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Fucoidan enhances the effect of chemotherapeutic drug against drug-resistant lung cancer cells

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Abstract: Development of adjuvant chemotherapy drugs against drug-resistant lung cancer cells is necessary. The use of non-toxic adjuvant natural product combined with chemotherapy drugs will be an important treatment mode in the future. The purpose of the study investigates that fucoidan enhances chemotherapy drug poisoning drug-resistant lung cancer cell. Drug-resistant lung cancer cells are established in the study. Cell culture, MTT assay, wound healing assay, gelatin zymography assay, DNA fragmentation assay, apoptosis assay, reverse transcription polymerase chain reaction (RT-PCR) western blot analysis was adopted. The results showed that fucoidan synergized with doxorubicin increased efficacy of poisoning drug-resistant lung cancer cells and enhanced the ability of doxorubicin to inhibit the migration of drug-resistant lung cancer cells. It was observed that fucoidan synergized with doxorubicin induced the increase of apoptosis and inhibited expression of MMP-9, LC3, Beclin-1 and β-catenin in drug-resistant lung cancer cells. Fucoidan synergized with doxorubicin significantly inhibited proliferation, migration and metastasis of drug-resistant lung cancer cells. Fucoidan strengthened doxorubicin to induce apoptosis and autophagy of drug-resistant lung cancer cells. This study confirms that the combined use of fucoidan and chemotherapeutic drugs can effectively poison drug-resistant lung cancer cells.

Keywords: Drug-resistant lung cancer cells, Fucoidan, doxorubicin, chemotherapy drug

INTRODUCTION

Cancer has been the leading cause of death in the world for many years and lung cancer ranks first. There are many ways to treat lung cancer, chemotherapy is commonly used in clinical treatment of lung cancer, but it still has serious side effects for the human body and the resistance of tumors to chemotherapy drugs is also increasing with the development and progress of chemotherapy (Shimomura *et al*. 2019). Cancer cells gradually develop resistance to chemotherapy drugs through various mechanisms and pathways, such as through tumor cell heterogeneity, or reducing drug uptake, increasing drug excretion and enhancing DNA repair, as well as abnormal apoptosis and autophagy, which is responsible for the development of chemoresistance (Gong *et al*. 2019).

Anthracyclines, especially Doxorubicin, have long been the mainstay of cancer treatment. Despite its broad antitumor activity, its side effects, especially cardiotoxicity, limit the clinical use of doxorubicin. This is especially true for patients requiring dose escalation at an advanced stage (Rivankar 2014)

Sorcin (soluble resistance related calcium binding protein) is a soluble calcium binding protein and plays a key role in regulation of multiple drug resistance (MDR). Many evidences show that sorcin is related to the development of drug resistance in various cancers (Battista *et al*. 2020). The studies indicate that sorcin is associated with resistance to paclitaxel in ovarian, breast and lung cancer cells and multidrug resistance in gastric cancer (Zhang *et al*. 2021; Zhou *et al*. 2019).

Due to tumor heterogeneity and increased drug resistance, chemotherapy is gradually losing their effectiveness. In addition, many recent studies have confirmed anticancer potential of dietary polyphenols. The choice of natural extracts to adjuvant chemotherapy is very important (Chen *et al*. 2018). A variety of natural extracts have been proven to prevent and treat cancer and slow down the toxicity of chemotherapeutic drugs to the gastrointestinal tract, liver, kidney, heart and nerves (Zhang *et al*. 2018).

Fucoidan, a natural sulfated polysaccharide mainly presents in the cell wall matrix of various brown seaweeds, has been described to have anti-cancer properties in different types of cancer. It can achieve anti-tumor effects by inducing cell cycle arrest, apoptosis and immune system activation through various mechanisms (Wang *et al*. 2019). Fucoidan has also been shown to induce apoptosis in human colon and breast cancer cells (Huang *et al*., 2021; He *et al*., 2019). Studies explore anti-lung cancer effects of fucoidan alone (Lin *et al*., 2020), but the effect of combining fucoidan and chemotherapy drug on drug-resistant lung cancer cell is still unknown.

MMPs play an important role in the migration and invasion of cancer cells (Mao *et al*. 2021). Some studies have shown that high expression of MMPs in tissues is usually associated with poor prognosis in non-small cell lung cancer (Huang *et al*. 2015).

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Autophagy has also been shown to protect tumor from necrosis caused by metabolic stress and recycle materials through the degradation of macromolecules to facilitate the survival of cancer cells (Mrakovcic and Frohlich, 2019). For MDR cancer cells, studies have reported that autophagy frequently occurs during tumorigenesis and cancer chemotherapy, mainly to protect cancer cells during chemotherapy, which also leads to the generation of drug resistance in cancer cells (Zhang *et al*. 2022).

The Wnt/β-catenin signaling pathway is related to embryonic development, cell proliferation, differentiation, survival and tumorigenesis. The Wnt signaling pathway depends on the expression of β-catenin in cells. When the expression level of β-catenin decreases, the Wnt signaling pathway is turned off; and when the expression level of β-catenin increases, the Wnt signaling pathway is turned on (Zhang and Wang, 2020). The dysregulation of this signaling pathway, especially the elevated expression of β-catenin, is frequently observed in human cancers, suggesting that aberrant activation of this pathway may be associated with cancer development. The literature confirmed that abnormal β-catenin activation in the Wnt/β-catenin signaling pathway can promote the occurrence and metastasis of non-small cell lung cancer (Yang *et al*. 2016).

