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Response of Subcutaneous Xenografts of Endometrial Cancer in Nude Mice to Inhibitors of Phosphatidylinositol 3-Kinase/Akt and Mitogen-Activated Protein Kinase (MAPK) Pathways: An Effective Therapeutic Strategy for Endometrial Cancer

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Abstract

Objective: This study was designed to explore whether inhibition of the extracellular-regulated kinase (ERK) and phosphatidylinositol-3-kinase (PI3K) signaling pathways can inhibit the growth of xenografts of endometrial cancer cell lines with different estrogen receptors (ER) profiles *in vivo* and to provide preliminary laboratory basis for the probability of endometrial adenocarcinoma treatment with blockage of the two pathways, especially to endometrial cancer with low ER status. Methods: Human endometrial cancer Ishikawa bearing ER and HEC-1A with low ER status cells were subcutaneously injected into BALB/c nude mice to establish endometrial cancer xenograft tumor models. The effects of PI3K/Akt inhibitor LY294002, MAPK/ERK1/2 inhibitor PD-98059 and their combinations on the growth of the xenograft tumors and apoptotic state of Ishikawa and HEC-1A cells were tested *in vivo* using the inhibitory rate, the terminal deoxynucleotidyl transferase-mediated nick-end labeling assay, H/E-stain. Western blot analysis was used to detect the alterations of activated ERK (P-ERK) and AKT (P-AKT) during this process. Results: LY294002, a PI3K/Akt pathway inhibitor, induced significant suppression in the growth of both Ishikawa and

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HEC-1A cell xenograft tumors, concomitant with increased apoptosis in xenografts as evidenced by TUNEL. A similar effect was also observed when the MAPK/ERK1/2 signaling pathway was inhibited by PD98059. Concurrent inhibition of the PI3K/Akt and MAPK/ERK1/2 pathways showed enhanced anti-tumor effects *in vivo* as indicated by increased apoptosis. At the same time, the levels of P-ERK and P-AKT in both xenograft tumors decreased, and their levels in combination group was the lowest. Conclusions: PD98059, LY294002 and their combinations showed remarkable inhibitory effects on xenograft tumors of endometrial carcinoma cell lines with different expression status of ER *in vivo* through blockage of PI3K/Akt and MAPK/ERK1/2 signaling pathways. This suggests that targeting these pathways may be an effective therapeutic strategy against endometrial carcinomas, especially for ER-negative cancers which show poor response to endocrinal therapy.

Keywords

Extracellular-Regulated Kinase (ERK), Proto-Oncogene Proteins, Akt, ERK Pathway Inhibitor, PD98059, Phosphatidylinositol-3-Kinase Pathway Inhibitor, LY294002, Endometrial Cancer Cell, Estrogen Receptor

1. Introduction

Endometrial carcinoma is one of the most common gynecological carcinomas. Two types of endometrial cancers have been described based on clinical and histopathology features. Type I endometrial carcinomas are usually found in patients with a high estrogen level that is unopposed by progestin. These well-differentiated tumors have good response to endocrinal therapy and a good prognosis. On the other hand, type II is generally associated with the normal estrogen state and usually shows poor response to endocrinal therapy. These tumors are usually poorly differentiated, highly malignant and present bad prognosis. Therefore, it is necessary to find a new strategy for the treatment of endometrial cancers, especially for type II.

Prior studies of endometrial carcinoma cell lines have shown that 17β-estradiol can activate the PI3K/Akt and MAPK/ERK signaling pathways [1]-[4]. It is widely accepted that MAPK/ERK and PI3K/Akt signaling cascades play critical roles in the transmission of signals that regulate gene expression, prevent apoptosis, and influence tumor progression and the effectiveness of anti-tumor therapy [5]-[8]. Mutation or aberrant expression of the components of these pathways has been observed in a variety of human cancers such as ovarian and endometrial cancers. For example, the levels of P-Akt and P-ERK found in endometrial adenocarcinoma and atypical endometrial hyperplasia were significantly higher than that in the proliferative phase endometrium [9] [10].

As a downstream target protein of PI3K, Akt is the core of the PI3K/Akt signaling pathway. Akt is also known as protein kinase B (PKB) and consists of three distinct domains: an aminoterminal pleckstrin homology (PH) domain containing a region for binding inositol phospholipids, a kinase domain, and a carboxyl terminal regulatory domain [8]. Akt is fully activated with phosphorylation Thr308 and Ser473, activated Akt is also called phosphorylated Akt (P-Akt). P-Akt induces the phosphorylation of its downstream targets, promotes tumor cell growth, hinders apoptosis, and contributes to tumor angiogenesis [11] [12].

