Original Article

Fucoxanthin induces growth arrest and apoptosis in human bladder cancer T24 cells by up-regulation of p21 and down-regulation of mortalin

Linbo Wang1, Yang Zeng1, Ye Liu1, Xuansheng Hu1, Shuhong Li1, Yuepeng Wang1,2, Ling Li1, Zhongfang Lei1, and Zhenya Zhang1*

1Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
2Natural Product Industry of Tsukuba Co., Ltd, E-26, 1187-80 (Kenkyugakuen C43-3) East Hiratuka, Tsukuba, Ibaraki 305-0812, Japan
*Correspondence address. Tel/Fax: +81-298-53-4712; E-mail: zhang.zhenya.fu@gmail.com

Fucoxanthin, a natural carotenoid, has been reported to have anti-cancer activity in human colon cancer cells, human prostate cancer cells, human leukemia cells, and human epithelial cervical cancer cells. This study was undertaken to evaluate the molecular mechanisms of fucoxanthin against human bladder cancer T24 cell line. MTT analysis results showed that 5 and 10 μM fucoxanthin inhibited the proliferation of T24 cells in a dose- and time-dependent manner accompanied by the growth arrest at G0/G1 phase of cell cycle, which is mediated by the up-regulation of p21, a cyclin-dependent kinase (CDK)-inhibitory protein and the down-regulation of CDK-2, CDK-4, cyclin D1, and cyclin E. In addition, 20 and 40 μM fucoxanthin induced apoptosis of T24 cells by the abrogation of mortalin–p53 complex and the reactivation of nuclear mutant-type p53, which also had tumor suppressor function as wild-type p53. All these results demonstrated that the anti-cancer activity of fucoxanthin on T24 cells was associated with cell cycle arrest at G0/G1 phase by up-regulation of p21 at low doses and apoptosis via decrease in the expression level of mortalin, which is a stress regulator and a member of heat shock protein 70, followed by up-regulation of cleaved caspase-3 at high doses.

Keywords  fucoxanthin; human bladder cancer T24 cells; G0/G1 phase; mortalin; p53

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Introduction

Bladder cancer is one of the most common urologic malignancies. It was estimated that there were ~70,000 cases of urinary bladder cancer in USA in 2013 [1]. Most of the malignant bladder cancers are transitional cell carcinoma which can be classified into two types: superficial bladder cancer (non-muscle invasive bladder cancer) and muscle invasive bladder cancer [2–4]. Most of the patients are suffering the non-muscle invasive bladder cancer which can be treated with endoscopic resection. However, the rate of recurrence is very high for tumors treated with resection only, and most deaths occur in patients with deeply invasive tumors and regional or distant metastases [5–8]. Many adjuvant therapies like chemotherapeutic agents have been used to cope with this problem. Traditional chemotherapeutic agents have strong systemic toxicity and low efficacy, which are intolerable and make the patients suffering [9,10]. Therefore, it is necessary to find some novel drugs and potential candidates of therapy drugs for the treatment of bladder cancer.

Fucoxanthin is found in edible brown algae such as Undaria pinnatifida, which together with β-carotene is one of the most abundant carotenoids in nature [11]. It has been reported to induce apoptosis in several human cancer cell lines such as PC-3 [12], colon cancer Caco-2 [13], and leukemia HL-60 cells [14] through down-regulating Bel-2 expression. In addition, fucoxanthin was found to inhibit the growth of tumor cells (hepatic carcinoma HepG2, colon adenocarcinoma WiDr, prostate cancer DU145 and leukemia cancer HL-60 cells), induce cell cycle arrest at the G1 phase by cyclin D, p21WAF1/Cip1, and MAPK regulation, and inactivate the Bel-xl pathway by the generation of reactive oxygen species (ROS), respectively [15–17]. Our previous research indicated that fucoxanthin can induce the apoptosis of human bladder cancer EJ-1 cells [18], which was identified to be the same cell line as T24 cells [19]. However, the inhibitory mechanism of fucoxanthin against human bladder cancer cells is still unclear.

