·Gynecologic Oncology·

The aromatase inhibitor letrozole combined with curcumin in the inhibition of xenografted endometrial carcinoma growth

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[Abstract] Background and Objective: Letrozole is an aromatase inhibitor that is used in the treatment of estrogen-sensitive tumors such as endometrial carcinoma. Tumor inhibition to a certain extent has been demonstrated, however, the therapeutic effects need improvement. Curcumin is reported to have antitumor capabilities and can enhance the sensitivity of tumor cells to anticancer agents. The present study promoted the inhibitory effect on implanted endometrial tumor growth by combining letrozole and curcumin. **Methods:** Endometrial carcinoma was implanted into nude mice. Tumor-laden mice were treated with the aromatase inhibitor letrozole (Let), curcumin (Cur), or both. The tumor growth was monitored. Tumor cell apoptosis was detected in both the control and treated groups. The expression of bcl-2 mRNA and Bcl-2 protein was detected with reverse transcription polymerase chain reaction (RT-PCR) and Western blot. **Results:** A total of 50 mice successfully received implants of endometrial tumors. Treatment with letrozole markedly inhibited tumor growth, and the inhibitory effect was enhanced by the combination of letrozole and curcumin. The inhibitory rates in the Let (1), Let (10), Cur, and Let+Cur groups were 15.95%, 22.49%, 21.57%, and 35.89%, respectively. Treatment with curcumin inhibited the expression of Bcl-2 in tumor cells at the mRNA and protein levels. The rates of apoptosis in the control group and the above-mentioned groups were 16.97%, 32.90%, 35.80%, 34.16%, and 47.24%, respectively. Tumor cell apoptosis was observed in mice treated with either letrozole or curcumin; however, the combination of letrozole and curcumin enhanced the inhibition rate in tumor growth. **Conclusions:** Treatment with either letrozole or curcumin could inhibit xenografted endometrial tumor growth by inducing apoptosis in tumor cells. The combination of letrozole and curcumin enhanced the inhibitory effect.

Key words: Endometrial carcinoma, RL-952 cell line, letrozole, curcumin, apoptosis, Bcl-2

Endometrial cancer, which is common in postmenopausal women, is etiologically unknown and usually considered to be related to excessive estrogen. In postmenopausal women, estrogen is mainly derived from androgen transformed by aromatase and can promote tumor growth through autocrine or paracrine signaling. Aromatase inhibitors can effectively impede this process and reduce estrogen levels, and thus can inhibit the growth of endometrial cancer. However, the aromatase inhibitor letrozole and progesterone were found to be similar in inhibiting tumor cell development in vitro-neither of them could completely inhibit the growth of cancer cells.[1] Recent studies have shown that curcumin has some antitumor effect. In this study, we developed an animal model of subcutaneous transplantation of human endometrial tumors in nude mice, and explored the antitumor effects on endometrial cancer of the third-generation aromatase inhibitor letrozole combined with curcumin.

Materials and Methods

Experimental materials

The human endometrial cancer cell line RL-952, an estrogen receptor-positive and medium-differentiated adenocarcinoma, was generously donated by the Institute of Obstetrics and Gynecology, Fudan University, Shanghai. BALB/C (nu/nu) nude mice were provided by Beijing Weitong Lihua Experimental Animal Technology Co. Ltd., with qualified number SCXK (Beijing) 2002-0003. The nude mice were female, aged 2–3 weeks, with weights of 13–16 g, and bred specific pathogen free. Letrozole was purchased from Hengrui Pharmaceutical Co. Ltd., Lianyungang, Jiangsu. β-actin and curcumin (Cur) with 99.5% purity were purchased from Sigma.