The mitogen-activated protein kinase pathway (MAPK/MEK/ERK1/2) is another important signaling pathway which converts extracellular stimulation to intracellular signals that control gene expression, cell proliferation, differentiation, and survival. It plays an important role in the process of cell malignant transformation. The MAPK signaling pathway activates ERK1/2 through multiple kinase cascades. The phosphorylation ERK1/2 (P-ERK1/2) is activated and then transfers signals from cell surface receptors to the nucleus: p-ERK1/2 transfers into nucleus to regulate various transcription factors such as e-Mye, sTArs, Jun, Fos, ATFZ and Max. These transcription factors further regulate their own target genes, causing specific changes in protein expression or activity, and ultimately regulate the cell metabolism and function to produce specific biological effects [13]. Blocking MAPK/ERK or PI3K/Akt signaling pathways could suppress cell proliferation of endometrial cancers *in vitro* [5] [14]. It has been recognized that the activation of MAPK/ERK or PI3K/Akt signaling pathways is closely related to the occurrence and development of endometrial cancers nowadays. As a result, blocking these

pathways might be supposed as a new molecular target therapy in endometrial carcinomas.

PD98059 and LY294002 can specifically block the MAPK/ERK1/2 or PI3K/Akt signaling pathways by inhibiting the activation of ERK1/2 or Akt, respectively. Here, we studied the effects of PD98059 and LY294002 on xenograft tumors of Ishikawa and HEC-1A endometrial carcinoma cell lines that have different estrogen receptor (ER) status. We found that blocking the MAPK/ERK and PI3K/Akt could effectively reduce tumor growth and enhance apoptosis in xenografts of Ishikawa and HEC-1A cell lines, suggesting that these pathways might be important targets for treating endometrial cancers.

2. Materials and Methods

2.1. Cell Lines and Culturing

The human endometrial carcinoma cell lines Ishikawa and HEC-1A were a kind gift from Prof. Wei, Peking University People's Hospital. The cell lines were maintained in DMEM (with 100 U/ml penicillin and 100 g/ml streptomycin, Gibco Company) supplemented with 10% FCS (Hangzhou Evergreen Company). Both cell lines were incubated in a humidified atmosphere of 5% CO_2 at 37˚C. Cultured cells were dissociated using 0.25% trypsin (Gibco Company), washed once in serum containing cell culture medium, once in PBS and thereafter resuspended in PBS at a concentration of 1×10^8 cells/ml for injection.

Female BALB/c nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and raised in the Experimental Animal Center of Henan Province, Clean Animal Room (SPF). A concentration of 1×10^8 cells in 1ml phosphate-buffered saline (PBS) was chosen as the suitable number of cells to be injected based on a pilot study. Mice at the age of 4 - 6 weeks (n = 48) were injected subcutaneously with human Ishikawa or HEC-1A cells (2×10^7 cells in 0.2ml PBS, n = 24 in each group) on the back. The cells were allowed to grow for 2 weeks to reach the proliferative phase. After two weeks, the mice injected with each cell type were randomly divided into four groups (n = 6 mice/group) to receive intraperitoneal injection of either saline (control), PD98059 (50 mg/kg, U.S. CST Corporation), LY294002 (50 mg/kg, U.S. CST Corporation) or PD98059 plus LY294002 twice a week for 3 weeks. The control mice were injected with the same volume of saline as the other drugs. The sizes of xenograft tumors were measured externally twice a week, and the tumor volume was calculated using the following formula: volume = $(a \times b^2)/2$, where a and b are the lengths of the major axis and the minor axis, respectively. The animals were monitored for 35 days after cell injection and sacrificed by cervical dislocation.

2.2. Pathological Analysis

Parts of the xenograft tumors were paraffin embedded, sectioned and H/E-stained for pathological examination.

2.3. Apoptosis Analysis

The terminal deoxy-UTP nick-end labeling (TUNEL) assays were carried out using an *in situ* apoptosis detection kit (KGI Biological Development Co., Ltd. Nanjing). Experiments were carried out following the manufacturer's instructions. In brief, three tissue biopsies were obtained from each individual paraffin-embedded tissue sample. The tissue biopsies were permeabilized with protein K at 37˚C for 20 min and washed twice with PBS. Terminal deoxynucleotidyl transferase solution was added to the tissue biopsies which were then incubated for 1 h in a 37˚C humidified incubator. They were then washed three times with PBS, and incubated in Streptavidin-HRP solution for 30 min at 37˚C. After washing thrice in PBS, the tissue biopsies were stained with DAB and Hematoxylin, and examined under an inverted microscope. The percentage of apoptotic nuclei was calculated as a percentage of the total counted nuclei from scoring at least 100 cells. Each count was repeated three times to ensure accuracy.

