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Mifepristone inhibited tumor progression by disrupting the stability of PD-L1 by miR-127-3p/VAMP2 in ovarian cancer

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Abstract: To investigate the effect of mifepristone on PD-L1 through miR-127-3p/VAMP2 axis to inhibit the malignant biological behavior of ovarian cancer cells. Western blotting was used to detect the protein expression of VAMP2, PD-L1, CyclinD1, Cl-caspase-3 and Bax; qRT-PCR was used to detect the expression of miR-127-3p; double luciferase reporter gene was used to verify the targeted binding of miR-127-3p to VAMP2. The results showed that mifepristone up-regulated the expression of miR-127-3p and mifepristone could significantly inhibit the proliferation of ovarian cancer SKOV3 cells and A2780 cells, promote apoptosis, inhibit the expression of PD-L1, down regulate the expression of CyclinD1, and up regulate the expression of cl-caspase-3 and Bax; silencing miR-127-3p could restore the effects of mifepristone on the proliferation and apoptosis of SKOV3 cells and A2780 cells, as well as the expression of PD-L1, CyclinD1, Cl-caspase-3 and Bax protein; our study confirmed that mifepristone can regulate the expression of VAMP2 and PD-L1 through miR-127-3p and VAMP2 can positively regulate the expression of PD-L1; finally, we found that mifepristone can down regulate PD-L1 through miR-127-3p/VAMP2 axis, inhibit proliferation and promote apoptosis of ovarian cancer cells. Mifepristone can down regulate PD-L1 through miR-127-3p/VAMP2 axis and inhibit the progression of ovarian cancer cells.

Keywords: Ovarian cancer, mifepristone, PD-L1, miR-127-3p, VAMP2.

INTRODUCTION

Ovarian cancer is the highest mortality in gynecological malignant tumor and is the main reason for pathogenesis and death among women (Singh *et al*., 2019). There are no obvious clinical symptoms in the early stage of ovarian cancer, and most patients are often in the late stage at the time of detection. Despite the progresses made in multidisciplinary surgical intervention and tumor therapy, the prognosis is still poor (De Angelis *et al*., 2014, Allemani *et al*., 2015). Therefore, it is important to find a new drug therapy in the clinical treatment of ovarian cancer. Studies have found that mifepristone has a significant effect on anti-tumor growth and the fact that mifepristone is effective against the proliferation of breast cancer cells has been confirmed (Liu *et al*., 2020, Liu *et al*., 2016). In addition, recent studies have proved that mifepristone can inhibit the development of ovarian cancer, and some studies have shown that it can be used as a preventative treatment medicine to reduce the risk of ovarian cancer (Zhang *et al*., 2016, Ponandai-Srinivasan *et al*., 2019). However, there are few studies on the regulation mechanism of mifepristone in ovarian cancer. PD-L1 is an essential immune-regulating factor (Oliveira *et al*., 2019), and studies have verified that PD-L1 is highly expressed in ovarian cancer patients (Bekos *et al*., 2021). PD-L1 plays an important role in the regulation of the progression of ovarian cancer, but whether mifepristone affects the stability of PD-L1 in the inhibition of ovarian cancer remains unknown. As a tumor suppressor gene, miR-127-3p is low-expressed in ovarian cancer and multiple studies have certified that high-expressed miR-127-3p can remarkably restrain the proliferation of ovarian cancer cells (Du *et al*., 2020, Zhang *et al*., 2021). However, the role of miR-127-3p mediated by mifepristone in the development of anti-ovarian cancer tumors is still unclear. This research was conducted to evaluate the expression level of miR-127-3p mediated by mifepristone in ovarian cancer cells and explore downstream target genes of miR-127-3p. Moreover, the regulation mechanism of mifepristone inhibiting the progression of ovarian cancer by mediating the stability of PD-L1 was investigated, striving to provide a basic theoretical basis for the clinical treatment of ovarian cancer.

MATERIALS AND METHODS

Drugs and reagents

Mifepristone was purchased from Meilun Biotechnology Co., Ltd.; RPMI-1640 and 10% fetal bovine serum were purchased from Thermo Fisher Scientific Co., Ltd.; Lipofectamine 2000, Trizol reagent and reverse transcription reagent kits were purchased from Invitrogen; cell count reagent kit, CCK8 reagent and cell apoptosis detection kit were purchased from Beyotime Biotechnology Co., Ltd.; Cyclin D1, Cl-caspase-3, Bax, PD-L1, VAMP2 and β-actin antibody were purchased

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from Abcom; miR-127-3p primer synthesis, anti-miR-NC, anti-miR-127-3p, miR-NC, miR-127-3p, VAMP2, si-PD-L1, and luciferase reporter gene vectors of WT-VAMP2 and MUT- VAMP2 were purchased from Sangon Biotechnology Co., Ltd.

