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Suppressive effects of lycopene and β-carotene on the viability of the human esophageal squamous carcinoma cell line EC109

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Abstract. The molecular mechanisms underlying the chemopreventive effects of carotenoids in different types of cancer are receiving increasing attention. In the present study, the role of peroxisome proliferator-activated receptor γ (PPARγ) in the effect of lycopene and β-carotene on the viability of EC109 human esophageal squamous carcinoma cells was investigated. The viability of EC109 cells was evaluated using MTT assays. The effects of lycopene and β-carotene on the expression of PPARγ, $p21^{WAF1/CIP1}$, cyclin D1 and cyclooxygenase-2 (COX-2) were analyzed by western blotting. Lycopene and β-carotene (5-40 μM) dose- and time-dependently reduced the viability of the EC109 cells. GW9662, an irreversible PPARγ antagonist, partly attenuated the decrease in EC109 cell viability induced by these carotenoids. Lycopene and β-carotene treatments upregulated the expression of PPARγ and $p21^{WAF1/CIP1}$, and downregulated the expression of cyclin D1 and COX-2. These modulatory effects of the carotenoid treatments were suppressed by GW9662, suggesting that the inhibition of EC109 cell viability by lycopene and β-carotene involves PPARγ signaling pathways and the modulation of $p21^{WAF1/CIP1}$, cyclin D1 and COX-2 expression.

Introduction

Epidemiologic research has consistently indicated an inverse association between the dietary levels of fruits and vegetables consumed and the risk of certain types of cancer (1,2). It has been suggested that carotenoids are chemopreventive constituents of fruit and vegetables (3,4). Esophageal cancer is a prevalent type of malignancy with a high mortality rate, which is increasing in incidence in China, according to a 2011 study (5). The northern area of Henan, China, including Linzhou, is an area of high incidence of esophageal and cervical cancer, and the mortality rate due to esophageal and gastric cancer is high in this region compared with the rest of the world (6). The results of nutrition intervention trials in Linxian demonstrated that the cancer-associated mortality rate over a 5.25-year period was significantly reduced among trial participants receiving β-carotene, vitamin E and selenium supplementation (7). The anti-cancer effects of crocetin, a carotenoid derived from saffron, have been reported in KYSE-150 human esophageal squamous cell carcinoma (ESCC) cells (8). Lycopene and β-carotene also occur in fruits and vegetables in relatively high concentrations. However, the effects of these carotenoids on the viability of esophageal cancer cells remain uncharacterized, despite the suspected etiological effects of chronic deficiencies of multiple micronutrients (9).

Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the nuclear hormone receptor superfamily. PPARγ is a transcription factor that serves crucial roles in the regulation of numerous physiological processes, including lipid metabolism and adipogenesis. PPARγ is expressed in various human tissues, and has been demonstrated to regulate cell proliferation, differentiation, and apoptosis (10,11). A deficiency in PPARγ may be a significant risk factor for carcinogenesis (12). It is established that PPARγ activation promotes antiproliferative, antiangiogenic and pro-differentiation pathways (13). Various *in vitro* studies have demonstrated that the activation of PPARγ leads to the growth inhibition of numerous types of neoplastic cell, including human esophageal adenocarcinoma cells (14-17). However, in Barrett's esophagus and esophageal adenocarcinoma, enhanced PPARγ expression has been associated with the development of carcinoma cells (18). Differential effects of the oral antidiabetic agent, pioglitazone, on PPARγ activation and the growth of the OE33 human Barrett's adenocarcinoma cancer cell line have been studied *in vitro* and *in vivo* (19). PPARγ activation by pioglitazone *in vitro* reduced OE33 cell growth by the induction of apoptosis, whereas systemic pioglitazone treatment of mice bearing transplantable Barrett's adenocarcinomas derived from OE33 cells increased the rate of cell proliferation (19).

Our previous studies demonstrated that the upregulation of PPARγ expression may be associated with the suppressive effects of β-carotene on the proliferation of MCF-7 breast cancer cells, and of carotenoids on the proliferation of K562 chronic myelogenous leukemia cells (20-22). However, to the best of

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our knowledge, the effects of lycopene and β-carotene on the viability of human ESCC cells, and the role of PPARγ in these effects, were not previously well defined.

