

Sensitization of TRAIL-resistant cervical cancer cells through combination of TRAIL and fucoxanthin treatments

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Abstract. – **OBJECTIVE:** Cervical cancer, the second most common cause of cancer death in women worldwide, is a malignant neoplasm arising from cells originating in the cervix uteri. Currently, surgery combined with chemo- and radiotherapy is the major therapeutic approach for women with early-stage cervical cancer. However, recurrent cervical cancers from acquired chemo-resistance remain a major cause of therapeutic failure.

MATERIALS AND METHODS: In this study, we assessed the effects of the combination of TRAIL with fucoxanthin, which has been reported to suppress the cervical cancer cells growth on the cervical cancer treatments. HeLa cells, SiHa cells, and CaSki cells were used as in vitro model. Mice xenograft was used as in vivo model. TRAIL-resistant cells were generated from CaSki cell line. The activity of PI3K/Akt pathway was detected by Western blot. Cell viability was measured by MTT assay.

RESULTS: We observed TRAIL-resistant cervical cancer cells were more sensitive to fucoxanthin treatments. By establishing a TRAIL-resistant cell line from CaSki, we found the TRAIL-resistant cells showed upregulated PI3K/Akt pathway. Moreover, CaSki TRAIL-resistant cells were more sensitive to the combination of TRAIL with either Akt inhibitor or fucoxanthin than treatment with TRAIL or fucoxanthin alone. Our in vitro and in vivo xenograft experiments demonstrate that the combination of TRAIL with fucoxanthin showed synergistically inhibitory effects on cervical cancer cells.

CONCLUSIONS: The findings of this study suggest that the combined use of fucoxanthin and TRAIL might be a useful strategy against TRAIL-resistant cervical cancer.

Key Words:

Cervical cancer, TRAIL, Fucoxanthin, PI3K/Akt pathway.

Introduction

Cervical cancer (CC), a malignant neoplasm arising from cells originating in the cervix uteri,

is the second most common cause of cancer death in women worldwide¹. Currently, surgery and chemo- and radiotherapy can cure 80-95% of women with early-stage cervical cancer². Effective cervical cancer therapeutic drug includes cisplatin, cyclophosphamide, ifosfamide, doxorubicin, and bleomycin neomycin³. Among them, the cisplatin-based chemotherapy was used by triple or quadruple⁴. However, the recurrent and metastatic cervical cancer from acquired chemo-resistance, remain a major cause of cancer death⁵. Therefore, the development of new diagnosis, prognostic, and therapeutic strategies is needed.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anti-cancer agent for cervical cancer therapy because it selectively induces cancer cell death⁶. TRAIL is a member of the tumor necrosis factor (TNF) family of ligands, which interact with the death receptors DR4 and DR5⁷. However, limitations exist in TRAIL therapy since a group of cancers gradually develop resistance toward TRAIL^{8,9}. Thus, investigating the molecular targets and signaling pathways responsible for TRAIL resistance is important for the development of effectively therapeutic strategies against TRAIL-resistant cervical cancers.

Recently, large amounts of algae extracts have been studied in the clinic for their antitumor effects. These results demonstrate that polysaccharides especially sulfate polysaccharides extracted from seaweeds have better antitumor activities because of their immune-stimulating activity, such as fucoïdan, carrageenan, and fucoxanthin¹⁰. Fucoxanthin is a carotenoid pigment that is commonly found in marine algae¹¹. Also, it has been shown to regulate diverse biological processes, including anti-oxidative, anti-inflammatory, and anti-cancer activities¹². PI3K/Akt (phosphoinositide 3-kinase and protein kinase B) pathway has

played an important role in the regulation of cell proliferation and apoptosis¹³. A previous publication from our lab demonstrated a tumor suppressive role of fucoxanthin in cervical cancer cells through inhibiting PI3K/Akt pathway¹⁴. In this study, we investigated the cytotoxic effects of the combination of TRAIL and fucoxanthin on cervical cancer cells using *in vitro* and *in vivo* models.

