Antimetastatic Effects of α-Carotene and Possible Mechanisms of Action in Human Hepatocarcinoma SK-Hep-1 Cells

Huei-Yan Chen,[†] Te-Cheng Yueh,[†,§] Yi-Chuan Chen,[#] Chien-Hao Huang,[†] Chih-Min Yang,*[,†] and Miao-Lin Hu*[,†,‡]

[†]Department of Food Science and Biotechnology, National Chung Hsing University, Taichung, Taiwan 402

[§]Division of Colon and Rectal Surgery, Department of Surgery, Taichung Armed Forces General Hospital, Taichung, Taiwan 411

[#]School of Post-Baccalaureate Chinese Medicine, Tzu Chi University, Hualien, Taiwan 970

[‡]Agricultural Biotechnology Center, National Chung Hsing University, Taichung, Taiwan 402

ABSTRACT: In vitro evidence suggests that α-carotene (AC) is an antimetastatic agent against cancer cells, but the mechanistic action is unclear. This study investigated the antimetastatic effect and possible mechanism of AC in comparison with β-carotene (BC) using human hepatocarcinoma SK-Hep-1 cells. Results reveal that treatment with AC (0.5−2.5 μM) for 48 h significantly inhibited invasion, migration, and adhesion of SK-Hep-1 cells in a concentration-dependent manner. These effects of AC were stronger than those of BC at the same concentration (2.5 μM). Mechanistically, AC significantly decreased activities of urokinase plasminogen activator and matrix metalloproteinases (MMP)-2 and -9, but increased protein expression of plasminogen activator inhibitor-1, tissue inhibitor of MMP (TIMP)-1 and -2, and nm23-H1, an antimetastatic protein. AC also attenuated focal adhesion kinase-mediated phosphorylation of mitogen-activated protein kinase family resulting in decreased protein expression of Rho and Rac 1. Overall, these data suggest that AC has potential as an antimetastatic agent.

KEYWORDS: α-carotene, β-carotene, metastasis, SK-Hep-1 cells

INTRODUCTION

Tumor metastasis, the acquisition of the capacity to leave a primary tumor to colonize distant sites of the body, is a major cause of death for cancer patients. Tumor metastasis involves a complex process, including invasion, intravasation, migration, colonization, and angiogenesis.[1] Metastasizing cells can degrade basement membrane and extracellular matrix (ECM) by matrix metalloproteinases (MMPs); among these MMPs, MMP-2 and MMP-9 are deeply involved in invasion and migration.[2] Inhibition of MMP activities by the endogenous tissue inhibitors of MMPs (TIMPs) plays an important role in the prevention of tumor metastasis.[3] Cancer cell migration requires the activation of Rho small GTPases, of which Rac1 increases actin polymerization at the leading edge through the formation of filopodia and larnellipodia, and Rho induces the actomyosin fiber contraction to pull the trailing edge forward during cell migration.[4] Notably, Rho and Rac1 are overexpressed in metastatic tumors and play an essential role in cell migration and adhesion.[5] Thus, inhibition of cancer metastasis may be a useful therapeutic strategy for alleviating cancer progression.

Epidemiological studies have indicated that elevated intake of carotenoids and increased serum levels of carotenoids are related to reduced risk of several chronic diseases, including cancer.[6] To date, more than 600 carotenoids are identified and approximately 40 carotenoids are found in our daily foods, whereas only 14 carotenoids can be found in human plasma and tissues.[6] α-Carotene (AC), one of the major carotenoids in plasma, is found in dark green and orange fruits and vegetables such as carrot, spinach, pumpkin, and tomato.[7] Serum concentration of AC is around 0.082 μM.[7] The structure of AC is similar to that of β-carotene (BC) except that AC has an

ε-ring and a β-ring, whereas BC has two β-rings; therefore, the converting ability of AC to vitamin A is about half that of BC.[8]

Elevated serum levels of AC were shown to be associated with decreased risks of cardiovascular diseases and cancer in healthy populations.[9] In vitro studies demonstrated that AC inhibits proliferation of human prostate cancer PC-3, DU145, and LNCaP cells[10] and of human neuroblastoma GOTO cells.[11] Zhang et al.[12] reported that AC enhances gap junctional intercellular communication in mouse embryo fibroblast C3H/10T1/2 cells in relation to its cancer chemoprevention. In animal models, Murakoshi et al.[13] reported protective effects of orally administered AC against carcinogenesis, including spontaneous liver tumor development in C3H/He mice and lung tumor formation in 4-nitroquinoline-1-oxide-initiated, and glycerol-promoted ddY strain mice as well as the formation of skin papillomas in ICR mice induced by 12-O-tetradecanoyl-phorbol-13-acetate and 7,12-dimethylbenz[α]anthracene. In Sprague−Dawley rats, supplementation by intragastric gavage with AC was shown to inhibit N-methylnitrosourea-induced colonic aberrant crypt foci formation.[14]

Limited evidence has indicated that AC may be antimetastatic against cancer cells in vitro by inhibition of cell invasion in rat ascites hepatoma AH109A cells.[15] Because liver cancer is the leading cause of death in Taiwan and in much of the world, we employed a highly invasive hepatocarocinoma SK-Hep-1 cell line to investigate the effects of AC on invasion,

migration, and adhesion in comparison with BC and to explore the possible molecular targets underlying such actions.

■ MATERIALS AND METHODS

Chemicals. All chemicals used in the present study are of the highest grade. Tetrahydrofuran (THF) and butylated hydroxytoluene (BHT) were obtained from Merck (Darmstadt, Germany). Dulbecco's modified Eagles medium (DMEM), nonessential amino acid, penicillin/streptomycin, sodium pyruvate, fetal bovine serum (FBS), trypsin, and Giemsa stain were obtained from Gibco/BRL (Grand Island, NY, USA). The antibody against MAPK/extracellular signal-regulated kinase (ERK) 1/2, c-Jun NH2-terminal kinase (JNK)/stress-activated protein kinase, p38 MAPK proteins, FAK, and p-FAK rabbit monoclonal antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-TIMP-1, anti-TIMP-2, anti-PAI-1, anti-Rho, and anti-Rac 1 rabbit monoclonal antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody were obtain from Epitomics (Burlingame, CA, USA).

AC and BC Preparation. AC and BC were purchased from Calbiochem (USA) and Wako (Japan), respectively. The purities of AC and BC were >97%, as claimed by the supplier. AC and BC were soluble in THF/BHT to form a 10 mM stock solution, and then diluted with FBS at the indicated ratio (1:9).[16] THF/BHT-FBS-AC or -BC was added to the culture medium at a calculated final concentration of 0.1−5 μM for AC and 2.5 μM for BC. THF at 0.2% (v/v) and FBS at 1.8% (v/v) served as the solvent control, which did not significantly affect the assays described below.

Cell Culture. The human hepatoma SK-Hep-1 (BCRC no. 67005) cell line was purchased from Food Industry Research and Development Institute, Hsin Chu, Taiwan. The SK-Hep-1 cells were cultured in DMEM containing 10% (v/v) FBS, 0.37% (w/v) NaHCO$_3$, penicillin (100 kU/L), and streptomycin (100 kU/L) in a humidified incubator under 5% CO$_2$ and 95% air at 37 °C.