Patients with cell lung cancer are mainly treated with chemotherapy in the clinic. Chemotherapy drugs have serious side effects and drug resistance. The use of lower doses of chemotherapy drugs combined with non-toxic and effective natural drugs can avoid the serious side effects and drug resistance of chemotherapy drugs and achieve the desired efficacy. Therefore, it is necessary to pay attention to choosing natural extracts to adjuvant chemotherapy drugs and alleviate complications. Therefore, in this study, we used the chemotherapy drug (doxorubicin) to stimulate lung cancer cells for a long time to establish drug-resistant lung cancer cells and studied fucoidan synergized with doxorubicin to effects of drug-resistant lung cancer cells.

MATERIALS AND METHODS

Cell culture

Lung cancer cells A549 cell line and normal human embryonic lung fibroblast MRC-5 cell line was cultured in DMEM / F12 (Gibco) medium at 37°C, containing 5% CO_2. The process of drug-resistant lung cancer cells (A549DoxR) was established from A549 cell line treated with low concentration of doxorubicin to induce its drug resistance.

MTT assay

1×10^4 cells were seeded to 96 wells and after 24 hours of cell adhesion, different concentrations of fucoidan and 8μM doxorubicin were added according to the experimental group. After 24 hours treatment, the cells were observed under a microscope, add 50μL MTT reagent, react at 37°C for 2-4 hours, remove the reagent from the incubator, add 100μL DMSO, make the MTT crystals completely dissolve in DMSO. Its absorbance was detected at O.D. 570nm using an ELISA reader.

Wound healing assay

Culture the cells in 6 wells to 100% full, take a 10μL tip, use the tip to draw a scar on the cells against the ruler and wash with 1 × PBS, according to experimental conditions. Add drugs, observe and photograph the cell movement at different times under a microscope.

Gelatin zymography assay

A549 and A549DoxR cells were cultured in density of 2.2 x 10^6 cells/ml, after 24 hours of cell adhesion, different concentrations of fucoidan and 8μM doxorubicin were added respectively. Collect supernatants separately, mix them with 5× non-reducing sample buffer and run the electrophoresis SDS-PAGE, stained proteins in the staining solution.

DNA fragmentation assay

The density of 2.2×10^6 cells/ml of A549 and A549DoxR were cultured. After 24 hours of cell adhesion, different concentrations of fucoidan and 8μM doxorubicin were added respectively. After treatment for 24-48 hours, cells were collected. Electrophoresis was performed using 2% agarose gel (100mV, 30 minutes).

Apoptosis assay

A549 and A549DoxR cells (1×10^4 cells/ml) were cultured in 96 wells. After 24 hours of cell adhesion, different concentrations of fucoidan and 8μM doxorubicin were added according to the experimental group. After 24 hours of treatment, the cell culture medium was removed, washed with 1×PBS, added 50μL of 1×annexin-binding buffer, 2μL of annexin V conjugate, reacted at room temperature for 15 minutes, washed with 1×annexin-binding buffer and photographed under a fluorescence microscope.

Reverse transcription polymerase chain reaction (RT-PCR)

After treatment of cells with drugs according to the experimental design, the cell culture medium was removed, washed with 1×PBS and TRIzol reagent (Sigma, St. Louis, MO) was added for cell lysis and converted to complementary DNA (cDNA) was synthesized with Super Script™ III reverse transcriptase (Invitrogen, USA), PCR conditions were set 30 amplification cycles consisting of 94˚C for 15 sec, 60˚C for 30 sec and 72˚C for 1min for 30 cycles, then final 72˚C extension for 7min. PCR products were electrophoresed in 2% agarose gel.

Western blot analysis

Cells were treated with drugs according to the experimental design. Cell lysates were separated by 8% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and were transferred to nitrocellulose membrane (PVDF membrane) using a wet blot format. The membranes were blocked with blocking buffer at room

temperature for 20min and were incubated primary antibody for 24h at 4□. Next, the membranes were washed three times with TBST (Tris-HCl, pH 7.4, NaCl and 0.1% Tween 20) for 10min and were incubated secondary horseradish peroxidase-conjugated goat anti-mouse antibodies for 1h. After washing, the membrane was detected by enhanced chemilluminescence (ECL) system and was measured densitometry by Image J software.

STATISTICAL ANALYSIS

Experiments were conducted independently for more than 3 times. Differences among groups were determined using one-way analysis of variance by the statistical analysis system (SAS 9.4) application package. All measurements are presented as means±SD. It was considered statistically significant from a difference with $p<0.05$.

RESULTS

Differences in cell morphology between drug-resistant lung cancer cells (A549DoxR) and lung cancer cells (A549)

The increase of doxorubicin concentration from 0.1μM to 8μM doesn't affected cells which continue to proliferate and changed their morphology from spindle-shaped to more elongated, compared with A549 without drug treatment. (fig. 1-1).

