Original Article

Docosahexaenoic Acid Reverses Epithelial-Mesenchymal Transition and Drug Resistance by Impairing the PI3K/AKT/Nrf2/GPX4 Signalling Pathway in Docetaxel-Resistant PC3 Prostate Cancer Cells

(docosahexaenoic acid / drug resistance / ferroptosis / GPX4 / autophagy / prostate)

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Abstract. Drug resistance is a serious problem in cancer therapy. Growing evidence has shown that docosahexaenoic acid has anti-inflammatory and chemopreventive abilities. Studies have shown that autophagy inhibition and ferroptosis are promising therapeutic strategies for overcoming multidrug resistance. This study was aimed to examine whether docosahexaenoic acid (DHA) could reverse docetaxel resistance in prostate cancer cells. Cell survival was examined by MTT and colony formation. Protein expression was determined by Western blot. Reactive oxygen species (ROS) production was measured by flow cytometry. DHA displayed anti-cancer effects on proliferation, colony formation, migration, apoptosis, autophagy and epithelial mesenchymal transition. Glutathione-S-transferase π is an enzyme that plays an important role in drug resistance. DHA inhibited GSTπ protein expression and induced cytoprotective autophagy by regulating the PI3K/AKT signalling pathway in PC3R cells. DHA combined with PI3K inhibitor (LY294002) enhanced apoptosis by alleviating the expression of LC3B, (pro-) caspase-3 and (uncleaved) PARP. DHA induced ferroptosis by attenuating the expression of glutathione peroxidase 4 (GPX4) and nuclear erythroid 2-related factor 2 (Nrf2). DHA-treated PC3R cells produced ROS. The ROS and cytotoxicity were reversed by treatment with ferrostatin-1. DHA combined with docetaxel inhibited EMT by regulating the expression of E-cadhein and N-cadherin. In summary, DHA reversed drug resistance and induced cytoprotective autophagy and ferroptosis by regulating the PI3K/AKT/Nrf2/GPX4 signalling pathway in PC3R cells. We propose that DHA could be developed as a chemosensitizer and that the PI3K/AKT /Nrf2/GPX4 signalling pathway might be a promising therapeutic target for overcoming cancer drug resistance.

Received November 8, 2021. Accepted April 22, 2022.

The present study was supported by "Yichun University Local Development Research Center" (Grant No. DF2019002), "PhD research foundation of Yichun University" (Grant No. 211-3360118006), and "Undergraduate Innovation and Entrepreneurship Training Program" (Grant No. 201910417007).

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Abbreviations: ARE – antioxidant responsive element, COX-2 – cyclooxygenase 2, CRPC – castration-resistant prostate cancer, CSC – cancer stem cells, DHA – docosahexaenoic acid, EMT – epithelial-mesenchymal transition, EPA – eicosapentaenoic acid, GPX4 – glutathione peroxidase 4, GPXs – glutathione peroxidases, GSH – glutathione, GSTπ – glutathione-S-transferase π, MRP1 – multidrug resistance associated protein 1, NQO1 – NADH quinone oxidoreductase 1, Nrf2 – nuclear erythroid 2-related factor 2, P-gp – P-glycoprotein, PUFA – ω-3 polyunsaturated fatty acids, ROS – reactive oxygen species.

Introduction

The low intake of ω-3 and high intake of ω-6 essential fatty acids increase the incidence of cancer (Simopoulos, 2002). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are ω-3 polyunsaturated fatty acids (PUFA). EPA and DHA are found and rich in fish oil. The chemical structure of DHA is 22 carbons and six double bonds. Previous studies have shown that intake of supplementary ω-3 fatty acids can reduce risk of cancer and prevent chronic diseases such as cardiovascular disease (Jain et al., 2015; Shahidi and Ambigaipalan,

2018). DHA has anticancer effects in cancer cells with many pharmacological activities (Jing et al., 2013). In *in vitro* studies, DHA caused cancer cell death by inducing reactive oxygen species (ROS) production, inhibiting cyclooxygenase 2 (COX-2) expression and inflammation (Poorani et al., 2016; Du et al., 2019). Studies have also shown that DHA enhances activity of chemotherapeutic agents (Shekari et al., 2020). A clinical study has demonstrated that DHA (1.8 g/day) combined with anthracycline-based chemotherapy improves the survival rate (Bougnoux et al., 2009). DHA has benefits and potential as a chemosensitizer with low side effects.

Prostate cancer is a common solid malignancy in western countries (Wang et al., 2018a). Hormone deprivation therapy is effective in the treatment of prostate cancer; however, many patients progress to a castration-resistant prostate cancer (CRPC) status (Mansinho et al., 2018). Docetaxel is a semi-synthetic taxane drug that specifically acts on the cell cycle and is used as the standard agent for CRPC treatment. It stabilizes microtubules and results in apoptosis and cell cycle arrest in prostate cancer cells (Nehme et al., 2001). Nonetheless, docetaxel also causes serious side effects during the treatment. Moreover, hormone deprivation therapy often results in multidrug resistance in prostate cancer patients due to an increased cellular metabolism of the drugs that is often associated with over-expression of P-glycoprotein (P-gp) or glutathione-S-transferase, leading to prostate cancer cells' resistance to apoptosis and autophagy (van Brussel et al., 1999; Curtin and Cotter, 2004; Kranzbuhler et al., 2019).

Epithelial-mesenchymal transition (EMT) is a cellular programme that plays an important role in inflammation, tissue remodelling and tumour metastasis (Dongre and Weinberg, 2019). EMT is a biological process causing that the phenotype of epithelial cell is transformed to mesenchymal cell. Transcription factors including Snail, Twist and Slug can increase the mesenchymal cell markers (N-cadherin and vimentin) but decrease the epithelial cell markers (E-cadherin) in drug-resistant cancer cells (Dongre and Weinberg, 2019). Recently, studies have also demonstrated that EMT is also involved in chemoresistance in multiple cancer cells by regulating the PI3K/AKT signalling pathway (Deng et al., 2019; Liu et al., 2019; Park et al., 2019; Vijay et al., 2019).

Autophagy is an intracellular process of degradation of damaged components in a lysosome-dependent pathway. At present, autophagy is a new target for chemoresistant cancer therapy (Smith and Macleod, 2019). Autophagy and apoptosis are involved in cell death. Ferroptosis is also a form of cell death, but the mechanism of action is different from apoptosis and autophagic cell death. Ferroptosis involves intracellular iron accumulation and lipid peroxidation. ROS overproduction is related to apoptosis, ferroptosis and autophagy, playing a role in activating or inhibiting signal transduction (Gao et al., 2020; Li et al., 2020). Inhibition of glutathione peroxidase 4 (GPX4) results in ROS production and ferroptosis. Studies have shown that ferroptosis agonists can induce cancer cell death. Inhibition of nuclear erythroid 2-related factor 2 (Nrf2) and GPX4 can overcome the resistance in head and neck cancer (Shin et al., 2018). Cancer stem cells (CSC) play an important role in chemotherapy resistance. Recently, it was shown that ferroptosis inducers could target CSC and overcome the drug resistance (Elgendy et al., 2020). Therefore, ferroptosis may be considered as a new strategy for overcoming drug resistance.

Docetaxel-resistant cells showed multidrug resistance, which involves several mechanisms including anti-apoptosis, decreased drug uptake, activation of detoxifying systems, and decreased ROS production. The strategy of overcoming the multidrug resistance lies in interfering in these mechanisms. Our previous study has shown that DHA can inhibit lipopolysaccharide-induced metastatic activities in prostate cancer by suppressing IL-6, IL-8, p-AKT, and COX-2 expression (Wu et al., 2019). In the current study, the results confirmed that DHA had docetaxel resistance reversal effects, and regulated autophagy and ferroptosis in androgen receptor-negative and drug refractory prostate cancer cells. Based on our current research, DHA may be used as an adjuvant agent to enhance chemosensitivity and overcome the drug resistance in prostate cancer cells.

Material and Methods

Cell lines and reagents

DHA was purchased from Sigma-Aldrich (St. Louis, MO), docetaxel, LY294002, MK2203 from Beyotime Institute of Biotechnology (Haimen, China), ferrostatin-1 from Aladdin Company (Shanghai, China), and the human prostate cancer line (PC3) from Shanghai Fuheng Biotechnology Co., Ltd. (Shanghai, China). The cells were cultured in DMEM/Ham's F12K medium with 10 % foetal bovine serum (FBS) and 100 U/ml penicillin + 100 µg/ml streptomycin and maintained at 37 °C in a humidified atmosphere with 5 % CO_2. FBS and the medium were obtained from LONSERA, ShangHai ShuangRu Biotech Co., Ltd (Shanghai, China).

Drug resistance prostate cell line

The drug-resistant prostate cancer cell line, PC3R, was established using a stepwise increase in the concentration of docetaxel from 0.1 nM to 5 nM during PC3 cells' treatment. When the cells adapted to a concentration, the concentration was increased. The cells were maintained in 5 nM docetaxel to maintain the phenotype.

Cell viability assay

Cells were grown in 96-well plates (1000 cells/well) with 100 µl medium and 10 % FBS. When the cells became 80% confluent, they were incubated with media containing various concentrations of DHA (1–150 µM), docetaxel (0.1–4 µM), LY294002 (10 µM), MK2206

(0.1 μM), and ferrostatin-1 (5 μM). The cells were co-treated with DHA (150 μM) in the presence of LY294002 (10 μM), MK2206 (0.1 μM), or ferrostatin-1 (5 μM). After 24 h or 48 h treatment, the cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay (Beyotime Institute of Biotechnology). The absorbance was measured using a microplate reader (iMark™ Microplate Absorbance Reader). Three independent experiments were performed in triplicate.

