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Omega-3 free fatty acids inhibit tamoxifen-induced cell apoptosis

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ABSTRACT

Fish oil, which contains omega-3 fatty acids mainly in the form of triglycerides, has benefits for reducing breast cancer risk, similar to tamoxifen action. However, it remains to be elucidated whether the combination of omega-3 free fatty acid (ω-3FFA) with tamoxifen leads to improved treatment in breast cancer. In this study, we observed that ω-3FFA induces MCF-7 cell apoptosis to suppress cell growth. The treatment of breast cancer cells with ω-3FFA attenuated tamoxifen-induced cell apoptosis. ω-3FFA and tamoxifen significantly increased Erk1/2 and Akt phosphorylation levels in a dose and time dependent manner. Compared to ω-3FFA alone, the combination of tamoxifen with ω-3FFA significantly increased Erk1/2 and Akt phosphorylation levels. Because Erk1/2 and Akt activation has been linked to tamoxifen-related anti-estrogen resistance in breast cancer patients, these results indicate that ω-3FFA may interfere with the effects of tamoxifen in the prevention of breast cancer risk.

1. Introduction

Fish oil, which contains omega-3 fatty acids (ALA, EPA and DHA) mainly in the form of triglycerides, is known to reduce the risk of breast cancer [1–3]. Much debate has been generated regarding fish oil bioavailability, in particular with respect to the different molecular forms (triglycerides, TG; ethyl esters, EE and free fatty acids, FFA) of fish oil supplements. The TG form of Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) modulates breast carcinogenesis through regulation of the apoptotic pathway and estrogen metabolism [4–6]. Alpha linolenic acid (ALA) in TG form was also shown to have a protective effect on breast cancer development [7]. Fish oil in TG form reduces obesity-induced breast cancer risk independently of the GPR120 signaling pathway [8]. The EE form of fish oil was less efficiently absorbed than the TG form, and has been used to treat patients with very high triglyceride levels [9]. The FFA form of omega-3 fatty acids had dramatically improved bioavailability over the EE form for the treatment of severe hypertriglyceridemia in overweight subjects with low fat consumption [10]. Omega-3 FFA also regulated inflammation and insulin sensitivity through the GPR120 signaling pathway [11].

Tamoxifen (TAM), a typical anti-estrogen drug, is administered as first-line treatment for advanced breast cancer patients and for the prevention of breast cancer in women at high risk of developing this disease [12,13]. Approximately 50% of ERα-positive breast cancer patients develop tamoxifen resistance in clinical treatment which leads to treatment failure [14,15]. Higher levels of phosphorylated Erk1/2 and Akt were detected in tamoxifen-resistant breast tumors than in surrounding tissue [16,17]. The elevated Erk1/2 and Akt activation result in tamoxifen-related anti-estrogen resistance in breast cancer patients [18–20].

Despite the reduction of breast cancer risk by fish oil, a diet rich in fish oil diet is not able to significantly enhance tamoxifen effect on prevention of carcinogenesis, tumor multiplicity and tumor volume, only the omega-3 fatty acids concentration of target tissue is correlated with carcinogenesis-related genes expression [21,22]. However, whether ω-3FFA is capable of improving tamoxifen effectiveness in the treatment of breast cancer remains uncertain. Here, we show that both ω-3FFA and tamoxifen induce MCF-7 cell apoptosis and activate the Erk1/2 and Akt signals in a dose-dependent manner. Omega-3FFA attenuated tamoxifen-induced cell apoptosis and enhanced Erk1/2 and Akt phosphorylation

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levels with tamoxifen incubation. These results suggest that ω-3FFA interfere with the effects of tamoxifen on the prevention of breast cancer development.

2. Materials and methods

2.1. Cell culture and DAPI staining

MCF-7 cells were obtained from ATCC and incubated in Dulbecco modified Eagle medium (DMEM; HyClone) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin-streptomycin, 1mM sodium pyruvate. MCF-7 cells were fixed with 40% paraformaldehyde for 15 min and incubated with diluted DAPI solution (300 nM) for 30 min.

2.2. Cell accounting and proliferation assay

Cells were counted with a cell counting chamber according to the manufacturer's instructions. MCF-7 were cultured at 5000 cells/well in a 96-well plate and incubated with drug or FFA for 24 h. CCK-8 substrate was then added. Plates were incubated for 3 h and measured the absorbance at 490 nm.