Effects of Doxorubicin on the survival of drug-resistant lung cancer cells (A549DoxR) and lung cancer cells (A549)

A549DoxR and A549 cells were treated with doxorubicin 8μM for 24 hours. A549DoxR cells were not affected, while A549 cells had been significantly poisoned by chemotherapy drugs (fig. 1-2). The effect of doxorubicin 8μM on the survival rate of A549 cells and A549DoxR cells was further observed by MTT assay. It was observed that the survival rate of A549 cells was significantly decreased compared with A549DoxR cells (fig. 2-1). Results showed that A549DoxR cells are highly resistant to doxorubicin.

The effect of Doxorubicin 8μM on the migration ability of drug-resistant lung cancer cells A549 (A549DoxR)

Using the wound healing assay analyzed A549DoxR treated with doxorubicin 8μM for 0, 8 and 24 hours, it was observed that the cell migration ability was not inhibited compared with the control group (fig. 2-2). Results showed that A549DoxR cells were significantly resistant to doxorubicin.

The difference of sorcin expression between drug-resistant lung cancer cell (A549DoxR) and lung cancer cell (A549)

Sorcin gene has been proved to be related to the resistance of various chemotherapeutic drugs. RT-PCR was used to analyze the expression of sorcin mRNA in A549 cells and A549DoxR cells. Our results showed that sorcin gene was overexpressed in A549DoxR cells compared with A549 cells (fig. 3A). Western blot analysis was used to analyze the expression of sorcin protein in A549 cells and A549DoxR cells and our we observed that sorcin protein was overexpressed in A549DoxR cells compared with A549 cells (fig. 3B). Result showed that there was a lot of sorcin in drug-resistant lung cancer cells.

Effects of different concentrations of fucoidan on the survival rate of lung cancer cells (A549)

The effect of different concentrations of fucoidan on the cell viability of A549 cells was analyzed by MTT assay. It was observed that the A549 cells were treated with fucoidan 25, 50, 100 and 300μg/mL for 24 hours and the cell viability was 55±3%, 52±4%, 38±3% and 35±3%, respectively. The A549 cell viability decreased significantly with increasing fucoidan concentration compared to the control group (fig. 4-1). This result showed that the fucoidan produced obvious toxicity to lung cancer cells (A549) and it was more obvious with the increase of fucoidan concentration.

Effects of different concentrations of fucoidan on the survival rate of drug-resistant lung cancer cells (A549DoxR)

The lowest dose of fucoidan 25μg/mL and the high dose of 100μg/mL were selected to treat the A549DoxR cells. Results of microscopic observation showed that cells were inhibited by fucoidan compared to the control group (fig. 4-2). Therefore, it was further analyzed by MTT assay that treated with fucoidan 25, 50, 100 and 300μg/mL, the cell viability was 90±3%, 87±2%, 79±3% and 75±4%, respectively. Compared to the control group, the doses of fucoidan 25, 50, 100 and 300μg/mL significantly decreased the A549DoxR cell viability, by 10%, 13%, 21% and 25%, respectively (fig. 4-3). Our results showed that the fucoidan can induce drug-resistant lung cancer cells death. The better result was obtained with the higher concentration of fucoidan.

Effects of different concentrations of fucoidan combined with doxorubicin on the survival rate of drug-resistant lung cancer cells (A549DoxR)

The effect of fucoidan combined with doxorubicin on the A549DoxR cells was observed by microscope. Compared to doxorubicin (8μM) group, the combined group showed an increase in cytotoxicity with increasing doses of fucoidan (fig. 4-4). The effect of fucoidan combined with doxorubicin on the survival rate of A549DoxR cells was analyzed by MTT assay. It was observed that the survival rate of A549DoxR cells treated with doxorubicin 8μM was 85%±2%, while the combination of doxorubicin 8μM and fucoidan 25, 50, 100 and 300μg/mL, induced a cell viability of 79±3%, 76±2%, 67±2% and 60±3%, respectively (fig. 5-1). The result showed that fucoidan synergized with doxorubicin to inhibit the viability of drug-resistant lung cancer cells.

Effects of different concentrations of fucoidan on the survival rate of human fetal lung fibroblast cell line (MRC-5)

MRC-5 was treated with fucoidan 25, 50, 100, 300μg/mL through MTT assay analysis. It was observed that the fucoidan dose of 300μg/mL significantly decreased the MRC-5 cell viability compared with the control group and the cell viability decreased by 17% (fig. 5-2).

This result showed that fucoidan 25, 50, 100μg/mL were not toxic to normal cells, we will use low concentration of fucoidan 25μg/mL and high concentration of fucoidan 100μg/mL as the exposure concentration of the subsequent experiments.

Effects of different concentrations of fucoidan on the migration ability of drug-resistant lung cancer cells (A549DoxR)

Wound healing assay was used to analyzed A549DoxR cells treated with fucoidan 25 and 100μg/ml. Our results showed that at doses of 25 and 100μg/mL, fucoidan induced an inhibition of cell migration ability more significantly compared with control. (fig. 5-3). The result showed that fucoidan had significant inhibitory effect on drug-resistant lung cancer cell migration and the higher the concentration, the more obvious the inhibitory effect.

Effects of doxorubicin combined with different concentrations of fucoidan on the migration ability of drug-resistant lung cancer cells (A549DoxR)

Fig. 5-4 showed the migration ability of A549DoxR cells treated with doxorubicin 8μM and doxorubicin 8μM combined with fucoidan 25 and 100μg/ml. Our results showed that A549DoxR cell migration ability was significantly inhibited at fucoidan doses of 25 and 100μg/mL combined with doxorubicin 8μM compared with control. This result shows that fucoidan has the effect of enhancing the effect of doxorubicin on the inhibition of metastatic ability of drug-resistant lung cancer cells.