2.4. Western Blot Analysis

Tumor tissues were homogenized in lysis buffer with a glass tissue homogenizer according to standard protocols. The lysates were centrifuged at 15,000 g for 10 minutes at 4˚C and the supernatants were collected. The protein concentration of the supernatants was then measured. The extracted proteins were denatured at 100˚C before being subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose

membranes. The membranes (PVDF) were incubated in a blocking solution containing 5% skim milk in Tris-buffered saline supplemented with 0.1% Tween 20 (TBST) for 1 hour at room temperature. The resulting membranes were incubated overnight at 4˚C with the appropriate primary antibodies diluted in the blocking solution. The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:1000 dilutions in TBST) at room temperature for 1 hour. The proteins were then detected with the enhanced chemiluminecence system (ECL, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.). The membrane was subsequently exposed to X-Kodak films for about 45s and then processed. The above procedures were repeated for Western blot analysis. All primary antibodies (Rabbit polyclonal antibodies against human phosphorylated Akt (Ser473) and Mouse anti-human ERK1/2 monoclonal antibody (sc7383), Santa Cruz Biotechnology) were used at 1:500 dilution and all secondary antibodies (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) were used at 1:1000 dilution. The activation of Akt and ERK1/2 was defined as the ratio of the p-Akt and p-ERK1/2 to β-actin and quantitatively evaluated after measuring the optical density of the protein bands.

2.5. Statistics

Data analysis was performed using the SPSS (17.0) software package. All data are expressed as mean ± standard deviation (X ± SD). Unpaired t test was used for analyzing pairs of data whereas comparison among various groups was performed by analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Results

To determine whether the effects of PD98059 and LY294002 on xenograft tumors of endometrial carcinoma cells were correlated with estrogen receptor (ER) status, we established xenograft tumor models with Ishikawa and HEC-1A cell lines that have distinct ER profiles (**Figure 1**).

3.1. Effects of PD98059 and LY294002 on Xenograft Tumors of Ishikawa Cells

Macroscopically, the mice began to show a tumor of palpable size about 8 days after being injected with Ishikawa cells,, but the growth rates of those tumors were different in the four groups (**Figure 2(A)**. F = 23.056, P = 0.000). Relative to the controls, LY294002 induced significant suppression in the growth of Ishikawa cell xenograft tumors. The mean volume of tumors treated with LY294002 (1874.56 ± 485.03 mm^3) was reduced by 39.73% compared with the controls (3110.6 ± 599.66 mm^3) at day 35 (F = 23.228, $P < 0.05$) (**Figure 2(A)**, **Figure 2(C)**). Parts of the xenograft tumors were paraffin embedded, sectioned and H/E-stained for pathological examination (**Figure 3**). According to TUNEL assays, the fraction of apoptotic cells in the tumor tissues of this group (apoptotic ratio = 19.37%) was much higher than that of the control group (apoptotic ratio = 3.33%, $P <$ 0.05) (**Figure 4**). Likewise, PD98059 significantly inhibited the growth of Ishikawa cell xenograft tumors. The mean volume of tumors (2072.33 ± 271.18 mm^3) was reduced by 34.82% (F = 23.228, $P < 0.05$) and the corresponding apoptotic ratio (18.57%) was much higher compared with the controls (apoptotic ratio = 3.33%, $P < 0.05$). Addi-

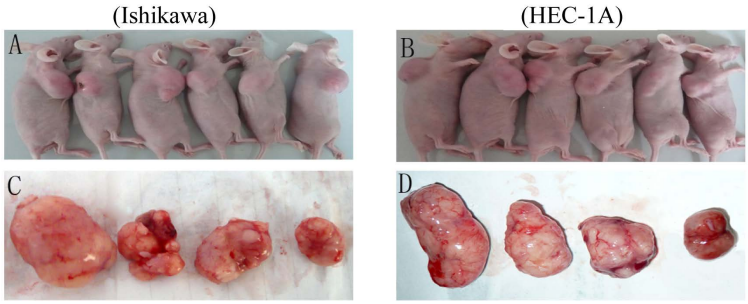

Figure 1. Establishment of xenograft tumors in nude mice. (A) representative xenograft models of the Ishikawa cell line. (B) representative xenograft models of the HEC-1A cell line. (C), (D) representative xenograft tumors in mice in different treatment groups.

