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Astaxanthin Inhibits Proliferation of Human Gastric Cancer Cell Lines by Interrupting Cell Cycle Progression

Jung Ha Kim*, Jong-Jae Park*, Beom Jae Lee*, Moon Kyung Joo*, Hoon Jai Chun†, Sang Woo Lee‡, and Young-Tae Bak*

*Division of Gastroenterology, Department of Internal Medicine, Korea University Guro Hospital, Korea University College of Medicine, †Division of Gastroenterology, Department of Internal Medicine, Korea University Anam Hospital, Korea University College of Medicine, Seoul, and ‡Division of Gastroenterology, Department of Internal Medicine, Korea University Ansan Hospital, Korea University College of Medicine, Ansan, Korea

Background/Aims: Astaxanthin is a carotenoid pigment that has antioxidant, antitumoral, and anti-inflammatory properties. In this *in vitro* study, we investigated the mechanism of anticancer effects of astaxanthin in gastric carcinoma cell lines. **Methods:** The human gastric adenocarcinoma cell lines AGS, KATO-III, MKN-45, and SNU-1 were treated with various concentrations of astaxanthin. A cell viability test, cell cycle analysis, and immunoblotting were performed. **Results:** The viability of each cancer cell line was suppressed by astaxanthin in a dose-dependent manner with significantly decreased proliferation in KATO-III and SNU-1 cells. Astaxanthin increased the number of cells in the G0/G1 phase but reduced the proportion of S phase KATO-III and SNU-1 cells. Phosphorylated extracellular signal-regulated kinase (ERK) was decreased in an inverse dose-dependent correlation with astaxanthin concentration, and the expression of $p27^{kip-1}$ increased the KATO-III and SNU-1 cell lines in an astaxanthin dose-dependent manner. **Conclusions:** Astaxanthin inhibits proliferation by interrupting cell cycle progression in KATO-III and SNU-1 gastric cancer cells. This may be caused by the inhibition of the phosphorylation of ERK and the enhanced expression of $p27^{kip-1}$. **(Gut Liver 2016;10:369-374)**

Key Words: Astaxanthin; Human gastric adenocarcinoma; Proliferation; Extracellular signal-regulated kinase; $p27^{kip-1}$

INTRODUCTION

Astaxanthin is a pinkish-orange carotenoid pigment that belongs to the xanthophylls family. It comprises the color of the crustacean, salmonid, and algae. Astaxanthin has antioxidant, photo-protective, antitumor, anti-inflammatory, and cardio-protective properties, with potential benefits for humans.[1,2] Astaxanthin is obtained from the green microalgae *Haematococcus pluvialis*, the red yeast *Phaffia rhodozyma*, and crustacean byproducts such as crab, shrimp, and lobster. Synthetic astaxanthin is commercially available as a chemical substance. It is commercially available as a dietary supplement.[2]

Free radicals and highly reactive oxygen species are produced after stimulation by air pollution, physiological stress, exposure to harmful chemicals, or exposure to ultraviolet (UV) light. These stresses can damage DNA, protein structures, and lipid membranes. This drives aging, inflammation, and carcinogenesis. Astaxanthin may have protective effects against diseases caused by oxidation.[3,4]

Effects of astaxanthin in gastric inflammation have been reported. In *Helicobacter pylori* infected mice, astaxanthin treatment can reduce the bacterial load and gastric inflammation by shifting the T lymphocyte response from the Th1-response to the Th1/Th2-response. Significant upregulation of CD4 and downregulation of CD8 in astaxanthin treated patients with *H. pylori* infection has been described.[3,5-7]

Previous investigators studied the effect of astaxanthin on various types of cancers in mammals. Astaxanthin inhibits carcinogenesis of the urinary bladder cancer.[8] In another study, rats fed with astaxanthin along with carcinogen had a significantly lower incidence of cancer in their oral cavity than the group of rats fed only with carcinogen.[9] Astaxanthin reportedly decreases the incidence of colon cancer in rats.[10] Dietary astaxanthin is also effective against breast cancer.[11] Astaxanthin could suppress cellular growth of prostate cancer by inhibiting the action of 5-α-reductase, and it also may attenuate liver

Correspondence to: Jong-Jae Park
Division of Gastroenterology, Department of Internal Medicine, Korea University Guro Hospital, Korea University College of Medicine, 148 Gurodong-ro, Guro-gu, Seoul 152-703, Korea
Tel: +82-2-2626-1027, Fax: +82-2-854-8453, E-mail: gi7pjj@yahoo.co.kr
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metastasis induced by stress in mice through the inhibition of lipid peroxidation.[12] Overall, the basis of the anticancer activity of astaxanthin might be that carotenoid slows growth of cancer cell by affecting cell communication at gap junction and by the modulation of immune systems.

