RESEARCH ARTICLE

Chemoprevention of colonic tumorigenesis by dietary hydroxylated polymethoxyflavones in azoxymethane-treated mice

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Scope: Hydroxylated polymethoxyflavones (PMFs), existing exclusively in citrus genus, have been reported to exhibit a broad spectrum of biological activity. Here we investigated the chemopreventive effects and underlying molecular mechanisms of dietary administration of hydroxylated PMFs in an azoxymethane (AOM)-induced colonic tumorigenesis model.

Methods and results: Male, Institute of Cancer Research (ICR), mice at age of 6 wk were injected with AOM twice weekly at a dose of 5 mg/kg for 2 wk and continuously fed control diet or diets containing 0.01 and 0.05% hydroxylated PMFs, respectively. Mice were then sacrificed at 6 and 20 wk, and colonic tissues were collected and examined. Hydroxylated PMFs feeding dose-dependently decreased the number of aberrant crypt foci in colonic tissues of mice. More importantly, we found that hydroxylated PMFs caused a strong reduction in numbers of large aberrant crypt foci and tumors in colonic tissue. Molecular analysis exhibited the anti-proliferative, anti-inflammatory, anti-angiogenic and pro-apoptotic activities of hydroxylated PMFs by significantly decreasing the levels of inducible nitric oxide synthase, cyclooxygenase, cyclin D1 and vascular endothelial growth factor through interfering with Wnt/β-catenin and epidermal growth factor receptor/Ras/mitogen-activated protein kinase signaling pathways as well as the activation of transcription factors NF-κB and STAT3 in colonic tissue, thus resulting in suppression of colonic tumorigenesis.

Conclusion: Taken together, these results demonstrated for the first time the *in vivo* chemopreventive efficacy and molecular mechanisms of dietary hydroxylated PMFs against AOM-induced colonic tumorigenesis.

Keywords:
5-Hydroxy polymethoxyflavones / Aberrant crypt foci / Apoptosis / Colonic tumorigenesis / Inflammation

1 Introduction

Colorectal cancer (CRC) is one of major causes of cancer-related mortality in both men and women in most developed countries [1]. The risk factors of CRC include age, family history, inflammatory bowel diseases and environmental and dietary procarcinogens [2]. Progression of this disease is commonly characterized by histologically distinct steps, *i.e.*

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Abbreviations: ACF, aberrant crypt foci; **AOM,** azoxymethane; **COX-2,** cyclooxygenase-2; **CRC,** colorectal cancer; **EGFR,** epidermal growth factor receptor; **ICR,** Institute of Cancer Research; **ERK,** extracellular signal-regulated kinase; **iNOS,** inducible nitric oxide synthase; **MMP-9,** matrix metalloproteinase 9; **STAT,** signal transducer and activator 3; **VEGF,** vascular endothelial growth factor

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colonic crypt hyperplasia, dysplasia, adenoma, adenocarcinoma and distant metastasis [3]. During this progression, formation of aberrant crypt foci (ACF) in early stage is believed to be a histological biomarker of colonic tumor development [4]. ACF also occurs at a higher frequency in colon cancer patients, which proposed as a putative preneoplastic lesion [5]. Moreover, increased number and multiplicity of ACF are thought to be associated with an increased risk for the development of CRC [4, 5].

The tumorigenesis of CRC involves various genetic and molecular changes in cell proliferation, inflammation, resistance to apoptosis and tumor angiogenesis [1, 2]. Mutation and constitute activation of β-catenin and K-ras lead to activation of Wnt/β-catenin/Tcf4 signaling pathway, which subsequently causes the transcription of downstream genes such as myc, cyclin D1, vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) that are involved in tumorigenesis [6, 7]. Oncogenic mutation of *K-ras* also results in activation of Ras and its downstream effectors, such as Raf/MEK/mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt pathways [8]. Inactivation of glycogen synthase kinase through phosphorylation by Akt caused stabilization of β-catenin and its nuclear accumulation [9]. Moreover, epidermal growth factor receptor (EGFR) signaling also involves in regulating colonocyte growth and differentiation, and is upregulated in hyperproliferative ACF as well as contributes to malignant growth of colon cancer [10, 11].

It is known that inflammation is causally linked to carcinogenesis and acts as a driving force in premalignant and malignant transformation [12, 13]. Expression of inflammation-associated enzymes such as inducible nitric oxide synthase (iNOS) and inducible-type cyclooxygenase (COX-2) contributes to colon tumorigenesis by production of nitric oxide (NO) and prostaglandin E2 (PGE_2) in azoxymethane (AOM)-induced rat and mouse colon cancers [14]. Inhibition of these two enzymes shows protective effects against colon tumor development in different animal models, suggesting they are crucial targets for mucosa inflammation and colon tumorigenesis [1].

Despite understanding of the process and mechanism in colonic carcinogenesis, present therapies including surgery, chemotherapy, radiotherapy and molecular-targeted therapy are still limited for advanced tumors. Hence, a growing amount of attention has been focused on investigating the potential of dietary substances for both prevention and control of colon cancer through chemopreventive strategies [15]. Epidemiological and laboratory studies suggest that the consumption of vegetables and fruits is correlated to decrease the risk of colon cancer [16, 17]. Intake of citrus fruits has been suggested to prevent the development of certain human cancers [18]. It is also commonly recognized that cancer induction can be prevented by ingestion of certain food phytochemicals, and flavonoids in citrus fruits and juices are one class of the most prominent cancer-preventing agents [17, 18].

The major phytochemicals in citrus peel include polymethoxylated flavonoids, terpenoids, such as limonene and linalool, and other volatile oils [19]. Among these, polymethoxyflavones (PMFs), particularly in the peel of sweet oranges (*Citrus sinensis*) and mandarin oranges (*Citrus reticulate*), have been demonstrated to have various biological activities including anti-carcinogenic, anti-inflammatory and antitumor activities [19, 20]. Our pervious studies also reported that 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone isolated from sweet orange peel extract shows the inhibitory effects against TPA-induced skin inflammation and tumor promotion in mice [21]. The most known and studied citrus PMF, nobiletin, has been reported in inhibition of AOM-induced colonic ACF formation in male F344 rats [22, 23]. However, the mechanisms underlying its actions have yet to be well characterized. In addition, recent studies have shown that some hydroxylated PMFs have more potent biological activities including anti-cancer and anti-inflammatory activities than their PMF counterparts [21, 24, 25]. This information indicated the hydroxylated PMFs may possess greater potency for colon cancer chemoprevention. In the present study, we investigated the effect of dietary hydroxylated PMF on AOM-induced ACF formation in mice. We also explored the potential mechanisms of their anti-colonic carcinogenesis actions such as proliferation, inflammation, apoptotic markers and possible signaling molecular pathways. Our results showed that dietary administration of hydroxylated PMFs significantly decreased ACF and tumor formation in AOM-treated mice, which is associated with a decrease in proliferation, inflammation and angiogenesis regulators and multiple signaling pathways but also an increase of apoptosis in the colonic tissue.

