European Review for Medical and Pharmacological Sciences 2014; 18: 2264-2269

Synergistic *in vitro* anti-tumor effect of letrozole and everolimus on human endometrial carcinoma Ishikawa cells

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Abstract. – OBJECTIVES: To investigate the in vitro effect of the use of combination of letrozole and everolimus on the proliferation and apoptosis of human endometrial carcinoma Ishikawa cells.

MATERIALS AND METHODS: Different concentrations of letrozole alone or in combination with everolimus were added to Ishikawa cell cultures *in vitro*. Cell viability was assessed using MTT assay. Double staining with Annexin-V-FITC was used to detect the cell apoptosis by the flow cytometry (FCM). The expression of PTEN and 4E-BP1 proteins was analyzed with Western blotting.

RESULTS: Letrozole by itself inhibited cell viability and decreased the invasiveness of Ishikawa cells. The anti-tumor effect of letrozole in combination with everolimus was improved compared to letrozole alone.

CONCLUSIONS: Letrozole and everolimus synergistically inhibit the proliferation and induce cell apoptosis by inhibiting the cell signal pathway PI3K/Akt/mTOR and inducing cell apoptosis, thus showing a promising anti-tumor effectiveness.

Key Words:
Endometrial carcinoma, Letrozole, Everolimus, Ishikawa cells.

Introduction

Endometrial carcinoma is one of the most common female genital tract malignancies and its incidence is increasing in recent years. The exact cause of endometrial carcinoma has not been fully understood, but most researchers believe that its pathogenesis is associated with long-term persistent stimulation of estrogen-like activities in absence of antagonistic activities of progesterone[1]. For post-menopausal women, estrogen biosynthesis is catalyzed by aromatase starting from androstenedione secreted by adrenal cortex[2]. The administration of aromatase inhibitors (AIs) is associated with suppression of aromatase activities, and reduction of androgen conversion into estrogens, resulting in blockage of tumor cell growth induced by estrogen stimulation. The PI3K-Akt-mTOR signal pathway is closely correlated to cell growth, proliferation and differentiation, and its overt importance has been demonstrated in the tumorigenesis and development of various common malignancies such as breast cancer, ovarian cancer, prostate cancer, liver cancer and colon cancer[3-7]. In this signal pathway, PTEN could suppress the conversion of PIP2 into PIP3, thereby, interfering with Akt and mTOR activation. Therefore, PTEN has been recognized to be one of the negative regulatory factors involved in the PI3K/Akt/mTOR signal pathway. It has been shown[8,9] that increased expression and activities of PTEN, as well as increased mutation rate, could be observed at high estrogen levels. Consequently, the synergic effect of letrozole (one of AIs) and everolimus (RAD001, one of mTOR specific inhibitors) on human endometrial carcinoma Ishikawa cells and relevant potential mechanism of action have been investigated in this study, to provide a theoretical basis for chemotherapies used for endometrial cancer.

Materials and Methods

Materials and Reagents

Human endometrial carcinoma Ishikawa cell lines and RPMI-1640 medium were purchased from KeyGEN Biotech, Nanjing; AnnexinV-FITC kit was purchased from Beyotime Institute of Biotechnology, Shanghai, China; MTT and DMSO were products of Sigma Co. Ltd. Letrozole and everolimus were supplied by Jiangsu Hengrui Medicine Co. Ltd and Novartis, respectively.

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Methods

Cell Culture and Grouping

Human endometrial carcinoma Ishikawa cell lines were inoculated into RPMI-1640 medium containing 10% fetal bovine serum and cultured under 37°C/5% CO_2 (v/v) in a humidified incubator. Three groups of cells were set up: negative control group, letrozole (Le) 10^{-7} mol/L, and letrozole (Le) 10^{-7} mol/L +RAD001 (Ra) 2 μg/ml treatment group.

