

Polyphyllin VII enhances the sensitivity of endometrial carcinoma cells to medroxyprogesterone acetate through upregulating miR-33a-5p expression

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Abstract. Endometrial carcinoma (EC) often exhibits resistance to hormone therapies, such as medroxyprogesterone acetate (MPA), highlighting the need for novel strategies to enhance therapeutic efficacy. The present study aimed to investigate the effects of polyphyllin VII (PPVII) on the efficacy of MPA in EC, focusing on the regulatory role of microRNA (miR)-33a-5p. Briefly, an MPA-resistant Ishikawa cell line (Ishikawa/MPA-R), maintained with 10 μ M MPA, was established and transfected with negative control (NC) and miR-33a-5p inhibitors. Following treatment with PPVII and MPA, the proliferation capacity and apoptosis levels of the Ishikawa and Ishikawa/MPA-R cells were evaluated using reverse transcription-quantitative polymerase chain reaction, MTT assay, clonogenic assay, flow cytometry, western blotting and dual-luciferase assay. Next, the expression levels of miR-33a-5p and F-box and leucine rich repeat protein 16 (FBXL16) were measured, and the regulatory relationship between miR-33a-5p and FBXL16 was analyzed. Significant reductions in cell viability were observed in all groups following treatment with increased concentrations of MPA and PPVII, with the greatest effect observed in the combined MPA + PPVII group ($P < 0.01$). The apoptosis levels of the Ishikawa/MPA-R cells were significantly increased in all drug treatment groups, particularly in the MPA + PPVII group ($P < 0.05$). PPVII treatment significantly increased the expression level of miR-33a-5p in Ishikawa/MPA-R cells

($P < 0.01$). In the PPVII + miR-33a-5p inhibitor group, the Ishikawa/MPA-R cells exhibited an upregulation in the viability ($P < 0.01$), colony formation ability ($P < 0.01$), proportion in the G1 phase ($P < 0.05$) and the protein expression levels of cyclin D1 ($P < 0.01$) and cyclin-dependent kinase 4 ($P < 0.01$), and a reduction in the miR-33a-5p expression ($P < 0.01$), apoptosis levels ($P < 0.05$), proportion in the S ($P < 0.05$) and G2 phases and the levels of Bcl-2-associated X protein ($P < 0.001$). The FBXL16 protein expression in Ishikawa/MPA-R cells was significantly higher compared with Ishikawa cells, and the mRNA and protein expression levels of FBXL16 were markedly elevated in the PPVII + miR-33a-5p inhibitor group compared with the PPVII + NC group ($P < 0.01$). These findings suggested that PPVII upregulated the expression of miR-33a-5p, enhanced the sensitivity of EC cells to MPA and potentially exerted anticancer effects in EC through the synergistic action of the miR-33a-5p/FBXL16 axis in combination with MPA.

Introduction

Endometrial carcinoma (EC), originating from the endometrial epithelium, is one of the four major reproductive system tumors that pose a risk to women's health. The main risk factors for EC are excessive endogenous or exogenous estrogens, coupled with a lack of sufficient progesterone (1). In addition to conventional surgery and radiochemotherapy, medroxyprogesterone acetate (MPA) is a principal conservative treatment for EC. MPA therapy is primarily administered through medication and intrauterine devices, making it particularly suitable for patients expecting to preserve fertility. MPA exerts its anticancer effects through progesterone receptor B (2). However, due to individual differences in progesterone receptor B among patients and the emergence of MPA resistance during treatment, the complete response rate is $< 70\%$ for patients with atypical endometrial hyperplasia or stage I EC, and the recurrence rate is $\sim 25\text{--}40\%$ (3,4). Therefore, further research is needed for the clinical resolution of the resistance of EC to MPA.

Paris polyphylla is a commonly used antitumor herb in Traditional Chinese Medicine, and saponins are its

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main active component. In addition to anthelmintic and anti-inflammatory effects, saponins also possess anticancer properties (5). Polyphyllin VII (PPVII), a saponin extracted from *Paris polyphylla*, exhibits anti-proliferative and pro-apoptotic effects on various tumor cells (6), including ovarian (7), breast (8) and liver cancer cells (9). However, the inhibitory effects of PPVII on EC have been rarely studied.

MicroRNAs (miRNAs) are short chain, non-coding RNAs that regulate the transcription and translation levels of target genes through binding to the 3' untranslated regions (3' UTRs) of target genes. Besides, miRNAs participate in the regulation of multiple biological processes, such as cell proliferation, apoptosis, differentiation and metabolism (10). A recent review has illustrated that miRNAs play a significant role in the occurrence and development of tumors and could serve as biomarkers for tumor diagnosis, prognosis assessment and treatment (11). As a member of the miR-33 family, miR-33a-5p is involved in regulating the proliferation, migration and invasion of various tumor cells such as breast and colorectal cancer (12,13). High-throughput genomics has demonstrated that miR-33a-5p may be a potential molecular regulator in the development of EC (14). In our preliminary, unpublished experiments, PPVII treatment significantly upregulated miR-33a-5p, which was associated with increased cell death. Additionally, miR-33a-5p has been reported to regulate key pathways involved in cell cycle control and apoptosis, underscoring its potential therapeutic relevance in EC (15).

Previous studies have suggested that F-box and leucine rich repeat protein 16 (FBXL16) plays a crucial role in the regulation of the cell cycle and apoptosis, and is associated with poor prognosis in EC (16). Given the connections between miR-33a-5p and cellular regulation, we hypothesize that miR-33a-5p may influence EC progression by targeting FBXL16, potentially offering antitumor effects.

PPVII has been proven to possess multiple biological activities, including anti-inflammatory, antioxidant and antitumor effects. Besides, PPVII can affect the growth and metastasis of tumor cells by regulating the expression of miRNAs (17). However, the role and regulatory mechanisms of PPVII in EC are still unclear. Notably, miR-33a-5p may be a key molecule in the treatment of EC with PPVII. Hence, the present study was designed to explore whether PPVII enhanced the sensitivity of EC to MPA by regulating the expression of miR-33a-5p. The findings of the present study are expected to provide new insights and strategies for the clinical treatment of EC.

Materials and methods

Cell culture, treatment and transfection. The human EC cell (ECC) line, Ishikawa (cat. no. CL-0283), was purchased from Wuhan Pricella Biotechnology Co., Ltd. Briefly, ECCs were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 12634010) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.; cat. no. A5669701) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 15140148) and maintained in an incubator at 37°C with 5% CO₂ (Thermo Fisher Scientific,

Inc.; cat. no. 51023126). Then, a progesterone-resistant cell line, referred to as Ishikawa/MPA-R, was established using MPA (MilliporeSigma). Cells were cultured in conventional medium with an initial concentration of 1 μ M MPA. Then, the cells were cultured and passaged in 0.02% ethylenediaminetetraacetic acid and 0.25% trypsin solution. The MPA concentration was increased by 2.5 μ M every 4 weeks. A stable Ishikawa/MPA-R cell line was established when the MPA concentration reached 10 μ M and the cell proliferation rate was comparable to that of the original Ishikawa cells using the MTT assay. The cells were then cultured in 10 μ M MPA to maintain resistance. The inhibition rate and half maximal inhibitory concentration (IC₅₀) for MPA in both Ishikawa and Ishikawa/MPA-R cells were determined using the MTT assay (16,18). In the present study, PPVII was procured from MedChemExpress (cat. no. HY-N0048).

