Establishment of a first-line second-line treatment model for human pulmonary adenocarcinoma

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Abstract. Lung cancer is one of the most prevalent types of cancer in the world. Surgery, chemotherapy and radiotherapy are used clinically as treatments for numerous cancers. Due to the appearance of drug resistance, the remission rate is limited to 40-50%. Docetaxel and pemetrexed are two drugs commonly used, and their effects in single-phase cell culture are well known. From the pharmacological point of view, it appears rational to hypothesize that sequential therapy effects can show better outcomes compared with traditional single-phase experiments. Considering this, the present study aimed to establish a first-line second-line adenocarcinoma treatment model, using the combination of cisplatin with docetaxel or pemetrexed *in vitro* in different sequential therapy timings. To test this, the human lung cancer A549 cell line was used. The inhibitory effect was determined by adding docetaxel following treatment with cisplatin and pemetrexed (Pem-Doc group) and comparing this with a group in which pemetrexed was added subsequent to treatment with cisplatin and docetaxel (Doc-Pem group). Additionally, the differences in the gene and protein expression levels of excision repair cross-completion gene 1 (ERCC1), a gene that promotes drug resistance to cisplatin, were compared between the two groups. The present results showed that the inhibitory effect of cell proliferation in the Pem-Doc group was increased compared with that of Doc-Pem group, while the gene expression and protein levels of ERCC1 in the Pem-Doc group were decreased compared with those of Doc-Pem group. The Pem-Doc treatment plan is more effective in inhibiting cell proliferation and in lowering the expression of the ERCC1 gene. Therefore, Pem-Doc may be a more effective adenocarcinoma treatment.

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Introduction

Non-small cell lung cancer (NSCLC) accounts for 75-80% of all primary lung cancers. A cisplatin-based chemotherapy regimen has been the main solution for treating advanced NSCLC (1-4). Traditionally the first-line chemotherapy regimen includes cisplatin combined with another third-generation drug, such as docetaxel, paclitaxel or gemcitabine (5-9). For the second-line treatment, pemetrexed, docetaxel and target drugs are constantly used (10-14). The association between histology and a first-line regimen had not been studied until recently, with certain studies indicating that overall survival (OS) correlates with histological subtype. For example, a trial containing 1,725 patients showed that pemetrexed is suitable to be used for tumors with non-squamous histology, as an improved OS time was recorded compared with patients with squamous histology, who experienced a shorter survival time (15-17). Therefore, establishing a treatment model to mimic the first-line second-line treatment is important, as it can be used to confirm the known clinical trial information, to analyze the mechanism and to test other drugs in the future. The inhibitory effects of docetaxel and pemetrexed on cell proliferation have been demonstrated (18,19). The present study compared the inhibitory effect of the docetaxel-pemetrexed (Doc-Pem) group with that of the pemetrexed-docetaxel (Pem-Doc) group. Due to certain factors, including the drug resistance of cisplatin during treatment, the remission rate is limited to 40-50%. Excision repair cross-completion gene 1 (ERCC1) is one of the drug resistance genes, and it plays an important role in DNA repair (20). Overexpression of ERCC1 can cause drug resistance to cisplatin, and lowering the expression of ERCC1 in A549 cells can enhance the sensitivity to cisplatin (21,22). The current study also detected if the combination plan can lower the expression of ERCC1.

Materials and methods

Materials. The materials used in the present study were as follows: Docetaxel (Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China); pemetrexed (National Institutes for Food and Drug Control, Beijing, China); cisplatin (National Institutes for Food and Drug Control, Beijing, China); the A549 cell line (JRDUN Biotechnology, Shanghai, China); Hyclone RPMI 1640 media (GE Healthcare; Logan, UT,

USA); fetal bovine serum (FBS; MRC, Jintan, Jiangsu, China); 0.25% EDTA-trypsin (Beijing Leagene Biotechnology Co., Ltd., Beijing, China); CellTiter 96 Aqueous One Solution Cell Proliferation assay (Promega Corporation, Madison, WI, USA); TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA); RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.); Real Time PCR Easy-SYBR Green I (Foregene Co., Ltd., Chengdu, Sichuan, China); primers (Shanghai Generay Biotech Co., Ltd., Shanghai, China); polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA); BCIP/NBT alkaline phosphatase color development kit (Beijing Huamaike Biotechnology, Beijing, China); and anti-ERCC1 (human) antibody, anti-β-actin (human) antibody and alkaline phosphatase-conjugated Affinipure Goat Anti-Rabbit IgG (H+L) antibody (Proteintech Group Inc., Rosemont, IL, USA).

