaling pathways (9-13). Other LncRNAs, such as WT1-AS and TUSC8 inhibit the proliferation, migration and invasion of cervical cancer cells (14, 15). RP11-284F21.9 is a new LncRNA defined by pan-cancer transcriptomic analysis (16). However, the function of RP11-284F21.9 in cervical cancer is not clear.

MiRNAs are small non-coding RNAs with ~22 nucleotide that post-transcriptionally regulate gene expression by binding to the 3’-UTR of their target mRNAs (17). Increasing studies have found that miRNAs play critical roles in the tumorigenesis of most human malignancies, including cervical cancer (18-20). Interestingly, LncRNAs can exert their functions as competing endogenous RNAs (ceRNAs) and sponge activity for
miRNAs (21-23). Multiple studies have revealed the lncRNA-miRNA-mRNA interaction network in cervical carcinoma (24, 25). LncRNA SOX21-AS1 could sponge miR-7/VDAC1 and promote cervical cancer development (26). Wang Q., et al. reported that lncRNA NOC2L-4.1 regulated miR-630/YAP1 pathway and functioned as a tumor oncogene in cervical cancer (27). Though much progress has been made, the understanding of the roles of lncRNA-miRNA-mRNA network in cervical carcinoma remains largely unclear.

In this study, we identified a novel lncRNA RP11-284F21.9 that was downregulated in cervical carcinoma tissue and cell line. We demonstrated that RP11-284F21.9 directly interacted with miR-769-3p as a ceRNA and bioinformatics analysis revealed that miR-769-3p regulated PPWD1 expression by targeting PPWD1 3’-UTR. We found that RP11-284F21.9-miR-769-3p-PPWD1 axis regulated proliferation, migration and invasion of cervical cancer cells both in vitro and in vivo. In conclusion, we indicate that lncRNA RP11-284F21.9 functions as a tumor suppressor in cervical carcinoma via targeting miR-769-3p/PPWD1, providing a potential promising therapeutic target for cervical carcinoma.

2 Materials and Methods

2.1 Clinical samples

In this study, cervical carcinoma tissues and the adjacent normal tissues were obtained from the patients who underwent surgical treatment at our hospital. All the tissues were stored in liquid nitrogen before RNA isolation. This study was approved by the Ethics Committee of our hospital. Written informed consent was signed before specimen collection.
2.2 Cell lines and culture

The cervical cancer cell lines Hela, SiHa, C33A, CaSki, and the normal human cervical cell line H8 were obtained from American Type Culture Collection (ATCC). All these cells were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were cultured in a humidified incubator at 37°C with 5% CO2. Mycoplasma detection was negative in all cell lines.

2.3 Transfection

Briefly, 3×10^5 cells were seeded in 6-well plates. When cells were approximately at 70%-80% confluence, the medium was changed to FBS-free DMEM and the transfection was performed using Lipofectamine™ 3000 (Invitrogen) according to manufacturer’s protocols. Cells were grouped as follows: (1) mock control and pcDNA3.1-RP11-284F21.9 group; (2) negative control (NC) group and miR-769-3p mimics group; (3) NC group, pcDNA3.1-RP11-284F21.9 group, miR-769-3p mimics group, miR-769-3p inhibitor group and pcDNA3.1-RP11-284F21.9+ miR-769-3p mimics group; (3) NC group, miR-769-3p mimics group, pcDNA3.1-PPWD1 group, miR-769-3p mimics+pcDNA3.1- PPWD1 group. Cells were harvested at indicated time points for further experiments.