Effects of different concentrations of fucoidan on the expression of MMP in drug-resistant lung cancer cells (A549DoxR)

Gelatin zymography assay was used to analyze the effect of fucoidan combined with doxorubicin on the expression of MMP of A549DoxR. A549DoxR cells were treated with doxorubicin 8μM or doxorubicin 8μM combined with fucoidan 25, 50 and 100μg/ml.

Doxorubicin 8μM combined with fucoidan treatment, with increasing fucoidan dose, induced a decrease of the expression of MMP-9 compared to doxorubicin 8μM (fig. 6-1).

The result showed that fucoidan synergized with doxorubicin to inhibit the migration viability of drug-resistant lung cancer cells.

Effects of different concentrations of fucoidan combined with doxorubicin on the apoptosis of A549DoxR cells

A549DoxR cells treated with doxorubicin 8μM or doxorubicin 8μM combined with fucoidan 25, 50 and 100μg/mL were analyzed by apoptosis assay. Our results showed that the apoptotic of A549DoxR cells (green fluorescence) treated with fucoidan combined with doxorubicin 8μM increased, compared to cells treated with doxorubicin 8μM alone.

The occurrence of apoptosis was more obvious as the dose of fucoidan increased. The result showed that fucoidan synergistically increased the apoptosis induced by doxorubicin (fig. 6-2).

Effects of Fucoidan combined with doxorubicin on apoptosis of drug-resistant lung cancer cells (A549DoxR)

Fig. 16 showed the apoptosis of A549 cells and A549DoxR cells. Our results showed that DNA fragments significantly increased in A549 cells treated with doxorubicin 8μM compared with the control group. DNA fragments of A549DoxR cells treated with doxorubicin 8μM combined with fucoidan 25μg/mL increased, compared to doxorubicin 8μM. The results showed that fucoidan synergistically increased doxorubicin-induced apoptosis (fig. 6-3).

Effects of different concentrations of fucoidan combined with doxorubicin on autophagy genes of drug-resistant lung cancer cells (A549DoxR)

RT-PCR analysis was used to analyze the effects of fucoidan combined with doxorubicin on the key genes LC3 and Beclin-1 in the autophagy pathway. A549DoxR cells were treated with doxorubicin 8μM or doxorubicin 8μM combined with fucoidan 25, 100μg/ml, it was observed that compared to doxorubicin 8μM, the expression level of LC3 and Beclin-1 genes of A549DoxR cells treated with doxorubicin 8μM combined with fucoidan decreased as the dose of fucoidan increased (fig. 7). The results showed that fucoidan cooperated with doxorubicin to inhibit the occurrence of autophagy.

Effects of different concentrations of fucoidan combined with doxorubicin on the proliferation genes and protein of drug-resistant lung cancer cells (A549DoxR)

The effect of fucoidan combined with doxorubicin on cell proliferation gene β-catenin was analyzed by RT-PCR. A549DoxR cells were treated with doxorubicin 8μM or doxorubicin 8μM combined with fucoidan 25 and 100 μg/ml. The expression level of β-catenin gene significantly decreased in A549DoxR cells treated with doxorubicin 8μM combined with fucoidan 25 and 100 μg/ml, compared with doxorubicin 8μM. The expression level of β-catenin significantly decreased with the increase of fucoidan dose (fig. 8A).

Western blot analysis was used to analyze the effect of fucoidan combined with doxorubicin on cell proliferation protein β-catenin.

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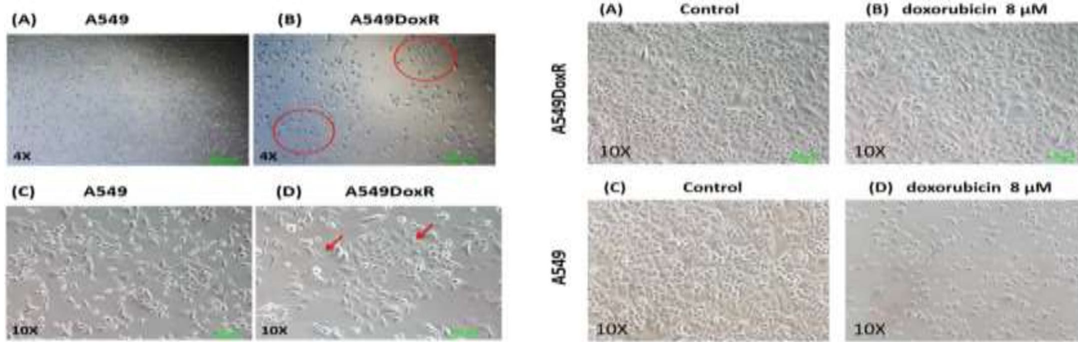

Fig. 1: 1-1: Observation of cell morphological differences between lung cancer cells (A549) and drug-resistant lung cancer cells (A549DoxR) by microscope. (A) (C) is A549 cells, (B) (D) is A549DoxR cells. The red circles are the aggregated A549DoxR cells (B) and the red arrows point to the morphologically altered and elongated A549DoxR cells (D). 1-2 Microscopic observation of the cell survival effects of drug-resistant lung cancer cells (A549DoxR) and lung cancer cells (A549) treated with Doxorubicin 8μM. (A) (B) is A549DoxR cells, (C) (D) is A549 cells. Comparison of cell viability between control (A) (C) and experimental (doxorubicin8μM) groups (B) (D) of treatment with doxorubicin 8μM by microscopy.