Cell culture. A549 cells were cultured in RPMI 1640 medium containing 10% FBS, and kept in a 37˚C incubator maintaining humidified air with 5% CO_2. When the cells reached 80-90% confluence, passaging was performed using 0.25% EDTA-trypsin.

Cell proliferation assay (CPA) to determine the half maximal inhibitory concentration (IC_{50}). The cells were seeded in 96-well plates at a density of $2x10^4$/ml (100 μl per well), and were kept in a 37˚C incubator maintaining humidified air with 5% CO_2. Subsequent to 24 h, drugs at different concentrations were added (docetaxel, 0.2, 0.3 and 0.4 mg/ml; pemetrexed, 0.4, 0.8 and 1.6 mg/ml; cisplatin, 0.265, 0.53 and 1.06 μg/ml), with no addition to the blank group (3 wells per drug). Following incubation in a 37˚C incubator for 72 h, 20 μl CPA solution was added. Subsequent to 2 h, 25 μl 10% SDS was added to stop the reaction. The optical density (OD) values were determined using a microplate reader (detection wave length, 492 nm; reference wave length, 630 nm; Multiskan MK3; Thermo Fisher Scientific, Inc.). The inhibition ratio was calculated as follows: Inhibition ratio (100%) = (1 - $OD_{experimental}$ / OD_{blank}) x 100. Graphs were created showing the log-concentration vs. inhibition ratio, and the IC_{50} values were calculated.

CPA for combination with cisplatin in different sequential therapy timings. The cells were cultured as aforementioned and four groups were then created (3 wells per drug): 0.2 mg/ml docetaxel plus 0.62 μg/ml cisplatin was added to form the Doc-Pem group; 1.42 mg/ml pemetrexed plus 0.62 μg/ml cisplatin was added to form the Pem-Doc group; 0.62 μg/ml cisplatin only was added to form the DDP group; and no addition was made to form the blank group. All cultures were maintained in an incubator, and subsequent to 24 h (or 48 h, 1st drug duration as shown in Table I) 1.42 mg/ml pemetrexed was added to the Doc-Pem group, and 0.2 mg/ml docetaxel was added to the Pem-Doc group. The medium was replaced for the DDP group and blank group, the incubation was continued for 24 h (or 48 h, 2nd drug duration as shown in Table I), and the OD and inhibition ratio calculated as aforementioned.

Quantitative polymerase chain reaction (qPCR). Cells of each group were collected and the total RNA was extracted using a TRIzol kit; the small amount of remaining genomic DNA was removed using DNase I (Beijing Huamaike Biotechnology, Beijing, China). The RNA quality was confirmed by Nanodrop spectrophotometry. Reverse transcription was performed as described in the manufacturer's instructions. Briefly, total RNA was mized with Random Hexamer primer (1 μl), made up to a total volume of 12 μl using nuclease-free water, and then 5X reaction buffer (4 μl), Ribolock RNase Inhibitor (1 μl), 10 mM dNTP mix (2 μl) and RevertAid M-MuLV RT (1 μl) were added. This was mixed gently and spun briefly, then incubated for 5 min at 25˚C, followed by 60 min at 42˚C. The reaction was terminated by heating at 70˚C for 5 min. The reverse transcription reaction products were stored at -80˚C. qPCR was performed using the StepOne system normalized to a reference gene by the comparative C_q method ($2^{-\Delta\Delta Cq}$) (23). The PCR was conducted using the Real Time PCR Easy SYBR Green kit (Foregene Co., Ltd.). The cycling conditions were 94˚C for 3 min, followed by 40 cycles at 94˚C for 10 sec, 65˚C for 10 sec and 72˚C for 30 sec, the signal of amplified ERCC1 or β-actin was collected at the end of each cycle. The PCR mixture containing no template was used as a negative control. This qPCR experiment was repeated twice. The primers were as follows: ERCC1 forward, 5'-CTT GTC TTC TGG CTC GAA GG-3' and reverse, 5'-ACT CAG GAG GCA GTG AAT GG-3'; β-actin forward, 5'-CAT CGT CCA CCG CAA ATG CTTC-3' and reverse, 5'-AAC CGA CTG CTG TCA CCT TCA C-3'.