2.4 RT-qPCR

Total RNA from tissues and cell lines was extracted by using TRIzol Reagent (invitrogen). cDNA was synthesized through the PrimeScript RT reagent Kit (TaKaRa) according to manufacturer’s instruction. Then real-time PCR assay was performed by employing SYBR PrimeScript™ PLUS RT-PCR Kit (TaKaRa) to detect the expression
level of LncRNA-RP11-284F21.9, miR-769-3p and PPWD1. The reaction condition of PCR is 95°C for 30 seconds, 60°C for 40 seconds and for 40 cycles. β-actin or U6 were used as an endogenous control to normalize. The relative expression levels were counted by $2^{-\Delta\Delta C_t}$ method. The primer sequence used in this study were as follows: LncRNA-RP11-284F21.9: forward, AGGATTGGCACTCActTCGG, reverse, TCTCTCACCACGTCTGGTCT; miR-769-3p: forward, 5′-GCGGCGGCTGGGATCTCCGGGGTC-3′, reverse, 5′-GTGCAGGGTGTCGAGGTT-3′; β-actin: forward, 5′-TGTCACCAACTGGGACGATA-3′, reverse, 5′-GGGGTGTTGAAGGTCTCAAA-3′; U6: forward, 5′-ATTGGAACGATACAGAGAAGATT-3′, reverse, 5′-GGAACGCTTCACGAATTTG-3′.

2.5 Cell proliferation assay

After transfection, cell proliferation ability was evaluated by CCK-8 assay (Dojindo). Cells were cultured for 0, 24, 48 or 72 h in 96-well plates, after that, 10 μl of CCK-8 (5 mg/mL) was added to the culture medium in each well. The absorbance at 450 nm was measured by Exl 800 microplate reader (Bio-tek).

EdU assay was performed to determine DNA synthesis in proliferating cells by using an EdU assay Kit (invitrogen). After transfection, cells were cultured for 48h, fixed with 4% paraformaldehyde and permeabilized by 0.3% Triton X-100. Then cells were incubated with 10 μM EdU for 2 h, and cell nuclei were stained with DAPI (5μg/ml). The number of Edu-positive cells was counted under a microscope in five random fields (Olympum). All assays were independently performed in triplicate.

2.6 Cell cycle analysis
Briefly, cells were gathered, and fixed with 70% ethanol at 4 °C overnight. After that, cells were treated with ribonuclease A (20 μg/ml, Sigma-Aldrich) and incubated with propidium iodide (50 μg/ml, Sigma-Aldrich) for 30 min at 37°C. Then, population in G2-M, S and G0-G1 phase was determined by flow cytometry (Becton Dickinson).

2.7 Wound-healing assay

After transfection, Hela or SiHa cells were seeded in 6-well plates and grown to 90% confluence. The wound was created by scratching with a sterile pipette tip (200 μl). The cells were cultured in DMEM medium with 10% FBS. The closure of wound was monitored by an inverted optical microscope (Olympus) at 0 and 48 h.

2.8 Cell migration/invasion assay

The 24-well transwell chambers (Costar) with Matrigel-coated membranes were used for invasion assay. 2×10⁵ Hela or SiHa cells in 100 μl FBS-free DMEM were added to the upper chamber and 500 μl DMEM medium with 10% FBS were added to the bottom chamber. After 48h, the invading cells in the bottom chamber were stained with 0.1% crystal violet. The cells were observed and calculated under the inverted microscope (Olympus).

2.9 Western blotting

Total protein from tissues or cells was extracted using RIPA lysis buffer with 1% protease inhibitors. BCA protein assay kit (Thermo) was used to determine the concentration of obtained total protein. 30 μg of proteins were separated on 10% SDS-PAGE and electro-transferred to PVDF membrane (Millipore). And then were incubated with anti-PPWD1 (Abcam) and anti-β-actin (Abcam) at 4°C overnight. After washed, the
membrane was incubated with a HRP-labelled secondary antibody IgG (Abcam) at room temperature for 1h. Immunolabelling was visualized by using the ECL system (Millipore).

2.10 Luciferase reporter assay

The 3’-UTR of RP11-284F21.9 containing the predicted miR-769-3p binding site was amplified by PCR. And then was cloned into a Dual-luciferase miRNA Target Expression Vector (Promega) for forming RP11-284F21.9-wild-type (RP11-284F21.9-Wt). The same approach was used to forming RP11-284F21.9-mutated-type (RP11-284F21.9-Mut). Similarly, PPWD1-wild-type (PPWD1-Wt) and PPWD1-mutated type (PPWD1-Mut) were set up. Then the luciferase activities were tested by Dual-luciferase reporter assay system (Promega).