Cell culture and transfection

SKOV3 and A2780 ovarian cancer cells and normal cells IOSE80, purchased from National Type Culture Collection (NTCC, Beijing, China), were stored in RPMI-1640 medium containing 1% penicillin-streptomycin with a volume fraction of 10%, which was preserved in an incubator (37℃, 5% CO_2). SKOV3 and A2780 cells were treated with 0, 0.1, 1, 10, 25 and 100μM mifepristone, respectively. Anti-miR-127-3p, miR-127-3p, VAMP2 and si-PD-L1 were transfected into SKOV3 and A2780 cells according to the instructions of Lipofectamine 2000 reagent.

The detection of the expression of miR-127-3p by Real-Time qPCR

Cells in each group were collected, and then total RNA was extracted with Trizol reagent. Subsequently, cDNA was synthesized with reverse transcription reagent kit and amplified. The primer sequences of miR-127-3p were as follows: F 5'-GGGTCGGATCCGTCTGAGC-3' and R 5'-CAGTGCGTGTCGTGGAGT-3'. The relative expression of miR-127-3p was calculated by $2^{-\Delta\Delta CT}$ method.

The detection of cell proliferation by CCK-8 method

According to the method of Zhang et al. (Zhang *et al*., 2021), transfected ovarian cancer cells were inoculated into 96-well plates and placed in an incubator at 37℃ with 5% CO_2, and 10Ml CCK-8 reagent was added to each well. After incubation for 4 hours, OD value was detected at 450nm using an enzyme calibration.

The detection of cell apoptosis by flow cytometry

Cells in each group (1×10^6 cells/mL) were collected and washed once with 3mLPBS. After centrifugation, PBS was removed and cells were re-suspended with 3mLPBS. After another centrifugation, PBS was abandoned and 5μL Annexin V-FITC labeling was added and the cells were incubated at room temperature and dark for 15 minutes. Add 5μLPI into the cells before operation and immediately detect cell apoptosis by flow cytometer.

The detection of the associated protein expression by Western blotting

Cells were collected in each group and RIPA lysis solution was added to lyse cells and extract total protein. After the protein concentration was determined, 50μg total protein were taken for protein isolate by polyacrylamide-gel electrophoresis. The isolated proteins were transferred to PVDF membrane and sealed with 5% skimmed milk powder at room temperature for 1h, and CyclinD1 (1:200), Cl-Caspase-3 (1:500), Bax (1:1000), PD-L1 (1:1000), VAMP2 (1:1000), β-actin (1:1000) first antibody were added respectively and incubated overnight at 4℃. The membrane was washed with TBST, followed by an hour of secondary antibody incubation and the gray scales was analyzed by Image J software.

Double luciferase reporter gene

Referring to the method of Kou et al. (Kou *et al*., 2021), VAMP2-3'UTR luciferase reporter vectors of WT-VAMP2 (wild-type) and MUT-VAMP2 (mutant) were constructed. MiR-NC and miR-127-3p were co-transfected with WT-VAMP2 and MUT-VAMP2, respectively, in SKOV3 and A2780 cells by Lipofectamine 2000. After 24h, the luciferase activity was detected by the double luciferase reporter detection system.

STATISTICAL ANALYSIS

All data analyses were conducted using SPSS19.0 software, and GraphPad Prism 8.0 software was used for drawing. The measurement data were expressed as the mean standard deviation of three experimental replicates. The *t*-test was performed for comparisons between two groups, and Dunnett's or Tukey's single factor analysis of variance was used for comparison among multiple groups, with a statistically significant difference ($P<0.05$).

RESULTS

Effects of different concentrations of mifepristone on the cell viability of ovarian cancer

It could be found that (fig. 1) mifepristone can remarkably reduce the cell viability of ovarian cancer cells ($P<0.05$). Since the cell viability of ovarian cancer cells treated with 25μM mifepristone is about 50%, the concentration of mifepristone was selected as 25μM for subsequent experiments.

