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ORIGINAL ARTICLE – CANCER RESEARCH

Naringin, a natural dietary compound, prevents intestinal tumorigenesis in $Apc^{Min/+}$ mouse model

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Abstract

Purpose Naringin is a natural dietary flavonoid compound. We aimed to evaluate the effects of naringin on intestinal tumorigenesis in the adenomatous polyposis coli multiple intestinal neoplasia ($Apc^{Min/+}$) mouse model.
Methods $Apc^{Min/+}$ mice were given either naringin (150 mg/kg) or vehicle by p.o. gavage daily for 12 consecutive weeks. Mice were killed with ether, and blood samples were collected to assess the concentrations of IL-6 and PGE_2. Total intestines were removed, and the number of polyps was examined. Tissue samples of intestinal polyps were subjected to the assays of histopathology, immunohistochemical analysis and Western blotting analysis.
Results $Apc^{Min/+}$ mice fed with naringin developed less and smaller polyps in total intestines. Naringin prevented intestinal tumorigenesis without adverse effects. Histopathologic analysis revealed the reduction of dysplastic cells and dysplasia in the adenomatous polyps. The treatments' effects might arise from its anti-proliferation, induction of apoptosis and modulation of GSK-3β

and APC/β-catenin signaling pathways. Naringin also exerted its effects on tumorigenesis through anti-chronic inflammation.
Conclusion Naringin prevented intestinal tumorigenesis likely through a collection of activities including anti-proliferation, induction of apoptosis, modulation of GSK-3β and APC/β-catenin pathways and anti-inflammation. Naringin is a potential chemopreventive agent for reducing the risk of colonic cancers.

Keywords Naringin · $Apc^{Min/+}$ mouse model · Intestinal adenomatous polyps · APC/β-catenin signaling pathway · Anti-proliferation · Apoptosis · Anti-inflammation

Introduction

Colorectal cancer (CRC) is a major cause of cancer-related deaths in many countries. Adenomatous polyps, or adenomas, have been considered as the major precursors for CRC, through a gradual series of histological changes, called 'adenoma–carcinoma' sequence. Mutation of a tumor suppressor gene, adenomatous polyposis coli (*APC*), is likely to be critical for initiating the 'adenoma–carcinoma' sequence (Bosman and Yan 2014; Liu et al. 2013). Clinical studies showed that half of population will develop at least one adenomatous polyp in their life, with 2–3 % of them going to develop CRC (Leoz et al. 2015). Fortunately, the progression of 'adenoma–carcinoma' sequence might last for more than 10 years (Arvelo et al. 2015). It has therefore provided an opportunity for chemoprevention of CRC through blocking the sequence of 'adenoma–carcinoma.'

Epidemiologic studies showed that the incidence of CRC is inversely correlated with the consumption of fruits and vegetables for containing considerable amounts of

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biologically active compounds (Annema et al. 2011). Dietary chemoprevention has thus received attention for reducing the risk of CRC. Flavonoids include a large class of natural polyphenolic compounds and are commonly found in fresh vegetables and fruits. Among naturally occurring flavonoids, naringin (4′, 5, 7-trihydroxy flavanone-7-rhamnoglucoside), a glycone form of naringenin, can be found in most citrus fruits (Cordenonsi et al. 2015). Naringin has been reported as a multifunctional agent. It mitigates cardiac hypertrophy by reducing oxidative stress and inactivating c-Jun nuclear kinase (JNK-1) protein in type I diabetes (Adeniyi et al. 2015). Naringin ameliorates sodium arsenite-induced renal and hepatic toxicity in rats by modulating the activities of KIM-1, caspase-3, TGF-β and TNF-α (Adil et al. 2015). Naringin ameliorates cognitive deficits via oxidative stress, proinflammatory factors and the PPARγ signaling pathway in a type 2 diabetic rat model (Qi et al. 2015). Naringin protects neural cells on 3-nitropropionic acid-induced mitochondrial dysfunction through modulation of Nrf2 signaling pathway (Kulasekaran and Ganapasam 2015). Naringin induces autophagy-mediated growth inhibition by downregulating the PI3K/Akt/mTOR cascade via activation of MAPK pathways in AGS cancer cells (Raha et al. 2015). Naringin prevents the cisplatin-induced oxidative stress, inflammatory response and apoptosis in rat striatum via suppressing the ROS-mediated NF-κB and P53 signaling pathways (Chtourou et al. 2015). Recently, naringin has been reported to have a function of anti-tumorigenesis. Naringin inhibited the growth of hepatocellular carcinoma HepG2 cells through the induction of apoptosis (Banjerdpongchai et al. 2015). Naringin induces apoptosis in human DU145 prostate cancer cell line (Lewinska et al. 2015). Naringin inhibits potential growth of human triple-negative breast cancer cells by targeting β-catenin signaling pathway (Li et al. 2013). These biological activities were all evaluated by in vitro assays. Herein, we aimed to evaluate the effects of naringin on tumorigenesis in the $Apc^{Min/+}$ mouse model, which represent the phenotypes of familial adenomatous polyposis (FAP) in humans (Merritt et al. 1997). This model is advantageous for evaluating the chemopreventive agents against the 'adenoma–carcinoma' sequence in intestines. Our results showed that $Apc^{Min/+}$ mice fed with naringin developed less and smaller intestinal polyps without adverse effects. Naringin prevented intestinal tumorigenesis through a collection of biological activities including anti-proliferation, induction of apoptosis and anti-inflammation. These biological activities of naringin characterized the basic requirements as a chemopreventive agent to reduce the risk of cancers.