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Establishing the animal model of endometrial carcinoma in nude mice

The RL-952 cells were grown in an RPMI-1640 medium with 10% fetal calf serum without penicillin and streptomycin incubated at 37℃ with 5% CO2 and saturated humidity. When cells were in the logarithmic growth phase, they were digested with trypsin and ethylenediaminetetraacetic acid (EDTA) before subculturing. After procuring the nude mice, they were allowed to adapt to the new environment for 5 days, then underwent bilateral ovariectomies via the back under a 10% chloral hydrate anesthesia administered by intraperitoneal injection. Another 5 days were given for postoperative recovery. RL-952 cells in the exponential growth phase were produced in 5×10^7/mL cell suspension after digestion, then injected subcutaneously at a dose of 0.1 mL in each bilateral castration of the nude mice (containing about 5×10^6 cells). The tumors were harvested when they reached 1.0 cm^3 and sliced into pieces measuring about 2.0 mm×1.5 mm×1.5 mm. These pieces were then subcutaneously implanted into 50 precastrated nude mice on the right back. When the tumor blocks grew to about 3–7 mm, the mice were randomly divided into four groups of 10 and treated as follows. The Let (1) group was administered 1 μg/d of letrozole. The Let (10) group was administered 10 μg/d of letrozole. The Cur group was treated with 300 mg/(kg·d) of curcumin. The Let+Cur group was treated with 10 μg/d of letrozole and 50 mg/(kg·d) of curcumin. The control group was given the same amount of triple-distilled water. Drugs were administered subcutaneously at the left back of the neck 6 days per week, for a total of 8 weeks. At 4–6 h after the last dose, the mice were sacrificed and the implanted tumors were removed and weighed. Based on the experiments in nude mice of Yoysungnoen *et al.*,[2] curcumin doses ≤50 mg/kg were considered low doses, ≥ 200 mg/kg were considered high doses, and doses between the two were considered mid-range doses. In this study, only a high-dose group [solo drug at 300 mg/(kg·d)] and a low-dose group [the drug combination of 50 mg/(kg·d)] were set for curcumin.

Observations

Growth monitoring Since the day the tumors were implanted, the mice were inspected daily for diet, activity, tumor formation, and whether there was redness, swelling, wounds, ulcers, and so on. After the block grew into a tumor, we checked its shape, texture, and activity. The length (a) and width (b) of tumors were measured weekly by Vernier caliper, and the tumor volume was calculated using the formula $V = ab^2/2$. The weights of the mice were measured weekly by electronic scale, and drug toxicity was assessed at the end of the experiments. An end mass/initial mass ≥0.8 was considered nontoxic; a ratio < 0.8 toxic. The tumor inhibition rate = (control group V - experimental group V) / control group V × 100%.

Using flow cytometry to detect the cell cycle and apoptosis in the tumors Pieces (about 1.5 mm × 1.5 mm × 1.5 mm) were cut from the near edge of the tumor, washed 2 times with a phosphate buffered saline (PBS) solution, then pulverized by mechanical grinding, and produced in cell suspension after filtration. The resulting cell suspension was washed 2 times with a PBS solution, fixed with cold 75% ethanol overnight, and resuspended in a PBS solution after centrifugation. RNase A trypsin was added for digestion at room temperature for 30 min, then propidium iodide (PI) was added for staining for 15–20 min. The cells were then analyzed with flow cytometry.

Real-time quantitative fluorescent RT-PCR Total RNA was extracted using Trizol, then reverse transcripted into complementary DNA (cDNA). The obtained cDNA was used as the template for quantitative RT-PCR (qRT-PCR) using the GoTaq Flexi DNA Polymerase Kit (US Promega Corporation), with β-actin as an internal control. Primers included 5'-TCTGGTCCCTTGCAGCTAGT-3' and reverse 5'-CAGG GAGGCTAAGGGGTAAG-3' for bcl-2 (annealing temperature 60℃, elongation time 30 s, 39 cycles, 196 bp; NCBI No. NM_138578); 5'-GCGGCCTGGACTCTCAACTG-3' and reverse 5'-AATGAATGGGGGTTGAATGA-3' for β-actin (annealing temperature 60℃, elongation time 30 s, 39 cycles, 187 bp; NCBI No. NG_007992).

Western blot analysis NuPAGE gel system was used to extract the total protein in each group, and the protein level was determined by the Bradford method. An appropriate mass of the total protein was applied to polyacrylamide gel electrophoresis, then transferred to a nitrocellulose membrane (Bio-Rad). After conventional blocking and membrane washing, the membrane was incubated overnight with 1:300 mouse anti-human Bcl-2 antibodies at 4℃. After that, the membrane was washed and incubated with 1:1000 goat anti-mouse IgG HRP at 37℃ for 2 h. The resulting membrane was stained with ECL, then photographed using the Bio-Rad image analysis system. Quantity One software was used for data analysis and relative protein quantity was denoted as the value of the gray value of the corresponding protein bands/the gray value of the β-actin protein bands.

