DOI 10.1002/mnfr.201200098 *Mol. Nutr. Food Res.* 2012, *56*, 1520–1531

RESEARCH ARTICLE

Concomitant consumption of lycopene and fish oil inhibits tumor growth and progression in a mouse xenograft model of colon cancer

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Received: February 13, 2012
Revised: June 2, 2012
Accepted: July 24, 2012

Scope: Our previous report showed that concomitant supplementation of lycopene and eicosapentaenoic acid synergistically inhibited the proliferation of human colon cancer HT-29 cells in vitro.

Methods and results: To validate our findings, the present study investigated whether consumption of lycopene and fish oil would help prevent tumor growth and progression in a mouse xenograft model of colon cancer. The inhibitory effects of lycopene and fish oil on tumor growth were verified by western blotting analysis, bioluminescent imaging, immunohistochemical (IHC) staining and ELISA. The results demonstrated that lycopene and fish oil synergistically inhibited the growth of colon cancer in tumor-bearing mice. The bioluminescent imaging, histopathological and IHC staining results indicated that lycopene and fish oil effectively suppressed tumor growth and progression of colon cancer in vivo. The chemopreventive effects of lycopene and fish oil were associated with augmented expression of the cell cycle inhibitors such as $p21^{CIP1/WAF1}$ and $p27^{Kip1}$, and suppression of proliferating cell nuclear antigen, β-catenin, cyclin D1 and c-Myc proteins. Furthermore, lycopene and fish oil inhibited tumor progression through suppression of MMP-7, MMP-9, COX-2 and PGE2.

Conclusion: These results show that lycopene and fish oil act synergistically as chemopreventive agents against tumor growth and progression in a mouse xenograft model of colon cancer.

Keywords:
β-catenin / Colon cancer / Fish oil / Lycopene / PCNA

1 Introduction

Colorectal cancer (CRC) is one of the prevalent types of cancer in many countries. In the United States alone, more than 50 000 deaths are attributed to this cancer every year [1]. CRC is characterized by the loss of genomic stability and acquisition of multiple tumor-associated mutations [2, 3]. These molecular mutations cause histopathologic changes and transform normal colonic epithelial cells into colorectal carcinoma. Mutational activation of oncogenic pathways or inactivation of tumor suppressor genes leads to tumor growth and progression, including angiogenesis, invasion, metastasis, and inflammation [4]. Malignant cancer cells detach from the primary tumor and migrate across structural barriers, including basement membranes and surrounding stromal extracellular matrix (ECM). Tumor invasion and metastasis require an increase in the expression of matrix metalloproteinases and the degradation of ECM [5, 6]. Matrix

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Abbreviations: **BW**, body weight; **CRC**, colorectal cancer; **DHA**, docosahexenoic acid; **ECM**, extracellular matrix; **EPA**, eicosapentaenoic acid; **FBS**, fetal bovine serum; **FO**, fish oil; **H&E**, hematoxilin–eosin; **PCNA**, proliferating cell nuclear antigen

metalloproteinases are zinc-dependent endopeptidases and capable of degrading ECM components. Enzymes such as MMP-7 and MMP-9 degrade ECM and create a microenvironment supporting maintenance of tumor development [7]. MMP-7 protein is able to trigger the cleavage of E-cadherin from cell membrane [8]. E-cadherin complexes are major constituents of the epithelial junctions in the normal colorectal epithelium. The loss of E-cadherin is considered to be a key step in the epithelial-to-mesenchymal transition. Epithelial-to-mesenchymal transition is a process whereby epithelial cells switch to a mesenchymal phenotype, enabling tumor invasion and metastasis. Several studies have reported MMP-7 is highly expressed in human colon cancer cells [9]. Recent studies have suggested a strong correlation between the MMP-7 level and colorectal carcinogenesis [9,10]. Clinical studies indicate that elevated expression of MMP-7 is significantly correlated with poor prognosis and low survival rate in CRC patients [7,11].

Aberrant activation of phosphatidylinositol-3-kinase/Akt signaling pathway is reportedly involved in tumor growth and progression. Upon the activation of phosphatidylinositol-3-kinase signaling pathway, the cytoplasmic β-catenin protein is translocated into the nucleus and capable of inducing the expression of MMP-7, c-Myc, and cyclin D1 in human colon cancer cells [12–14]. The upregulation of MMP-7 and c-Myc is highly correlated with tumor progression of colon cancer [15]. During the proliferation of CRC cells, cell cycle-related proteins, such as cyclin D1 and proliferating cell nuclear antigen (PCNA), function as major regulators of cell-cycle progression and DNA replication, respectively. Recent studies have indicated that $p21^{CIP1/WAF1}$ and $p27^{Kip1}$ proteins serve as cell-cycle inhibitors and suppress the activity of cyclin D1 and PCNA proteins.

Epidemiological studies have shown that people consuming traditional Mediterranean diets with good amounts of vegetables, especially tomato, fish, fruits, olive oil, grains and beans, have lower rates of chronic diseases such as cancer. Lycopene, a major component in tomato, has potential as a chemopreventive agent against several types of cancer cell lines [16–19]. Epidemiologic studies reported statistically significant inverse associations between tomato consumption and risk of several types of cancer such as lung and prostate cancer [20–24]. Previous studies suggest that diets enriched in *n*-3 PUFAs suppresses the tumor growth [25–28]. These PUFA such as eicosapentaenoic acid (EPA) with five double bonds or docosahexenoic acid (DHA) with six double bonds have been shown to inhibit the inflammatory response and proliferation of cancer cells [25].

Consumption of lycopene at high doses reduces malignant lesion in a rodent model [29]. Our previous report demonstrates that lycopene could inhibit the expression of MMP-7, cell invasion, and proliferation of human colon cancer HT-29 cells in vitro [30,31]. Furthermore, low doses of lycopene and EPA synergistically inhibit the proliferation of human colon cancer HT-29 cells in vitro [32]. In the current study, we extended our investigation to an in vivo xenograft tumor model to validate the significance of the in vitro findings. The chemopreventive effects of lycopene and fish oil on the growth and progression of colon cancer cells in a mouse xenograft tumor model were investigated.

2 Materials and methods

2.1 Reagents and antibodies

Lycopene dry powder (Lycovit 10%) was kindly provided by Dr. Xiang–Dong Wang (Tufts University, USA). Fish oil (obtained from Jung–Nan Chemicals, Taiwan) contains approximately 32% of total *n*-3 PUFA (including 18% EPA, 12% DHA, and 2% of ALA) and 5% total *n*-6 PUFA. Corn oil was purchased commercially. Corn oil contains 54% of n-6 PUFA and 1% of *n*-3 PUFA. The ratio of *n*-6/*n*-3 PUFA for fish oil and corn oil used are estimated as 0.15 and 54, respectively. The anti-β-catenin, anti-$p21^{CIP1/WAF1}$, anti-cyclin D1, anti-E-cadherin, anti-MMP-7, anti-PCNA, and anti-lamin A/C antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-c-Myc, anti-COX-2, and anti-$p27^{kip1}$ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-actin antibody and RPMI 1640 medium were purchased from Sigma (St. Louis, MO, USA). The MMP-9 and PGE2 ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). Human colon cancer HT-29 cell line with luciferase reporter gene was purchased from Caliper Life Sciences (Hopkinton, MA, USA). Lycopene was dissolved in either corn oil or fish oil at dosages of 3 and 6 mg/kg of body weight (BW).