2 Materials and methods

2.1 Reagents

AOM was purchased from Sigma Chemical (St. Louis, MO, USA). Hydroxylated PMFs were prepared according to the method of Li *et al.* [26]. Briefly, 10 g of orange peel extract (40% PMFs, Danisco, Lakeland, FL, USA) was dissolved in 95% ethanol and to the solution was added 3 M aqueous hydrochloric acid. The resulted solution was heated to reflux for 12 h. The reaction progress was monitored by TLC and LC/MS. The reaction was cooled and the ethanol was removed *in vacuo*. Ethyl acetate and water were added, and the organic layer was collected. The aqueous layer was extracted with ethyl acetate twice. The combined organic layer from the three extractions were washed with dilute sodium bicarbonate, water and brine and dried over anhydrous sodium sulfate. After filtration and concentration, the residue was lyophilized to give 3.85 g of light yellow solid of hydroxylated PMFs. The experimental diets contained 0.01 or 0.05% of hydroxylated PMFs in basal diet that were prepared once weekly and stored at 4°C for use.

2.2 HPLC analysis

The Dionex UltiMate 3000 LC Modules equipped with a pump (model: LPG-3400 pump, Sunnyvale, CA), UV-Vis detector (model: VWD-3400 detector) and an autosampler (model: WPS-3000 SL) were used. A Supelco's RP-Amide C16 column (150 × 4.6 mm id, 3 μm particle size) was used for hydroxylated PMF analysis. The column temperature was maintained at 35°C in a column oven (Dionex model: STH 585). The mobile phase for the HPLC system consisted of HPLC grade water with 0.05% v/v trifluoroacetic acid (solvent A) and 80% acetonitrile with 20% tetrahydrofuran (solvent B) with a constant flow rate set at 1 mL/min. The optimized HPLC condition is as follows: a 17 min program was started with 50% of solvent B, and then kept this gradient till 17 min. Hydroxylated PMF was detected with a UV wavelength at 326 nm and the injection volumes were 5 μL. The external standard quantification method was applied in this study. Every single peak area for the quantification was laid in the linear range of standard curves.

2.3 Animals

Male Institute of Cancer Research (ICR) mice at 5 wk of age were purchased from the BioLASCO Experimental Animal Center (Taiwan Taipei, Taiwan). After 1 wk of acclimation, animals were randomly distributed into control and experimental groups. All animals were housed in a controlled atmosphere (25 ± 1°C at 50% relative humidity) and with a 12-h light/12-h dark cycle. Animals had free access to food and water at all times. Food cups were replenished with fresh diet everyday. All animal experimental protocol used in this study was approved by Institutional Animal Care and Use Committee of the National Kaohsiung Marine University (IACUC, NKMU).

2.4 Experimental procedure

The experimental protocol for this study is shown in Fig. 1. Briefly, mice were randomly divided into four groups of 15 animals each. At 6 wk of age, mice in groups 2, 3 and 4 were given AOM at a dose of 5 mg/kg *via* an i.p. injection twice a week for 2 wk, and the group 1 was received injection of saline. Groups 1 and 2 were fed with standard AIN-76 diet and composition as described before [27], while groups 3 and 4 were fed diets containing hydroxylated PMFs at dose levels of 0.01 and 0.05%, respectively, continued until the end of study. The diet intake of animals was monitored every day. All animals were sacrificed by CO_2 asphyxiation at 6 or 20 wk for evaluation of aberrant crypts or tumors in colonic tissues. The liver, kidneys and spleen were removed and weighed. The entire colons were excised, cut longitudinally, rinsed with PBS and fixed flat between sheets of filter paper with 10% buffered formalin overnight. All fixed colonic tissues were subjected to ACF and tumor number evaluation or for immunohistochemistry study (Fig. 2).

2.5 Determination of ACF and tumors

The formalin-fixed colonic tissues were stained in 0.2% methylene blue solution for 10 min and dipped in distilled water. The total number of ACF in each focus were counted under a microscope (× 40) and expressed as mean ± SE. ACF were classified with the following morphological characteristics, the enlarged and elevated crypts than in normal mucosa and increased pericryptal space and

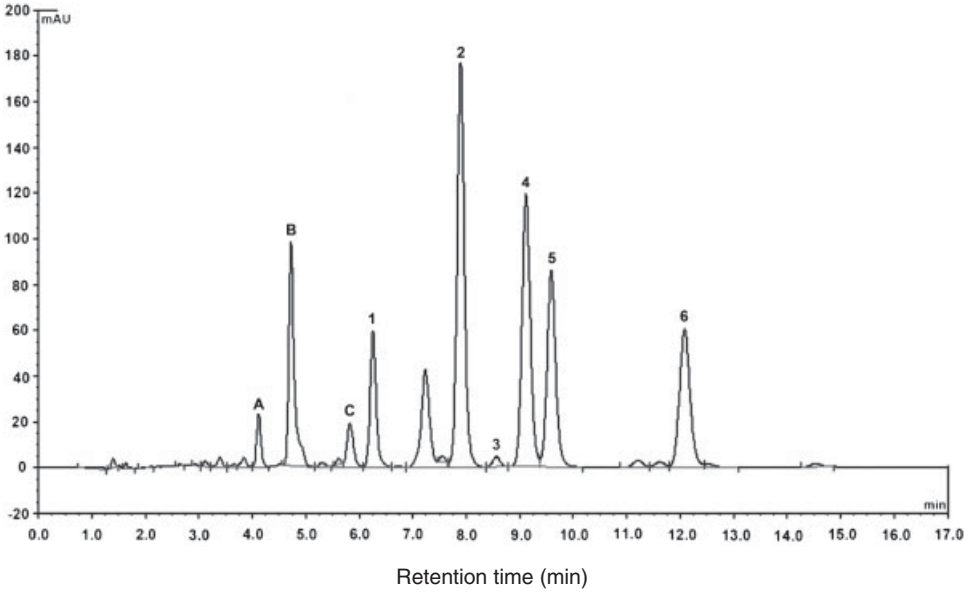

Figure 1. HPLC peaks of three PMFs and six hydroxylated PMFs. PMFs: (A) nobiletin, (B) heptamethoxyflavone, (C) tangeretin. Hydroxylated PMFs: (1) 5-hydroxy-6,7,3′,4′-tetramethoxyflavone, (2) 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone, (3) 5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone, (4) 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone, (5) 5-hydroxy-6,7,4′-trimethoxyflavone, (6) 5-hydroxy-6,7,8,4′-tetramethoxyflavone.

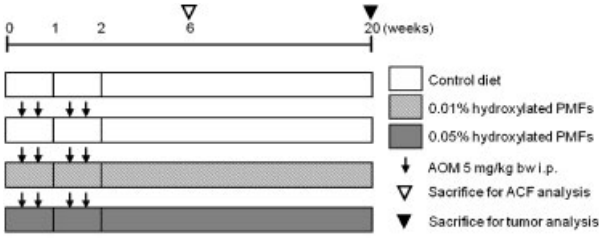

Figure 2. Experimental treatment protocol in AOM-induced colon carcinogenesis.

irregular lumen. The ACF location (distance from anus) and size (number of aberrant crypts) were recorded. Larger ACF were defined as ACF with six or more component crypts. The crypt multiplicity of lesions was determined by transforming the diameter (mm) to crypt multiplicity. Diameters were scored with an eyepiece graticule. The lesions containing >32 aberrant crypts or diameters $\geqq 1$ mm were defined as tumors and confirmed by histological H&E stain.