Cellular Uptake of AC and BC. SK-Hep-1 cells were incubated with 5 μM AC or BC for 12, 24, and 48 h in a 6-well plate containing 2 mL of medium per well. After incubation, the culture medium was removed, and the cells were washed with PBS twice. For measurement of cellular AC, the attached cells per well (10^5 cells) were scraped and resuspended in 300 μL of a mixture of methylene chloride and methanol (3:4, v/v). The mixture was sonicated at room temperature for 1 min and centrifuged at 1200g for 5 min. The supernatant was transferred to a new tube and stored at −80 °C until use. For measurement of cellular BC, the condensation of cells was necessary because the absorbance of BC at 450 nm (50 μM with an absorbance of 0.22) is much lower than that of AC (10 μM with an absorbance of 0.88) (data not shown). Thus, attached cells from 12 wells (10^5 cells/well) were scraped and resuspended in 1 mL of a mixture of *n*-hexane and absolute ethanol (2:1, v/v) followed by sonication at room temperature for 1 min and centrifugation at 1200g for 5 min. The supernatant was evaporated with a gentle stream of nitrogen and redissolved in 200 μL of a mixture of *n*-hexane and absolute ethanol (2:1, v/v) and then transferred to a new tube and stored at −80 °C until use. The cellular levels of AC or BC were analyzed using a Hitachi HPLC system (Hitachi, Japan) monitored at 450 nm. The mobile phase (acetonitrile and methanol: 75:25 by vol) was set at a flow rate of 1.5 mL/min, and the injection volume was 20 μL for each sample onto a 4.6 mm × 250 mm Navi C18-5 column (Wakopak). The cellular uptake of AC or BC is defined as the difference of total amounts of a carotenoid absorbed by SK-Hep-1 cells (incubated at 37 °C for 24 h) minus the amounts of AC or BC adherent to the cell surface (incubated at 4 °C for 24 h).[17] The uptake efficiency of AC or BC was calculated by the formula [(nmol AC or BC)/10^5 cells/(nmol AC or BC/well] × 100%,[17] in which the numerator is the cellular uptake of AC or BC and the denominator is the amount of AC or BC added to the medium (5 μM or 10 nmol AC or BC in 2 mL of medium per well).

Cell Invasion and Migration Assay. Cell invasion and migration were determined using transwell chambers (Costar) with 6.5 mm polycarbonate filters of 8 μm pore size according to the method reported by Repesh[18] with minor modifications. The major difference between cell invasion and migration assay is that each filter for the invasion assay was additionally coated with 100 μL of a 1:20 diluted matrigel in cold DMEM to form a thin continuous film on the top of the filter. After preincubation with AC (0.5−5 μM) or BC (2.5 μM) for 12, 24, or 48 h, SK-Hep-1 cells (5×10^4 cells/400 μL for invasion and 10^5 cells/400 μL for migration) were suspended in serum-free DMEM and placed in the upper chamber followed by incubation for an additional 24 h for the invasion assay and 6 h for the migration assay. DMEM (600 μL) containing 10% FBS was placed in the lower chamber. After incubation, the cells on the upper surface of the filter were completely wiped away with cotton swabs. The cells on the lower surface of the filter were fixed in methanol, stained with Giemsa, and then counted under a microscope. For each replicate, the cells in five randomly selected fields were photographed, and the counts were averaged.

Cell Adhesion Assay. The 24-well plates were precoated with 100 μL of a 1:20 diluted matrigel in cold DMEM to form a thin continuous film and dried in a laminar hood overnight. After preincubation with AC (0.5−5 μM) or BC (2.5 μM) for 12, 24, or 48 h, cells were adjusted to 5×10^4/mL in DMEM followed by incubation for an additional 2 h. After incubation, cells were washed twice in PBS and then incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 1 h. Supernatant were removed, and DMSO was added to dissolve the solid residue cells. The optical density at 570 nm of each well was then measured using a microplate reader (FLUOstar OPTIMA, BMG Labtechnologies).

Determination of MMP-2 and -9 and uPA Activities by Zymography. The activities of MMP-2 and -9 and uPA in culture medium were measured by gelatin and casein−plasminogen zymography. Briefly, SK-Hep-1 cells (5×10^4 cells/mL) were treated with AC (0.5−5 μM) or BC (2.5 μM) for 24 h in DMEM containing 10% (v/v) FBS and were incubated for another 24 h in serum-free medium. The serum-free medium was collected and electrophoresed (80 V; 120 min) in a 10% sodium dodecyl sulfate (SDS)−PAGE gel containing 0.1% (w/v) gelatin or casein−plasminogen. The gel was washed for 30 min at room temperature in a washing buffer containing 2.5% (v/v) Triton X-100 followed by incubation in reaction buffer containing 40 mM Tris-HCl, 10 mM CaCl$_2$, and 0.01% NaN$_3$ for 12−15 h at 37 °C. The gel was stained with Coomassie brilliant blue R-250 for 30 min and then destained in 10% acetic acid (v/v) and 50% methanol (v/v). The relative activities of MMP-2 and -9 and uPA were quantified using Matrox Inspector 2.1 software.

Western Blotting. Protein expression of MMP-2, MMP-9, TIMP-1, TIMP-2, nm23-H1, Rho, Rac 1, PAI-1, FAK, and MAPK family was determined by Western blotting. The cellular proteins were extracted using RIPA buffer containing protease inhibitors and were centrifuged for 30 min at 4 °C. A portion of the protein (50 μg) from the supernatant was resolved by SDS−polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with TBS buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 5% nonfat milk for 1 h and then was incubated with different primary antibodies. The membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 1 h followed by visualization using an ECL chemiluminescent detection kit (Amersham Co, Bucks, UK). The relative density of protein expression was quantitated by densitometry (Matrox Inspector 2.1 software).

Statistical Analysis. Values are expressed as means ± SD and analyzed using one-way ANOVA followed by Fisher's protected least significant difference test for comparisons of group means. All statistical analyses were performed using SPSS for Windows, version 10 (SPSS, Inc.); a P value <0.05 is considered statistically significant.

■ RESULTS

Cellular Uptake of AC and BC in SK-Hep-1 Cells. The cellular uptake of AC (5 μM) and BC (5 μM) in SK-Hep-1 cells was measured using HPLC. After incubation of SK-Hep-1 cells with 5 μM AC or BC for 12 h, a small amount of AC (0.18
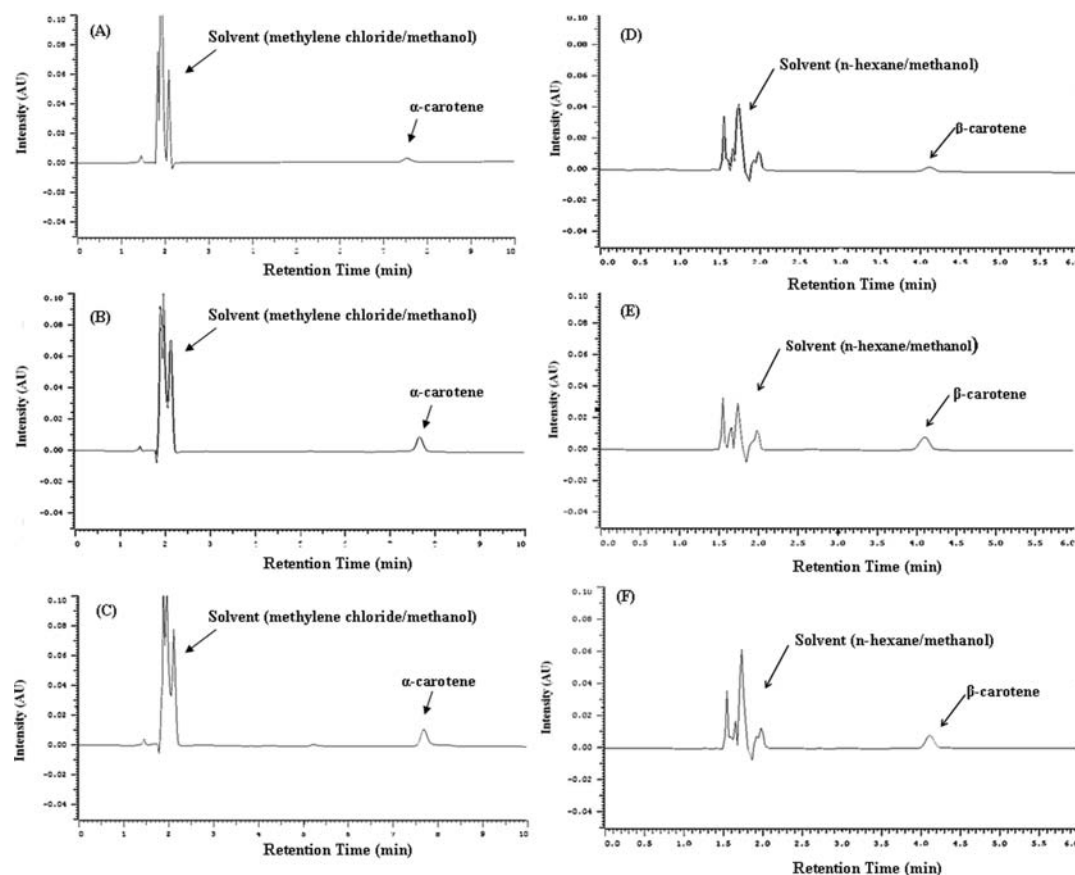
Figure 1. Cellular uptake of α-carotene (5 μM) and β-carotene (5 μM) in SK-Hep-1 cells determined using HPLC analysis monitored at 450 nm. Typical HPLC chromatograms of α-carotene in SK-Hep-1 cells were obtained at incubation times of 12 h (A), 24 h (B), and 48 h (C). Typical HPLC chromatograms of β-carotene in SK-Hep-1 cells were obtained at incubation times of 12 h (D), 24 h (E), and 48 h (F).