2.2 Cell culture

Human colon cancer HT-29 cells with luciferase reporter gene were cultured in a 37°C humidified incubator with 5% CO_2 and grown to confluency using fetal bovine serum (FBS) supplemented RPMI 1640 media. Cells used in different experiments have the similar passage number. RPMI 1640 medium was supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 3.7 g/L sodium bicarbonate in the absence of antibiotics. The expression of luciferase was monitored by reacting with the luciferin substrate. Luciferase transfected human colon cancer HT-29 cells were able to convert luciferin into oxy-luciferin with an emission of bioluminescence. The levels of bioluminescence intensities were represented as photons s^{-1} cm^{-2}. Using bioluminescent imaging allows reliable, rapid, sensitive, noninvasive, and longitudinal monitoring of tumor growth in vivo.

2.3 Xenograft implantation of tumor cells

To establish the mouse xenograft model, subconfluent cultures of colon cancer HT-29 cells were given fresh

medium 24 h before being harvested by a brief treatment with 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% FBS, and the cells were washed twice and resuspended in serum-free RPMI 1640 medium. Only single-cell suspensions with a viability of greater than 90% were used for the injections.

2.4 Animals, diet, and lycopene/fish oil supplementation

Adult (3–4-week-old) BALB/C AnN-Foxn1 nude mice (19–22 g) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Mice were maintained under specific pathogen-free conditions in facilities approved by the National Laboratory Animal Center in accordance with current regulations and standards (animal protocol no. 97–27-N). During the entire experimental period, mice were fed a standard Lab 5010 diet purchased from LabDiet (St. Louis, MO, USA). The standard diet contains crude fat (13.5% total diet energy), protein (27.5% total diet energy), and carbohydrate (59% total diet energy), and had no detectable amounts of lycopene and fish oil as indicated by the supplier. Mice that had been anesthetized with an inhalation of isofluorane were placed in a supine position. The mice were subcutaneously (s.c.) injected with human colon cancer HT-29 cells (1×10^6/0.1 mL medium) into the right flank of each BALB/C AnN-Foxn1 nude mouse. A well-localized bleb was considered to be a sign of a technically satisfactory injection.

After the inoculation, mice were divided into six subgroups. Lycopene and fish oil were given to experimental animals by gavage once a day at a total volume 0.15 mL. Low-dose (Low_Lyc) or high-dose (High_Lyc) lycopene group received a daily oral consumption dose of lycopene dissolved in corn oil (4% w/w) at 3 or 6 mg/kg of BW once per day, respectively. Fish oil group (FO) received fish oil (4% w/w of diet) once per day. Other groups (FO_Low_Lyc or FO_High_Lyc) received FO (4% w/w) and lycopene at an oral dose of 3 or 6 mg/kg of BW once per day, respectively. The tumor control group received corn oil (4% w/w) once per day only. Body weights were determined once weekly. During the study, blood samples for ELISA were collected from tail vein at weeks 3, 4, and 5. At the end of the experimental period, animals were euthanized with CO_2 inhalation; tumor tissues were then excised, weighed, and frozen immediately. These tumor tissues were sectioned, and stained with Mayer's hematoxilin–eosin (H&E) for light microscopy. The remaining tissues of liver, lung, spleen, pancreas, and intestine were also excised, weighed, and frozen for further experiments. Blood samples were collected from the heart in a 1-mL vacutainer tube in the presence or absence of heparin and centrifuged for 10 min at $1000 \times g$ to obtain plasma or serum, respectively.

2.5 Bioluminescent imaging of colon cancer

Documentation of bioluminescent imaging was also performed weekly during the 5-week experimental period by using a noninvasive in vivo imaging system 200 (IVIS 200) (Caliper Life Sciences, Inc.). These experimental animals were anesthetized by staying in a chamber with isoflurane gas. To measure the expression of luciferase in vivo and image the tumor growth, nude mice received D-luciferin, the substrate of luciferase, at a dose of 150 mg/kg BW. Briefly, D-luciferin (15 mg/mL), the substrate of luciferase, was injected into the tumor-bearing nude mice peritoneally. Results of bioluminescent imaging were collected and analyzed by accessory software. The bioluminescence intensities were determined by selecting the region of interest (regions with bioluminescence emission) and adjusted with the size of the selecting area (cm^2). The intensity of bioluminescence was expressed as photons/sec/cm^2. Bioluminescence intensities compared to the tumor control group (tumor-bearing mice without lycopene or FO supplementation at week 2) represented proliferation indices.

2.6 Histopathological, immunohistochemical (IHC), and immunofluorescent staining of tumor tissues

Frozen tumor tissues were cut in 5 μm sections and immediately fixed with 4% paraformaldehyde. Sections were stained with Mayer's H&E for light microscopy. Negative controls did not exhibit any staining. In a blinded manner, three hot spots were examined per tumor section (high-power fields X 400) of six different tumors in each group. For IHC staining, frozen tissue sections were treated with 0.3% hydrogen peroxide to block the endogenous peroxide activity. Nonspecific protein bindings were blocked with 10% normal goat serum for 1 h followed by incubation with either anti-β-catenin or anti-COX-2 primary antibodies (1:300). Tissue sections were washed with 0.1 M PBS and incubated with biotinyated immunoglobin G (1:300 secondary antibody) at room temperature for 1 h. Tissue sections were stained with Avidin-Biotin complex, diaminobenzidine, and hydrogen peroxide. Cell nuclei were stained with hematoxilin. Imaging was performed at 400× magnifications. Images of tumor sections were acquired on an Olympus BX-51 microscope using an Olympus DP-71 digital camera and imaging system (Olympus, Tokyo, Japan).

2.7 Protein extraction and Western blotting analysis

Briefly, animal tissues were prepared using a Tissue Nuclear Extract Reagent Kit containing protease and phosphatase inhibitors according to the manufacturer's instructions (Pierce Biotechnology, Lackford, IL, USA). After centrifugation for 10 min at $12\,000 \times g$ to remove cell debris, the supernatants

were further separated and retained as cytoplasmic and nuclear fraction extracts, respectively. Cross contamination between the nuclear and cytoplasmic fractions was not found (data not shown).

The nuclear fraction of tissue proteins (100 μg) was fractioned on 10% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with anti-cyclinD1 monoclonal antibody, according to the manufacturer's instructions. The blots were stripped and reprobed with a lamin A/C antibody as the loading control. The levels of PCNA, $p21^{CIP1/WAF1}$, $p27^{Kip1}$, β-catenin, and c-Myc in the nuclear fractions of the tumor tissues were measured using a similar procedure described above. The cytoplasmic fraction of tissue proteins (100 μg) was fractioned on 10% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with anti-MMP-7 or anti-E-cadherin monoclonal antibody, according to the manufacturer's instructions. The blots were stripped and reprobed with a β-actin antibody as the loading control.