Materials and methods

Ethics statement

The research protocol was approved by the Animal Care and Use Committee at Capital Medical University. The permit number is AEEI-2014-101. We declared that the following experiment complied with the principles of laboratory animal care issued by NIH.

Naringin

Naringin (purity ≥98 %) was purchased from Sigma Chemical Company and was solved in 5 % sodium carboxymethyl cellulose (CMC-Na) (Sigma Chemical Co).

Mice model and drug administration

$Apc^{min/+}$ male mice (5 weeks old) were purchased from Model Animal Research Center of Nanjing University. Mice were crossed with wild-type C57BL/6 female mice to generate $Apc^{Min/+}$ mice (Liu et al. 2013). A total of 18 mice (both male and female, 4 weeks old) were randomly divided into two groups. They were fed with standard mouse pellet diet, which contains 52 % carbohydrate, 12 % fat, 23 % protein, 4 % fiber, 6 % ash and 3 % moisture (Meidenbauer et al. 2014). After 1 week of acclimation, $Apc^{Min/+}$ mice (6 weeks old) were administrated either vehicle (CMC-Na 5 %, v/v) or naringin (150 mg/kg) by p.o. gavage daily (0.2 ml/10 g body weight). Administrations were performed for 12 consecutive weeks. Mice were weighed weekly and checked daily for any health problem. At age of 18 weeks, mice were anesthetized with ether and blood samples were collected by exsanguination from inferior vein.

Intestinal polyps score

Total intestines were removed from each mouse and were sliced longitudinally and rinsed with saline. Total number and size of intestinal polyps were examined under a dissecting microscope. Intestinal polyps were categorized by size into 1–2 mm, 2–3 mm and >3 mm. After number counting, parts of small and colonic intestines were frozen in liquid nitrogen for Western blotting assay. Other parts of intestines were fixed in 10 % phosphate-buffered formalin for histopathology/immunohistochemical (IHC) analysis.

Histopathology and immunohistochemical analysis (IHC)

The paraffin-embedded intestinal polyps were cut into sections with into 5-μm-thick sections. Sections were stained with hematoxylin and eosin (H&E) for histopathology. IHC analysis was performed as previously described (Huang et al. 2015). Sections were deparaffinized, and endogenous peroxidase was blocked by exposure to 3 % hydrogen peroxide in methanol. Primary antibodies included cyclin D1 (2922), β-catenin (9582), p-NFκB (4810, Cell Signaling Technology), Cox-2 (sc-23984, Santa Cruz), GSK3β (ab32391) and PCNA (ab29, Abcam). Second antibodies were anti-mouse IgG and anti-rabbit IgG (Santa Cruz). Positive staining with PCNA, cyclin D1, GSK3β and β-catenin was defined as brown staining in nuclei of adenomatous cells. Positive staining with COX-2 and p-NFκB was identified based on brown intensity of membrane, cytoplasm and nuclei of adenomatous cells (Liu et al. 2013; Huang et al. 2015). The stained adenomatous cells were compared with total number cells at five selected fields from each polyp.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining assay was performed to identify apoptotic cells in the adenomatous polyps. TUNEL kit was purchased from Roche Diagnostics (Germany). Sections were prepared as described in the immunohistochemical analysis (IHC). The staining assay was performed according to the manufacturer's instruction. TUNEL-positive cells were recognized by their dark brown nuclear staining. The proportion of apoptotic cells in six mice was scored in randomly chosen fields under a microscope.

Western blotting assay

The intestinal polyps were excised and pooled together based on groups. Three–five polyps were incubated with 80 μl RIPA lysis buffer at 4 °C for 30 min to obtain lysates of adenomatous cells as previously described (Liu et al. 2013). The supernatants were diluted, and the concentration of protein was determined by using bicinchoninic acid kit (Pierce). Lysates (30 μg of protein per lane) were fractionated by SDS-PAGE. Protein was electro-transferred onto poly (vinylidene fluoride) (PVDF) membranes and was then determined by using the primary antibodies. The primary antibodies included β-catenin (9582), p-β-catenin Ser[37] (9561), cleaved caspase-3 (Asp175) (9664), Bcl-xL (2764), cyclin D1 (2922), p-NFκB (4810, Cell Signaling Technology), p-GSK3β Ser[9] (ab75814), TNF-α (ab6671), PCNA (ab29), β-actin (ab6276, Abcam), c-Myc (sc-42) and Bax (sc-493, Santa Cruz). PVDF membranes were washed in 0.05 % Tween-20/Tris-buffered saline and incubated with horseradish peroxidase-conjugated secondary antibody. The bound antibodies were visualized and quantified under FluorChem FC3 image analyzer (molecular devices). Densitometric analyses of bands were adjusted with β-actin functioning as a loading control.

ELISA

Plasma IL-6 was detected by using sandwich ELISA kits according to manufacturer's protocol (EZMIL6, Meck Millipore). Plasma PGE$_2$ was quantified by an enzyme immunoassay (EIA) kit (ADI-900-001, Enzo Life Science). Briefly, serum and PGE2 conjugates were added to a 96-well plate pre-coated with goat anti-mouse IgG for 2 h. Plate was washed with PBS to remove unbound antibody–enzyme reagent. The substrate solution was added, and the intensity of color developed was read at a wavelength of 405 nm.

Statistical analysis

Statistical analysis was done with SPSS/Win13.0 software (SPSS, Inc., Chicago, Illinois). Data were described as mean ± SD. Comparisons between control and treatment group were conducted by two-tailed Student's t test. A p value less than 0.05 was considered statistically significant.