Colony formation assay

The total number of 5×10^2 PC3R cells were seeded in 6-well plates for 4 h and then treated with different concentrations of DHA (25, 50 μM), docetaxel (0.1 μM), or combination of treatments. After incubation for seven days without changing the medium, colonies were formed. The colony is defined as a group > 50 cells. The cells were counted manually by an inverted microscope. Three independent experiments were performed.

Western blotting

An amount of 20 μg of total protein, which was obtained using M-PER® (Thermo Fisher Scientific, Waltham, MA), was boiled with sample buffer (100 mM Tris, pH 6.8, 20 % glycerol, 4 % SDS, and 0.2 % bromophenol blue). The proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked with Tris-buffered saline (pH 7.6) containing 0.1 % Tween 20 (TTBS) and 5 % non-fat milk at room temperature for 1 h, washed with TTBS, and then incubated at 4 °C with the following primary antibodies overnight: LC3B (Abways Technology, China), GST-π (1 : 1000; Abways Technology), β-actin (1 : 8000; Sigma-Aldrich, Burlington, MA), GPX4 (1 : 1000; Abways Technology), Nrf2 (1 : 1000; Abcam, Shanghai, China), E-cadherin (1 : 1000; Cell Signaling Technology. Beverly, MA), N-cadherin (1 : 1000; Cell Signaling Technology), caspase-3 (1 : 1000; Cell Signaling Technology), PARP (1 : 1000; Cell Signaling Technology). The membranes were washed in TTBS before being incubated with horseradish peroxidase (HRP)-linked antibody. The secondary antibody incubations were performed using anti-rabbit IgG HRP-linked antibody (1 : 1000; Cell Signaling Technology), and anti-mouse IgG HRP-linked antibody (1 : 1000; Cell Signaling Technology) at room temperature for 1 h. The blotted membrane was exposed to an enhanced chemiluminescent kit to reveal the protein expression pattern (Epizyme Biotech, China). The bands were detected by ChemiScope 3300 Mini (Clinx Science Instruments, Shanghai, China). The densitometry quantification of the blots was determined by ImageJ software. Three independent experiments were performed.

Intracellular ROS determination

The total number of 1×10^5 PC3R cells were treated with different concentrations of DHA or combination treatment of DHA with ferrostatin-1. After treatment for 24 h, the cells were detached and treated with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) in PBS for 30 minutes. After washing, cells were resuspended in PBS for ROS detection by flow cytometry (NovoCyte Flow Cytometer). Data were analysed by the NovoExpress software. Three independent experiments were performed in duplicate.

The concentration of cellular GSH

The intracellular GSH contents of PC3R cells were determined by using a Glutathione Assay kit (Nanjing Jiancheng Bioengineering Institute, China). PC3R cells were seeded in 24-well plates with DMEM/Ham's F12K medium and incubated overnight at 37 °C. After the treatment, the cells were collected and the concentration of cellular GSH was determined following the manufacturer's protocol. Three independent experiments were performed in triplicate.

Migration assay

The cell migration was measured by the wound healing assay. PC3R and PC3 cells were seeded in 6-well plates. When the cells reached 90% confluence, they were scratched by a 200 μl pipette tip. The cell debris was washed by PBS. The PC3R cells were treated with 0.1 % FBS containing 0.1 μM docetaxel, 25 μM DHA or 0.1 μM docetaxel + 25 μM DHA for 24 h and 48 h. Images were captured with a phase-contrast microscope. Migration was assessed by measuring the surface area that the cells occupied and the gap area. The area was calculated by the ImageJ software. Three independent experiments were performed in duplicate.

Statistical analysis

The data normality test was conducted with Shapiro-Wilk test. When the data normality test assumptions were fulfilled, the test could be continued with one-way analysis of variance (ANOVA) or *t*-test.

A two-tailed Student *t*-test was employed to analyse the differences between two groups, and Pearson's correlation analysis was used to analyse the correlation. One-way analysis of variance (ANOVA) was used for comparing the control group with more than one treatment group, and the Tukey's post hoc test was then performed. All data are presented as mean ± standard error of mean (SEM). A P-value of < 0.05 was regarded as statistically significant. Microsoft Excel 2016 MSO (16.0.4266.1001) software was used to carry out the statistical analysis.

Results

Development of the docetaxel-resistant PC3 cell line

We established a docetaxel-resistant cell line, PC3R, from the parental PC3 cell line using a stepwise exposure to increasing concentrations of docetaxel (from 0.1 nM up to 4 nM). PC3R has a higher IC_{50} compared

with parental PC3 cells (15.4 μM versus 0.245 μM, respectively) at 48 h. The IC_{50} values were calculated by the CompuSyn software. The cytotoxicity of docetaxel in PC3 and PC3R cells is shown in Fig. 1A. The resistance index (RI) was 62.85, and the results indicated that PC3R was resistant to docetaxel.

Docetaxel-resistance reversal effect of DHA on proliferation and colony formation

We examined the anticancer activities of DHA in PC3 and PC3R cell lines using different treatment concentrations of DHA (25–150 μM) for 24 h and 48 h. The cell survival rates of PC3 and PC3R following 48 h treatment with a concentration of 150 μM of DHA were 36 ± 11.89 % and 31 ± 7.129 % (% of control), respectively. High concentrations of DHA (100–150 μM) had cytotoxic effects on both cell lines (Fig. 1B). DHA groups (25 and 50 μM) showed 16 % and 31 % reduction in cell survival rate at 48 h in PC3R cells. Docetaxel at the concentration of 0.1 μM had little cytotoxicity in PC3R cells. The combination groups (25 or 50 μM DHA + 0.1 μM docetaxel) displayed more cytotoxicity in PC3R cells than that of the docetaxel group (Fig. 2A). Further, we determined the values of combination index (CI) by the CompuSyn software. The value of CI below 1 means that the combination drugs have synergistic effects. The values of CI of 25 μM DHA combined with 0.1 μM docetaxel were 0.42 and 0.44 at 24 h and 48 h, respectively, in PC3R cells. The values of CI of 50 μM DHA combined with 0.1 μM docetaxel were 0.63 and 0.55 at 24 h and 48 h, respectively, in PC3R cells. These results showed that DHA could enhance the sensitivity to docetaxel in PC3R cells. Moreover, the colony number in combination treatment was much lower than that in the docetaxel or DHA treatment alone group (Fig. 2B). Taken these results together, DHA had a reversal effect on docetaxel resistance of prostate cancer cells.

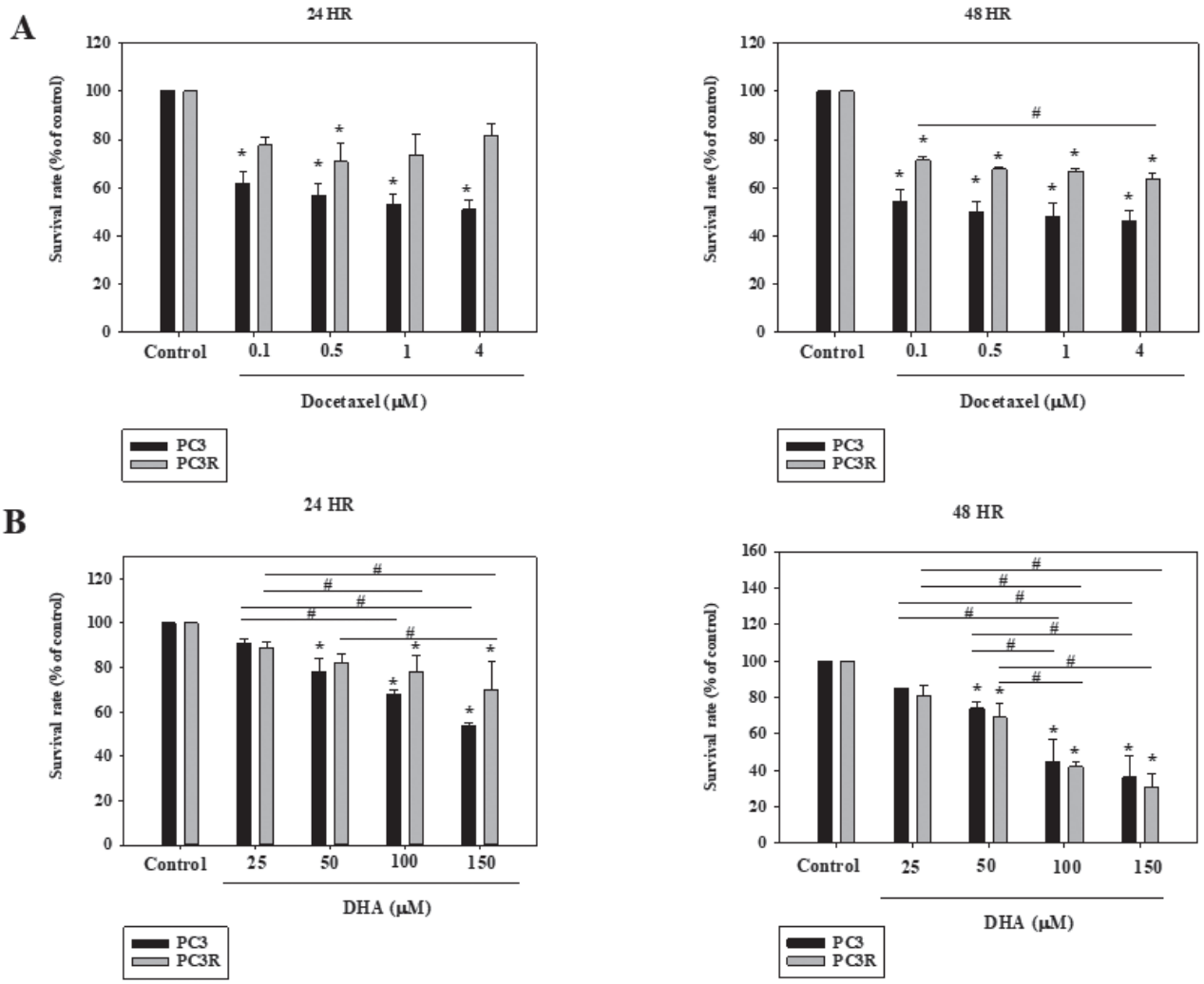

Fig. 1. Cytotoxicity of docetaxel (0.1–4 μM) and DHA (25–150 μM) in PC3 and PC3R cell lines for 24 h and 48 h (**A, B**). Cell viability was determined by the MTT assay. Data are represented as mean ± SEM in three separate experiments. P* < 0.05 compared with control. # indicates P < 0.05.