The present study aimed to evaluate the inhibitory effects of lycopene and β-carotene on the viability of the EC109 human ESCC cell line, and to elucidate the association between PPARγ signaling and effect on viability. Subsequently, the expression levels of cell proliferation regulators were explored in the context of the carotenoid effects.

Materials and methods

Reagents and antibodies. The ESCC cell line, EC109, was a gift from the College of Public Health, Zhengzhou University (Zhengzhou, China). Fetal bovine serum (FBS) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China), and RPMI-1640 medium was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). MTT, penicillin, streptomycin, and β-carotene (purity, 97%) were all purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). 2-Chloro-5-nitro-N-phenylbenzamide (GW9662) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA) and lycopene was purchased from Chengdu Herbpurify Co., Ltd. (http://www.herbpurify.com; Chengdu, China; purity, 90%) identified by high performance liquid chromatography following re-purification as described by the study of Nguyen *et al* (23). Rabbit anti-PPARγ (cat. no. H-100), mouse anti-p21 (cat. no. F-5), mouse anti-cyclin D1 (cat. no. A-12) and mouse anti-COX-2 (cat. no. H-3) antibodies were all purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit anti-β-actin (cat. no. D110007), and goat anti-rabbit IgG (cat. no. D110058) and goat anti-mouse IgG (cat. no. D110087) secondary antibodies were from Sangon Biotech Co., Ltd. (Shanghai, China).

Cell culture. EC109 cells were cultured in RPMI-1640 medium supplemented with FBS (10%, v/v), penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were incubated at 37˚C in a humidified atmosphere of 5% CO_2. Cells were grown to 80-90% confluence and sub-cultured.

Drug treatment and MTT assays. EC109 cells were plated in 96-well plates at $5x10^3$ cells/well for MTT assays, or 6-well plates at $1.5x10^5$ cells/well for protein extraction. After 24 h, β-carotene or lycopene were applied at final concentrations of 0, 1, 5, 10 or 20 μmol/l, and incubated for 0, 24, 48 or 72 h. Each treatment was repeated in 6 separate wells. For GW9662 treatments, GW9662 was added to the medium at 2 h prior to carotenoid treatment. Dimethyl sulfoxide (DMSO) was used as a vehicle to deliver lycopene, β-carotene and GW9662 to the cells. The concentration of DMSO was consistent in all experiments, at 0.1% (w/v). Cell viability was measured using an MTT assay, as previously described (20).

Western blot analysis. Western blotting was performed as previously described (20). Briefly, subsequent to 72 h carotenoid treatment, the cells were harvested and lysed in ice-cold lysis buffer [50 mM Tris-Cl, 150 mM NaCl, 0.02% (w/v) NaN_3, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 2 μg/ml leupeptin, and 1% (v/v) Triton X-100]. The protein concentration was determined using a BCA kit (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Pall Life Sciences, Port Washington, NY, USA). The membranes were then incubated with primary antibodies against PPARγ, p21, cyclin D1, COX-2 and β-actin, then subsequently with secondary antibodies. The proteins were visualized by enhanced chemiluminescence using the Super Signal West Pico Chemiluminescent system (Pierce; Thermo Fisher Scientific, Inc.) and visualized by autoradiography on Kodak-XAR film. The intensity of protein bands was digitized and analyzed with ImageJ 1.46r software (National Institutes of Health, Bethesda, MA, USA).

Statistical analysis. All experiments were performed ≥3 times. Statistical analysis was performed using one-way analysis of variance followed by Tukey's test. Data are presented as the mean ± standard error. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of carotenoids on EC109 cell viability. Fig. 1 demonstrates the effect of altering carotenoid concentration on the viability of EC109 cells, as determined by MTT assays. Treatment with lycopene or β-carotene reduced the cell viability compared with the control group. The suppression was dose-dependent; cell viability was significantly reduced from 5 μM carotenoid treatment. Fig. 2 shows that the reduction in EC109 cell viability was induced in a time-dependent manner. β-Carotene appeared to be more effective than lycopene in reducing cell viability.