2.3. Reagents and immunoblotting

Omega-3 free fatty acids (EPA, DHA and ALA) were purchased from NU-CHEK (USA) and dissolved in ethanol. Tamoxifen was purchased from Sigma. MCF-7 cells were lysed with RIPA lysis buffer. The protein content was quantified by Coomassie brilliant blue staining approach. Immunoblot analysis was performed with standard protocols and visualized with ECL or ECL plus [23]. Some blots were visualized using the FluorChem (ProteinSimple, USA) system. Akt, p-Akt, Erk, p-Erk, p38 and p-p38 antibodies (Cell Signaling Technology) were used to recognize each corresponding protein, respectively.

2.4. Statistical analysis

Each experiment was repeated at least three times. Data are presented as mean ± S.D. The statistical significance was determined using Student's T-test. The value for $P \leq 0.05$ was regarded as significant.

3. Results

3.1. Omega-3 free fatty acid (ω-3FFA) suppresses cell growth

Previous studies have indicated that DHA induced cell apoptosis by activating reactive oxygen species formation and caspase-8 signaling pathways [24]. To examine the role of ω-3 fatty acids in the form of FFA (ω-3FFA) in MCF-7 cells apoptosis, EPA, DHA and ALA in FFA form were added into medium to stimulate cells for 24 h, respectively. The number of viable cell after incubation with 100 μM ω-3FFA was dramatically decreased (Fig. 1A and B). Similarly, quantitation of viable cell number in proliferation and cytotoxicity assays also showed a significant reduction of viable cell number after ω-3FFA treatment in a dose-dependent manner (Fig. 1C). Using DAPI nuclear staining to visualize nuclear changes and assess apoptosis, we observed chromosome condensation and DNA fragmentation in ω-3FFA treated cells (Fig. 1D). All of these data suggest that EPA, DHA and ALA in FFA form suppress cell growth and induce cell apoptosis.

3.2. Tamoxifen induces cell apoptosis

Tamoxifen (TAM) is an anti-estradiol drug and widely used in breast cancer chemoprevention and chemotherapy [14,25,26]. To examine the effects of TAM on cell apoptosis, estrogen-receptor positive MCF-7 cells were incubated with tamoxifen for 24 h. Consistent with literature, cell apoptosis was triggered dramatically by TAM at 50 μM (Fig. 1E and F). DAPI nuclear staining analysis also showed that cell apoptosis was induced 6 h after TAM treatment (Fig. 1G). These data verify that TAM induces breast cancer cell apoptosis.

3.3. Omega-3 FFA prevents tamoxifen-induced cell apoptosis

As described above, ω-3FFA and tamoxifen each induce breast cancer cell apoptosis. To investigate if there is a synergistic effect of ω-3FFA and tamoxifen or a possible reduction in toxicity of one agent by another, we performed cell counting and apoptosis analysis after tamoxifen and tamoxifen combined with ω-3FFA treatment, and compared the results with control treatment. 50 μM tamoxifen and 50 μM tamoxifen combined with low dose ω-3FFA induced significant cell apoptosis. Strikingly, cell apoptosis resulting from treatment with 50 μM tamoxifen was markedly attenuated with high dose ω-3FFA (Fig. 2A and B). Moreover, inhibition of ω-3FFA to tamoxifen-induced cell death was dependent on the dose of tamoxifen (Fig. 2C). Together, these results indicate that ω-3FFA inhibits tamoxifen-induced cell death in a dose-dependent manner.

3.4. Omega-3FFA increases Akt and Erk1/2 phosphorylation levels

To further identify signaling events downstream of tamoxifen and ω-3FFA treatment, we detected Akt, Erk1/2 and p38 phosphorylation levels after ω-3FFA and tamoxifen treatment. Immunoblotting results revealed increased phosphorylation levels of Akt and Erk1/2 after tamoxifen incubation in a dose dependent manner (Fig. 3B and C). Compared to tamoxifen, ω-3FFA dose-dependently enhanced not only Akt and Erk1/2 phosphorylation levels but also p38 activation (Fig. 3A). Similar to tamoxifen, ω-3FFA also dramatically enhanced Erk1/2 and Akt phosphorylation levels from 5 min to 12 h after stimulation (Fig. 3D).

