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.

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); Hyclone RPMI 1640 media (GE Healthcare; Logan, UT, USA).
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); BCP/NBT alkaline phosphatase color development kit (Beijing Huamai 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% CO2. 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 (IC50). The cells were seeded in 96-well plates at a density of 2x10^3/ml (100 µl per well), and were kept in a 37˚C incubator maintaining humidified air with 5% CO2. 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 - ODExperimental / ODBlank) x 100. Graphs were created showing the log-concentration vs. inhibition ratio, and the IC50 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 Huamai 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 mixed 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 Cq method (ΔΔCq) (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 CAG 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 with 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. SA00022-2; 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) 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-way analysis 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...
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 resistance enzyme, was evaluated.

**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.
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)
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 glycaminide 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 approved as the 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 has 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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