$\pm$ 0.01 nmol/10^5 cells) and BC (0.23 $\pm$ 0.02 nmol/10^5 cells) was detected (Figure 1A,D). After incubation of SK-Hep-1 cells with 5 μM AC or BC for 24 and 48 h, the amounts of AC increased to 0.48 $\pm$ 0.01 and 0.54 $\pm$ 0.09 nmol/10^5 cells, respectively (Figure 1B,C) whereas those of BC increased to 0.84 $\pm$ 0.12 and 0.69 $\pm$ 0.05 nmol/10^5 cells, respectively (Figure 1E,F). As summarized in Table 1, the uptake efficiency of AC in SK-Hep-1 cells was 2.34, 9.50, and 10.9% at 12, 24,

Table 1. Cellular Uptake of α-Carotene and β-Carotene in SK-Hep-1 Cells Incubated with 5 μM α-Carotene and β-Carotene for 12, 24, and 48 h[a]

	α-carotene		β-carotene	
incubation time (h)	nmol/10^5 cells[b]	efficiency[c] (%)	nmol/10^5 cells[b]	efficiency[c] (%)
12	0.18 $\pm$ 0.01	2.3	0.23 $\pm$ 0.02	4.7
24	0.48 $\pm$ 0.01	9.5	0.84 $\pm$ 0.12	16.8
48	0.54 $\pm$ 0.09	10.9	0.69 $\pm$ 0.05	13.8

[a]SK-Hep-1 cells were incubated with 5 μM α-carotene and β-carotene for 12, 24, and 48 h in a 6-well plate containing 2 mL of medium. Data are from three or four separate experiments. [b]The cellular uptake of α-carotene or β-carotene is defined as the difference of total amounts of α-carotene or β-carotene absorbed by SK-Hep-1 cells (incubated at 37 °C for 24 h) minus the amounts of α-carotene or β-carotene adherent to cell surface (incubated at 4 °C for 24 h). [c]The uptake efficiency of α-carotene or β-carotene was calculated by the formula [(nmol α-carotene or β-carotene)/10^5 cells]/[(nmol α-carotene or β-carotene)/well] $\times$ 100%.

and 48 h of incubation, respectively, whereas that of BC was 4.7, 16.8, and 13.8% at 12, 24, and 48 h, respectively.

Effects of AC and BC on Invasion, Migration, and Adhesion in SK-Hep-1 Cells. Neither AC (0.5–5 μM) nor BC (2.5 μM) affected cell morphology and cell viability of SK-Hep-1 cells (data not shown). However, incubation of SK-Hep-1 cells with AC (0.5–5 μM) for 12, 24, and 48 h inhibited migration (Figure 2A) and adhesion (Figure 2C) in U-shaped manners; that is, AC concentration-dependently inhibited migration and adhesion up to 2.5 μM, whereas the effect of 5 μM AC weakened to some extent. AC added at 2.5 μM and incubated for 24 h produced the strongest inhibition on migration and adhesion, with an inhibition of 45% ($P < 0.05$) and 34% ($P < 0.05$), respectively (Figure 2). Results from the invasion assay (Figure 2B) reveal that AC concentration-dependently inhibited invasion at 12 h of incubation, although the effects of AC were only significant at 2.5 and 5 μM, at which the inhibitions of the two concentrations of AC were similar. At 24 and 48 h of incubation, AC added at 0.5–2.5 μM significantly inhibited cell invasion, whereas AC added at 5 μM did not affect cell invasion. Importantly, AC was more effective than BC at the same concentration (2.5 μM) in inhibition of invasion, migration, and adhesion (Figure 2). On the basis of the time course response, we chose an incubation time of 24 h for the following experiments.

Effects of AC and BC on Activities of MMP-9, MMP-2, and uPA in SK-Hep-1 Cells. AC (0.5–2.5 μM) treatment significantly decreased activities of MMP-9, MMP-2, and uPA at 24 h of incubation in a concentration-dependent manner
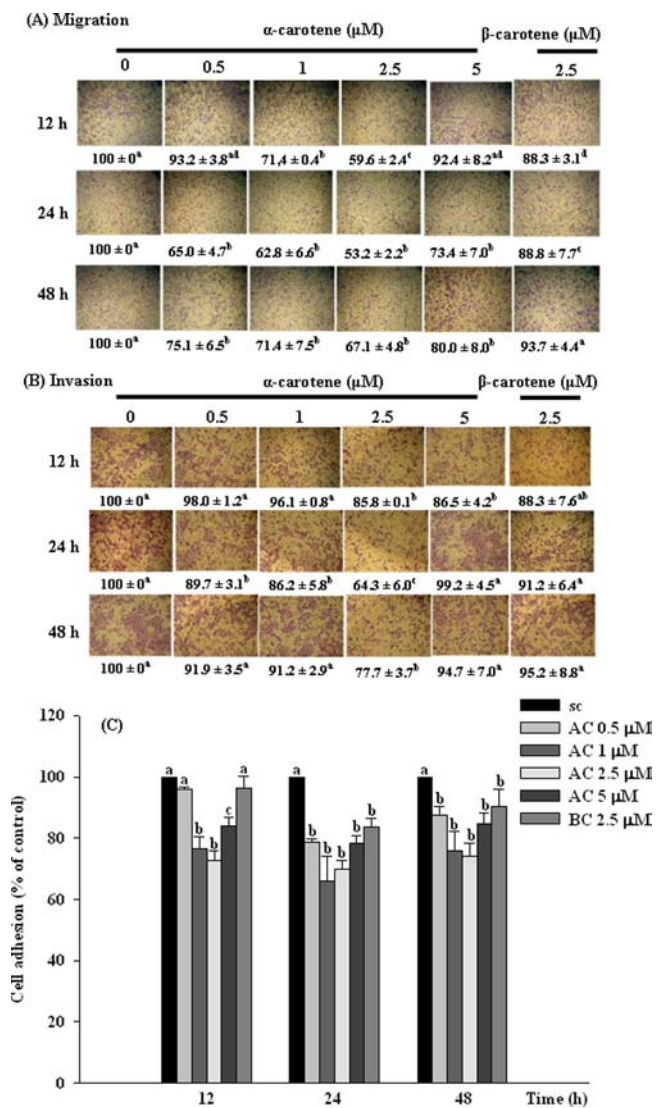
Figure 2. Effect of α-carotene (0.5−5 μM) or β-carotene (2.5 μM) on migration, invasion, and adhesion in SK-Hep-1 cells: (A) images of cell migration; (B) images of cell invasion; (C) cell adhesion. Cells were incubated with α-carotene or β-carotene for 12, 24, and 48 h before the assay. Data are expressed as means ± SD, n = 3−4; means at each time point without a common letter differ significantly (P < 0.05).