It has been well recognized that enhanced Akt or Erk1/2 activation leads to tamoxifen resistance in breast cancer cells [27,28]. To determine if ω-3FFA-induced tamoxifen resistance was caused by elevated Akt and Erk1/2 activation, we examined Akt and Erk1/2 phosphorylation levels in cells co-treated with ω-3FFA plus tamoxifen. Indeed, higher Erk1/2 phosphorylation levels were observed in tamoxifen with ω-3FFA co-treated cells than in ω-3FFA treated cells (Fig. 3E). In particular, FFA form EPA plus tamoxifen dramatically improved higher Akt phosphorylation levels compared to tamoxifen or ω-3FFA stimulation alone. Together, these data indicate that elevated Erk1/2 and Akt phosphorylation levels triggered by ω-3FFA treatment could possibly result in tamoxifen resistance of MCF-7 cells.

4. Discussion

Fish oil mainly containing ω-3 polyunsaturated fatty acids (PUFA) is able to reduce cellular proliferation and increase cell apoptosis to prevent breast cancer development [29,30]. However, which molecular forms of ω-3 PUFAs perform anti-breast cancer function is not fully understood. The results present here indicate that ω-3FFA plays a protective role of against breast carcinogenesis by inducing cell apoptosis. Despite the promotion of breast cancer apoptosis by ω-3FFA, the effects on breast cancer of ω-3FFA combined with tamoxifen