Materials and Methods

Cell Culture

HeLa cells, SiHa cells, and CaSki cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in log growth phase in cell culture incubator at 37°C in humidified air with 5% CO₂. HeLa and SiHa cells were cultured with Dulbecco's Modified Eagle Medium (DMEM), and CaSki cells were cultured with Roswell Park Memorial Institute 1640 (RPMI 1640) medium, respectively. Medium for all cells was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cell culture reagents were obtained from Thermo Scientific (Waltham, MA, USA).

Antibodies and Reagents

Fucoxanthin and TRAIL were purchased from Sigma-Aldrich (Shanghai, China). The rabbit monoclonal antibodies against phosphor-Akt (#4060), total Akt (#9272), phosphor-PI3K (#4228), total PI3K (#4292) and β -actin (#4970) were purchased from Cell Signal Technology (Danvers, MA, USA). The horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Sigma-Aldrich (Shanghai, China).

Cell Viability Assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the previous report¹⁴. Briefly, after treatments, cells were seeded in 96-well dishes at 1×10^4 to 2×10^4 cells per well. Each well was then supplemented with 10 μ L MTT (Sigma-Aldrich, Shanghai, China) and incubated for 4 h at 37°C. The medium was then removed, and 150 μ L dimethyl sulfoxide (DMSO) were added to solubilize the MTT formazan. The optical density was read at 490 nm.

Clonogenic Assay

Cells were seeded in a 6-well plate at a density of 3,000 cells per well in growth medium and in-

culated for 24 h. Then the cells were treated with TRAIL at 800 ng/ml for 2 weeks to allow survival colonies formation. The colonies were stained with 0.25% crystal violet in ethanol.

Western Blot

The whole cell proteins were extracted by RIPA Lysis and Extraction Buffer (Thermo Fisher, Waltham, MA, USA). Protein concentrations were determined by Bradford assay. Equal amount proteins of each sample were separated on a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto nitrocellulose membrane. Membranes were incubated with blocking buffer for 1 h at room temperature. The membrane was probed with primary antibodies for overnight at 4°C. After washing with phosphate-buffered saline and Tween 20 (PBST), membranes were probed with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000 dilution). The protein expressions were detected using the Phototope-HRP Western Detection kit (Cell Signal Technology, Danvers, MA, USA).

Mice and Tumor Xenografts

The tumor xenograft experiments were performed according to our previous report¹⁴. Five-week-old nude BALB/c mice and their diet were all purchased from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China). The mice were allowed *ad libitum* access to food and water. Nude BALB/c mice were used for subcutaneous implantation of human cervical tumor cells HeLa (2×10^6 per site). Tumor-bearing mice were randomly divided into four groups with eight mice per group. Mice with control, TRAIL alone (10 mg/kg), fucoxanthin alone (10 mg/kg) and the combination of TRAIL and fucoxanthin (10 and 10 mg/kg, respectively) were administered intragastrically once per day starting on day 10 after inoculation. The control group was treated with the same volume of 5% sodium carboxymethylcellulose. Mice were sacrificed after treatment for 5 weeks. All animal experiments were conducted in accordance with the Bioethics Committee guidelines in Bengbu Medical College. General reactions were observed every day after drug administration. Tumor growth was measured with a microcaliper every 5 days throughout the experiment. Tumor volume was calculated as follows: $\text{volume} = A \times B^2/2$, where A is the longer and B is the shorter diameter (mm), as described previously.

Statistical Analysis

Statistical analysis was conducted using one-way ANOVA or student *t*-test by GraphPad 5.0 software for Windows (GraphPad Software, San Diego, CA, USA). We used Dunnett tests for the comparison of every mean to a control mean. All experiments were repeated three times and the *p* less than 0.05 was considered statistically significant.

