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Inhibited effects of veliparib combined doxorubicin for BEL-7404 proliferation of human liver cancer cell line

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ABSTRACT

Objective: To explore inhibition effects of veliparib as PARP inhibitor combined doxorubicin for BEL-7404 proliferation of human liver cancer cell line. **Methods:** BEL-7404 was taken as the object of study and conventional culture was performed. It wastreated by doxorubicin and (or) veliparib after 24 h. Cell proliferation rate was detected by four methyl thiazolyl tetrazolium (MTT) assay, cell apoptosis was measured with annexin V-FITC/PI double staining method by flow cytometry, DNA damage degree evaluation by single cell gel electrophoresis assay, and cytosolic C levels of the mitochondrial and cytosol by polyacrylamide gel electrophoresis (Western blotting). **Results:** Cell proliferation rate of doxorubicin combined veliparib group was lower than that of the control group and doxorubicin alone treated group significantly (P<0.01), the apoptosis rate was significantly higher than that of the control group and doxorubicin alone treated group (P<0.05). At the same time, DNA damage level of doxorubicin combined with veliparib group was significantly higher than doxorubicin alone treatment group and the control group (P<0.01), and cytochrome C in the cytosol was significantly higher than that of control group and doxorubicin alone treated group (P<0.01). **Conclusions:** Veliparib, PARP inhibitor could inhibit PARP activity, block tumor cell DNA repair, and have significant sensitizing effect for hepatocellular carcinoma cell line BEL-7404 treated with doxorubicin. This might provide a new target for clinical treatment of hepatic carcinoma.

1. Introduction

Doxorubicin (also named as adriamycin, ADR) has broad antitumor effect as the typical DNA intercalating agent, which is often used as first-line anticancer drugs in treatment of acute leukemia, malignant lymphoma, multiple myeloma, breast cancer, osteosarcoma, soft tissue sarcoma and liver cancer[1]. Research showed that ADR was a kind of the cell cycle non-specific drugs, which could combine with the DNA topoisomerase Ⅱ, strain close re-process of bridge fracture instantly double chain enzyme caused by the inhibition of topoisomerase Ⅱ. And initiatation of programmed cell apoptosis is not easy due to the recovery of the double strand breaks generated DNA replication and transcription[2,3]. ADR is considered to be one of the most effective chemotherapy for treatment of liver cancer, which reaches the highest concentration in the liver and stay longer in the body. The pharmacokinetic characteristics are very conducive to the treatment of liver cancer. Research proves that it's markedly effective and effective rate is more than 10%-30%, which could make the liver cancer issue shrink or fade. However, it is still limited in the clinical application due to the adverse reactions, such as hair loss, bone marrow suppression and cardiac toxicity, *et al*. Therefore it is of great scientific significance to explore other factors related to the effect of anti tumor.

Poly adenosine double phosphate ribose polymerase (PARP) is a catalytic ribozyme for catalytic poly ADP ribose existing in eukaryotic cells, which plays the key role in DNA damage repair. It could recognize and bind the DNA chain fracture, then recruitment ADP, the histone and the auxiliary factor related to DNA damage repair, then

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complete DNA repair through the adjustment process of a series of catalysis[4]. The clinical studies have demonstrated that the inhibitor of PARP could initiate DNA damage repair by inhibition of anti tumor drugs, and lead to the apoptosis of tumor cells. Therefore, as a kind of the clinical treatment for cancer around PARP, those PARP inhibitors combined with chemotherapy or radiotherapy can repair the process of DNA damage and enhance the sensitivity of tumor cells. And then further it can reduce the clinical side effects of by reducing chemotherapy or the radiation dose[5,6]. Large number of clinical trials hve found that PARP inhibitors have small toxic and side effect, while it is effective and well tolerated chemotherapy or radiotherapy sensitizer, so it has broad prospects for cancer treatment.