Based on different treatments, Ishikawa/MPA-R cells were divided into the following groups: i) The Control group, cells were cultured with medium only; ii) the MPA group, cells were cultured with medium containing MPA (30 μ M); iii) the PPVII group, cells were cultured with medium containing PPVII (2 μ M); iv) the MPA + PPVII group, cells were cultured with medium containing both MPA (30 μ M) and PPVII (2 μ M); v) the PPVII + negative control (NC) inhibitor group, Ishikawa/MPA-R cells were transfected with a NC inhibitor and cultured with PPVII (2 μ M); and vi) the PPVII + miR-33a-5p inhibitor (miR-33a-5p in) group, Ishikawa/MPA-R cells were transfected with a miR-33a-5p inhibitor and cultured with PPVII (2 μ M).

Before performing the transfection experiment, ECCs were allocated to different treatment groups according to the experimental design and seeded in culture dishes at a density of 5x10⁵ cells/well. The transfection experiment was conducted using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.; cat. no. 11668500) according to the manufacturer's protocol. Briefly, 5 μ l Lipofectamine 2000 was diluted in 250 μ l Opti-MEM (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated for 5 min at room temperature. Separately, 1 μ g of miR-33a-5p in or NC was diluted in 250 μ l Opti-MEM and then mixed with the Lipofectamine 2000 mixture. The final mixture was added to the cells and incubated at 37°C for 6 h. Following transfection, cells were incubated for 48 h before proceeding with subsequent experiments. The sequences for the miR-33a-5p in and negative control were as follows: 5'-UGCAAUGCAACUACAUGCAC-3' and 5'-UUGCUUACGUAGCACUUACGU-3', respectively (Shanghai GenePharma Co., Ltd.).

MTT assay for cell viability. The experiment was conducted according to the MTT assay kit protocol (Merck KGaA; cat. no. 475989). First, cells were seeded into a 96-well plate at a density of 5,000 cells/well, with a culture volume of 100 μ l. After treatment of the cells, the medium was replaced and 10 μ l MTT solution was added for 4 h of incubation. The medium in each well was removed and 100 μ l DMSO was added to dissolve the formazan crystals. The plate was shaken for 10 min to ensure complete dissolution. Subsequently, the optical density at 570 nm was measured using a microplate reader, and the cell viability and half maximal inhibitory concentration values for each group were calculated (19,20).

Clonogenic assay. Cells from each group were grown to the logarithmic phase, seeded into 6-well plates at a density of 300 cells/well and cultured under the aforementioned conditions. The observation lasted for 7-14 days and the medium was replaced every 3 days during the culture period. At the end of the culture, cells were fixed with 4% paraformaldehyde for 20 min at room temperature and then stained with 1% crystal violet dye (Sangon Biotech, Co., Ltd.) for 5 min at room temperature. A digital camera (Canon, Inc.) was used to collect images and the number of cell colonies (>50 cells as one colony) was counted using a light microscope (Olympus Corporation). The experiment was independently performed in triplicate.

Flow cytometry. Ishikawa/MPA-R cells were cultured in 6-well plates at a density of 5×10^5 cells/well for 24 h. Next, cells in the logarithmic growth phase were collected and washed 2 or 3 times with phosphate-buffered saline (PBS) for the following experiments. i) Cell cycle analysis: Cells were fixed with 70% ethanol at -20°C for at least 2 h. Then, the cells were washed with PBS and centrifuged ($300 \times g$, 5 min, 4°C) to remove the ethanol. Subsequently, the cells were stained with propidium iodide (Abcam; cat. no. ab14083) for 30 min at 37°C . Then, flow cytometry (LSRFortessa; BD Biosciences) was performed to analyze the stained cells to determine the proportion of cells in different cell cycle stages (G0/G1, S and G2/M). Lastly, the flow cytometry data were analyzed using FlowJo v10.0 software (FlowJo LLC). ii) Cell apoptosis analysis: Early apoptotic cells were labeled with fluorescein isothiocyanate-conjugated annexin V (Abcam; cat. no. ab14085) and late apoptotic and necrotic cells were stained with propidium iodide. The staining was performed at room temperature in the dark for 15 min. Then, the cells were gently washed with PBS to remove unbound markers, followed by resuspension in 1X binding buffer provided in the Annexin V-FITC Apoptosis Detection Kit (Abcam; cat. no. ab14085). The apoptosis rate of cells was analyzed using a flow cytometer (LSRFortessa; BD Biosciences) and calculated with FlowJo software.

Western blotting. Cells from each group were lysed on ice using radioimmunoprecipitation assay lysis buffer (Biosharp Life Sciences; cat. no. BL504A), and the supernatant was collected to measure protein concentration using the bicinchoninic acid assay. Equal amounts of denatured proteins ($20 \mu\text{g}$ per lane) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then, the proteins were transferred onto a polyvinylidene fluoride membrane. Subsequently, the membrane was blocked with 5% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.; cat. no. A5256701) at room temperature for 1 h and then incubated with the primary antibodies on a shaker at 4°C overnight. The next day, the membrane was washed three times with TBST (containing 0.1% Tween 20), 10 min each time. Subsequently, the membrane was incubated with HRP-conjugated secondary antibodies for 1-2 h at room temperature and washed three times for 10 min each with TBST. Then, bands were developed using an enhanced chemiluminescence reagent (Abcam; cat. no. ab133406) on the ImageQuant LAS 500 system (GE Healthcare Life Sciences), and the band intensities were

Table I. Reverse transcription-quantitative polymerase chain reaction primer sequences.