2.8 Quantitative real-time PCR (qPCR)

Tissue RNA samples were prepared, extracted from each group, and reversely converted into cDNA for further analysis. Briefly, cDNA samples were used in PCR reaction mix containing gene-specific primers: COX-2 forward, 5′-CGCTCAGCCATACAGCAA-3′ and reverse, 5′-GAATCCTGTCCGGGTACAATC-3′; and GAPDH (was used as an internal control gene) forward: 5′-ACGGATTTGG TCGTATTGGG-3′, reverse: 5′-TGATTTTGGAGGGATC TCGC-3′. The cDNA was amplified in the following conditions: denaturation: 95°C for 2 min; amplification: 40 cycles (94°C for 30 s); annealing (1 min for 30 s); and extension (1 min for 30 s). Quantitative PCR experiments were performed using the real-time PCR detection system (ABI 7900, Applied Biosystems, Carlsbad, CA, USA). The relative COX-2 mRNA expression levels in different treatment groups were presented as folds of the corresponding negative control group.

2.9 Detection of plasma MMP-9 and serum PGE_2 by ELISA

The MMP-9 plasma level (from week 3 and week 5) was measured by ELISA according to the manufacturer's instructions (R&D Systems). Briefly, a 100 μL diluted plasma sample (1:8 dilution) from each group was added to each well and analyzed.

The PGE_2 serum level (from week 3 and week 5) was measured by ELISA using a similar procedure described above. Briefly, a 150 μL diluted serum sample (1:15 dilution) from each group was added to each well and analyzed. Upon completion of the ELISA process, the plate was read at 450/570 nm wavelength using a microplate reader (Tecan, Mannedorf, Switzerland).

2.10 Gelatin zymogram

Measurement of MMP-9 enzymatic activity from each plasma group was performed as follows. Gels were copolymerized with 0.1% gelatin. For each group, equal amount of plasma sample was added to each well and analyzed. Electrophoresis was carried out using the minigel slab apparatus Mini Protean 2 (Bio-Rad, Hercules, CA, USA) at a constant voltage of 150 V, until the dye reached the bottom of the gel. Following electrophoresis, gels were washed in buffer solution (2.5% Triton X-100 in 50 mM Tris–HCl (pH 7.5)) for 1 h in an orbital shaker. The zymograms were incubated for 24 h at 37°C in incubation buffer (0.15 M NaCl, 10 mM $CaCl_2$, 0.02% NaN_3 in 50 mM Tris–HCl (pH 7.5)). Gels were then stained with Coomassie Blue and destained with 7% methanol and 5% acetic acid. Areas of enzymatic activity appeared as clear bands over the dark background.

2.11 Statistical analysis

Quantitative analysis was used to determine whether there were differences in the tumor growth among the experimental groups and tumor control groups ($n = 6$ for each group) in CRC cell bearing mice. Statistical analyses of the differences in tumor growth among the experimental and control conditions were performed using SYSTAT software (SYSTAT Software Inc., Chicago, IL, USA). Confirmation of difference in tumor growth as statistically significant required a rejection of the null hypothesis of no difference between the mean proliferation indices obtained from different sets of experimental and control groups at the $p = 0.05$ level using one-way ANOVA. The Bonferroni post hoc test was used to determine differences between the groups.

3 Results

3.1 Lycopene and fish oil synergistically inhibited tumor growth of colon cancer in a mouse xenograft model

To prove a correlation between bioluminescent intensity (photon counts) and the tumor growth, human colon cancer HT-29 cells with luciferase reporter gene were implanted into the right flank of nude mice in a pilot study. The results from the pilot study showed a correlation between tumor size measuring by callipers and the bioluminescent intensity (photon counts $= 208 \pm 18 \times$ size $(mm)^3$; $r^2 = 0.99$) (data not shown). Our previous studies demonstrated that lycopene and EPA synergistically inhibited the proliferation of human colon cancer HT-29 cells in vitro. We therefore extended our

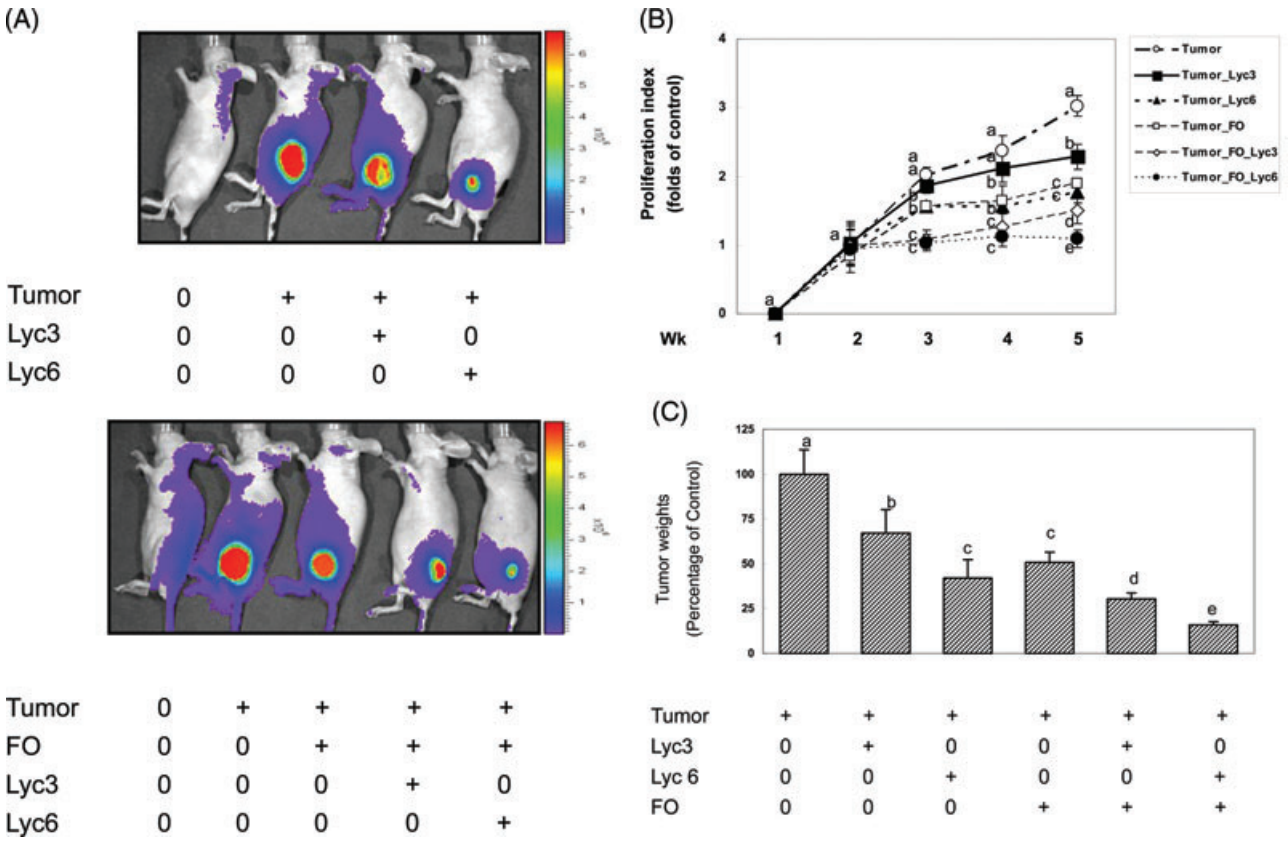

Figure 1. Lycopene and fish oil synergistically inhibited tumor growth of colon cancer in a mouse xenograft model. (A) Xenograft nude mice ($n = 6$ for each group) were divided into six groups (tumor, tumor with low lycopene, tumor with high lycopene) and given fish oil (0.4% w/w) for 5 weeks. The extent of tumor growth was evaluated by using bioluminescent imaging system. Photos of primary tumors from different groups were shown. (B) Data of bioluminescent intensity represented the proliferation index in primary tumor tissues. Different letters represent statistically significant difference at the same time point, $p < 0.05$. (C) Data represent the change of tumor weight (at week 5) among tumor control group and experimental groups. Different letters represent statistically significant difference, $p < 0.05$. ($p < 0.05$ at week 5).