Results

TRAIL Sensitive and Resistant Cervical Cancer Cells show Different Sensitivity to Fucoxanthin

To explore the effects of fucoxanthin on the TRAIL sensitivity of cervical cancer cells, we focused on two cervical cancer cell lines: CaSki, which is sensitive to TRAIL treatment, and SiHa, which is resistant to TRAIL. As we expected, results demonstrated CaSki cells have a significant low IC₅₀ to TRAIL (147.31 ng/ml) than that of the SiHa cells (2.31 μg/ml) (Figure 1A). Moreover, we observed treatment with fucoxanthin induces apoptosis in both cells. Interestingly, CaSki cells showed significantly resistant to fucoxanthin compared to SiHa cells (Figure 1B), suggesting a reverse relation between fucoxanthin and TRAIL sensitivities in cervical cancer cells. Therefore, we hypothesized fucoxanthin could increase the cytotoxicity of cervical cancer cells with TRAIL treatments.

Generation of TRAIL-Resistant Cells from CaSki

To investigate the mechanisms of the TRAIL sensitivity in cervical cancer cells, we established TRAIL-resistant cell line originating from CaSki

since it is a relative TRAIL sensitive cell line. The CaSki cells were treated with gradually elevated concentrations of TRAIL for the selection of survival cells in three months. After selection, survival colons were pooled and used for this work. Due to characteristic of the TRAIL resistance, we treated with TRAIL at elevated concentrations for 48 h. Results (Figure 2A-B) illustrated the TRAIL-resistant cells could tolerate higher concentrations of TRAIL compare with CaSki parental cells. The IC₅₀ of resistant cells was 815.9 ng/ml, which is about 5-fold higher than that of the parental cells (Figure 2A).

TRAIL-Resistant Cells Show Increased PI3K/Akt Pathway

Our previous publication showed the fucoxanthin treatment could suppress PI3K/Akt pathway¹⁴. Moreover, the activated PI3K/Akt signaling pathway has been reported in human cervical cancer¹⁵, presenting molecular targets for the effective treatment of advanced cancers. To access the potentially dysregulated intracellular signaling pathways in the TRAIL-resistant cervical cancer cells, we examined the PI3K/Akt pathway. Parental and TRAIL-resistant cells were analyzed by Western blotting. As we expected, TRAIL-resistant cells showed increased phosphorylation of PI3K and Akt (Figure 3A). To further support the above results, we treated TRAIL-resistant cells with TRAIL alone, MK-2206, which is an Akt pathway inhibitor alone or with the combination of TRAIL and MK-2206 for 48 h. The cells were more sensitive to TRAIL with the combined treatments than TRAIL or MK-2206 alone (Figure 3B). Taken together, these results suggest targeting the upregulated PI3K/Akt pathway may be an efficient approach to sensitize cervical cancer cells to TRAIL.