Ferroptosis and autophagy inhibition mediate cell death

Activation of the PI3K/AKT signalling pathway is associated with cell survival and drug resistance. To determine the DHA-induced cell death via the PI3K/AKT signalling pathway and ferroptosis, we used a PI3K inhibitor (LY294002, 10 μM), AKT inhibitor (MK2206, 0.1 μM) and ferroptosis inhibitor (ferrostatin-1, 5 μM) in this study. LY294002 is not only a PI3K inhibitor, but also an autophagy inhibitor that inhibits the early stage of autophagy (Ryabaya et al., 2017). The MTT assay indicated that inactivation of the PI3K/AKT signalling pathway increased the cytotoxicity of DHA in PC3R cells. Moreover, the DHA-induced cell death was blocked by ferrostatin-1. These results have shown that the DHA-induced cell death by ferroptosis and the cytotoxicity was slightly enhanced when the PI3K/AKT signalling pathway was blocked (Fig. 2C).

DHA decreased GSTπ expression, and triggered autophagy and apoptosis through the PI3K/AKT pathway in PC3R cells

Up-regulation of GSTπ expression is associated with drug resistance in cancer cells. We further examined GSTπ protein expression after treatment of PC3R cells with DHA. The results indicated that treatments with different concentrations of DHA (1–100 μM) inhibit GSTπ expression in a dose-dependent manner (Fig. 3A). To assess whether DHA induces autophagy in PC3R cells, we examined the protein expression level of autophagic marker LC3B protein. The results indicated that DHA (100 μM) markedly increased the expression level of the LCB3-II/LC3B-I ratio 24 h following treatment of PC3R cells (Fig. 3A). The PI3K/AKT signalling pathway is involved in many cellular processes, including proliferation, migration, and multidrug resistance. To determine whether the PI3K/AKT signalling

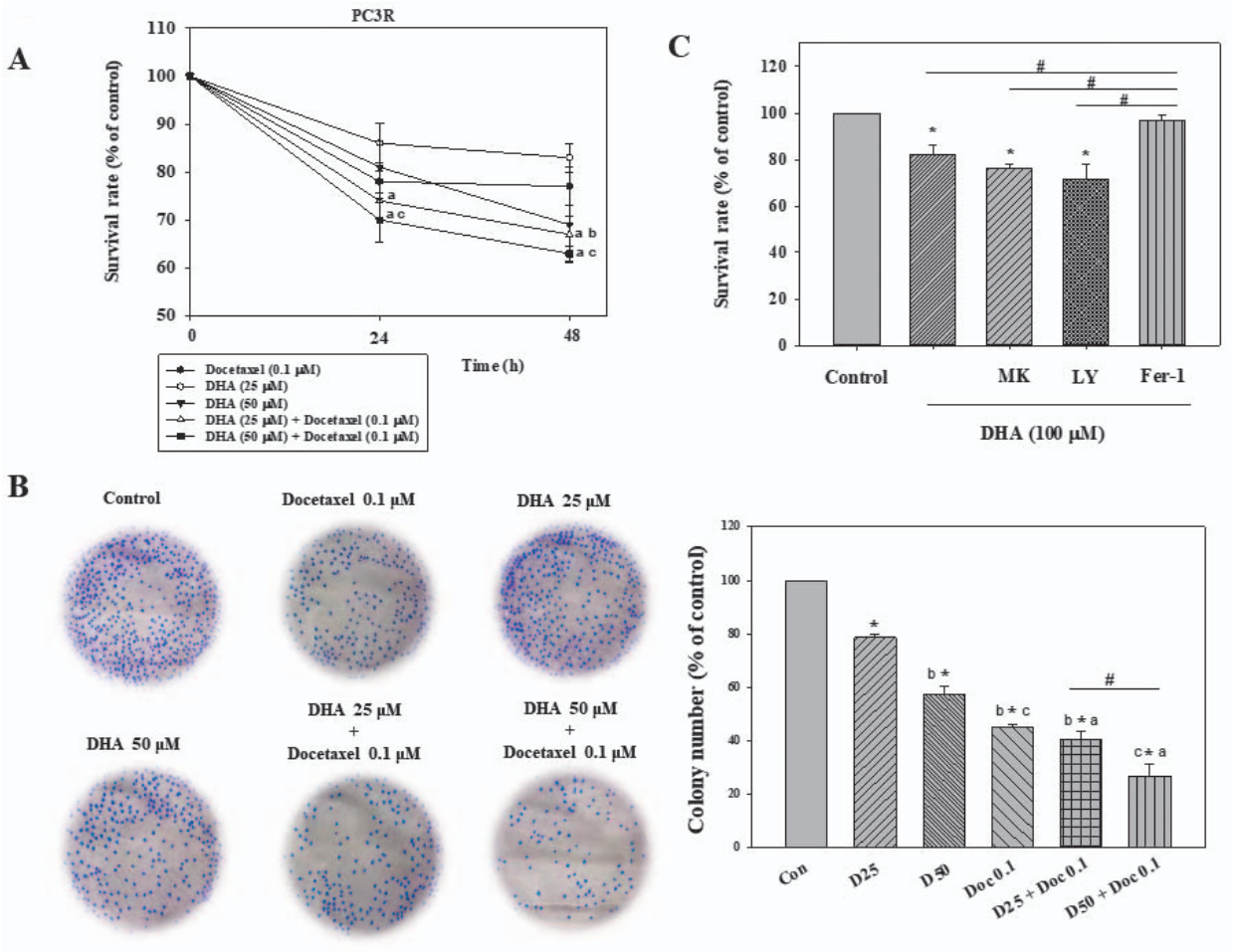

Fig. 2. Cytotoxicity and mechanism of action of DHA on colony formation and proliferation. (**A**) Cytotoxicity of DHA (25–50 μM), docetaxel (0.1 μM) and combination treatments in PC3R cells. (**B**) The indicated treatments were examined with respect to their effects on the colony formation. (**C**) Cytotoxicity of DHA (100 μM) in the presence or absence of LY294002 (10 μM, LY), MK2206 (0.1 μM, MK) and ferrostatin-1 (5 μM, Fer-1) for 24 h in PC3R cells. Cell viability was determined by the MTT assay. Data are represented as mean ± SEM in three separate experiments. P* < 0.05 compared with control. P^a < 0.05 compared with docetaxel (0.1 μM). P^b < 0.05 compared with DHA (25 μM). P^c < 0.05 compared with DHA (50 μM). # indicates P < 0.05.

pathway reverses drug resistance, we used a PI3K inhibitor (LY294002, 10 μM) or AKT inhibitor (MK2206, 0.1 μM). The results demonstrated that PI3K or AKT inhibitors decrease GSTπ and LC3B expression (Fig. 3B). We further found that the combination of DHA (100 μM) and LY294002 (10 μM) affected the GSTπ protein expression. Therefore, inactivation of the PI3K/AKT signalling pathway can overcome the drug resistance in prostate cancer.

Inhibition of autophagy induced apoptosis by DHA

PARP and caspase-3 regulate apoptosis. The protein expression of (pro-) caspase-3 and (uncleaved) PARP was higher in PC3R than that in PC3 cells (Fig. 4A). Further, we examined the relationship between apoptosis and autophagy by DHA treatment. The apoptotic effects of DHA in PC3R cells increased significantly when combined with autophagy inhibitor LY294002. As shown in Fig. 4B, the (uncleaved) PARP was reduced after co-treatment with DHA and LY294002. These results demonstrated that DHA induced protective autophagy in PC3R cells.