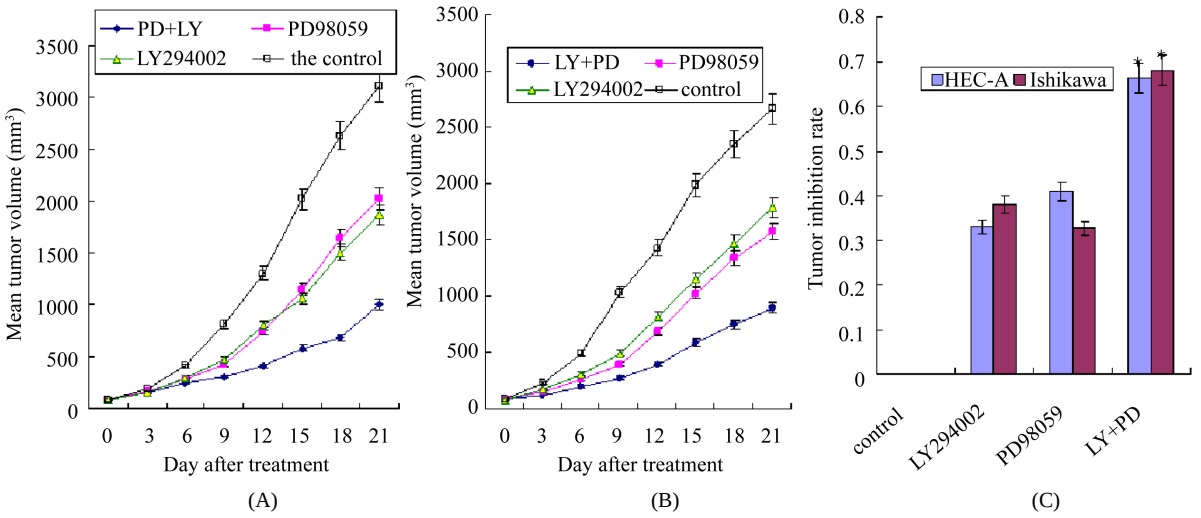

Figure 2. Effect of PD98059, LY294002 and their combination on the growth of human endometrial carcinoma xenografts. Ishikawa (A) and HEC-1A (B) cells were grown as xenografts in nude mice. After tumor establishment (14 days after cells were injected, tumor volume reached about 80 mm^3), mice were treated with PD98059, LY294002, PD98059 + LY294002 or saline by intraperitoneal injection for 3 weeks. Tumors were measured twice a week using calipers. Both PD98059 and LY294002 significantly delayed tumor growth in both Ishikawa (A) and HEC-1A (B) cell xenograft models when compared with the control, and their inhibitory effect was enhanced when they were used simultaneously. (C) Bar diagram representing mean ± SD of tumor inhibition ratios, $*P < 0.01$ compared between groups treated with PD98059 or LY294002 only.

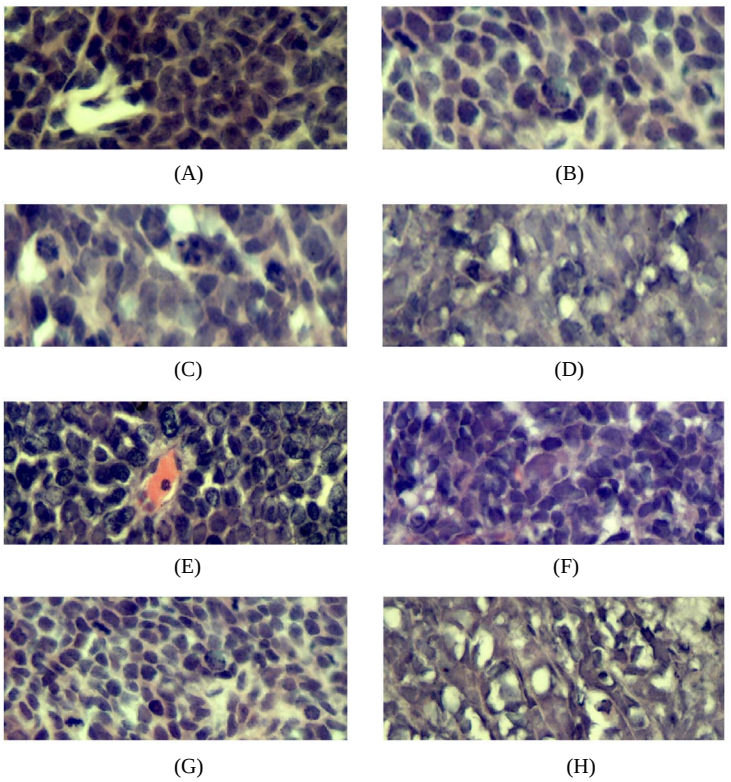

Figure 3. H/E staining (×40) of xenograft tumors. Representative H/E-stained sections of Ishikawa (A)-(D) and HEC-1A cell (E)-(H) xenografts in different groups. Compared with the controls, the number of tumor cells decreased and the number of nuclei decreased by varying degrees in groups treated with PD98059, LY294002 or a combination of both.