Effects of mifepristone on proliferation, apoptosis and PD-L1 expression of ovarian cancer cells

In comparison to the Control group, CCK-8 results suggested that the proliferation of ovarian cancer cells in the mifepristone group was markedly reduced ($P<0.05$) (fig. 2A, B), while the apoptosis ratio of cells in the mifepristone group was observably increased ($P<0.05$) (fig. 2C, D). Western blotting results implied that the expression levels of CyclinD1 and PD-L1 in the mifepristone group were notably decreased ($P<0.05$), while the expression levels of apoptosis-inducing related proteins Cl-caspase-3 and Bax were conspicuously increased ($P<0.05$) (fig. 2E, F).

The above results have indicated that mifepristone dramatically inhibited the proliferation of ovarian cancer cells and the expression of PD-L1 and promoted apoptosis.

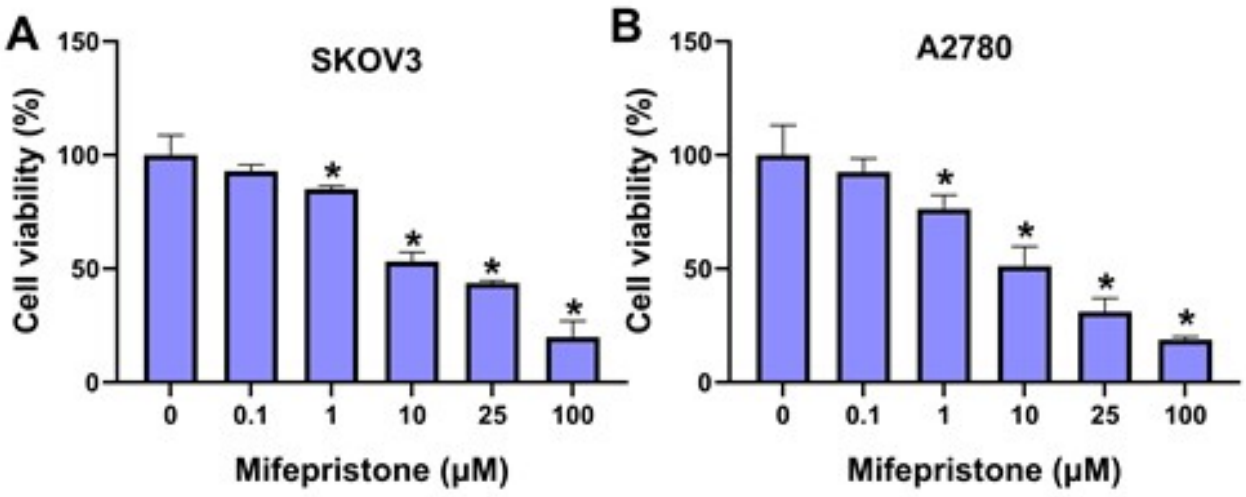

Fig. 1: Effects of different concentrations of mifepristone on the cell viability of ovarian cancer SKOV3 cells (A) and A2780 cells (B). $^{*}P<0.05$