Ferroptosis of PC3R cells after DHA treatment

The protein expression levels of GPX4 and Nrf2 in PC3 and PC3R cells were investigated following treatment with DHA (1–100 μM). The GPX4 expression level was significantly up-regulated in PC3R cells compared with PC3 cells (Fig. 5A). However, the Nrf2 expression level was slightly increased in PC3R cells (Fig. 5A). The degree of ferroptosis in PC3R cells following 24 h treatment with DHA was evaluated by determining the protein expression levels of GPX4 and Nrf2. We observed that the expression levels of GPX4 and Nrf2 were decreased, while their expression levels increased after co-treatment with DHA and ferroptosis inhibitor,

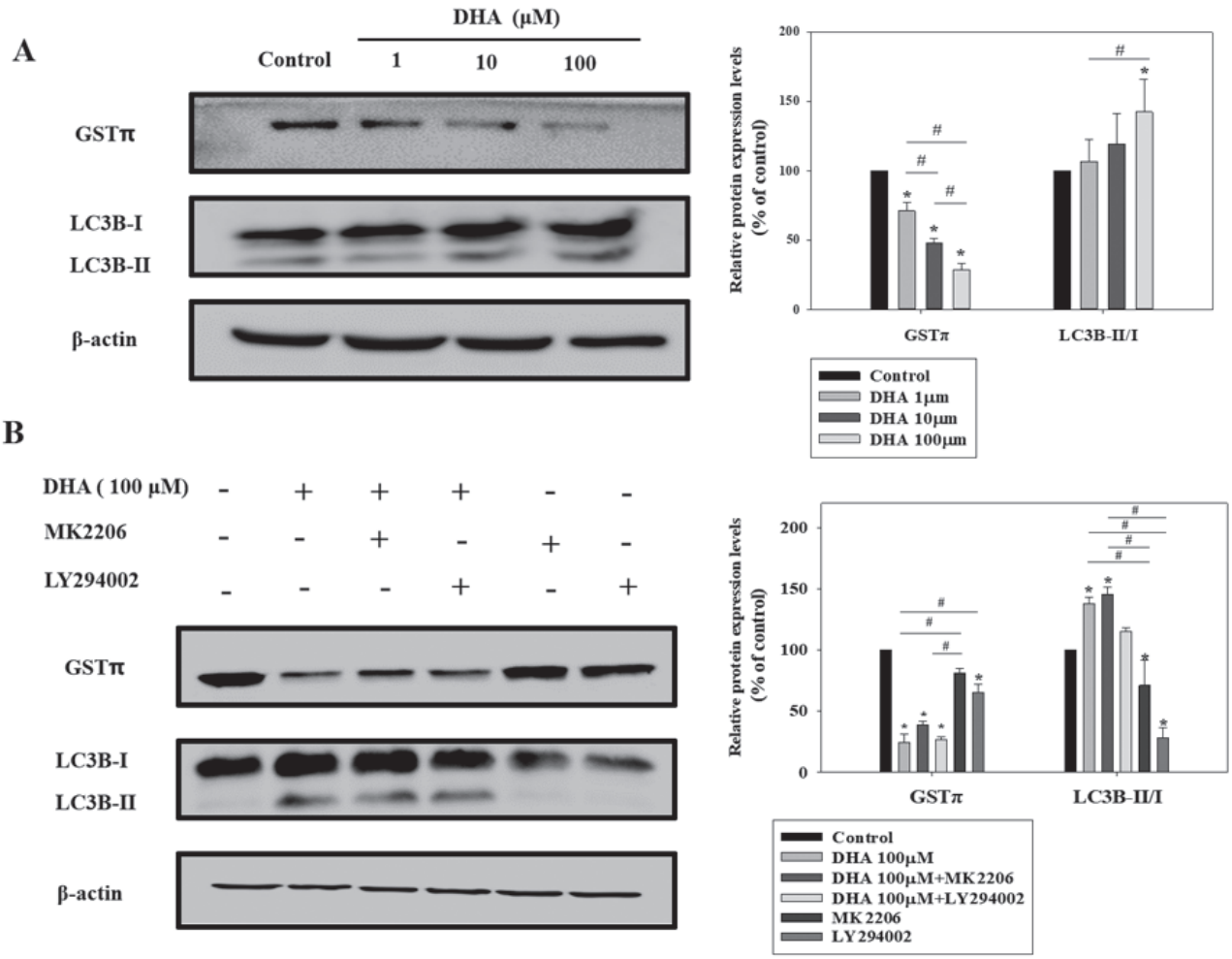

Fig. 3. Autophagic effects and GSTπ inhibition were detected after DHA, PI3K inhibitor (LY294002, LY), or AKT inhibitor (MK2206, MK) treatment in PC3R cells. Protein expression of GSTπ, LC3B-I and LC3B-II was detected by Western blot. (**A**) PC3R cells were treated with DHA (1–100 μM) for 24 h. (**B**) PC3R cells were treated with DHA (100 μM) in the presence or absence of LY294002 (10 μM) and MK2206 (0.1 μM) for 24 h. Data are represented as mean ± SEM in three separate experiments. P* < 0.05 compared with control. # indicates P < 0.05.

ferrostatin-1 (5 μM) (Fig. 5B). Ferroptosis is associated with accumulation of lipid peroxidation products, ROS production and depletion of cellular GSH. GSH and ROS levels were determined. We found that ROS production was increased under DHA (100, 150 μM) treatment. The ROS production was reduced by a combination of ferrostatin-1 (5 μM) with DHA treatment (150 μM) (Fig. 6A, 6B). Moreover, GHS was reduced after DHA (1–150 μM) treatments at 24 h. The GSH level of PC3R following 4 h treatment with a concentration of 150 μM was 60.37 ± 9.67 % (% of control) (Fig. 6C).

Docetaxel-resistant reversal effect of DHA on EMT

To evaluate the migration capacity, the wounding assay was performed. The results showed that DHA (50 μM), docetaxel (0.1 μM), and combination treatment had an anti-migration effect on PC3R cells for 48 h (Fig. 7A). EMT also plays an important role in multidrug resistance and tumour metastasis. The results have shown that the protein expression of E-cadherin was significantly down-regulated, but N-cadherin was up-regulated in PC3R cells (Fig. 7B). The combination treatment of DHA and docetaxel reversed the E-cadherin and N-cadherin protein expression (Fig. 7C). These results indicated that DHA possesses anti-metastatic activity.

Discussion

The anti-cancer effects of ω-3 PUFA have been observed in prostate cancer. DHA inhibits cancer cell growth by inducing apoptosis and autophagy through inhibition of the PI3K/AKT pathway (Song and Kim, 2016; Tsai et al., 2017; El-Ashmawy et al., 2020). Docetaxel is a chemotherapeutic agent that belongs to the taxane family, which functions as an inhibitor of beta-tubulin binding and as a promoter of cell cycle arrest at G2/M phase. Docetaxel is used for the treatment of hormone-refractory prostate cancer (HRPC) or androgen-independent prostate cancer (AIPC). However, docetaxel treatment may cause drug resistance in prostate cancer cells. Several studies have demonstrated that the treatment combination of DHA and docetaxel results in synergistic effects on several cancer cells, including gastric, prostate, and breast cancers (Shaikh et al., 2008; Newell et al., 2019; Shekari et al., 2020). In the present study, we reported an inhibitory effect of DHA on cancer

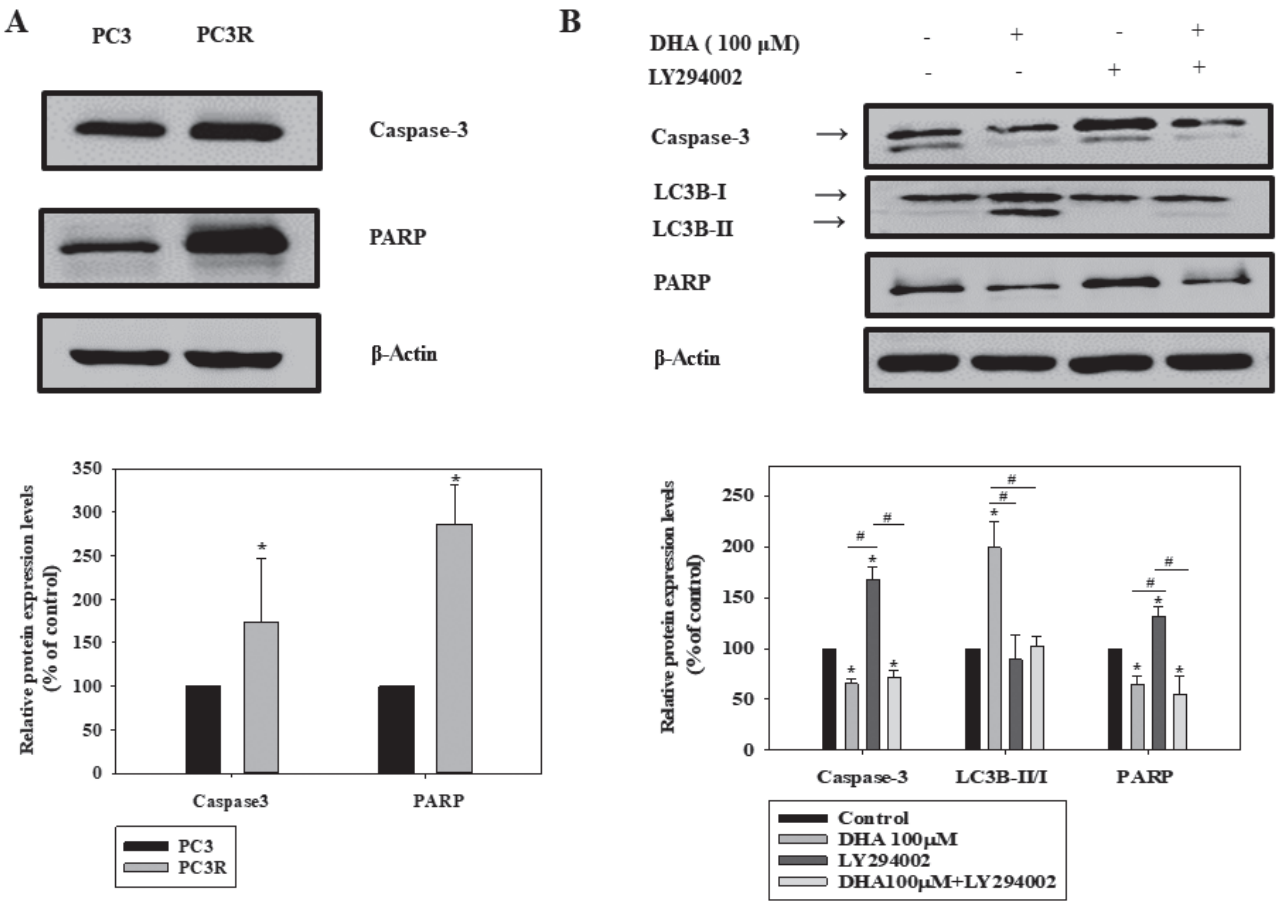

Fig. 4. Protein expression of caspase-3 and PARP in PC3 and PC3R cells (**A**). Protein expression of caspase-3 and PARP in PC3R cells after DHA (100 μM) with or without LY294002 treatments for 24 h. Data are represented as mean ± SEM in three separate experiments. P* < 0.05 compared with control. # indicates P < 0.05.

cell growth in PC3 and docetaxel-resistant PC3R cells. The combination therapy is often applied in cancer treatment to achieve greater cytotoxicity, a decrease in drug resistance and low side effects.