Figure 3. Effect of α-carotene (0.5−5 μM) or β-carotene (2.5 μM) on activities of MMP-9, MMP-2, and uPA in SK-Hep-1 cells: (A) gelatin zymography for MMP-9 and MMP-2 and casein−plasminogen zymography for uPA; (B) densitometric analysis of panel A. Cells were incubated with α-carotene or β-carotene for 24 h and incubated for another 24 h in serum-free medium. Data are expressed as means ± SD, n = 3−4; means without a common letter differ significantly (P < 0.05).

Figure 4. Effect of α-carotene (0.5−5 μM) or β-carotene (2.5 μM) on protein expression of TIMP-1, TIMP-2, PAI-1, and nm23-H1 in SK-Hep-1 cells: (A) Western blots of TIMP-1, TIMP-2, PAI-1, nm23-H1, and β-actin; (B) densitometric analysis of panel A. Cells were incubated with α-carotene or β-carotene for 24 h before the assay. Data are expressed as means ± SD, n = 3−4; means without a common letter differ significantly (P < 0.05).

with inhibitions of 30% (P < 0.05) for MMP-9, 35% (P < 0.05) for MMP-2, and 30% (P < 0.05) for uPA at 2.5 μM AC (Figure 3). However, these inhibitory effects of 5 μM AC were less effective than those of 2.5 μM AC (Figure 3). In contrast, BC (2.5 μM) significantly inhibited the activity of uPA without affecting the activities of MMP-9 and MMP-2, and the inhibitory effect of BC on uPA activity was less effective than that of AC at the same concentration (2.5 μM) (Figure 3).

Effects of AC and BC on Protein Expression of TIMP-1, TIMP-2, PAI-1, and nm23-H1 in SK-Hep-1 Cells. In contrast, AC (0.5−2.5 μM) treatment significantly increased protein expression of TIMP-1, TIMP-2, PAI-1, and nm23-H1 at 24 h of incubation in a concentration-dependent manner up to 2.5 μM, at which concentration there were increases of 60% (P < 0.05) for TIMP-1, 99% (P < 0.05) for TIMP-2, 52% (P < 0.05) for PAI-1, and 80% (P < 0.05) for nm23-H1 (Figure 4). However, these effects of 5 μM AC were less effective than those of 2.5 μM AC (Figure 4). BC (2.5 μM) also significantly
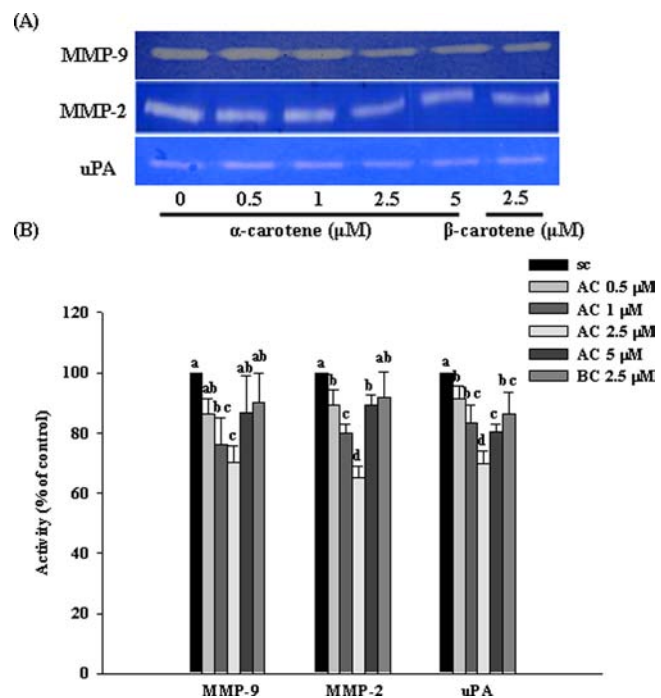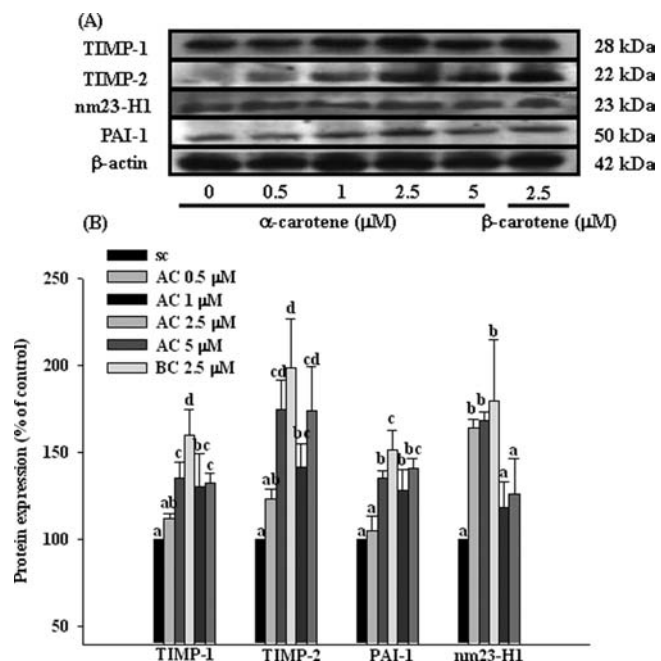
increased protein expression of TIMP-1, TIMP-2, and PAI-1 but did not affect protein expression of nm23-H1, and these effects of BC were less effective than those of AC at the same concentration (2.5 μM) (Figure 4).

Effects of AC and BC on Protein Expression of Rho and Rac 1 in SK-Hep-1 Cells. Both AC and BC treatments significantly decreased protein expression of Rho and Rac 1 in SK-Hep-1 cells (Figure 5). These inhibitory effects of AC (0.5−
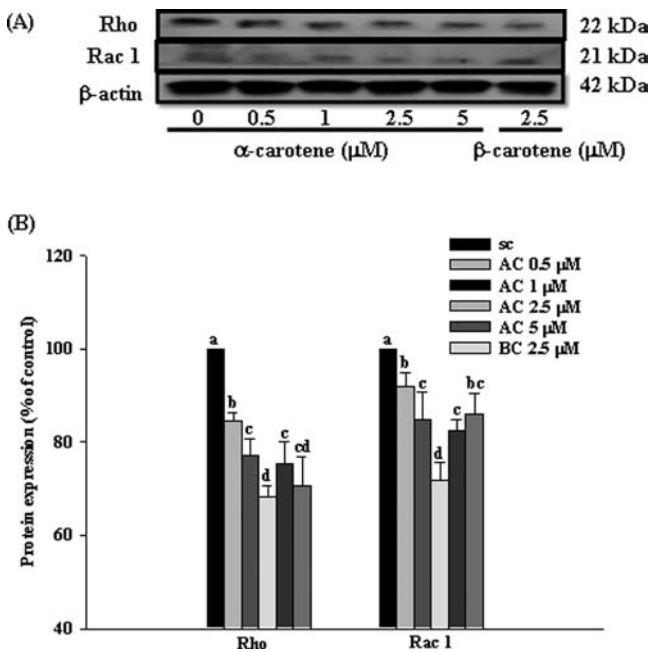

Figure 5. Effect of α-carotene (0.5−5 μM) or β-carotene (2.5 μM) on protein expression of Rho and Rac 1 in SK-Hep-1 cells: (A) Western blot of Rho, Rac 1, and β-actin; (B) densitometric analysis of panel A. Cells were incubated with α-carotene or β-carotene for 24 h before assay. Data are expressed as means ± SD, $n = 3-4$; means without a common letter differ significantly ($P < 0.05$).