Gene	Primer (5'-3')
miR-33a-5p (human)	Forward: TGCAATGCAACTACAATGCAAA Reverse: CTCAACTGGTGTCTGGAGTCG GCAATTCAGTTGAG
U6 (human)	Forward: CTCGCTTCGGCAGCACA Reverse: AACGCTTCACGAATTTGCGT
FBXL16 (human)	Forward: TCTGGTATTTCTCGGCCTGC Reverse: ACGTTGTAGAGCTCCTTGGC
GAPDH (human)	Forward: ATGGGCAGCCGTTAGGAAAG Reverse: ATCACCCGGAGGAGAAATCG

miR, microRNA; FBXL16, F-box and leucine rich repeat protein 16.

analyzed using ImageJ software (version 1.54; National Institutes of Health). Specific information on the antibodies (all from Abcam) used in this experiment were as follows: B-cell lymphoma 2 (Bcl-2; cat. no. ab182858; 1:2,000); Bcl-2-associated X (Bax; cat. no. ab32503; 1:1,000), Cyclin D1 (cat. no. ab134175; 1:1,000); Cyclin-dependent kinase 4 (CDK4; cat. no. ab108357; 1:400); FBXL16 (cat. no. ab272898; 1:100); GAPDH (cat. no. ab9485; 1:2,500); and secondary antibody (cat. no. ab288151; 1:10,000).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells using the TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The quality and quantity of the extracted RNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). Then, $1 \mu\text{g}$ of total RNA was reverse-transcribed into cDNA using HiScript III 1st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd.; cat. no. R312) according to the manufacturer's instructions. qPCR was conducted using Taq Pro Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd.; cat. no. Q712) following the kit protocol. The reaction conditions were set as follows: Stage 1, 95°C for 30 sec; stage 2, 40 cycles of denaturation at 95°C for 10 sec and annealing at 60°C for 10 sec; stage 3, extension at 72°C for 30 sec. The results were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method to determine the relative expression levels of the target genes (21), with U6 or GAPDH serving as the internal control. The primers for all genes were designed based on sequences obtained from miRBase (Table I).

Dual-luciferase reporter gene assay. The TargetScan database (https://www.targetscan.org/vert_72/) was used to predict the potential binding sites of miR-33a-5p in the FBXL16 3'UTR. The sequences of wild-type or mutated miR-33a-5p targeting the FBXL16 3'UTR were cloned into the pMIR-REPORT luciferase vector (Shanghai GenePharma Co., Ltd.). Subsequently, the cells were cultured and co-transfected with the reporter vectors and miR-33a-5p mimics or NC miRNAs using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After 48 h, the

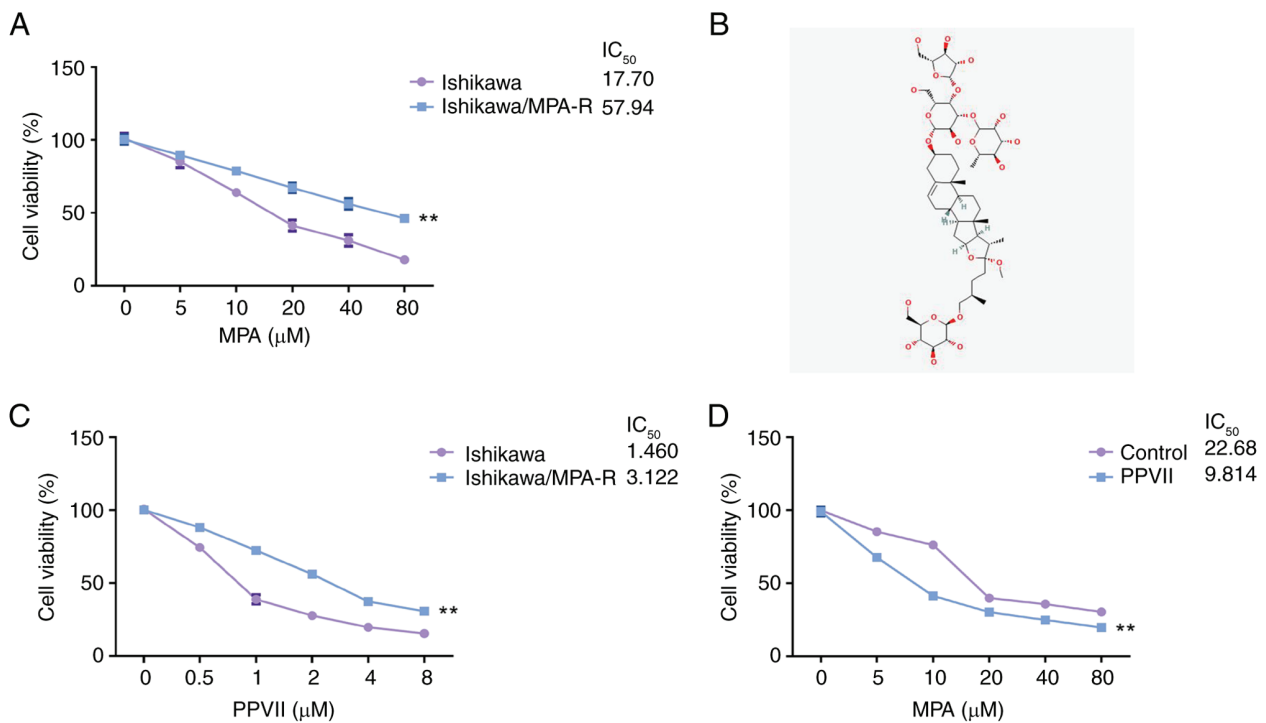


Figure 1. Effect of MPA and PPVII on the viability of ECCs. (A) The viability of Ishikawa and Ishikawa/MPA-R cells treated with different concentrations of MPA. (B) The chemical structure of PPVII. (C) The viability of Ishikawa and Ishikawa/MPA-R cells treated with different concentrations of PPVII. (D) The viability of Ishikawa/MPA-R cells treated with different concentrations of MPA in the presence of 2 μ M PPVII. Data are presented as the mean $\pm$ standard deviation (n=3), **P<0.01. PPVII, polyphyllin VII; MPA, medroxyprogesterone acetate; ECC, endometrial carcinoma cell.

luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega Corporation). The results were normalized to *Renilla* luciferase activity to control for transfection efficiency (15). The sequences for the miR-33a-5p mimics and NC were as follows: 5'-GUGCAUUGUAGUUGCAUUGCA-3' (sense) and 5'-UGCAAUGCAACUACA AUGCAC-3' (anti-sense); 5'-UGUACGUAUCGUAGCAUGUCA-3' (sense) and 5'-ACAUGCUACGAUACGUACAGU-3' (anti-sense), respectively.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8.0 (Dotmatics) and SPSS 26.0 (IBM Corp.). All data are presented as the mean $\pm$ standard deviation. A two-tailed independent samples t-test was adopted for comparisons between two groups. For comparisons among multiple groups, a one-way analysis of variance followed by Tukey's post-hoc test was used. P<0.05 was considered to indicate a statistically significant difference.

Results

PPVII significantly increases the sensitivity of ECCs to MPA. The MTT results showed a decrease in both Ishikawa and Ishikawa/MPA-R cell viability as the concentrations of MPA increased (0, 5, 10, 20, 40 and 80 μ M), with a more notable decline in the Ishikawa cells. Notably, the higher viability of Ishikawa/MPA-R cells compared with Ishikawa cells at increasing MPA concentrations confirmed the successful construction of the MPA-resistant Ishikawa cell line (P<0.01; Fig. 1A). Following incubation with PPVII (Fig. 1B), the viability of the Ishikawa and Ishikawa/MPA-R cells exhibited

a downward trend with increasing concentrations of PPVII (0, 0.5, 1, 2, 4 and 8 μ M), with a more significant reduction in Ishikawa cell viability (P<0.01; Fig. 1C). Compared with the untreated Ishikawa/MPA-R control group, Ishikawa/MPA-R cells treated with PPVII (2 μ M) displayed a more notable decrease in cell viability at various concentrations of MPA (0, 5, 10, 20, 40 and 80 μ M; P<0.01; Fig. 1D). These outcomes indicate that PPVII significantly increased the sensitivity of ECCs to MPA.