Cell Proliferation by MTT Assay

The cells were harvested in their logarithmic growth phase. The yielded cell suspension was adjusted to 3×10^4/ml in concentration and was inoculated into a 96-well plate. After cultivation for 24 hours, the culture was divided into three groups; negative control group, letrozole (Le) 10^{-7}mol/L, and letrozole (Le) 10^{-7} mol/L +RAD001 (Ra) 2 μg/ml treatment group. Five replicates of each group were treated for 24h, 48h and 72h. Afterwards, 20 μl of MTT solution (5 mg/ml) was added to each well and the resultant culture was incubated in a 37°C/5% CO_2 incubator for another 4 hours. With the supernatant discarded, 150 μl of dimethylsulfoxide (DMSO) was added to each well, followed by oscillation in darkness for 10 min. The absorbance values (OD) was determined at the wavelength of 492 nm on a microplate reader. Inhibition rate was calculated as: inhibition rate = (1- OD of treatment/OD of control)*100% and the plot graphs were depicted.

Annexin V/PI double-staining Apoptosis assay

Subsequent to treatment for 24h and 48h and cell lysis of each group, the resultant culture was centifuged at 1200 RPM for 5 min to remove medium and was twice washed with PBS. The supernatant was discarded, and the cells were harvested. Cells were re-suspended in 500 μl of Binding Buffer and cells then cultivated with Annexin V-FITC (5 μl) and PI (5 μl) at ambient conditions in darkness for 30 min before flow cytometry analysis within 1 hour.

Changes in Western Blot Expression Profiles of PTEN and 4E-BP1

Ishikawa cells were harvested in their growth and division phase, and trypsinized. Approximately $1x10^5$ cells per 5 mL in each well were inoculated into 6-well plates. Following cultivation for 24 hours and development of cell adherence, all supernatant was removed and the residual cultures (final volume 5 ml) were treated according to the group schedule, and protein lysate was added following repeated frozen-thawing procedures. Cell cultures were then harvested to extract proteins. Protein concentrations were determined quantitatively. Protein samples (15 μg) were loaded for SDS-PAGE gel assays, followed by wet transfer to NC membrane. After addition of primary PTEN (1:1000), 4E-BP1 (1:1000), Actin (1:5000), the culture was shaken overnight at 4°C. Cultured cells were washed and the secondary goat anti-rabbit antibody (1:5000) was added before incubation at room temperature for 30 min. Samples were washed with washing buffer before transferring to NC membrane and examined using scanner examination Image J analysis program to analyze the results. The relative content of target proteins was determined based on the absorbance ratio of target protein strip versus actin strip.

Results

MTT Assays

A certain growth inhibition was observed in Ishikawa cells during the course of treatment with letrozole monotherapy or in combination with everolimus. The growth inhibition rate for letrozole was 27.5%, 31.1% and 38.0% at 24h, 48h and 72h respectively in a time-dependent manner ($r = 0.984$, $p < 0.05$). The inhibitory effect of letrozole in combination with everolimus on cell growth was significantly higher than letrozole monotherapy at same concentration ($p < 0.05$). The maximal inhibitory effect was observed to be 54.7% at 72h (Figure 1 and Table I).

Ishikawa Apoptosis Rate

Significant apoptosis induction effect of letrozole monotherapy or in combination with everolimus on Ishikawa cells was observed after treatment for 24h and 48h ($p < 0.05$). The effect was greater for combination therapy at the same concentration ($p < 0.05$) and also to some extent the effect was time-dependent ($p < 0.05$) (Figure 2 and Table II).

Expression Profiles of PTEN and 4E-BP1 in Ishikawa Cells

As shown by Western blot assays, the expression profiles of PTEN in Ishikawa cells were increased after the treatment of letrozole monother-

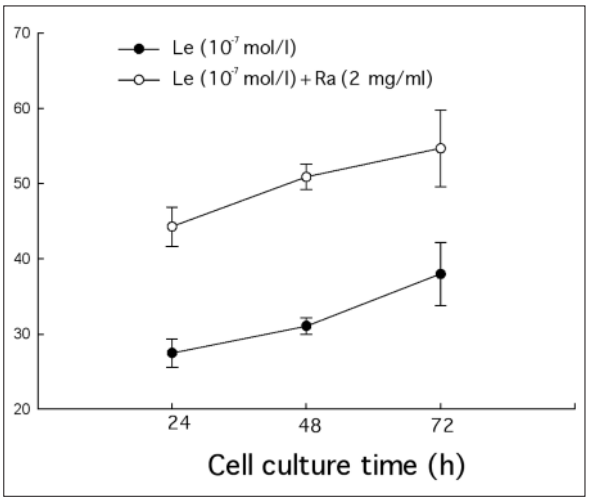

Figure 1. Comparison of the growth inhibition rate of Ishikawa cell.