2.6 Western blot analysis and Ras activation assay

For protein analyses, total scraped colon mucosa was homogenized on ice for 15 s with a Polytron tissue homogenizer and lysed in 0.5 mL ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM NaF, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1% NP-40, and 10 mg/mL leupeptin) on ice for 30 min, followed by centrifugation at 10 000 *g* for 30 min at 4°C. The samples (50 μg of protein) were mixed with 5 × sample buffer containing 0.3 M Tris-HCl (pH 6.8), 25% 2-mercaptoethanol, 12% SDS, 25 mM EDTA, 20% glycerol and 0.1% bromophenol blue. The mixtures were boiled at 100°C for 5 min, subjected to stacking gel and then resolved by 12% SDS-polyacrylamide minigels at a constant current of 20 mA. Subsequently, electrophoresis was carried out on SDS-polyacrylamide gels. For Western blot analysis, proteins on the gel were electrotransferred onto a 45 micron immobilon membrane (PVDF; Millipore, Bedford, MA) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine and 20% methanol. The membranes were blocked with blocking solution (20 mM Tris–HCl, pH 7.4, 0.2% Tween 20, 1% BSA and 0.1% sodium azide) and probed overnight at 4°C with primary antibody (diluted 1:1000 in blocking solution). The primary antibodies used were as follows: iNOS, p50, p65, VEGF, MMP-9, E-cadherin and phospho-phosphatidylinositol 3-kinase (Tyr508) polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); ornithine decarboxylase and COX-2 monoclonal antibodies (Transduction Laboratories, BD Biosciences, Lexington, KY); phospho-p65 (Ser536), phospho-signal transducer and activator 3(STAT3) (Ser727), phospho-STAT3 (Tyr705), phospho-p38 (Thr180/Tyr182), phospho-extracellular signal-regulated kinase (ERK)1/2 (Thr202/Tyr204), phospho-EGFR (Tyr845,Tyr992, Tyr1045, Tyr1068), phospho-GSK3β (Ser 9), β-catenin, STAT3, ERK, p38, EGFR, GSK3β, and cyclin D1 polyclonal antibodies (Cell Signaling Technology, Beverly, MA); phospho-Akt (Ser473) and Akt polyclonal antibodies (Upstate Biotechnology, Lake Placid, NY); poly(ADP-ribose) polymerase (Upstate Biotechnology, Lake Placid, NY) and caspase-3 (Transduction Laboratories). The membranes were subsequently probed with anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase (Transduction Laboratories) and visualized using enhanced chemiluminescence (Amersham). The densities of the bands were quantitated with a computer densitometer (AlphaImagerTM 2200 System). All the membranes were stripped and reprobed for β-actin (Sigma Chemical) or lamin B (Santa Cruz Biotechnology) as loading control. To measure the level of activated Ras (Ras-GTP) in colon mucosa, 100 μg of cellular lysates were determined using a Ras activation assay kit (Upstate Biotechnology) following the recommendation of the manufacturer.

2.7 Preparation of cytosolic and nuclear extracts from colonic tissue

Cytosolic and nuclear protein extractions were performed as described previously [21]. In brief, the colonic tissues were scrapped and extracted by homogenization in 0.5 mL of ice-cold hypotonic buffer A containing 10 mM *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA and 0.1 mM PMSF and then homogenized in a Polytron for 1 min. The homogenates were incubated on ice with gentle shaking for 15 min and centrifuged at 1000 rpm. for 5 min to remove tissue debris. The supernatant were collected and stored at −70°C. The pellet was resuspended in buffer A supplemented with 50 μL of 10% NP-40, vortexed and centrifuged for 2 min at 14 000 rpm. The nuclear pellet was resuspended in 200 μL of high salt extraction buffer C (50 mM *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 10% glycerol. It was kept on ice for 30 min and then centrifuged at 14 000 rpm. for 5 min. The supernatant that constituted nuclear proteins was transferred into a new tube and stored at −70°C after determination of protein concentration with a protein assay kit (Bio-Rad, São Paulo, Brazil).

2.8 RT-PCR

Total RNA was isolated from scraped colon mucosa using Trizol Reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). Changes in the steady-state concentration of mRNA in iNOS, COX-2 and β-actin were assessed by RT-PCR. Total of 2 μg RNA was transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Renfrewshire, UK) in a final volume of 20 μL. RT reactions were performed at 50°C for

50 min and 70°C for 15 min in Gene Cycler thermal cycler (Bio-Rad). The thermal cycle conditions were initiated at 95°C for 1 min, and 30 cycles of amplification (94°C for 30 s, 58°C for 25 s and 72°C for 1 min), followed by extension at 72°C for 3 min. The cDNA was amplified by PCR with the following primers: iNOS, forward primer 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ (2944-2968), reverse primer 5′-GGCTGTCAGAGAGCCTCGTGGCTTTGG-3′ (3416-3440); COX-2, forward primer 5′-GGAGAGACTATCAAGATAGTGATC-3′ (1094-1117), reverse primer 5′-ATGGTCAGTAGACTTTTACAGCTC-3′ (1931-1954); β-actin, forward primer 5′-AAGAGAGGCATCCTCACCCT-3′, reverse primer 5′-TACATGGCTGGGGTGTTGAA-3′. PCR products were analyzed by 1% agarose gel and visualized by ethidium bromide staining.

2.9 Immunohistochemical analysis

Three micrometer section of colonic mucosa in ACF and tumor segments were incubated with 0.3% hydrogen peroxide (H_2O_2) in PBS to quench the endogenous peroxidase activity. For antigen retrieval, sections were heated in 10 mM citrate buffer (pH 6.0) (Immuno DNA retriever with citrate, BIO SB, Santa Barbara, CA) in microwave oven for 7 min at reaching full power. Sections were then incubated with β-catenin (1:200), COX-2 (1:100 dilute), iNOS (1:50) and VEGF (1:100) primary antibody in PBS for 1 h at room temperature. After washing with PBS, the sections were incubated with a biotin-conjugated horseradish peroxidase secondary antibody (1:200). Immunoreactivity was visualized by standard biotin-labeled secondary antibody and streptavidin-biotinperoxidase for 30 min each. 3,3′-diaminobenzidine tetrahydrochloride (0.05%, DAB) was used as the substrate, positive signal was detected as a brown color under a light microscope. The sections also counterstained with hematoxylin, dehydrated and mounted.

2.10 Activity of caspase-3

The caspase activity in protein extractions of colon tissue was determined by a fluorogenic assay (Promega's CaspACE Assay System, Madison, WI). As described previously [22], briefly, 50 μg of total protein, as determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories), was incubated with 50 μM substrate Ac-Asp-Glu-Val-Asp-methylcoumaryl-7-amine (caspase-3 specific substrate) at 30°C for 1 h. The release of methylcoumaryl-7-amine was measured by excitation at 360 nm and emission at 460 nm using a fluorescence spectrophotometer (ECLIPSE, Varian, Palo Alto, CA).

2.11 Statistical analysis

Relative expression values are given as mean ± SE for the indicated fold of expression in colon mucosa of mice. A one-way Student's *t*-test was used to assess the statistical significance between the AOM and the hydroxylated PMFs plus AOM-treated groups. A *p*-value of < 0.05 was considered statistically significant.