2.11 RNA immunoprecipitation (RIP) assay

RIP assay was performed by Imprint RNA immunoprecipitation kit (Sigma-Aldrich) according to manufacturer’s instruction. Briefly, IgG-induced Hela cells were collected and resuspended in RIP lysis buffer (Solarbio), subsequently centrifuged at 12 000 g for 5 minutes. Then, cell lysate was incubated with anti-Argonaute2 (anti-Ago2) or anti-IgG (negative control) overnight at 4°C, followed by the addition of Protein A magnetic beads to get the immunoprecipitation complex. Lastly, the relative enrichment of RP11-284F21.9 and miR-769-3p were detected by RT-qPCR.

2.12 Tumor xenograft model

The posterior flank of the 6-week-old male BALB/c nude mice (n=10) were subcutaneously injected with SiHa (5×10⁷) cells transfected with mock or pcDNA3.1-RP11-284F21.9. Tumor volumes were examined every 4 days, and tumor tissues were photographed and weighed on day 13. At 13 days after inoculation, mice were mice were
euthanized by intraperitoneal injection of tripled 3% pentobarbital sodium. The expression level of miR-769-3p in tumor tissues was detected by qRT-PCR. The protein level of PPWD1 in tumor tissues was measured by western blot. The expression of Ki-67 was analyzed by immunohistochemical (IHC) staining. All animal work was performed at Xi’an Jiaotong University, and all animal protocols were approved by the ethics committee of the Affiliated Cancer Hospital & Institute of Xi’an Jiaotong University.

2.13 Statistical analysis

Statistical analyses were performed with GraphPad Prism 6.0 software and data were expressed as mean ± SD. Statistical comparisons were made by one-way analysis of variance (ANOVA) and P < 0.05 indicated a statistically significant difference.

3. Results

3.1 RP11-284F21.9 is downregulated in cervical carcinoma tissues and cell lines

To investigate the function of lncRNA RP11-284F21.9 in cervical carcinoma, we first assessed the expression levels of lncRNA RP11-284F21.9 in cervical carcinoma tissues and adjacent normal tissues. The results showed that lncRNA RP11-284F21.9 expression was significantly lower in cervical carcinoma tissues in comparison with that in adjacent normal tissues (p < 0.01, Figure 1A). Furthermore, we demonstrated that the expression levels of lncRNA RP11-284F21.9 were also remarkably downregulated in four different cervical cancer cell lines (Hela, SiHa, C33A, and CaSki) compared with that in normal human cervical cell line H8 (Figure 1B). Hela and SiHa, which had relative lower lncRNA RP11-284F21.9 expression, were selected for the subsequent experiments.
3.2 Overexpression of RP11-284F21.9 inhibits proliferation, invasion and migration of cervical carcinoma cells in vitro