There has been no research concerning the anticancer effect of astaxanthin in human gastric adenocarcinoma. This study was performed to evaluate the effect of astaxanthin on the growth of gastric cancer cell lines and to investigate the mechanism of anticancer properties of astaxanthin.

MATERIALS AND METHODS

1. Cell culture and reagents

Human gastric adenocarcinoma cell lines AGS, KATO-III, MKN-45, and SNU-1 were purchased from Korean Cell Line Bank (Seoul, Korea). All four cell lines were maintained in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin G and 100 µg/mL streptomycin, and were maintained at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. Astaxanthin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in dimethyl sulfoxide (DMSO) solution and stored at 4°C.

2. Assessment of proliferation of cancer cell lines

The effect of astaxanthin on cell viability in gastric cancer cell lines was investigated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (methyl-tetra-zolium, MTT; Sigma- Aldrich).[13] Cells were washed with antibiotic-free culture medium in wells of a 96-well plate. Each cell line was exposed to 0, 10, 50, and 100 µM astaxanthin in 1% DMSO for 24 hours in the culture medium. After the removal of culture medium, 50 µL of MTT solution (2 µg MTT per 1 mL phosphate buffered saline) was added to each well and incubated for 4 hours at 37°C. The absorbance was indicated on an enzyme-linked immunosorbent assay reader at 490 nm. Cell viability (%) was calculated using the following formula:

% Survival=(sample optical density [OD]–total OD)/
(spontaneous OD–total OD)×100

3. Flow cytometric analysis of cell cycle status

The cell lines were serum-starved for 24 hours and then incubated in culture medium with 0, 10, 50, or 100 µM astaxanthin for 24 hours. All gastric cancer cells were reversibly blocked with aphidicolin and subsequently rinsed and replated. Cells were sampled and incubated with propidium iodide and RNAse (Cycle Test Plus; Becton Dickinson, Franklin Lakes, NJ, USA). Cell cycle distribution was analyzed using a flow cytometer (Becton Dickinson). Cytofluorometric analysis was carried out using CellQuest software (Becton Dickinson). The percentage of cells in G0/G1, S and M phases was determined using Modfit software version 2.0 (Becton Dickinson).[14]

4. Assessment of cell cycle machinery protein by immunoblotting

Specific primary antibody to phospho-extracellular signal-regulated kinase (p-ERK, sc-7383), phospho-serine/threonine-specific protein kinase (p-Akt, sc-135650), $p27^{Kip-1}$ (sc-528), phospho-retinoblastoma protein (p-Rb, sc-169028) and cyclin D1 (sc-753), and horseradish peroxidase IgG as the secondary antibody were purchased from Santa-Cruz Biotechnology (Santa Cruz, CA, USA). Each cultured cell line was lysed on ice in 0.5 mL of a solution containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 5 mM NaF, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM Na_3VO_4, and 1% protease inhibitor cocktail. The lysates were centrifuged at 15,000 *g* for 15 minutes at 4°C. The resulting supernatants were subjected to 10% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a nitrocellulose membrane, which was exposed to a blocking buffer (20 mM Tris-HCl [pH 7.4], 5% skim milk, and 0.1% Tween 20) for 1 hour at room temperature. The separated proteins were incubated overnight with antibody to p-ERK, p-Akt, $p27^{Kip-1}$, p-Rb or cyclin D1 diluted 1:1,000 in blocking buffer. The membrane was rinsed with washing buffer (20 mM Tris-HCl [pH 7.4] and 0.1% Tween 20), incubated for 1 hour at room temperature with horseradish peroxidase-conjugated IgG secondary antibody diluted 1:1,000 in blocking buffer, washed again, incubated with electrochemiluminescence plus detection reagent (PerkinElmer, Waltham, MA, USA) for 5 minutes, and