PPVII synergizes with MPA to inhibit the viability of ECCs. Compared with the control group, a significant decrease was observed in the viability of Ishikawa/MPA-R cells treated with MPA and PPVII alone (P<0.01). Furthermore, the combined treatment of MPA + PPVII resulted in a more notable reduction in cell viability (P<0.01), indicating a synergistic effect (Fig. 2A). As shown in Fig. 2B, the clonogenic assay further confirmed the synergistic effects of MPA and PPVII in the Ishikawa/MPA-R cells. The number of colonies formed in the control group was significantly higher relative to the treatment groups. Treatment with MPA and PPVII alone markedly reduced the number of colonies (P<0.01 or P<0.001), while the combined treatment of MPA + PPVII led to an even greater decrease (P<0.001; Fig. 2B). These results highlight the potential of the combined treatment to more effectively inhibit ECC proliferation and colony formation as opposed to the treatment with either agent alone.

PPVII combined with MPA increases apoptosis and inhibits the cell cycle in ECCs. Flow cytometry analysis showed a significant increase in the apoptosis rates of Ishikawa/MPA-R

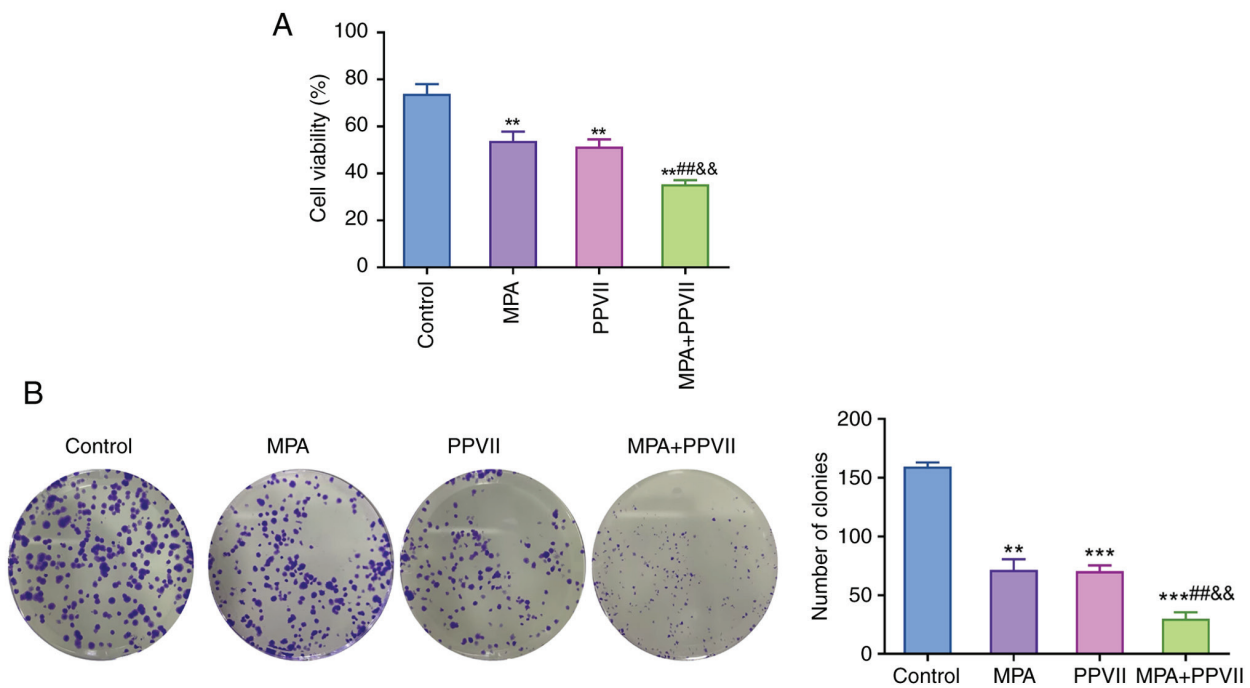


Figure 2. PPVII combined with MPA inhibits the viability and clonogenic capacity of endometrial carcinoma cells. (A) The viability of Ishikawa/MPA-R cells treated with PPVII, MPA and their combination, as determined using MTT assay. (B) Clonogenic assay showing the clonogenic capacity of Ishikawa/MPA-R cells under the same treatment conditions. Data are presented as the mean $\pm$ standard deviation (n=3). **P<0.01, ***P<0.001 vs. Control group; ##P<0.01 vs. MPA group; &&P<0.01 vs. PPVII group. PPVII, polyphyllin VII; MPA, medroxyprogesterone acetate.

cells treated with MPA and PPVII alone (P<0.01; Fig. 3A). The combined treatment of MPA + PPVII further enhanced the apoptosis levels compared with the MPA and PPVII alone groups (P<0.01; Fig. 3A). These findings indicated that the synergistic effect involved the promotion of apoptotic cell death. Notably, the observed 10% apoptotic cells in the Control group without treatment could be attributed to baseline apoptosis, which is common in cell cultures due to natural cell turnover and stress conditions. Moreover, the cell cycle analysis displayed in Fig. 3B demonstrated the impact of MPA and PPVII on cell cycle distribution. Both MPA and PPVII treatments led to significant cell cycle arrest at the G0/G1 phase compared with the Control group (P<0.05). The combined treatment resulted in an even greater accumulation of cells in the G0/G1 phase and a reduction of cells in the S phase (P<0.01), indicating enhanced cell cycle arrest. This suggests that the combination treatment of MPA and PPVII effectively halted cell cycle progression.

In addition, western blot analysis was performed to investigate the synergistic effects of MPA and PPVII on cell apoptosis and cell cycle regulation (Fig. 3C and D). The results revealed that, compared with the Control group, the expression levels of Bax protein were significantly higher in the treatment groups (P<0.001), while the expression levels of Bcl-2, Cyclin D1 and CDK4 were significantly lower (P<0.01 or P<0.001). Notably, the changes in the MPA + PPVII group were the most notable (P<0.01 or P<0.001). These results indicate that the combination of PPVII and MPA enhanced apoptosis and inhibited the cell cycle in ECCs.

PPVII increases the sensitivity of ECCs to MPA by upregulating miR-33a-5p. Next, the mechanisms by which PPVII

regulates the expression of miR-33a-5p were investigated. miR-33a-5p was chosen for investigation due to its known role in regulating cell proliferation, apoptosis, and drug sensitivity in various cancers. Given PPVII's antitumor properties, it was hypothesized that it might influence miR-33a-5p expression as a potential mechanism for enhancing MPA sensitivity in endometrial cancer cells. Quantitative analysis revealed that the miR-33a-5p expression was significantly decreased in Ishikawa/MPA-R cells compared with Ishikawa cells (P<0.01; Fig. 4A). Additionally, treatment with PPVII significantly increased the expression of miR-33a-5p in Ishikawa/MPA-R cells compared with the Control group (P<0.01; Fig. 4B).