Results

Naringin prevented intestinal adenomatous polyps without causing health problems to mice

In control group, $Apc^{Min/+}$ mice at age of 18 weeks developed 31.2 and 8.3 polyps, respectively, on average in small intestine and colon (Fig. 1a). Mice fed with naringin developed less and smaller intestinal polyps (Fig. 1b). Total intestinal polyps were reduced by 52.6 % ($p < 0.01$ vs. vehicle control) in small intestines and 62.5 % ($p < 0.01$ vs. vehicle control) in colons (Fig. 1c). Further analysis showed differential naringin effects depending upon polyp sizes and segments. Naringin reduced larger polyps (>2 mm) more significantly than smaller polyps. Naringin reduced the number of polyps between 1 and 2 mm by 58.8 % ($p < 0.01$ vs. vehicle control) and the number of polyps between 2 and 3 mm by 66.5 % ($p < 0.01$ vs. vehicle control), in small intestine (Fig. 1d). Naringin reduced the colonic polyps by 64.1 % ($p < 0.01$ vs. vehicle control) in the number of polyps between 1 and 2 mm,

Fig. 1 Naringin prevented the intestinal tumorigenesis in *Apc*-*Min/+* mice. At age of 18 weeks, mice were killed and intestinal polyps and size were counted. **a** Vehicle control intestines. **b** Mice fed with naringin developed less and smaller intestinal