To evaluate the function of lncRNA RP11-284F21.9 on cervical carcinoma cells, we constructed pcDNA3.1-RP11-284F21.9 to overexpress lncRNA RP11-284F21.9. The overexpression efficiency was confirmed in both Hela and SiHa cells (Figure 2A). Cell proliferation assay showed that the proliferation of Hela or SiHa cervical carcinoma cells transfected with pcDNA3.1-RP11-284F21.9 was remarkably decreased in comparison with that in cells transfected with mock control ($p < 0.01$, Figure 2B and 2C). The inhibition of cell proliferation by lncRNA RP11-284F21.9 overexpression was further confirmed in Hela or SiHa cells by using EDU staining assay (Figure 2D and 2E). We also performed cell cycle analysis in Hela or SiHa cells transfected with mock control or pcDNA3.1-RP11-284F21.9. Overexpression of RP11-284F21.9 significantly arrested cervical carcinoma cells at G2 phase with less cells in G1/S phase (Figure 2F and 2G). Moreover, transwell assay revealed that overexpressing lncRNA RP11-284F21.9 drastically inhibited cell invasion in Hela or SiHa cervical carcinoma cells ($p < 0.01$, Figure 2H-2I). In addition, we also conducted wound-healing assay and found that lncRNA RP11-284F21.9 overexpression decreased the relative migration distance of Hela or SiHa cells ($p < 0.01$, Figure 2J and 2K). Consistently, overexpression of lncRNA RP11-284F21.9 inhibited the expression of epithelial-mesenchymal transition (EMT) markers and proliferation marker Ki-67 in Hela or SiHa cells (Figure S4A and S5A). In summary, our results suggested that lncRNA RP11-284F21.9 overexpression inhibited proliferation, invasion and migration of cervical carcinoma cells in vitro.
3.3 RP11-284F21.9 directly interacts with miR-769-3p and functions as the miR-769-3p sponge

To explore the underlying mechanisms that RP11-284F21.9 regulates cervical carcinoma cell proliferation, invasion and migration, we used the DIANA tool combined the dataset of miRNAs involved in cell cycle regulation (GO:0000086) to search for the target of RP11-284F21.9 (Figure S1). Emerging evidence has shown that IncRNAs function as competing endogenous RNAs (ceRNAs) in regulating gene expression as miRNA sponges (28, 29). There are 11 miRNAs potentially interacting with RP11-284F21.9 while overexpression of RP11-284F21.9 significantly inhibited the expression of miR-769-3p (Figure S1). MiR-769-3p was identified to have the complementary binding sites with RP11-284F21.9 (Figure 3A). Luciferase reporter assay was conducted and the results showed miR-769-3p mimics significantly inhibited the luciferase activity in HEK293 cells transfected with pmirGLO-RP11-284F21.9-WT, but not in HEK293 cells transfected with pmirGLO-RP11-284F21.9-Mut (Figure 3B). We also performed RNA immunoprecipitation experiment and found that antibody against argonaute-2 (Ago2) could specifically enrich both RP11-284F21.9 and miR-769-3p (Figure 3C). Pearson correlation analysis revealed that the expression of IncRNA RP11-284F21.9 was negatively associated with the expression of miR-769-3p (Figure S3A). These findings suggested that RP11-284F21.9 could specifically and directly interact with miR-769-3p.

We further verified the interaction between RP11-284F21.9 and miR-769-3p using mi769-3p mimics or inhibitor. As shown in Figure 3D, Hela or SiHa cells transfected with pcDNA3.1-RP11-284F21.9 or miR-769-3p inhibitor significantly inhibited miR-769-3p expression. While miR-769-3p mimics markedly increased the miR-769-3p
levels, overexpression of RP11-284F21.9 partially reversed the increase of miR-769-3p.

Functionally, compared with that of negative control group, overexpression of RP11-284F21.9 or miR-769-3p inhibitor transfection had similar biological functions on Hela and SiHa cells, such as cell proliferation suppression, cell cycle arrested at G2 phase and inhibition of cell invasion (Figure 3E-3K). MiR-769-3p mimics enhanced cell proliferation and cell invasion, with more cells proceeding to S phase of cell cycle; however, simultaneously overexpression RP11-284F21.9 reversed the function effects of miR-769-3p overexpression (Figure 3E-3K). Thus, we demonstrated that RP11-284F21.9 directly interacts with miR-769-3p and functions as the miR-769-3p sponge.