Liver cancer is a major threat to human health in the world, ranked sixth in the global incidence of malignant tumors. New cases is more than 626 000 every year, about half of whom occurrs in our country and the mortality rate ranks the second in tumor mortality. The majority of liver cancer patient lost the best time for treatment at the time of diagnosis due to surgical contraindication or transform. Chemotherapy is the main treatment measurement for them, while its efficacy is limited to drug resistance and chemotherapy side effects. Therefore it is necessary to explore chemotherapeutic drugs of the efficient, targeting and low toxicity for liver cancer. Veliparib is a kind of efficient PARP inhibitor and is the representative of the third generation. Research showed that veliparib used alone is tolerated well and activity is high in ovarian cancer patients carrying the mutation gene of BRCA[7]. Experiment of the mouse breast tumor model showed that tumor volume is significantly significant reduced combined with veliparib, compared with chemotherapy alone[8]. This study is designed to investigate the inhabition effect of veliparib combination with ADR on the proliferation of hepatocellular carcinoma cell lines BEL-7404. This study aims to observe DNA damage and cytochrome c release by comet assay and Western blotting methods, explore its possible mechanism and provide the experimental basis for the clinical treatment of liver cancer.

2. Materials and methods

2.1. Cell lines and the reagents

Human liver cancer cell line BEL-7404 was purchased from America type culture collection. Fetal bovine serum, RPMI1640 was from American product of Gibco Company. Injection of doxorubicin was from Pfizer Pharmaceutical (Wuxi) Co.ltd. Veliparib was from America Selleck Company. Mitochondria/cytosolic protein extraction, isolation kit was provided by the biotechnology research Institute of Biyuntian from Jiangsu. BCA protein assay kit was from Wuhan boster Biological Engineering Co., Ltd. (AR0146). Annexin V-FITC/PI double staining cell apoptosis detection kit was from Nanjing keygen biotech development Co., Ltd. P roduction (KGA105), methylthiaLzolyItetraLzolium (MTT) were from America Sigma-Aldrich Company. Mouse anti human β -actin antibody, mouse anti human cytochrome C antibody and Goat anti mouse two anti were from Nanjing Bioworld Biotechnology Co.ltd. Other common chemicals were analytically pure made in China.

2.2. Method

2.2.1. Cell culture

EL-7404 cell were cultured with 10% fetal bovine serum, 50 U/mL penicillin and RPMI1640 with 50 μg/mL streptomycin, and cultured with 5% CO_2, 37 ℃ in the incubator. They were digested by 0.25% Trypsin, and passaged one day at the proportion of 1:2.

2.2.2. MTT test

BEL-7404 cells of logarithmic growth period were selected and made into single cell suspension and counted. 4×10^3 cells each hole was put into 96 well culture plate before 24 h. 5 μg/mL ADR was made based on our preliminary experimental results and other reports of the literature. Cells were divided into 5 groups randomly: control group (control) (without any treatment), ADR group (5 μg/mL ADR treatment alone), combined group of A (5 μg/mL ADR+5 μmol/L veliparib), combined group of B (5 μg/mL ADR+ 10 μmol/L veliparib), Group C (5 μg/mL ADR+20 μmol/ L veliparib). 6 holes in each group were cultured for 24 h, and added with 100 μL MTT solution after the supernatant discarded (final concentration was 0.05 mg/mL). They were cultured for 4 h at 37℃ free from light, and the supernatant was discarded. Then it was added with 150 μL dimethyl sulfoxide (DMSO) in each hole, the absorbance value of Synergy 2 type multifunctional enzyme mark instrument (A570) was read. Cell proliferation rate (%)=OD value of the experimental group/OD value of the control group / ×l00].

2.2.3. BEL-7404 cell apoptosis analyzed by Annexin V-FITC/PI double staining method

The single cell suspension was prepared from BEL-7404 cell of logarithmic growth period and counted, which were seeded in 6 well culture plates, 3×10^6 cells each hole, 24 h before the experiment.

Cells were treated for 24 h, and washed 2 times with pre-cold PBS, digested by 0.25% trypsin. Cells were collected to made cells suspension with PBS after centrifugation at 1 000 *g* for 5 min. After counting, 5.0×10^5 suspension cells was centrifuged for 5 min, supernatant was discard, and it was added with 500 μL binding buffer mixture gently.

Then it was added with 5 μL Annexin V-FITC and 5 μL PI solution and mixed for 10 min (20-25 ℃, free from light). The apoptosis rate was detected by flow cytometry and the detection results were analyzed by FACSC alibur software .