Statistical methods

Experimental data was expressed as mean ± standard deviation, and variance was analyzed with SPSS13.0. Least significant difference (LSD) analysis was performed when variances were homogenous; and Tamhane analysis was performed when variances were heterogeneous. P<0.05 indicated statistical significance.

Results

Growth conditions of transplanted tumors in each group

Once the tumor was implanted, daily observation of the tumor block was performed. During the first few days, slight redness and swelling at regional parts of the implanted blocks were observed and then they gradually increased. After they grew into tumors, they were round or oval in shape, with a hard texture and good activity. When the tumor block grew to a certain size, a few tumors had local ulcerations in the shape of a rat bite. Except for a few mice that were restrained from free activity because of the quick growth of the implanted tumor cells, the rest of the mice had no significant changes in diet or activity. During the

experiments, the tumors experienced necrosis and slow growth and finally disappeared in 2 mice in the experimental groups. Compared with body weight before treatment, the mice in all groups had different levels of weight loss due to larger tumors and energy consumption, although the overall body weight in the mice increased. In 1 mouse, weight decreased significantly, the lowest at 14 g, and it died afterward. In the final evaluation of drug toxicity, because the end weight/beginning weight > 0.8, letrozole and curcumin were confirmed as nontoxic.

In the control group, tumor volume increased significantly, while in the treatment groups, tumors grew at slower speeds. At the 8th week of medication, each treatment group had significant differences in tumor volume and tumor inhibition rates compared with the control group ($P < 0.01$). The Let (10) group had a higher tumor inhibition rate in a concentration-dependent manner. The Cur group also had a high inhibition rate. But the highest inhibition rate was shown when the two medicines were combined compared with the two drugs alone, and the statistical difference was significant ($P < 0.05$). The tumor inhibition rates and volume changes of the implanted tumors in each group after treatment are shown in Table 1.

Table 1 The effects of letrozole and curcumin on the weight of tumor-bearing nude mice and the tumor inhibition rates

Group	Tumor volume (mm^3)		Nude mice weight (g)		Inhibition rate (%)
	Before treatment	After treatment	Before treatment	After treatment	
Control	66.44±25.30	3787.94±697.54	19.70±1.42	24.90±2.28	–
Letrozole (1 μg/d)	60.93±28.49	3183.76±421.23[a]	20.50±1.65	24.33±1.66	15.95±12.18[ab]
Letrozole (10 μg/d)	64.84±23.14	2936.03±446.84[a]	20.10±1.73	25.75±2.92	22.49±13.12[ab]
Curcumin	65.09±27.80	2970.50±414.53[a]	20.10±1.85	24.50±2.80	21.57±12.00[ab]
Letrozole+Curcumin	66.01±22.39	2428.45±440.89[a]	20.80±1.62	25.11±2.93	35.89±11.64[a]

All values are presented as mean ± SD. [a]Comparison between the experimental groups and the control group, $P < 0.01$; [b]compared with the combined use of letrozole and curcumin, $P < 0.05$.

Cell apoptosis and the cell cycle in the tumor tissue of nude mice

According to the results of flow cytometry, compared with the control group, the letrozole and the curcumin groups had significantly higher ratios of G_1 phase cells, but G_1-, S-, and G_2-phase cell ratios of the transplanted tumors in each group had no significant difference ($P > 0.05$). Compared with the control group, the treatment groups had significant differences in the rates of apoptosis, with the Let+Cur group having the highest rate of cell apoptosis ($P < 0.05$), as shown in Table 2. The determination of apoptosis in each group is shown in Figure 1.

Table 2 The effects of letrozole and curcumin on the cell cycle and apoptotic index of tumor tissue (%)

Group	Cell cycle			Apoptosis rate
	G_0/G_1	S	G_2/M	
Control	59.33±10.28	23.36± 3.40	17.31± 7.00	16.97± 4.80
Letrozole (1 μg/d)	59.58± 6.69	34.63±11.81	5.79± 5.14	32.90± 5.76[ab]
Letrozole (10 μg/d)	55.60± 9.47	39.07±13.83	5.34± 4.52	35.80± 5.12[ab]
Curcumin	56.36± 4.31	31.99± 6.43	9.98±17.28	34.16±12.39[ab]
Letrozole+Curcumin	66.51±12.78	38.41±16.78	3.08± 5.33	47.24±10.90[a]

All values are presented as mean ± SD. [a]Comparison between the experimental groups and the control group, $P < 0.05$; [b]compared with the combined use of letrozole and curcumin, $P < 0.05$.