tionally, in xenograft tumors arising from these cell lines, there was no significant difference between the efficiency

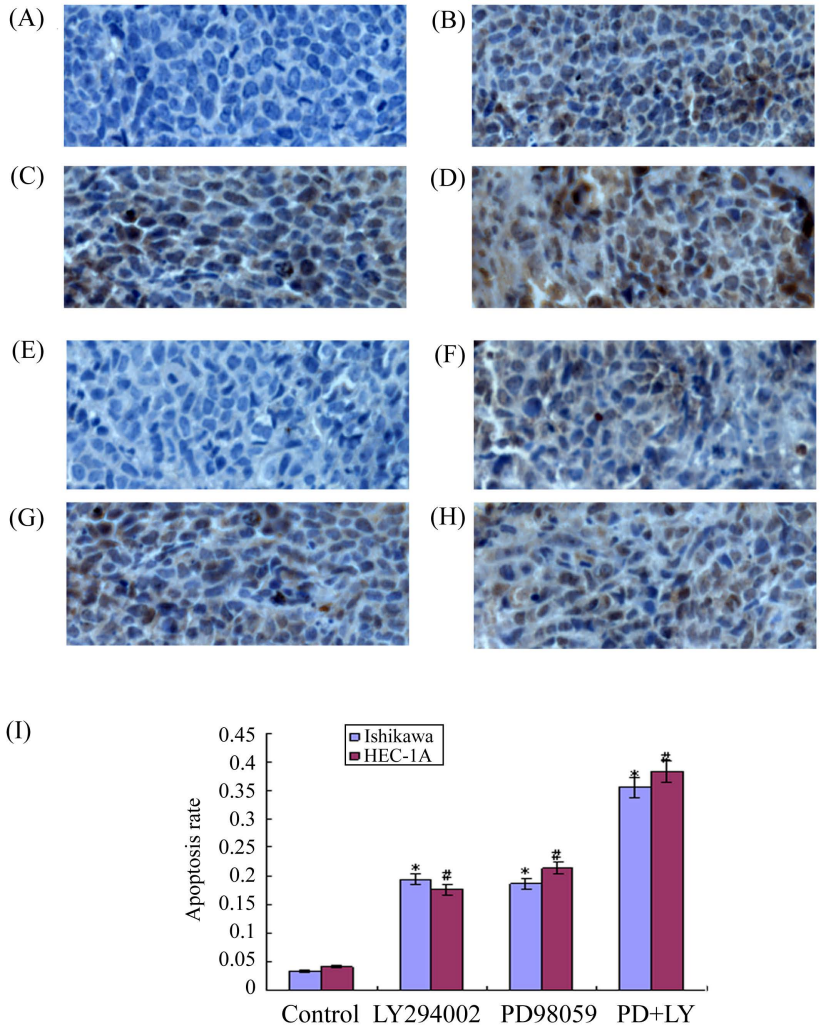

Figure 4. The effects of PD98059 and LY294002 on the rate of apoptosis. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was conducted on the control, LY294002, PD98059 and PD98059 + LY294002 groups. Apoptosis was measured in paraffin-embedded xenograft tumors of Ishikawa and HEC-1A cells (×40). The apoptosis rate in the xenografts of different groups (I), presented as mean ± SD, **P* < 0.01, #*P* < 0.01 compared with the control group of their corresponding cell line.

of LY294002 and PD98059 in inhibiting the growth of the xenograft (F = 23.228, *P* = 0.555). However, the response of xenograft tumors to a combination of the two inhibitors was significantly stronger than to PD-98059 alone (F = 23.228, *P* = 0.003) or LY294002 alone (F = 23.056, *P* = 0.001). The mean volume of tumors (3110.6 ± 599.7 mm^3) was reduced by 67.98% compared with the controls, and the rate of apoptosis reached 35.60%, which was much higher than that caused by PD98059 alone (F = 71.010, *P* < 0.05) or LY294002 alone (F = 71.010, *P* < 0.05) (**Figure 4**).