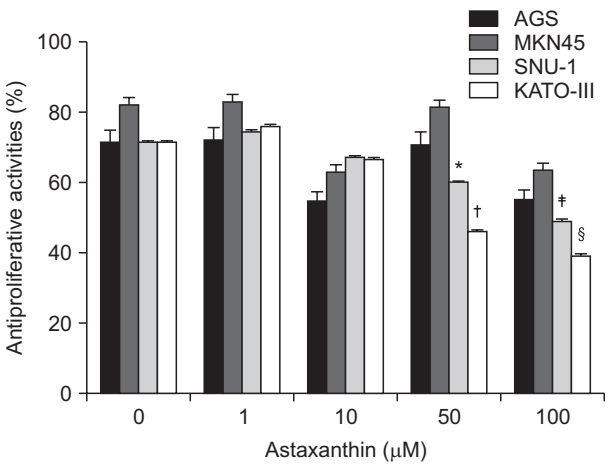

Fig. 1. Cell proliferation assay using gastric cancer cell lines. The cells were starved for 24 hours and then exposed to the vehicle or to 1, 10, 50, or 100 µM astaxanthin for 48 hours. The rate of cell proliferation was measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Preincubation with astaxanthin resulted in a dose-dependent inhibition of cell proliferation in KATO-III and SNU-1 cells. However, there was no significant effect on cell viability in AGS and MKN-45. *p=0.046; †p=0.038; ‡p=0.041; §p=0.031, versus vehicle alone.