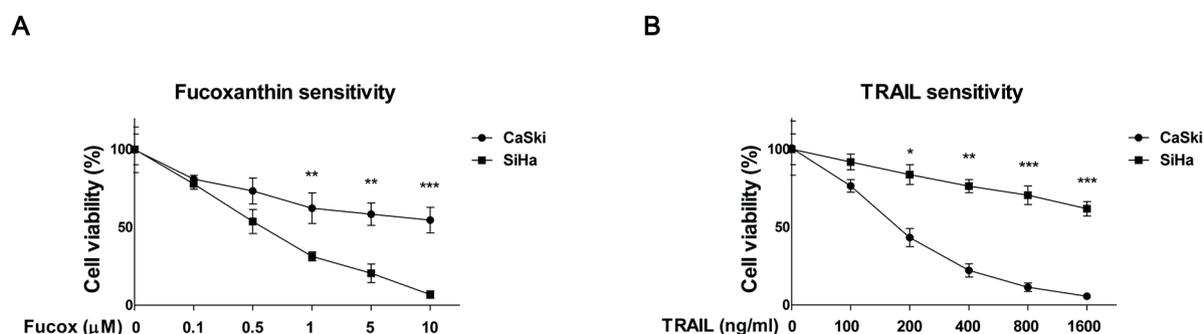


Figure 1. Cervical cell lines exhibit different sensitivities to TRAIL or fucoxanthin treatments. (A) CaSki and SiHa cells were treated with TRAILs at 0, 100, 200, 400, 800 or 1600 ng/ml for 24 h, followed by the measurements of cell viability using MTT assay. (B) CaSki and SiHa cells were treated with fucoxanthin at 0, 0.1, 0.5, 1, 5 or 10 μM for 24 h, followed by the measurements of cell viability using MTT assay. Fucox: Fucoxanthin. Data are mean±SD. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

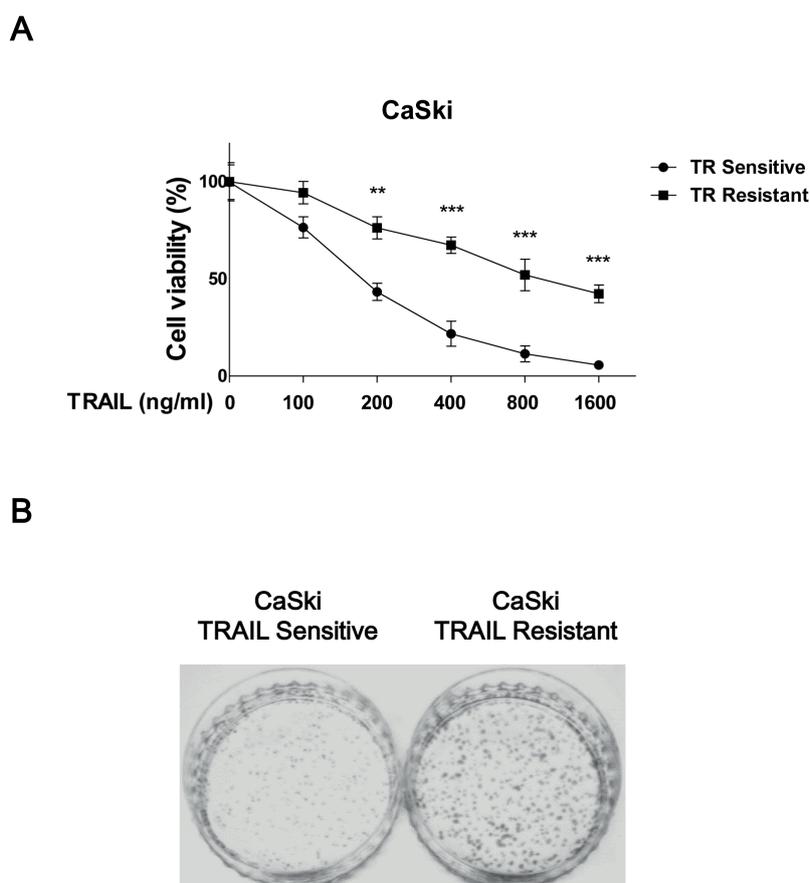


Figure 2. Establishment of TRAIL-resistant cervical cancer cell line. **(A)** CaSki TRAIL sensitive and resistant cells were treated with the indicated concentrations of TRAIL for 24 h; the cell viabilities were measured by MTT assay. **(B)** The clonogenic assay showed the more survival clones in TRAIL-resistant cells than sensitive cells under TRAIL treatments for one week. TR Sensitive: TRAIL Sensitive; TR Resistant: TRAIL-resistant. Data are mean±SD. ** $p < 0.01$, *** $p < 0.001$.