3.2. Effects of PD98059 and LY294002 on Xenograft Tumors of HEC-1A Cells

We found that LY294002 effectively inhibited the growth of xenograft tumors (mean volume = 1791.5 ± 296.1 mm^3) by 32.77% compared with the controls (mean volume = 2664.7 ± 280.59 mm^3) (F = 41.841, *P* = 0.000). The apoptotic rate in the LY294002-treated tumors was 17.53%, it is higher than that in the controls (apoptotic ratio = 4.23%, F = 79.632, *P* < 0.05). In the group treated with PD98059 alone, volumes of xenografts in mice (mean volume = 1573.2 ± 264.7 mm^3) was reduced by 40.96% (F = 41.841, *P* = 0.000) compared with the control. The apoptotic ratio (21.33% in) was also higher than the controls (F = 79.632, *P* < 0.05) in this group.

However, the anti-tumor effect of PD98059 was not significantly different when compared with the effect of LY294002 (F = 41.841, P = 0.186), their apoptotic ratios showed no significantly difference (F = 79.632, P = 0.103), too. Additionally, the combination of the two inhibitors induced a stronger suppression of the growth of the xenografts. The mean volume (900.2 ± 260.1 mm^3) in the LY294002 + PD98059 group was reduced by 66.22% compared with the controls (F = 41.841, P = 0.000), and the mean apoptotic ratio in the group was 38.33% (**Figure 2**; **Figure 4**).

3.3. PD98059 and LY294002 Reduced the Activation of PI3K and MAPK Pathways in Ishikawa and HEC-1A Cells

To study the constitutive activation of PI3K/Akt and MAPK/ERK1/2 in the xenografts of the two endometrial carcinoma cell lines, we firstly analyzed the basal p-Akt and p-ERK1/2 levels by Western blot analysis (**Figure 5**). Our results showed that HEC-1A cell lines had weaker p-Akt (t = −12.280, $P < 0.05$) and relatively high p-ERK1/2 levels (t = 10.851, $P < 0.05$), while Ishikawa cell lines had weaker p-ERK1/2 and relatively high p-Akt levels. This indicates that there are inherent differences between the p-Akt and p-ERK1/2 levels in the xenograft tumors of different endometrial carcinomal cell lines.

We tested the effects of PI3K inhibitors LY294002, the MAPK inhibitors PD98059 and their combinations on the activation of Akt and ERK1/2 by western blot analysis in vivo. It revealed that LY294002 induced decreased levels of p-Akt protein expression in xenografts of both Ishikawa (F = 462.084, $P < 0.05$) and HEC-1A (F = 78.287, $P < 0.05$) cell lines compared with the controls. PD98059 induced decreased levels of p-ERK but have no effect on levels of p-Akt in xenografts of Ishikawa (F = 462.084, P = 0.057) and HEC-1A cells (F = 78.287, P = 0.831) (**Figure 6**). Combinations of the two inhibitors showed an enhance effect. The single treatment with LY294002 reduced levels of p-Akt by 72.4% in Ishikawa and 53.57% in HEC-1A cell group, While PD98059 inhibit the p-ERK1/2 level by 48.51% in Ishikawa and 67.21% in HEC-1A cell group compared with the controls. Suppression of p-Akt(inhibition rate,86.28% in Ishikawa and 81.26% in HEC-1A cell group) and p-ERK1/2 (inhibit rate 79.24% in Ishikawa and 80.87% in HEC-1A cell group)expression levels was enhanced by the concurrent injection of PD98059 or LY294002.this finding was matched with the apoptosis analysis and the rate of tumor growth.

4. Discussion

In this study, we found that intraperitoneal injection of the specific PI3K inhibitor LY294002 or MAPK inhibitor PD98059 effectively suppresses endometrial cancer xenograft tumors growth by inducing apoptosis *in vivo*. Our observations are consistent with previous studies on endometrial cancer cells *in vitro* [5] [14]. We also found that the inhibitory effect on these xenografts were more significant when the PI3K/Akt and MAPK/ERK1/2 pathways were inhibited simultaneously.

Akt and ERK1/2 have previously been shown to be constitutively active in premalignant and malignant human endometrial cancers [9] [10]. They are known to stimulate cell proliferation, inhibit apoptosis and thus prolong cell survival endometrial cancer *in vitro* upon activation by stimuli such as estradiol (E2) and SDF-1α