On the other hand, p53 is a transformation-related protein and a cellular protein that accumulates in the nuclei of cancer cells and binds tightly to the simian virus 40 large T antigens. Well known as the ‘guardian of the genome’, p53 is multifunctional and regarded to play a direct role in DNA repair, cell cycle arrest, and cellular apoptotic initiation [20]. It is estimated that >50% of human cancers correlate with...
mutation of p53 [21]. p53 augmentation is also associated with aging-related senescence in multiple organisms mostly due to its role in terminal cell cycle arrest [22]. It was also reported that p53 protein would localize to the mitochondrion during stress and directly influence mitochondrial-derived cellular apoptosis [23]. Mortalin, a member of heat shock protein 70 (Hsp70) first identified in 1993, was reported to be able to interact with and against tumor suppressor p53, induce the ROS signaling, induce cell differentiation, etc. [24]. Mortalin is predominantly present in mitochondria and involved in mitochondrial import, control of membrane permeability, and ROS production [25]. Most recently, Wadhwa et al. [26] pointed out that p53 also exists in the mitochondria and interacts with mortalin/mtHsp70, Bcl-2, and Hsp60, and these interactions are involved in p53-mediated apoptosis independent of its nuclear function.

Generally speaking, abrogation of mortalin–p53 interaction leads to the translocation and transcriptional p53 function, which results in the inhibition on cancer cells. Transcriptional p53 induced apoptosis and cell cycle arrest in G0/G1 phase on cancer cells. Fucoxanthin has been reported to regulate the p53 expression level by cell cycle arrest and apoptosis in several human cancer cell lines [27]. Thus, it is speculated that fucoxanthin might induce cell cycle arrest and apoptosis in human bladder cancer cells, providing a potential new candidate of therapy drugs for the treatment of human bladder cancer. Up to now, however, no information could be found in the literature.

This study aims to investigate the effect of fucoxanthin on cell proliferation in human bladder cell line T24 and examine the cell cycle regulatory proteins and mortalin–p53 interaction. The involved molecular mechanisms were also discussed.

Materials and Methods

Cell culture and treatment
T24 cells from American Type Culture Collection (ATCC, Manassas, USA) were grown in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS; Sigma) at 37°C in an atmosphere of 5% CO2 and 95% air in a humidified incubator (Astec, Fukuoka, Japan).

Cytotoxicity assay
Fucoxanthin (Sigma) was dissolved in dimethylsulfoxide (Sigma) and stored at −20°C. The effect of fucoxanthin on cell proliferation was analyzed using the MTT assay kit purchased from Roche (Tokyo, Japan). In detail, T24 cells were seeded in 96-well plate at 5 × 104 cells/ml. After 24 h incubation, T24 cells were treated with different concentrations of fucoxanthin (5, 10, 20, and 40 μM) for 24, 48, or 72 h, and the medium was removed, then 100 μl of phosphate buffered saline (PBS) and 10 μl of MTT labeling reagent was added to each well according to the manufacturer’s instructions. After 4 h of incubation, 100 μl of the solubilization solution was added to each well and mixed thoroughly using a pipette, and the plate was incubated in humidified atmosphere for overnight. An iMark microplate reader (Bio-Rad, Hercules, USA) was used to measure the spectrophotometric absorbance of the sample at wavelength of 570 nm.

Colonies assay
T24 cells were seeded at 500 cells/well into 6-well culture plate, in which three replicate wells were used for control and treatment (20 and 40 μM fucoxanthin for 48 h, respectively) and left to form colonies in the next 15 days with a regular change of medium every day. Colonies were fixed in methanol, stained with 0.1% crystal violet solution (Sigma), photographed by a scanner (Canon, Tokyo, Japan) and counted.

