ASTAXANTHIN INHIBITS CELL PROLIFERATION, MIGRATION, INVASION AND INDUCED APOPTOSIS VIA AMPK-mTOR SIGNALING PATHWAY IN HEPATOCELLULAR CARCINOMA HEP 3B CELLS

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Hepatocellular carcinoma (HCC) is a fatal malignancy with a poor prognosis. There is an urgent need to study the molecular mechanisms of HCC development and explore potential drugs to improve survival. This study aims to investigate the antitumor effects of astaxanthin on HCC proliferation, migration, invasion and apoptosis through regulation of adenosine-activated protein kinase (AMPK). CCK8, wound healing, transwell and flow cytometry assays were used to evaluate Hep 3B cell viability, migration, invasion and apoptosis after astaxanthin treatment. Protein expression was determined by Western blot. CCK8 assays showed that all concentrations (200 μM, 400 μM, 500 μM) of astaxanthin used in this study significantly inhibited the proliferation of Hep 3B cells ($P < 0.05$). Wound healing, transwell and flow cytometry showed that astaxanthin inhibited Hep 3B cell migration and invasion and induced apoptosis. Western blot showed that astaxanthin increased the expression of p-AMPK ($P <0.05$) and inhibited its downstream p-mTOR protein expression ($P < 0.05$). The effects of astaxanthin on cell proliferation, migration, invasion and apoptosis was attenuated after addition of the AMPK blocker Compound C. To conclude, astaxanthin inhibits the proliferation, migration and invasion of HCC by regulating AMPK, and promotes tumor cell apoptosis in a dose-dependent manner.

Keywords: astaxanthin, hepatocellular carcinoma, Hep 3B; AMPK, m-TOR

INTRODUCTION

Hepatocellular carcinoma (HCC) is a fatal malignancy with a poor prognosis. The incidence of HCC has continued to rise in recent years. HCC is the fifth most common cancer and is the third leading cause of cancer-related death worldwide.[1] The occurrence and development of HCC is characterized by multiple genes, multi-factors, multi-stage and fast evolution. Liver cancer is often difficult to detect in the early stage, its onset is insidious, easy to metastasize, and it is not sensitive to chemotherapy drugs.[2] Currently, the preferred strategy for patients diagnosed with HCC is surgical resection. According to the diagnostic and therapeutic strategy of Barcelona clinical liver cancer (BCLC), the range of benefits of the primary treatment is small, because it is aimed at early asymptomatic patients rather than those with vascular invasion and distant metastasis.[3] For these reasons, investigators have directed their attention to molecular targets and transcatheter arterial chemoembolization (TACE), which has been shown to improve survival in patients with HCC. However, the effects of these treatments remain suboptimal due to their high recurrence rate.[4,5] Therefore, there is an urgent need to study the molecular mechanisms of HCC development and explore potential drugs to improve survival.

Astaxanthin (3,3'-dihydroxy-b, b'-carotene-4,4'-dione, ASX) is a pinkish-orange carotenoid pigment that belongs to the xanthophylls family. It comprises the color of the crustaceans, salmonids, and algae. As a dietary carotenoid, ASX is extracted from algae, shrimp, salmon,

lobster, and some other organisms.[6] ASX has antioxidant, photo-protective, immune-modulatory, anti-inflammatory, and cardio-protective properties, with potential benefits for humans.[7] Most importantly, no apparent side effects have been reported for ASX.[8] Therefore, ASX has been considered as a potential treatment for various diseases, such as inflammatory diseases, metabolic diseases, and neurodegenerative diseases.[9]

Recently, the anti-tumor role of ASX has been attracting attention. This powerful antioxidant may be a novel and potential drug for inhibiting the proliferation of carcinoma cells.[10-11] Emerging data indicate that ASX induced intrinsic apoptosis not only in oral cancer cells, but in skin cancer, breast cancer, and neuroblastoma SH-SY5Y cells.[12-14] Digestive tumors can be fatal, and research focused on colon cancer showed that ASX could inhibit tumor invasion by regulating the expression of ERK-2, NF-κB, and COX-2.[15] A large number of studies have confirmed that ASX can effectively inhibit hepatoma cells *in vitro*. In 2010, Tripathi D. N. explored the effects of ASX on early hepatocarcinogenesis in rats.[16] In addition, Song and colleagues demonstrated that ASX induced mitochondria-mediated apoptosis in rat hepatocellular carcinoma CBRH-7919 cells with an IC50 of 39 μM through inhibition of the JAK/STAT3 signaling pathway.[17,18] Although the effects of ASX in human hepatocellular carcinoma have been reported, the corresponding molecular mechanisms that may result in the development of ASX for HCC patients remain to be studied.