To further elucidate the role of miR-33a-5p in ECCs, the expression of miR-33a-5p was manipulated and its effects were assessed. Transfection of Ishikawa/MPA-R cells with miR-33a-5p in was confirmed to be successful, as evidenced by a significant downregulation of miR-33a-5p expression compared with the in NC group (P<0.01; Fig. S1A). As showed in Fig. 5A, cells transfected with miR-33a-5p inhibitor resulted in a notable downregulation of miR-33a-5p expression compared with the PPVII + in NC group (P<0.01). This inhibition of miR-33a-5p led to a significant elevation in cell viability (P<0.01; Fig. 5B) and clonogenic capacity (P<0.01; Fig. 5C), a reduction in apoptosis levels (P<0.05; Fig. 5D), accompanied by an increase in the proportion of cells in the G1 phase and a decrease in the proportion of cells in the S phase compared with the PPVII + in NC group (P<0.05; Fig. 5E). Western blot analysis further supported these findings, showing that the PPVII + miR-33a-5p in group exhibited a significant reduction in the Bax protein level (P<0.001) and a notable elevation in the protein expression levels of Bcl-2, Cyclin D1, and CDK4 compared with the PPVII + in NC group (P<0.01; Fig. 5F).

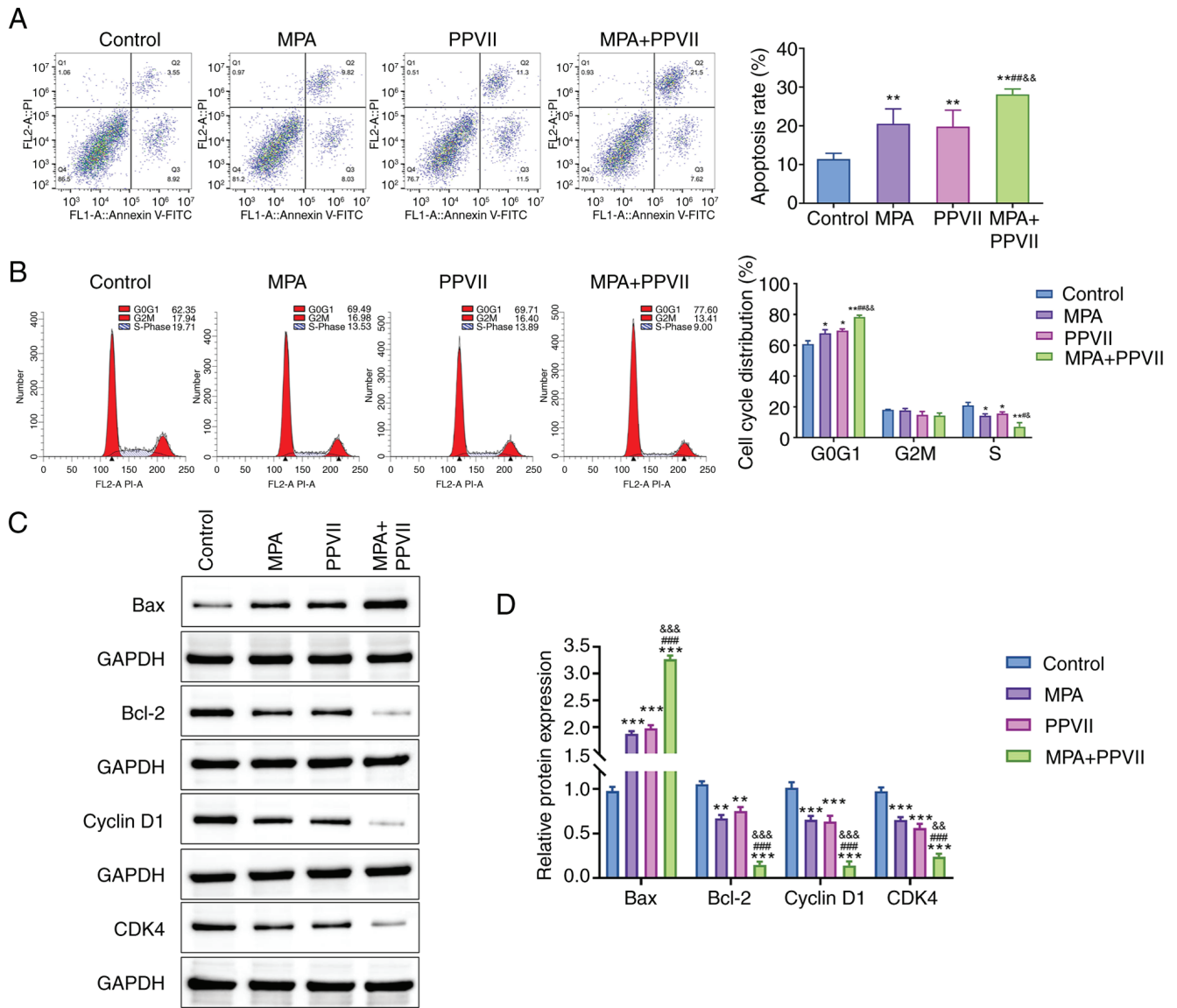


Figure 3. PPVII combined with MPA promotes the apoptosis of endometrial carcinoma cells. (A) The apoptosis level of Ishikawa/MPA-R cells in each group, as determined using flow cytometry. (B) The cell cycle changes in Ishikawa/MPA-R cells in each group, as determined using flow cytometry. (C) Western blotting was used to detect the protein expression of Bax, Bcl-2, Cyclin D1 and CDK4 in Ishikawa/MPA-R cells in each group, which was (D) semi-quantified. Data are presented as the mean $\pm$ standard deviation ($n=3$); * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. Control group; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ vs. MPA group; & $P<0.05$, && $P<0.01$, &&& $P<0.001$ vs. PPVII group. PPVII, polyphyllin VII; MPA, medroxyprogesterone acetate; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X; CDK4, Cyclin-dependent kinase 4.