3 Results

3.1 The composition of hydroxylated PMFs samples

The composition and content of hydroxylated PMFs and PMFs are listed in Table 1 and Fig. 1. HPLC fingerprint showed three PMFs and six hydroxylated PMFs in orange peel extract after conversion under acidic conditions, including nobiletin (**A**), heptamethoxyflavone (**B**), tangeretin (**C**), 5-hydroxy-6,7,3′,4-tetramethoxyflavone (**1**), 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (**2**), 5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone (**3**), 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (**4**), 5-hydroxy-6,7,4′-trimethoxyflavone (**5**) and 5-hydroxy-6,7,8,4′-tetramethoxyflavone (**6**). Among these identified hydroxylated PMFs, 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (396.42 ± 4.62 mg/g), 5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone (254.78 ± 4.95 mg/g), and 5-hydroxy-6,7,4′-trimethoxyflavone (115.52 ± 2.35 mg/g) are the most abundant. The total content of hydroxylated PMFs in the mixture is 893.25 ± 12.27 mg/g.

3.2 General observation

During the experiment, all mice were monitored to investigate whether hydroxylated PMFs feeding caused any adverse effects. As shown in Tables 2 and 3, the body weight in each group did not differ or show any unhealthy symptoms throughout the study. Furthermore, no significant difference of the mean weights of liver and spleen, and no pathologic alternations were found among the groups (data not shown). The results suggested no any noticeable

Table 1. The composition and contents of PMF and hydroxylated PMF in orange peel extract

OH-PMFs and PMFs	Concentration (mg/g OH-PMFs)
5-Hydroxy-3,6,7,8,3′,4′hexamethoxyflavone	254.78 ± 4.95
5-Hydroxy-6,7,8,3′,4′-pentamethoxyflavone	396.42 ± 4.62
5-Hydroxy-3,6,7,3′,4′-pentamethoxyflavone	7.08 ± 0.11
5-Hydroxy-6,7,8,4′-tetramethoxyflavone	74.96 ± 0.16
5-Hydroxy-6,7,4′-trimethoxyflavone	115.52 ± 2.35
5-Hydroxy-6,7,3′,4′-tetramethoxyflavone	44.5 ± 0.08
Nobiletin	10.41 ± 0.14
Heptamethoxyflavone	55.04 ± 0.46
Tangeretin	20.34 ± 0.62

Table 2. Effects of dietary hydroxylated PMFs on AOM-induced ACF formation in ICR mice[a)]

Group	No. of mice	Body weight (g)	No. ACF/colon		Incidence of ACF formation
			ACF	Large ACF	
AOM	15	37.9±2.3	47±3[b)]	23±3[b)]	15/15 (100%)
AOM + 0.01% hydroxylated PMFs	15	38.4±2.4	29±2[b)]	13±4[b)]	15/15 (100%)
AOM + 0.05% hydroxylated PMFs	15	38.5±2.9	29±6[b)]	10±2[b)]	15/15 (100%)

a) All mice of each group were killed by decapitation at the end of wk 6. The colons were removed and fixed in 10% buffered formalin. ACF in formalin-fixed colons were identified as crypts with increased methylene blue staining and expanded pericryptal spaces ($n = 15$). The average number of ACF and large ACF (≧ 6 component crypts/focus) were expressed as mean±SE *per* colon.
b) $p < 0.01$, compared with AOM-treated alone.

Table 3. Effects of dietary hydroxylated PMFs on the AOM-induced colonic tumor in male ICR mice[a)]

Group	No. of mice	Body weight (g)	Liver (mg)	Spleen (mg)	Microadenomas/colon	
					Tumor multiplicity	Tumor incidence (%)
AOM	15	46.6±3.2	2.5±0.4	0.22±0.04	7.8±1.3[b)]	15/15 (100%)
AOM + 0.01% hydroxylated PMFs	15	40.6±3.2	2.1±0.4	0.23±0.01	4.0±0.8[c)]	15/15 (100%)
AOM + 0.05% hydroxylated PMFs	15	42.4±4.2	2.2±0.3	0.23±0.02	1.5±0.6[b)]	15/15 (100%)

a) Animals were treated as described in Fig. 2. All mice of each group were killed by decapitation at the end of week 20. Colon tissues were analyzed by H&E stain for microadneoma. The average number of tumor were expressed as mean±SE per colon.
b) $p < 0.01$, compared with AOM-treated alone.
c) $p < 0.01$, compared with AOM-treated alone.

side effect or toxicity caused by dietary hydroxylated PMFs treatment.

3.3 Dietary hydroxylated PMFs treatment inhibited ACF formation

The efficacy of dietary administration of hydroxylated PMFs on inhibiting AOM-induced ACF formation was determined. Colonic ACF were identified and analyzed under a light microscope after methylene blue staining. Table 2 summarizes the number of ACF *per* mice, multiplicity and incidence after 6 wk treatment. All mice developed ACF in the colon after AOM treatment. The mean number of ACF per colon in AOM alone group was 47±3, whereas mice treated AOM and fed with 0.01 or 0.05% hydroxylayed PMFs diet showed a significantly lower number of ACF at 29±2 ($p < 0.05$) and 29±6 ($p < 0.05$), respectively. It has been suggested that larger ACF (containing six or more crypts *per* focus) have higher risk for malignant tumor progression [28]. We also found that hydroxylated PMFs notably reduced the number of large ACF to 13±4 and 10±2 at 0.01 or 0.05%, respectively in hydroxylated PMF-treated groups compared to 23±3 in AOM-treated group ($p < 0.05$). However, the incidence of ACF in 0.01 and 0.05% hydroxylayed PMF-treated groups was the same as the AOM-treated group that showed a 100% incidence.

3.4 Dietary hydroxylated PMFs modulated AOM-induced β-catenin signaling and E-cadherin expression

β-catenin is a downstream effector of Wnt/APC/β-catenin signaling pathway that controls colonic epithelial cell proliferation, and commonly dysregulated in colon ACF and tumor [29, 30]. Inhibition of glycogen synthase kinase function through phosphorylation by Akt results in cytosolic β-catenin accumulation and subsequent translocates into the nucleus that regulates transcription of oncogenes, such as cyclin D1 and VEGF [31, 32]. To determine the effect of dietary hydroxylated PMFs on β-catenin expression, the colonic tissues were collected and analyzed by Western blot and IHC analysis. As presented in Fig. 3A, the nuclear accumulation of β-catenin was markedly reduced in hydroxylated PMF-treated groups as compared to the AOM-treated group. Similarly, IHC examination showed that cytoplasmic and nuclear β-catenin intensity was increased when mice were treated with as evidenced AOM alone as by dark brown staining (Fig. 3B). In contrast, hydroxylated PMF groups had lighter nuclear staining of β-catenin as compared with AOM-alone group. We also analyzed the expression of cyclin D1, a major downstream target of β-catenin. The results showed that cyclin D1 was strongly expressed in AOM-alone group, and its expression level was reduced in hydroxylated PMF-treated mice (Fig. 3C). Moreover, phosphorylation of Akt at Ser473 and GSK3β at Ser9 (as an inactive form) were observed in AOM alone group,