In eukaryotic cells, adenosine monophosphate-activated protein kinase (AMPK) is a key regulator of energy balance that is expressed ubiquitously. AMPK is closely related to the tumor suppressor function of liver kinase B1 (LKB1) and tumor protein p53, thereby regulating the activity of cell survival signals, such as mammalian target of rapamycin (mTOR) and protein kinase B (Akt), leading to inhibition of cell growth and cell cycle arrest.[19] It has been reported that the overexpression of large tumor suppressor gene 2 (LATS2) can attenuate the treatment resistance of HCC HepG2 cells to sorafenib-mediated death by suppressing the AMPK-mitofusin 2 (Mfn2) signaling pathway.[19] Inhibition of glycogen synthase kinase-3 beta (GSK3β) activity inhibits the HCC malignant phenotype by suppressing glycolysis by enhancing AMPK/mTOR signaling.[20] Importantly, AMPK may serve as a novel target to overcome chemoresistance in HCC.[21] Based on the foregoing research, this study aims to explore the effect of ASX on the AMPK signaling pathway in HCC.

EXPERIMENTAL

Cell culture

Human hepatocellular carcinoma (HCC) cell line Hep 3B was purchased from Chinese Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 U/mL streptomycin at 37 °C under a humidified atmosphere of 5% CO_2 and 95% air and subcultured when cell density reached to 90% every 2-3 days. Astaxanthin ((3S,3′S)-3,3′- dihydroxy-β,β′-carotene-4,4′-dione) was purchased from Sigma-Aldrich (St. Louis, MO, USA, Cat. No. 41659), dissolved in dimethyl sulfoxide (DMSO) solution and stored at 4 °C.

Cell counting Kit-8 (CCK-8) assays

Cell viability was evaluated using a CCK-8 kit (Dojindo Molecular Technologies Inc., Kumamoto, Japan) according to the manufacturer's instructions. Finally, the absorbance was read using a microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm.

Cell migration assays

Cell migration was assessed using the wound healing assays. The Hep 3B cells were seeded in a 6-well plate at a density of 1×10^5/mL and incubated to reach 70%–80% confluence. A sterile 10 μL micropipette tip was then used to make a scratch on the cell layer. The cells in each well were washed three times with PBS to remove floating cells, then treated as described for 6 h. After treatments, the drugs were removed, cell culture was continued in medium containing 10% FBS, and cells were photographed at an identical location at 0 h, 12 h later, 24 h later and 48 h later, respectively. Wound closure was analyzed by comparing areas of each scratch field, using ImageJ software. The migration assays were performed independently in triplicate.

Transwell assays

Transwell assays were carried out according to the manufacturer's instructions. BioCoat Matrigel invasion chambers (8-μM polycarbonate Nucleopore filters, BD BioSciences, San Jose, CA, USA) were used to examine cell invasion. After treatment with carvacrol (500 μM) or vehicle control (0.1% DMSO) for 24 h, 100 μL of cells (2.5×10^4 cells/mL) in FBS free DMEM were added to the top chamber. 600 μL of complete medium was added in the bottom chamber as a chemoattractant. Invading cells degraded the Matrigel and moved on to the lower membrane surface

in the chamber. Cells were fixed in 75% ethanol and stained with crystal violet (0.1%). Images of the invaded cells were captured with a digital camera connected to an Olympus microscope (CKX41). The number of invading cells was counted using Image-Pro Plus software with the cell counter tool.