polyps. **c** Naringin prevented intestinal polyps. **d** Naringin reduced sizes in different sections of intestines. *$p < 0.05$; **$p < 0.01$ versus vehicle control. *Bars* represent mean ± SD of 9 mice
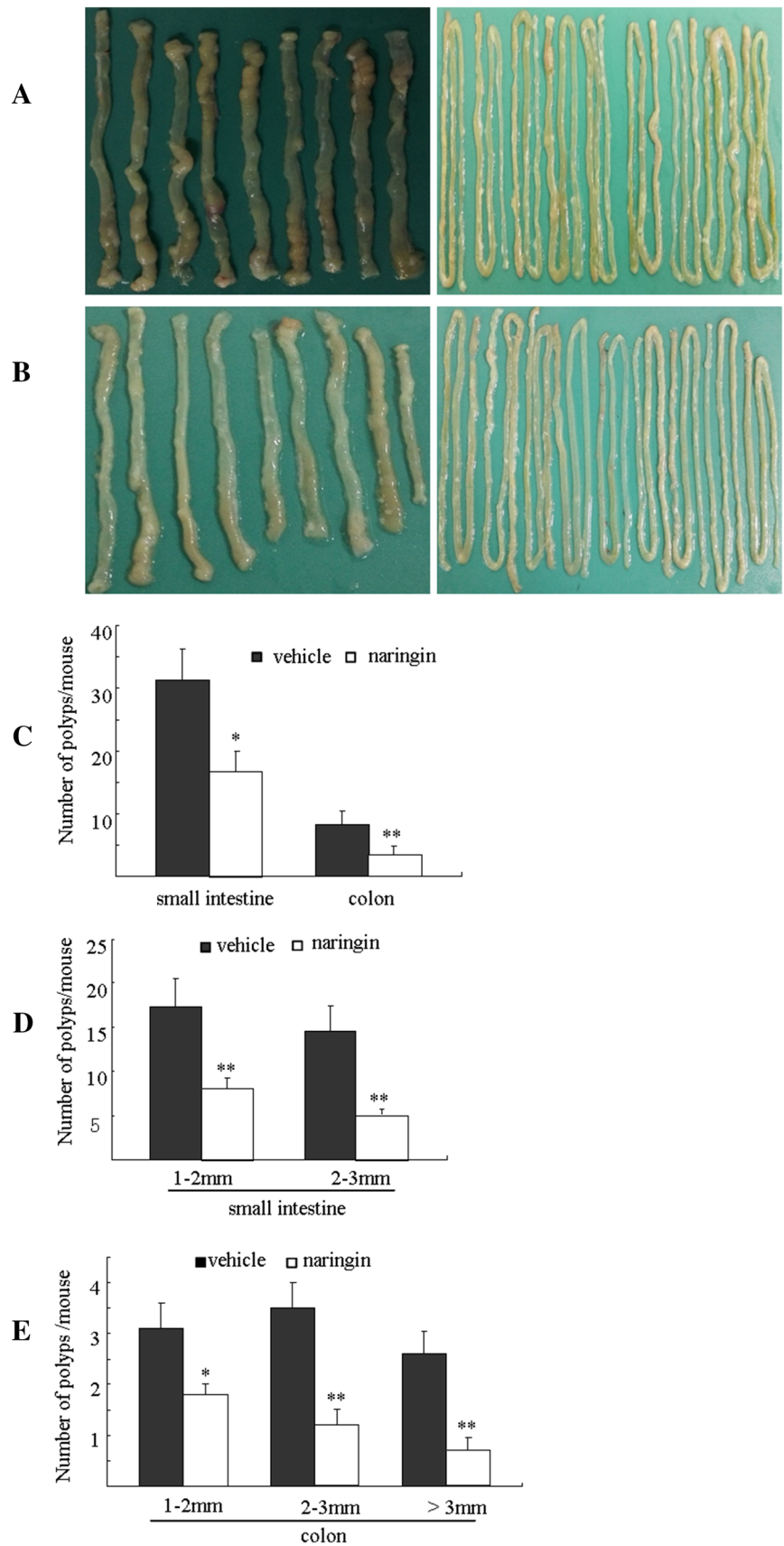
Fig. 2 H&E-stained intestinal sections from $Apc^{Min/+}$ mice. **a** Control mice with crater-shaped adenomatous polyps in small intestine (×100). *a* (*Inset*) adenomatous epithelium shows the enlarged, hyperchromatic, elongated and crowded dysplastic nuclei (×400). **b** Advanced adenomatous polyps with focal high-grade dysplasia in colonic section of control mice (×100). *b* (*Inset*) crypt architecture appears more complex, and the nuclei are more pleomorphic with frequent mitoses. There is a lack of nuclear polarization in glands (×400). **c** Small intestine in the naringin-fed mice (×100). *c* (*Inset*) crypt architecture is essentially normal with unremarkable epithelial nuclei (×400). **d** Colon from naringin-fed mice (×100). *d* (*Inset*) structure and nuclei of epithelium are basically normal (×400)
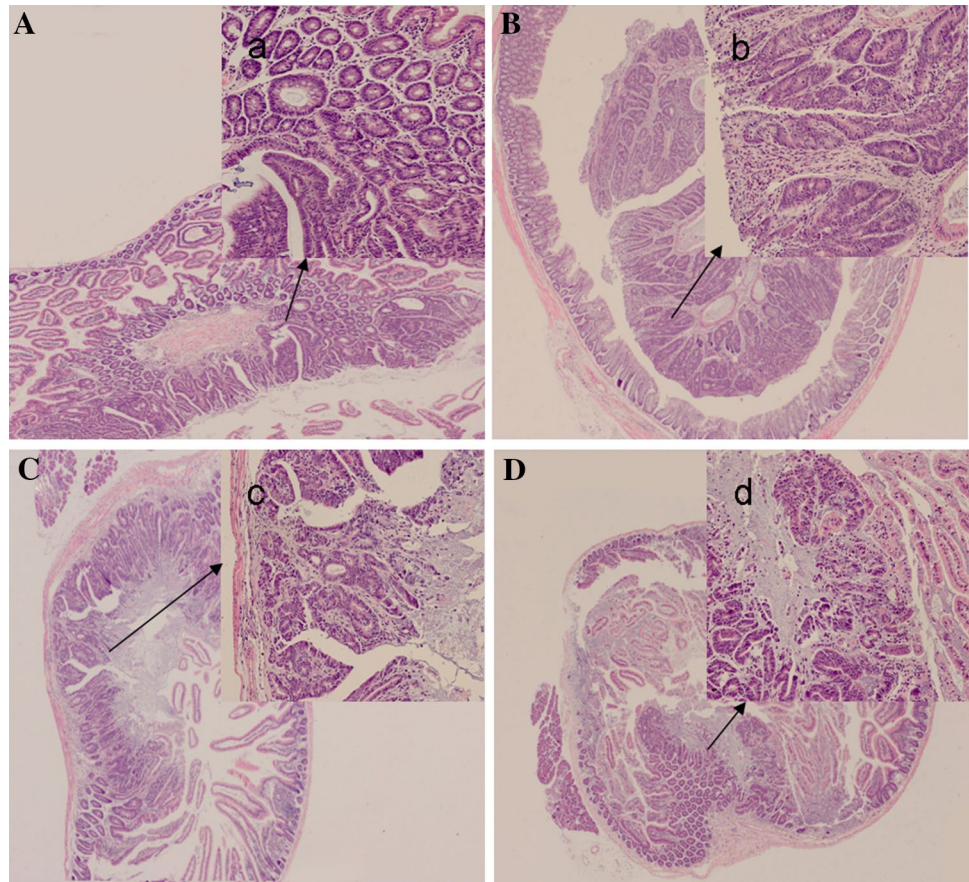
72.4 % ($p < 0.01$ vs. vehicle control) in the number of polyps between 2 and 3 mm and 84.1 % ($p < 0.01$ vs. vehicle control) in the number of polyps >3 mm (Fig. 1e).