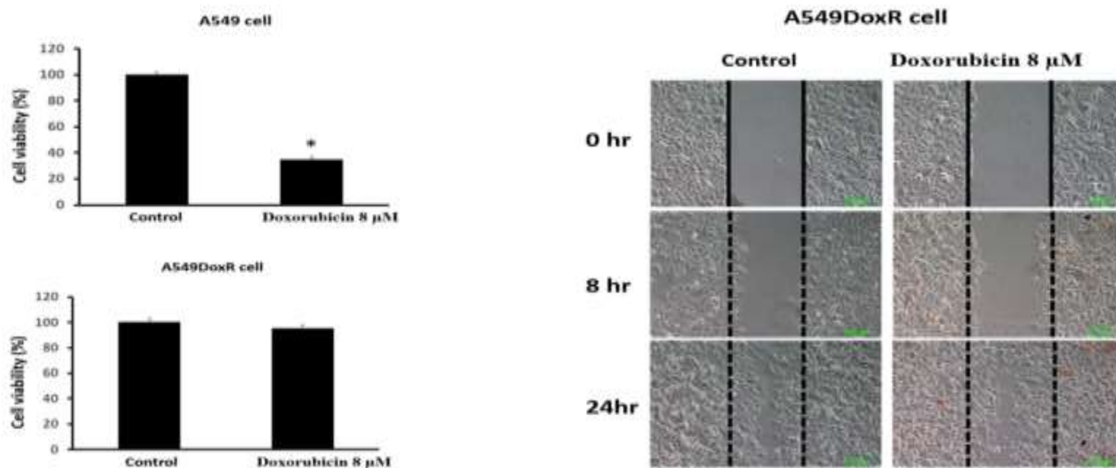

Fig. 2: 2.1: The effects of lung cancer cells (A549) and drug-resistant lung cancer cells (A549DoxR) treated with Doxorubicin 8μM were analyzed by MTT assay. After treatment of A549 cells and A549DoxR cells with Doxorubicin 8μM, the difference of cell viability was measured by MTT assay. * P<0.01 vs control. Values expressed as mean±SEM of three independent experiments. 2-2: Analysis of the effect of cell migration in drug-resistant lung cancer cells (A549DoxR) treated with doxorubicin 8μM using the wound healing assay. The difference in A549DoxR cells migration ability between the control group and the experimental group (doxorubicin 8μM) was compared at 0, 8 and 24 hours by wound healing assay.

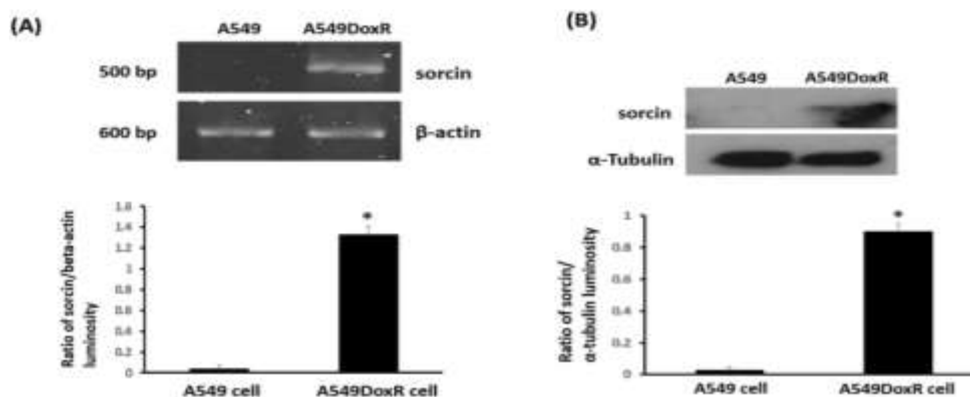

Fig. 3: (A) Analysis of sorcin mRNA expression in lung cancer cells (A549) and drug-resistant lung cancer cells (A549DoxR) by RT-PCR. (B) Western blot analysis was used to analyze the expression of sorcin protein in lung cancer cells (A549) and drug-resistant lung cancer cells (A549DoxR). *P<0.01 vs A549 cells. Values expressed as mean ±SEM of three independent experiments.