Chemoresistance is a serious problem in cancer therapy, which is associated with several genes or proteins whose functions are involved in multidrug resistance, such as multidrug resistance associated protein 1 (MRP1) and GSTπ. GSTπ is a phase II detoxification enzyme in the liver metabolism, and a high level of GSTπ expression is associated with drug resistance. PC3 and DU145 (castration-resistant) prostate cancer cell lines have been shown to have higher GSTπ expression compared with the LNCaP prostate cancer cell line (Hokaiwado et al., 2008). In our previous study, docetaxel-resistant PC3 cells had higher GSTπ expression compared with parental PC3 cells (Liu et al., 2017). It is believed that down-regulation of GSTπ expression can overcome drug resistance. In the current study, we show that DHA inhibits GSTπ expression in PC3R cells, and we suggest that DHA may possess the ability to overcome the drug resistance. The PI3K/AKT signalling pathway is associated with cell proliferation and survival. The activation of this pathway contributes to drug resistance in prostate cancer cells (Kosaka et al., 2011). A study indicated that the PI3K/AKT pathway is up-regulated in TLR4-mediated docetaxel resistance. DHA can induce autophagy and apoptosis by inactivating the PI3K/AKT pathway in non-small cell lung cancer cells (Zhang et al., 2012; Kim et al., 2015). Moreover, our previous study reported that DHA inhibits p-AKT expression in LPS-treated PC3 cells (Wu et al., 2019). In this study, we determined whether the inactivation of PI3K/AKT expression inhibits GSTπ expression in PC3R cells and used a PI3K (LY294002) inhibitor and AKT (MK2206) inhibitor to examine their involvement in this resistance. We observed that DHA, LY294002, or combination treatment groups inhibited GSTπ expression by inactivating the PI3K/AKT signalling pathway in docetaxel-resistant prostate cancer cells.

Autophagy plays an important role in inducing cancer cell death or in maintaining cell survival. Studies report-

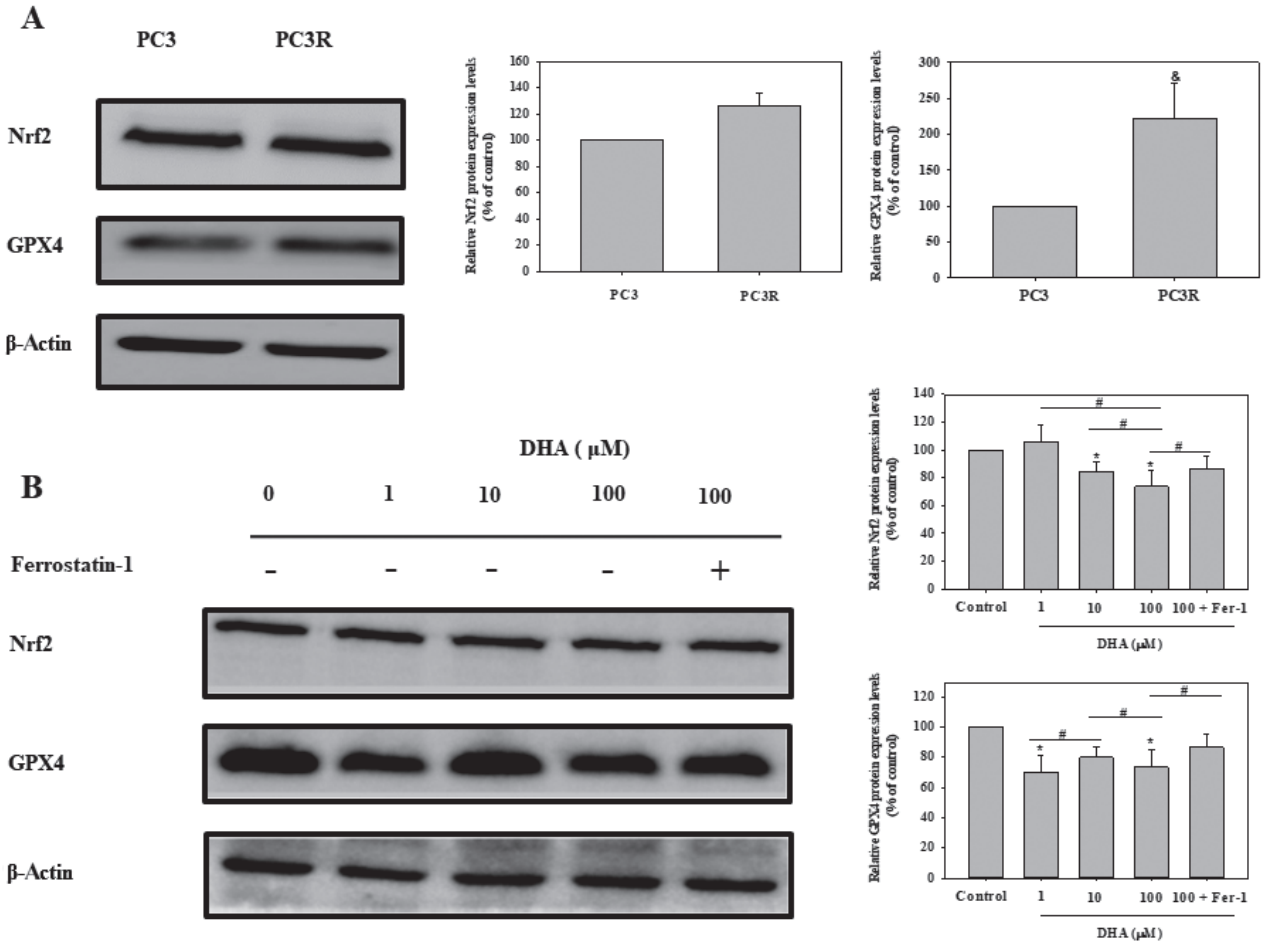

Fig. 5. Protein expression of Nrf2 and GPX4 in PC3 and PC3R cells. (**A**). The protein expression of Nrf2 and GPX4 was detected in PC3R cells after DHA treatment (1–100 μM) in the presence or absence of 5 μM of ferrostatin-1 (Fer-1) for 24 h (**B**). Data are represented as mean ± SEM in three separate experiments. $P^{*} < 0.05$ compared with control. $P^{\#} < 0.05$ compared with DHA (100 μM). $P^{\&} < 0.05$ compared with PC3.

ed that the inhibition of autophagy overcomes drug resistance and enhances cell death in response to chemotherapy and radiotherapy (Xu et al., 2012; Wright et al., 2013; Rosenfeld et al., 2014). DU145 and PC3 are castration-resistant prostate cancer cells. We established docetaxel-resistant PC3 cells for the study because autophagy is impaired in DU145 cells due to the loss of ATG5 protein expression (Ouyang et al., 2013). Therefore, DU145 cells are not suitable for examining the relationship between autophagy and apoptosis. Several studies have reported that autophagy is a self-protective mechanism resisting apoptosis (Esteve and Knecht, 2011; Kim et al., 2017). DHA induces autophagy by increasing the expression of LC3B-II. Autophagy inhibition and PI3K inhibition by LY294002 increased DHA-induced apoptosis in PC3R cells. The cell growth inhibition was observed in the co-treatment of DHA with LY294002 (PI3K inhibitor and autophagy inhibitor). Based on these results, the inhibition of autophagy causes cell death by DHA and is associated with inhibition of the PI3K/AKT signalling pathway. In the current study, DHA-induced autophagy played a protective role against apoptosis. Our work demonstrated that by inhibiting autophagy via adding LY294002 to the DHA treatment, GSTπ, LC3B-II, and (uncleaved) PARP were decreased, but the anti-tumour effect was increased. These results supported our assumption that inactivation of the PI3K/AKT signalling pathway and autophagy inhibition can overcome drug resistance in cancer cells.

EMT is a process in which cells lose the epithelial character and transit to invasive mesenchymal cells. EMT can be controlled by several transcription factors including Snail and Twist. Proinflammatory cytokines can promote tumour progression and metastasis in local tumour tissue. When EMT occurs in tumour cells, the expression level of E-cadherin is decreased, but the expression level of N-cadherin is increased. Recently, a link between EMT and drug resistance was reported (Du and Shim, 2016). EMT was observed in docetaxel-resistant cancer cells (Ashrafizadeh et al., 2021; Chen et al., 2021). EMT was also observed in the current study. DHA treatment up-regulated the expression of E-cadherin, but down-regulated the expression of N-cadherin. DHA reversed docetaxel resistance by regulating EMT. Based on these results, DHA might have anti-metastatic activity.