2.2.4. Evaluation of DNA damage degree of BEL-7404 cell by single cell gel electrophoresis assay

According to method of the literature[9], by three layer of the gel method, 3 parallel plates were prepared at each concentration. Gel-Gel film-cell disruption-DNA selection-electrophoresis-neutralization was performed, then propidium iodide (PI) staining was carried out. Single cell gel electrophoresis images were observed under a fluorescence microscope (400 times), 25-50 cells was counted each layer, not less than 100 cells of three layers in each group. The comet tail length, the tail area, tail torque, tail torque arm and olive tail of the image were calculated by CASP comet image analysis software. In view of the olive tail moment was a comprehensive index of DNA damage evaluation, and it was selected as the final index for cell DNA damage evaluation.

2.2.5. Cytochrome C protein in mitochondrion and cytoplasm of BEL-7404 cell by Western blotting detection

Mitochondria/cytosolic protein extraction, separation and quantitative measurement were performed according to kit instructions. 30 μg was taken to measure protein samples with protein sample buffer at the proportion of 4:1, and 95 ℃ water bath degeneration was carried out for 10 min. Electrophoresis was performed 2.5 h in 15% SDS- polyacrylamide gel, transferred to 0.45 μm PVDF membrane, closed for 1 h at room temperature in 5% bovine serum albumin buffer solution. It was washed with PBST swing 3 times, 10 min for every time. The first antibody was incubated at 4 ℃ overnight. The next day, PBST membrane was washed 3 times, 10 min for every time. It was added with the second antibody labeled horseradish peroxidase (HRP). They were incubated them for 1 h, added with PBST and washed. Then it was added with ECL sensitivity luminous liquid, and a protein expression level was detected with multiple function gel imaging analyzer. With β-actin as equal protein control, the gray value of protein band in each group were analyzed by gel image analysis software (Image J).

2.3. Statistical processing

All data were presented as mean±standard deviation, the experimental results was analyzed with SPSS11.0 software package. The variance homogeneity test and *t* test were used between the two groups, $P<0.05$ showed that they had significant difference.

3. Result

3.1. Results of the cell proliferation rate

As shown in Figure 1, the proliferation rate of BEL-7404 cells in all the treatment groups were significantly lower than those of the control group ($P<0.01$). When veliparib concentration was 5 μmol/L, the inhibitory effect on the proliferation rate of BEL-7404 was more significant than that with ADR alone ($P<0.05$). When the veliparib concentration was 10 μmol/L and 20 μmol/L, the proliferation rate of BEL-7404 was significantly increased ($P<0.01$). The proliferation rates of ADR group, group A, group B and group C cell were veliparib dose-dependent.

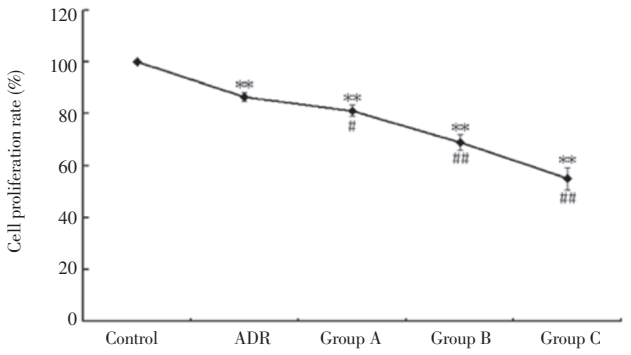

Figure 1. MTT test analysis.

The cell proliferation rate of BEL-7404 was changed after 24 h treated with ADR and (or) veliparib. Compared with the control group, *$P<0.05$. Compared with ADR group, ##$P<0.01$. Compared with ADR group, #$P<0.05$.

3.2. Results of the apoptosis rate

As shown in Figure 2, BEL-7404 cells were treated with different methods after 24 h, the apoptosis was the main form of cell early death in all treatment groups. And whether the early apoptosis rate or late apoptosis rate, they were significantly higher than those of the control group ($P<0.01$). When veliparib and ADR was used together (combined group A, B and C), with the dose increased, the cell apoptotic rate was increased, which showed Veliparib dose dependent change,and the early and late apoptosis apoptosis rate was higher than that in ADR group significantly ($P<0.05$).