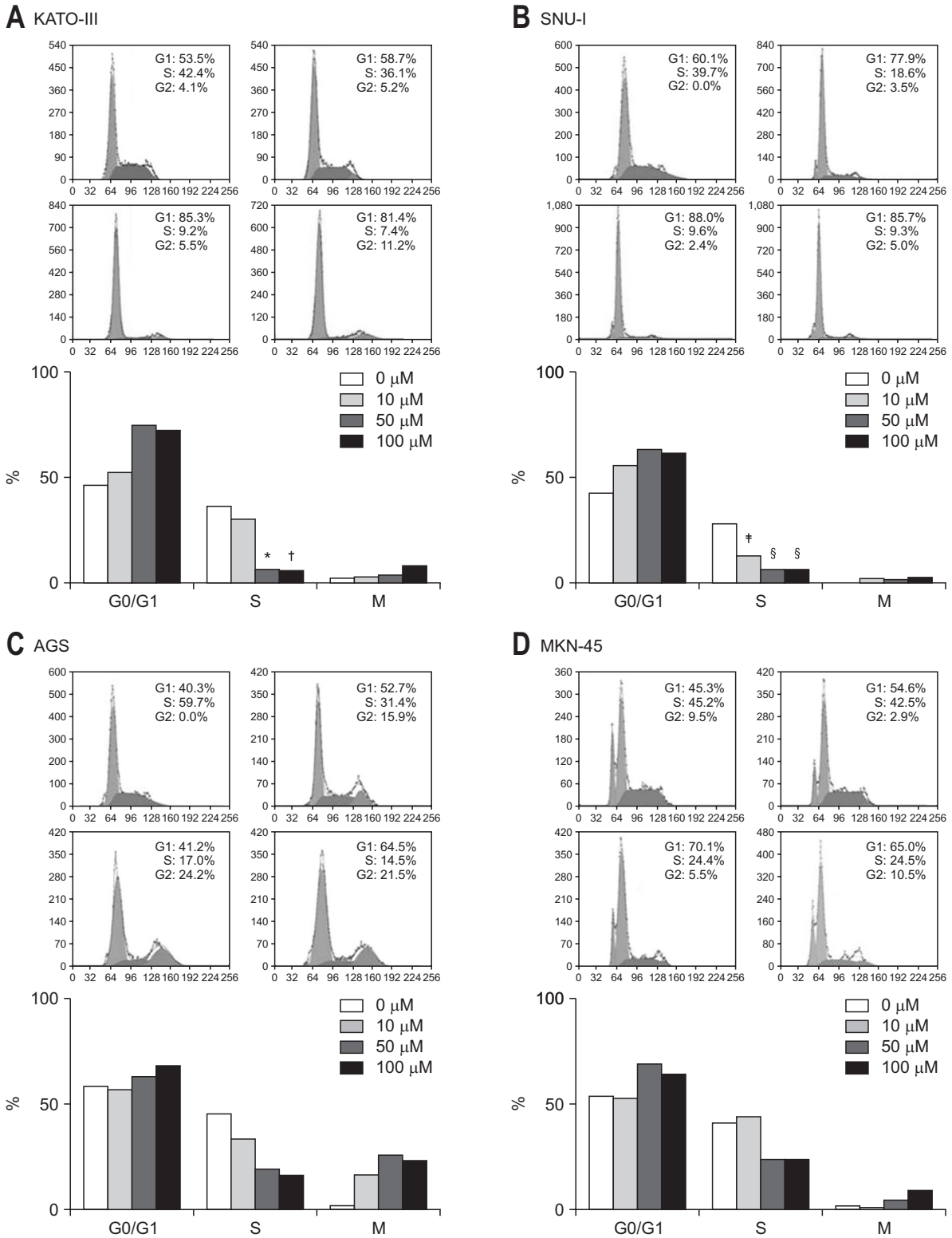

Fig. 2. Cell-cycle progression analysis. (A) KATO-III, (B) SNU-1, (C) AGS, and (D) MKN-45 were serum starved for 24 hours and then preincubated with astaxanthin for 48 hours. The cells were collected and stained with 0.5 mg/mL propidium iodide, and the cell fluorescence was measured with a FACScan apparatus. Compared with the control cells, the cells exposed to astaxanthin showed an increase in the number of cells in the G0/G1 phase and a decrease in the S phase at higher concentrations (50 and 100 μM). Preincubation with astaxanthin decreased the number of cells in the S phase, which indicated the induction of cell-cycle arrest at G0/G1. *p=0.015; †p=0.010; ‡p=0.046; §p=0.021, versus vehicle alone (each group for n=3 experiments).

exposed to film.[15]

5. Statistical analysis

Statistical significance was investigated by the Mann-Whitney U test. Differences were considered statistically significant at a p-value <0.05.

RESULTS

1. Astaxanthin inhibits proliferation of KATO-III and SNU-1 cells

The gastric cancer cell lines were cultured in the presence of different concentrations of astaxanthin. A modified MTT assay was performed to examine the effects of astaxanthin on cell proliferation. The ratio of proliferation of gastric cancer cells in medium with astaxanthin depended on the sensitivity of cancer cells and the concentration of the drug. Dose-dependent inhibition of proliferation of KATO-III and SNU-1 cells was evident. Astaxanthin at 50 and 100 μM significantly suppressed cell proliferation as compared with control, although it could not reach IC50 even under 100 μM. Viability of AGS and MKN-45 cells was unaffected (Fig. 1).

2. Astaxanthin induces cell cycle arrest in the G0/G1 phase in KATO-III and SNU-1 cells

Fluorescence activated cell sorting analysis was done to ascertain the cell cycle distribution of each cell line. In KATO-III and SNU-1 cell, the percentage of cells arresting in the G0/G1 phase increased with higher concentrations of astaxanthin (50 and 100 μM). However, astaxanthin did not affect cell cycle of AGS and MKN-45 cell lines. From these results, we considered that cell cycle arrest had occurred at the G0/G1 phase in KATO-III and SNU-1 cell lines, and that astaxanthin suppressed cell proliferation at the S phase in KATO-III and SNU-1 (Fig. 2).

3. High concentrations of astaxanthin modulate p-ERK level in KATO-III and SNU-1 cells

ERK and Akt have key roles in cell proliferation and survival of cells including human gastric cancer. We evaluated the level of p-ERK and p-Akt in KATO-III and SNU-1 cells according to the concentration of astaxanthin, to determine whether astaxanthin could influence the intracellular signaling pathway. The proliferation of KATO-III and SNU-1 cells was significantly suppressed by astaxanthin administration in a dose-dependent manner. Astaxanthin at 100 μM inhibited the p-ERK level, indicating the inhibition of protein synthesis and suppression of cell proliferation.[16] However, p-Akt was unaffected by astaxanthin treatment (Fig. 3).

4. Astaxanthin regulates $p27^{Kip-1}$ protein level in a dose-dependent manner in KATO-III and SNU-1 cells

KATO-III and SNU-1 cells were incubated with vehicle or 10, 50, 100, or 200 μM astaxanthin for 24 hours. The protein level of $p27^{Kip-1}$, an inhibitor of cyclin dependent kinases, increased in a dose-dependent manner in both cell lines. However, p-Rb and cyclin D1 were not influenced by astaxanthin (Fig. 3).