3.4 MiR-769-3p regulates PPWD1 expression by targeting PPWD1 3’-UTR.

Previous study has shown that miR-769-3p could regulate breast cancer cell apoptosis via downregulating NDRG1 (30). To search for the target of miR-769-3p in cervical carcinoma, bioinformatics analysis was performed and PPWD1 was predicted as a direct target of miR-769-3p (Figure 4A). Luciferase reporter experiment confirmed the interaction between miR-769-3p and WT 3’-UTR of PPWD1 (Figure 4B). Consistently, the expression of PPWD1 was significantly downregulated in cervical cancer tissues and cell lines compared with that in control tissues or cell line (Figure S2). Overexpression miR-769-3p inhibited the expression of PPWD1 in Hela or SiHa cells, and transfection with pcDNA3.1-PPWD1 recused PPWD1 expression in Hela or SiHa cells transfected with miR-769-3p mimics (Figure 4C). In addition, Pearson correlation analysis indicated that the expression of miR-769-3p was negatively correlated with the expression of PPWD1 in cervical cancer tissues (Figure S3B). We also evaluated the effect of miR-769-3p and PPWD1 overexpression on biological function of Hela and SiHa cells. As
shown in Figure 4D-4J. miR-769-3p overexpression promoted cell proliferation and
invasion, with more cells proceeding to S phase of cell cycle. However, overexpression
of PPWD1 had the reverse effects in Hela or SiHa cells. Intriguingly, overexpression of
miR-769-3p, together with PPWD1 overexpression in Hela or SiHa cells, resulted in
similar cell proliferation, cell cycle distribution and cell invasion compared with those
cells transfected with NC (Figure 4D-4J). Consistently, miR-769-3p mimics inhibited
PPWD1 protein expression while pcDNA3.1-PPWD1 transfection could rescue the
protein expression levels of PPWD1 (Figure 4K and 4L). In addition, overexpression of
PPWD1 inhibited the expression of epithelial-mesenchymal transition (EMT) markers
and proliferation marker Ki-67 in Hela or SiHa cells, while miR-769-3p mimics exhibited
the opposite function (Figure S4 and S5). Taken together, our data indicated that MiR-
769-3p directly regulated PPWD1 expression and miR-769-3p/PPWD1 axis was critical
for proliferation and invasion of cervical cancer cells.

3.5 Overexpression of RP11-284F21.9 inhibits tumor growth of cervical carcinoma
in vivo

We further evaluated the function of RP11-284F21.9 on tumor growth in vivo using
xenograft tumor model. SiHa cells were transfected with mock control or pcDNA3.1-
RP11-284F21.9 and implanted into nude mice. Overexpression of RP11-284F21.9
remarkably decreased cervical carcinoma growth and development (Figure 5A).
Compared with the tumors in Mock control group, the tumor growth was significantly
inhibited in pcDNA3.1-RP11-284F21.9 group with drastically smaller xenograft tumor
size/weight (Figure 5B-5D). We also checked the miR-769-3p and PPWD1 expression in
tumor tissues. As shown in Figure 5E-5H, tumor tissues from pcDNA3.1-RP11-
284F21.9 group showed remarkably lower expression levels of miR-769-3p, with significantly higher expression levels of PPWD1. Consistently, overexpression of RP11-284F21.9 inhibited cell proliferation, with a lower expression of proliferation marker Ki-67 in xenograft tumor tissues (Figure 5I-5J). Overall, our data suggests that Overexpression of RP11-284F21.9 inhibits tumor growth of cervical carcinoma in vivo via regulating miR-769-3p/PPWD1.

4. Discussion

Accumulating evidence has shown that lncRNAs can function as oncogene or tumor suppressors in various cancers (6, 31). LncRNAs can exert their functions as ceRNAs by sponging miRNAs in regulating target mRNAs (21-23). Here we further extended the understanding of lncRNA-miRNA-mRNA network in cervical cancer by showing that RP11-284F21.9 regulates PPWD1 expressing by competitively binding to miR-769-3p.