Flow cytometry

$1x10^6$ cells/mL were seeded in 6-well plates and exposed to astaxanthin. Suspended and adherent cells were collected and washed with cold PBS. Cells were then fixed with chilled 70% alcohol overnight. The fixation fluid was washed with PBS before the test. Twenty microliters RNase A was added and incubated for 30 min at 37 °C and 0.5% propidium iodide staining solution was added for overnight staining at 4 °C away from light. The apoptosis rate was measured by flow cytometry (Beckman, Brea, CA, USA).

Western blot

Cells were rinsed with PBS, fully lysed on ice, and placed in a 4 °C overspeed centrifuge. The supernatant was removed and quantified with the BCA protein quantification kit. After boiling for 5 min, protein samples were separated by SDS-PAGE, transferred to PVDF membrane, and incubated with horseradish-rabbit resistance (1:800 dilution) for 1 h at room temperature. ECL color was developed, and the relative optical density value was measured with the graphic analysis software system of high-definition color pathological cell measurement program.

Statistical analysis

All data were expressed as mean $\bar{x}$±SD. Statistical analysis was performed with SPSS 26.0. Comparisons between groups were performed by one-way ANOVA, a p value < 0.05 being considered significant.

RESULTS AND DISCUSSION

Astaxanthin inhibited HCC cell proliferation

The structure of the astaxanthin used in this study was shown in Figure 1A. To evaluate the effect of astaxanthin on cell proliferation of human liver cancer cell line Hep 3B, we carried out CCK8 assays with following groups: A: control (equal volume saline); B: 200 μM astaxanthin; C: 400 μM astaxanthin; D: 500 μM astaxanthin; E: 200 μM astaxanthin and Compound C (50 μM); F: 400 μM astaxanthin and Compound C (50 μM); G: 500 μM astaxanthin and Compound C (50 μM).

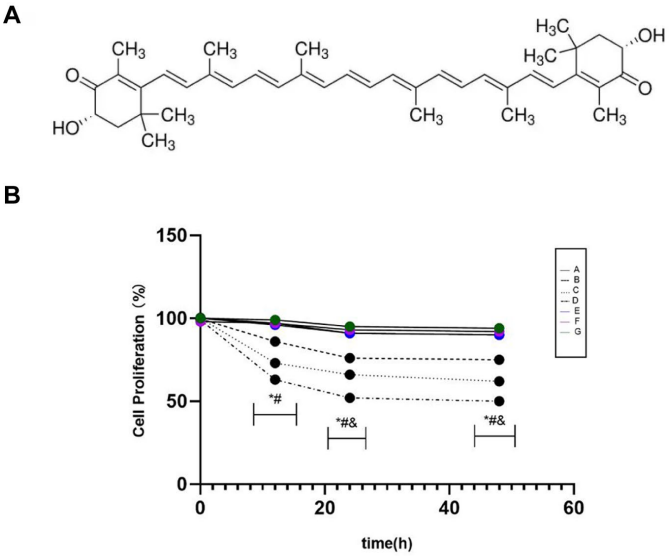

Figure 1: A. Structure of astaxanthin ((3S,3′S)-3,3′- dihydroxy-β,β′-carotene-4,4′-dione); B. CCK8 assays: Group A: control (equal volume saline); B: 200 μM astaxanthin; C: 400 μM astaxanthin; D: 500 μM astaxanthin; E: 200 μM astaxanthin and Compound C (50 μM); F: 400 μM astaxanthin and Compound C (50 μM); G: 500 μM astaxanthin and Compound C (50 μM). Compared with groups A, E, F, G, $^{*}P < 0.05$; compared with group B at the same time point, $^{\#}P < 0.05$; compared within group with 12 h, $^{\&}P < 0.05$

Compound C is also called Dorsomorphin, a selective and ATP-competitive AMPK inhibitor. As shown in Figure 1B, each concentration of ASX inhibited the growth of Hep 3B cells. The inhibitory effect of cell growth in groups C and D was more obvious than in group B ($^{\#}P < 0.05$), and there was no significant difference between group C and group D ($P > 0.05$). In groups E, F and G, after adding the AMPK blocker Compound C, the cell viability showed no

significant difference with the control group A ($P > 0.05$). The inhibitory effect of cell growth in groups B, C and D was more obvious than in groups E, F and G, respectively ($^{*}P < 0.05$). These results demonstrated that ASX inhibited HCC cell proliferation, and this effect could be attenuated by the AMPK blocker Compound C.