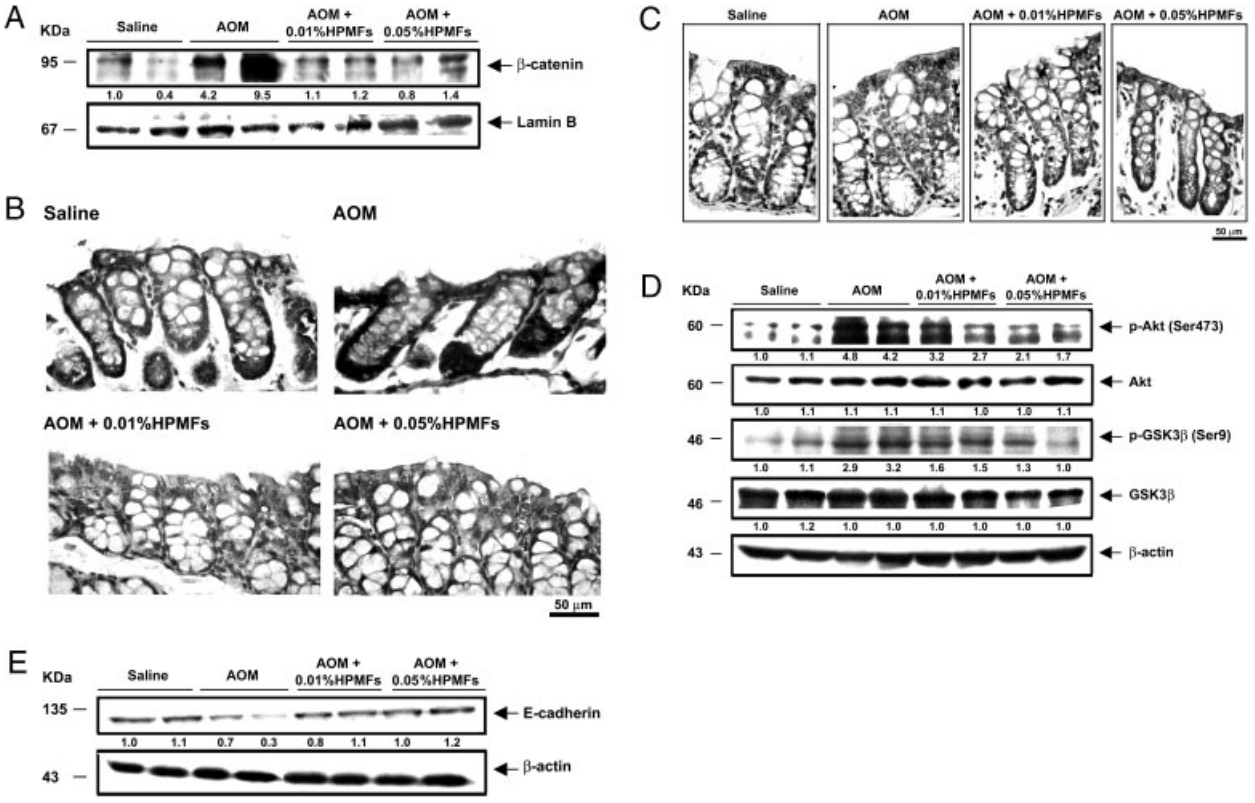

Figure 3. Effects of dietary hydroxylated PMFs on β-catenin signaling and E-cadherin expression in colonic tissues. Mice were treated as described in Section 2. After 6 wk treatment, colonic tissues were excised and collected. Total or nuclear lysates from normal colonic mucosa and ACF were prepared and analyzed for β-catenin (A), p-Akt and p-GSK3β (C), and E-cadherin (E) by Western blot analysis. Lamin B and β-actin were used as the control for loading. Expression of β-catenin (B) and cyclin D1 (D) in nomal and ACF crypt were assessed by immunohistochemical analysis that demonstrated as dark stain in nuclei (magnification, × 400).

but were also markedly decreased in hydroxylated PMF group (Fig. 3D). Loss of E-cadherin has been implied to involve in stimulation of colonic cell proliferation. We also found that AOM-treated animals showed decreased levels of E-cadherin and this phenomenon was reversed by hydroxylated PMFs treatment (Fig. 3E). These results demonstrated that dietary hydroxylated PMF treatment prevented aberrant β-catenin expression and signaling in the colon of the AOM-treated mice.

3.5 Dietary hydroxylated PMFs inhibited AOM-induced iNOS and COX-2 expression through down-regulating NF-κB and STAT3 signaling

The inflammatory molecules, iNOS and COX-2 have been considered involved in colonic carcinogenesis [14]. Therefore, we next investigated the effects of dietary hydroxylated PMFs on AOM-induced iNOS and COX-2 expression in mouse colon. As shown in Fig. 4A, dietary hydroxylated PMFs resulted in a dramatic reduction of iNOS, COX-2 and ornithine decarboxylase protein levels in colonic mucosa compared with AOM-alone group. The results from RT-PCR (Fig. 4B) also showed that dietary hydroxylated PMFs treatment markedly decreased iNOS and COX-2 mRNA expression. iNOS and COX-2 are frequently regulated by activating NF-κB signaling pathway and NF-κB activation is critical for inflammation and cancer development [33]. The translocation of NF-κB was measured using extracts of nucleus and cytosol of colon mucosa by Western blot analysis. We found that AOM-treatment caused NF-κB subunits, p50 and p65, as well as the phospho-p65 to translocate the nucleus (Fig. 4C). Dietary hydroxylated PMFs feeding significantly suppressed the nuclear levels of p50, p65 and phospho-p65 in colon mucosa of AOM-treated mice. STAT3 is another key signaling molecule involved in inflammatory response and has been reported involved in inflammation-related colon carcinogenesis [34, 35]. Western blot analysis has shown that AOM-treatment induced a dramatic increase in the phosphorylation of STAT3 at Ser727 and Tyr705, and also its nuclear translocation, which is necessary for STAT3 transcriptional activity. However, feeding with 0.01 and 0.05% hydroxylated PMFs dose-dependently decreased the phosphorylation and nuclear translocation of STAT3. These results suggested that dietary hydroxylated PMF treatment inhibited inflammatory