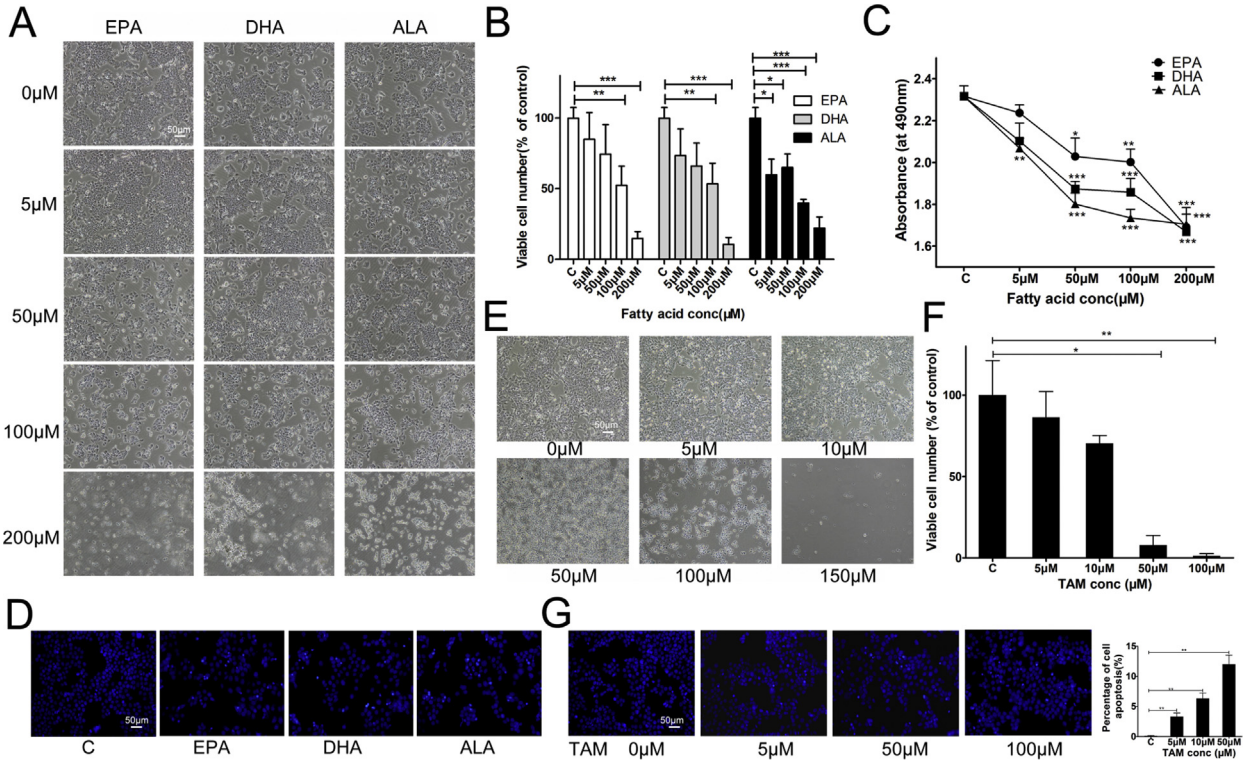

Fig. 1. Effects of ω-3FFA and TAM on cell growth. (A–D) MCF-7 cells were treated with omega-3 free fatty acid at different concentrations (5, 50, 100 and 200 μM) for 24 h. Cell morphology (A); viable cell number (B) and cell counting kit assays (CCK-8) (C); Nuclear staining by DAPI (D). (E–G) MCF-7 cells were treated with TAM at different concentrations (5, 10, 50, 100 and 150 μM) for 24 h. Cell morphology (E); viable cell number (F); cell nuclear was stained by DAPI (G) 6 h after TAM (5, 50 and 100 μM) treatment. Data are shown as the means ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001, as determined by Student's T-test.

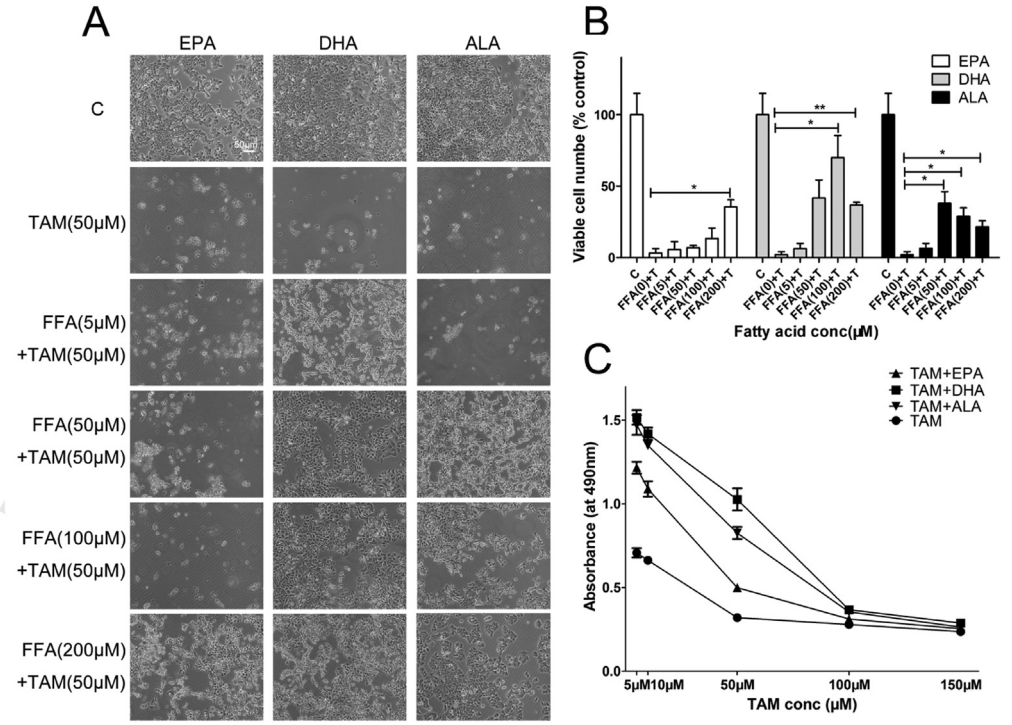

Fig. 2. Effects of ω-3FFA on TAM-induced cell apoptosis. (A–B) Cells were treated with ω-3FFA at different concentrations (5, 50, 100 and 200 μM) with or without TAM (50 μM) for 24 h. Cell morphology (A) and viable cell number (B). (C) MCF-7 cells were treated with TAM at different concentrations (5, 10, 50, 100 and 200 μM) with or without ω-3 FFAs (100 μM). Cell counting kit assay CCK-8 (C) Data are shown as the means ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 as determined by Student's T-test.