Western blot analysis. Cells of each group were collected (cells from five 10-cm dishes per group), and 1/4 volume of 5X loading buffer was added. The cells were subjected to 98˚C denaturation for 5 min, prior to separation by 10% SDS-PAGE. The proteins were transferred to a PVDF membrane by the semi-dry method, and were blocked for 30 min by 5% skimmed milk. The primary antibodies anti-ERCC1 (Proteintech Group Inc.; catalog no. 14586-1-AP; dilution, 1:300) and anti-β-actin (Proteintech Group Inc.; catalog no, 20536-1-AP; dilution, 1:500) were added, and the sheets were incubated at 4˚C overnight, prior to being washed by Tris-buffered saline with Tween-20 (TBST) 4 times, for 5 min each. The ALP-conjugated Affinipure Goat Anti-Rabbit IgG(H+L) secondary antibody (Proteintech Group Inc.; catalog no. SA00002-2; dilution, 1/500) was then added and incubated at room temperature for 1 h, prior to being washed by TBST 4 times, for 5 min each. The color was developed with a BCIP/NBT kit. The bands were analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA) and the ERCC1/β-actin ratios were calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA) to measure the grayscale value of each band.

Statistical analysis. All analyses were performed with the Statistical Package for the Social Sciences version 22 (IMB SPSS, Armonk, NY, USA). One-wayanalysis of variance and the least significant difference test were used to evaluate the differences between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inhibitory effect on cell proliferation is stronger in the Pem-Doc group. The CPA (Fig. 1) showed that all 3 drugs, including docetaxel, pemetrexed and cisplatin, could inhibit

Table I. Duration of drug treatment in 4 methods.

Method	1st drug regime and duration	2nd drug regime and duration	Change to fresh media
1	Doc + DDP for 24 h	Pem for 24 h	
	Pem + DDP for 24 h	Doc for 24 h	
	DDP for 24 h	Blank for 24 h	
	Blank for 24 h	Blank for 24 h	
2	Doc + DDP for 24 h	Pem for 24 h	24 h
	Pem + DDP for 24 h	Doc for 24 h	24 h
	DDP for 24 h	Blank for 24 h	24 h
	Blank for 24 h	Blank for 24 h	24 h
3	Doc + DDP for 48 h	Pem for 24 h	
	Pem + DDP for 48 h	Doc for 24 h	
	DDP for 48 h	Blank for 24 h	
	Blank for 48 h	Blank for 24 h	
4	Doc + DDP for 24 h	Pem for 48 h	
	Pem + DDP for 24 h	Doc for 48 h	
	DDP for 24 h	Blank for 48 h	
	Blank for 24 h	Blank for 48 h	

Doc, docetaxel; Pem, pemetrexed; DDP, cisplatin.

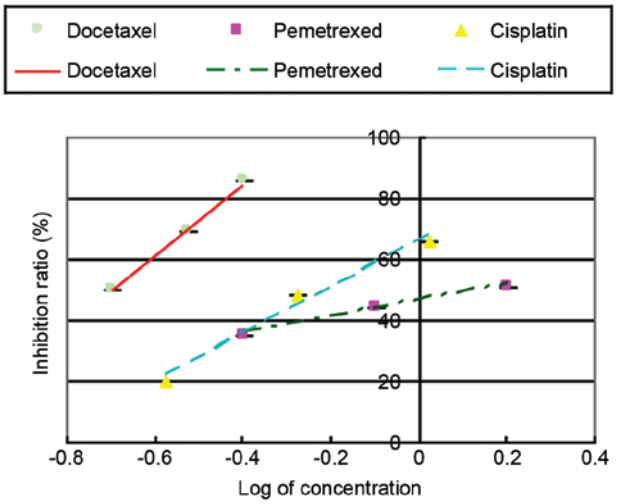

Figure 1. Inhibitory effect of A549 cells by cell proliferation assay with the addition of three drugs. X-axis, log of concentration of each drug; Y-axis, inhibition ratio compared with the blank group (without the addition of drugs). The inhibition ratio increased with the increase in drug concentration.