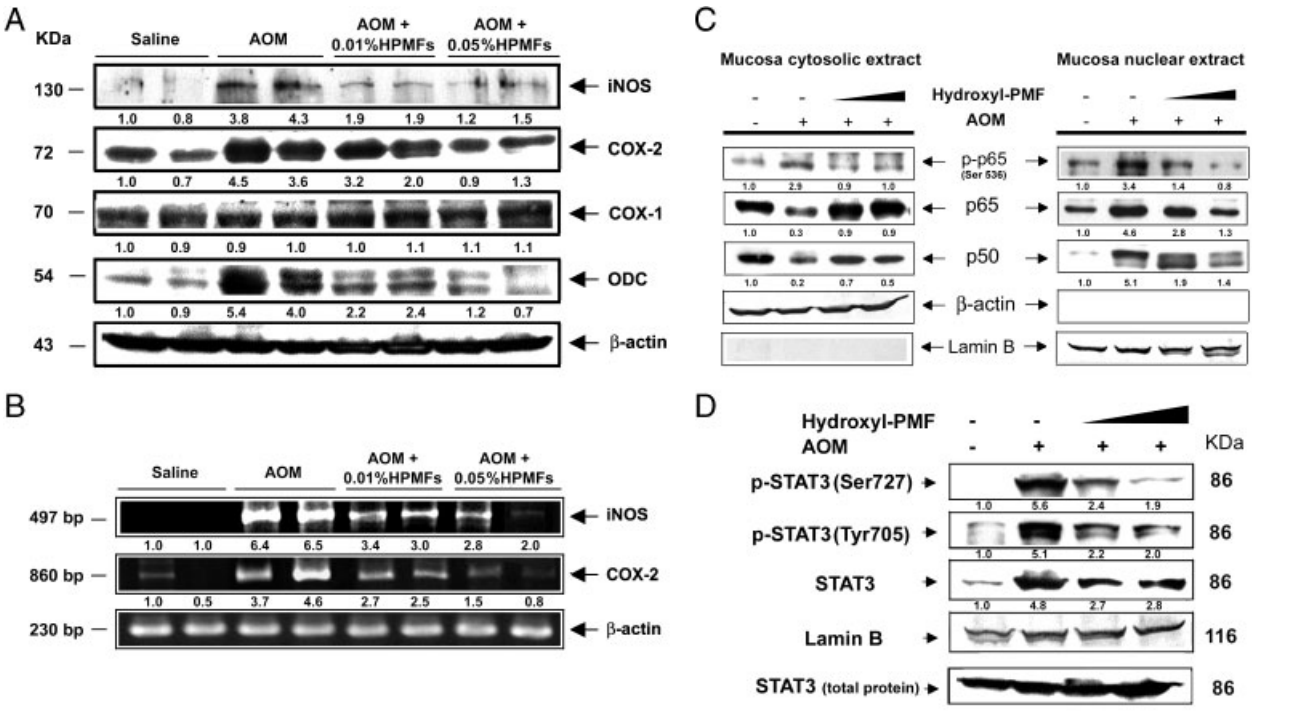

Figure 4. Inhibitory effects of dietary hydroxylated PMFs on AOM-induced expression of inflammatory enzymes and down-regulation of NF-κB and STAT3 signaling in colonic tissue. Protein lysates and mRNA from normal colonic mucosa and ACF were extracted and subjected to Western blot analysis and RT-PCR for iNOS and COX-2 protein (A) and gene expression (B) as described in Section 2. Nuclear and cytosolic extracts from normal colonic mucosa and ACF were assayed for p-p65 (Ser536), p65, p50 (C), p-STAT3 (Ser727 and Tyr705) and total STAT3 (D) by Western blot analysis. The values under each lane indicate relative density of the band normalized to β-actin or lamin B. Data are representative of at least three independent experiments, which showed a similar result.

molecules iNOS and COX-2 expression in mouse colon might be through targeting NF-κB and STAT3 signaling pathways.

3.6 Inhibitory effects of dietary hydroxylated PMFs on AOM-upregulated EGFR and Ras signaling

Recent studies suggested overexpression of EGFR is associated with the promotion of ACF proliferation and tumor progression [10, 11]. Using antibody and receptor tyrosine kinase antagonists to block EGFR signaling is effective for decreasing the formation of ACF [11, 36]. To determine the effects of dietary hydroxylated PMFs on AOM-induced EGFR signaling, we examined phosphorylation of EGFR and its downstream effectors including ERK and Ras in mouse colon. As shown in Fig. 5, AOM significantly induced phosphorylation of EGFR (Tyr845, Tyr992 and Tyr1068) and ERK1/2 in mouse colon (Figs. 5A and B). Dietary hydroxylated PMFs treatment markedly inhibited AOM-induced phosphorylation of EGFR and ERK1/2 in a dose-dependent manner. We also analyzed activation of Ras by performing kinase assay. Treatment with AOM significantly increased activation of Ras (Fig. 5C). Dietary hydroxylated PMFs also dose-dependent suppressed AOM-induced Ras activation but did not affect total Ras content in colon mucosa. These results indicated that the decrease in numbers and development of ACFs by dietary hydroxylated PMFs might be through inhibiting EGFR/Ras/ERK signaling pathway.

3.7 Dietary hydroxylated PMFs suppressed AOM-induced colonic tumor formation.

We further evaluated the anti-colonic tumorigenesis activity of long-term feeding of hydroxylated PMFs. Mice were fed 0.01 or 0.05% hydroxylated PMFs for 20 wk, the colonic tissues were collected and tumors identified were examined by H&E staining. As shown in Table 3, long-term feeding of hydroxylated PMFs did not cause any effects on body weight. The mean number of microadenoma in AOM-treated group was 7.8 whereas were decreased in both 0.01 and 0.05% hydroxylated PMFs groups that showed the number of 4.0 and 1.5, respectively. Thus, dietary hydroxylated PMFs significantly reduced the number of microadenoma by 48% in 0.01% hydroxylated PMFs group and 80% in 0.05% hydroxylated PMFs group, compared with AOM-alone group. We subsequently analyzed the levels of inflammatory, proliferative and angiogeneic molecules in colonic

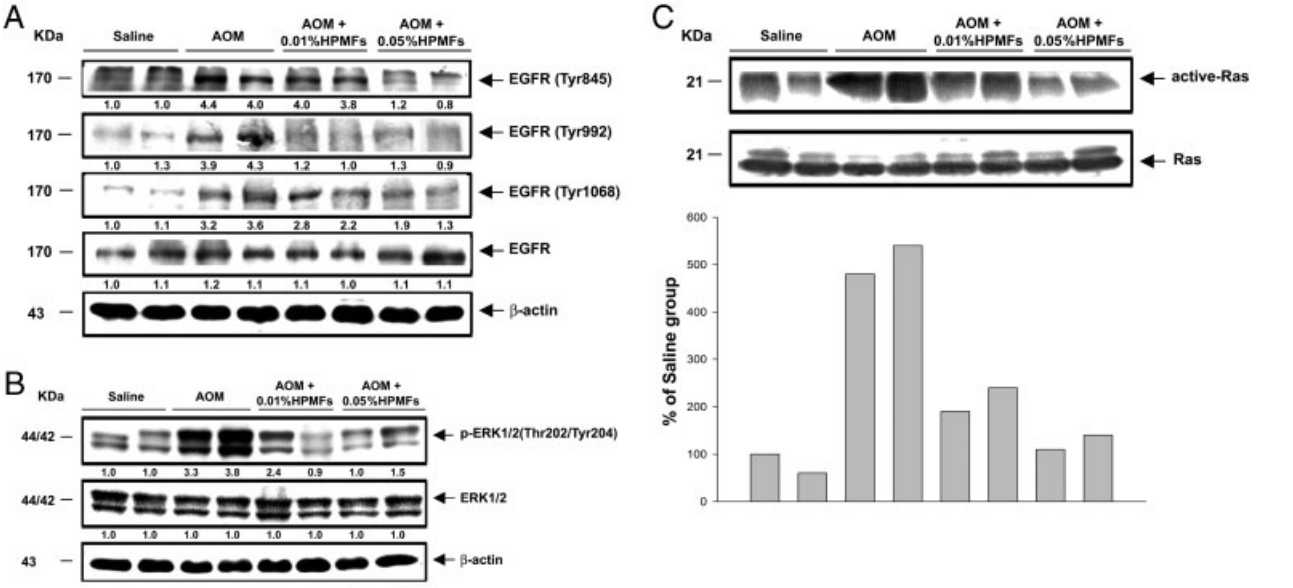

Figure 5. Dietary hydroxylated PMFs down-regulated AOM-induced EGFR signaling in colonic tissue. Mice treatment and protein lysates preparation was as described in Section 2. Levels of p-EGFR (Tyr845, 992 and 1068), total EGFR (A), p-ERK1/2 and ERK1/2 (B) were analyzed by Western blot analysis. The values under each lane indicate relative density of the band normalized to β-actin. Ras-GTP was affinity-purified with Raf-RBD-agarose and detected by anti-Ras immunoblotting (C). Total Ras levels from the same samples are shown below. Data are representative of two independent experiments.

tumors. As shown in Fig. 6A, the protein expression of inflammatory enzymes iNOS and COX-2 were increased in AOM-treated group. However, long-term dietary hydroxylated PMFs feeding strongly reduced iNOS and COX-2 protein expression in AOM-induced colonic tumor tissue (Figs. 6A and B).