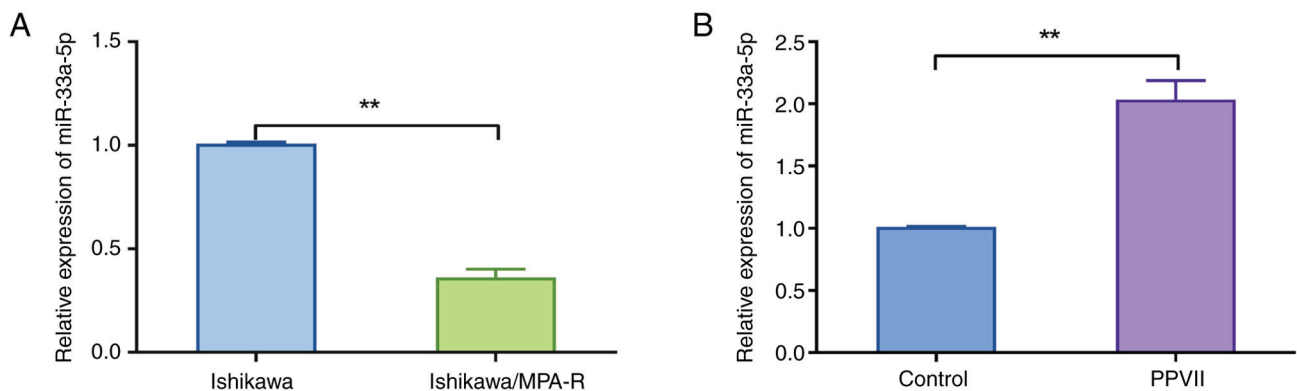


Figure 4. Expression of miR-33a-5p in endometrial carcinoma cells. (A) Assessment of miR-33a-5p expression in Ishikawa and Ishikawa/MPA-R cells using RT-qPCR. (B) Assessment of miR-33a-5p expression in Ishikawa/MPA-R cells treated with PPVII using RT-qPCR. Data are presented as the mean $\pm$ standard deviation ($n=3$). ** $P<0.01$. PPVII, polyphyllin VII; MPA, medroxyprogesterone acetate; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR, microRNA.

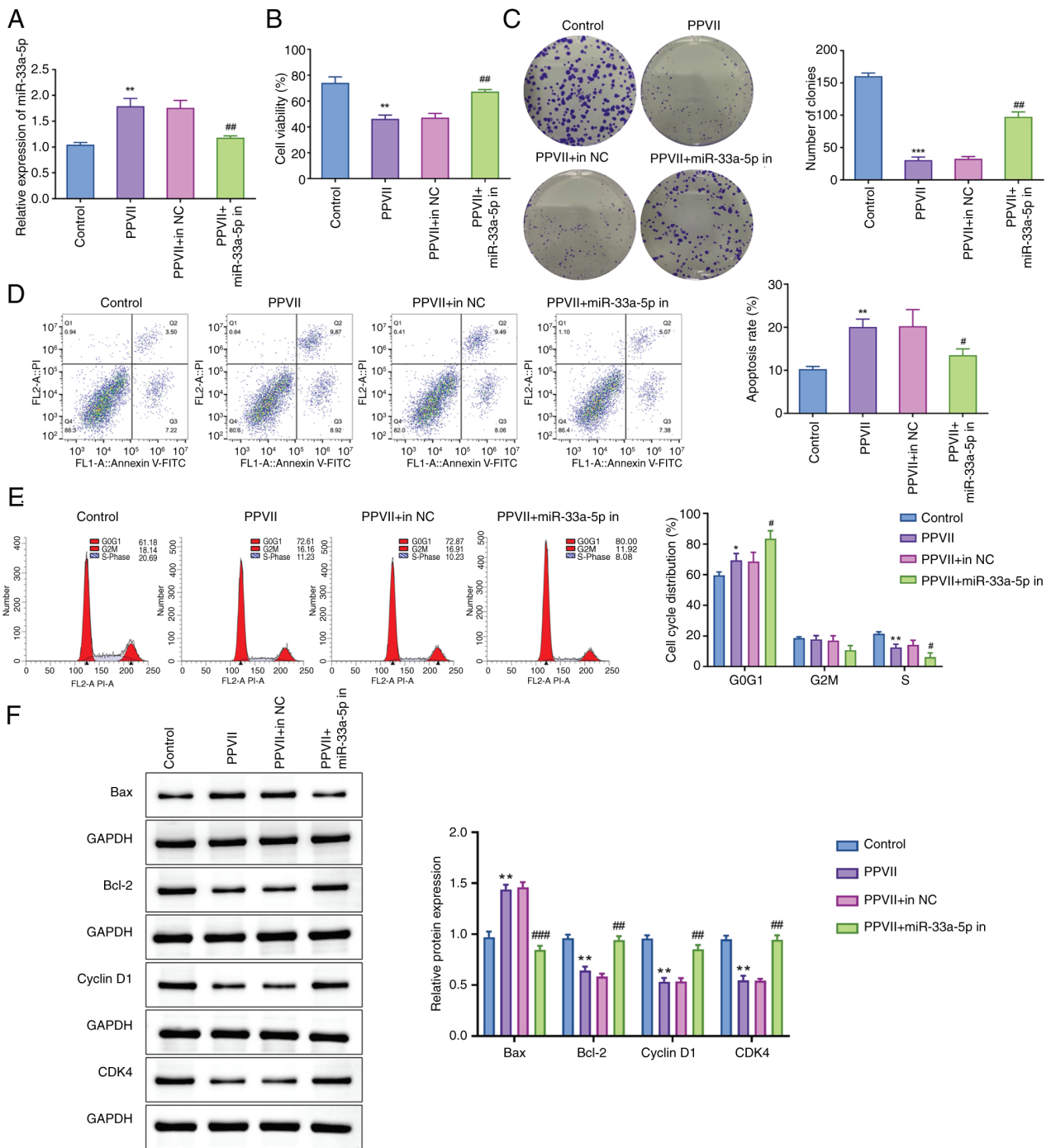


Figure 5. Inhibition of miR-33a-5p suppresses the anticancer effect of PPVII on endometrial carcinoma cells. (A) The detection of miR-33a-5p in Ishikawa/MPA-R cells in each group using reverse transcription-quantitative polymerase chain reaction. (B) The viability of Ishikawa/MPA-R cells, as determined using MTT assay. (C) Clonogenic assay to assess the clonogenic capacity of Ishikawa/MPA-R cells in each group. (D) Flow cytometry to measure the apoptosis level of Ishikawa/MPA-R cells in each group. (E) Flow cytometry to analyze the cell cycle phase proportions of Ishikawa/MPA-R cells in each group. (F) Western blotting to assess the protein expression levels of Bax, Bcl-2, Cyclin D1 and CDK4 in Ishikawa/MPA-R cells in each group. Data are presented as the mean $\pm$ standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. Control group; #P<0.05, ##P<0.01, ###P<0.001 vs. PPVII + in NC group. PPVII, polyphyllin VII; MPA, medroxyprogesterone acetate; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X; CDK4, Cyclin-dependent kinase 4; miR, microRNA; NC, negative control; in, inhibitor.

