Mifepristone sensitizing cisplatin for cervical adenocarcinoma HeLa cell sensitivity to chemotherapy and its mechanism

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Summary

Objective: The study was designed to investigate proliferation inhibition for cervical adenocarcinoma HeLa cell treated with cisplatin combined with mifepristone and access its possible mechanism. *Materials and Methods:* HeLa cell was processed by different concentrations of mifepristone, cisplatin, and their combination respectively. Cell's proliferation inhibition rate and induction apoptosis ability were detected by MTT assay, FCM; the expression of P53, survivin and HPV E6 protein were measured by Western Blot. *Results:* The results showed that cisplatin inhibits proliferation of HeLa cells in different concentrations ($p < 0.01$). Mifepristone had no effect on HeLa cell proliferation inhibition rate during 24 and 48 hours ($p > 0.05$). Mifepristone at low concentrations (≤ 10 μmol/l) combined with cisplatin can significantly enhance the inhibitory effect of cisplatin on HeLa cell line. Flow cytometry showed that mifepristone at low concentrations (≤ 10 μmol/l) combined with cisplatin can induce apparent apoptosis of HeLa cell line in concentration dependent manner. Western blotting demonstrated that the expression of P53 protein increased and the expression of HPV E6 survivin protein decreased in HeLa cells treated with MIF at low concentrations (≤ 10 μmol/l) combined with cisplatin. *Conclusion:* Mifepristone at low concentrations (≤ 10 μmol/l) can enhance chemosensitivity and capability of inducing apoptosis of cisplatin to HeLa cells. The strengthening effect of growth inhibition and chemosensitivity to cisplatin of mifepristone are associated with down-regulating HPV E6 survivin protein and upregulating p53 protein.

Key words: Mifepristone; Cervical cancer; Cisplatin; Chemotherapy sensitivity.

Introduction

Cervical cancer is a common gynecological malignancy, although its overall morbidity and mortality is declining, but the incidence of cervical adenocarcinoma has increased significantly in recent years and worldwide detection rate reached 10% to 22% [1]. Surgery and radiotherapy are the traditional treatments of cervical cancer and cisplatin-based concurrent chemoradiation became the standard mode of treatment for more than Stage IIb in advanced cervical cancer [2-4]. Some patients will be cured or alleviated with these treatments. However, the mortality rate of patients with cervical adenocarcinoma is 50% due to early metastasis, high recurrence, no sensitivity to chemotherapy, and poor prognosis. Therefore, it is particularly important to improve the sensitivity of cervical adenocarcinoma to chemotherapeutic drugs. Mifepristone is a progesterone and glucocorticoid hormone receptor antagonist. An in vitro study found that mifepristone inhibited the growth of human cervical squamous cell carcinoma [5], but was still in laboratory stage. However, there is no two-drug combination treatment of cervical cancer reported. This study was designed to investigate the effect of cisplatin combined with mifepristone to treat cervical adenocarcinoma HeLa cells including the sub-cell cycle and apoptosis of HeLa cells, explore its possible mechanism, and provide a theoretical basis for clinical application.

Materials and Methods

Cells and their culture

The human cervical adenocarcinoma HeLa cells were supplied by the Wuhan University Type Culture Preservation Center and were then sent to the Institute of Biology, the Three Gorges University. Monolayer culture were established in RPMI 1640 medium with 10% dialyzed fetal calf serum. The cells were grown for three days (showing approximate density of 1×10^6 cells/ml) with daily medium change before they were used in the experiment.

Drug and reagents

Cisplatin was supplied by Shandong Qilu Pharmaceutical Co., Ltd. (batch number: 031106), paired with saline two mg/ml stock solution, and diluted to the desired concentration with complete culture solution when used. Mifepristone was provided by the Shanghai Hualian Pharmaceutical Factory. Mifepristone was added from an ethanol stock to the cell culture medium to obtain a final concentration of 10 mg/ml, was diluted to the desired concentration with culture medium when temporarily used. The final ethanol concentration in the culture medium was lower than 0.2%. MTT, propidium iodide (PI) was obtained from Sigma. Mouse anti-human p53 monoclonal antibody, rabbit anti-human survivin, HPV16 E6 protein antibody,

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mouse anti-human β-actin monoclonal antibody, goat anti-mouse IgG-HRP secondary antibody and sheep anti-rabbit IgG-HRP secondary antibodies were supplied by Santa Cruz Biotechnology Company.