The mechanism of inducing cell death is very complex, and it is not controlled by one signalling pathway

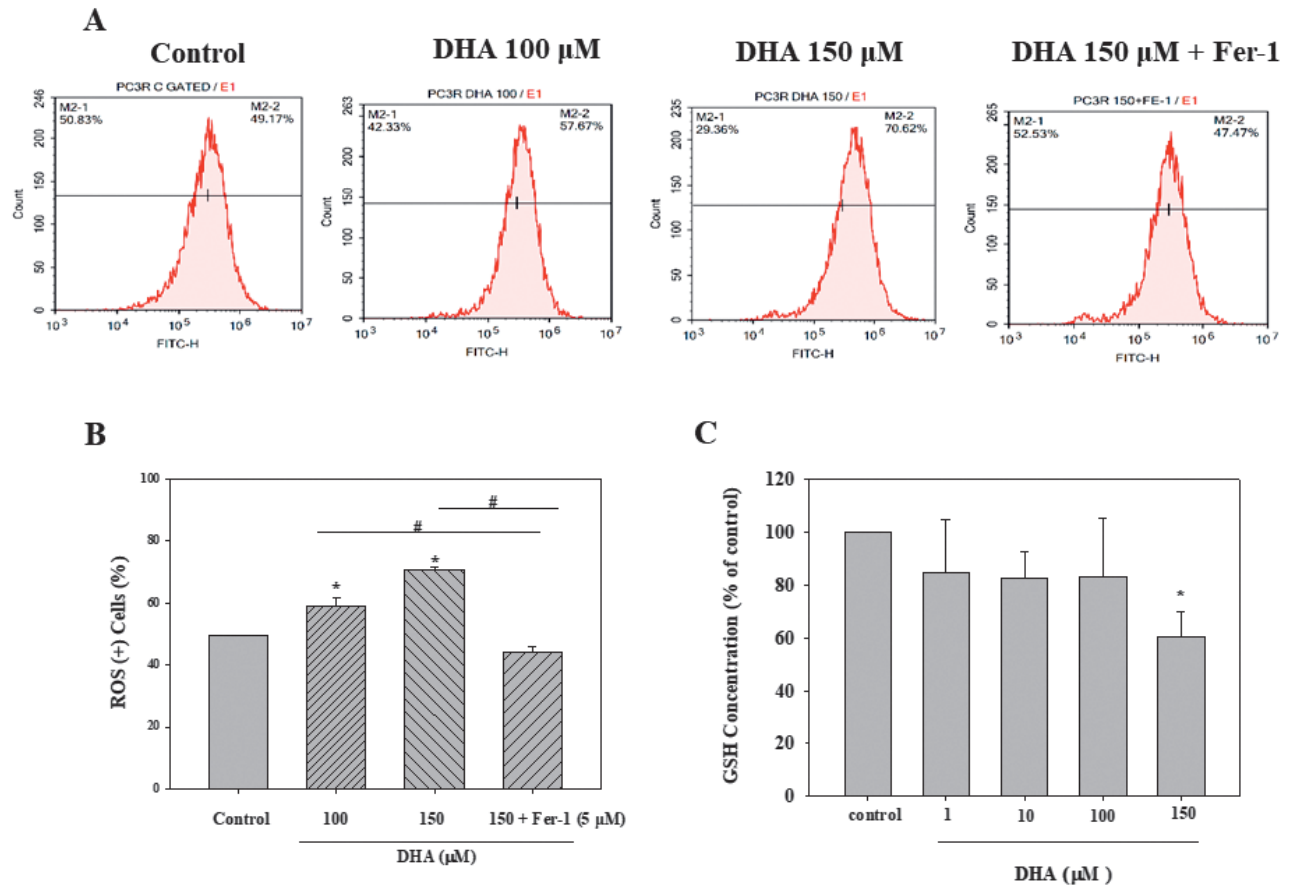

Fig. 6. ROS patterns and the concentration of GSH in DHA-treated PC3R cells for 24 h. Results are representative of three experiments. Data are represented as mean ± SEM in three separate experiments. P* < 0.05 compared with control. # indicates P < 0.05.

only. A high level of ROS causes DNA damage and oxidative stress in cancer cells. DHA treatment of Paca-44 pancreatic cancer cells resulted in oxidative stress that originated from depletion of intracellular glutathione (GSH) (Merendino et al., 2003). In the prostate cancer cell lines PC3 and DU145, oxidative stress and apoptosis were observed following DHA treatment (Shin et al., 2013). The DHA-induced apoptosis and cytotoxicity were blocked by the treatment with an antioxidant. These findings indicated that DHA causes oxidative stress and induces cancer cell death. Ferroptosis is considered as an autophagic cell death process, and glutathione peroxidases (GPXs) use GSH to reduce peroxides. Ferroptosis is triggered by blocking GSH synthesis or via inhibiting GPX4 activity. The Nrf2/ARE (antioxidant responsive element) signal transduction plays an important role in anti-oxidation as ARE regulates the expression of NQO1, GSTπ, and MRP1. Nrf2 regulates several anti-oxidation enzymes, and GPX4 is also regulated by Nrf2. A study has reported that drug resistance is associated with ferroptosis inhibition in cancer cells (Yamaguchi et al., 2013). The inhibition of Nrf2 and GPX4 can reverse resistance to ferroptosis in head and neck cancer (Shin et al., 2018). A study indicated that wogonoside reverses cisplatin resistance in human gastric cancer cells through the PI3K/Akt/Nrf2/ARE signalling pathway (Wang et al., 2018b). In this study, we demonstrated that DHA induces ferroptosis and overcomes drug resistance through down-regulating Nrf2/GPX4 signalling in prostate cancer cells. Moreover, the cell death was reduced in co-culture of DHA and ferrostatin-1. These data support the idea that DHA caused docetaxel-resistant prostate cancer cell death by inducing ferroptosis.

In conclusion, DHA can reverse drug resistance by down-regulating GSTπ expression and inducing cytoprotective autophagy through the PI3K/AKT/Nrf2/GPX4 signalling pathway in docetaxel-resistant PC3 cancer cells (Fig. 8). Above all, the chronic use of docetaxel can cause drug resistance by inhibiting ferroptosis. The PI3K/AKT/Nrf2/GPX4 signalling pathway plays an important role in drug resistance, and therefore, could be

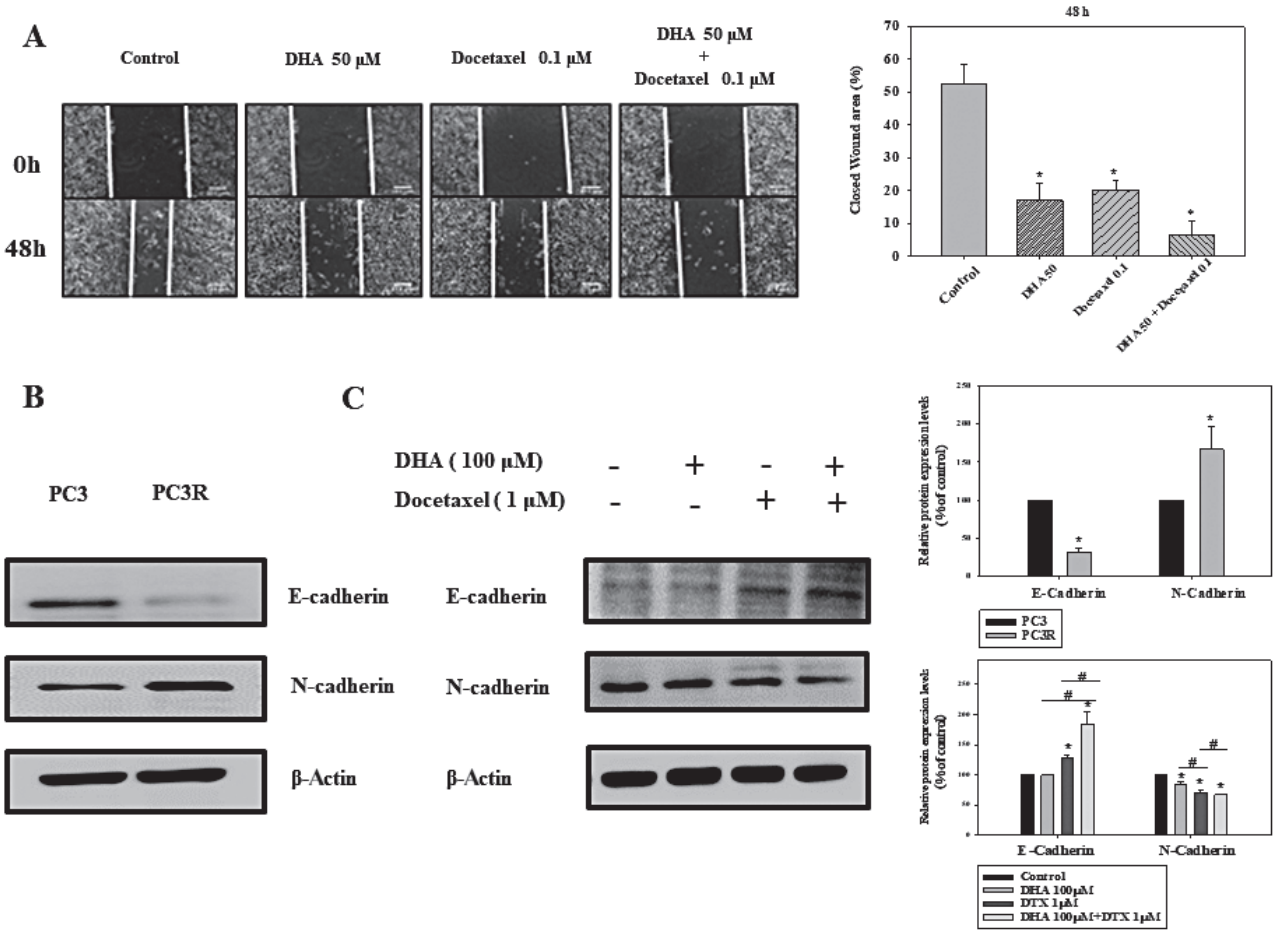

Fig. 7. Docetaxel resistance reversal effect of DHA on migration and EMT (**A**) DHA, docetaxel or combination treatments displayed anti-migration activity. (**B**) Protein expression of E-cadherin and N-cadherin in PC3 and PC3R cells. (**C**) E-cadherin and N-cadherin were examined in PC3R cells after DHA treatment with or without docetaxel treatment for 24 h by Western blot. Data are represented as mean ± SEM in three separate experiments. P* < 0.05 compared with control. # indicates P < 0.05.

considered a therapeutic target in overcoming prostate cancer drug resistance. This study also provides a molecular mechanism and a therapeutic target for DHA in inducing ferroptosis and apoptosis in drug-resistant prostate cancer cells.