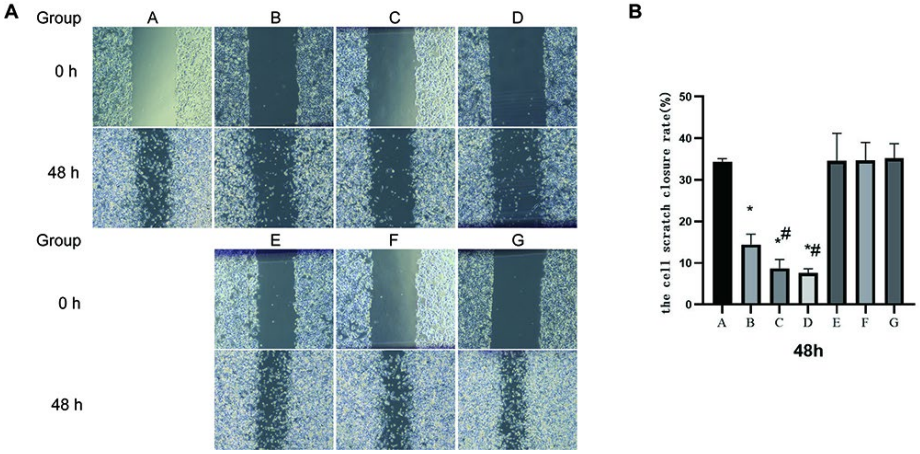

Figure 2: Wound healing assays (A) and scratch closure rate (B); Group A: control (equal volume saline); B: 200 μM astaxanthin; C: 400 μM astaxanthin; D: 500 μM astaxanthin; E: 200 μM astaxanthin and Compound C (50 μM); F: 400 μM astaxanthin and Compound C (50 μM); G: 500 μM astaxanthin and Compound C (50 μM). Compared with groups A, E, F, G, $^{*}P < 0.05$; compared with group B, $^{\#}P < 0.05$

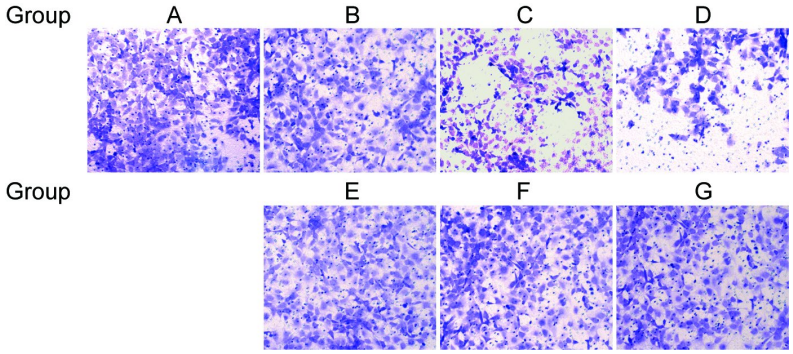

Figure 3: Transwell assays; Group A: control (equal volume saline); B: 200 μM astaxanthin; C: 400 μM astaxanthin; D: 500 μM astaxanthin; E: 200 μM astaxanthin and Compound C (50 μM); F: 400 μM astaxanthin and Compound C (50 μM); G: 500 μM astaxanthin and Compound C (50 μM)

Astaxanthin inhibited HCC cell migration and invasion

To further evaluate the effect of astaxanthin on cell migration and invasion of Hep 3B cells, we carried out wound healing and transwell assays with the same groups in Figure 1. In the wound healing assays, the cell scratch closure rate of the astaxanthin-treated group (200 μM, 400 μM, 500 μM) was dose-dependent and significantly reduced compared with the control group (Fig. 2). In the transwell assays, compared with the group without astaxanthin, the number of invasive cells in each treated group was significantly reduced, and the greater the concentration of astaxanthin, the less the number of invasive cells (Fig. 3). After the addition of AMPK blocker Compound C, astaxanthin had no significant effect on the migration and invasion ability of Hep 3B cell (Figs. 2, 3).