DISCUSSION

Chemoprevention using materials from natural products may be an ideal treatment modality of cancer due to their relatively inexpensive cost, ready availability, and decreased toxicity. Preexisting experimental studies using antioxidants or extracts from plants have shown blockade of specific pathways involving carcinogenesis, but clinical data and large scale cohort studies have proven disappointing.[17] Organic foods appear to

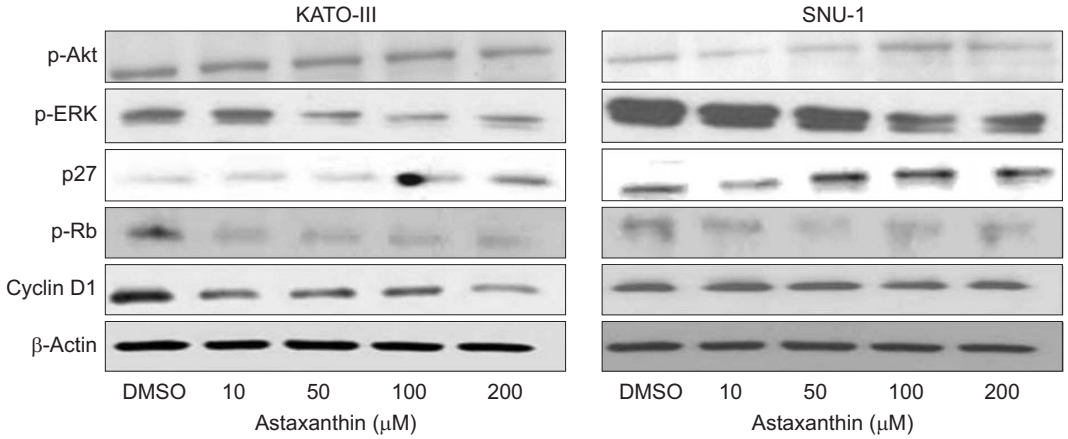

Fig. 3. Effects of astaxanthin on the cell proliferative and cell cycle machinery profiles. KATO-III and SNU-1 cells were treated with and without astaxanthin (0, 10, 50, 100, and 200 μM) for 24 hours. Western blots showed decreased p-ERK at 50 and 100 μM in both cell lines. Nevertheless, the lack of effect of astaxanthin on the activation of p-Akt was observed in both cell lines. Astaxanthin upregulated $p27^{kip-1}$ in dose-dependent manners in both cell lines. The cyclin D1 and p-Rb protein levels were not affected by astaxanthin (each group for n=3 experiments).
p-Akt, phospho-serine/threonine-specific protein kinase; p-ERK, phospho-extracellular signal-regulated kinase; p-Rb, phospho-retinoblastoma protein; DMSO, dissolved in dimethyl sulfoxide.

possess many tumor suppression mechanisms of organic foods; the mechanisms of chemoprevention through analysis of cell signaling pathways have been studied. Most of the studies have aimed to discover the tumor suppression effect using natural products. However, the structural diversity makes it difficult to assess the exact mechanism.[18] From these natural products, many important dietary antioxidants including vitamin C, E, carotenoid, and selenium have been identified and studied. The results concerning cancer prevention have been inconsistent. Still, developing antitumor agents with these natural substances would carry the advantage of reduced side effects compared to chemical compounds.

Astaxanthin has various effects including antioxidation, protection from UV light, anti-inflammation, and anticancer activities.[19,20] The anticancer property of astaxanthin has received much attention. Several previous studies demonstrated that astaxanthin could prevent carcinogenesis of colon, urinary bladder, prostate, breast, and oral cavity.[8-12] However, the exact mechanism of carcinogenesis remains unknown. This study focused on the effect of astaxanthin on the proliferation and survival of gastric cancer cells from four cell lines.