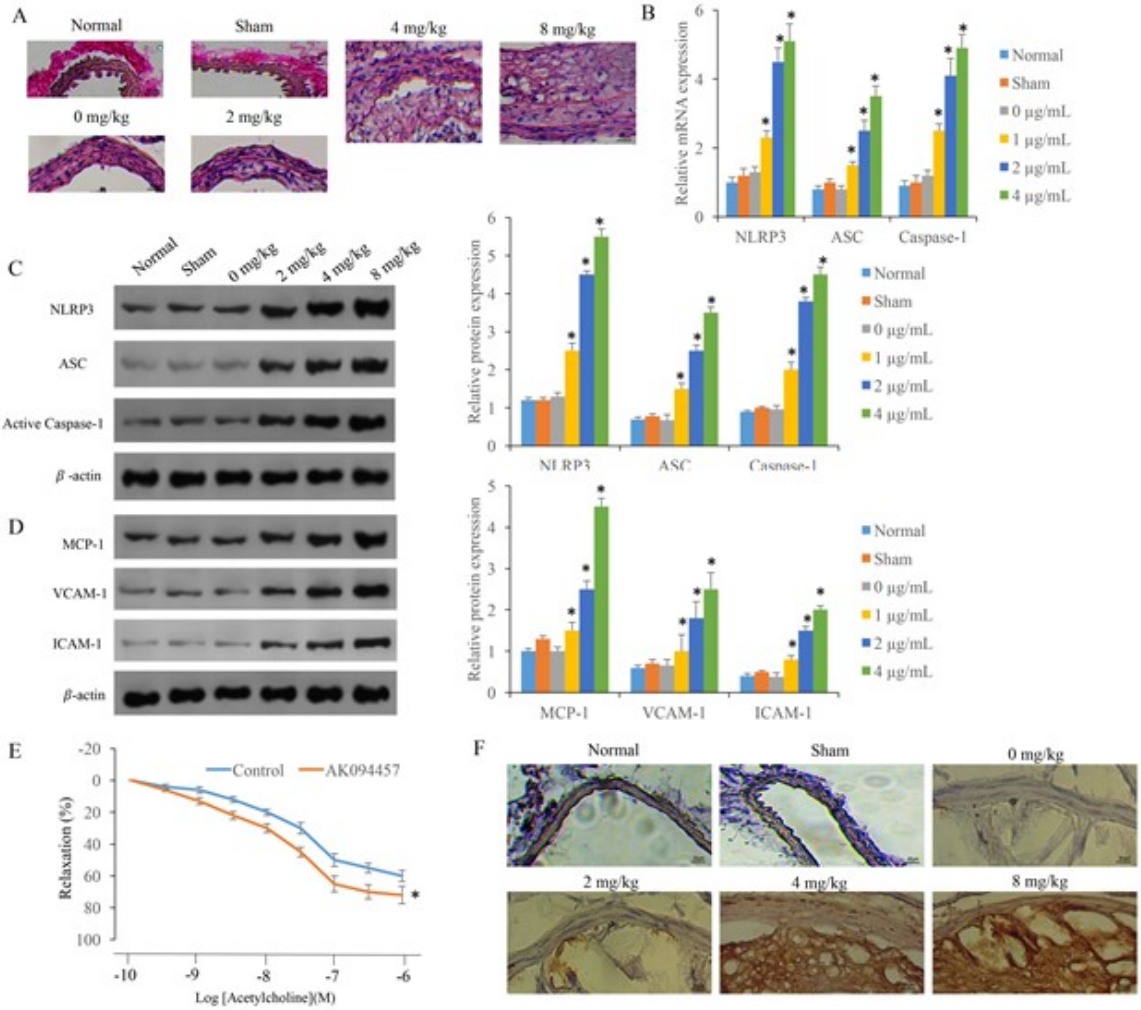

Fig. 2: Effects of mifepristone on proliferation, apoptosis and PD-L1 expression of ovarian cancer cells. A-B: CCK-8 was used to detect the proliferation of SKOV3 cells and A2780 cells; C-D: flow cytometry was used to detect the apoptosis of SKOV3 cells and A2780 cells; E-F: Western blotting was used to detect the expressions of CyclinD1, Cl-caspase-3, Bax, PD-L1 and β-actin. $^{*}P<0.05$