investigation to an in vivo xenograft tumor model to validate the significance of the in vitro findings. The inhibitory effects of lycopene and fish oil on the growth of colon cancer cells in a mouse xenograft tumor model were investigated. A mouse xenograft tumor model was established by subcutaneously inoculating human colon cancer HT-29 cells into the right flank of each nude mouse. Bioluminescent imaging results demonstrated that consumption of lycopene effectively suppressed tumor growth of CRC in tumor-bearing mice (Fig. 1A). The results showed that lycopene consumption significantly suppressed the growth of colon cancer in tumor-bearing mice ($p < 0.05$). As shown in Fig. 1A, lycopene consumption at 3 mg/kg of BW (Low_Lyc) and 6 mg/kg BW (High_Lyc) significantly inhibited the tumor growth ($p < 0.05$). Consumption of fish oil (4% w/w diet) also effectively inhibited the tumor growth in vivo ($p < 0.05$). Furthermore, concomitant consumption of lycopene and fish oil significantly suppressed CRC tumor growth in vivo ($p < 0.05$) (Fig. 1A). We further quantified and analyzed the proliferation index of CRC in mouse xenograft model to demonstrate these important findings ($p < 0.05$) (Fig. 1B). Compared with the tumor control group, the weight of tumor tissues per mouse significantly decreased in the Low_Lyc, High_Lyc, FO, FO_Low_Lyc, and FO_High_Lyc groups, respectively, accounting for a 33%, 58%, 50%, 70%, and 85% inhibition at the end of the study (5th week) ($p < 0.05$) (Fig. 1C). Our previous report suggested that consumption of lycopene and EPA synergistically inhibited the proliferation of human colon cancer HT-29 cells in vitro [33]. In the present study, we demonstrated that consumption of lycopene and fish oil significantly inhibited CRC tumor growth in this mouse xenograft model ($p < 0.05$).

3.2 Lycopene and fish oil significantly modulated the expression of $p21^{CIP1/WAF1}$, $p27^{Kip1}$, and PCNA proteins in the mouse xenograft tumor model

Previous studies indicated that the cyclin D1 and PCNA proteins play crucial roles in the regulation of cell proliferation. Moreover, the $p21^{CIP1/WAF1}$ and $p27^{Kip1}$ inhibited the activities