In addition, the metastasis marker MMP-9, angiogenic molecule VEGF as well as the proliferative marker cyclin D1 were also up-regulated in AOM-induced tumor tissue. Long-term dietary hydroxylated PMFs feeding markedly reduced above protein expression in tumor tissue. Together, these results suggested the *in vivo* anti-tumor activity of dietary hydroxylated PMFs, evidenced by the significant lower number of microadenima in the hydroxylated PMFs treated mice in comparison with the control group

3.8 Apoptosis-inducing effects of dietary hydroxylated PMFs

We next investigated whether the dietary hydroxylated PMFs induced suppression on colonic tumor formation was associated with its pro-apoptotic effect. As shown in Fig. 7A, dietary hydroxylated PMFs resulted in the degradation of 116 kDa poly(ADP-ribose) polymerase to 85 kDa fragments and the cleavage of caspase-3 in a dose-dependent manner. The apoptosis-inducing activity was also confirmed by analyzing the caspase activity. AOM-treated group showed a slight increase of caspase-3 activity (Fig. 7B) compared with normal colonic mucosa. However, dietary hydroxylated PMFs treatment strongly increased the activation of caspase-3, by 3.6- and 5.2-fold at 0.01 and 0.05%, respectively, compared with the AOM-treated group. These results indicated that the dietary hydroxylated PMFs suppressed AOM-induced colonic tumorigenesis possibly through the induction of apoptosis.

4 Discussion

In the present study, for the first time, we showed that dietary consumption of hydroxylated PMFs at doses of 0.01 and 0.05% resulted in significant inhibition of AOM-induced ACF formation in male ICR mice. Long-term (20 wk) dietary consumption of hydroxylated PMFs also caused reduction of colon tumor multiplicity without any noticeable effects, indicating long-term safety and chemopreventive efficacy of dietary hydroxylated PMFs. We also demonstrated that the chemopreventive effect of hydroxylated PMFs was associated with a decrease of proliferation and inflammation as well as modulation of the intermediate signaling pathways in colon of mice. These findings strongly suggested the chemopreventive potential of dietary administration of hydroxylated PMFs against colonic tumorigenesis.

Aberrant β-catenin expression and signaling play a important role in colonic tumorigenesis by increasing transcription of a number of genes, such as cyclin D1, VEGF and MMPs [31, 32, 37]. In our study, we found that hydroxy-

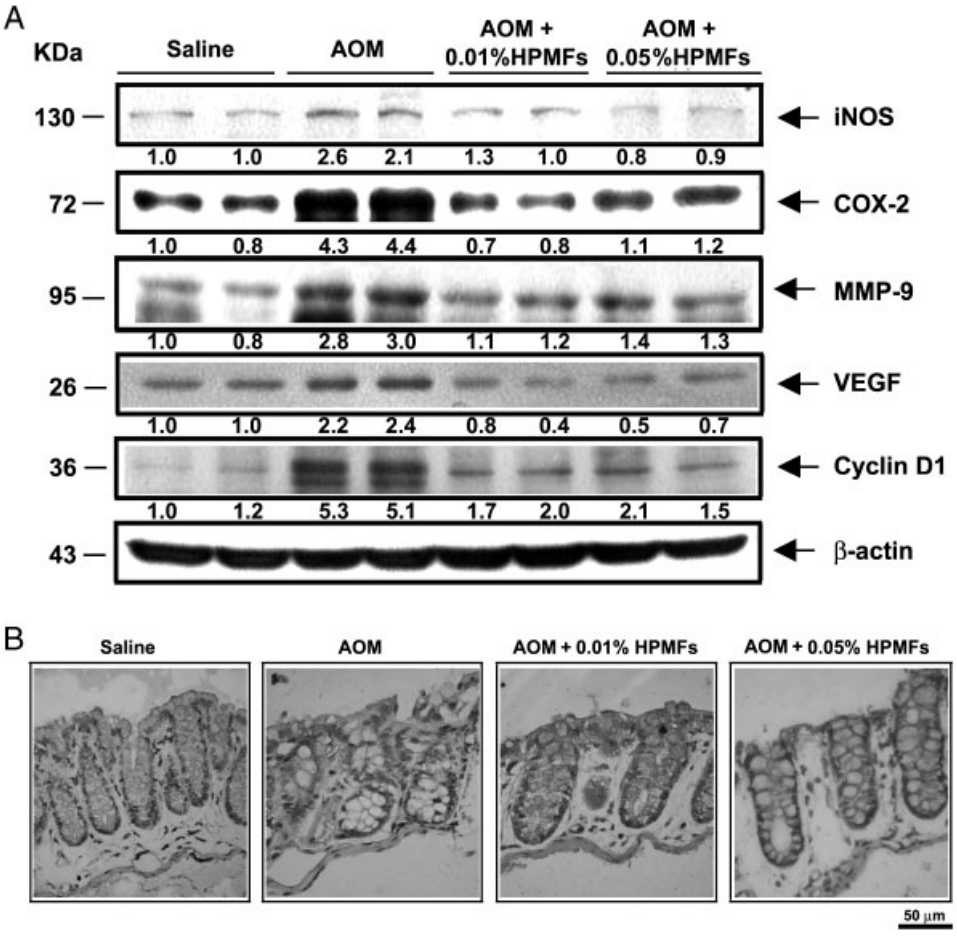

Figure 6. Effects of dietary hydroxylated PMFs on AOM-induced expression of inflammatory enzymes, angiogenesis- and proliferation-related proteins in colonic tumor tissues. Mice were treated as described in Section 2. After 20 wk treatment, colonic tissue were excised and protein lysates of tumor tissue were prepared for measurement of iNOS, COX-2, MMP-9, VEGF and cyclin D1 (A) expression by Western blot analysis. Colonic tissues were embedded in paraffin and detected by immunohistochemical analysis using COX-2 antibody (B), demonstrated as dark stain (magnification, × 400).

lated PMFs inhibited AOM-induced nuclear accumulation of β-catenin through decreasing the phosphorylation of Akt that caused loss of function of GSK3β, subsequently down-regulated expression of cyclin D1 in colonic tissue, and thus reduced the aberrant crypt cell proliferation and ACF formation (Fig. 3). In addition to cyclin D1, E-cadherin is another important molecule in controlling crypt cell growth, proliferation and cell–cell adhesion [38], which is also often down-regulated in colon cancer [39, 40]. Since E-cadherin is critical for maintaining morphology of normal crypt [41], suppressed loss of E-cadherin by hydroxylated PMFs indicates another possible mechanism for inhibition of ACF formation (Fig. 3).

Overexpression of iNOS and COX-2 enzymes contributes to promote tumorigenesis by induction of inflammation, abnormal cell proliferation and decreasing apoptosis [42]. In the current study, we showed that dietary hydroxylated PMFs significantly inhibited AOM-induced increase of both mRNA and protein levels of iNOS and COX-2 (Figs. 4A and B). Dietary hydroxylated PMFs also had caused marked reduction of the phosphorylation and nuclear translocation of NF-κB and STAT3, both of which are major regulators of iNOS and COX-2 expression (Figs. 4C and D). These results suggested that anti-inflammatory efficacy of hydroxylated PMFs might be one of the mechanisms for its chemopreventive activity in inhibiting AOM-induced colonic tumorigenesis.