2.5 μM) were in concentration-dependent manner up to 2.5 μM AC, with reductions of 32% ($P < 0.05$) for Rho and 28% ($P < 0.05$) for Rac 1 at 2.5 μM AC (Figure 5). However, AC at 5 μM was less inhibitory than AC at 2.5 μM on Rho and Rac 1 protein expression (Figure 5). AC and BC had similar inhibitory effects on Rho protein expression, whereas BC was less effective on Rac 1 protein expression than AC at the same concentration (2.5 μM) (Figure 5).

Time Course and Concentration Effects of AC on Phosphorylation and Protein Expression of FAK and MAPK Family in SK-Hep-1 Cells. SK-Hep-1 cells were treated with AC (2.5 μM) for 0−6 h to examine the upstream signaling molecules, including FAK and the MAPK family. Results reveal that AC transiently but significantly decreased phosphorylation of FAK at 0.5−1 h of incubation without affecting protein expression of FAK during 6 h of incubation and that the maximal inhibition occurred at 0.5 h of incubation with a reduction of 49% ($P < 0.05$) (Figure 6A). Similarly, AC transiently but significantly reduced phosphorylation of ERK, p38, and JNK at 0.5−3 h of incubation without reducing their protein expression during 6 h of incubation, and the strongest inhibition occurred at 2 h of incubation with reductions of 35% ($P < 0.05$) for pERK, 50% ($P < 0.05$) for p-p38, and 24% ($P < 0.05$) for pJNK (Figure 6A). On the basis of the time course experiment, we chose incubation times of 0.5 h for pFAK and 2
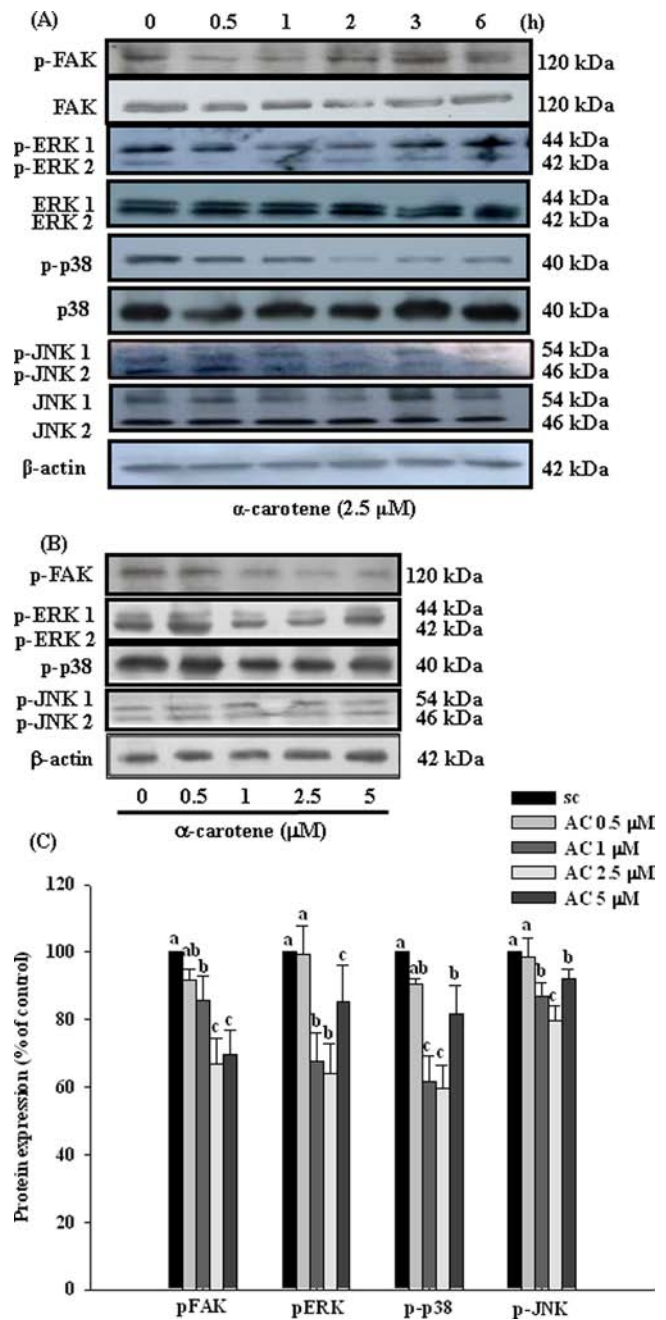

Figure 6. Time course and concentration effects of α-carotene on phosphorylation and protein expression on FAK and the MAPK family (ERK1/2, p38, and JNK 1/2) in SK-Hep-1 cells: (A) Western blot of phosphorylation and protein expression of FAK and the MAPK family; (B) Western blot of phosphorylation of FAK (0.5 h) and the MAPK (2 h) family; (C) densitometric analysis of panel B. Cells were treated with α-carotene (2.5 μM) for 0.5−6 h for time course effect and with α-carotene (0.5−5 μM) for different incubation times, that is, 0.5 h for phosphorylation of FAK and 2 h for phosphorylation of the MAPK family. Data are expressed as means ± SD, $n = 3-4$; means without a common letter differ significantly ($P < 0.05$).

h for the pMAPK family for determining the concentration effects of AC. We found that AC (0.5−2.5 μM) significantly and concentration-dependently inhibited phosphorylation of FAK at 0.5 h of incubation and the MAPK family at 2 h of incubation, whereas the effects of 5 μM AC were less effective than those of 2.5 μM AC (Figure 6B,C).

To further determine whether the inhibitory effects of AC on activities of MMP-9 and MMP-2 occur primarily through the inhibition of MAPK signaling pathways, SK-Hep-1 cells were pretreated with an ERK inhibitor (PD98059; 10 or 20 μM), a p38 inhibitor (SB203580; 10 or 20 μM), or a JNK inhibitor (SP600125; 10 or 20 μM) for 1 h and then incubated with or without AC (1 μM) for 24 h. This concentration of AC (1 μM) was chosen because it produced only a slight inhibition of MMP-9 and MMP-2 activities (Figures 3 and 7). Similarly, treatment with each of the three inhibitors only slightly decreased the activities of MMP-9 and MMP-2 (Figure 7). Under such conditions, the combined treatment enhanced the inhibition of MMP-9 (Figure 7B) and MMP-2 (Figure 7C) activities.

DISCUSSION

The present study investigated the antimetastatic effect of AC and determined its possible mechanisms of action in SK-Hep-1 cells, a highly metastatic human hepatoma cell line. We found that AC treatment significantly inhibited metastasis of human hepatocarcinoma SK-Hep-1 cells, as evidenced by inhibition of invasion, migration, and adhesion in a concentration-dependent manner up to 2.5 μM. These effects of AC were stronger than those of BC at the same concentration (2.5 μM). We also showed that AC and BC were well-absorbed by SK-Hep-1 cells and that the cellular uptake of AC and BC in SK-Hep-1 cells reached 9.5 and 10.9% for AC as well as 16.8 and 13.8% for BC at 24 and 48 h of incubation, respectively. The results indicate that AC and BC are both well-absorbed by SK-Hep-1 cells and that the cellular uptake of AC and BC is not associated with their antimetastatic actions in SK-Hep-1 cells. To the best of our knowledge, the present study is the first to demonstrate that AC exhibits antimetastatic effects on a human hepatoma cell line.