Cell cycle assay
T24 cells were seeded at 1 × 105 cells/ml into 6-well plate and treated with 5 and 10 μM fucoxanthin for 48 h when the cell confluences reached around 70%. The cell pellets were harvested by centrifugation at 1500 g for 10 min and then suspended into 250 μl of cold PBS. The cells were fixed in 500 μl of 70% alcohol for at least 2 h at −20°C. The fixed cells were washed with PBS and incubated with 20 μg/ml propidium iodide (PI) (Dojindo, Tokyo, Japan) in PBS for 30 min at 4°C in the dark. Guava PCA PLATFORMS system (Merck Milipore, Billerica, USA) was used for cell cycle assay. The data were analyzed by Flowjo software (Tree Star, Ashland, USA).

Nuclear staining and morphological study
When T24 cells cultured in 6-well plate were treated with 20 μM fucoxanthin for 24 and 48 h, respectively, nuclear staining analysis was performed. In brief, T24 cells were fixed with 4% paraformaldehyde for 15 min and then treated with 0.1% Triton X-100 for 30 min. After PBS washing, cells were treated with 2 μg/ml Hoechst 33342 solution for 15 min at room temperature.

In order to acquire quantified apoptosis data, a mass of nuclear staining images were automatically obtained by IN Cell Analyzer 1000 (GE Healthcare UK Ltd, Buckinghamshire, UK). The fluorescent microphotographs of nine fields were taken automatically for each well. Hoechst-positive nuclei were recognized by IN Cell Developer Software (GE Healthcare UK Ltd). Parameters such as nuclei shape and nuclei fluorescence signal density for each well were calculated. Viable cells and apoptotic cells were classified by these parameters, and cells with condensed nuclei were considered as apoptosis. The cell numbers were counted.
using the IN Cell Developer Tool Box 1.7 (GE Healthcare UK Ltd).

**Western blot analysis**

The antibodies anti-p53 DO-1, CDK-2, CDK-4, cyclin D1, cyclin E, p21, caspase-3, and cleaved caspase-3 were purchased from Santa Cruz (Santa Cruz, USA), anti-β-actin from Chemicon (Billerica, USA), and anti-mortalin from Sigma. T24 cells were seeded in 6-well plate and treated with fucoxanthin when cells were well attached. Treatment of 5 and 10 μM fucoxanthin was used for determining the expression levels of CDK-2, CDK-4, cyclin D1, cyclin E, and p21, and 20 and 40 μM fucoxanthin was used for determining the expression levels of p53, mortalin, caspase-3, and cleaved caspase-3. After 48 h, cells were lysed in radio immunoprecipitation assay lysis buffer. The protein sample (20 μg) separated on 10% sodium dodecyl sulfate polyacrylamide gel (Sigma) was electroblotted onto a nitrocellulose membrane (BA85; Schleicher and Schuell, Dassel, Germany) using a semidry transfer blotter (Bio-Rad). Immunoassays were done with anti-p53 DO-1, anti-mortalin, anti-caspase-3, anti-cleaved caspase-3, anti-CDK-2, anti-CDK-4, anti-cyclin D1, anti-cyclin E, anti-p21, and anti-β-actin antibodies, which were all diluted into 1 : 1000. Relative expression level was normalized to β-actin by using software Image J (National Institutes Health, Bethesda, USA).

**Immunostaining**

Cells were grown to ~70% confluency on coverslips in 12-well culture plate, and then treated with 40 μM fucoxanthin. After 48 h, the cells were washed with cold PBS and fixed with a pre-chilled methanol/acetone (1 : 1, v/v) mixture for 10 min. Fixed cells were washed with cold PBS twice, permeabilized with 0.2% PBS-T (Sigma) (Triton X-100 in PBS) for 10 min, and blocked with 2% bovine serum albumin (Sigma) in PBS for 30 min. Cells were probed with anti-p53 and anti-mortalin primary antibodies on coverslips. The antibody staining was visualized with secondary antibodies donkey anti-rabbit IgG (Alexa Fluor 488-conjugated) and goat anti-mouse IgG (Alexa Fluor 594-coujugated). After three washes in PBS-T, the cells on coverslips were overlaid onto frosted slides with Fluoromount (Difco; Becton, USA). The cells were examined on a Carl Zeiss Microscope (Axiovert 200M; Zeiss, Jane, Germany).