apy or in combination with everolimus for 24 hours, in comparison to baseline profile ($p < 0.05$). The extent of increase was higher in the group of letrozole in combination with everolimus compared with that of letrozole monotherapy ($p < 0.05$). The expression profile of 4E-BP1 was decreased in comparison to the pretreatment. Moreover, the extent of decrease was higher in the combination group compared to letrozole monotherapy group ($p < 0.05$).

Discussion

Endometrial carcinoma is one of the most common malignancies of female genital tract and the endocrine therapy against this cancer has become of extensive concern in recent years. Endometrial hyperplasia follows a progressive course characterized by mild hyperplasia, sac gland hyperplasia, adenomatous hyperplasia, dysplasia, carcinoma *in situ*, invasive carcinoma[10]. It has been proposed that the most endometrial carcinomas are estrogen dependent and their tumorigenesis and development are associated with long-term persistent stimulation of estrogen-like activities in the absence of antagonistic activities of progesterone[1]. The PI3K-Akt-mTOR signal pathway is closely correlated to various common malignancies. In this signal pathway, PTEN could suppress the conversion of PIP2 into PIP3, thereby, interfering with Akt and mTOR activation. Therefore, PTEN has been recognized to be one of the negative regulatory factors involved in the PI3K/Akt/mTOR signal pathway. As shown by some studies[9], an elevated expression profile of PTEN was observed in epithelial and mesenchymal cells in the proliferative phase, while decreased PTEN expression profile was identified in glandular epithelium in the secretary phase. Thus, stimulation of estrogen at high concentration was associated with PTEN amplification. Therefore, in case of PTEN deletion mutation and functional loss, an atypical endometrial proliferation is more likely to occur under high estrogen levels. The mutation rate of PTEN gene was identified to be 32%-83% in endometrial carcinoma and a vital role of PTEN gene was proposed in the mechanism of action of endometrial cancer[11,12].

As an inhibitor interfering with mTOR signal pathway, everolimus could form a complex with intracellular protein FKBP12, which subsequently binds to mTORC1 to exert its inhibitory effects. As a result, the phosphorylation process of downstream 4EBP1 was suppressed, thereby interfering with cell growth and proliferation[13]. While letrozole, as a nonsteroidal aromatase inhibitor, could significantly suppress estrogen synthesis from external re-

Table I. Effect of Letrozole monotherapy or in combination with everolimus on the proliferation of Ishikawa cells at different time points (OD value, $\bar{x} \pm s$).

Group	24h		48h		72h	
	OD value	Inhibition rate (%)	OD value	Inhibition rate (%)	OD value	Inhibition rate (%)
Control group	1.086 ± 0.089	0	1.120 ± 0.012	0	1.153 ± 0.003	0
Letrozole monotherapy group	0.787 ± 0.076	0.275 ± 0.019	0.771 ± 0.057	0.311 ± 0.011	0.715 ± 0.042	0.380 ± 0.042
Letrozole plus everolimus group	0.604 ± 0.034	0.443 ± 0.026	0.549 ± 0.012	0.509 ± 0.017	0.522 ± 0.027	0.547 ± 0.051

Note: n=5 (number of wells). $p < 0.05$ for all treatment groups when compared with the control group $p < 0.05$ when both groups were compared at the same time point $p < 0.05$ when two time points were compared within the same group.