Acknowledgements

The authors would like to express their gratitude to EditSprings (https://www.editsprings.com/) for the expert linguistic services provided.

Competing interests

The authors have no conflict of interest to declare.

References

Ashrafizadeh, M., Mirzaei, S., Hashemi, F., Zarrabi, A., Zabolian, A., Saleki, H., Sharifzadeh, S. O., Soleymani, L., Daneshi, S., Hushmandi, K., Khan, H., Kumar, A. P., Aref, A. R., Samarghandian, S. (2021) New insight towards development of paclitaxel and docetaxel resistance in cancer cells: EMT as a novel molecular mechanism and therapeutic possibilities. *Biomed. Pharmacother.* **141**, 111824.

Bougnoux, P., Hajjaji, N., Ferrasson, M. N., Giraudeau, B., Couet, C., Le Floch, O. (2009) Improving outcome of chemotherapy of metastatic breast cancer by docosahexaenoic acid: a phase II trial. *Br. J. Cancer* **101**, 1978-1985.

Chen, B., Zhang, Y., Li, C., Xu, P., Gao, Y., Xu, Y. (2021) CNTN-1 promotes docetaxel resistance and epithelial-to-mesenchymal transition via the PI3K/Akt signaling pathway in prostate cancer. *Arch. Med. Sci.* **17**, 152-165.

Curtin, J. F., Cotter, T. G. (2004) JNK regulates HIPK3 expression and promotes resistance to Fas-mediated apoptosis in DU 145 prostate carcinoma cells. *J. Biol. Chem.* **279**, 17090-17100.

Deng, J., Bai, X., Feng, X., Ni, J., Beretov, J., Graham, P., Li, Y. (2019) Inhibition of PI3K/Akt/mTOR signaling pathway alleviates ovarian cancer chemoresistance through reversing epithelial-mesenchymal transition and decreasing cancer stem cell marker expression. *BMC Cancer* **19**, 618.

Dongre, A., Weinberg, R. A. (2019) New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer. *Nat. Rev. Mol. Cell. Biol.* **20**, 69-84.

Du, B., Shim, J. S. (2016) Targeting epithelial-mesenchymal transition (EMT) to overcome drug resistance in cancer. *Molecules* **21**, 965.

Du, J., Wang, T., Li, Y., Zhou, Y., Wang, X., Yu, X., Ren, X., An, Y., Wu, Y., Sun, W., Fan, W., Zhu, Q., Wang, Y., Tong, X. (2019) DHA inhibits proliferation and induces ferroptosis of leukemia cells through autophagy dependent degradation of ferritin. *Free Radic. Biol. Med.* **131**, 356-369.

El-Ashmawy, N. E., Al-Ashmawy, G. M., Amr, E. A., Khedr, E. G. (2020) Inhibition of lovastatin- and docosahexaenoic acid-initiated autophagy in triple negative breast cancer reverted resistance and enhanced cytotoxicity. *Life Sci.* **259**, 118212.

Elgendy, S. M., Alyammahi, S. K., Alhamad, D. W., Abdin, S. M., Omar, H. A. (2020) Ferroptosis: an emerging approach for targeting cancer stem cells and drug resistance. *Crit. Rev. Oncol. Hematol.* **155**, 103095.

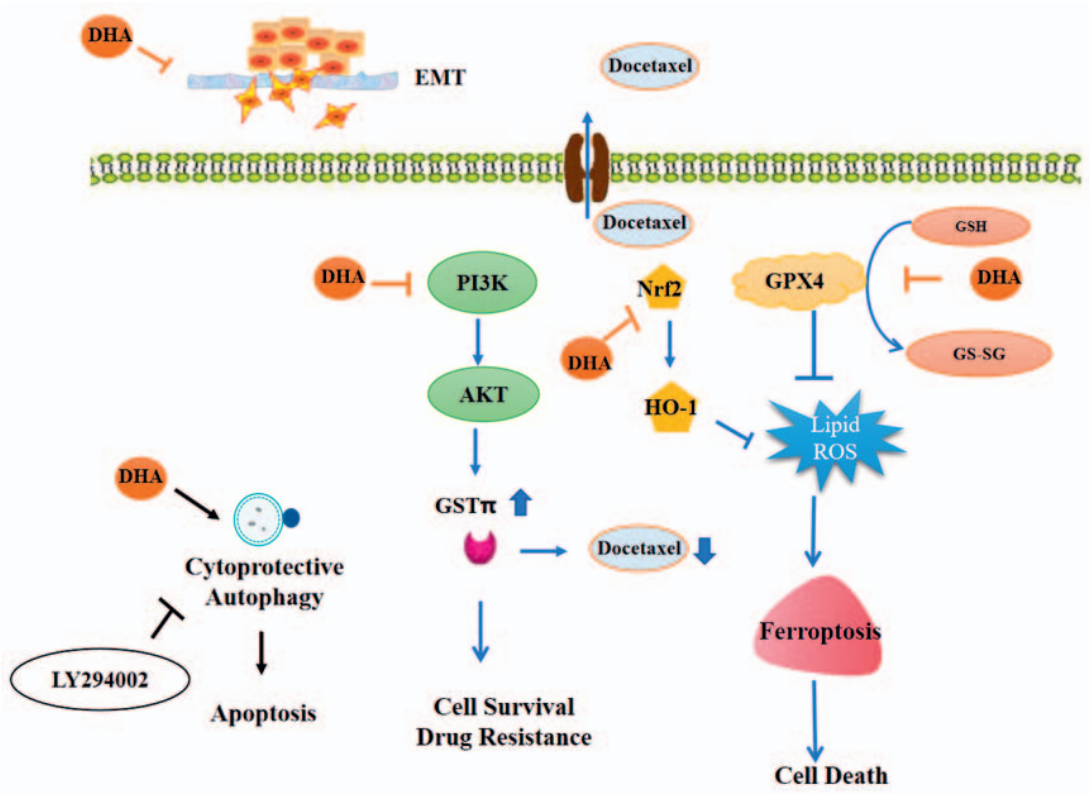

Fig. 8. The mechanism of action of DHA overcomes the drug resistance in prostate cancer cells.

Esteve, J. M., Knecht, E. (2011) Mechanisms of autophagy and apoptosis: recent developments in breast cancer cells. *World J. Biol. Chem.* **2**, 232-238.

Gao, L., Loveless, J., Shay, C., Teng, Y. (2020) Targeting ROS-mediated crosstalk between autophagy and apoptosis in cancer. *Adv. Exp. Med. Biol.* **1260**, 1-12.

Hokaiwado, N., Takeshita, F., Naiki-Ito, A., Asamoto, M., Ochiya, T., Shirai, T. (2008) Glutathione S-transferase pi mediates proliferation of androgen-independent prostate cancer cells. *Carcinogenesis* **29**, 1134-1138.

Jain, A. P., Aggarwal, K. K., Zhang, P. Y. (2015) Omega-3 fatty acids and cardiovascular disease. *Eur. Rev. Med. Pharmacol. Sci.* **19**, 441-445.

Jing, K., Wu, T., Lim, K. (2013) Omega-3 polyunsaturated fatty acids and cancer. *Anticancer Agents Med. Chem.* **13**, 1162-1177.

Kim, K. Y., Kim, S. H., Yu, S. N., Park, S. G., Kim, Y. W., Nam, H. W., An, H. H., Yu, H. S., Kim, Y. W., Ji, J. H., Seo, Y. K., Ahn, S. C. (2017) Lasalocid induces cytotoxic apoptosis and cytoprotective autophagy through reactive oxygen species in human prostate cancer PC-3 cells. *Biomed. Pharmacother.* **88**, 1016-1024.

Kim, N., Jeong, S., Jing, K., Shin, S., Kim, S., Heo, J. Y., Kweon, G. R., Park, S. K., Wu, T., Park, J. I., Lim, K. (2015) Docosahexaenoic acid induces cell death in human non-small cell lung cancer cells by repressing mTOR via AMPK activation and PI3K/Akt inhibition. *Biomed. Res. Int.* **2015**, 239764.

Kosaka, T., Miyajima, A., Shirotake, S., Suzuki, E., Kikuchi, E., Oya, M. (2011) Long-term androgen ablation and docetaxel up-regulate phosphorylated Akt in castration resistant prostate cancer. *J. Urol.* **185**, 2376-2381.

Kranzbuhler, B., Salemi, S., Mortezavi, A., Sulser, T., Eberli, D. (2019) Combined N-terminal androgen receptor and autophagy inhibition increases the antitumor effect in enzalutamide sensitive and enzalutamide resistant prostate cancer cells. *Prostate* **79**, 206-214.