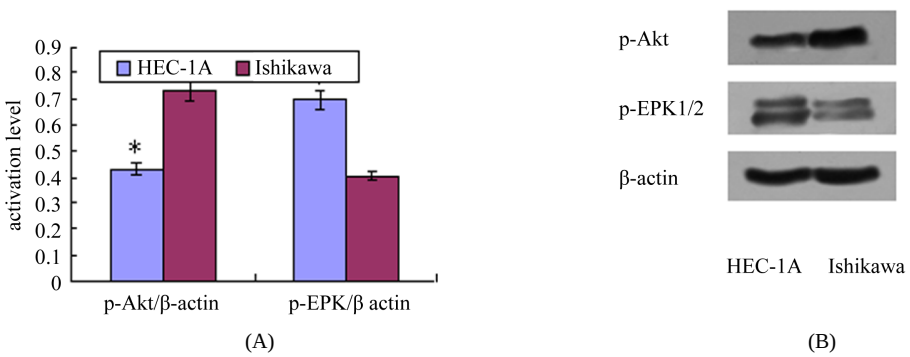

Figure 5. The p-Akt and p-ERK1/2 levels in control xenografts of Ishikawa and HEC-1A cell was analyzed by western blot. Xenografts of Ishikawa and HEC-1A cell lines have different p-Akt, p-ERK1/2 levels (A-B).*$P < 0.01$.

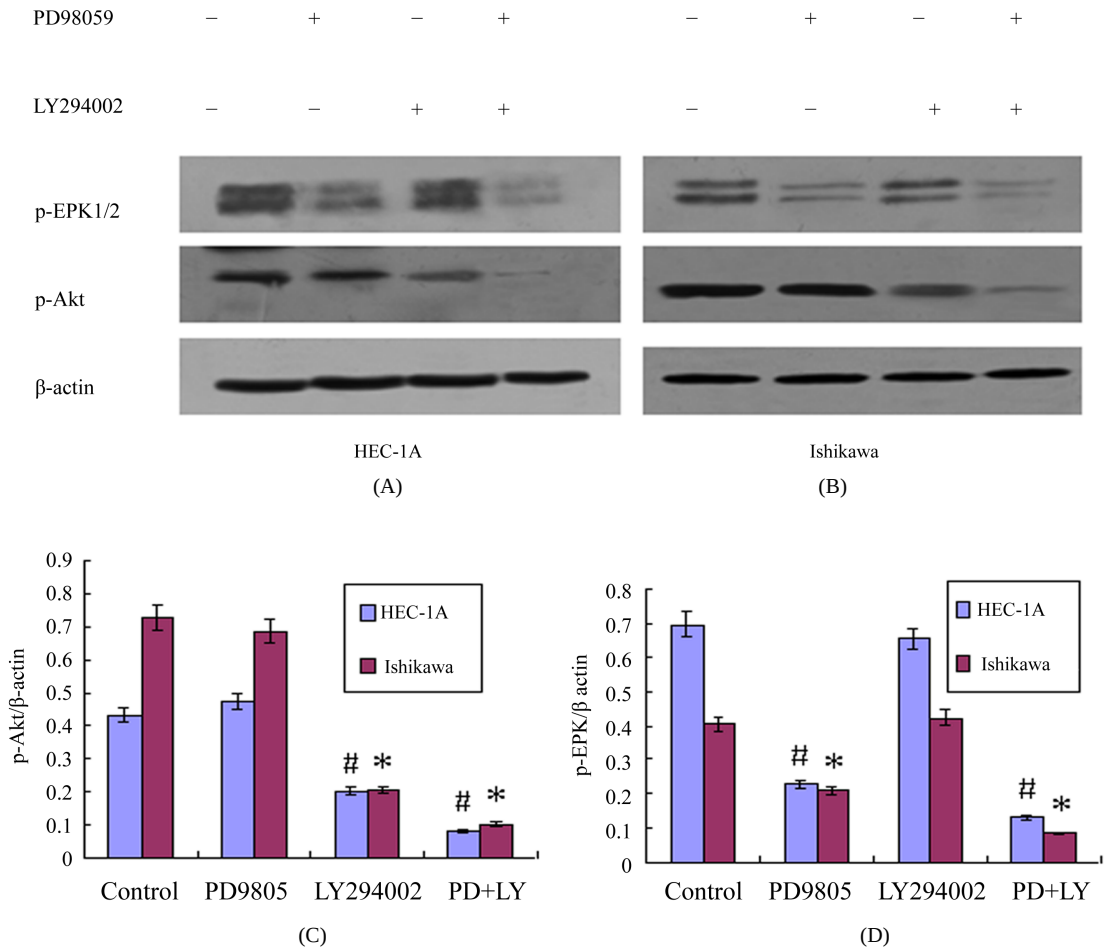

Figure 6. The p-Akt and p-ERK1/2 levels in xenografts of different groups were detected by western blot. (A): Levels of p-Akt and p-ERK1/2 in xenografts of HEC-1A cell lines. (B): Levels of p-Akt and p-ERK1/2 in xenografts of Ishikawa cell lines. (C), (D): bar diagram of the ratios of p-Akt:*β*-actin and p-ERK1/2:*β*-actin in the two endometrial cancer cell groups. The results represent mean ± SD of three independent experiments each performed in triplicate (*$P < 0.01$; #$P < 0.01$ compared with the controls).