MTT assay

Digesting the HeLa cells in logarithmic growth phase, percussing into a single cell suspension to adjust cell concentration to 3×10^4 Ge / ml, to 100 μl / well were seeded in 96-well plates. The culture medium was replaced with the medium containing serial dilutions of various chemotherapeutic drugs. Experimental groups were as follows: each concentration of the dosing groups had five parallel wells. The final concentration of the single-agent dosing group was as follows: mifepristone: 0.625, 1.25, 2.5, 5, 10, 20 μg/ml; cisplatin: 0.625, 1.25, 2.5, 5, 10, 20 μg / ml. The final concentration of mifepristone and cisplatin in combined group were accordance with the experimental results of single-agent program as follows: 0, 0.625, 1.25, 2.5, 5, 10, 20 μg / ml + 2.5 μg of / ml. Indwelling zero holes (containing only culture medium used in the absorbance of zero), and control well (without any intervention agent) and the vehicle group (cell culture system by adding the drug dissolution media). Set 37.5% CO_2 humidified incubator to continue to foster 24, 48, and 72 hours. Each interval adding a final concentration of 0.2 g / μl of MTT solution 100 μl, cultured for four hours, discard supernatant, add 200 μl / hole dimethyl sulfoxide (DMSO), mixed on the vortex oscillator for 30 min, and detected on a microplate reader at 570 nm with the measured absorbance (A) values. The inhibition rate of tumor cells to each drug with different concentrations was calculated as follows: inhibition rate = 100% × [1-(Adrug treated - Ablank)/(Acontrol - Ablank)]. The IC50 value resulting from 50% inhibition of cell growth was calculated. Each concentration of drugs was measured in triplicate wells on the same plate in three independent experiments.

Flow cytometric

After 24 hours of drug incubation, the cells simultaneously stained with PI stain, according to the IC50 value of different drugs, to discriminate viable cells from early apoptotic cells and cells that had lost membrane integrity as a result of very late apoptosis. Cells were fixed in pre-cooling 80% ethanol at 4°C for one hour, performed by the 0.1% Triton X-100, digested with RNase at 37°C for one hour, stained with propidium iodide (0.5 mg/ml) in the dark for 30 min and detected on the flow cytometric after filtering in 300-mesh nylon mesh. All experiments were performed in triplicate.

Western blot test analysis of Beclin 1

Cells were incubated for 24 hours after drug treatment, collected by adding the cell lysate, washed twice with PBS, harvested in PBS, and pelleted by centrifugation. The pellet was weighed and suspended in 2 × laemmli buffer. Protein were loaded on a 10% SDS-polyacrylamide gel electrophoresis and then transferring to PVDF membrane. After blocking, membranes were incubated for one h with mouse anti-human P53, rabbit anti-human survivin and anti-HPV16 E6 antibody, respectively, and incubated in Shaker at 4°C overnight. Anti-actin was used to ensure equal loading. Then membranes were added to sheep anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP to react for two hours, washed twice with TBST, then develop in the ECL pressure film.

Statistical analysis

Using SPSS 13.0 software for statistical analysis. The Student's *t*-test was used within the group. Data between the two groups were compared using ANOVA analysis. Statistical significance occurred if $p < 0.05$.

Results

This present experiment showed that different concentrations of cisplatin inhibited the growth of the cervical adenocarcinoma cells HeLa and the contrast of growth inhibition rate had statistical significance whether within the groups or between the groups ($p < 0.05$, $p < 0.01$). The inhibitory effect of cispaltin to HeLa cells strengthened with increased drug concentration and the extension of time (Figure 1).

Mifepristone single drug results showed that mifepristone inhibits the proliferation of HeLa cells when the concentration was greater than 10 μg/ml less than 48 hours with a time-dependent manner ($p < 0.05$). The inhibition rate of mifepristone to HeLa cells was not significant ($p > 0.05$) in HeLa cell vehicle group, low concentrations of mifepristone, (0.625, 1.25, 2.5, 5, 10 μg / ml) and blank control group. HeLa cell proliferation inhibition rate enhanced with the concentration of mifepristone increased in 72 hour group in concentration-dependent manner ($p < 0.05$). The relationship between different doses of mifepristone, duration of action, and cell inhibition rate are shown in Figure 2.