Several mechanisms may be involved in the antimetastatic effect of AC. One is that the balance between MMPs and TIMPs is destroyed by AC in SK-Hep-1 cells. TIMPs, the endogenous inhibitors of MMPs, bind to the active site of MMPs in a 1:1 stoichiomertic ratio resulting in inhibition of MMPs activities.[19] It has been shown that MMP-2 and MMP-9 activities are regulated extracellularly by TIMP-2 and TIMP-1, respectively.[20] In addition, overexpression of MMP-2 and MMP-9 is known to be one of the characteristics in most metastatic tumors,[21] whereas TIMP-1 and TIMP-2 overexpression is known to inhibit tumor growth and metastasis.[22] Therefore, the reduction of MMP-2 and MMP-9 activity and promotion of TIMP-1 and TIMP-2 expression by AC treatment, as observed in this study, may inhibit metastasis of SK-Hep-1 cells.

Another mechanism involved in the antimetastatic effect is the up-regulation of nm23-H1 expression by AC. Nm23-H1, one of the metastatic suppressor genes, is most deeply involved in phenotype of highly metastatic malignant tumors, including hepatocarcinoma.[23] Overexpression of nm23-H1 was shown to inhibit invasion, migration, and colonization in hepatocarcinoma.[23] The nm23-H1 gene was also known to down-regulate expression of MMP-2, MMP-9[24] and Rho small GTPases.[25] In accordance, we showed that AC decreased activities of MMP-2 and MMP-9 and expression of Rho and Rac 1 through promotion of nm23-H1 expression, leading to inhibition of metastasis in SK-Hep-1 cells. Interestingly, MMP-2 and MMP-9 activities and nm23-H1 expression are not affected by BC
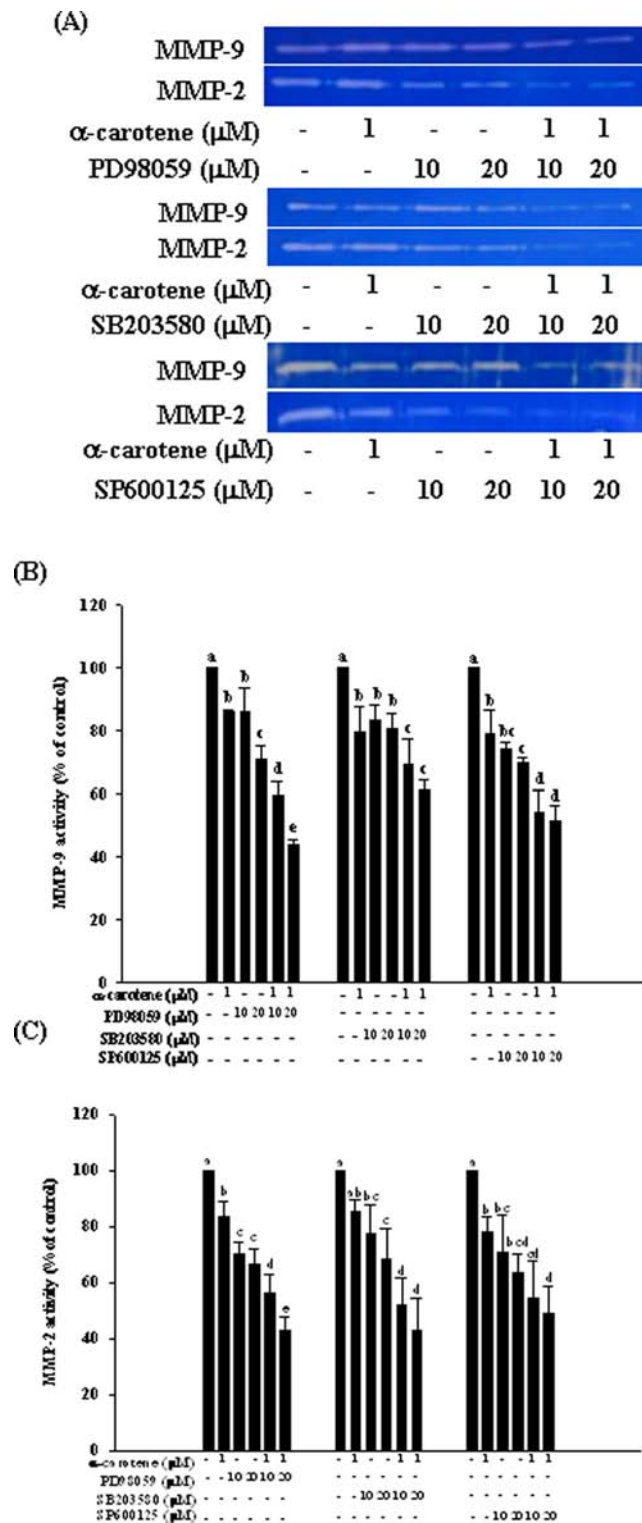

Figure 7. Effects of ERK inhibitor (PD98059), p38 inhibitor (SB203580), and JNK inhibitor (SP600125) with or without α-carotene on the activities of MMP-9 and MMP-2 in SK-Hep-1 cells: (A) gelatin zymography of MMP-9 and MMP-2; (B) densitometric analysis of MMP-9; (C) densitometric analysis of MMP-2. Cells were plated in 24 wells and pretreated with PD98059 (10 or 20 μM), SB203580 (10 or 20 μM), or SP600125 (10 or 20 μM) for 1 h and then incubated in the presence or absence of α-carotene (1 μM) for 24 h. Data are expressed as means $\pm$ SD, $n = 3-4$; means without a common letter differ significantly ($P < 0.05$).

treatment, suggesting that AC and BC may have different mechanisms on the antimetastasis of SK-Hep-1 cells.

Still another mechanism is the down-regulation of the uPA system by AC. The uPA system, consisting of uPA, PAI, and membrane-linked receptor uPAR, has been shown to play an important role in cancer metastasis.[26] uPA activates the conversion of inactive plasminogen to active plasmin followed by inducing the conversion of proMMPs to MMPs, leading to degrading and remodeling of the ECM.[27] PAI-1, an endogenous inhibitor of uPA, modulates cellular adhesion and migration through reacting with uPA, forming a stable complex with a 1:1 stoichiometry.[26] Therefore, inhibition of uPA catalytic activity may reduce tumor metastasis. Consistent with these aspects, our findings indicate that induction of PAI-1 expression and attenuation of uPA activity followed by inhibition of MMP-2 and MMP-9 activities are involved in the antimetastatic effects of AC in SK-Hep-1 cells.

FAK, a nonreceptor tyrosine kinase, plays a critical role in cell proliferation and metastasis.[28] FAK phosphorylation has been shown to help transduction of integrin-generated signals to the downstream MAPK cascade, leading to invasion, migration, and cytoskeletal rearrangement in cancer cells.[29] Indeed, knockdown of FAK was demonstrated to inhibit invasion and migration through reduction of MMP-9 secretion and Rho A expression in lung cancer cells.[30] In tumor progression and metastasis, phosphorylation of MAPK can be detected in many malignant cells including breast cancer, colon cancer, and lung cancer cells.[31] Therefore, inhibition of MAPK phosphorylation was found to prevent metastasis in several types of cancer cells.[32,33] In the present study, we found that the inhibitory effects of AC on phosphorylation of FAK and MAPK family were rapid and transient, and the most effective times were at 0.5 h (for phosphorylation of FAK) and 2 h (phosphorylation of the MAPK family) but disappeared at 2 or 6 h of incubation. We did not determine the effect of AC on protein expression and phosphorylation of FAK and MAPK at 12, 24, and 48 h of incubation because the activation of these protein kinases was known to be rapid and transient.[34] In addition, using specific inhibitors for ERK, p38, and JNK, we confirmed that AC in combination with these inhibitors enhanced the inhibition of MMP-9 and MMP-2 activities. These results suggest that AC inhibits activities of MMP-9 and MMP-2 in SK-Hep-1 cells through attenuation of FAK-mediated ERK/p38/JNK pathways.