3.3. Experimental results of single cell gel electrophoresis

As shown in Figure 3, the BEL-7404 cells were treated for 24 h, compared to the control group, DNA damage degree of all treated group were increased significantly ($P<0.01$). Treated with veliparib and ADR, BEL-7404 cell DNA damage level was increased with veliparib dose increasing, and it was veliparib dose dependent. Compared with ADR group, cell DNA damage in all treated group was also increased

significantly (P<0.05).

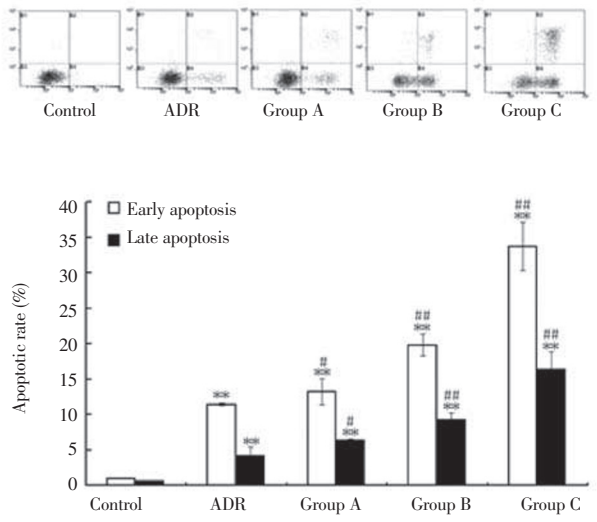

Figure 2. Change of apoptosis rate of BEL-7404 cells treated with ADR and (or) veliparib by flow cytometry after 24 h.
Compared with the control group, **P<0.01. Compared with the control group, *P<0.05. Compared with ADR group, ##P<0.01. Compared with ADR group, #P<0.05.

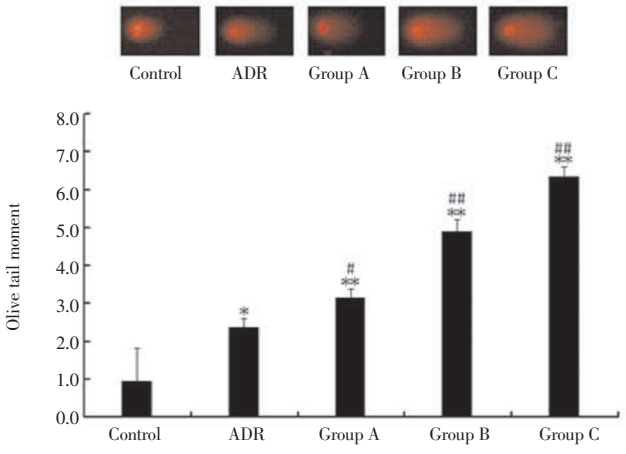

Figure 3. DNA damage degree of BEL-7404 cell detected by single cell gel electrophoresis assay treated with ADR and (or) veliparib for 24 h.
Compared with the control group, *P<0.05. Compared with ADR group, ##P<0.01.Compared with ADR group, #P<0.05.

3.4. Results of Western blotting

As shown in Figure 4, BEL-7404 cells were treated for 24 h, mitochondrial C level of cytochrome in the treatment group were lower significantly, while those in cytoplasm was increased significantly (P<0.05). With veliparib and ADR together, except (ADR+5 mol/L veliparib) group, cytochrome C levels of mitochondrion in the other combination group were significantly lower than those in ADR alone treated group (P<0.05). Cytochrome C level of intracellular in all the combined treatment groups were significantly higher than those of ADR alone treatment group (P<0.01).

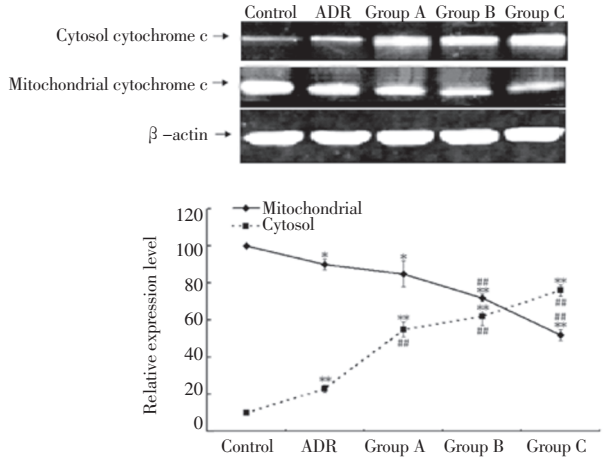

Figure 4. Cytochrome c distribution changes of BEL-7404 cell in the mitochondria and cytoplasm after 24 h.
Compared with the control group, *P<0.05. Compared with ADR group, ##P<0.01. Compared with ADR group, #P<0.05.