The authors obtained the values of IC50 of cisplatin to HeLa cells for 24, 48 and 72 hours and were 9.86, 2.95, and 0.96 μg/ml, respectively, based on the concentration of cisplatin and the corresponding inhibition rate with cisplatin in HeLa cells. Therefore the authors chose 2.5 μg/ml as cisplatin fixed concentration in combined group and less than or equal to 10 μg/ml as mifepristone follow-up experimental drug dose in accordance with inhibitory effect of single-agent mifepristone to HeLa cells. The authors observed the effect of 2.5 μg/ml cisplatin with different concentrations of mifepristone to HeLa cells in 24, 48, and 72 hours. It was found that the inhibitory effect on HeLa cells became increasingly apparent ($p < 0.05$) while the mifepristone concentration was gradually increased (1.25 - 10 μg/ml). The combined effects of cisplatin with different concentrations of mifepristone enhanced the inhibition of cisplatin on the proliferation of HeLa cells (Figure 3).

The authors detected an apoptosis rate by measuring sub-G1 peak and chose 2.5 μg/ml of cisplatin combined with different concentrations of mifepristone (2.5, 5, and 10 μg / ml) in the following experiments according to the result of MTT's results in HeLa cells for 24 hours. The results showed that the apoptotic rate differences had a statistical significance compared to the combination, monotherapy, and control groups. The statistic values are shown in Table 1. There were no significant differences in cell cycle in each group (Figure 4).