[1]-[4] [15]. Thus, activation of the PI3K/Akt and MAPK/ERK1/2 pathways likely play important roles in the occurrence and development of endometrial cancer. Additionally, these signaling pathways may be important potential targets for chemoprevention and treatment of endometrial cancer.

Ishikawa and HEC-1A cells are both endometrial carcinoma cell lines. Ishikawa cells with rich-expressed ER, in contrast, HEC-1A cells either completely lack or show low levels of ER expression [16] [17]. Previous research has shown that anti-estrogen agents and estrogen receptor antagonist ICI182780 can inhibit proliferation of Ishikawa cells, promote its apoptosis, and arrest its cell cycle at the G1 phase, but have no significant effects on ER-negative HEC-1A cells [18]. This suggests that anti-estrogen treatment on ER-negative endometrial cancer may not be effective.

In order to further investigate whether the activation of PI3K/Akt and MAPK/MEK/ERK1/2 signaling pathways and the inhibitory effects of their inhibitors are related to ER expression profiles, we selected both the Ishikawa and HEC-1A cell lines in our study.

Our results show that LY294002 and PD98059 showed remarkable anti-tumor effects on both Ishikawa and HEC-1A cell lines *in vivo*. Concurrent inhibition of both pathways further enhanced apoptosis in xenografts of Ishikawa and HEC-1A cells. It indicated that the inhibitory effect of LY294002 and PD98059 on endometrial carcinomas was independent with the ER profiles. Thus, blocking these signaling pathways may be an effective alternative treatment for endometrial cancer, especially for ER-negative cancers.

Furthermore, we evaluated levels of p-Akt and p-ERK1/2 in the xenografts of animals treated by the two in-

hibitors by Western blot analysis. We observed that p-Akt level was reduced by LY294002 and p-ERK1/2 level was reduced by PD98059 in xenografts of the two endometrial carcinoma cell lines. In addition, compared with Ishikawa cells, the level of p-Akt was lower but that of p-ERK1/2 was slightly higher in xenografts of HEC-1A cells. Similar results have been reported in studies of these cell lines *in vitro* [15]. We speculate that the differences in expression level of p-Akt and p-ERK1/2 may be related to the differences in expression of ER and PTEN between the two cell types. PTEN is known as a tumor suppressor gene. Mutation or deletion of the PTEN gene enhance the activation of the PI3K/AKT signal pathway but it can inhibit the activation MAPK/ERK1/2 pathway mediated by the growth factor [19] [20]. Ishikawa cells have increased PI3K/Akt activity due to lack of PTEN expression [21], which may explain the relatively higher p-Akt level we observed in the xenografts of Ishikawa cells. Interestingly, although the baseline level of p-Akt and p-ERK1/2 were different in the two cell lines, we did not detect any significant difference between the effects of blocking the two pathways by LY294002 and PD98059.

Several studies have reported evidence suggesting extensive cross-talk between the PI3K/Akt and MAPK/ERK1/2 signaling pathways. Some studies suggest that the activity of PI3K plays an important role in the induction of the Ras/MAPK pathway. In addition，inhibitor of the PI3K has been reported to attenuate the activity of MAPK, and this effect could occur at different levels of the Ras/MAPKERK pathway. For example, wortmannin has been shown to block the ERK1/2 activation induced by CD3 antibody in T cells and MAPK activation in the skeletal muscle of rabbits [22]. Lee *et al.* found that the PI3K/Akt signaling pathway has a regulatory role on the activation of ERK1/2 [23]. Our observation that concurrent inhibition of the PI3K/Akt and MAPK/ERK1/2 pathways had synergistic effects and showed enhanced anti-proliferative effects *in vivo* by increasing apoptosis, also suggests that complex interactions may exist between the two pathways. However, the nature and mechanism of the interaction is unclear. Further research on the interactions between these pathways may lead us to more effective treatments for cancer.

5. Conclusion

In summary, we found that LY294002 and PD98059 both inhibited the growth of xenografts of cells with different ER profiles by blocking the PI3K/Akt and MAPK/ERK1/2 signaling pathways, respectively, *in vivo*. Our findings suggest that targeting these pathways may be an effective alternative for treatment of endometrial cancer, especially for ER-negative endometrial cancer.

Acknowledgements

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