Li, J., Cao, F., Yin, H. L., Huang, Z. J., Lin, Z. T., Mao, N., Sun, B., Wang, G. (2020) Ferroptosis: past, present and future. *Cell Death Dis.* **11**, 88.

Liu, C. M., Kao, C. L., Tseng, Y. T., Lo, Y. C., Chen, C. Y. (2017) Ginger phytochemicals inhibit cell growth and modulate drug resistance factors in docetaxel resistant prostate cancer cell. *Molecules* **22**, 1477.

Liu, T., Zhang, X., Du, L., Wang, Y., Liu, X., Tian, H., Wang, L., Li, P., Zhao, Y., Duan, W., Xie, Y., Sun, Z., Wang, C. (2019) Exosome-transmitted miR-128-3p increase chemosensitivity of oxaliplatin-resistant colorectal cancer. *Mol. Cancer* **18**, 43.

Mansinho, A., Macedo, D., Fernandes, I., Costa, L. (2018) Castration-resistant prostate cancer: mechanisms, targets and treatment. *Adv. Exp. Med. Biol.* **1096**, 117-133.

Merendino, N., Molinari, R., Loppi, B., Pessina, G., M, D. A., Tomassi, G.,Velotti, F. (2003) Induction of apoptosis in human pancreatic cancer cells by docosahexaenoic acid. *Ann. N. Y. Acad. Sci.* **1010**, 361-364.

Nehme, A., Varadarajan, P., Sellakumar, G., Gerhold, M., Niedner, H., Zhang, Q., Lin, X., Christen, R. D. (2001) Modulation of docetaxel-induced apoptosis and cell cycle arrest by all-trans retinoic acid in prostate cancer cells. *Br. J. Cancer* **84**, 1571-1576.

Newell, M., Goruk, S., Mazurak, V., Postovit, L., Field, C. J. (2019) Role of docosahexaenoic acid in enhancement of docetaxel action in patient-derived breast cancer xenografts. *Breast Cancer Res. Treat.* **177**, 357-367.

Ouyang, D. Y., Xu, L. H., He, X. H., Zhang, Y. T., Zeng, L. H., Cai, J. Y., Ren, S. (2013) Autophagy is differentially induced in prostate cancer LNCaP, DU145 and PC-3 cells via distinct splicing profiles of ATG5. *Autophagy* **9**, 20-32.

Park, J. H., Kim, Y. H., Park, E. H., Lee, S. J., Kim, H., Kim, A., Lee, S. B., Shim, S., Jang, H., Myung, J. K., Park, S., Lee, S. J., Kim, M. J. (2019) Effects of metformin and phenformin on apoptosis and epithelial-mesenchymal transition in chemoresistant rectal cancer. *Cancer Sci.* **110**, 2834-2845.

Poorani, R., Bhatt, A. N., Dwarakanath, B. S., Das, U. N. (2016) COX-2, aspirin and metabolism of arachidonic, eicosapentaenoic and docosahexaenoic acids and their physiological and clinical significance. *Eur. J. Pharmacol.* **785**, 116-132.

Rosenfeld, M. R., Ye, X., Supko, J. G., Desideri, S., Grossman, S. A., Brem, S., Mikkelson, T., Wang, D., Chang, Y. C., Hu, J., McAfee, Q., Fisher, J., Troxel, A. B., Piao, S., Heitjan, D. F., Tan, K. S., Pontiggia, L., O'Dwyer, P. J., Davis, L. E., Amaravadi, R. K. (2014) A phase I/II trial of hydroxychloroquine in conjunction with radiation therapy and concurrent and adjuvant temozolomide in patients with newly diagnosed glioblastoma multiforme. *Autophagy* **10**, 1359-1368.

Ryabaya, O. O., Inshakov, A. N., Egorova, A. V., Emelyanova, M. A., Nasedkina, T. V., Zasedatelev, A. S., Khochenkov, D. A., Stepanova, E. V. (2017) Autophagy inhibitors chloroquine and LY294002 enhance temozolomide cytotoxicity on cutaneous melanoma cell lines in vitro. *Anticancer Drugs* **28**, 307-315.

Shahidi, F., Ambigaipalan, P. (2018) Omega-3 polyunsaturated fatty acids and their health benefits. *Annu. Rev. Food Sci. Technol.* **9**, 345-381.

Shaikh, I. A., Brown, I., Schofield, A. C., Wahle, K. W., Heys, S. D. (2008) Docosahexaenoic acid enhances the efficacy of docetaxel in prostate cancer cells by modulation of apoptosis: the role of genes associated with the NF-κB pathway. *Prostate* **68**, 1635-1646.

Shekari, N., Javadian, M., Ghasemi, M., Baradaran, B., Darabi, M., Kazemi, T. (2020) Synergistic beneficial effect of docosahexaenoic acid (DHA) and docetaxel on the expression level of matrix metalloproteinase-2 (MMP-2) and microRNA-106b in gastric cancer. *J. Gastrointest. Cancer* **51**, 70-75.

Shin, D., Kim, E. H., Lee, J., Roh, J. L. (2018) Nrf2 inhibition reverses resistance to GPX4 inhibitor-induced ferroptosis in head and neck cancer. *Free Radic. Biol. Med.* **129**, 454-462.

Shin, S., Jing, K., Jeong, S., Kim, N., Song, K. S., Heo, J. Y., Park, J. H., Seo, K. S., Han, J., Park, J. I., Kweon, G. R., Park, S. K., Wu, T., Hwang, B. D., Lim, K. (2013) The omega-3 polyunsaturated fatty acid DHA induces simultaneous apoptosis and autophagy via mitochondrial ROS-

mediated Akt-mTOR signaling in prostate cancer cells expressing mutant p53. *Biomed Res. Int.* **2013**, 568671.

Simopoulos, A. P. (2002) The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed. Pharmacother.* **56**, 365-379.

Smith, A. G., Macleod, K. F. (2019) Autophagy, cancer stem cells and drug resistance. *J. Pathol.* **247**, 708-718.

Song, E. A., Kim, H. (2016) Docosahexaenoic acid induces oxidative DNA damage and apoptosis, and enhances the chemosensitivity of cancer cells. *Int. J. Mol. Sci.* **17**, 1257.

Tsai, C. H., Shen, Y. C., Chen, H. W., Liu, K. L., Chang, J. W., Chen, P. Y., Lin, C. Y., Yao, H. T., Li, C. C. (2017) Docosahexaenoic acid increases the expression of oxidative stress-induced growth inhibitor 1 through the PI3K/Akt/Nrf2 signaling pathway in breast cancer cells. *Food Chem. Toxicol.* **108**, 276-288.

van Brussel, J. P., van Steenbrugge, G. J., Romijn, J. C., Schroder, F. H., Mickisch, G. H. (1999) Chemosensitivity of prostate cancer cell lines and expression of multidrug resistance-related proteins. *Eur. J. Cancer* **35**, 664-671.

Vijay, G. V., Zhao, N., Den Hollander, P., Toneff, M. J., Joseph, R., Pietila, M., Taube, J. H., Sarkar, T. R., Ramirez-Pena, E., Werden, S. J., Shariati, M., Gao, R., Sobieski, M., Stephan, C. C., Sphyris, N., Miura, N., Davies, P., Chang, J. T., Soundararajan, R., Rosen, J. M., Mani, S. A. (2019) GSK3β regulates epithelial-mesenchymal transition and cancer stem cell properties in triple-negative breast cancer. *Breast Cancer Res.* **21**, 37.

Wang, G., Zhao, D., Spring, D. J., DePinho, R. A. (2018a) Genetics and biology of prostate cancer. *Genes Dev.* **32**, 1105-1140.

Wang, L. J., Wang, S. G., Deng, T. X. (2018b) Wogonoside reverses cisplatin resistance in SGC7901/cDDP cells through inhibition of PI3K/Akt/Nrf2/ARE signaling pathway. *Sheng Li Xue Bao* **70**, 397-405. (in Chinese)

Wright, T. J., McKee, C., Birch-Machin, M. A., Ellis, R., Armstrong, J. L., Lovat, P. E. (2013) Increasing the therapeutic efficacy of docetaxel for cutaneous squamous cell carcinoma through the combined inhibition of phosphatidylinositol 3-kinase/AKT signalling and autophagy. *Clin. Exp. Dermatol.* **38**, 421-423.

Wu, Z., Chen, C. Y., Kao, C. L., Jiang, Y., Liu, C. M. (2019) Docosahexaenoic acid inhibits lipopolysaccharide-induced metastatic activities by decreasing inflammation on prostate cancer cell. *Pharmazie* **74**, 675-679.

Xu, Y., Yu, H., Qin, H., Kang, J., Yu, C., Zhong, J., Su, J., Li, H., Sun, L. (2012) Inhibition of autophagy enhances cisplatin cytotoxicity through endoplasmic reticulum stress in human cervical cancer cells. *Cancer Lett.* **314**, 232-243.

Yamaguchi, H., Hsu, J. L., Chen, C. T., Wang, Y. N., Hsu, M. C., Chang, S. S., Du, Y., Ko, H. W., Herbst, R., Hung, M. C. (2013) Caspase-independent cell death is involved in the negative effect of EGF receptor inhibitors on cisplatin in non-small cell lung cancer cells. *Clin. Cancer Res.* **19**, 845-854.

Zhang, Y., Wang, Y., Yuan, J., Qin, W., Liu, F., Wang, F., Zhang, G., Yang, X. (2012) Toll-like receptor 4 ligation confers chemoresistance to docetaxel on PC-3 human prostate cancer cells. *Cell Biol. Toxicol.* **28**, 269-277.