Effects of carotenoids in combination with GW9662 on EC109 cell viability. To evaluate the effect of GW9662, an irreversible PPARγ antagonist, on the viability of EC109 cells, a dose-response experiment was performed. GW9662 was used at a concentration of 1-20 μM for 72 h prior to an MTT assay. As shown in Fig. 3, GW9662 alone exerted a suppressive effect on EC109 cell viability. At low concentrations (1-3 μM) the suppressive effect of GW9662 was insignificant. However, at a concentration ≥5 μM, GW9662 significantly reduced cell viability. Therefore, a final concentration of 3 μM was used in the subsequent experiments of the present study.

EC109 cells were treated with 20 and 40 μM lycopene and β-carotene, subsequent to GW9662 pre-treatment for 2 h. As shown in Fig. 4, GW9662 treatment partly attenuated the reduced viability of EC109 cells induced by carotenoid treatment. This implies that PPARγ was at least partially involved in the suppression of EC109 cell viability by lycopene and β-carotene.

The effect of GW9662 on PPARγ expression is mediated by carotenoids. The modulatory effects of carotenoids and GW9662 on the expression of PPARγ were examined by investigating the changes in the PPARγ protein levels following carotenoid treatment with GW9662 pre-treatment. Fig. 5 demonstrates that EC109 cells treated with 40 μM lycopene or β-carotene for 72 h exhibited significantly increased levels of PPARγ protein compared with untreated cells. GW9662 treatment attenuated the inhibitory effects of carotenoids on EC109 cell viability

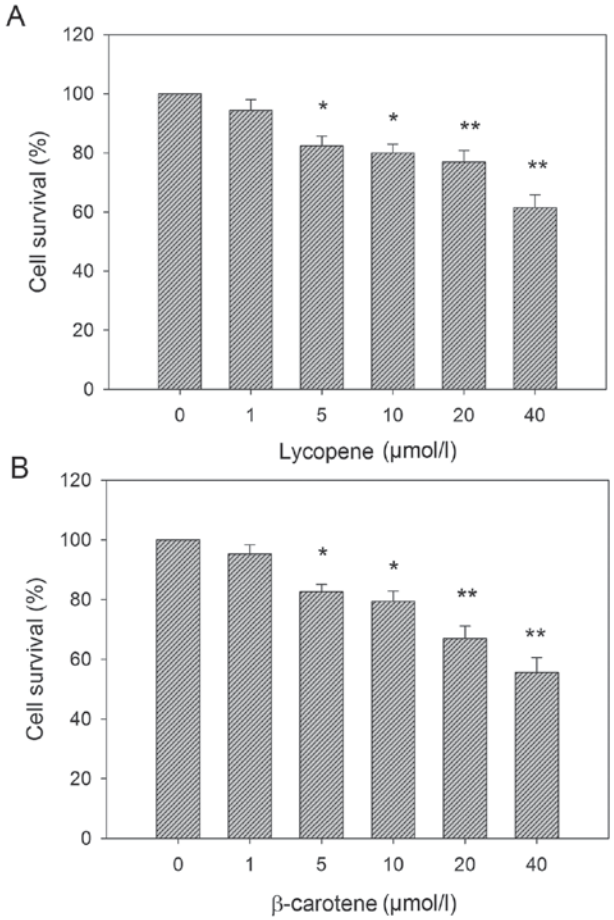

Figure 1. Effect of carotenoids on the viability of EC109 cells. EC109 cells were exposed to (A) lycopene or (B) β-carotene for 72 h, and viability was measured by an MTT assay. Data are presented as the mean percentage cell survival with standard error bars (n=6). *P<0.05, **P<0.01 vs. untreated control group.