Other carotenoids including lycopene and canthaxanthin decrease growth of tumor cells by modulating cell cycle progression.[13,21-23] Astaxanthin could inhibit cell growth related to cell cycle arrest and also induce apoptosis in cancer cells. Colon cancer cells treated with *H. pluvialis* exhibited a dose-dependent decrease of cyclin D1 and a dose-dependent increase in p53, $p21^{WAF-1/CIP1}$, and $p27^{Kip-1}$. These results proved the blockage of cell cycle in G1/G1 phase.[10] Moreover, *H. pluvialis* extract could induce apoptosis of tumor cells by decreasing expression of Bcl-2 and Bcl-XL.[10] The present data are consistent with these prior findings. Astaxanthin downregulated p-ERK and upregulated $p27^{Kip-1}$ protein level by dose-dependent manner in two gastric cancer cell lines. Though *H. pluvialis* extract was able to modulate p-Akt pathway, pure astaxanthin did not decrease p-Akt in gastric cancer cell lines. More research will be needed.

The possible mechanism of the anticancer effect of astaxanthin is as follows. Cyclin D, the extracellular signal, is the first cyclin produced in the cell cycle, which binds to cyclin-dependent kinase 4 (CDK4) and becomes active. Cyclin D/CDK4 complex phosphorylates Rb and p-Rb separates from the E2F/DP1/Rb complex. Activation of E2F results in transcription of cyclin E, which binds to CDK2 and drives cells from G1 to S phase. The mitogen-activated protein kinases/ERK pathway communicates an extracellular signal from a receptor on the cell surface to the DNA in the nucleus. Astaxanthin downregulates p-ERK level in tumor cells, inhibiting the cyclin D1/CDK4 complex. On the other hand, increased $p27^{Kip-1}$ by astaxanthin induces suppression of cyclin E/CDK2 complex. These processes could cause cell cycle arrest at the G1/G0 phase.[24]

This study showed inconsistent results in the four gastric cancer cell lines. One reason is that each gastric cancer cell line has different characteristics. AGS and SNU-1 originated from primary human gastric adenocarcinoma, KATO-III from a specimen of pleural metastasis and MKN-45 from a liver metastatic tissue. They express different histologic cell types and also have different molecular characteristics including genetic and epigenetic changes in tumor suppressor genes, oncogenes, cell cycle regulator genes, growth factors, and their receptors. For example, both AGS and MKN-45 have the wild-type *P53* gene. SNU-1 also expresses *P53*. On the other hand, gross genetic deletion of *P53* is evident in KATO-III dells.[25-28] Furthermore, KATO-III does not express *P16* although no genetic alteration was detected. Presently, astaxanthin suppressed p-ERK but enhanced the $p27^{Kip-1}$ protein level in KATO-III and SNU-1 cells. The following findings may be associated with the results of our experiments. MKN-45 induces moderate elevation of p21 and relatively low level of CDK2 and cyclins. p-Rb is also detected in MKN-45, but not in KATO-III. In MKN-45, there is the rearrangement or a polymorphism of *P27* gene.[29]

There are several limitations in this study. First, astaxanthin could act as a cellular growth inhibitor at high concentration. The concentration of astaxanthin was four to 10 times higher than used in other studies. We did not use a natural product but rather a semi-synthetic compound, which was more oxidized than the natural one. Therefore, semisynthetic astaxanthin might show weaker oxidative power than natural one. A recent clinical study administered astaxanthin to heavy smokers; each subject was given daily either 5.0 (low dose), 20.0 and 40.0 mg of astaxanthin (high dose). There was no significant toxicity or side effects from the 21-day astaxanthin supplementation.[30] According to this research, high-dose oral astaxanthin may be given safely. Second, several pivotal proteins involved in cell cycle progression, survival and apoptosis, such as p53 and p21, were not evaluated in this study. It will be supplemented in additional experiment.

We have demonstrated that astaxanthin acts as a potential inhibitor of cellular growth in gastric cancer cell lines. It may mediate anticancer effect through decreasing p-ERK and increasing the $p27^{Kip-1}$ protein level. Although additional experiments are mandatory, our data support an adjuvant role of astaxanthin as a chemotherapeutic agent.

In conclusion, astaxanthin inhibits the proliferation of KATO-III and SNU-1 gastric cancer cells, which is explained by the findings that astaxanthin suppresses p-ERK and increases $p27^{Kip-1}$ in a dose-dependent manner. Our data also suggest that astaxanthin could affect cell cycle machinery proteins, resulting in inhibition of the growth of gastric cancer cells.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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