EGFR family proteins are important for regulating cell growth and proliferation but also contribute to malignant transformation and tumor progression [43]. It has also been reported EGFR signaling is up-regulated in carcinogen-induced colonic tumor and human ACF [10, 11]. Blockage of EGFR signaling effectively inhibits ACF formation and neoplastic progression [11, 36]. We found that mice treated with AOM at 6 wk markedly increased the phosphorylation of EGFR at Tyr845, 992 and 1068 that are required for its downstream signaling c-Src, PLCγ and GRB2 adaptor protein [44–46]. Moreover, AOM-treatment also increased the phosphorylation of ERK1/2 and Ras activity that both are downstream effectors of EGFR. However, dietary hydroxylated PMFs strongly reduced the phosphorylation of EGFR and its downstream signaling as well as the activation of Ras in mouse colon. In addition to β-catenin, cyclin D1 and COX-2 also are important targets for EGFR signaling that are involved in the increase of cell proliferation [47, 48]. Thus, dietary hydroxylated PMFs inhibited AOM-induced ACF formation possibly not only through lowered β-catenin expression but also interfered EGFR signaling. Importantly,

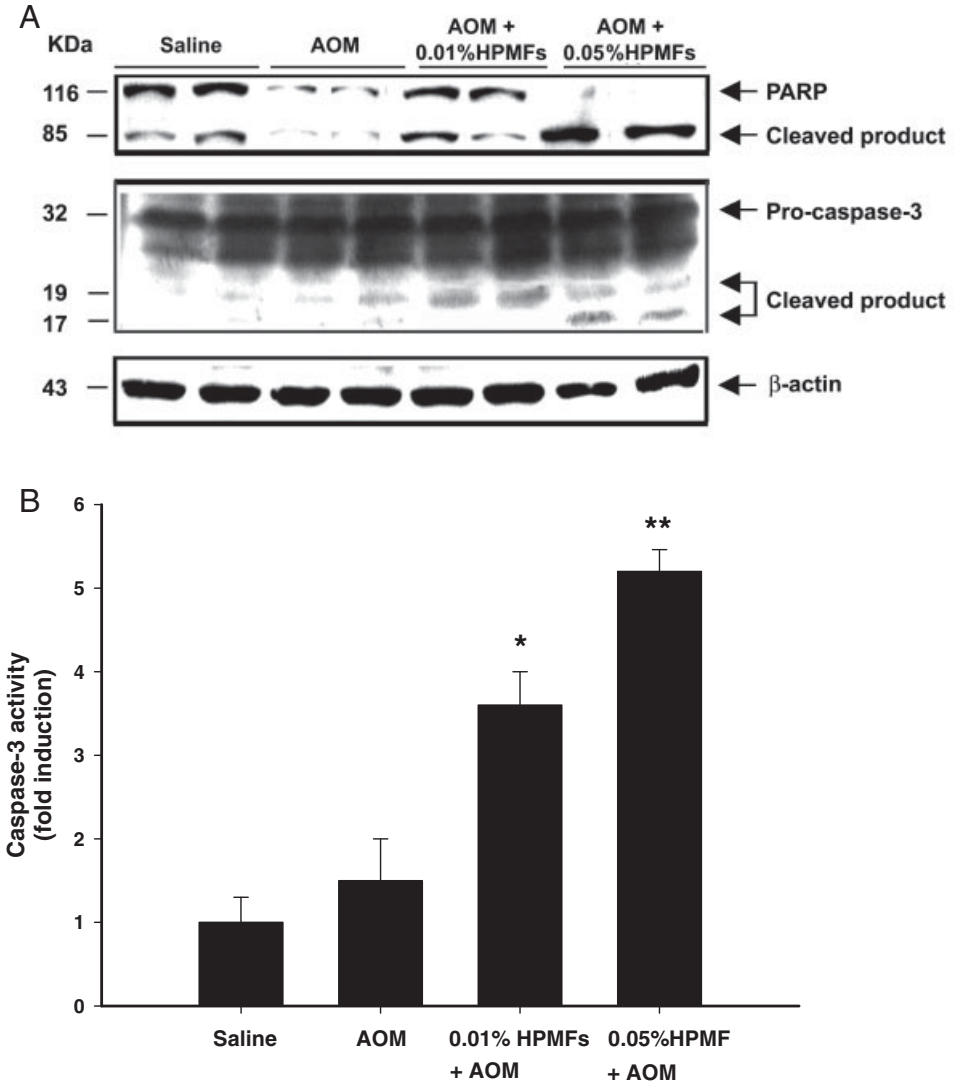

Figure 7. Dietary hydroxylated PMFs increased apoptosis in AOM-treated male ICR mice. After 20 wk treatment, colonic tissues were excised and protein lysates of tumor tissue were prepared for measurement of poly(ADP-ribose) polymerase (PARP) and caspase-3. Western blot analysis of poly (ADP-ribose) polymerase in colonic tumor tissue (A). Caspase-3 activity was analyzed as described under Section 2 (B). $p<0.05$ and $p<0.01$ were compared with AOM-alone group. Data are representative of three independent experiments.

our study showed that hydroxylated PMFs significantly decreased the number of large ACF that had higher risk progress to adenoma. Regarding the role of EGFR in progression of ACF and development of microadenoma, downregulation of growth signaling of EGFR might be a major target for hydroxylated PMFs on reduction of large ACF.

Another important finding in this study is the observation that long-term dietary hydroxylated PMFs treatment had decrease the number of colonic tumors in mice. As the significant role of iNOS and COX-2 in colonic tumorigenesis [14], we also found that the expression of these two enzymes was decreased in colonic tumor by hydroxylated PMFs treatment. Angiogenesis is the process of forming new blood vessels from preexisting vessels and is an essential process for tumor growth, invasion and metastasis [49]. Several proangiogenic factors are implied in tumorigenesis, such as VEGF and COX-2 [50]. Here, we also observed that hydroxylated PMFs suppressed the expression of VEGF and MMP-9 in colonic tumors. It therefore suggests that hydroxylated PMFs suppress the colonic tumor formation possibly through inhibiting inflammation and tumor angiogenesis. Moreover, decreased apoptosis and excessive cell proliferation are correlated to the increase in tumor development and progression [51]. This anti-proliferative and pro-apoptotic activity could be an important mechanism for hydroxylated PMFs to inhibit AOM-induced colon tumor formation. Previous *in vitro* studies demonstrated that hydroxylated PMFs and PMFs induce apoptosis in mature adipocytes and various cancer cells *via* activation of Ca^{2+}-dependent calpain and Ca^{2+}/calpain-dependent caspase-12 [52–54], implying the pro-apoptotic effects of dietary hydroxylated PMFs in AOM-treated colonic tumorigenesis through Ca^{2+}-medicated pathway.

In summary, our study showed *in vivo* chemopreventive efficacy including anti-inflammatory, anti-proliferative, anti-angiogenic and apoptosis-inducing effects of dietary hydroxylated PMFs in AOM-induced colonic tumorigenesis.

Treatment with hydroxylated PMFs suppressed colonic ACF and tumor formation through down-regulation of Wnt/β-catenin and EGFR signaling pathways as well as activation of STAT3 and NF-κB transcription factors, thus blocking expression of iNOS, COX-2 VEGF and MMP-9. Above all, this is the first investigation with evidence that hydroxylated PMFs have great potential as a novel chemopreventive agent to be used in the treatment of inflammation associated with tumorigenesis, especially in the prevention and treatment of CRC.

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The authors have declared no conflict of interest.

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