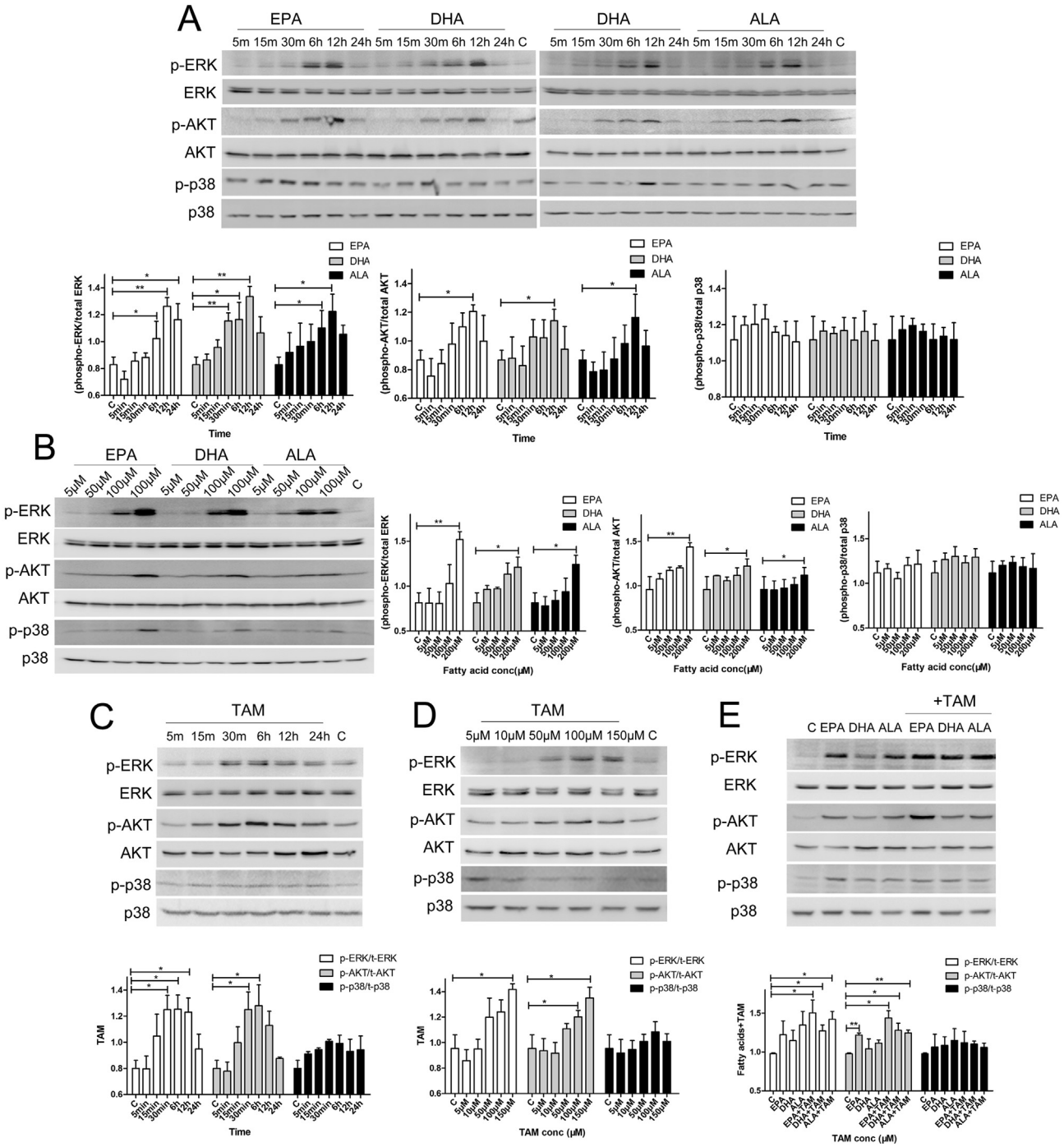

Fig. 3. Erk1/2 and Akt phosphorylation levels in MCF-7 cells. (A) Erk1/2 and Akt phosphorylation levels in MCF-7 cells at different time point (5 min, 15 min, 30 min, 6 h, 12 h and 24 h) after omega-3FFA (50 μM) treatment. (B) Effects of omega-3FFA (5, 50, 100 and 200 μM) on Erk1/2 and Akt phosphorylation 6 h after treatment. (C) Erk1/2 and Akt phosphorylation levels were determined by immunoblotting cell lysates at different time point (5 min, 15 min, 30 min, 6 h, 12 h and 24 h) after TAM (10 μM) stimulation. (D) Erk1/2 and Akt phosphorylation levels in cells 6 h after TAM treatment at different dose (5, 10, 50, 100 and 150 μM). (E) Erk1/2 and Akt phosphorylation levels in cells were detected by immunoblotting 6 h after incubation with ω-3FFA (100 μM) with and without TAM (50 μM). Data are shown as the means ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ as determined by Student's T-test.

is unclear. This report presents biochemical and cellular data suggesting that ω-3FFA inhibits tamoxifen-induced cell apoptosis and enhances tamoxifen-stimulated Erk1/2 and Akt phosphorylation levels, suggesting that consumption of FFA may interfere with tamoxifen treatment for breast cancer patients.

There are several lines of evidence indicating that the administration of omega-3 fatty acids or its metabolites is able to reduce cellular proliferation and increase apoptosis *in vitro* and *in vivo* [24,29]. Consistently, our results strongly suggest that ω-3FFA induced cell apoptosis in a dose-dependent manner to suppress cell growth of MCF-7 cells (Fig. 1C and D). Similarly, flaxseed oil with high triglyceride form of ALA content inhibits MCF-7 cells growth with high E2 level [31]. Fish oil suppresses breast cancer invasion through the CD44 signal pathway in a mouse model [32] and reduces obesity-induced breast cancer progression independent of GPR120 signaling pathway [8].

However, it is interesting that ω-3FFA attenuates tamoxifen-induced cell apoptosis in MCF-7 cells (Fig. 2B and C). Similarly, intake of fish oil-rich diets is not able to increase tamoxifen effects on prevention of carcinogenesis and tumor multiplicity and volume, but it is able to improve the chemopreventive efficacy of tamoxifen against MNU-induced mammary carcinogenesis