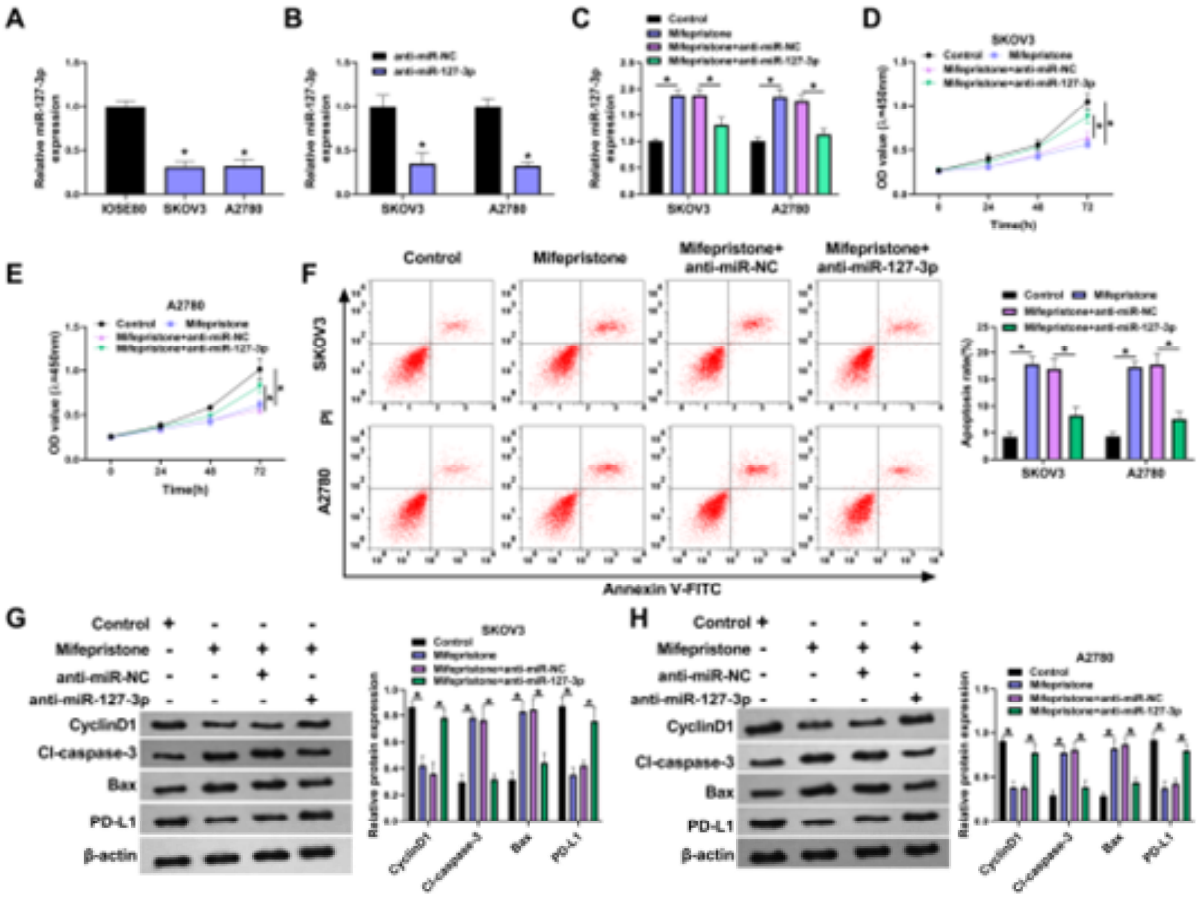

Fig. 3: Effects of silencing miR-127-3p reversed mifepristone on proliferation, apoptosis and PD-L1 expression of ovarian cancer cells A-C: qRt-PCR was used to detect the expression of miR-27-3p; D-E: CCK-8 was used to detect the proliferation of SKOV3 cells and A2780 cells; F: apoptosis of SKOV3 cells and A2780 cells was detected by flow cytometry; G-H: Western blotting was used to detect the expression of CyclinD1, Cl-caspase-3, Bax, PD-L1 and β-actin. **P*<0.05.

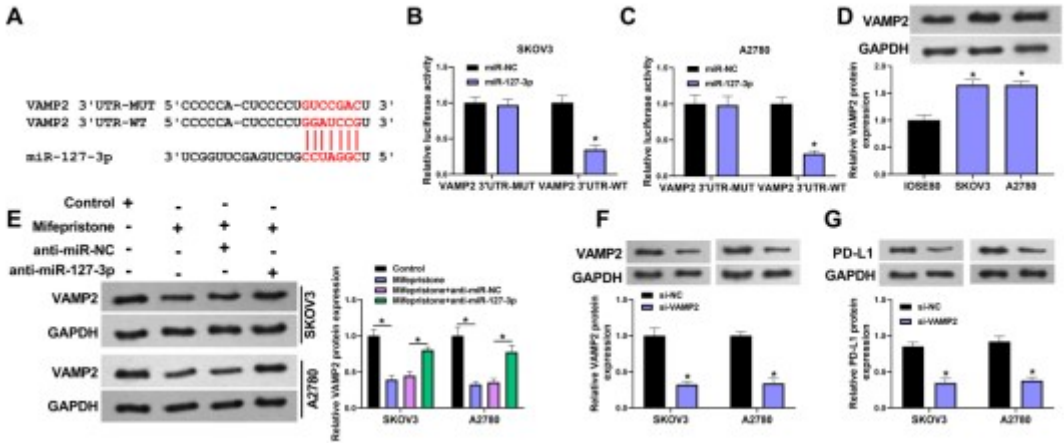

Fig. 4: The targeted binding of miR-127-3p and VAMP2 A: Starbase database predicted the binding site of miR-127-3p and VAMP2; B-C: double luciferase reporter gene verified the targeting relationship between miR-127-3p and VAMP2; D: Western blotting was used to detect the expression of VAMP2 in ovarian cancer cells; E: Western blotting was used to detect the expression of VAMP2; F: Western blotting was used to detect the knockdown efficiency of VAMP2; G: Western blotting was used to detect the expression of PD-L1. **P*<0.05