The authors observed the expression level of HPV E6,

Fig. 1

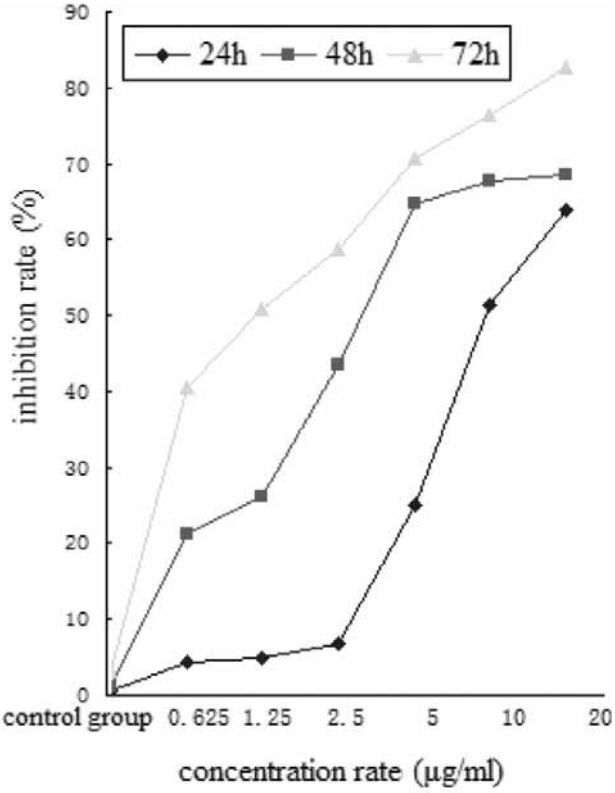

Fig. 2

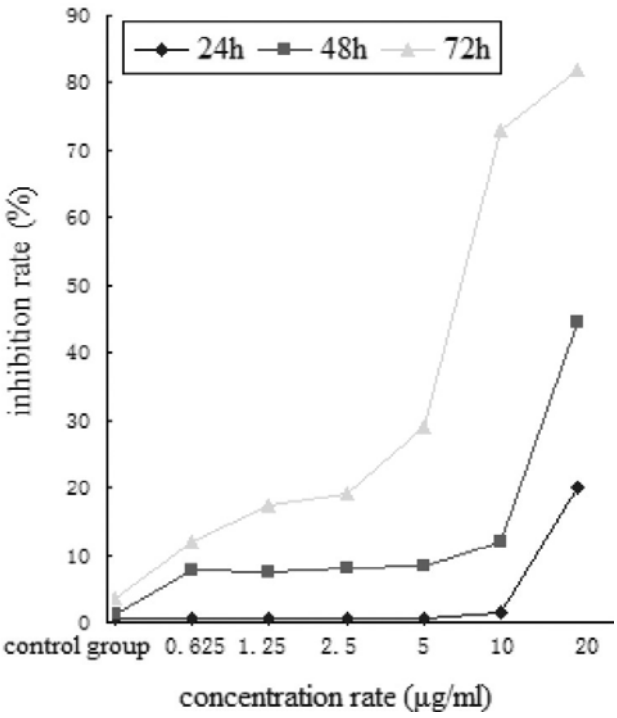

Fig. 3

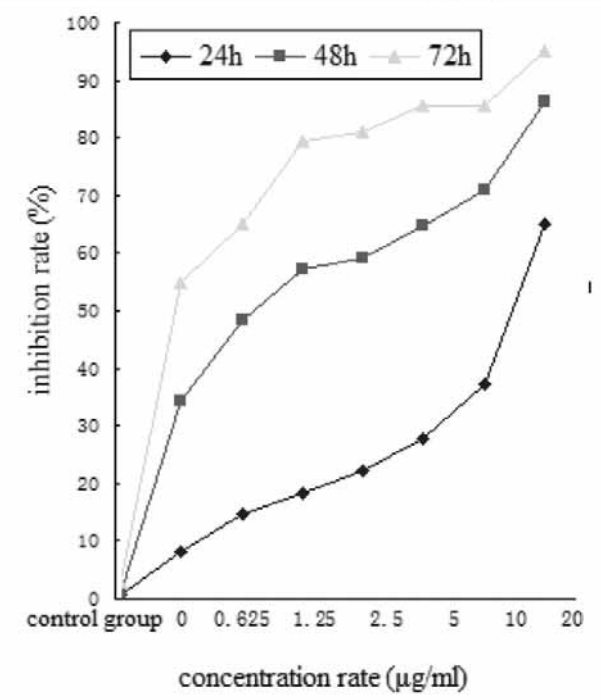

Figure 1. — Inhibiting effects of cisplatin on the proliferation of HeLa cells in dose and time dependant manner.
Figure 2. — Inhibiting effects of mifepristone on the proliferation of HeLa cells in dose and time dependant manner.
Figure 3. — Inhibiting effects of mifepristone and cisplatin on the proliferation of HeLa cells in dose and time dependant manner.

p53, and survivin protein in HeLa cells after incubation with 2.5 μg/ml cisplatin combined with different concentrations of mifepristone (2.5, 5, and 10 μg / ml) for 24 hours by Western Blot. The detection showed that the expression of HPV E6 and survivin protein decreased whereas the P53 expression gradually increased (Figure 5).

Discussion

Cisplatin is the most-widely used single-agent chemotherapy in cervical cancer and its efficacy is the most positive. Cisplatin is the main drug program in combination chemotherapy for cervical cancer, but the results