These findings suggest that PPVII enhanced the sensitivity of ECCs to MPA, and the inhibition of miR-33a-5p reversed this effect, which indicated the critical role of miR-33a-5p in mediating the response of ECCs to PPVII and MPA.

miR-33a-5p affects the viability and proliferation of ECCs through targeted regulation of FBXL16. Preliminary

bioinformatics analysis suggested that miR-33a-5p might regulate FBXL16, guiding our investigation into its functional role in ECCs. As shown in Fig. S2A, publicly available miRNA sequencing data revealed a distinct miRNA expression profile in PPVII-treated Ishikawa/MPA-R cells, with miR-33a-5p significantly upregulated among other miRNAs. Pathway enrichment analysis indicated that miR-33a-5p was involved in

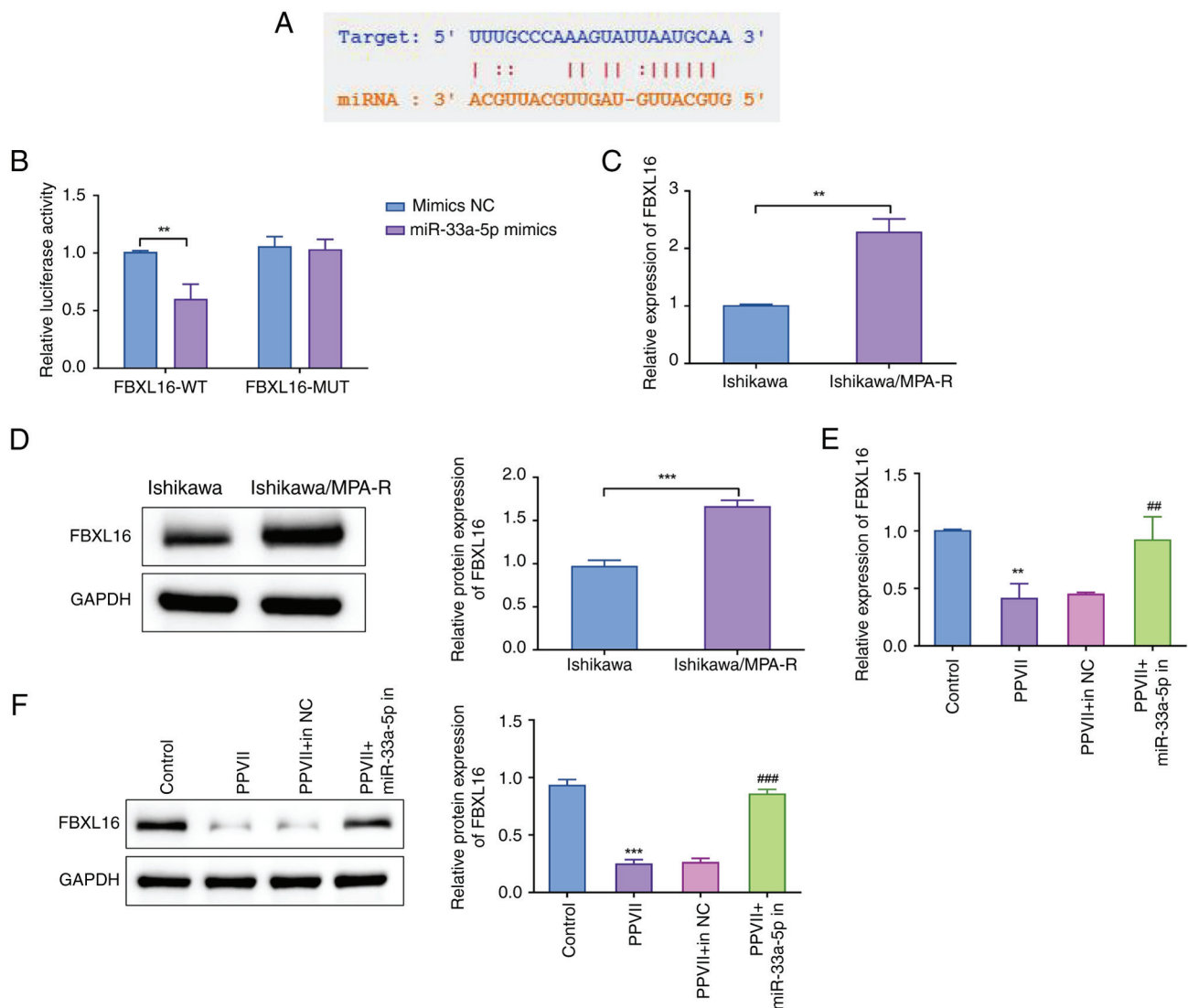


Figure 6. Negative regulation of FBXL16 by miR-33a-5p in endometrial carcinoma cells. (A) Prediction of the potential binding site of miR-33a-5p and the FBXL16 3' untranslated region using the TargetScan database. (B) Dual-luciferase reporter assay to investigate the targeting relationship between miR-33a-5p and FBXL16; ** $P < 0.01$ vs. mimics NC group. Detection of the (C) mRNA and (D) protein expression levels of FBXL16 in Ishikawa and Ishikawa/MPA-R cells using RT-qPCR and western blotting. Detection of the (E) mRNA and (F) protein expression levels of FBXL16 in Ishikawa/MPA-R cells in each group using RT-qPCR and western blotting. Data are presented as the mean $\pm$ standard deviation ($n=3$). ** $P < 0.01$, *** $P < 0.001$ vs. Ishikawa or Control group; ## $P < 0.01$, ### $P < 0.001$ vs. PPVII + in NC group. PPVII, polyphyllin VII; FBXL16, F-box and leucine rich repeat protein 16; MPA, medroxyprogesterone acetate; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR, microRNA; NC, negative control; in, inhibitor; WT, wild-type; MUT, mutated.

critical cellular processes, including cell cycle regulation and mitotic progression (Fig. S2B). Furthermore, miR-33a-5p was predicted to target the FBXL16 gene as illustrated in Fig. S2C.

To experimentally confirm whether miR-33a-5p directly targets FBXL16, dual-luciferase reporter assays were conducted. Firstly, the transfection efficiency of Ishikawa/MPA-R cells with miR-33a-5p mimic was verified using RT-qPCR. As shown in Fig. S1B, cells transfected with the miR-33a-5p mimic exhibited a significant increase in miR-33a-5p levels compared with the NC mimic group ($P < 0.01$), which the confirmed successful transfection with high efficiency. Next, the dual-luciferase reporter assay results confirmed that miR-33a-5p bound to a specific sequence within the 3'UTR of FBXL16 (Fig. 6A). Specifically, miR-33a-5p significantly reduced the activity of the wild-type FBXL16 3'UTR reporter gene ($P < 0.01$), indicating direct binding and regulation by miR-33a-5p. In contrast, cells with a mutated

FBXL16 3'UTR showed no significant change in luciferase activity, confirming the specificity of miR-33a-5p binding to the wild-type sequence (Fig. 6B).