Astaxanthin induced HCC cell apoptosis

In order to determine whether astaxanthin induced apoptosis in Hep3B cells, we used flow cytometry. The results showed that after astaxanthin treatment (200 μM, 400 μM, 500 μM) for 48 h, the percentage of early and late apoptosis in Hep3B cells was significantly higher than that in the control (Fig. 4).

Astaxanthin increased p-AMPK expression and reduced m-TOR expression

In the above-mentioned groups, we detected whether astaxanthin regulated AMPK and mTOR expression by Western blot (Fig. 5). Total AMPK

expression showed no significant change in all groups. P-AMPK expression in groups B, C and D was significantly increased compared with group A, and P-AMPK expression in groups C and D was significantly increased compared with group B. Compared with group A, the expression of p-mTOR in groups B, C and D was significantly reduced, while the reduction in groups C and D was more obvious than in group B. No significant differences were observed between p-AMPK and p-mTOR expression between groups E, F and G and the control group.

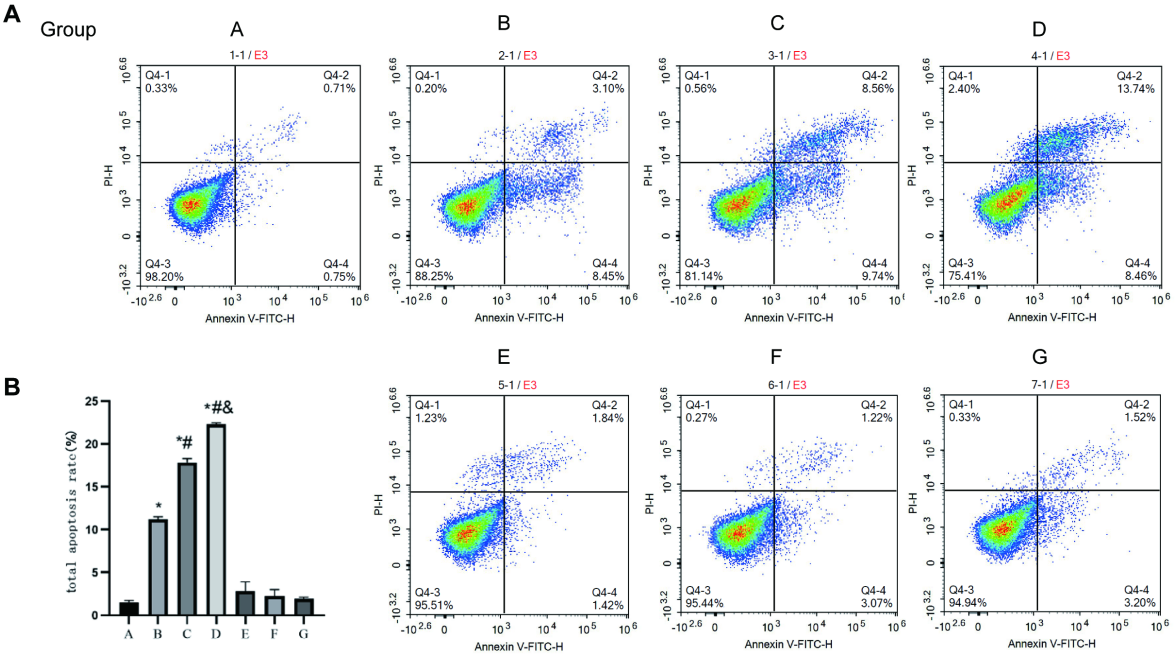

Figure 4: Cell apoptosis analysis by flow cytometry after astaxanthin treatment of Hep3B cells (A) and total apoptosis rate (B). Compared with groups A, E, F, G, *P <0.05; compared with group B, #P <0.05; compared with group C, &P <0.05