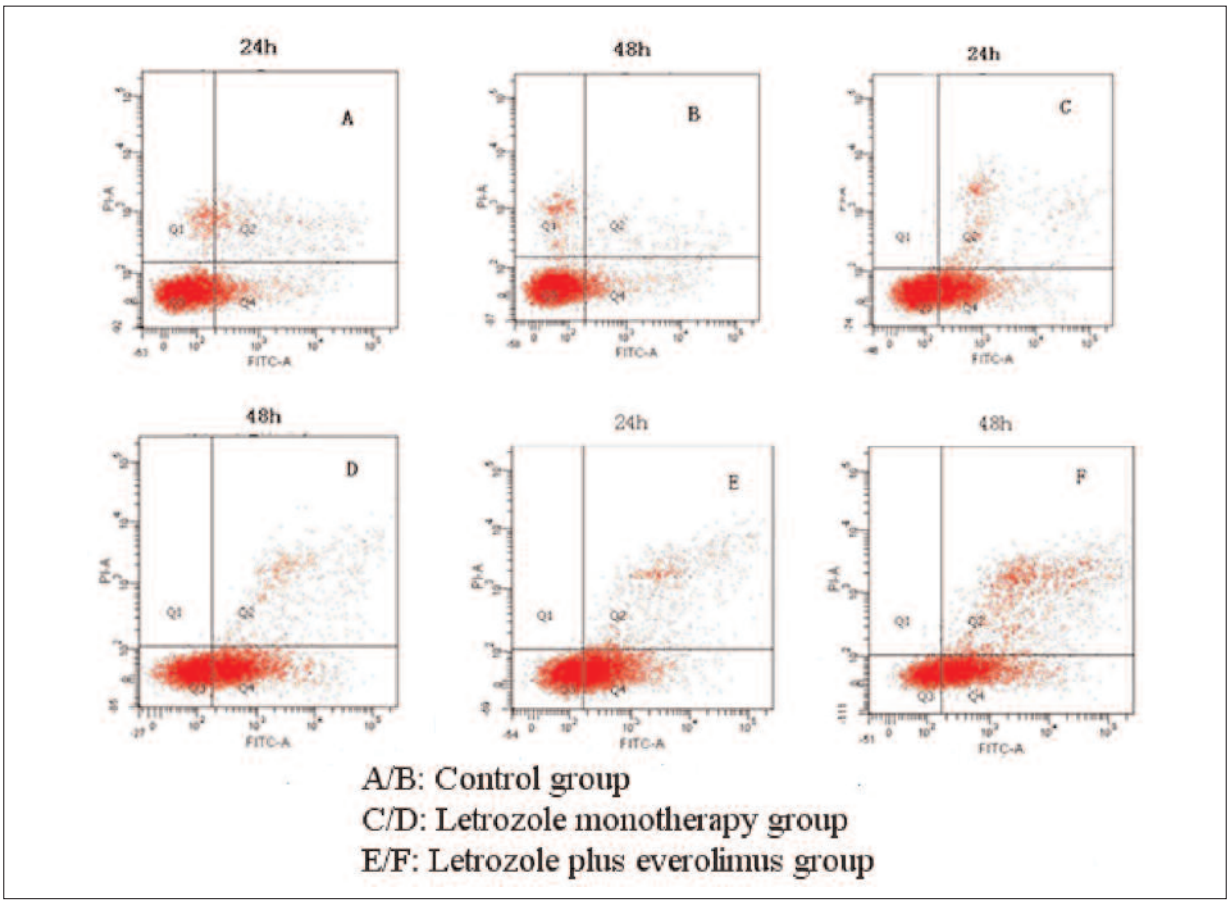

Figure 2. Effect of Letrozole monotherapy or in combination with everolimus on the apoptosis in Ishikawa cells after treatment.

sources except those of glandular origin, thereby, able to block estrogen-induced tumor growth. In this study, the administration of letrozole was associated with decreased cell proliferation. The growth of Ishikawa cells was significantly suppressed and a certain time-response effect was demonstrated. The effect of letrozole in combination with everolimus on cell growth and induction apoptosis was considerably higher than letrozole monotherapy at same concentration. These results show the synergetic effect of letrozole and everolimus. The administration of letrozole was associated with increased expression profile of PTEN protein and reduced expression profile of 4EBP1. This demonstrates the suppression effect of letrozole on the mutation and deletion of intracellular PTEN gene, resulting in inactivation of

Table II. Apoptosis rate in Ishikawa cells after 24h and 48 h of the treatment with Letrozole monotherapy or in combination with everolimus ($\bar{x} \pm s$, n = 3).