**Statistical analysis**

All experiments were performed at least three times. Statistical difference in multiple groups was determined by one-way analysis of variance with Graphpad Prism 5 software (San Diego, USA). *P < 0.05, **P < 0.01, and ***P < 0.001 were considered of statistically significant difference.

**Results**

**Inhibitory effect of fucoxanthin on proliferation of T24 cells**

Fucoxanthin treatment induced a reduction on the cell viability of T24 cells in a dose- and time-dependent manner (Fig. 1A). The viability of T24 cells was not altered by incubation with 40 μM fucoxanthin for 24 h, but was reduced to 55.1% ± 6.27% at 48 h, and to 15.6% ± 4.28% at 72 h compared with the control. The analysis of colony-forming efficiency, a reliable indicator of rapid in vitro growth and colony forming characteristic of T24 cells, also showed a reduction of proliferation of T24 cells with 20 and 40 μM fucoxanthin for 15 days incubation compared with the control (Fig. 1B). The colony numbers were decreased to 94.48% ± 1.55% and 85.93% ± 4.10% at 20 and 40 μM fucoxanthin treatment, respectively (Fig. 1C). These results indicated that fucoxanthin could inhibit the proliferation of T24 cells in a dose- and time-dependent manner.

**Fucoxanthin induces cell cycle arrest at G0/G1 phase**

G1 phase plays an important role on cell cycle and it determines whether a cell commits to division or to leave the cell cycle [28]. The effect of fucoxanthin on cell cycle was investigated by flow cytometry in PI-stained T24 cells. It was found that the accumulation of G0/G1 phase was significantly increased by the incubation with 5 and 10 μM fucoxanthin for 48 h as compared with control, while the G2/M and S phase accumulations were greatly decreased, and the sub-G1 phase was not altered (Fig. 2A,B).

p21, cyclin-dependent kinases (CDKs), and cyclins play a crucial role in the regulation of the cell cycle [29], so the effect of fucoxanthin on the expressions of these factors was investigated. It was found that 5 and 10 μM fucoxanthin decreased CDK-2, CDK-4, cyclin D1, and cyclin E after 48 h incubation, while 5 and 10 μM fucoxanthin could significantly increase the expression level of p21 (Fig. 2C). These results indicated that the low doses (5 and 10 μM) of fucoxanthin induced the inhibitory effect via cell cycle arrest at G0/G1 phase.

**Induction of apoptosis by fucoxanthin treatment**

The cell nuclei were stained with Hoechst 33342 and then classified according to their morphology. Remarkable increase of concentrated nuclei of T24 cells was observed after 24 and 48 h incubation with 20 μM fucoxanthin (Fig. 3A). Quantified data showed that 20 μM fucoxanthin induced significant increase of apoptotic cell numbers to 40.27% ± 2.33% after 48 h incubation (Fig. 3B).

Caspase-3 is one of the known effector caspases that, once activated, irreversibly executes cell death through...
inducing apoptosis on cells [30]. The effect of fucoxanthin on the expression of caspase-3 and cleaved caspase-3 was investigated by western blotting analysis. It was found that 20 and 40 μM fucoxanthin significantly increased cleaved caspase-3 in a dose-dependent manner after 48 h incubation (Fig. 4B). These results suggested that fucoxanthin induced apoptosis on T24 cells at high doses (20 and 40 μM).