LncRNAs show great diagnostic and prognostic values in multiple tumors (32, 33). In this study, we found that lncRNA RP11-284F21.9 was downregulated in cervical cancer tissues and cell lines (Figure 1), which could be used a novel biomarker for diagnosis and prognosis of cervical cancer. Consistent with our findings, comprehensive network analysis revealed that RP11-284F21.9 was a promising prognostic signature in lung adenocarcinoma (34). Xiaoshun Shi et. al reported that RP11-284F21.9 could predict lung adenocarcinoma-specific overall survival (35). Functionally, we also demonstrated that overexpression of RP11-284F21.9 inhibited cervical cancer cell proliferation, invasion and migration in vitro (Figure 2) and suppressed cervical tumor development in vivo (Figure 5).
MiR-769-3p was predicted to have the complementary binding sequences of lncRNA RP11-284F21.9 (Figure 3). Mir-769-3p was demonstrated to aberrant expressed in pediatric gliomas by a microRNA microarray assay (36). Also, miR-769-3p could inhibit cell proliferation and enhance apoptosis in breast cancer MCF-7 cells (30). We confirmed that miR-769-3p promoted cervical carcinoma Hela and SiHa cells proliferation, migration and invasion in our study. Moreover, overexpression of miR-769-3p could abolish the inhibitory effect of RP11-284F21.9 overexpression (Figure 3). Thus, our results suggest that RP11-284F21.9 and miR-769-3p functionally antagonize each other in cervical cancer cells.

Bioinformatics analysis found that miR-769-3p directly regulated PPWD1 expression by targeting 3’-UTR of PPWD1 (Figure 4). To our knowledge, this is the first report demonstrating the tumor suppressor role of PPWD1 in cervical cancer. Previous studies have shown that PPWD1 might functional relate to cancer pathogenesis as it has the well-characterized WD40 domain which has critical functions in tumorigenesis (37). Taken together, the regulatory network of RP11-284F21.9-miR-769-3p-PPWD1 could be a reliable drug target for cervical cancer therapy.

5. Conclusion

In summary, our findings reveal that lncRNA RP11-284F21.9 might be a tumor suppressor in cervical carcinoma and function as a ceRNA in regulating PPWD1 through competitively binding to miR-769-3p. The RP11-284F21.9/miR-769-3p/PPWD1 axis could serve as a promising prognostic biomarker and therapeutic target for cervical carcinoma.

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Statement of Human and Animal Rights

The experimental procedures in this study were conducted in accordance with the Ethics Committee of the Xi’an Jiaotong University’s approved protocols.

Data Availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Conflict of interest The authors declare that they have no competing interests.

Author Contributions

Wen-Wei Zhao conceived and designed the experiments; Hong-Fang Han, Qian Chen performed the experiments, analysis the data and wrote the manuscript. All authors read and approved the final manuscript.

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Figure legends

**Figure 1.** RP11-284F21.9 is downregulated in cervical carcinoma tissues and cell lines. (A) Relative expression levels of RP11-284F21.9 in cervical carcinoma tissues compared with that in adjacent normal tissues were analyzed by RT-PCR. (B) Relative expression levels of RP11-284F21.9 in human cervical carcinoma cell lines (Hela, SiHa, C33A and CaSki) and normal human cervical cell line H8 was analyzed by RT-PCR. Data were presented as mean ± SD. *p < 0.05, **p < 0.01.

**Figure 2.** Overexpression of RP11-284F21.9 inhibits proliferation, invasion and migration of cervical carcinoma cells in vitro. (A) Hela or SiHa cells were transfected with mock control or pcDNA3.1-RP11-284F21.9. The overexpression efficiency was examined by RT-PCR. (B, C) Cell proliferation of Hela (B) or SiHa (C) cells transfected with mock control or pcDNA3.1-RP11-284F21.9 was assessed by CCK-8 assay at indicated time points. (D, E) Cell proliferation of Hela (D) or SiHa (E) cells transfected with mock control or pcDNA3.1-RP11-284F21.9 was assessed by immunofluorescence staining of EDU and DAPI. (F, G) Cell cycle analysis of Hela or SiHa cells transfected with mock control or pcDNA3.1-RP11-284F21.9 was analyzed by flow cytometry. (H-I) Cell invasion capability of Hela or SiHa cervical carcinoma cells transfected with mock control or pcDNA3.1-RP11-284F21.9 was analyzed by transwell assay. (J, K) Wound-healing assay was conducted to determine the relative migration distance of Hela or SiHa cells transfected with mock control or pcDNA3.1-RP11-284F21.9. Data were presented as mean ± SD. *p < 0.05, **p < 0.01.