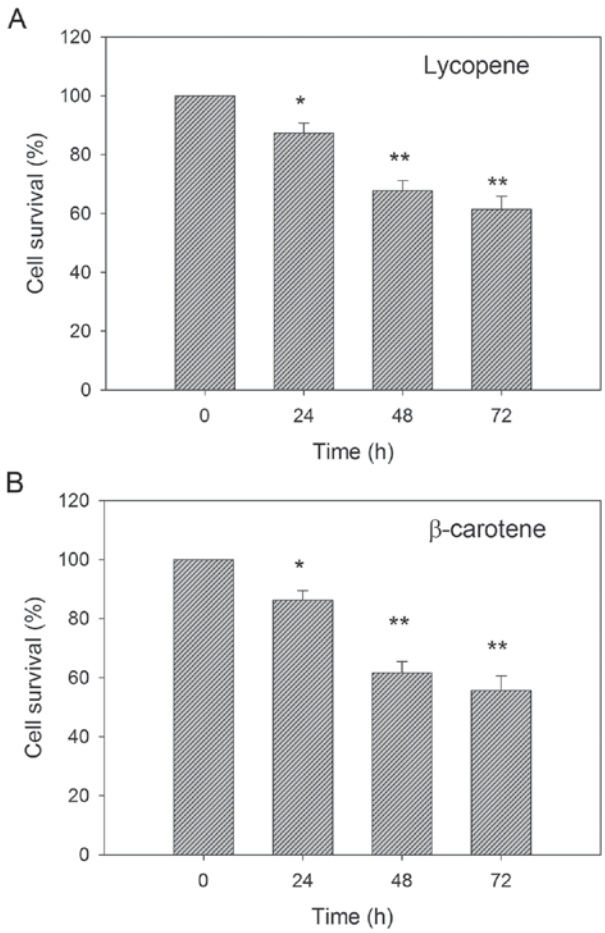

Figure 2. Effect of carotenoid treatment duration on the viability of EC109 cells. The cells were exposed to 40 mM (A) lycopene or (B) β-carotene, and viability over time was measured by an MTT assay. Data are presented as the mean percentage cell survival with standard error bars (n=6). *P<0.05, **P<0.01 vs. untreated control group.

(Fig. 4), and the increase in PPARγ protein levels induced by carotenoids was reduced with GW9662 pre-treatment in EC109 cells.

Effects of GW9662 on p21, cyclin D1 and COX-2 expression modulated by carotenoids. Our previous studies demonstrated that carotenoids can modulate the expression of the cell cycle control protein cyclin D1, the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ and COX-2 (16-18). GW9662 attenuated the modulation of p21 and cyclin D1 expression by carotenoids, suggesting that p21 and cyclin D1 participate in the PPARγ-dependent pathway, which executes the antiproliferative effects of carotenoids (18). In order to understand whether the growth-inhibitory effects of carotenoids on EC109 cells is dependent on these three regulators, the cells were treated with carotenoids in the absence or presence of GW9662, and p21, cyclin D1 and COX-2 protein expression levels were measured by western blotting. Fig. 6 shows that the protein levels of p21, cyclin D1 and COX-2 were altered by carotenoid and GW9662 treatment. P21 protein levels were significantly increased by carotenoids in the absence of GW9662. However, the inhibition of PPARγ by GW9662 resulted in a significant reduction in p21 protein levels. The downregulation of cyclin D1 and COX-2 expression induced by carotenoids was also diminished in the presence of GW9662 pre-treatment.

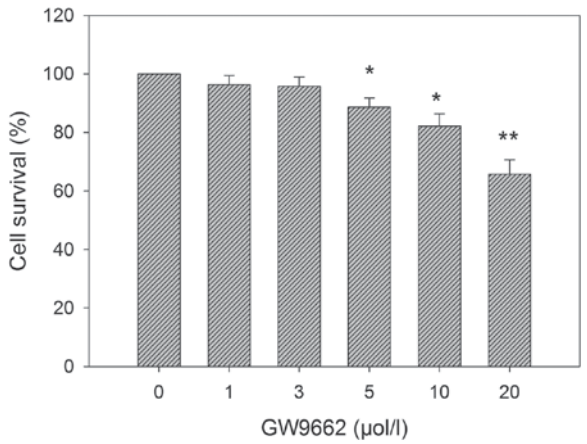

Figure 3. Effect of GW9662 on the viability of EC109 cells. Cells were exposed to varying concentrations of GW9662 for 72 h, and viability was measured by an MTT assay. Data are presented as the mean percentage cell survival with standard error bars (n=3). *P<0.05, **P<0.01 vs. untreated control group.