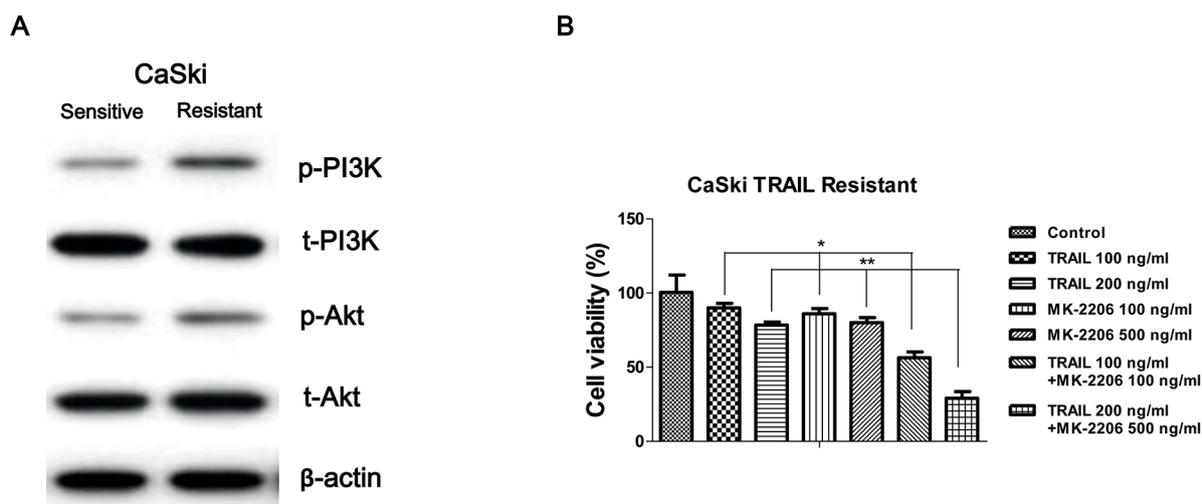


Figure 3. TRAIL-resistant cells show increased PI3K/Akt pathway. **(A)** The expressions of phosphorylated PI3K, total PI3K, phosphorylated Akt, total Akt were measured in CaSki TRAIL sensitive and resistant cells by Western blot. β-actin was a loading control. **(B)** CaSki TRAIL-resistant cells were treated with TRAIL alone at 100 or 200 ng/ml, MK-2206 alone at 100 or 500 ng/ml or the combination of TRAIL with MK-2206 at the indicated concentrations for 24 h, the cell viabilities were measured by MTT assay. Data are mean±SD. * $p < 0.05$, ** $p < 0.01$.