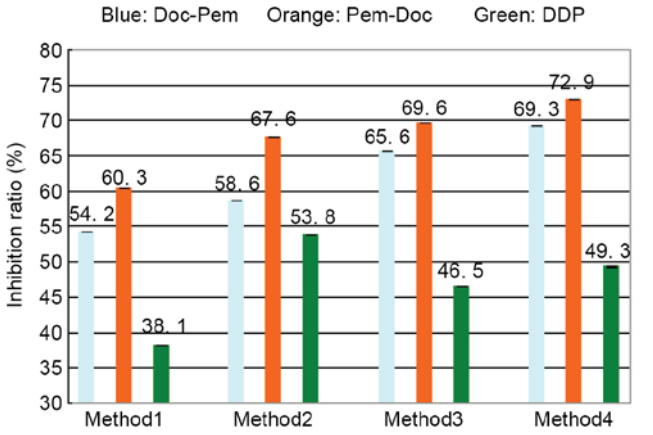

Figure 2. Inhibition ratios of 4 groups using 4 methods, as determined by cell proliferation assay. The 4 methods differ by duration time in the 1st and 2nd stages, as shown in Table I. Doc, docetaxel; Pem, pemetrexed; DDP, cisplatin.

the proliferation of the cells; this inhibitory effect became stronger with increased drug concentration. The IC_{50} of docetaxel was 0.2 mg/ml, the IC_{50} of pemetrexed was 1.42 mg/ml and the IC_{50} of cisplatin was 0.62 μg/ml. To test the effect of sequential combination, four methods were tested (Table I). The results (Fig. 2) show that in method 1, the inhibition ratio of the Pem-Doc group (60.3%) and that of the Doc-Pem group (54.2%) was increased compared with that of the DDP group (38.1%) (both P<0.001). Additionally, the Pem-Doc group was significantly increased compared with the Doc-Pem group (P=0.020). In methods 2, 3 and 4, the inhibition ratio of the Pem-Doc group and the Doc-Pem group was increased compared with that of the DDP group (P<0.001, P=0.002, P<0.001, P<0.001, P<0.001 and P<0.001, respectively), and there were significant differences between the Pem-Doc group and the Doc-Pem group (P<0.001, P=0.040 and P=0.040, respectively). This indicated that the inhibitory effect on cell proliferation in the Pem-Doc group was stronger compared with that of the Doc-Pem group; furthermore, this tendency was not affected by the different treatment duration of the drugs.

ERCC1 gene expression in the Pem-Doc group is lower than that in the Doc-Pem group. To investigate the association between the inhibitory effects of two regimes on the cell proliferation and drug resistance of cisplatin, ERCC1, a drug