and preneoplastic lesions [21,22]. Of note, resolvin D, a metabolite of fish oil, regulates ERα localization to promote MCF-7 cell proliferation [33]. Ethyl ester form omega-3 fatty acids are involved in the regulation of triglyceride levels, but not breast cancer development [9]. These conflicting data implicate that different molecular forms or metabolites of omega-3 PUFA have different physiological functions in preventing breast cancer progression.

Akt and Erk1/2 phosphorylation levels in TAM and ω-3FFA treated cells are higher than those in control cells (Fig. 3A, B and C), suggesting that the induction of cell apoptosis by TAM and ω-3FFA is associated with Erk1/2 and Akt signaling. Although activated Erk1/2 is considered to promote cell proliferation, there are also reports showing that enhanced activation of Erk1/2 and Akt is involved in increasing cell apoptosis [34,35]. Moreover, tamoxifen enhanced FFA effects on promoting Erk1/2 and Akt activation (Fig. 3D). Thus, we propose that enhanced Erk1/2 and Akt phosphorylation levels by FFA leads to tamoxifen resistance. Consistently, recent studies showed that high Erk1/2 and Akt activity in breast carcinoma is associated with a poor patho-phenotype, as well as hormone and TAM resistance [36]. And Gαs, a fatty acid receptor (GPCR) associated protein, is involved in Erk1/2 and Akt-mediated tamoxifen resistance in the breast cancer cells [37]. Together, these results suggest that enhanced Erk1/2 and Akt activation by ω-3FFA result in the prevention of tamoxifen-induced cell apoptosis.

Despite being safe and well-tolerated in health, side effects of fish oil still need to be considered. Indeed, it has been well recognized that taking more than 3 g of fish oil daily may increase the risk of bleeding, stomach upset and nausea. Of note, our results indicate that ω-3FFA attenuates tamoxifen-induced apoptosis in MCF-7 cells (Fig. 2B and C), suggesting ω-3FFA may present an unwanted drug–drug interaction in the prevention of tamoxifen for breast cancer risk. Elucidating the effect of ω-3FFA on tamoxifen signaling in cell apoptosis may provide novel therapeutic target for improving treatment of breast cancer patients.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgments

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