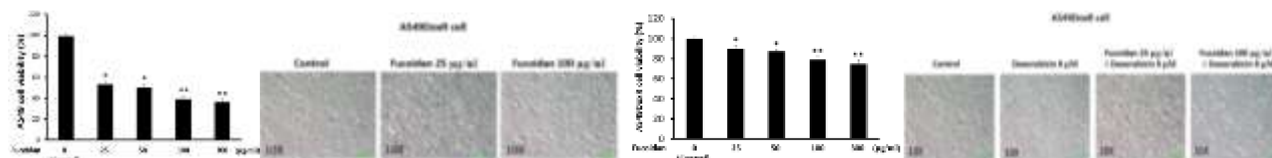

Fig. 4: 4-1: MTT assay is used to analyze the effect of cell viability of lung cancer cells (A549) treated with fucoidan 25, 50, 100 and 300μg/ml. *P<0.01 vs control. **P<0.01 vs control, fucoidan 25 and 50μg/ml. Values expressed as mean ±SEM of three independent experiments. 4-2: Observation of the cell survival effect of drug-resistant lung cancer cells (A549DoxR) treated with fucoidan 25 and 100μg/ml by microscope. 4-3: MTT assay was used to analyze the effect of cell viability of drug-resistant lung cancer cells (A549DoxR) treated with fucoidan 25, 50, 100 and 300μg/ml. * P<0.01 vs control. ** P<0.01 vs control, fucoidan 25 and 50μg/ml. Values expressed as mean ±SEM of three independent experiments. 4-4: Microscopic observation of the cell survival effect of drug-resistant lung cancer cells (A549DoxR) treated with doxorubicin 8μM, doxorubicin 8μM combined with fucoidan 25 and 100μg/ml.

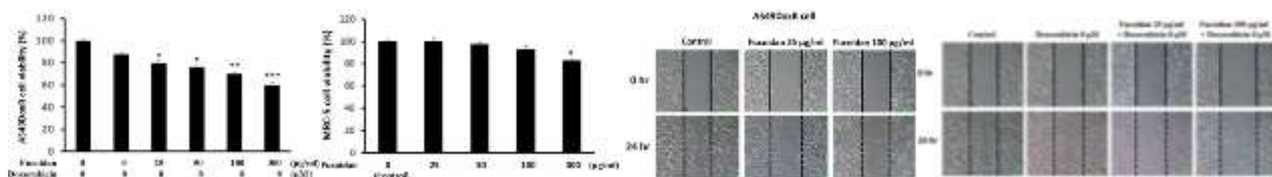

Fig. 5: 5-1: The effect of cell viability of drug-resistant lung cancer cells (A549DoxR) treated with Doxorubicin 8μM, Doxorubicin 8μM combined with fucoidan 25, 50, 100 and 300μg/ml was analyzed by MTT assay. * P<0.01 vs control, Doxorubicin 8μM. ** P<0.01 vs control, Doxorubicin 8μM, doxorubicin 8μM combined with fucoidan 25 and 50μg/ml. *** P<0.01 vs control, Doxorubicin 8μM, doxorubicin 8μM combined with fucoidan 25, 50 and 100μg/ml. Values expressed as mean± SEM of three independent experiments. 5-2: The effect of human normal fetal lung fibroblast (MRC-5) treated with fucoidan 25, 50, 100 and 300μg/ml on the cell viability was analyzed by MTT assay. * P<0.01 vs control, fucoidan 25, 50 and 100μg/ml. Values expressed as mean ±SEM of three independent experiments. 5-3: Analysis of the effect of cell migration ability of drug-resistant lung cancer cells (A549DoxR) treated with fucoidan 25 and 100μg/ml for 0 and 24 hours using wound healing assay. 5-4: Analysis of the effect of drug-resistant lung cancer cells (A549DoxR) on the migration ability of cells treated with doxorubicin 8μM, doxorubicin 8μM combined with fucoidan 25 and 100μg/mL for 0 and 24 hours using the wound healing assay.