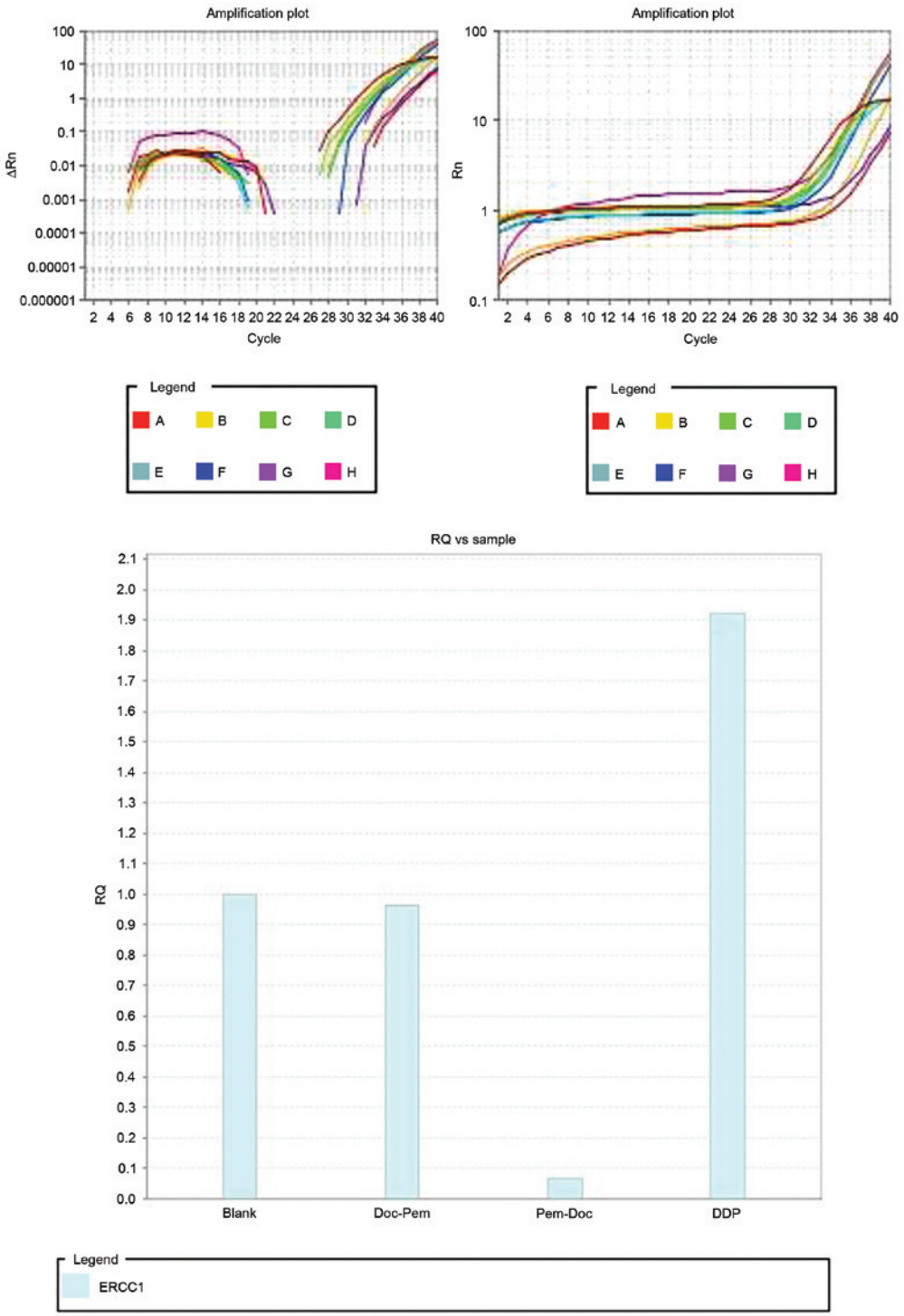

Figure 3. Expression of ERCC1 by quantitative polymerase chain reaction. The internal control was β-actin. A to H represent the samples in row A (numbers 1 to 12) to row H (numbers 1 to 12), respectively (A1, ERCC1 primers, no template; B1, actin primers, no template; C1, ERCC1 primers, template from blank group; D1, actin primers, template from blank group; E1, ERCC1 primers, template from Doc-Pem group; F1, actin primers, template from Doc-Pem group; G1, ERCC1 primers, template from Pem-Doc group; H1, actin primers, template from Pem-Doc group; A2, ERCC1 primers, template from DDP group; B2, actin primers, template from DDP group. Blank, without addition of drugs in the 1st and 2nd stages; RQ, relative quantification; Rn, normalized reporter (fluorescence of the reporter dye divided by the fluorescence of a passive reference dye (ROX)]; ΔRn, Rn minus the baseline.

resistance gene, was studied by qPCR, with β-actin as the internal control. qPCR (Fig. 3) showed that the expression of the ERCC1 gene in the DDP group was ~1.9 times that of the blank group (without the addition of drugs). The expression in the Doc-Pem group was slightly decreased compared with that of the blank group, while the expression in the Pem-Doc group was 0.066 times that of the blank group. This indicates that the Pem-Doc plan may increase the inhibitory effect by inhibiting the expression of ERCC1.