During the process of the experiment, mice did not show considerable change in food consumption and body weight. There was no any significant difference in liver function, blood element counts and other biological signs between naringin-fed and vehicle control mice (data not shown).

Naringin blocked the progression of intestinal tumorigenesis

In $Apc^{Min/+}$ mice, all intestinal polyps were histologically identified as adenocarcinoma, which demonstrates an early stage of colonic tumorigenesis. With size growth, these intestinal polyps might gradually progress from benign adenoma to adenocarcinoma with high-grade dysplasia and develop intramucosal carcinoma. Small intestinal polyps showed the crowded pencil-shaped hyperchromatic nuclei with preserved polarity and diminished mucin (Fig. 2a). Larger polyps were seen in colons, showing intramucosal adenocarcinoma with focal high-grade dysplasia and nuclear pleomorphism, lack of nuclear polarity, frequent mitoses and architectural distortion (Fig. 2b). Naringin blocked the progression of intestinal polyps. The number

of dysplastic cells obviously decreased, and the degree of high-grade dysplasia was significantly reduced in small intestinal (Fig. 2c) and colonic polyps (Fig. 2d). Normal intestinal villi were seen in most small and colonic intestines in the naringin-fed mice.

Naringin inhibited the proliferation and induced apoptosis in the intestinal polyps

Excessive proliferation mostly contributed to the formation and progression of adenocarcinoma polyps. We examined the expression levels of β-catenin targets, cyclin D1, PCNA and c-Myc, which are considered as the major markers of tumorigenesis. Naringin selectively inhibited the proliferation of intestinal adenocarcinomas. Naringin did not significantly affect the crypt architecture and the proliferation of epithelia on the normal intestines (data not shown). IHC analysis showed a reduction of cyclin D1 staining cells by 61.2 % ($p < 0.01$ vs. vehicle control) and 72.9 % ($p < 0.01$ vs. vehicle control) (Fig. 3a) and a reduction of PCNA staining cells by 57.8 % ($p < 0.01$ vs. vehicle control) and 70.4 % ($p < 0.01$ vs. vehicle control) (Fig. 3b), respectively, in small intestinal polyps and colonic polyps. Further, Western blotting showed the decrease in cyclin D1 by 49.6 % ($p < 0.05$ vs. vehicle control) and 42.2 % ($p < 0.05$
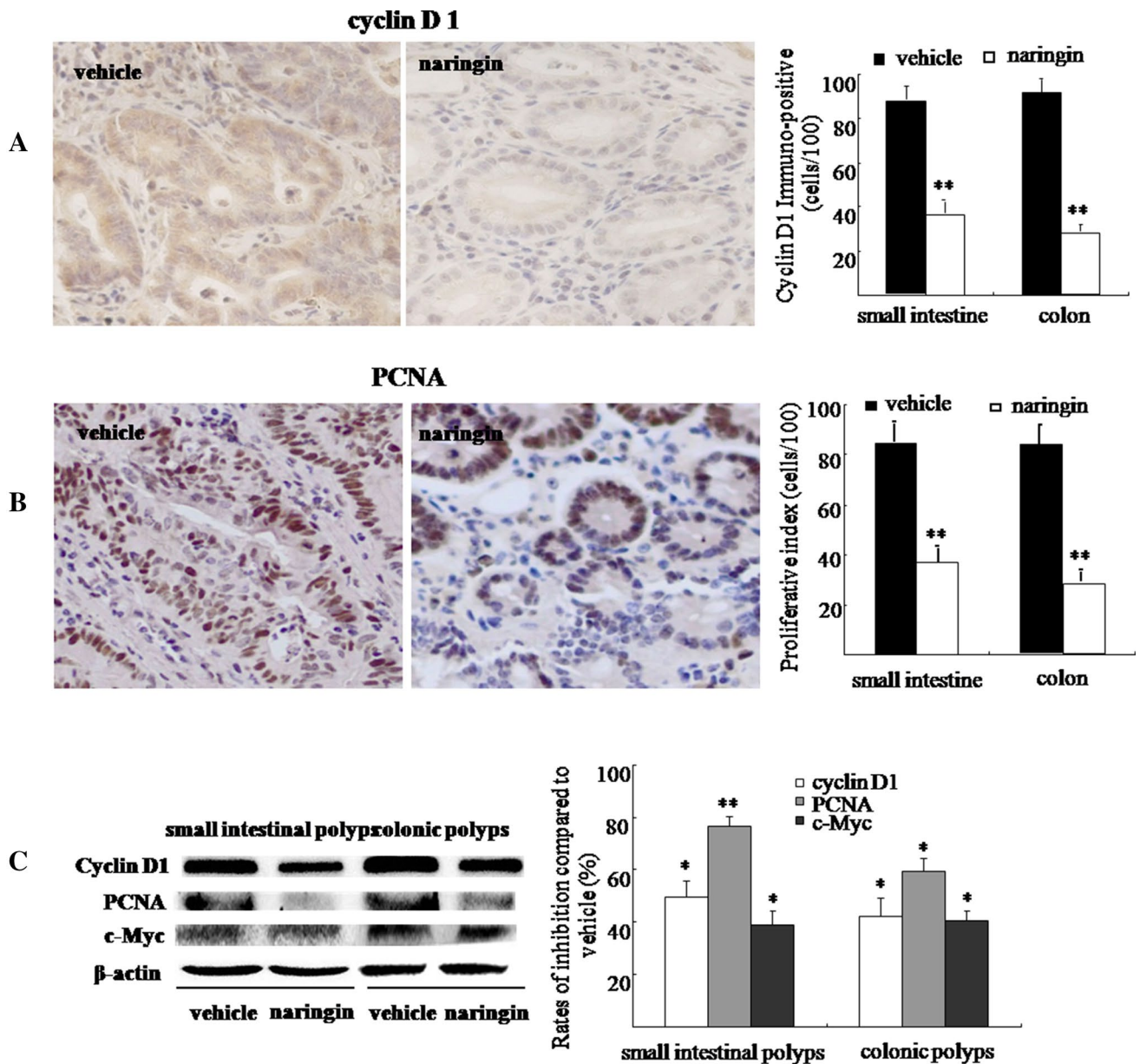
cyclin D 1

A

PCNA

B

C

Fig. 3 Naringin inhibited expression of cyclin D1 and PCNA in epithelia of intestinal polyps. IHC showed the expressions of cyclin D1 (**a**) and PCNA-positive cells (**b**) in colonic polyps (×400). The stained adenomatous cells were compared with total number cells at five selected fields from each polyp. Results were denoted as a percentage of stained cells in each figure. **c** Western blotting showed the expressions of cyclin D1, PCNA and c-Myc in small intestinal polyps and colonic polyps. Densitometric bands were adjusted with β-actin and compared with vehicle control. *Bars* represent mean ± SD of triplicate experiments. *$p < 0.05$, **$p < 0.01$ versus vehicle control

vs. vehicle control) (Fig. 3c), PCNA by 76.6 % ($p < 0.01$ vs. vehicle control) and 59.3 % ($p < 0.05$ vs. vehicle control) and c-Myc by 39.1 % ($p < 0.05$ vs. vehicle control) and 40.7 % ($p < 0.05$ vs. vehicle control), respectively, in small intestinal polyps and colonic polyps.