The expression levels of the mRNA and protein of Bcl-2 in each group

The results showed that Bcl-2 mRNA and protein expression was significantly less in the Cur group and the Let+Cur group, compared with the control group ($P < 0.05$) (Figures 2 and 3).

Discussion

Endometrial cancer is one of the three most malignant tumors in the female reproductive system. Generally, long-term stimulation of exogenous or nonresistant-endogenous estrogen and no progesterone resistance are considered to be related to its occurrence and development. Therefore, antiestrogen therapies have attracted more and more attention in recent years. Studies have shown that for some reason aromatase expression increases in the body or in endometrial cancer tissue, and the accelerated local concentration of estrogen stimulated tumor-cell proliferation. In addition, estrogen levels are positively related to clinical stage and a tumor-infiltrating condition.[3]

Letrozole is a nonsteroidal aromatase inhibitor, which can significantly and selectively inhibit estrogen synthesis in tissues other than glands, blocking estrogen-induced tumor growth.

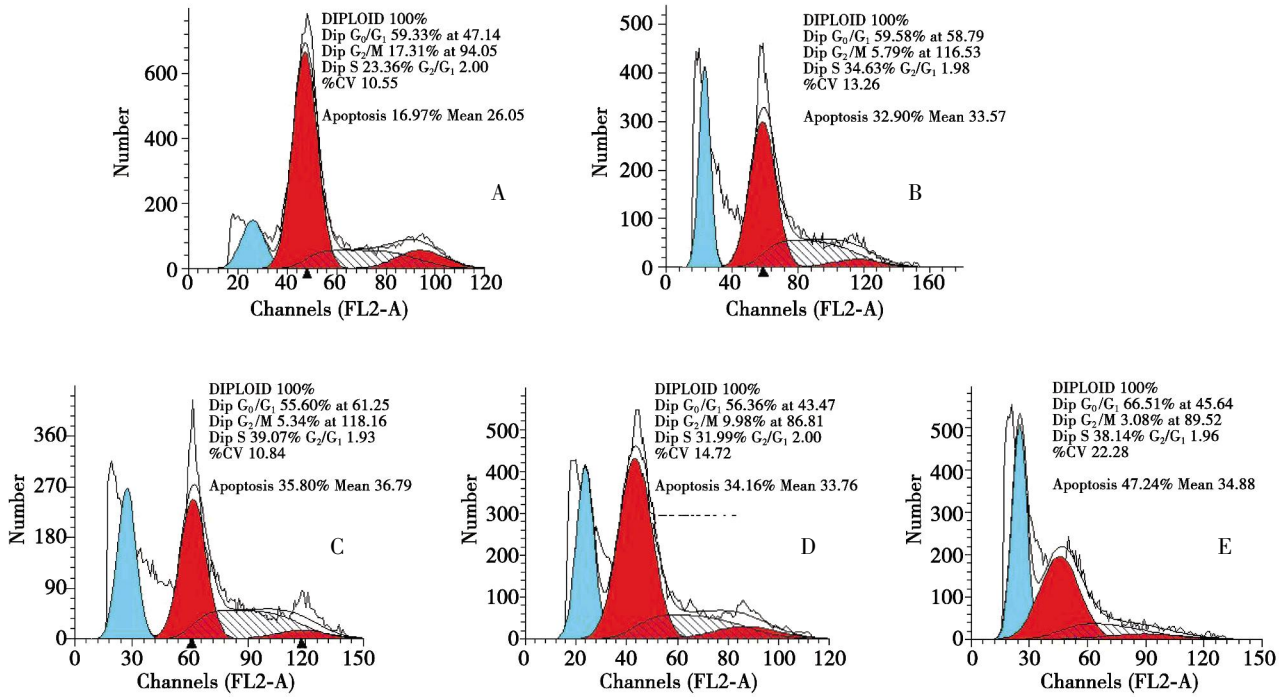

Figure 1 The apoptosis of tumor cells detected by flow cytometry in both control and treated groups

A, control; B, 1 μg/d letrozole; C, 10 μg/d letrozole; D, 300 mg/(kg·d) curcumin; E, 10 μg/d letrozole plus 50 mg/(kg·d) curcumin.