Group	24h	48h
	Apoptosis rate (%)	Apoptosis rate (%)
Control group	12.3 ± 0.72	15.4 ± 0.16
Letrozole monotherapy group	36.9 ± 0.60	40.3 ± 0.73
Letrozole plus everolimus group	52.7 ± 0.81	59.6 ± 1.03

Note: $p < 0.05$ in treatment groups when compared with the control group. $p < 0.05$ when both treatment groups were compared at the same time point. $p < 0.05$ when two time points are compared within the same group.

Table III. Expression of PTEN and 4EBP1 proteins 24 hours after the treatment of letrozole alone or in combination with everolimus in Ishikawa cells ($\bar{x} \pm s$, n = 3).

Group	Relative absorbance ratio of PTEN/actin	Relative absorbance ratio of 4E-BP1/actin
Control group	0.321 ± 0.009	1.041 ± 0.175
Letrozole monotherapy group	0.684 ± 0.033	0.794 ± 0.017
Letrozole plus everolimus group	0.993 ± 0.160	0.272 ± 0.032

Note: $p < 0.05$ in treatment groups when compared with the control group. $p < 0.05$ when both treatment groups were compared at the same time point. $p < 0.05$ when two time points are compared within the same group.

PI3K/Akt/mTOR signal pathway, reduction of downstream 4EBP1 expression and subsequent inhibition of tumor cell proliferation. Everolimus is associated with inhibition of PI3K/Akt/mTOR signal pathway. The suppression effect on the expression profile of the downstream effector 4EBP of mTOR signal pathway of letrozole in combination with everolimus was decreased significantly. Thus combined administration of letrozole and everolimus could produce more pronounced tumor cell suppression effect by inhibiting PTEN/PI3K/Akt/mTOR signal pathway.

Currently, chemotherapy remains to be one of the comprehensive interventions in the treatment of advanced or recurrent endometrial cancer. However, current available chemotherapies only demonstrate limited efficacy against endometrial cancer and poor prognosis for patients in advanced stages of this disease. Therefore, further research and development and introduction of novel therapies remain important for patients with advanced or recurrent endometrial cancer.

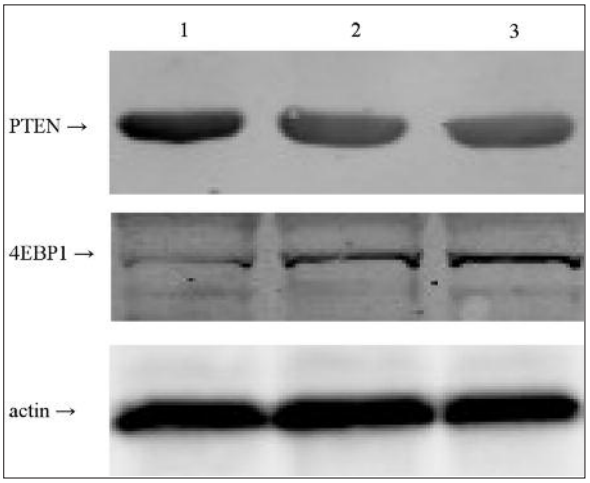

Figure 3. Expression of PTEN and 4EBP1 proteins after 24 hours of treatment of letrozole alone or in combination with everolimus 1 Letrozole plus everolimus group; 2 Letrozole monotherapy group; 3 Control group.

Conclusions

In this study, the administration of letrozole in combination with everolimus was shown to be associated with a significant proliferation suppression and apoptosis promotion in Ishikawa endometrial cancer cells, by interfering with PTEN/PI3K/Akt/mTOR signal pathway. This study was conducted to provide a theoretical basis for targeted therapies for endometrial cancer.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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