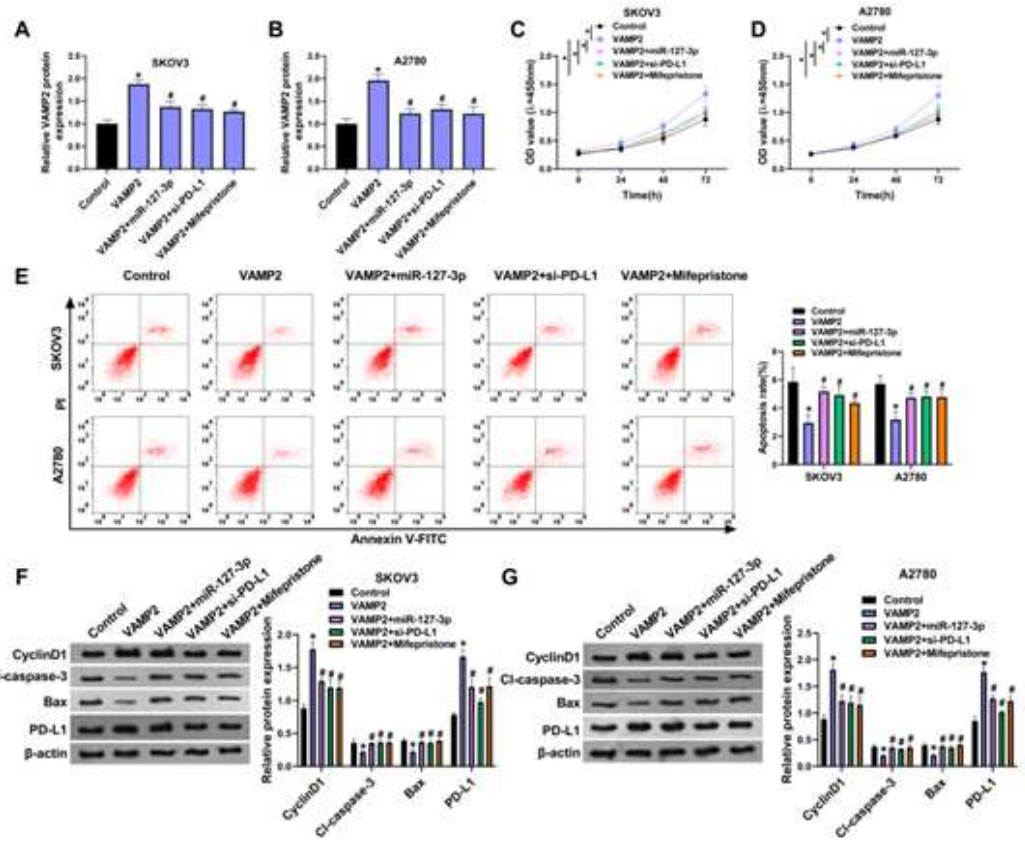

Fig. 5: Mifepristone can down regulate PD-L1 through miR-127-3p/VAMP2 axis, inhibit proliferation and promote apoptosis of ovarian cancer cells. A-B: Western blotting was used to detect the expression of VAMP2; C-D: CCK-8 was used to detect the proliferation of ovarian cancer cells: E: apoptosis of ovarian cancer cells was detected by flow cytometry; F-G: Western blotting was used for detecting the expression of CyclinD1, Cl-caspase-3, Bax, PD-L1 and β-actin. Compared with the Control group, $^{*}P<0.05$; relative to VAMP2 group, $^{\#}P<0.05$.