**Figure 3.** RP11-284F21.9 directly interacts with miR-769-3p and functions as the miR-769-3p sponge.
(A) The potential binding sites between RP11-284F21.9 and miR-769-3p predicted using the DIANA tools. Mutated RP11-284F21.9 binding sites were shown. (B) HEK293 cells were transfected with pmirGLO-RP11-284F21.9-WT or pmir-GLO-RP11-284F21.9-Mut, together with miR-769-3p mimic or negative control miR-NC. 72 h post transfection, the relative luciferase activity was measured. * p < 0.05 vs. miR-NC. (C) RNA immunoprecipitation assay was performed using Anti-Ago2 or Anti-IgG antibodies; the relative enrichment of RP11-284F21.9 or miR-769-3p in Hela cells was analyzed. ** p < 0.01 vs. Anti-IgG control. (D) The expression levels of miR-769-3p in Hela or SiHa cells transfected with negative control (NC), pcDNA3.1-RP11-284F21.9, miR-769-3p mimics, miR-769-3p inhibitor or pcDNA3.1-RP11-284F21.9+miR-769-3p mimics were analyzed by RT-PCR 72 hours later. * p < 0.05, ** p < 0.01 vs. NC; # p < 0.05 vs. pcDNA3.1-RP11-284F21.9; & p < 0.05, && p < 0.01 vs. miR-769-3p mimics. (E, F) Cell proliferation of Hela (B) or SiHa (C) cells in different groups was assessed by CCK-8 assay at indicated time points. * p < 0.05 vs. NC; # p < 0.05 vs. pcDNA3.1-RP11-284F21.9; (G-I) Cell cycle of Hela or SiHa cells in different groups was analyzed by flow cytometry. (J, K) Cell invasion capability of Hela or SiHa cervical carcinoma cells was analyzed by transwell assay. * p < 0.05, ** p < 0.01 vs. NC; # p < 0.05 vs. pcDNA3.1-RP11-284F21.9; & p < 0.05, && p < 0.01 vs. miR-769-3p mimics.

**Figure 4. MiR-769-3p regulates PPWD1 expression by targeting PPWD1 3’-UTR.**

(A) Diagram of the predicted binding sites of miR-769-3p on the 3’-UTR of PPWD1 and the mutated sequence of 3’-UTR of PPWD1. (B) Relative luciferase activity in HEK293 cells transfected with mock control, luciferase reporter vector containing PPWD1 3’-UTR WT sequence (WT) or mutated sequence (Mut), together with miR-NC or miR-769-
3p mimics was analyzed. (C-L) Hela or SiHa cells were transfected with NC, miR-769-3p mimics, pcDNA3.1-PPWD1 or miR-769-3p mimics + pcDNA3.1-PPWD1. (C) The mRNA expression levels of PPWD1 in Hela or SiHa cells were analyzed by qRT-PCR. * \( p < 0.05 \), ** \( p < 0.01 \) vs. NC; # \( p < 0.01 \) vs. miR-769-3p mimics; && \( p < 0.01 \) vs. pcDNA3.1-PPWD1. (D, E) Cell proliferation of Hela or SiHa was analyzed by CCK-8 assay. * \( p < 0.05 \) vs. NC; # \( p < 0.05 \) vs. pcDNA3.1-PPWD1; (F-H) Cell cycle of Hela or SiHa cells in different groups was analyzed by flow cytometry. (I, J) Cell invasion capability of Hela or SiHa cervical carcinoma cells was analyzed by transwell assay. * \( p < 0.05 \), ** \( p < 0.01 \) vs. NC; # \( p < 0.01 \) vs. miR-769-3p mimics; && \( p < 0.01 \) vs. pcDNA3.1-PPWD1.