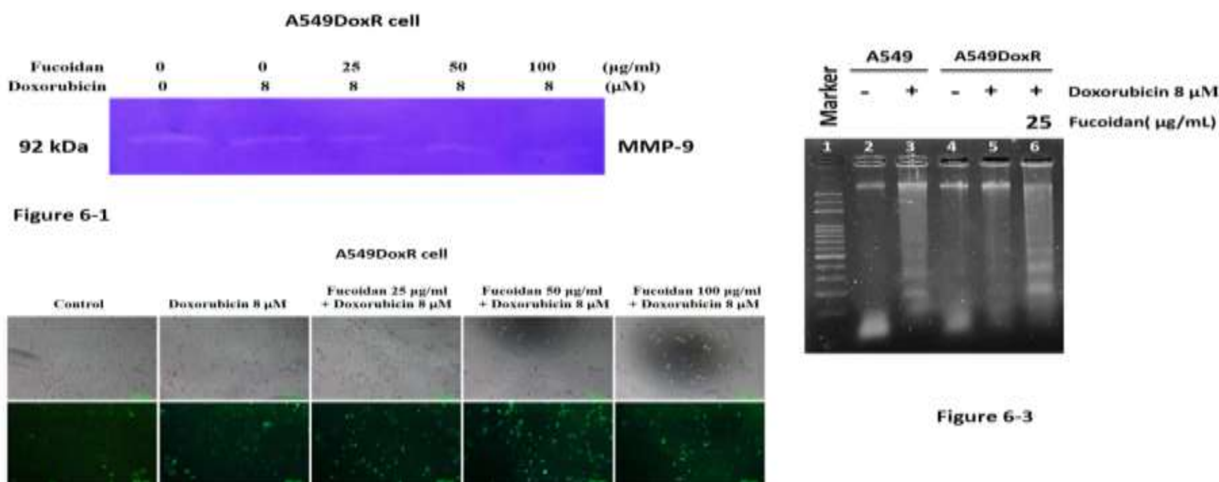

Fig. 6: 6-1: Gelatin zymography assay was used to analyze the effect of MMP-9 expression on drug-resistant lung cancer cells (A549DoxR) treated with doxorubicin 8μM, doxorubicin 8μM combined with fucoidan 25, 50 and 100μg/ml. 6-2: Apoptosis assay was used to analyze the apoptosis effect of drug-resistant lung cancer cells (A549DoxR) treated with doxorubicin 8μM, doxorubicin 8μM combined with fucoidan 25, 50 and 100μg/ml. 6-3: DNA fragmentation assay was used to analyze the apoptosis effect of lung cancer cells (A549) treated with doxorubicin 8μM and drug-resistant lung cancer cells (A549DoxR) treated with doxorubicin 8μM, doxorubicin 8μM combined with fucoidan 25μg/ml. Lane 1 is a 100bp DNA ladder marker, lanes 2 (control) is A549 cells, lanes 3 is A549 cells treated with doxorubicin 8μM, lanes 4 (control) is A549DoxR cells, lane 5 is A549DoxR cells treated with doxorubicin 8μM, lane 6 is A549DoxR cells treated with doxorubicin 8μM combined with fucoidan 25μg/ml.

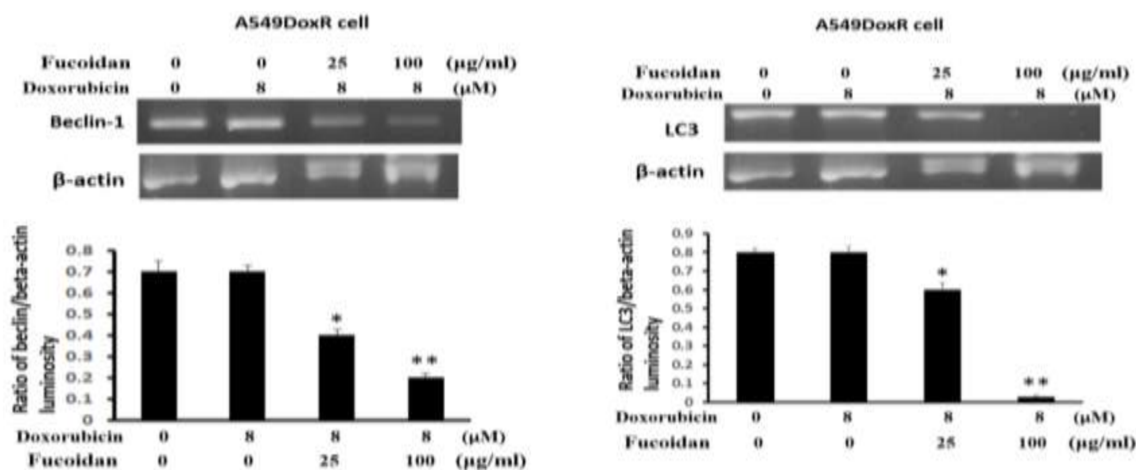

Fig. 7: The expression of beclin-1 and LC3 genes in drug-resistant lung cancer cells (A549DoxR) treated with doxorubicin 8μM, doxorubicin 8μM combined with fucoidan 25 and 100 μg/ml by RT-PCR analysis. * P<0.01 vs control and Doxorubicin 8μM. **P<0.01 vs control, Doxorubicin 8μM, doxorubicin 8μM combined with fucoidan 25μg/ml. Values expressed as mean ±SEM of three independent experiments.

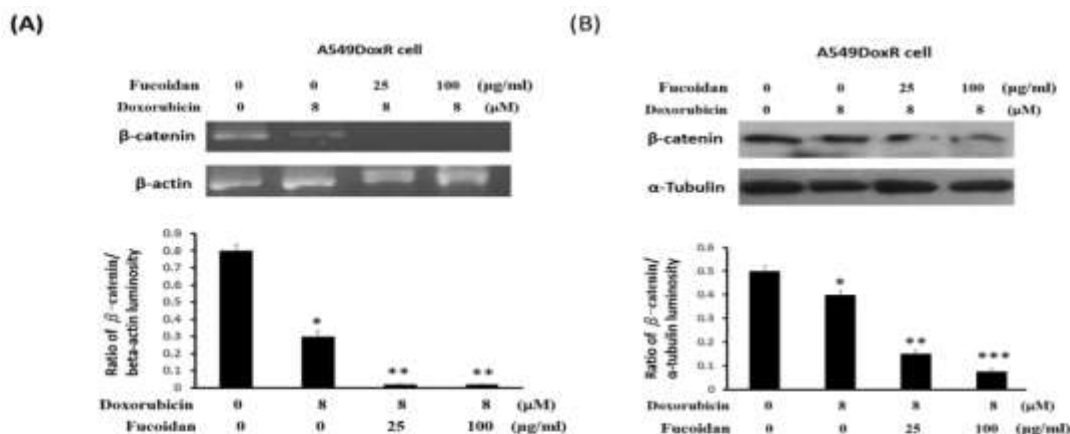

Fig. 8: (A) The effect of β-catenin gene expression in drug-resistant lung cancer cells (A549DoxR) treated with doxorubicin 8μM, doxorubicin 8μM combined with fucoidan 25 and 100μg/ml by RT-PCR analysis. (B) The effect of β-catenin protein expression in drug-resistant lung cancer cells (A549DoxR) treated with doxorubicin 8μM, 8μM doxorubicin combined with fucoidan 25 and 100μg/ml by western blot analysis. *P<0.01 vs control. **P<0.01 vs control, doxorubicin 8μM. *** P<0.01 vs control, doxorubicin 8μM, doxorubicin 8μM combined with fucoidan 25μg/ml. Values expressed as mean± SEM of three independent experiments.