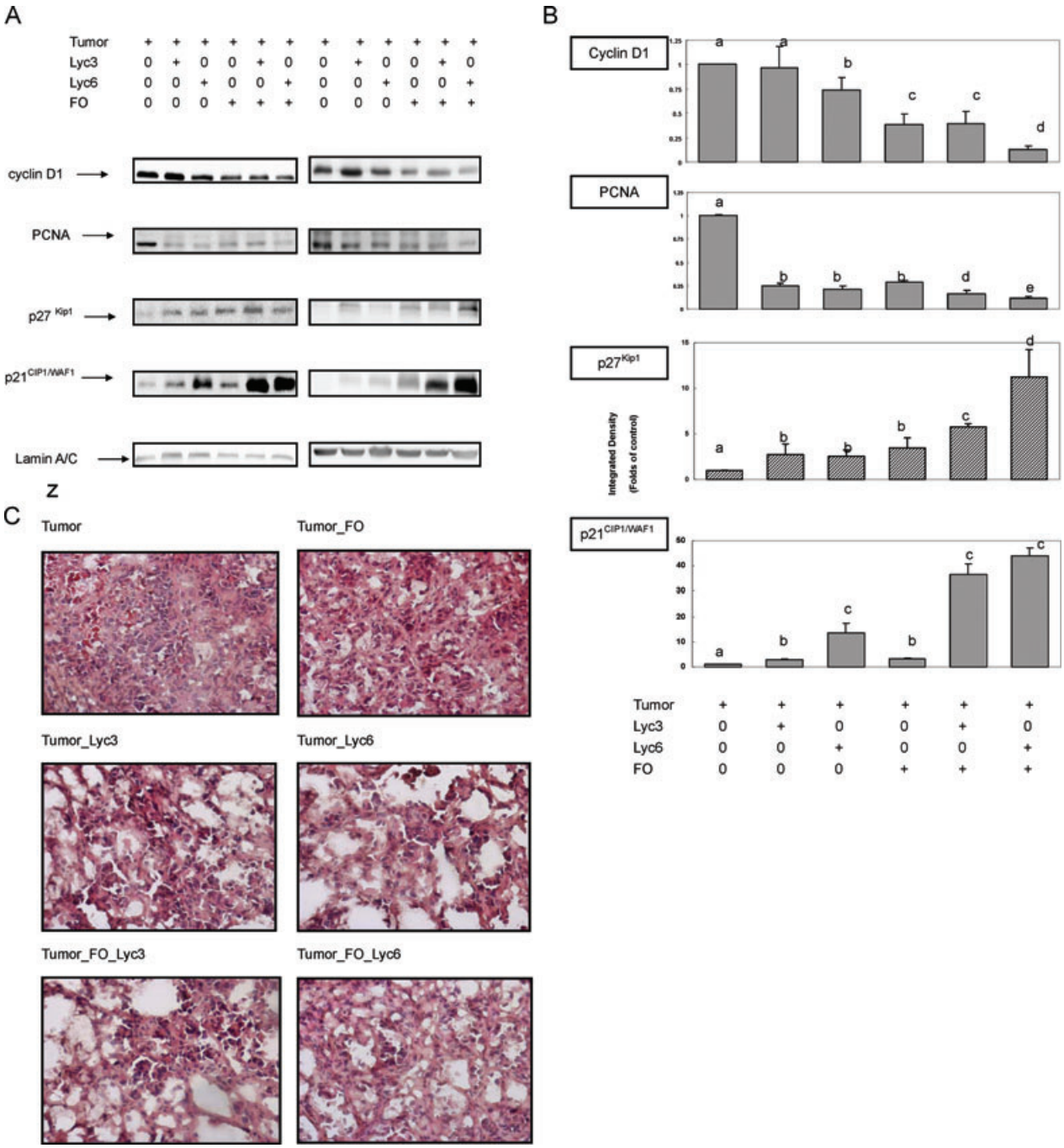

Figure 2. Lycopene and fish oil significantly modulated the expression of $p21^{CIP1/WAF1}$, $p27^{Kip1}$, and PCNA proteins in the mouse xenograft tumor model. (A) Cell lysates from animal tissues (at week 5) were prepared using Tissue Nuclear Extract Reagent Kit containing protease inhibitor and phosphatase inhibitors according to the manufacture's instruction. After centrifugation for 10 min at 12 000 × *g* to remove cell debris, the supernatants were retained as a whole tissue extract. Cross contamination between nuclear and cytoplasma fractions were not found (data not shown). Cell lysates were blotted with either anti-cyclin D1, anti-PCNA, anti-$p21^{CIP1/WAF1}$, or anti-$p27^{kip1}$ monoclonal antibody as described in Materials and methods. The levels of detection in cell lysate represent the amount of cyclin D1, PCNA, $p21^{CIP1/WAF1}$, or $p27^{kip1}$ in human colon cancer cells. The blots were stripped and reprobed with anti-lamin A/C antibody as loading control. The results presented are representative of six different experiments. The immunoreactive bands are noted with arrow. (B) The integrated densities of cyclin D1, PCNA, $p21^{CIP1/WAF1}$, and $p27^{kip1}$ proteins adjusted with internal control protein (lamin A/C) were shown in the panel. Different letters represent statistically significant difference, $p < 0.05$. (C) Tumor tissues (at week 5) were formalin fixed, embedded in paraffin, sectioned, and subjected to H&E staining. Imaging was documented at 400× magnification. Blue spots represented the nuclei stained with hematoxylin. Red spots represented cytoplasma stained with eosin.

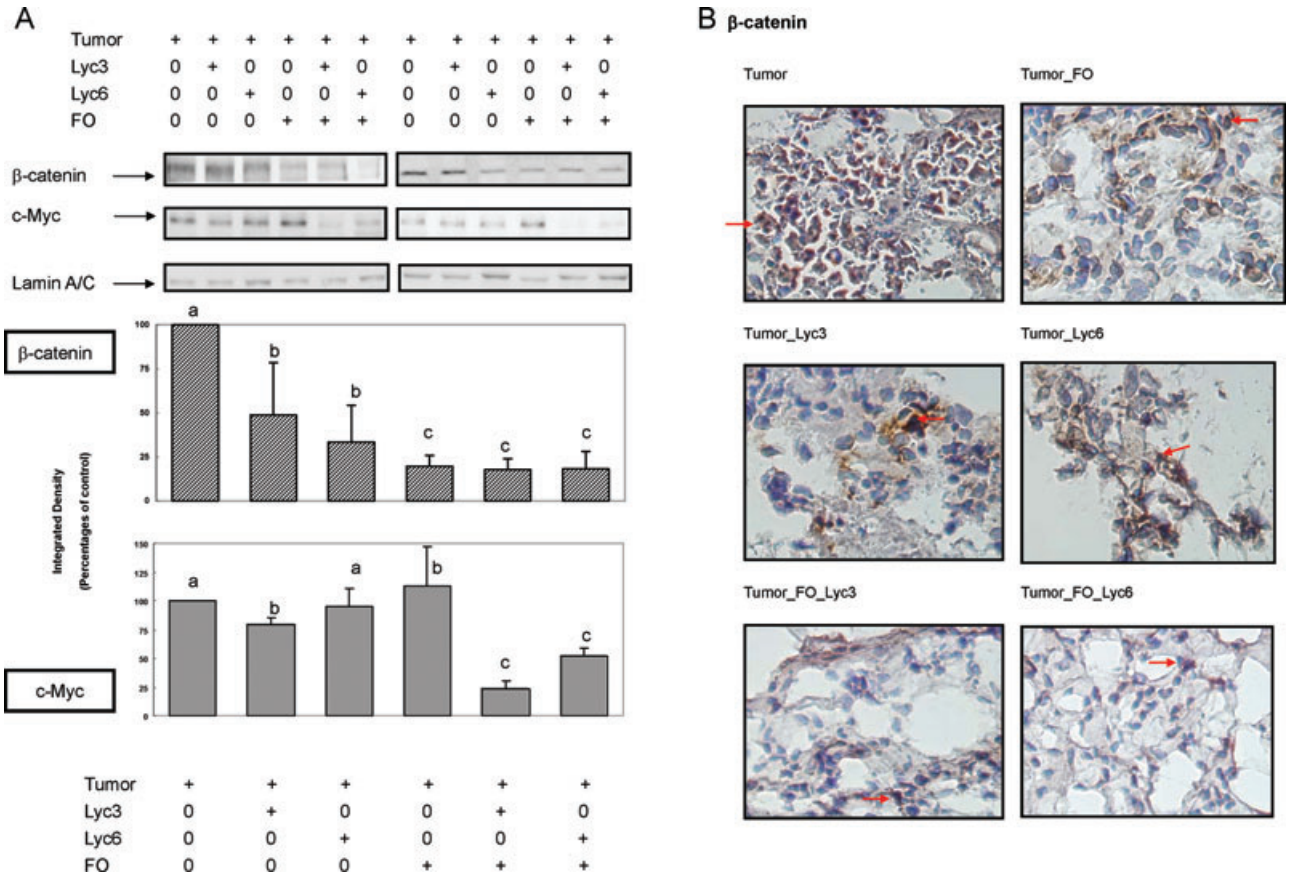

Figure 3. Lycopene and fish oil suppressed the expression of β-catenin and c-Myc proteins in tumor-bearing mice. (A) Nuclear fractions of cell lysates (at week 5) were blotted with either anti-β-catenin or anti-c-Myc monoclonal antibodies as described in Materials and methods. The levels of detection in cell lysate represent the amount of β-catenin or c-Myc in human colon cancer cells. The blots were stripped and reprobed with anti-lamin A/C antibody as loading control. The results presented are representative of six different experiments. The immunoreactive bands are denoted with an arrow. The integrated density of β-catenin or c-Myc protein adjusted with internal control protein (lamin A/C) was shown in the bottom panel. Different letters represent statistically significant difference, $p < 0.05$. (B) Tumor tissues (at week 5) were frozen, sectioned, and subjected to anti-β-catenin antibody by immunohistochemical (IHC) staining described in Material and methods. Imaging was documented at 200× magnification. Brown area represented distribution of β-catenin proteins (indicated with red arrows) in HT-29 cells stained with monoclonal antibody. Blue spots represented the location of cell nuclei stained with hematoxylin. The results presented are representative of six different experiments.

of cyclin D1 and PCNA. Therefore, we investigated whether lycopene and fish oil would modulate the expression of these proteins in vivo. As shown in Fig. 2A, consumption of lycopene and fish oil synergistically inhibited the nuclear levels of cyclin D1 and PCNA through the induction of $p21^{CIP1/WAF1}$ and $p27^{Kip1}$ proteins in tumor-bearing mice. The quantitative results demonstrated that FO_Low_Lyc and FO_High_Lyc suppressed the expression of PCNA by up to 84% and 89%, respectively (Fig. 2B). The Mayor H&E staining results also showed that lycopene and fish oil significantly inhibited tumor growth and progression in tumor-bearing mice (Fig. 2C). The results suggested that the chemopreventive effects of lycopene and fish oil on tumor growth and progression of CRC were associated with the suppression of cyclin D1 and PCNA. The molecular mechanisms of actions were in part through the augmented expression of $p21^{CIP1/WAF1}$ and $p27^{Kip1}$ proteins in these tumor-bearing mice. Therefore, lycopene and fish oil could potentially function as synergistic agents for chemoprevention of colon cancer, as demonstrated here in this mouse xenograft tumor model.