Several in vitro studies have indicated that AC is more potent than BC at the same concentration against proliferation of human prostate cancer cells[10] and human neuroblastoma GOTO cells.[11] In vivo studies have confirmed that AC has a stronger potency in inhibiting tumorigenesis in liver, lung, skin, and colon than BC, when AC and BC were orally administered to mice or rats at the same doses.[13,14] These results suggested that the antiproliferative effect of AC and BC is not related to their pro-vitamin A activity, as has long been indicated by Schwartz et al.[35] Our present findings extend this notion to the antimetastatic activities of AC, which has a lower conversion rate to vitamin A than BC[8] and was found to possess a stronger antimetastatic activity than that of BC at the same concentration in SK-Hep-1 cells.

Although the antimetastatic effects of AC were stronger than those of BC, the plasma level of the latter is somewhat higher than that of the former. The physiological plasma BC and AC concentrations in healthy humans are around 0.42 and 0.1 μM, respectively.[7] After supplementation with a 30 mg BC capsule/day or consuming carrot for 6 weeks, plasma levels increased from 0.3 to 7.9 μM for BC and from 0.08 to 0.97 μM for AC in healthy men.[36] Therefore, the concentrations of AC (0.5–5 μM) and BC (2.5 μM) used in the present study are supraphysiological. It should be noted that the concentration effects of AC on most experiments in this study were either U- or bell-shaped; that is, the effects of AC were often lower at 5 μM than at 2.5 μM. These effects are consistent with our previous findings on lycopene research.[37] A possible explanation for the U- or bell-shaped response is that the antioxidant activity of carotenoids may shift to prooxidant activity at higher concentrations of carotenoids.[38]

In conclusion, we have demonstrated that AC significantly inhibits the metastasis of SK-Hep-1 cells and that this effect may involve the regulation of gene expression and signaling transduction pathway related to cell invasion and migration, as summarized in Figure 8. Although results from the present cell
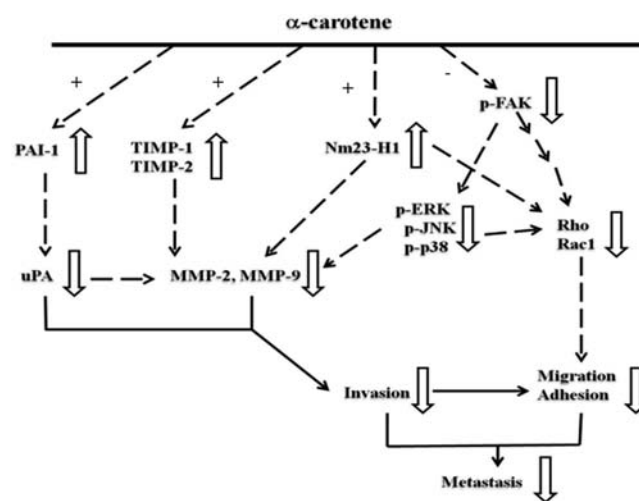

Figure 8. Proposed schematic diagram for the mechanistic action of α-carotene on SK-Hep-1 cell metastasis: increase ($\uparrow$) or decrease ($\downarrow$) as a result of up-regulation (+) or down-regulation (−) by α-carotene.

culture study suggest that AC could be used as an antimetastatic agent or a cancer therapeutic adjuvant, in vivo studies are needed to determine such a prospect.

■ AUTHOR INFORMATION

Corresponding Authors
*(M.-L.H.) Mailing address: Department of Food Science and Biotechnology, National Chung Hsing University, 250 Kuo Kuang Road, Taichung, Taiwan, 402 ROC. Phone: +886-4-2281-2363. Fax: +886-4-2281-2363. E-mail: mlhuhu@nchu.edu.tw.
*(C.-M.Y.) E-mail: s143524@hotmail.com.

Notes
The authors declare no competing financial interest.

■ ABBREVIATIONS USED

AC, α-carotene; BC, β-carotene; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMP; THF, tetrahydrofuran;

BHT, butylated hydroxytoluene; DMEM, Dulbecco's modified Eagles medium; FBS, fetal bovine serum

■ REFERENCES

(1) Weinstat-Saslow, D.; Steeg, P. S. Angiogenesis and colonization in the tumor metastatic process: basic and applied advances. *FASEB J.* **1994**, *8* (6), 401−407.

(2) Bjorklund, M.; Koivunen, E. Gelatinase-mediated migration and invasion of cancer cells. *Biochim. Biophys. Acta* **2005**, *1755* (1), 37−69.

(3) Lambert, E.; Dasse, E.; Haye, B.; Petitfrere, E. TIMPs as multifacial proteins. *Crit. Rev. Oncol. Hematol.* **2004**, *49* (3), 187−198.

(4) Friedl, P.; Wolf, K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat. Rev. Cancer* **2003**, *3* (5), 362−374.

(5) Vega, F. M.; Ridley, A. J. Rho GTPases in cancer cell biology. *FEBS Lett.* **2008**, *582* (14), 2093−2101.

(6) Nishino, H.; Murakosh, M.; Ii, T.; Takemura, M.; Kuchide, M.; Kanazawa, M.; Mou, X. Y.; Wada, S.; Masuda, M.; Ohsaka, Y.; Yogosawa, S.; Satomi, Y.; Jinno, K. Carotenoids in cancer chemo-prevention. *Cancer Metastasis Rev.* **2002**, *21* (3−4), 257−264.

(7) Tucker, K. L.; Chen, H.; Vogel, S.; Wilson, P. W.; Schaefer, E. J.; Lammi-Keefe, C. J. Carotenoid intakes, assessed by dietary questionnaire, are associated with plasma carotenoid concentrations in an elderly population. *J. Nutr.* **1999**, *129* (2), 438−445.

(8) Bauernfeind, J. C. Carotenoid vitamin A precursors and analogs in foods and feeds. *J. Agric. Food Chem.* **1972**, *20* (3), 456−473.

(9) Li, C.; Ford, E. S.; Zhao, G.; Balluz, L. S.; Giles, W. H.; Liu, S. Serum α-carotene concentrations and risk of death among US adults: the Third National Health and Nutrition Examination Survey Follow-up Study. *Arch. Intern. Med.* **2011**, *171* (6), 507−515.

(10) Kotake-Nara, E.; Kushiro, M.; Zhang, H.; Sugawara, T.; Miyashita, K.; Nagao, A. Carotenoids affect proliferation of human prostate cancer cells. *J. Nutr.* **2001**, *131* (12), 3303−3306.

(11) Murakoshi, M.; Takayasu, J.; Kimura, O.; Kohmura, E.; Nishino, H.; Iwashima, A.; Okuzumi, J.; Sakai, T.; Sugimoto, T.; Imanishi, J.; et al. Inhibitory effects of α-carotene on proliferation of the human neuroblastoma cell line GOTO. *J. Natl. Cancer Inst.* **1989**, *81* (21), 1649−1652.

(12) Zhang, L. X.; Cooney, R. V.; Bertram, J. S. Carotenoids enhance gap junctional communication and inhibit lipid peroxidation in C3H/10T1/2 cells: relationship to their cancer chemopreventive action. *Carcinogenesis* **1991**, *12* (11), 2109−2114.