A549DoxR cells were treated with doxorubicin 8μM or doxorubicin 8μM combined with fucoidan 25 and 100μg/ml. The expression of β-catenin protein significantly decreased in A549DoxR cells treated with doxorubicin 8μM combined with fucoidan 25μg/ml and 100μg/ml, compared to doxorubicin 8μM, the protein expression significantly decreased with the increase of fucoidan dose (fig. 8B). The results confirmed that fucoidan cooperates with doxorubicin to inhibit the proliferation of A549DoxR cells by reducing the expression of β-catenin protein.

DISCUSSION

Although there have been many articles on the effect of fucoidan on cancer, our work is the first study that mainly focuses on the effect of fucoidan combined with chemotherapy drugs on drug-resistant lung cancer cells as the subject of discussion.

Chen *et al*. (2021) indicated that fucoidan inhibited the proliferation and angiogenesis of NSCLC cells through the mTOR pathway and promote their apoptosis by increasing the Bax/Bcl-2 ratio. The study suggested that the Oversulfation of fucoidan is the main reason for enhancing the activity of lung cancer and the study also shows that its underlying mechanism may be related to the Akt/mTOR/S6 pathway (Hsiao *et al*., 2021). The study points out that fucoidan is a potential preventive and therapeutic agent for lung cancer, which inhibiting cancer cell by activating the TLR4/ROS/ER and PERK-ATF4-CHOP pathway, resulting in the progression of lung cancer

cell apoptosis (Hsu *et al*., 2017). Hsu *et al*. (2018) demonstrated that the fucoidan-induced TLR4-mediated endoplasmic reticulum stress molecule CHOP advanced caspase-3 activation. In human lung cancer cells A549, apoptosis was induced by downregulating p38, PI3K/Akt and activating the ERK1/2 MAPK pathway (Zhao *et al*. 2015).

The results of this study confirm that fucoidan can inhibit the cell viability of lung cancer cell A549 and has a significant inhibitory effect at a low dose of 25μg/mL (fig. 4-1). At present, the related research of fucoidan on drug-resistant cancer cells is not very clear, our study investigated the effect of fucoidan on drug-resistant lung cancer cells A549/Doxo. It has been pointed out that a new target named soluble resistance-related calcium-binding protein (Sorcin) has been discovered, which is involved in the development of multi-drug resistance (MDR) in cancer (Sun *et al*. 2019). Sorcin can directly interact with adriamycin (doxorubicin) binds with high affinity and the expression of sorcin confers the development of tumor resistance to various chemotherapeutic drugs (Genovese *et al*. 2017). Our results of this study showed that drug-resistant lung cancer cell line (A549/Doxo) had the expression of sorcin mRNA by RT-PCR (fig. 3A) and the expression of sorcin protein by Western blot analysis (fig. 3B). Fucoidan can also inhibit the cell survival rate of A549/Doxo, with a significant inhibitory effect at a dose of 50μg/mL (fig. 4-3) and synergizes with doxorubicin to inhibit the cell survival of A549/Doxo. The combination of 8μM doxorubicin and 100μg/mL fucoidan had a significant inhibitory effect and with the increase of the dose of fucoidan, the A549/Doxo cell survival rate also decreased (fig. 5-1).

CONCLUSIONS

In our study, the results of the wound healing assay first found that with the increase of the dose of fucoidan, the migration ability of A549/Doxo was inhibited (fig. 5-3) and the combination of doxorubicin and fucoidan also showed a synergistic effect (fig. 5-4). Zhang *et al*. (2021) suggested that the expressions of MMP-9 played important roles in lung cancer and were closely related to clinicopathology and provided help for the diagnosis. Our results showed that fucoidan can synergize with doxorubicin to inhibit the expression of MMP-9 in A549/Doxo (fig. 6-1). The results of apoptosis assay and DNA fragmentation assay found that fucoidan enhancing doxorubicin increase the occurrence of apoptosis (fig. 6-2 and fig. 6-3). Autophagy can protect drug-resistant cancer cells from chemotherapeutic drug-induced death (Li *et al*. 2017). In this study, RT-PCR results showed that fucoidan combined with doxorubicin reduced the expression of autophagy marker molecules LC3 and Beclin-1 mRNA (fig. 7). The literature has confirmed that fucoidan can inhibit the growth of breast cancer cells by down-regulating β-catenin (Xue *et al*. 2017). In this study, it was observed that fucoidan and doxorubicin inhibited the expression of β-catenin through RT-PCR and Western blot analysis (fig. 8A and fig. 8B). Based on the above experimental results, we believe that fucoidan can achieve the effect of adjuvant chemotherapy drugs to inhibit drug-resistant lung cancer cells. We hope that in the future clinical cancer treatment, fucoidan can synergize with chemotherapeutic drugs to enhances the sensitivity of chemotherapy drugs for drug-resistant lung cancer cells and reduces the side effects caused by chemotherapy drugs.

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