3.3 Lycopene and fish oil suppressed the expression of β-catenin and c-Myc proteins in tumor-bearing mice

Previous investigation had indicated that the β-catenin protein plays an important role in the regulation of cyclinD1, c-Myc, and MMP-7 expression. Moreover, the expression of c-Myc and MMP-7 is strongly correlated with tumor progression of CRC. Therefore, we investigated whether lycopene and fish oil would synergistically suppress the expression of

the β-catenin and c-Myc proteins in vivo. As shown in Fig. 3A, lycopene and fish oil significantly inhibited the expression of β-catenin. Furthermore, lycopene and fish oil synergistically suppressed the expression of c-Myc protein in vivo. To examine the chemopreventive effects of lycopene and fish oil, we further identified the distribution of β-catenin proteins by using IHC staining analysis in these tumor-bearing mice. As shown in Fig. 3B, β-catenin proteins (brown areas indicated with red arrows) were accumulated in the nuclei of tumor control group. However, consumption of lycopene and fish oil significantly blocked nuclear translocalization and reduced nuclear levels of β-catenin proteins in tumor tissues.

These results suggest that the inhibitory effects of lycopene and fish oil on tumor growth and progression were associated with the suppression of β-catenin, cyclinD1, and c-Myc proteins in this mouse xenograft model of CRC.

3.4 Lycopene and fish oil significantly suppressed the expression of MMP-7 and MMP-9 in tumor-bearing mice

Previous studies suggested that expression of MMP-9 and MMP-7 was associated with tumor invasion and progression of CRC. In the current studies, we investigated whether consumption of lycopene and fish oil could modulate the expression of MMP-9 and MMP-7 proteins in the mouse xenograft model. At week 3, the basal MMP-9 plasma levels in tumor-free mice were approximately 4.2 ng/mL (Fig. 4A). Mice inoculated with colon cancer cells had plasma levels of MMP-9 (mean ± SEM: 13.5 ± 3.2 ng/mL). Moreover, the MMP-9 plasma levels decreased from 13.5 ng/mL in the tumor control group to 11.7 ng/mL, 6.8 ng/mL and 6.1 ng/mL in the Low_Lyc, High_Lyc, and FO–fed groups, respectively. Furthermore, the Low_Lyc_FO and High_Lyc_FO had MMP-9 plasma levels at 5 ng/mL and 4.2 ng/mL, respectively.

By the end of the study, the basal MMP-9 plasma levels in tumor-free mice were approximately 6.1 ng/mL (Fig. 4A). Mice inoculated with colon cancer HT-29 cells had high plasma levels of MMP-9 (mean ± SEM: 23 ± 8.1 ng/mL). However, consumption of lycopene and fish oil significantly decreased the MMP-9 plasma level in these tumor-bearing mice. The MMP-9 plasma levels decreased from 23 ng/mL in the tumor control group to 16.4 ng/mL, 9.1 ng/mL and 8.9 ng/mL in the Low_Lyc, High_Lyc, and FO-fed groups, respectively. Furthermore, the Low_Lyc_FO and High_Lyc_FO had MMP-9 plasma levels at 6.7 ng/mL and 6.2 ng/mL, respectively. Quantitative results of MMP-9 activities were identified by using zymogram analysis (Fig. 4B). Studies indicated that an expression of MMP-7 molecule was inversely correlated with the expression of E-cadherin protein. Therefore, we further investigated whether consumption of lycopene and fish oil would inhibit the expression of MMP-7 proteins in these tumor-bearing mice. As shown in Fig. 4C, MMP-7 was highly expressed in the tumor tissues. Consumption of lycopene and fish oil significantly suppressed the expression of MMP-7 protein in these tumor-bearing mice. The quantitative results demonstrated that lycopene and fish oil significantly inhibited the expression of MMP-7 by up to 90% and 91% in Low_Lyc_FO and High_Lyc_FO, respectively. Moreover, lycopene and fish oil synergistically induced the expression of E-cadherin protein in tumor-bearing mice (Fig. 4C). These results suggested that the inhibitory effects of lycopene and fish oil on tumor growth and progression in colon cancer were associated with suppression of MMP-7 and MMP-9 proteins. Therefore, lycopene and fish oil could potentially act as synergistic agents to prevent tumor growth and progression of CRC, as demonstrated here in this mouse xenograft tumor model.

3.5 Lycopene and fish oil significantly suppressed tumor inflammation in tumor-bearing mice

A recent study indicated that MMP-9 expression is associated with upregulation of COX-2 proteins in several types of cancer. Several clinical studies indicated that COX-2 expression is associated with the tumor inflammation and progression in CRC. Therefore, these evidences suggest that COX-2 expression and its major metabolite, PGE2, could play crucial roles in tumor progression of CRC. In the present study, we further examined the inhibitory effects of lycopene and fish oil on the expression of COX-2 and PGE_2 in these tumor-bearing mice. As shown in Fig. 5A, the results indicated that the tumor control group had augmented the gene expression of *COX-2*. The IHC staining results also demonstrated a prevalent expression of COX-2 in the tumor control group (Fig. 4B). However, consumption of lycopene and fish oil significantly suppressed COX2 expression in these tumor-bearing mice (Fig. 4A & 4B). The results further demonstrated that mice inoculated with colon cancer HT-29 cells had increased serum PGE_2 levels (Fig. 5C). At week 3 and week 5, consumption of lycopene and fish oil significantly reduced PGE_2 serum levels in vivo. These results suggested that consumption of lycopene and fish oil significantly suppressed the expression of COX-2 and its major metabolite, PGE2, in tumor-bearing mice. Taken together; it is probable that lycopene and fish oil could inhibit tumor inflammation and progression of CRC in part through suppression of COX-2 and PGE_2 in these tumor-bearing mice.

4 Discussion

Many studies suggested that lycopene and fish oil might exert anti-cancer effects and inhibit the expression of oncoproteins associated with cancer prognosis. In the current study, the doses of lycopene and fish oil were based on the physiological relevance and daily intakes of fish oil or lycopene for humans. Due to the physiological relevance of the diets used in this study, the dose of fish oil was determined based on the typical consumption of EPA and DHA at 1–2% of total diet energy