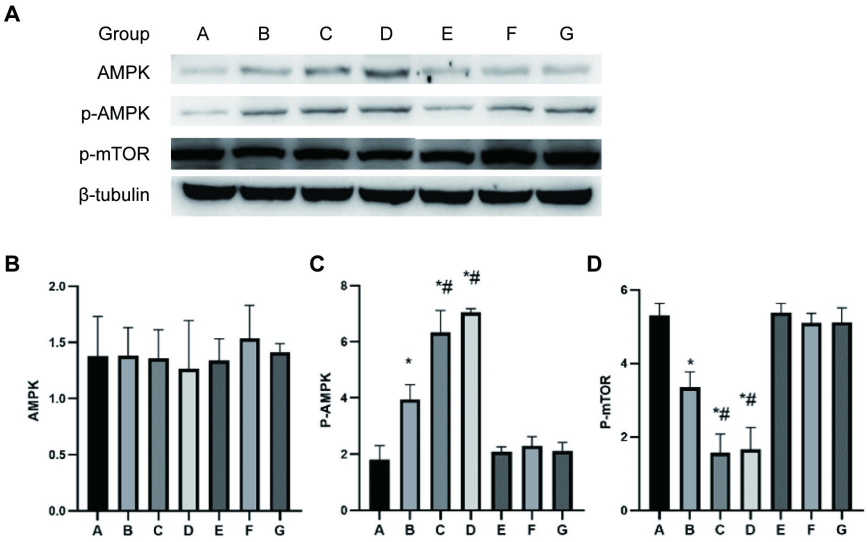

Figure 5: Astaxanthin regulates the expression of AMPK, P-AMPK and P-mTOR; (A) Western blot analysis to measure effects of astaxanthin on protein expression of AMPK, P-AMPK and P-mTOR in Hep3B cells; Statistical analysis of (B) AMPK, (C) P-AMPK and (D) P-mTOR; Compared with groups A, E, F, G, *P <0.05; compared with group B, #P <0.05

HCC seriously threatens human health, and its occurrence and development involve complex mechanisms. Clinical routine treatment methods, including major surgical treatment and interventional therapy, are only applicable for 30%-40% of patients with early liver cancer.[22]

Therefore, current domestic and foreign studies will focus on effective drug therapy, such as alkylating agents, antimetabolites, antibiotics, and hormones. Emerging data suggest that antioxidant drugs also play an important role in cancer therapy.[23]

Preventive interventions using natural plants and their extracts may be an ideal way to combat cancer due to their relatively inexpensive costs, clinical utility and lower toxic side effects. Current studies on the use of antioxidants or plant extracts to treat cancer have shown that there are many substances that can block specific pathways of cancer, but clinical data and large-scale cohort studies have been disappointing.[24,25] By studying the effects of cell signaling pathways on chemical prevention mechanisms, we suggest that organic foods may have multiple mechanisms to inhibit the development of tumor safety.[26] Most studies aimed to discover tumor inhibition using natural products. However, the diversity of structures makes it difficult to assess the exact mechanism. From these natural products, many important dietary antioxidants, including vitamins C, E, carotenoids, and selenium, have been identified and studied.[27,28] The findings on cancer prevention are inconsistent. Nonetheless, the development of antineoplastic agents containing these natural substances would have the advantage of reducing side effects compared with chemical compounds.

Astaxanthin has various effects, such as antioxidant, anti-ultraviolet, anti-inflammatory and anticancer effects. The antitumor effect of astaxanthin has received much attention. Several previous studies have shown that astaxanthin prevents carcinogenesis in the colon, bladder, prostate, breast, and oral cavity.[13,29] However, the exact oncogenic mechanism remains unclear. This study focuses on the effects of astaxanthin on the proliferation and survival of human liver cancer.

The proliferation and differentiation of tumor cells are involved in tumor invasion, therefore, inhibition of growth and promotion of apoptosis in HCC cells are important targets in cancer treatment.[30] In our study, we chose CCK8, wound healing, transwell, flow cytometry and western blotting to comprehensively study the proliferation and apoptosis of the HCC cell lines Hep3B. To assess proliferation, we measured the growth of HCC cells within 48 h treated with different concentrations of ASX (200-500 μM) using the CCK8 kit. The results showed that ASX inhibited tumor cell proliferation in a time- and dose-dependent manner. In cells treated with high dose ASX, strong inhibition was seen at 48 h.