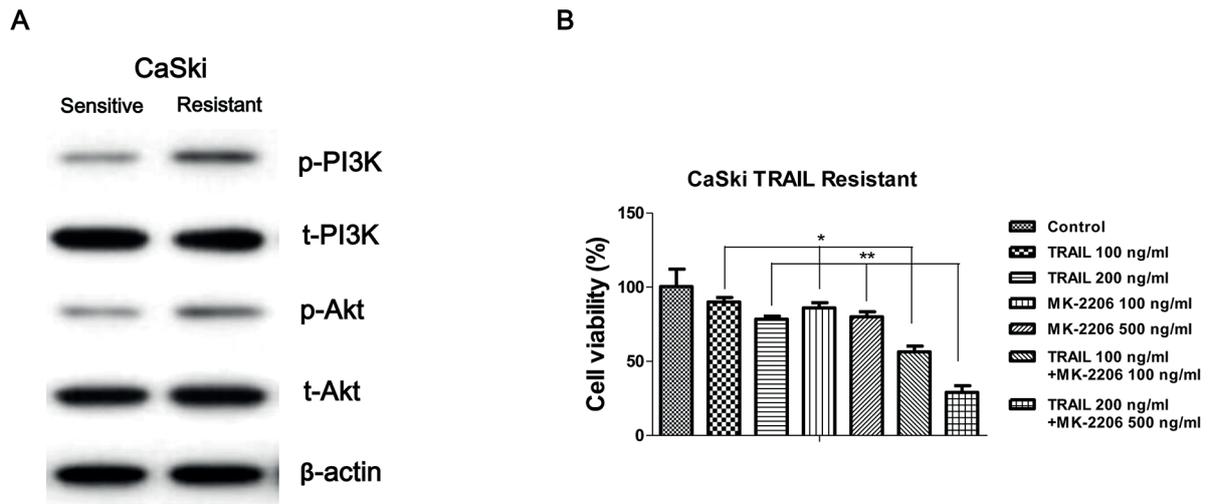


Figure 4. Fucoxanthin suppresses PI3K/Akt pathway of cervical cancer cells. (A) CaSki and (B) SiHa cells were treated with fucoxanthin at 100 nM for 24 h; cells were collected and subjected to Western blot analysis. β-actin was a loading control. Fucox: Fucoxanthin.

Resensitization of TRAIL-resistant Cells by Fucoxanthin

We then assessed the effects of fucoxanthin on the TRAIL sensitivity of cervical cancer cells. Previously, we demonstrated fucoxanthin inhibited PI3K/Akt signaling pathway to suppress HeLa cell growth. Similar results showed in figure 1B that fucoxanthin could suppress CaSki cells proliferation. Moreover, the PI3K/Akt pathway was

significantly inhibited by fucoxanthin treatments in CaSki cells (Figure 4). To investigate whether fucoxanthin could improve the anti-cancer effects of TRAIL in cervical cancer cells, we treated the CaSki TRAIL-resistant cells with TRAIL or fucoxanthin alone or the combination of both. As we expected, fucoxanthin resensitized CaSki TRAIL-resistant cells, compared with CaSki parental cells (Figure 5A). Consistently, the PI3K/

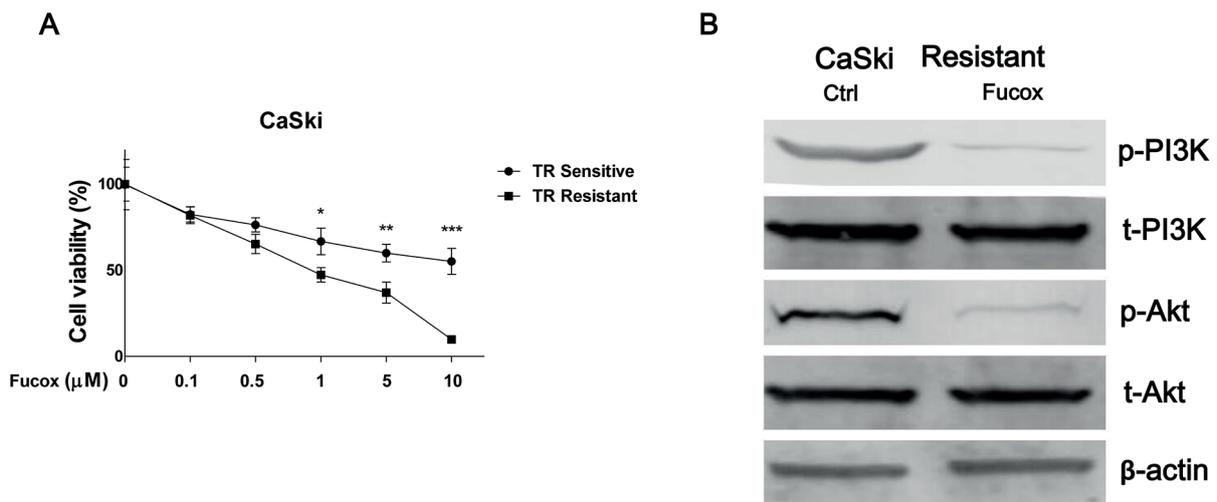


Figure 5. Fucoxanthin sensitizes TRAIL-resistant cervical cancer cells through inhibition of PI3K/Akt pathway. (A) CaSki parental and TRAIL-resistant cells were treated with fucoxanthin at 0, 0.1 0.5, 1, 5 or 10 μM for 24 h, cell viabilities were measured by MTT assay. (B) CaSki parental and TRAIL-resistant cells were treated with fucoxanthin at 0, or 0.5 μM for 24 h. The phosphorylation of Akt, total Akt, phosphorylation of PI3K, total PI3K were measured by Western blot. β-actin was a loading control. Fucox: fucoxanthin. Data are mean±SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Akt pathway of TRAIL-resistant cervical cancer cells was more significantly downregulated by fucoxanthin treatments (Figure 5B).