Intestinal adenocarcinoma is mostly characterized by insufficient apoptosis. Naringin selectively induced adenocarcinoma cells to apoptosis. TUNEL assay showed the increase in apoptotic cells by 49.4 % ($p < 0.05$ vs.

vehicle control) in small intestinal polyps (Fig. 4a) and 154.1 % ($p < 0.01$ vs. vehicle control) in colonic polyps (Fig. 4b). Western blotting showed the modulation of proapoptotic Bax, anti-apoptotic Bcl-xL and apoptotic executor c-caspase-3 in naringin-treated intestinal polyps (Fig. 4c). Naringin treatment resulted in an increase in c-caspase-3 by 193.5 % ($p < 0.01$ vs. vehicle control) and 246.3 % ($p < 0.01$ vs. vehicle control) and an increase in Bax by 89.6 % ($p < 0.01$ vs. vehicle control)

Fig. 4 Naringin induced apoptosis of intestinal polyps. Naringin increased the TUNEL-positive cells in the small (**a**) and colonic intestinal polyps (**b**) (×200). TUNEL-positive cells with *dark brown* nuclear staining were compared with vehicle control. **c** Western blotting showed the expression of c-caspase-3, Bax, Bcl-2 and Bcl-xL in small intestinal polyps and colonic polyps. Densitometric bands were adjusted with β-actin and compared with vehicle control. *Bars* represent mean ± SD of triplicate experiments. $*p < 0.05$, $**p < 0.01$ versus vehicle control
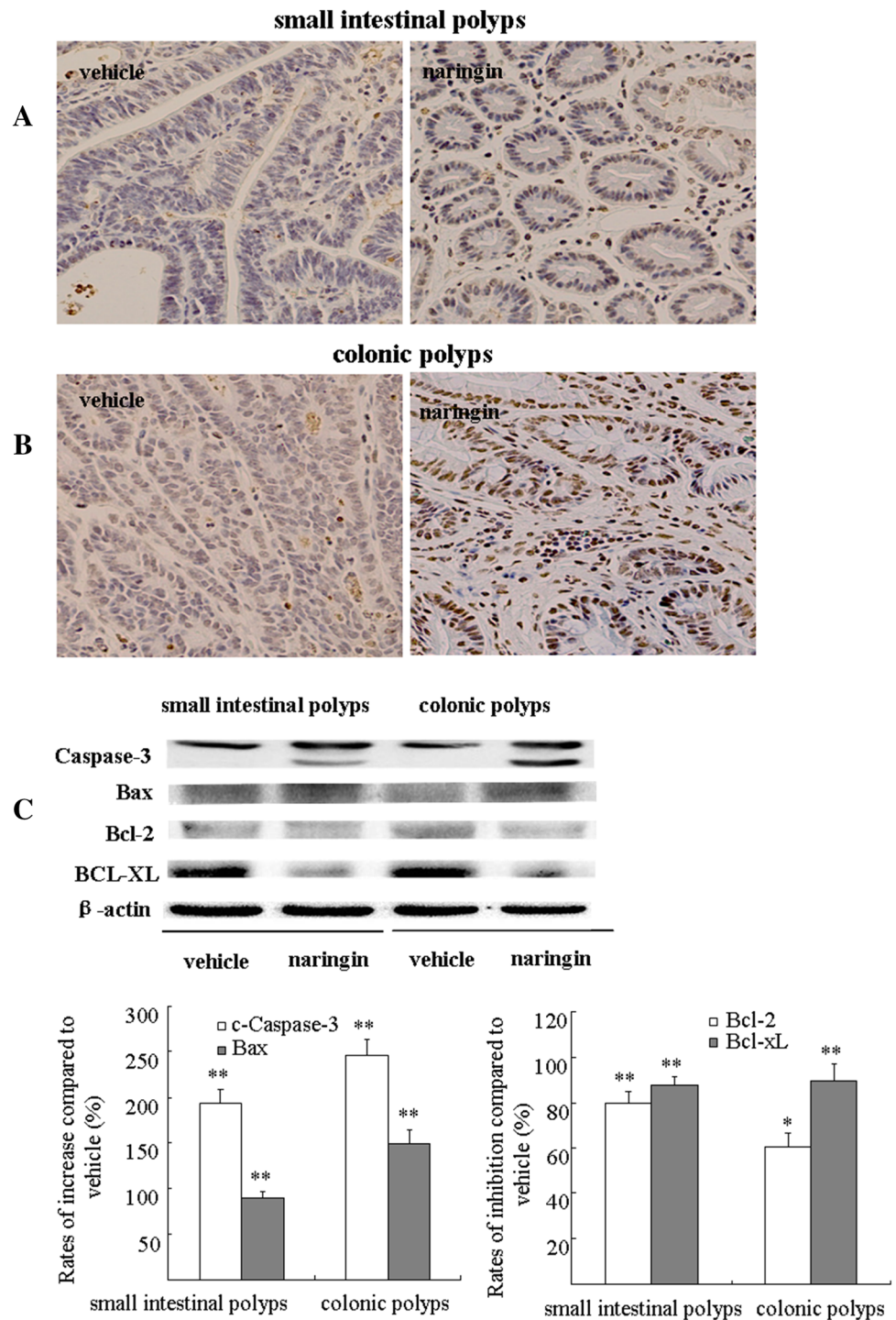
and 149.5 % ($p < 0.01$ vs. vehicle control), respectively, in small intestinal and colonic polyps. Naringin reduced the level of Bcl-2 by 79.3 % ($p < 0.01$ vs. vehicle control) and 60.3 % ($p < 0.05$ vs. vehicle control) and Bcl-xL by 87.8 % ($p < 0.01$ vs. vehicle control) and 89.3 % ($p < 0.01$ vs. vehicle control), respectively, in small intestinal polyps and colonic polyps.