4. Discussion

PARP is an important gene in eukaryotic cells, and play a central role in the DNA damage and repair process[10]. PARP-1 is one of the most important members of PARP family and a kind of ribozyme in eukaryotic cells. When the DNA damage occurs, its conservative sequence can combined with single or double stranded DNA rapidly and directly, and activate DNA- protein complex form, thereby raising more DNA damage repair factor. It can transfer cell damage signal and complete the DNA repair process by receptor protein poly adenosine diphosphate glycosylation modification, self modification and poly adenosine diphosphoric acid glycosyl hydrolase role[4]. At the same time, the excessive activation of PARP-1 initiate the cells apoptosis by promoting mitochondrial apoptosis inducing factor release and the fracture of double stranded DNA[11]. Deletion of PARP-1 can make DNA injury of tumor cell be susceptible to chemotherapy drug, which may involved in certain tumors occur directly[12]. In addition, the function of PARP-1 is inhibited by chemical inhibitors or gene silencing, and which can reduce DNA damage repair efficiency and enhance the effect of radiotherapy or chemotherapy for some DNA damage[13]. Recently, the application of PARP inhibitors alone achieved good effect in the specific treatment of tumor. Bryant study found that a PARP-1 inhibitor alone could inhibit significantly the tumor cells growth with deletion of BRCA-1 and BRCA of-2 gene in vitro experiment[14]. In vivo test, Martin found that PARP-1 inhibitors alone could inhibit the growth of skin tumor cells[15]. In addition, the research that PARP-1 inhibitors with chemotherapy drugs for cancer treatment has entered clinical trials, and some progress has been made. Zhang *et al* found the epithelial ovarian cancer cell

line resistant to cisplatin exposure to PARP-1 inhibitor PJ34 could be against ovarian cancer cell proliferation, and the sensitivity of cells to cisplatin increased significantly[16]. Veliparib is a kind of PARP-1 inhibitors of oral potent, and now has entered clinical trial, and have synergistic effects with chemotherapy drugs including temozolomide. And it combined with radiotherapy could enhance significantly the killing effect on tumor and had good tolerance[4], bioavailability in mice, cats, dogs after took them could be reached at 92%, 61% and 72%[17].

In this study, human liver cancer cell line BEL-7404 as the research object, to investigate the effect of veliparib inhibition on ADR induced cell proliferation. The experimental results showed that cell mortality rate of ADR group, ADR combined veliparib group increased significantly compared with the control group. At the same time, mortality rate of ADR combined with veliparib group was significantly higher than that of ADR alone group. This suggested that the use of veliparib made BEL-7404 cells to chemotherapeutic drug susceptibility to ADR. Further proved cells DNA damage of ADR group, ADR combined veliparib group were significantly increased, DNA damage level of ADR combined veliparib group was significantly higher than that of ADR alone group. Results of protein levels showed cytochrome c release of mitochondrion in ADR combined with veliparib group was higher than that of control group and ADR alone group. Cytochrome c release was a key event in apoptosis of mitochondrial dependent[18]. Therefore, MTT, cell apoptosis and single cell gel electrophoresis experiment results showed in this experimental system, veliparib aggravate the damage tumor cells DNA induced ADR through DNA repair inhibition of PARP-1, and promote the inducing effect of ADR on mitochondrial dependent apoptosis.

In conclusion, the results of this study showed that, 5-20 μmol/L concentration of veliparib combined with routine dosage of ADR could inhibit the proliferation of liver cancer cell line BEL-7404 and induce its apoptosis. This study on veliparib as sensitizer for chemotherapy ADR provide a preliminary experimental basis for adjuvant treatment of liver cancer. With the deep research on veliparib as adjuvant chemotherapy, veliparib may play the key role for treatment of various cancer in the future clinical.

Conflict of interest statement

We declare that we have no conflict of interest.

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