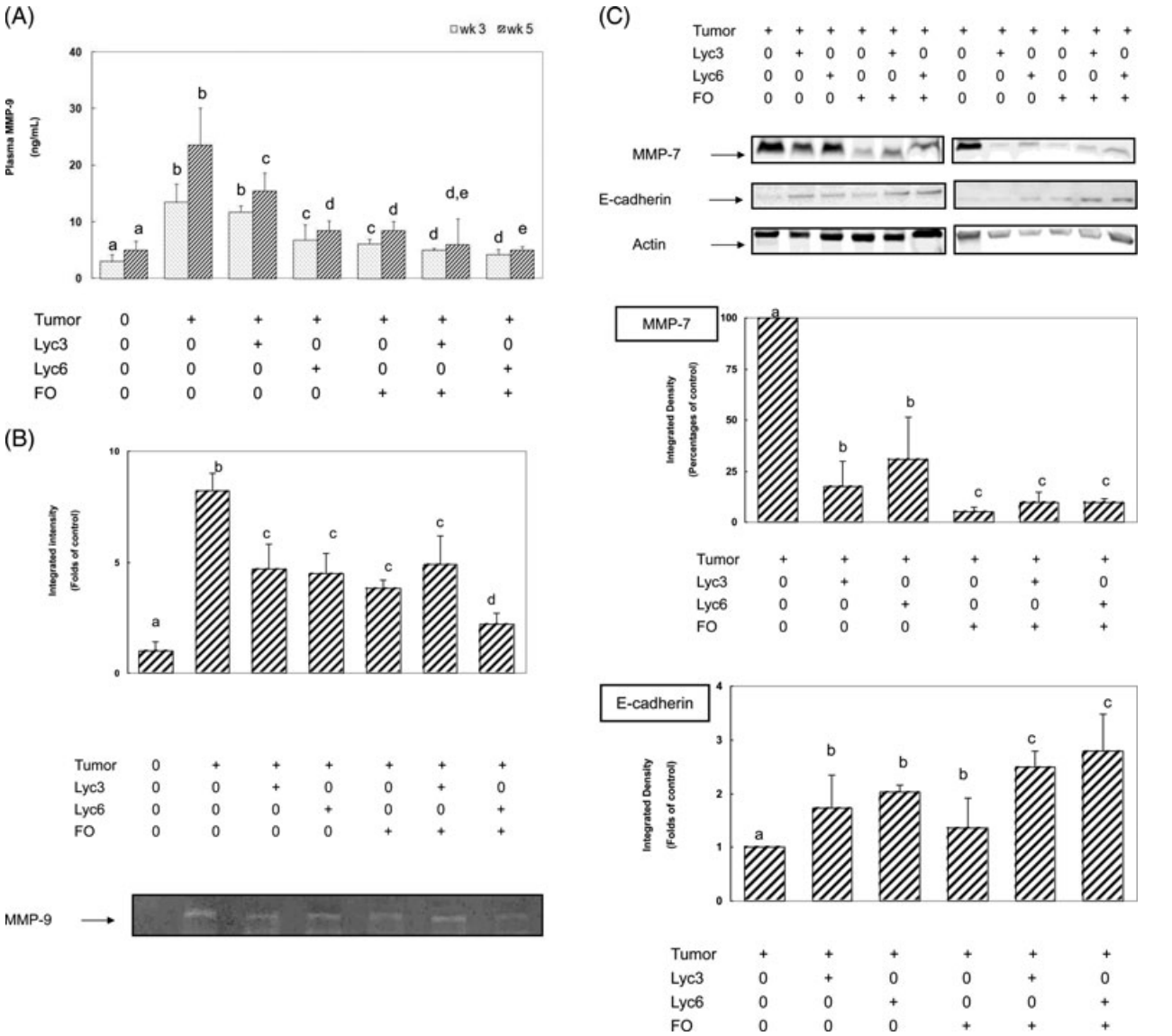

Figure 4. Lycopene and fish oil significantly suppressed the expression of MMP-7 and MMP-9 in tumor-bearing mice. (A) The plasma levels of MMP-9 were determined by using ELISA Kit (R&D Systems Inc.). Briefly, equal amounts of diluted plasma sample (100 μL) from each group (at week 3 and week 5) was added to each well and reacted with MMP-9 antibody according to the manufacturer's instructions. Upon completion of the ELISA process, fluorescence intensities were read using a wavelength of 450/570 nm. These results presented are representative of six different experiments and presented as plasma MMP-9 levels. Different letters represent statistically significant difference at the same time point, $p < 0.05$. (B) Equal amounts of plasma samples (at week 5) from each group were loaded into gelatin-containing gel. After incubation and staining of gelatin gel, the photographs of zymogram bands are denoted with arrows. Areas of enzymatic activity appeared as clear bands over the dark background. These results presented are representative of six different experiments. Different letters represent statistically significant difference, $p < 0.05$. (C) Cytoplasma fractions of cell lysates (at week 5) were blotted with either anti-MMP7 or anti-E-cadherin monoclonal antibody as described in Materials and methods. The levels of detection in the cell lysate represent the amount of MMP7 or E-cadherin in human colon cancer cells. The blots were stripped and reprobed with anti-actin antibody as loading control. The results presented are representative of six different experiments. The immunoreactive bands are denoted with an arrow. The integrated densities of MMP7 and E-cadherin proteins adjusted with internal control protein (actin) were shown in the bottom panel. Different letters represent statistically significant difference, $p < 0.05$.

in the Japanese diet [34]. In the current study, around 2 and 1.4% of total diet energy are from EPA and DHA, respectively.

In this study, the doses of lycopene consumption at 3 mg/kg of BW per day and 6 mg/kg of BW per day in mice are equivalent to consumption of lycopene at doses of 1.7 and 3.4 mg lycopene per day for humans (with 70 kg BW), respectively [35]. The average daily intake of lycopene for humans is highly variable from 0.6 to 6.2 mg in many countries,