Naringin modulated GSK-3β activity and inhibited β-catenin expression in adenomatous cells

In colonic cancer cells, loss of APC function leads to dysfunction of GSK-3β and results in an accumulation of β-catenin. Naringin modulated the activity of GSK-3β and inhibited β-catenin expression in intestinal adenomatous

cells. IHC assay showed an increase in active GSK-3β staining cells by 63.4 % ($p < 0.05$ vs. vehicle control) and 125.6 % ($p < 0.01$ vs. vehicle control), respectively, in small intestine and colon (Fig. 5a). Naringin decreased the expression of β-catenin staining cells by 68.1 % ($p < 0.01$ vs. vehicle control) and 66.9 % ($p < 0.01$ vs. vehicle control), respectively, in small intestine and colon (Fig. 5b). Further analysis by Western blotting assay showed an increase in active GSK-3β by 46.8 % ($p < 0.05$ vs. vehicle control) and 42.7 % ($p < 0.05$ vs. vehicle control), while phosphorylated GSK-3β was decreased by 80.2 % ($p < 0.01$ vs. vehicle control) and 85.8 % ($p < 0.01$ vs. vehicle control), respectively, in small intestinal polyps and colonic polyps (Fig. 5c). The expression of β-catenin significantly reduced by 68.3 % ($p < 0.01$ vs. vehicle control) and 79.2 % ($p < 0.01$ vs. vehicle control) and phosphorylated β-catenin by 77.8 % ($p < 0.01$ vs. vehicle control) and 80.4 % ($p < 0.01$ vs. vehicle control), respectively, in small intestinal polyps and colonic polyps (Fig. 5c).

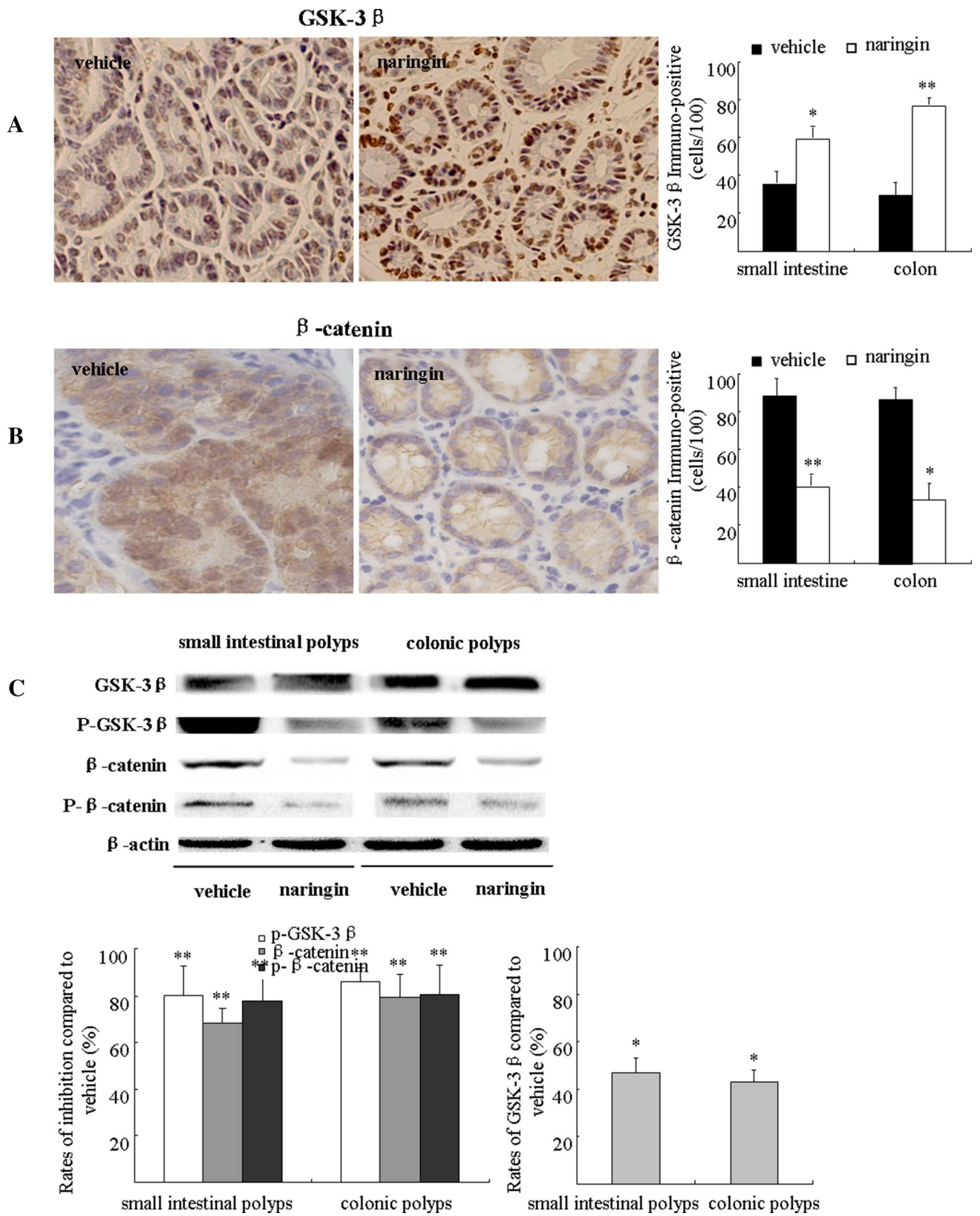## Naringin reduced inflammatory cytokines in intestinal polyps and plasma