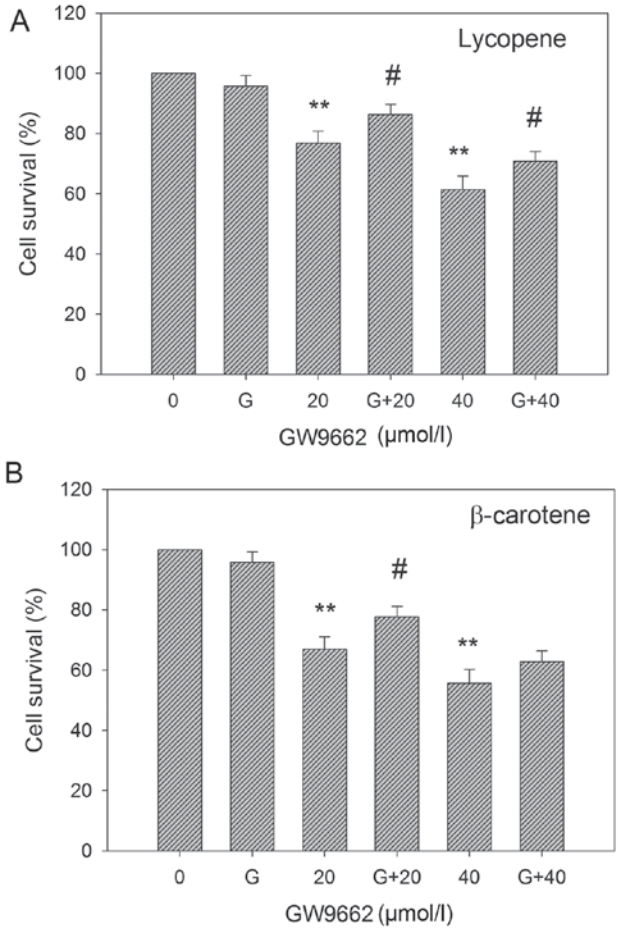

Figure 4. Effect of lycopene and β-carotene on the viability of EC109 cells pre-treated with GW9662. EC109 cells were exposed to (A) lycopene and (B) β-carotene following a GW9662 pre-treatment for 2 h. MTT assay results are presented as the mean percentage cell survival with standard error bars. n=3. $^{\#}$P<0.05, **P<0.01 vs. untreated control group. G, 3 μM GW9662 treatment; 20, 20 μM carotenoid treatment; 40, 40 μM carotenoid treatment.

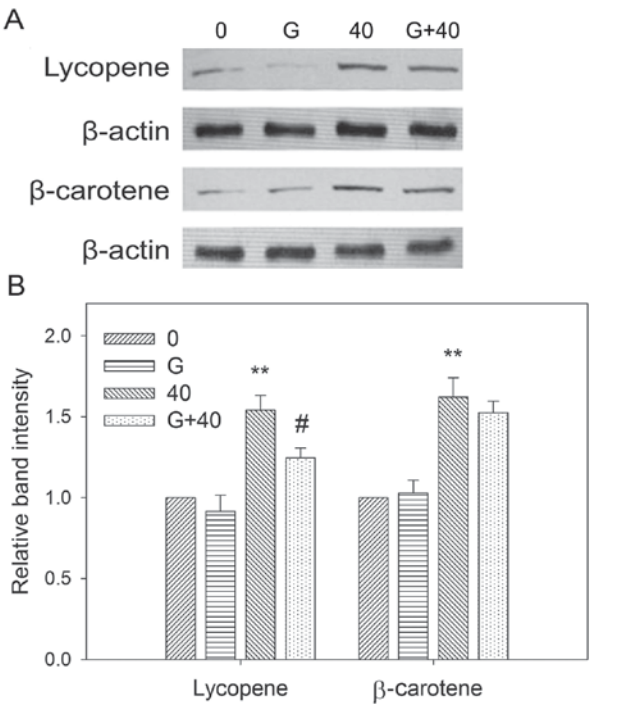

Figure 5. Effect of carotenoid and GW9662 treatment on the expression levels of PPARγ protein. EC109 cells were exposed to (A) lycopene or (B) β-carotene in the absence or presence of GW9662 and the effect was analyzed with western blotting. The quantified western blot results are presented as the mean band intensity, relative to untreated control cells, with standard error bars. n=3. $^{\#}$P<0.05, **P<0.01 vs. untreated control group. PPARγ, peroxisome proliferator-activated receptor γ; G, 3 μM GW9662 treatment; 40, 40 μM carotenoid treatment.