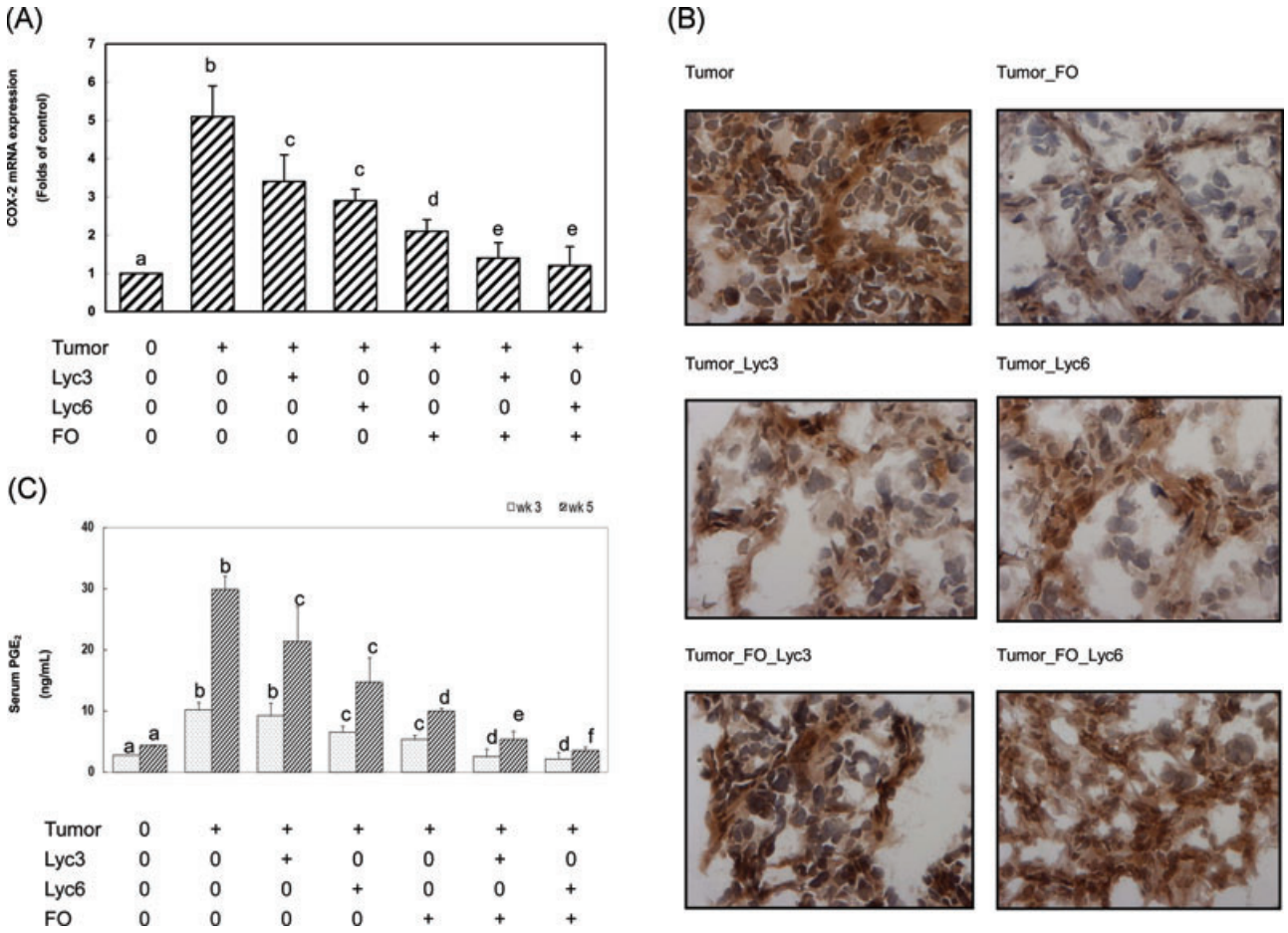

Figure 5. Lycopene and fish oil significantly suppressed tumor inflammation in tumor-bearing mice. (A) The relative COX-2 mRNA expressions in tumor tissues (at week 5) were analyzed by using qRT-PCR. Relative COX-2 mRNA levels in different experimental groups were compared to the corresponding negative control group (presented as folds of control). Different letters represent statistically significant difference, $p < 0.05$. (B) Tumor tissues (at week 5) were frozen, sectioned, and subjected to anti-COX-2 antibody by IHC staining described in Material and methods. Imaging was documented at 400× magnification. Brown area represented distribution of COX-2 protein in HT-29 cells stained with monoclonal antibody. Blue spots represented the location of cell nuclei stained with hematoxylin. The results presented are representative of six different experiments. (C) The serum levels of PGE2 were determined by using ELISA Kit (R&D Systems, Inc.). Briefly, equal amounts of diluted serum sample (150 μL) from each group (at week 3 and week 5) was added to each well and reacted with PGE_2 conjugate molecule according to the manufacturer's instructions. Upon completion of the ELISA process, fluorescence intensities were read using a wavelength of 450/570 nm. These results presented are representative of six different experiments and presented as serum PGE_2 levels. Different letters represent statistically significant difference at the same time point, $p < 0.05$.

including North America [36]. Thus, the doses of fish oil and lycopene were within the range consumed by humans.

Our previous studies demonstrated that lycopene and fish oil inhibited the proliferation of human colon cancer HT-29 cells in vitro. The present study demonstrated that consumption of lycopene and fish oil inhibited tumor growth in significant or even synergistic ways. The current study demonstrated that concomitant consumption of lycopene and fish oil significantly inhibited the growth of colon cancer in a mouse xenograft model (Fig. 1). At low dosage (3 mg/kg BW), lycopene and fish oil (FO_Low_Lyc) effectively suppressed the growth of colon cancer up to 70%. At a high dosage (6 mg/kg BW), lycopene and fish oil (FO_High_Lyc) effectively suppressed the proliferation of colon cancer cells up to 85% in vivo. In our previous study, we already demonstrated that lycopene inhibited the proliferation of human colon cancer HT-29 cells through suppression of β-catenin signaling pathways and cell-cycle progression in a dose-dependent manner (0, 2, 5, and 10 μM) [31]. Our results further demonstrated that lycopene and EPA synergistically inhibited cell proliferation in human colon cancer HT-29 cells [32]. We further demonstrated that lycopene and fish oil significantly suppressed the expression of cyclin D1 and PCNA proteins. The molecular actions of lycopene and fish oil on the suppression of tumor growth were associated with the upregulation of $p21^{CIP1/WAF1}$ and $p27^{Kip1}$ cell-cycle inhibitor proteins.

The results suggested that lycopene and fish oil effectively inhibited the cell-cycle progression and cell proliferation in tumor-bearing mice. In the present study, we demonstrated that consumption of lycopene and fish oil significantly suppressed the level of malignant biomarker β-catenin protein in vivo. Our novel evidences also demonstrated that lycopene and fish oil effectively inhibited the expression of cyclin D1 and c-Myc proteins in colon cancer cells in tumor-bearing mice. Previous studies indicated that MMP-7 was mostly overexpressed in human colon cancer patients. Here, we further demonstrated that consumption of lycopene and fish oil synergistically inhibited MMP-7 expression and the tumor progression of colon cancer cells in tumor-bearing mice. Moreover, consumption of lycopene and fish oil stabilizes the adherent junction E-cadherin molecules through the suppression of MMP-7 proteins (Fig. 4). The β-catenin has come into the scene and reached central status as an important regulator of cyclin D1, c-Myc, and MMP-7 proteins during tumor development. Increasing evidence implicates β-catenin is an important biomarker of malignant colon cancer. It is plausible that lycopene and fish oil synergistically inhibit tumor growth through the modulation of β-catenin signaling pathways. These results from the present study were consistent with our previous findings and suggested chemopreventive roles of lycopene and fish oil against human colon cancer. Previous reports suggested that overexpression of biomarkers such as MMP-9, COX-2, and PGE2 may play crucial roles in the tumor progression and inflammation of colon cancer. Suppression of COX-2, MMP-9, and PGE2 would hinder the progression of tumor. A recent study indicated that fish oil inhibited the expression of COX-2 and prostaglandin E2 (PGE2) in human colon cancer HT-29 cells [37]. We further examined whether consumption of lycopene and fish oil could help prevent tumor inflammation and progression in tumor-bearing mice. The results demonstrated that lycopene and fish oil significantly inhibit the expression of MMP-9, COX-2, and PGE2 in vivo at week 3. The results also demonstrated that lycopene and fish oil still significantly suppressed the expression of MMP-9 and PGE2 at week 5. Moreover, the PGE2 levels in both Low_Lyc_FO and High_Lyc_FO were even much lower than the ones observed in the tumor control group at week 5. It suggested that the combined effects of lycopene and fish oil might provide an additional way to effectively inhibit the tumor inflammation and progression.

In conclusion, lycopene and fish oil significantly inhibit the tumor growth, progression, and inflammation in tumor-bearing mice. Here, we proposed a synergistic effect of lycopene and fish oil on the growth of human colon cancer HT-29 cells in vivo. Lycopene and fish oil may potentially act as chemopreventive agents to suppress tumor growth in a mouse xenograft model of CRC.

This material is based upon work supported, in part, by the National Science Council grant, under agreement No. NSC-97–2320-B-039–043-MY3, NSC-100–2320-B-039–003, and Taiwan Department of Health Clinical Trial and Research Center of Excellence, under Agreement No. DOH 100-TD-B-111–004, DOH 101-TD-B-111–004, DOH-100-TD-C-111–005, DOH-101-TD-C-111–005. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the National Science Council and Department of Health.

The authors have declared no conflict of interest.

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