Combination of TRAIL and Fucoxanthin shows Synergistic Effects on Cervical Cancer Cells

To expand our results from bench to clinic, we investigated whether the combination of TRAIL and fucoxanthin has synergistically inhibitory effects on cervical cancer cells, we treated CaSki, and SiHa cells with TRAIL or fucoxanthin alone or the combination of both. Results (Figure 6A-B) demonstrated a significantly increased cytotoxicity to cervical cancer cells with the combination of both drugs. To explore the roles of TRAIL plus fucoxanthin in tumor growth, we employed an ectopic implantation model in nude mice¹⁴. CaSki cells were subcutaneously injected into posterior flanks of mice. When tumors reached about 5 mm in diameter, mice were divided into three groups (n=8) and administered per os with TRAIL or fucoxanthin alone or the combination of both. Compared with control, either TRAIL or fucoxanthin alone suppressed tumor growth (Figure 6C).

Moreover, injection of TRAIL plus fucoxanthin showed much higher inhibitory effects on tumor weights and volumes than either alone (Figure 6C). These findings demonstrated that the combination of TRAIL and fucoxanthin could suppress the growth of the cervical cancer cells-derived mice tumors *in vivo*.

Discussion

In this investigation, we compared the sensitizing effect of the fucoxanthin in combination with TRAIL on human cervical cancer cells. Although TRAIL is a widely studied anticancer agent applied in multiple cancers, recent studies have shown that human cervical cancer cells, acquired resistant to TRAIL¹⁶. Thus, identification of the therapeutic enhancing agents, which could overcome TRAIL resistance, represents an important therapeutic approach for the clinical treatments of cervical cancer. We found TRAIL sensitive cells were more resistant to fucoxanthin, a type of carotenoid found naturally in edible brown seaweed such as wakame and hijiki and TRAIL-resistant cervical cancer cells were