Currently letrozole has been successfully used to treat breast cancer, endometriosis, and estrogen-dependent tumors.[4] In humans and mice, only one gene CY19 encodes aromatase, and interference of CY19 or inhibitors of its products can be effective in reducing the biosynthesis of estrogen.[5] In this study, both ovaries of nude mice were removed to establish postmenopausal human xenografted endometrial carcinoma. We preliminarily studied in vivo the antitumor effect of letrozole through its interference with the expression of aromatase in vitro. It was found that in the control group, tumor volume increased significantly, however, in the treatment group, tumor volume growth was relatively slow, and the rate of cell apoptosis increased significantly. According to the above results, letrozole could significantly inhibit endometrial cancer tumor growth in a dose-dependent way.

In overseas phase 3 clinical trials of letrozole treatment for the patients with breast cancer, letrozole (2.5 mg/d) had the same effect with progesterone in the overall response rate.[6] Other similar studies implied that when treatment failed, regarding time and the rate of overall survival, there was no significant difference between letrozole (2.5 mg/d) and progesterone.[6,7] Bellone et al.[8] reported that a case of advanced and recurrent endometrial cancer achieved complete remission with treatment of anastrozole, an aromatase inhibitor. Despite the Canadian Gynecologic Oncology Group report that the overall remission rate of anastrozole- and letrozole-treated advanced and recurrent endometrial cancer was only about 9%, because they are well tolerated, they still have prospective application.[9,10]

In recent years, the antitumor effects of curcumin have received attention. Studies have shown that curcumin has an antitumor effect on bladder cancer, colorectal cancer, prostate cancer, and lung cancer.[11] The antitumor mechanism of curcumin may be that it: (1) inhibits the expression of oncogenes to promote the expression of tumor suppressor genes; (2) induces apoptosis of tumor cells; (3) induces tumor cell differentiation; (4) inhibits matrix metalloproteinases and tumor angiogenesis; (5) suppresses the activities of ring oxygenase (cyclooxygenase-2, COX-2) and inducible nitric oxide synthase (iNOS); (6) inhibits a variety of protease activities related to tumor cell proliferation; (7) inhibits the activities of proto-oncogenes (activator protein-1, AP-1) and nuclear factor Kappa B (NF-κB); or (8) counters oxidative damage.[11]

In addition, curcumin can increase tumor cell sensitivity to other chemotherapeutic drugs and reduce the toxic side effects of chemotherapy. Studies have found that curcumin can significantly increase the cell-killing effects of adriamycin on the human hepatocellular carcinoma SMMC-7721 cell, so that a small dose of doxorubicin could have a good effect, avoiding adriamycin-induced toxicity by the application of high doses. Curcumin could also improve the sensitivity to cisplatin of the human lung adenocarcinoma A549 cell.[12,13] Xu *et al.*[14] found that curcumin can inhibit the invasion and metastasis of the human cervical carcinoma cell CaSki in vitro, the function of which might be through down-regulation of the expression of matrix metalloproteinases 2 and type-Ⅰ matrix metalloproteinase membrane protein. Curcumin had a significant growth-inhibitory effect on the human ovarian cancer cell line HO-8910 subcutaneously transplanted in nude mice. The inhibition rates of the low-dose and the high-dose groups reached 53.08% and 70.47%, respectively. Detection through pathology, electron microscopy,