Abrogation of mortalin–p53 complex and reactivation of nuclear p53 tumor suppression protein by fucoxanthin p53, the ‘guardian of the genome’, is a major player in the cell apoptosis in response to the diverse endogenous and exogenous stress signals [31]. Mortalin binds to the mutant p53 in cancer cells and inhibits its apoptotic functions. It thus acts as an anti-apoptotic factor contributing to the continued survival of cancer cells [32,33]. We found that after 48 h incubation, 40 μM fucoxanthin significantly inhibited the expression of mortalin, while 20 and 40 μM fucoxanthin up-regulated the expression of p53 in dose-dependent manner as revealed by western blot assay (Fig. 4B). After fucoxanthin treatment, the expression of p53 was increased in T24 cell nuclei and released from the mortalin–p53 complex. These results were further confirmed by visualization of p53 (green) and mortalin (red) by immunocytochemistry in T24 cells incubated with 40 μM fucoxanthin for 48 h. It was found that after 40 μM fucoxanthin treatment, mortalin (red) showed strong decrease and p53 (green) showed strong increase in the nuclei of T24 cells (Fig. 4A). These results suggested that fucoxanthin induced apoptosis in T24 cells by the abrogation of mortalin–p53 complex and the reactivation of p53 followed by the up-regulation of cleaved caspase-3.

**Discussion**

To the best of our knowledge, this paper is the first to report that low dose (5 and 10 μM) of fucoxanthin inhibited the proliferation via fucoxanthin-induced G0/G1 growth arrest through up-regulation of p21 followed by down-regulation of CDK-2, CDK-4, cyclin D1, and cyclin E, and high dose (20 and 40 μM) of fucoxanthin induced apoptosis through reactivation of p53 tumor suppression protein by abrogation.
of mortalin–p53 complex followed by up-regulation of cleaved caspase-3 in T24 cells.

The results of this study demonstrated that fucoxanthin inhibited the proliferation of T24 cells in a dose- and time-dependent manner. In the results of MTT assay, the decreasing tendencies of proliferation of T24 cells with 5 and 10 μM fucoxanthin treatment were gentle, while the decreasing tendencies of proliferation with 20 and 40 μM fucoxanthin treatment were rapid. Therefore, with respect to cell cycle analysis, nuclear staining, western blot analysis, and immunostaining, results suggested that low dose (5 and 10 μM) of fucoxanthin could inhibit the expression of CDK-2, CDK-4, cyclin D1, cyclin E, and p21 in T24 cells and obviously induced cycle arrest at G0/G1 phase, which is a critical role in anti-proliferation of T24 cells [38]. In addition, low dose (5 and 10 μM) of fucoxanthin was observed to induce growth arrest at G0/G1 phase on SK-Hep-1 cells. It was found that the same dose of fucoxanthin could induce growth arrest of T24 cells [39].


It was reported that CDKs could regulate the progression of cell cycle. Human cells contain more than nine CDKs. CDK-2 and CDK-4 are directly involved in G1/S transition [36]. Inhibition of CDK-2 and CDK-4 could contribute to the phosphorylation of cyclin E and cyclin D, thus cell cycle arrest at G0/G1 phase [37]. In this study, we found that low dose (5 and 10 μM) of fucoxanthin could inhibit the expression of CDK-2, CDK-4, cyclin D1, cyclin E, and p21 in T24 cells and obviously induced cycle arrest at G0/G1 phase, which is a critical role in anti-proliferation of T24 cells [38]. In addition, low dose (5 and 10 μM) of fucoxanthin was observed to induce growth arrest at G0/G1 phase on SK-Hep-1 cells. It was found that the same dose of fucoxanthin could induce growth arrest of T24 cells [39]. Fucoxanthin could up-regulate p21WAF/Cip1 resulting in G0/G1 phase arrest from 25 to 70 μM on HCT116 cells. We also claimed that fucoxanthin induced growth arrest of T24 cells by up-regulation of p21 protein at 5 and 10 μM. Therefore, the growth arrest effect of fucoxanthin on T24 cells is much stronger than that on human colon HCT116 cells [34].
High dose (20 and 40 μM) of fucoxanthin was determined to have significant cytotoxicity on T24 cells by MTT assay. Through morphological observation with Hochezt 33342 staining and endpoint apoptosis analysis, the apoptotic phenotype of T24 cells was observed with high dose of fucoxanthin. This study focused on both fucoxanthin-induced cell cycle arrest at G0/G1 phase and fucoxanthin-induced apoptosis, and the expression levels of p53, caspase-3, cleaved caspase-3 were determined, which are relative to apoptosis of cells [30,33,36]. High dose of fucoxanthin was found to induce apoptosis on T24 cells by increasing the expression level of p53 and cleaved caspase-3. The mitochondrial mortalin can interact with and against Bcl-2 family proteins and p53 to mediate apoptosis on cells [25]. Mortalin is a chaperone that can negatively combine with p53 [33,36].