Discussion

The antiproliferative effects of carotenoids have been demonstrated in numerous tumors and cell lines. Our previous results showed that β-carotene inhibited the growth of MCF-7 breast cancer and H1299 lung cancer cells, and induced apoptosis (20), and that β-carotene, astaxanthin, capsanthin and bixin suppressed the proliferation of K562 cells, induced apoptosis, and affected cell cycle progression (21,22). Crocetin has also been demonstrated to inhibit cell proliferation and migration, and induce apoptosis, in ESCC KYSE-150 cells (8). The present study demonstrates that lycopene and β-carotene significantly decreased the viability of EC109 cells in a dose- and time-dependent manner. To the best of our knowledge, this is the first report of lycopene and β-carotene inhibiting the viability of human ESCC cells. This may be helpful for understanding the results of the nutrition intervention trials in Linxian, and indicates the potent chemopreventive effects of carotenoids on human ESCC.

It has been demonstrated that the activation of PPARγ expression by agonists inhibited the growth of ESCC cells (13-17). However, in Barrett's esophagus and esophageal adenocarcinoma, enhanced PPARγ expression has been associated with differential effects on the proliferation of carcinoma cells (18,19). In the present study, the antiproliferative effects of lycopene and β-carotene on ESCC EC109 cells were associated with the PPARγ pathway, as GW9662 treatment not only attenuated the upregulation of PPARγ expression, but also the reduction in EC109 cell viability induced by lycopene and β-carotene. These data are in accord with our previous observations that the upregulation of PPARγ expression contributed to the inhibition of MCF-7 breast cancer and K562 cell growth by carotenoids (16-18). PPARγ activation has also been associated with the antiproliferative effects of lycopene in LNCaP and DU145 human prostate cancer cells (24,25). Together, these studies suggest that PPARγ may serve important roles in the anti-cancer effects of carotenoids, which may be of universal significance for chemoprevention strategies against cancer. However, GW9662 pre-treatment did not abolish the growth-inhibitory effects of the tested carotenoids on EC109 cells completely, suggesting that the anti-cancer effects of the carotenoids are separate from those of conventional thiazolidinedione PPARγ agonists.

$p21^{WAF1/CIP1}$ and cyclin D1 are well characterized key regulators of cell cycle progression (26). P21 negatively modulates cell cycle progression (27), and the upregulation of p21 has been demonstrated to inhibit the proliferation and colony formation of lung cancer cells (28). Cyclin D1 is overexpressed in various types of human cancer, and its overexpression is positively associated with tumor progression (26,28). Cyclin D1 has also been associated with aggressive tumor behavior in ESCC (29). COX-2 has been