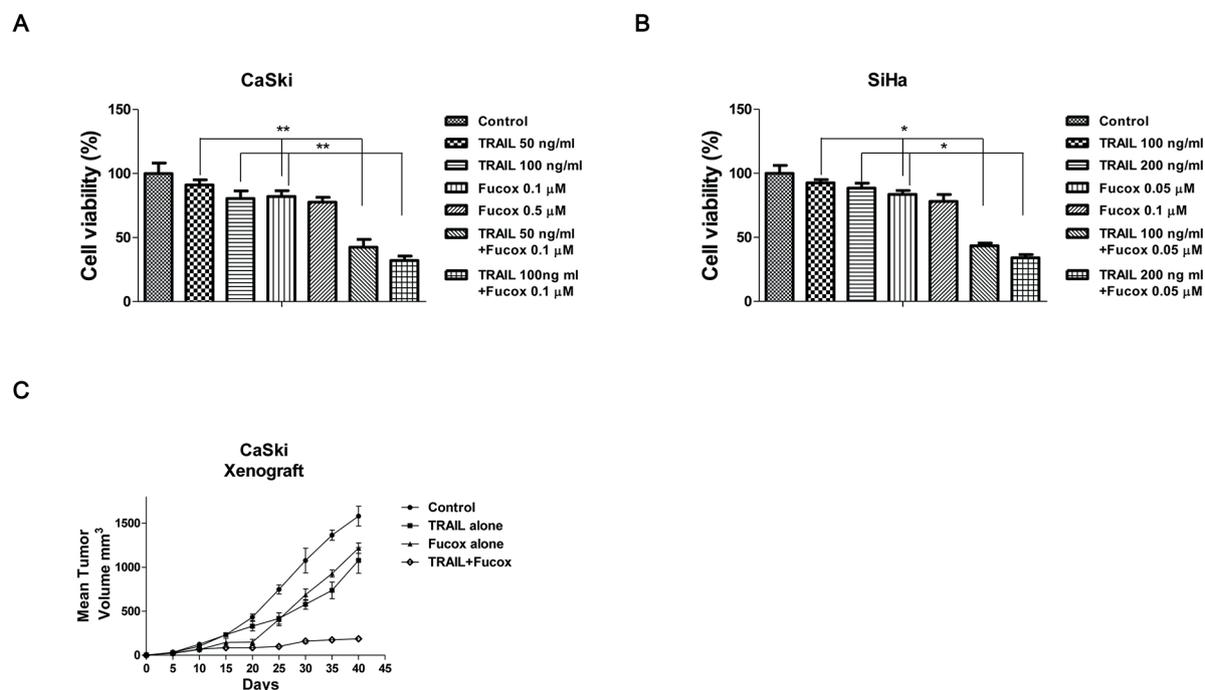


Figure 6. *In vitro* and *in vivo* synergistically inhibitory effects of the combination of TRAIL and fucoxanthin on cervical cancer. (A) CaSki or (B) SiHa cells were treated with TRAIL alone, fucoxanthin alone or the combination of TRAIL and fucoxanthin for 24 h; cell viabilities were measured by MTT assay. (C) The effect of TRAIL alone, fucoxanthin alone and the combination of both on the growth of human xenograft tumor model in mice. After ending 4-week treatment, the tumor volumes were measured. Fucox: fucoxanthin. Tumor volume=(width²×length)/2. Data are mean±SD. **p* < 0.05, ***p* < 0.01.

more sensitive to fucoxanthin, suggesting treatments of TRAIL-resistant cells with fucoxanthin might increase the sensitivity. By establishing TRAIL-resistant cells, we found CaSki TRAIL-resistant cells exhibited upregulated PI3K/Akt pathway compared with their parental cells. In addition to fucoxanthin, previous reports have demonstrated the synergistic effect of another anticancer agent genistein plus TRAIL in multiple TRAIL-resistant cancers such as hepatocellular carcinoma¹⁷, pancreatic cancer¹⁸, gastric cancer¹⁹ and malignant glioma²⁰. Moreover, a recent study demonstrated daidzein could promote the TRAIL-induced apoptosis of malignant glioma cells²¹. Our results support a new therapeutic approach using fucoxanthin plus TRAIL treatments against cervical cancer.

PI3K and its downstream molecules of Akt signaling pathways are tightly related to proliferation and survival of human tumor cells¹³. Moreover, a dysregulated activity of PI3K/Akt pathway results in malignant transformation, tumor cell migration, adhesion and tumor angiogenesis, rendering PI3K/Akt signaling pathway inhibitors as targets for therapeutic strategies being developed¹³. Here we explored the possible mechanisms of a combination of fucoxanthin and TRAIL. Our results showed, to our knowledge, for the first time, fucoxanthin could suppress the upregulated PI3K/Akt pathway in TRAIL-resistant cervical cancer cells. Consistently, a previous publication from our lab demonstrated the PI3K/Akt pathway was inhibited in HeLa cells with fucoxanthin treatments¹⁴. Also, we found the inhibited Akt phosphorylation was in a dose-dependent manner and induced caspase-dependent apoptosis in human cervical cancer cell line HeLa¹⁴.

Conclusions

Our results showed that TRAIL-resistant cervical cancer cells exhibit upregulated PI3K/Akt pathway, which could be the target effectively inhibited by fucoxanthin. The combination of TRAIL and fucoxanthin potentiates TRAIL-mediated cell death through the suppression of PI3K/Akt pathway. The findings of this study suggest that the combined use of fucoxanthin and TRAIL might be a useful strategy against TRAIL-resistant cervical cancer.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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