Pem-Doc group inhibits the expression of the ERCC1 protein more efficiently. To obtain further information on ERCC1 expression, cells from 4 groups underwent western blotting. The result (Fig. 4) show that the DDP group (0.62 μg/ml)

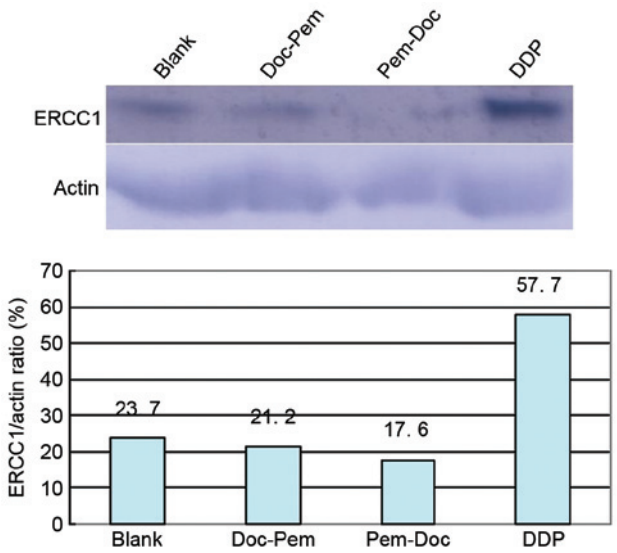

Figure 4. Expression of ERCC1 by western blot analysis. Internal control, β-actin. The grayscale values of each band were analyzed using ImageJ software.

markedly promoted the expression of the ERCC1 protein; the ERCC1/β-actin ratio was 57.7%, which was 2.4 times that of the blank group. The values of the combination groups were lower than that of blank group; the value in the Doc-Pem group was 0.89 times that of the blank group, while it was 0.74 times that of the blank group in the Pem-Doc group. This indicates that the Pem-Doc plan may have a better inhibitory effect through inhibiting the expression of the ERCC1 protein.

Discussion

Docetaxel promotes microtubule polymerization and inhibits depolymerization, which results in the death of cancer cells. The combination of docetaxel with cisplatin is constantly used in the clinic, and patients with non-small cell lung cancer can benefit from the prolongation of survival (7,10,14). Pemetrexed is a novel drug that can inhibit the metabolism of folic acid by inhibiting the activity of required enzymes, including thymidylate, dihydrofolate reductase and phosphoribosyl glycinamide transtormylase (17). This process results in abnormalities in folic acid metabolism and nucleotide synthesis, which leads to the death of cancer cells. In a large trial containing 571 patients (12), results showed that pemetrexed had the same effect as docetaxel in treating patients with advanced stage NSCLC. Additionally, fewer side effects were observed and pemetrexed may therefore be a useful drug for second-line treatment. In 2004, pemetrexed was approved for use as a second-line treatment. In previous years, it was reported that the combination of pemetrexed with cisplatin was well tolerated and was the approved standard first-line therapy (24,25). Although pemetrexed is an ineffective drug for squamous carcinomas, it has been found to be effective in non-squamous NSCLC (15-17). Adenocarcinoma is a non-squamous subtype that accounts for ~50% of lung cancer cases. The inhibitory effect of chemical drugs on cell proliferation have been researched widely (21,22), and the present study was designed to establish an adenocarcinoma treatment model, which includes the combination of cisplatin with docetaxel or pemetrexed *in vitro* in different sequential therapy timings, so that the effect and mechanism could be studied. The present model can be used to test other drugs, and to compare the effect against the Pem-Doc group, which acts as a control.

The present results showed that the inhibitory effect of the combination groups (Doc-Pem and Pem-Doc groups) was stronger than that of the DDP group. Among the combination groups, the effect in the Pem-Doc group was stronger compared with that in the Doc-Pem group. Furthermore, the tendency did not change with different treatment durations (24→24 h; 48→24 h; 24→48 h). The drug resistance of cisplatin is an important affecting factor in chemotherapy, qPCR showed that the expression of the ERCC1 gene in the Pem-Doc group was inhibited compared with that of the Doc-Pem group, and the same pattern was identified for the protein level by western blot analysis. This indicated that Pem-Doc plan may increase the inhibitory effect more efficiently by inhibiting the expression of ERCC1, thus lowering the drug resistance of cisplatin.

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