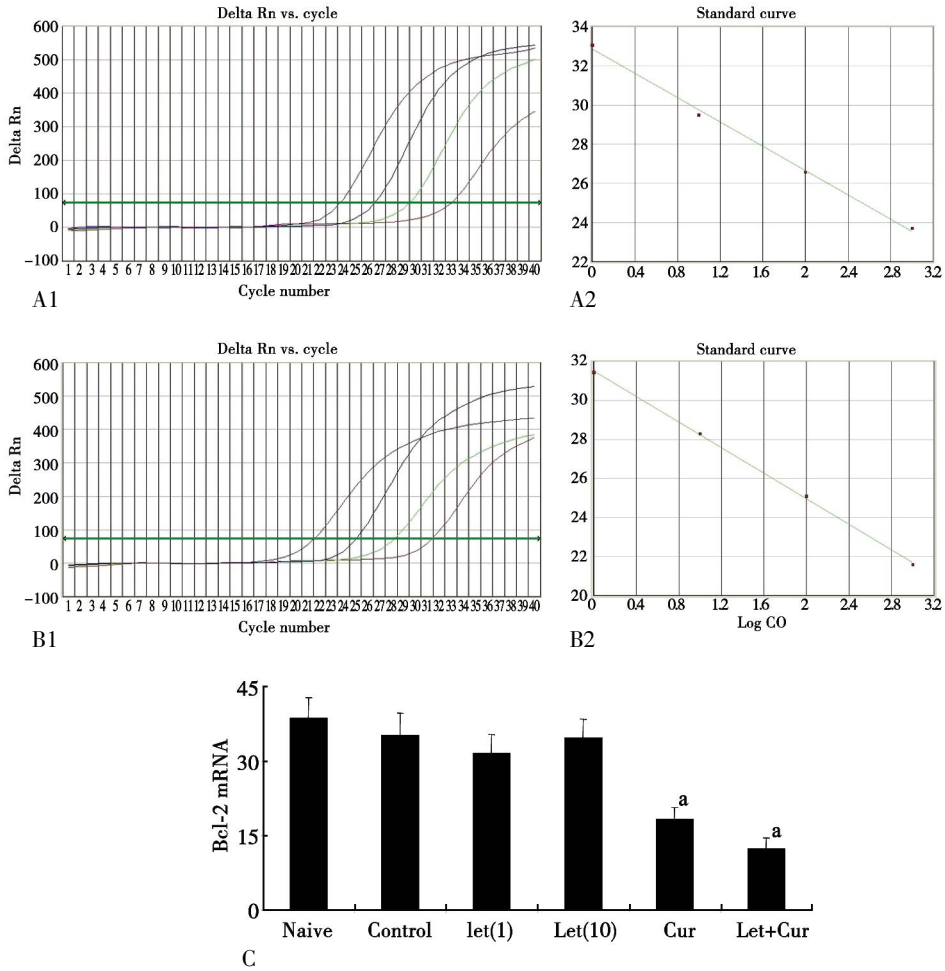

Figure 2 Bcl-2 expression in xenografted tumor tissues

Bcl-2 expression in tumor tissue was determined by real-time RT-PCR. A1 and A2 are the standard curves of Bcl-2 mRNA; B1 and B2 are the standard curves of β-actin; C indicates the level of Bcl-2 mRNA in tumor tissues. Data were normalized by the internal control β-actin and are presented as means (SD). Let(1), letrozole, 1 μg/d; Let (10), letrozole, 10 μg/d; Cur, curcumin, 300 mg/(kg·d); Let+Cur, mice were treated with both letrozole (10 μg/d) and curcimin [50 mg/(kg·d)]. $^{a}P<0.05$, vs. naive group (uterine tissue).

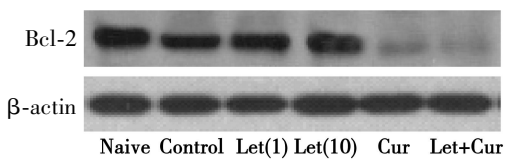

Figure 3 Bcl-2 protein expression in tumor tissues detected by Western blot

Let (1), letrozole, 1 μg/d; Let (10), letrozole, 10 μg/d; Cur, curcimin, 300 mg/(kg·d); Let+Cur, mice were treated with both letrozole (10 μg/d) and curcimin [50 mg/(kg·d)].

and flow cytometry suggested that curcumin prompted the occurrence of apoptosis in tumor cells after treatment.[15] As a new type of plant resource for chemopreventive cancer drugs and antineoplastic agents with wide accessibility, inexpensive cost, and nontoxic side effects, curcumin is receiving increasing attention from tumor experts.

In this study, the combination therapy of curcumin and letrozole showed significant inhibition in tumor growth, compared to treatment with either agent alone, and the dose was lower. Bcl-2 expression was found to be significantly lower in the Cur group and the Let+Cur group, according to results of qRT-PCR and Western blot analysis, indicating the inhibitory mechanism of curcumin on endometrial tumor growth might be through its inhibition of Bcl-2 expression, and thus promoted apoptosis of cancer cells. Throughout the course of drug therapy, because there was no effect on the body weight of the mice, letrozole and curcumin were illustrated to have no toxicity on the growth of the mice.