**Figure 5. Overexpression of RP11-284F21.9 inhibits tumor growth of cervical carcinoma in vivo.**

Cervical carcinoma SiHa cells were transfected with mock or pcDNA3.1-RP11-284F21.9 and then implanted subcutaneously into nude mice to develop tumor. (A) Growth curve of tumor volume in nude mice were measured at indicated time points. (B-D) The volume and weight of tumors from mock or pcDNA3.1-RP11-284F21.9 group were measured at day 13. (E, F) The expression levels of miR-769-3p and PPWD1 in the tumor tissues from mock or pcDNA3.1-RP11-284F21.9 group were analyzed by RT-PCR. (G, H) The protein expression levels of PPWD1 in the tumor tissues from mock or pcDNA3.1-RP11-284F21.9 group were analyzed by Western blot. (I, J) The proliferation
marker Ki-67 expression in xenograft tumor tissues was analyzed by IHC staining. ** p < 0.01 vs. Mock control group.

**Figure S1. Bioinformatics analysis predicted RP11-284F21.9 interacted with miR-769-3p.** (A) Bioinformatics analysis was performed using DIANA and G2/M transition of mitotic cell cycle|GO:0000086 dataset to predict the potential miRNAs interacting with RP11-284F21.9. (B) The pcDNA3.1-RP11-284F21.9 or mock vector was transfected into HEK293 cells and the expression of 11 miRNAs was analyzed 48 hours later by qPCR. * p < 0.05 vs. Mock vector control.

**Figure S2. PPWD1 is downregulated in cervical carcinoma tissues and cell lines.** (A) Relative expression levels of PPWD1 in cervical carcinoma tissues compared with that in adjacent normal tissues were analyzed by RT-PCR. (B) Relative expression levels of PPWD1 in human cervical carcinoma cell lines (Hela, SiHa, C33A and CaSki) and normal human cervical cell line H8 were analyzed by RT-PCR. Data were presented as mean ± SD. ** p < 0.01.

**Figure S3. Pearson correlation analysis between the lncRNA RP11-284F21.9, miR-769-3p, and PPWD1.** (A) Pearson correlation analysis was performed between the expression of lncRNA RP11-284F21.9 and miR-769-3p in cervical cancer samples. (B) Pearson correlation analysis was performed between the expression of PPWD1 and miR-769-3p in cervical cancer samples.

**Figure S4. The effect of RP11-284F21.9/miR-769-3p/PPWD1 axis on EMT was evaluated.** (A) Hela or SiHa cells were transfected with pcDNA3.1-RP11-284F21.9 or mock vector. The protein expression levels of EMT-related markers E-cadherin, N-
cadherin, Vimentin, β-catenin and Snail were analyzed 48 hours later. (B) Hela or SiHa cells were transfected with miR-769-3p mimics or miR-NC. The protein expression levels of EMT-related markers E-cadherin, N-cadherin, Vimentin, β-catenin and Snail were analyzed 48 hours later. (C) Hela or SiHa cells were transfected with pcDNA31.-PPWD1 or mock vector. The protein expression levels of EMT-related markers E-cadherin, N-cadherin, Vimentin, β-catenin and Snail were analyzed 48 hours later. ** p < 0.01.

Figure S5. The effect of RP11-284F21.9/miR-769-3p/PPWD1 axis on expression of cell proliferation marker Ki-67 was evaluated. (A) Hela or SiHa cells were transfected with pcDNA3.1-RP11-284F21.9 or mock vector. The protein expression levels of Ki-67 were analyzed 48 hours later. (B) Hela or SiHa cells were transfected with miR-769-3p mimics or miR-NC. The protein expression levels of Ki-67 were analyzed 48 hours later. (C) Hela or SiHa cells were transfected with pcDNA31.-PPWD1 or mock vector. The protein expression levels of Ki-67 were analyzed 48 hours later. ** p < 0.01.