Effects of silencing miR-127-3p reversed mifepristone on proliferation, apoptosis and PD-L1 expression of ovarian cancer cells

As shown in fig. 3A, compared with normal cells, the expression of miR-127-3p in ovarian cancer cells was prominently decreased ($P<0.05$), indicating that miR-127-3p was low-expressed in ovarian cancer cells. From fig. 3B, it can be noticed that the expression of miR-127-3p was observably decreased after anti-miR-127-3p transfection ($P<0.05$), suggesting that the transfection was successful. Thereafter, ovarian cancer cells treated with mifepristone were transfected with anti-miR-127-3p. QRT-PCR results proved that anti-miR-127-3p markedly decreased the expression of miR-127-3p in mifepristone-treated ovarian cancer cells ($P<0.05$) (fig. 3C). CCK-8 results indicated that anti-miR-127-3p enhanced the proliferation of mifepristone-treated ovarian cancer cells (fig. 3D, E). Flow cytometry results confirmed that anti-miR-127-3p significantly reduced the apoptosis rate of ovarian cancer cells after mifepristone treatment ($P<0.05$) (fig. 3F). Western blotting results revealed that anti-miR-127-3p remarkably increased the protein expression levels of CyclinD1 and PD-L1 in ovarian cancer cells after mifepristone treatment, but notably decreased the protein expression levels of Cl-caspase-3 and Bax ($P<0.05$) (fig. 3G, H). The above results suggested the effects of silencing miR-127-3p reversed mifepristone on proliferation, apoptosis and PD-L1 expression of ovarian cancer cells.

The targeted binding of miR-127-3p and VAMP2

Starbase online database predicted that miR-127-3p had binding sites with VAMP2 (fig. 4A). At the same time, the results of double luciferase reporter gene experiment suggested that co-transfection of miR-127-3p conspicuously reduced the luciferase intensity of wild-type VAMP2 (WT-VAMP2) ($P<0.05$), while the luciferase intensity of mutant VAMP2 (MUT-VAMP2) showed no significant change ($P>0.05$) (fig. 4B, C), indicating that miR-127-3p and VAMP2 could be targeted bound. As shown in fig. 4D, compared with normal cells, the protein expression level of VAMP2 in ovarian cancer cells was

remarkably increased (P<0.05). Western blotting results implied that anti-miR-127-3p prominently increased the protein expression level of VAMP2 in ovarian cancer cells treated with mifepristone (P<0.05) (fig. 4E). Meanwhile, it can be seen from fig. 4F and fig. 4G that after knocking down VAMP2, the protein expression level of PD-L1 was observably inhibited, indicating that mifepristone may regulate the expression of PD-L1 through the miR-127-3p/VAMP2 axis and play a role in tumor suppression.

Mifepristone can down regulate PD-L1 through miR-127-3p/VAMP2 axis, inhibit proliferation and promote apoptosis of ovarian cancer cells

The cells were divided into Control, VAMP2, VAMP2+MIRI-127-3P, VAMP2+si-PD-L1, and VAMP2+Mifepristone groups. In comparison with the Control group, the expression of VAMP2 protein in the VAMP2 group was remarkably increased, while the expression of VAMP2 protein in other groups was recovered (P<0.05) (fig. 5A, B). The results of CCK-8 experiments demonstrated that the over-expression of VAMP2 markedly enhanced the proliferation of ovarian cancer cells (P<0.05) (fig. 5C, D). Flow cytometry results showed that over-expression of VAMP2 notably reduced the apoptosis rate of ovarian cancer cells (P<0.05) (fig. 5F); Western blotting results indicated that compared with the Control group, the expression levels of CyclinD1 and PD-L1 proteins in the VAMP2 group were observably increased and the protein levels of Cl-caspase-3 and Bax proteins were significantly decreased (P<0.05) (fig. 5F, G). However, VAMP2+miR-127-3p, VAMP2+si-PD-L1 and VAMP2+Mifepristone could restore the effects of over-expression of VAMP2 on the proliferation and apoptosis of ovarian cancer cells as well as on the expression of PD-L1 protein. The above results implied that mifepristone can down regulate PD-L1 through miR-127-3p/VAMP2 axis, inhibit proliferation and promote apoptosis of ovarian cancer cells.