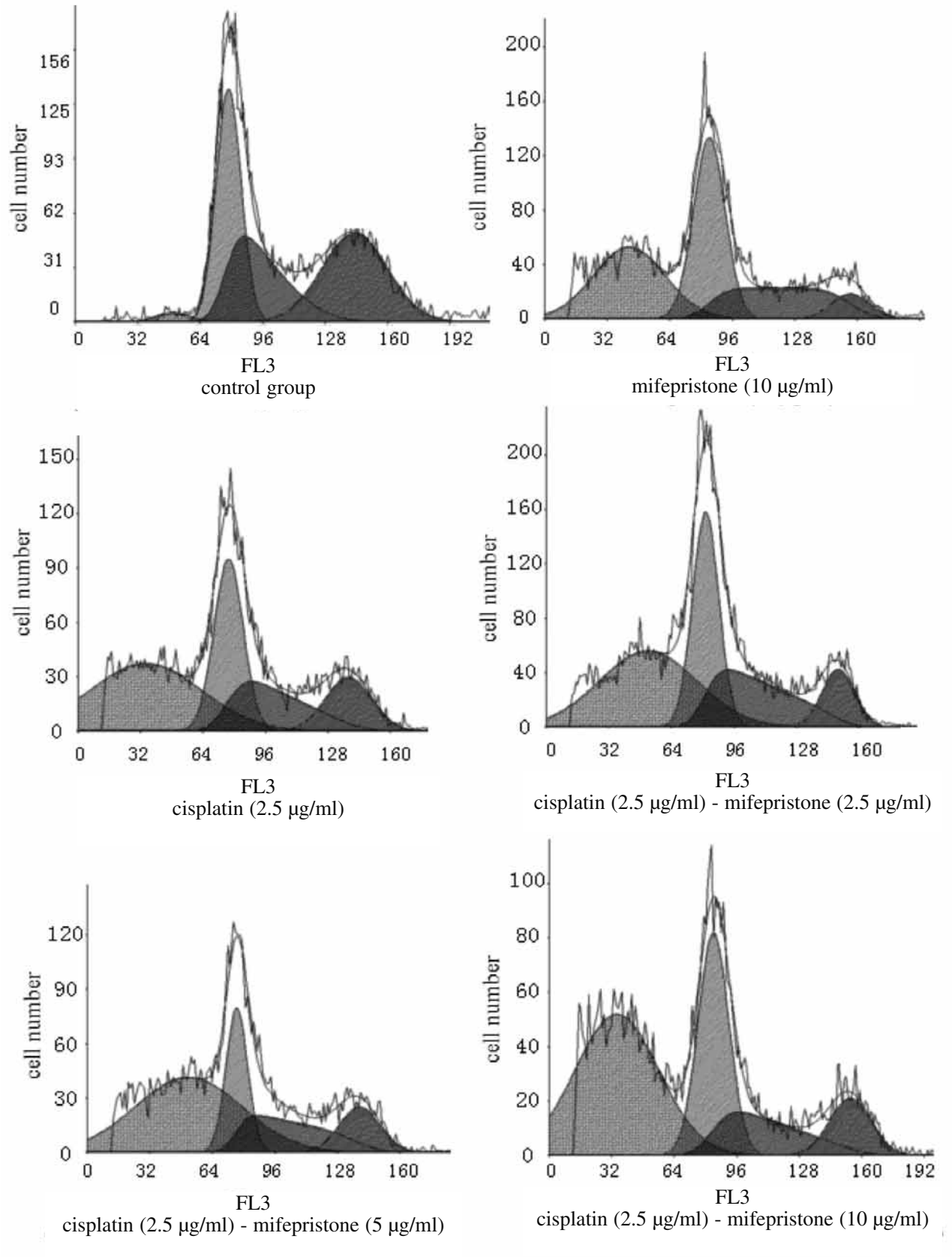

Figure 4. — Apoptosis rates and cell cycle distribution of HeLa cells after treatment with cisplatin and different concentrations of mifepristone evaluated by PI staining.

Table 1. — *Cell cycle and apoptosis of the cells after different treatments studied (mean ± SEM, ANOVA F, and p values).*

Groups (ug/ml)	Cell Cycle			Apoptosis	*F*	*p*
	$G_1 \sim G_0$	S	$G_2 \sim M$	($subG_1$)		
Control group	72.27 ± 2.49	12.93 ± 0.31	14.26 ± 0.39	0.54 ± 0.04		
mifepristone (10)	50.30 ± 0.26	39.37 ± 0.76	9.91 ± 0.09	0.42 ± 0.03	13.92	0.02
Cisplatin (2.5)	77.27 ± 0.47	10.47 ± 0.25	8.67 ± 0.16	3.59 ± 0.18	1925.606	0.000
cisplatin (2.5) + Mifepristone (2.5)	77.33 ± 0.76	8.62 ± 0.29	9.23 ± 0.28	4.82 ± 0.15	198.579	0.000
cisplatin (2.5) + Mifepristone (5)	80.87 ± 0.85	6.57 ± 0.25	7.55 ± 0.13	5.01 ± 0.12	10.438	0.032
cisplatin (2.5) + Mifepristone (10)	76.97 ± 0.80	6.31 ± 0.18	10.63 ± 0.15	6.09 ± 0.80	222.548	0.000

p values 0.05 and corresponding *F* values are highlighted and vs the former group.

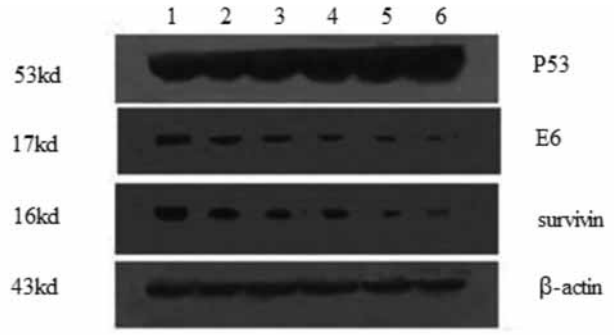

Figure 5. — Protein expression status of p53, E6, and survivin with different treatments 1: control group 2: mifepristone (10) 3: cisplatin (2.5) unit: μg/ml 4: cisplatin (2.5) + mifepristone (2.5) 5: cisplatin (2.5) + mifepristone (5) 6: cisplatin (2.5) + mifepristone (10).

are not effective because its adverse effects and the resistance of tumor cells to cervical cancer. Therefore, it is extremely necessary to find an efficient chemosensitizer and study its anti-tumor mechanism in combination with chemotherapeutic drugs. Chemosensitizer is a class of drug which can improve the efficacy of chemotherapeutic drugs through synergy with chemotherapeutic drugs whereas its effect on tumor treatment is relatively weak.