To sum up, the third-generation aromatase inhibitor letrozole could significantly inhibit endometrial cancer tumor growth and delay tumor progression, with fewer adverse reactions. In addition, it showed good synergy with curcumin, thus providing an experimental basis for exploring new ways of combining Chinese and Western medicine to cure patients with endometrial cancer.

References

[1] Wang QL, Liang YJ, Wu YZ, et al. Effects of letrozole on endometrial carcinoma cells [J]. Yi Xue Yan Jiu Sheng Xue Bao, 2007,20 (6):627. [in Chinese]

[2] Yoysungnoen P, Wirachwong P, Changtam C, et al. Anti-cancer and anti-angiogenic effects of curcumin and tetrahydrocurcumin on implanted hepatocellular carcinoma in nude mice [J]. World J Gastroenterol, 2008,14 (13):2003-2009.

[3] Berstein LM, Tchernobrovkina AE, Gamajunova VB, et al. Tumor estrogen content and clinico-morphological and endocrine features of endometrial cancer [J]. J Cancer Res Clin Oncol, 2003,129(4):245-249.

[4] Bulun SE, Lin Z, Imir G, et al. Regulation of aromatase expression in estrogen-responsive breast and uterine disease: from bench to treatment [J]. Pharmacol Rev, 2005,57(3):359-383.

[5] Simpson ER, Clvne C, Rubin G, et al. Aromatase—a brief overview [J]. Ann Rev Physiol, 2002,64:93-127.

[6] Dombernowsky P, Smith I, Falkson G, et al. Letrozole, a new oral aromatase inhibitor for advanced breast cancer: double-blind randomized trial showing a dose effect and improved efficacy and tolerability compared with megestrol acetate [J]. J Clin Oncol, 1998,16(2)453-461.

[7] Buzdar A, Douma J, Davidson N, et al. A phase III, multicenter, double-blind, randomized study of letrozole, an aromatase inhibitor, for advanced breast cancer versus megestrol acetate [J]. J Clin Oncol, 2001,19(14): 3357-3366.

[8] Bellone S, Shah HR, McKenney JK, et al. Recurrent endometrial carcinoma regression with the use of the aromatase inhibitor anastrozole [J]. Am J Obstet Gynecol. 2008,199(3):e7-e10.

[9] Ma BB, Oza A, Eisenhauer E. The activity of letrozole in patients with advanced or recurrent endometrial cancer and correlation with biological markers—a study of the National Cancer Institute of Canada Clinical Trials Group [J]. Int J Gynecol Cancer, 2004,14(4):650-658.

[10] Rose PG, Brunetto VL, VanLe L et al. A phase II trial of anastrozole in advanced recurrent or persistent endometrial carcinoma: a Gynecologic Oncology Group study [J]. Gynecol Oncol 2000,78(2):212-216.

[11] Anand P, Sundaram C, Jhurani S, et al. Curcumin and cancer: An "old-age" disease with an ´´age-old" solution [J]. Cancer Lett, 2008,267:133-164.

[12] Xue Y, Liu BR, Mu LM. Inhibition effects of curcumin combined adriamycin on the human liver cancer cell line SMMC27721 [J]. Zhong Liu Yan Jiu Yu Lin Chuang, 2002,12(6):372-373. [in Chinese]

[13] Cao H, Diao LM, Xia D. Effects of curcumin combined with cisplatin on the proliferation and apoptosis of human lung cancer cell line A549 in vitro [J]. Wu Han Da Xue Xue Bao:Yi Xue Ban, 2008,29 (2):213-217. [in Chinese]

[14] Xu F, Mou XL, Zhao J. Effects of curcumin on invasion and metastasis of human cervical cancer cells caski [J]. Zhong Shan Da Xue Xue Bao: Yi Xue Ke Xue Ban, 2009,30(1):92-95. [in Chinese]

[15] Wang Q, Jia XM, Wang SY, et al. Growth effect of cuecumin on human ovarian carcinoma xenografted in nude mice [J]. Jiang Su Yi Yao, 2005,31(3):217-218. [in Chinese]