(13) Murakoshi, M.; Nishino, H.; Satomi, Y.; Takayasu, J.; Hasegawa, T.; Tokuda, H.; Iwashima, A.; Okuzumi, J.; Okabe, H.; Kitano, H.; et al. Potent preventive action of α-carotene against carcinogenesis: spontaneous liver carcinogenesis and promoting stage of lung and skin carcinogenesis in mice are suppressed more effectively by α-carotene than by β-carotene. *Cancer Res.* **1992**, *52* (23), 6583−6587.

(14) Narisawa, T.; Fukaura, Y.; Hasebe, M.; Ito, M.; Aizawa, R.; Murakoshi, M.; Uemura, S.; Khachik, F.; Nishino, H. Inhibitory effects of natural carotenoids, α-carotene, β-carotene, lycopene and lutein, on colonic aberrant crypt foci formation in rats. *Cancer Lett.* **1996**, *107* (1), 137−142.

(15) Kozuki, Y.; Miura, Y.; Yagasaki, K. Inhibitory effects of carotenoids on the invasion of rat ascites hepatoma cells in culture. *Cancer Lett.* **2000**, *151* (1), 111−115.

(16) Lin, C. Y.; Huang, C. S.; Hu, M. L. The use of fetal bovine serum as delivery vehicle to improve the uptake and stability of lycopene in cell culture studies. *Br. J. Nutr.* **2007**, *98* (1), 226−232.

(17) Williams, A. W.; Boileau, T. W.; Clinton, S. K.; Erdman, J. W., Jr. β-Carotene stability and uptake by prostate cancer cells are dependent on delivery vehicle. *Nutr. Cancer* **2000**, *36* (2), 185−190.

(18) Repesh, L. A. A new in vitro assay for quantitating tumor cell invasion. *Invasion Metastasis* **1989**, *9* (3), 192−208.

(19) Han, X.; Zhang, H.; Jia, M.; Han, G.; Jiang, W. Expression of TIMP-3 gene by construction of a eukaryotic cell expression vector and its role in reduction of metastasis in a human breast cancer cell line. *Cell Mol. Immunol.* **2004**, *1* (4), 308−310.

(20) Patterson, M. L.; Atkinson, S. J.; Knauper, V.; Murphy, G. Specific collagenolysis by gelatinase A, MMP-2, is determined by the hemopexin domain and not the fibronectin-like domain. *FEBS Lett.* **2001**, *503* (2−3), 158−162.

(21) Liabakk, N. B.; Talbot, I.; Smith, R. A.; Wilkinson, K.; Balkwill, F. Matrix metalloprotease 2 (MMP-2) and matrix metalloprotease 9 (MMP-9) type IV collagenases in colorectal cancer. *Cancer Res.* **1996**, *56* (1), 190−196.

(22) Cruz-Munoz, W.; Khokha, R. The role of tissue inhibitors of metalloproteinases in tumorigenesis and metastasis. *Crit. Rev. Clin. Lab. Sci.* **2008**, *45* (3), 291−338.

(23) Nakayama, T.; Ohtsuru, A.; Nakao, K.; Shima, M.; Nakata, K.; Watanabe, K.; Ishii, N.; Kimura, N.; Nagataki, S. Expression in human hepatocellular carcinoma of nucleoside diphosphate kinase, a homologue of the nm23 gene product. *J. Natl. Cancer Inst.* **1992**, *84* (17), 1349−1354.

(24) Ohba, K.; Miyata, Y.; Koga, S.; Kanda, S.; Kanetake, H. Expression of nm23-H1 gene product in sarcomatous cancer cells of renal cell carcinoma: correlation with tumor stage and expression of matrix metalloproteinase-2, matrix metalloproteinase-9, sialyl Lewis X, and c-erbB-2. *Urology* **2005**, *65* (5), 1029−1034.

(25) Murakami, M.; Meneses, P. I.; Lan, K.; Robertson, E. S. The suppressor of metastasis Nm23-H1 interacts with the Cdc42 Rho family member and the pleckstrin homology domain of oncoprotein Dbl-1 to suppress cell migration. *Cancer Biol. Ther.* **2008**, *7* (5), 677−688.

(26) Andreasen, P. A.; Kjoller, L.; Christensen, L.; Duffy, M. J. The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int. J. Cancer* **1997**, *72* (1), 1−22.

(27) Carmeliet, P.; Moons, L.; Lijnen, R.; Baes, M.; Lemaitre, V.; Tipping, P.; Drew, A.; Eeckhout, Y.; Shapiro, S.; Lupu, F.; Collen, D. Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat. Genet.* **1997**, *17* (4), 439−444.

(28) Gilmore, A. P.; Romer, L. H. Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation. *Mol. Biol. Cell* **1996**, *7* (8), 1209−1224.

(29) Schlaepfer, D. D.; Hanks, S. K.; Hunter, T.; van der Geer, P. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* **1994**, *372* (6508), 786−791.

(30) Meng, X. N.; Jin, Y.; Yu, Y.; Bai, J.; Liu, G. Y.; Zhu, J.; Zhao, Y. Z.; Wang, Z.; Chen, F.; Lee, K. Y.; Fu, S. B. Characterisation of fibronectin-mediated FAK signalling pathways in lung cancer cell migration and invasion. *Br. J. Cancer* **2009**, *101* (2), 327−334.

(31) Hoshino, R.; Chatani, Y.; Yamori, T.; Tsuruo, T.; Oka, H.; Yoshida, O.; Shimada, Y.; Ari-i, S.; Wada, H.; Fujimoto, J.; Kohno, M. Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene* **1999**, *18* (3), 813−822.

(32) Huang, G. J.; Yang, C. M.; Chang, Y. S.; Amagaya, S.; Wang, H. C.; Hou, W. C.; Huang, S. S.; Hu, M. L. Hispolon suppresses SK-Hep1 human hepatoma cell metastasis by inhibiting matrix metalloprotei-nase-2/9 and urokinase-plasminogen activator through the PI3K/Akt and ERK signaling pathways. *J. Agric. Food Chem.* **2010**, *58* (17), 9468−9475.

(33) Westermarck, J.; Kahari, V. M. Regulation of matrix metal-loproteinase expression in tumor invasion. *FASEB J.* **1999**, *13* (8), 781−792.

(34) Yang, C. M.; Huang, S. M.; Liu, C. L.; Hu, M. L. Apo-8′-lycopenal induces expression of HO-1 and NQO-1 via the ERK/p38-Nrf2-ARE pathway in human HepG2 cells. *J. Agric. Food Chem.* **2012**, *60* (6), 1576−1585.

(35) Schwartz, J.; Shklar, G. Regression of experimental oral carcinomas by local injection of β-carotene and canthaxanthin. *Nutr. Cancer* **1988**, *11* (1), 35−40.

(36) Micozzi, M. S.; Brown, E. D.; Edwards, B. K.; Bieri, J. G.; Taylor, P. R.; Khachik, F.; Beecher, G. R.; Smith, J. C., Jr. Plasma carotenoid response to chronic intake of selected foods and β-carotene supplements in men. *Am. J. Clin. Nutr.* **1992**, *55* (6), 1120−1125.

(37) Huang, C. S.; Fan, Y. E.; Lin, C. Y.; Hu, M. L. Lycopene inhibits matrix metalloproteinase-9 expression and down-regulates the binding

activity of nuclear factor-κB and stimulatory protein-1. *J. Nutr. Biochem.* **2007**, *18* (7), 449−456.

(38) Palozza, P.; Calviello, G.; Serini, S.; Maggiano, N.; Lanza, P.; Ranelletti, F. O.; Bartoli, G. M. β-Carotene at high concentrations induces apoptosis by enhancing oxy-radical production in human adenocarcinoma cells. *Free Radical Biol. Med.* **2001**, *30* (9), 1000−1007.