AMP-activated protein kinase (AMPK) is a very important key enzyme in energy metabolism in eukaryotic cells, so it is called the “master switch of energy metabolism”. AMPK acts as the master switch of energy metabolism and can be activated under hypoxia and ischemia. Studies have confirmed that AMPK is closely related to tumor growth, proliferation and apoptosis.[31] Phosphorylate mammalian target of rapamycin (p-mTOR) is an atypical serine-threonine protein kinase, and mTOR responds to insulin, nutrients, growth factors and other stimuli; it plays an important role in cell growth and proliferation and cell apoptosis. Hyperactivation of mTOR signaling pathway is closely related to tumor occurrence and development.[32] AMPK activation can inhibit mTOR phosphorylation and exert an antitumor effect. Previous studies have confirmed that the AMPK activator epigallocatechin gallate (EGCG) can downregulate the expression of phosphorylated mammalian target of rapamycin and exert the inhibitory effect of hepatoma cells.[33]

One of the direct upstream regulators of the mTOR pathway, which acts in response to nutrients and cellular energy status, is AMP-activated protein kinase (AMPK). Kimura *et al*. reported that activation of AMPK inhibits p70 S6 kinase (p70 S6K) of the mTOR pathway, and that glucose or amino acid withdrawal activates AMPK and inhibits mTOR signaling.[34] In particular, upon cellular energy starvation, AMPK directly phosphorylates and thereby activates the mTOR inhibitor tuberous sclerosis complex 2 (TSC2). AMPK-activated TSC2 inhibits the mTOR pathway that involves cell growth and survival.[35] Oxygen deprivation was observed to promote LC3 processing and autophagosome formation in tumor cells, which are mediated by AMPK activation and by downregulation of mTOR.[36]

The effect of the antioxidant astaxanthin on AMPK signaling has not been extensively studied. However, the findings from the few studies that have been reported indicate that astaxanthin acts as a positive AMPK regulator. Specifically, astaxanthin was observed to inhibit lipogenesis and fat accumulation in the liver and to inhibit hepatic apoptosis in oleic acid-induced hepatic steatosis. Astaxanthin’s anti-steatotic properties are attributed to its ability to activate AMPK

signaling, as reflected by the observed increase in the ratio of phosphorylated vs unphosphorylated AMPK (p-AMPK/AMPK). Astaxanthin-induced AMPK activation was found to negatively regulate lipogenesis and promote fatty acid oxidation.[37] Furthermore, it has been shown that the intake of esterified astaxanthin that was extracted from *Haematococcus pluvialis* increases the running time of mice to exhaustion as a result of the increased level of total AMPK in the skeletal muscle.[38]

Combined with the above research background, this study cultured HCC cells *in vitro* and administered different concentrations of ASX on HCC cells for different times to detect the inhibition rate and apoptosis of cancer cells.[39] Our study found that ASX can inhibit the growth and proliferation of HCC cells and promote its apoptosis. The above effects are concentration- and time-dependent. However, there is an upper limit platform for the concentration of ASX. No significant changes were observed in the tumor suppression of groups C and D. Combined with the expression of p-AMPK (activation status of AMPK), the strength of anti-liver cancer effect of ASX was positively correlated with p-AMPK expression. Its concentration-dependent anticancer effect may be related to the saturation of AMPK phosphorylation. After the addition of AMPK blocker Compound C, the anti-liver effect of ASX was blocked (manifested as the mean inhibition rate of cancer cells in groups E, F and G was 0 and no apoptosis), which further confirmed that the anti-HCC effect of ASX in vitro was achieved through the activation of AMPK. A thorough study of the expression of downstream protein p-mTOR of AMPK found that ASX exerts anti-cancer effect through AMPK-mTOR signaling.

CONCLUSION

This study was carried out to investigate the antitumor effects of astaxanthin on HCC proliferation, migration, invasion and apoptosis through regulation of adenosine-activated protein kinase (AMPK). CCK8, wound healing, transwell and flow cytometry assays were performed to evaluate Hep 3B cell viability, migration, invasion and apoptosis after astaxanthin treatment. The results of the study suggest that ASX can inhibit the growth and proliferation of HCC and promote its apoptosis *in vitro*. The mechanism is related to the activation of adenylate-activated protein kinase and thus the inhibition of mammalian target of rapamycin. This study further clarified the role of ASX in anti-HCC and its non-immune mechanism, and provided experimental and theoretical basis for ASX to become a new anti-HCC drug and the discovery of new anti-HCC targets.

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