DISCUSSION

Ovarian cancer is a highly heterogeneous malignant tumor. Due to the difficulties in early diagnosis, ovarian cancer is more common in the middle and late stages and it has a high mortality (Momenimovahed *et al*., 2019, La, 2017). Although advances have been made in molecular prediction index diagnosis, surgery and chemotherapy in the treatment of advanced ovarian cancer in the past decades (Krzystyniak *et al*., 2016, Dochez *et al*., 2019), the clinical results of ovarian cancer patients are not optimistic due to the increase in chemotherapy resistance and recurrence of patients. Therefore, it is very important to develop a new effective treatment strategy. Progestogen in ovarian cancer is a crucial endogenous factor that can induce the primary tumor to develop into metastatic ovarian cancer. Mifepristone is the first synthetic progesterone receptor regulator in the world. Mifepristone has an antagonistic effect, and can effectively bind to the progesterone receptor PGR, causing conformational changes of carboxyl terminus of PGR to block the progesterone signaling pathway, thus effectively inhibiting the development of ovarian cancer (Bygdeman *et al*., 1993, Vegeto *et al*., 1992, Kim *et al*., 2020). Previous scholars have confirmed that mifepristone can prominently inhibit the proliferation of ovarian mesenchymal stem cells in BRCA carriers, thus reducing the risk of ovarian cancer (Ponandai-Srinivasan *et al*., 2019). In their research, Zhang et al. (Zhang *et al*., 2016) found that mifepristone up-regulated GRP78 and CHOP in ovarian cancer cells, and at the same time, three pathways related to unfolded protein response-PERK, IRE1α and ATF6, were all activated by mifepristone, verifying that mifepristone could trigger unfolded protein response and kill ovarian cancer cells. Goyeneche et al. (Goyeneche *et al*., 2007) also confirmed the inhibitory effect of mifepristone on ovarian cancer in vivo and vitro experiments. This study found that mifepristone could inhibit the proliferation of ovarian cancer SKOV3 cells and A2780 cells, and promote the apoptosis of them, once again proving that mifepristone had a significant inhibitory effect on the development of ovarian cancer.

PD-L1, encoded by CD274 gene, is an immunosuppressive molecule (Oliveira *et al*., 2019). It has been reported that PD-L1 can inhibit the activation of CD+T cells in lymph nodes (Filippova *et al*., 2018, Kythreotou *et al*., 2018, Wang *et al*., 2018, Wu*et al*., 2019) and the interaction of PD-L1 and PD1 to restore anti-tumor immunity (Llosa *et al*., 2015, Guo *et al*., 2019) by binding to programmed cell death receptor 1(PD1). More and more people have proved the role of PD-L1 in ovarian cancer. Anastasiadou et al. (Anastasiadou *et al*., 2021) confirmed in the previous study that PD-L1 was highly expressed in ovarian cancer and the vitro experiment showed that the over-expression of miR-200c-3p could exert the anti-cancer effect by reducing the expression of PD-L1. Bekos et al. (Bekos *et al*., 2021) and Yun et al. (Yun *et al*., 2021) also confirmed this view in their studies. However, the regulation of mifepristone on PD-L1 is still unclear. In our previous study, we found that mifepristone could significantly inhibit the expression of PD-L1 in ovarian cancer cells, indicating that mifepristone could exert its anti-cancer effect by destroying the stability of PD-L1. However, the mechanism of mifepristone in regulating PD-L1 remains unknown.

In the previous study, mifepristone was found to up-regulate the expression of miR-127-3p. Du et al. (Du *et al*., 2020) and Zhang et al. (Zhang *et al*., 2021) have confirmed in their studies that miR-127-3p plays an important role in ovarian cancer. This study found that silencing miR-127-3p reversed mifepristone has the regulatory effect on ovarian cancer cells, indicating that

miR-127-3p is also crucial in the inhibition of ovarian cancer by mifepristone. Next, we explored the downstream target of miR-127-3p, and found the targeted binding of VAMP2 and miR-127-3p. It has been proved that VAMP2 is highly expressed in ovarian cancer cells, and the high expression of VAMP2 promotes the proliferation of ovarian cancer cells (Sun *et al*., 2019, Li *et al*., 2020, Lin *et al*., 2020, Li *et al*., 2019, Zhang *et al*., 2019). This study has once again confirmed that the over-expression of VAMP2 promotes the proliferation of ovarian cancer cells and inhibits apoptosis. At the same time, this study has also revealed that mifepristone can remarkably inhibit the expression of VAMP2, indicating that mifepristone can exert the anti-cancer effect by regulating the expression of VAMP2 through miR-127-3p.

CONCLUSION

In this study, we also found that knocking down the expression of VAMP2 effectively inhibited the expression of PD-L1. At the same time, the anaplerosis experiment also found that knocking down miR-127-3p and over-expression of PD-L1 could reverse the effects of VAMP2 on ovarian cancer cells. The above results indicated that the regulation of PD-L1 stability by mifepristone was achieved through miR-127-3p/VAMP2 pathway. This study revealed a new regulatory mechanism of mifepristone in the anti-ovarian cancer progression, and provided a powerful basic theoretical basis for the anti-ovarian cancer research.

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