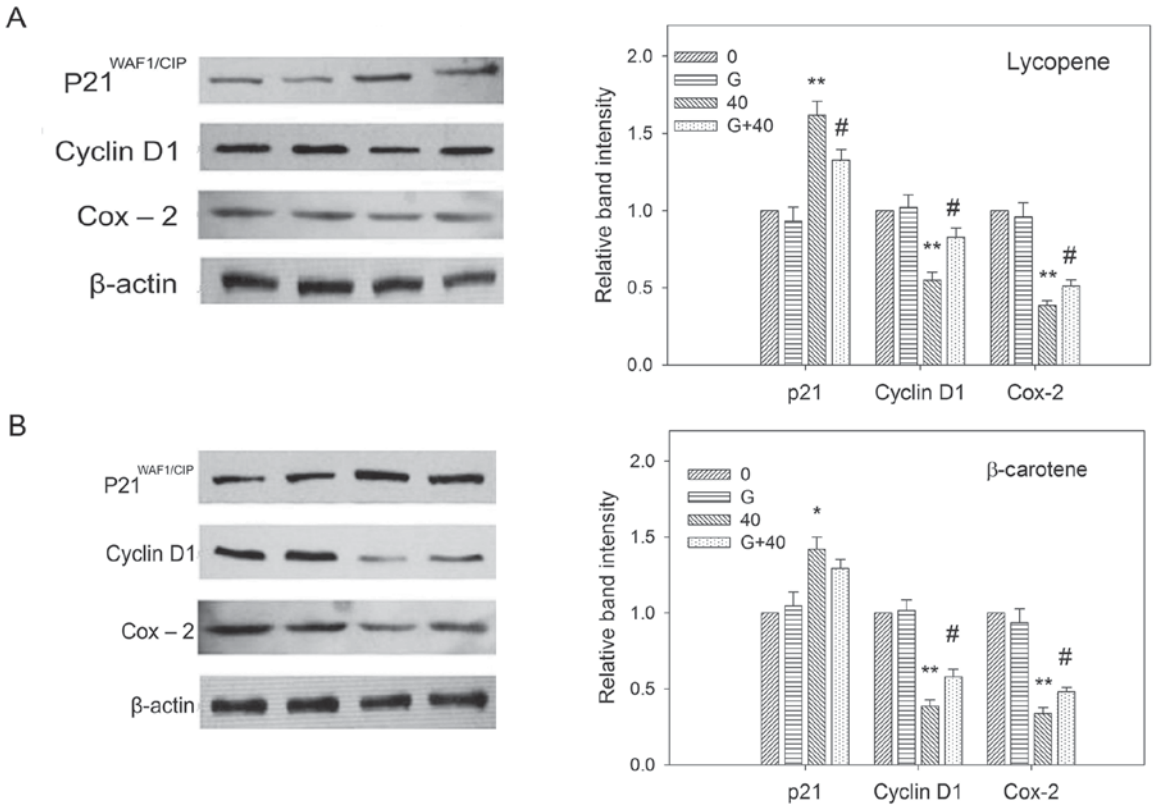

Figure 6. Effect of GW9662 on p21, cyclin D1 and COX-2 expression modulated by carotenoids. (A) EC109 cells were exposed to lycopene in the absence or presence of GW9662. (B) EC109 cells were exposed to β-carotene respectively in the absence or presence of GW9662. The quantified western blot results are presented as the mean band intensity, relative to untreated control cells, with standard error bars. n=3. $^{\#}$P<0.05, **P<0.01 vs. untreated control group. COX-2, cyclooxygenase-2; G, 3 μM GW9662 treatment; 40, 40 μM carotenoid treatment.

demonstrated to be upregulated in several types of cancer, including esophageal carcinomas, and serves an important role in ESCC carcinogenesis (30,31). Our previous studies demonstrated that the tested carotenoids upregulated the expression of p21 and downregulated the expression of cyclin D1 and COX-2 in MCF-7 breast cancer and K562 cells (20-22). GW9662 treatment significantly weakened the regulatory effects of the carotenoids on cyclin D1 and p21 expression (22). The present study demonstrated that lycopene and β-carotene upregulated the expression of PPARγ and p21$^{WAF1/CIP1}$, and downregulated the expression of cyclin D1 and COX-2 in EC109 cells. The modulatory effects on the expression of p21, cyclin D1 and COX-2 by the carotenoids were attenuated by GW9662. This suggests that p21, cyclin D1 and COX-2 may be involved in a PPARγ-dependent pathway that executes the growth-inhibitory effects of lycopene and β-carotene on EC109 cells. The activation of PPARγ has been reported to be associated with an alteration in the cell cycle in tumors, and may be associated with the altered expression of p21 and cyclin D1 (13-15,21,32-34). Thus, carotenoids may exert antitumor effects on ESCC by upregulating p21 and downregulating cyclin D1 and COX-2 through activating PPARγ, which in turn reduces cell proliferation.

In summary, lycopene and β-carotene may act as potent anti-proliferative agents in ESCC cells. The present study may offer novel insights into improved dietary or supplementation strategies and future therapeutic interventions in esophageal cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Author's contributions

NBN repurified and measured lycopene, performed EC109 cell culture and drug treatment, measured cell viability and protein levels, and made substantial contributions to the experimental design and data collection. PL participated in measurements of cell viability and protein levels, and made substantial contributions to data analysis and interpretation, and wrote the manuscript. WEZ made substantial contributions to experimental design, data analysis and interpretation, and drawing the conclusions. WEZ also participated in writing and submitting the manuscript, revising it critically for important intellectual content and gave final approval of the version to be published.

Each author agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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