Mifepristone, a potent progesterone and glucocorticoid antagonist, can bind progesterone receptor, prevent the receptor complex with DNA progesterone response element binding, and stop DNA transcription. Few observations have demonstrated that progesterone and sex hormone regulated HPV gene expression, and concerned with the conversion of the cancerous cell [6-8]. In this experiment, the inhibitory rate of mifepristone on cervical cancer HeLa cells indicated that mifepristone had no effect on cell obvious proliferation inhibition in low-dose (concentration lower than 10 μg/ml) and in a short time (less than 24h). However, mifepristone showed the trend of inhibition of proliferation of HeLa cells if extending the duration of action and increasing its concentration. This result indicates that mifepristone possesses a potential to become a chemosensitizer.

The killing effect of chemotherapeutic drugs on tumor is divided into the direct result of cell necrosis and induction of apoptosis. Analysis results of FCM showed that cisplatin, cisplatin combined with low concentrations of mifepristone had the ability of inducing apoptosis of Hela cell. Presumably, mifepristone's sensitizing effect to cisplatin may be mainly manifested in its ability to enhance the ability of cisplatin-induced apoptosis. In addition, the antiglucocorticoid properties of mifepristone have not only been effective in treating acute psychotic depression, but also helpful in high stress-related conditions including HIV. Mifepristone has helped in other conditions with progesterone receptors such as uterine leiomyomas, endometriosis, and some breast cancer [9]. Cisplatin combined with mifepristone does not cause adverse reactions superimposed on the body. The combination treatment may provide a new idea for cervical cancer chemotherapy.

In the study of apoptotic mechanism of mifepristone-reinforced cisplatin's inhibitory effect on HeLa cells showed that expression of HPV E6 protein gradually reduced with an increasing concentration of mifepristone. The occurrence of cervical adenocarcinoma with high-risk HPV infection is inseparable. The main mechanism of HPV carcinogenic effects is generally believed to be a transformation of the genes E6 and E7 genes encoding the protein to induce cell oncogene activation and tumor suppressor gene inactivation; therefore, E6 and E7 oncogene expression regulation and its variation become the focus of the carcinogenic mechanism of HPV. Lee *et al*. [6,10] found that the E6 and E7 affected tumor suppressor factor p53 and Rb respectively, inhibited their apoptosis, led to the oncoprotein expression of increased high-risk HPV, resulting in cell cycle disorders. This is the most important step in the carcinogenic process. Bartholomew *et al*. [11] found that progesterone, glucocorticoids, and other steroid hormones may down-regulate the level of expression of type I leukocyte antigen (HLA) on cervical cancer cell surface in HPV (+). The regulation of HLA-1 expression of steroid hormones on tumor cells was dependent on the integration and transcription of the HPV genome, but HPV can be blocked by mifepristone. Prompt mifepristone may play a sensitizing role through inhibition of HPV E6 protein interactions in cervical cancer.

Survivin gene is the strongest inhibitor of apoptosis IAP family, which is an important factor for the contact interface of the cell cycle and apoptosis with dual function of inhibition of apoptosis and regulation of mitosis. The Beardsmore and Temme [12, 13] studies' results showed that high expression of survivin in various tumors are not only associated with poor prognosis and resistance to chemotherapy. Branca *et al*. [14] study had shown that survivin is a sign of an early cervical cancer, and its anti-apoptotic function is achieved by blocking the normal transcription of the wild-type p53 by HPV E6. In this present study, the authors detected that the expression of HPV16 E6, survivin decreased and P53 protein was gradually increased as the concentration of mifepristone increased in combined group compared with the control group and the monotherapy group. The authors presume that mifepristone impacted the synthesis of DNA, affected the integration of HPV16 E6 gene and the expression

of HPV16 E6 protein reduced, reducing the inhibition of p53 protein so that the expression of p53 protein increased, and consist with the Reedy [15] results. Of course, this is only a relative preliminary study of cisplatin combined with mifepristone mechanism of action. Therefore further experiments in the future are required, such as detection of the gene expression of these proteins, transcriptional activity, the changes of target DNA binding capacity, and related protein expression levels.

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