Then attempts were made to determine whether fucoxanthin-induced apoptosis is dependent on the suppression of mortalin. Our results showed that fucoxanthin-induced apoptotic T24 cells were accompanied by low expression level of mortalin followed by increase of p53. Thus, it is believed that mortalin might be the mediated pathway of fucoxanthin-induced apoptosis of T24 cells. A previous report indicated that wild-type p53 initiated apoptosis if DNA damage was irreparable [40]. Interestingly, T24 cells were found to contain p53 mutant having an in-frame deletion of tyrosine [41]. T24 cells are with mutant-type p53 which exist in half of human cancers and do not represent only the mere loss of wild-type p53 tumor suppressor activity [41], but high dose of fucoxanthin treatment can lead to nuclear translocation and transcriptional reactivation of p53 function, resulting in apoptosis of T24 cells. In addition, mutant p53 could be activated by releasing p53 into nuclei from ubiquitin-like-domain-containing protein—mortalin complex, which could induce apoptosis on HT-29 cells [42]. Therefore, we deduced that the fucoxanthin-induced apoptosis of T24 cells in this study was attributable to p53 gene reparation or the transcriptional active mutant-type p53.

Considering the safety of fucoxanthin, previous researchers stated that fucoxanthin and its metabolites fucoxanthinol had less adverse effect on normal and uninfected cells both in vitro and in vivo [43,44]. Beppu et al. [45] conducted a single-dose toxicity study at doses of 1000 and 2000 mg/kg and a repeated oral dose toxicity with 500 and 1000 mg/kg for one month on 93% fucoxanthin in Imprinting Control Region mice. No mortalities and no abnormalities were found in gross appearance in these two studies, and no abnormalities were observed in liver, kidney, spleen, and gonadal tissues induced by fucoxanthin. These studies suggested that fucoxanthin is a safe compound under the experimental conditions [45,46]. In addition, the metabolic products of fucoxanthin in human could be deacetylated into fucoxanthinol at intestinal epithelium. Research also showed that fucoxanthin could be hydrolysed into fucoxanthinol during the uptake by differentiated cell lines and a tissue culture model. The resultant fucoxanthinol can be traced into the blood circulation system in mammals, which was reported to have higher bioavailability in body than fucoxanthin [47–49].

Our previous research indicated that fucoxanthin had inhibitory effect on the proliferation of human bladder cancer EJ-1 cells by inducing apoptosis [18]. This study disclosed the mechanism of fucoxanthin-induced inhibitory effect. In conclusion, our data revealed that T24 cells were greatly sensitive to proliferation inhibition and apoptosis induction by fucoxanthin. Low dose (5 and 10 μM) fucoxanthin-induced proliferation inhibition was associated with growth arrest at G0/G1 phase via up-regulation of p21 followed by down-regulation of CDK-2, CDK-4, cyclin D1 and cyclin E. On the other hand, high dose (40 μM) fucoxanthin-induced
apoptosis contributed to down-regulation of Hsp70/mortalin, which also released p53 from cytoplasm to nuclear translocation, meanwhile the fucoxanthin-induced apoptosis was followed by up-regulation of cleaved caspase-3. Hence, these results demonstrated that fucoxanthin might be a potential candidate of therapy drugs for the treatment of bladder cancer.

References

Fucoxanthin induced growth arrest and apoptosis on T24 cells


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