The expression of FBXL16 in Ishikawa and Ishikawa/MPA-R cells was then further examined. The expression of FBXL16 was significantly higher in Ishikawa/MPA-R cells compared with Ishikawa cells ($P < 0.01$ or $P < 0.001$; Fig. 6C and D). This indicated that FBXL16 was upregulated in MPA-resistant cells. To assess the effect of PPVII and miR-33a-5p on FBXL16 expression, the mRNA and protein levels of FBXL16 were measured in Ishikawa/MPA-R cells under different treatment conditions. In the presence of PPVII, the mRNA ($P < 0.01$) and protein expression levels of FBXL16 ($P < 0.001$) were significantly downregulated compared with the control group (Fig. 6E and F). Conversely, in the PPVII + miR-33a-5p in group, there was a notable elevation in both the mRNA ($P < 0.01$) and protein expression levels

of FBXL16 ($P<0.001$) compared with the PPVII + in NC group (Fig. 6E and F). These results suggest that PPVII and miR-33a-5p inhibited the expression of FBXL16, whereas the inhibition of miR-33a-5p led to the upregulation of FBXL16.

Discussion

EC ranks as the second most common gynecological malignant tumor after cervical cancer, accounting for 20-30% of gynecological malignant tumors. In some developed cities, the incidence of EC has surpassed that of other gynecological malignant tumors (22). PPVII is one of the main active components with anticancer properties derived from plants in the Paris polyphylla family. Generally, PPVII exerts its anticancer effects by promoting apoptosis and necrosis, inhibiting proliferation and migration, inducing autophagy and increasing drug sensitivity in cancer cells. PPVII has been reported to treat various tumors, such as gastric and small cell lung cancer (23,24). It is also worth noting that PPVII may exert anticancer effects through multiple pathways (25). For instance, PPVII can not only reduce pro-inflammatory cytokines by inhibiting nuclear factor- κ B and mitogen-activated protein kinase (26), but also activate the c-Jun N-terminal kinase pathway by inhibiting the phosphorylation of phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin, thereby inducing the autophagy of tumor cells (23,27). In addition, PPVII can lead to cancer cell death by inducing mitochondrial dysfunction (28). Furthermore, PPVII may synergize with chemotherapeutic drugs to increase the expression of p52 and activate the signaling pathways such as mitogen-activated protein kinase, thereby inducing cancer cell apoptosis (27) and inhibiting cancer cell growth (29). At present, the studies on the anticancer effects of PPVII on EC are limited. In the present study, PPVII could effectively inhibit ECC viability, and PPVII combined with MPA could inhibit the proliferation of ECCs and promote apoptosis.

Epithelial-mesenchymal transition (EMT) refers to the transformation of epithelial cells into mesenchymal cells to acquire the differentiation and invasive abilities of mesenchymal cells. EMT is also a crucial mechanism in the development of EC (30). PPVII can attenuate EMT by inhibiting the cancerous inhibitor of protein phosphatase 2A/protein kinase B/mechanistic target of the rapamycin axis, thereby increasing the sensitivity of tumor cells to chemotherapeutic agents (31). The long non-coding RNA just proximal to the X-inactive specific transcript/miR-33a-5p/twist-related protein 1 axis affects the EMT process by activating the wingless-related integration site/ β -catenin signaling pathway, thus inhibiting cancer cell metastasis (32). Hypoxia-inducible factor expression is activated in cancer cells under hypoxic conditions, which in turn upregulates miR-33a-5p expression, affecting the EMT process and further inhibiting the invasive ability of cancer cells (33). To the best of our knowledge, no studies have thus far directly linked PPVII and miR-33a-5p. However, in the present study, PPVII and MPA treatments elevated the expression of miR-33a-5p in ECCs, suggesting a potential regulatory relationship. Hence, more detailed mechanistic studies, including validation of key pathways and *in vivo* studies, are necessary to provide comprehensive mechanistic insights into the synergistic effects of PPVII and

MPA in inhibiting EC growth. Furthermore, in the present study, the inhibition of miR-33a-5p function weakened the anticancer effects of PPVII and MPA in ECCs, indicating that miR-33a-5p might act as a tumor suppressor and could play a key role in EC treatment.

FBXL16 is an important target gene of miR-33a-5p (34,35), and its high expression is associated with MPA resistance and poor prognosis in EC (16). FBXL16 promotes the dephosphorylation of Cyclin D1 via the AKT serine/threonine kinase 1/glycogen synthase kinase 3 β /Cyclin D1 pathway, thus enhancing MPA resistance in ECCs (16). Based on the studies of FBXL16 in other tumor cells (16,36), it can be speculated that FBXL16 may be involved in key biological processes in EC, such as apoptosis, invasion and metastasis. The present study demonstrated that FBXL16 was negatively regulated by miR-33a-5p, which was consistent with the previous findings. However, in addition to FBXL16, miR-33a-5p may regulate multiple downstream target genes. Therefore, it is of significance to expand future studies to other downstream targets of miR-33a-5p, providing a more comprehensive understanding of the role of miR-33a-5p in the biological pathways under investigation.

Despite the significant findings of the present study, several limitations should be acknowledged. First, although miR-33a-5p and its target gene, FBXL16, play a role in regulating ECC proliferation, the precise regulatory mechanisms remain to be explored. Specifically, the pathway through which miR-33a-5p modulates FBXL16 expression and the biological significance of this modulation warrant further investigation. Second, the present study primarily focused on *in vitro* experiments. Future research should incorporate *in vivo* studies to validate the findings in a more complex biological context. These studies should involve the use of animal models to evaluate the efficacy and safety of the combined treatment of PPVII and MPA in EC. Moreover, the implications of the present study suggest that PPVII could enhance the sensitivity of ECCs to MPA, potentially offering a novel therapeutic strategy. Therefore, it is imperative to conduct clinical trials to confirm the effectiveness and safety of this combination therapy for patients. In addition, previous studies have shown that miR-33a-5p often collaborates with other miRNAs to modulate key signaling pathways involved in cell growth and survival (37,38). Notably, miR-33a-5p interacts with miR-128-3p in lung cancer (37), influencing processes such as cell proliferation, apoptosis and metastasis. This interaction suggests a complex regulatory network that may contribute to the resistance mechanisms observed in EC. Thus, additional experiments are necessary to investigate the relationship between miR-33a-5p and other miRNAs in EC, providing a more comprehensive understanding of the therapeutic potential of targeting miR-33a-5p alongside other miRNAs.

In conclusion, the present study demonstrated that PPVII, alone and in combination with MPA, effectively inhibited ECC proliferation by promoting apoptosis and inducing cell cycle arrest. The upregulation of miR-33a-5p by PPVII enhanced the sensitivity of MPA-resistant ECCs. The miR-33a-5p/FBXL16 axis may play a crucial role in this regulatory mechanism. While the findings of the present study provide significant insights, further research is needed to explore the detailed mechanisms and validate these results in *in vivo* and clinical settings.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

Conception and design of the research and drafting of the manuscript was conducted by HL; acquisition of data and revision of the manuscript for important intellectual content was conducted by YP and XZ; analysis and interpretation of data was conducted by HL and XZ; statistical analysis was conducted by YP. All authors contributed to editorial changes in the manuscript. All authors read and approved the final version of the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work. HL and XZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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