Chronic inflammation stimulates intestinal tumorigenesis by altering the microenvironment in ways that stimulate stroma and epithelial cells. High levels of Cox-2, NF-κB, TNF-α, PGE$_2$ and IL-6 are considered to be the most valuable malignant indicators in adenomatous cells in $Apc^{Min/+}$ mice. Naringin possessed the activity of suppressing chronic inflammatory cytokines in both intestinal adenomatous polyps and plasma. IHC analysis showed that naringin reduced the level of Cox-2 by 56.6 % ($p < 0.01$ vs. vehicle control) and 67.8 % (Fig. 6a, $p < 0.01$ vs. vehicle control) and p-NF-κB by 83.5 % ($p < 0.01$ vs. vehicle control) and 86.7 % (Fig. 6b, $p < 0.01$ vs. vehicle control), respectively, in small intestinal polyps and colonic polyps. Further, Western blotting showed the inhibition Cox-2 by 40.4 % ($p < 0.05$ vs. vehicle control) and 50.8 % ($p < 0.05$ vs. vehicle control), p-NF-κB by 90.2 % ($p < 0.01$ vs. vehicle control) and 78.6 % ($p < 0.01$ vs. vehicle control) and TNF-α by 75.6 % ($p < 0.01$ vs. vehicle control) and 86.3 % ($p < 0.01$ vs. vehicle control), respectively, in small intestinal polyps and colonic polyps (Fig. 6c). The levels of PGE$_2$ and IL-6 in serum were assayed by ELISA. High levels of PGE$_2$ and IL-6 were determined in $Apc^{Min/+}$ mice. Naringin reduced the concentration of PGE$_2$ from 4898.1 ± 222.1 pg/mL to 2927.8 pg/mL ± 270.3 pg/mL (Fig. 6d, $p < 0.05$ vs. vehicle control). The level of serum IL-6 reduced from 19.5 ± 1.4 pg/mL to 11.7 ± 1.2 pg/mL (Fig. 6e, $p < 0.05$ vs. vehicle control).

Fig. 5 Naringin modulated the expressions of GSK-3β and▶ β-catenin in intestinal adenomatous cells. **a** IHC showed the expression of GSK-3β and p-GSK-3β in small and colonic intestinal polyps (×400). **b** IHC showed the expression of β-catenin in small and colonic intestinal polyps (×400). **c** Western blotting analyzed the expression of GSK3β and p-β-catenin (Ser 37) in small and colonic intestinal polyps. Densitometric bands were adjusted with β-actin and compared with vehicle control. *$p < 0.05$, **$p < 0.01$ versus vehicle control. *Bars* represent mean ± SD of triplicate experiments

Discussion

$Apc^{min/+}$ mouse model represents the phenotypes of familial adenomatous polyposis (FAP) in humans with spontaneous intestinal tumorigenesis. In this model, tumors in small intestine are composed of dysplastic crypts surrounded by hyperplastic villi and crypts. Colonic tumors are peduncular, forming a spherical mass of dysplastic cells supported by a stromal stalk and displaying a characteristic 'rose' shape in larger polyps (McAlpine et al. 2006). This model is advantageous for evaluating the chemopreventive agents against the 'adenoma–carcinoma' sequence in intestines. In this study, $Apc^{Min/+}$ mice were fed with naringin from 6 weeks of age and administrated for 12 consecutive weeks. At 18 weeks of age, mice with naringin developed less and smaller polyps with lower degree of cytological dysplasia in intestines. Molecular analysis showed that naringin selectively inhibited proliferation and induced intestinal adenoma to apoptosis. The effects of treatments were associated with naringin's activities of blocking the progression of tumorigenesis and reducing the proliferation of adenomatous polyps, leading to a chemoprevention in colonic cancer. Further, naringin possessed the activity of anti-proinflammatory cytokines, such as Cox-2, NF-κB, TNF-α, PGE$_2$ and IL-6 in the adenomatous polyps and plasma. Naringin inhibited intestinal tumorigenesis without adverse effects to mice. These properties of naturally food component characterized the basic requirements for a chemopreventive agent. We therefore suggested that naringin might develop as a potential agent for reducing the risk of colonic cancers.

Chemopreventive effect of naringin was accompanied by its anti-proliferative and proapoptotic mechanisms. It has been widely considered that excessive cell proliferation and insufficient apoptosis are associated with tumorigenesis (Rigby et al. 2007; Wang et al. 2014). In $Apc^{min/+}$ mouse model, different polyp sizes represent different stages of tumorigenesis. An early stage of intestinal tumorigenesis is the benign adenoma that could develop to adenoma with high-grade dysplasia (Cheung et al. 2014; Kwong and Dove 2009; Shih et al. 2001). Intramucosal carcinomas with ulceration frequently occurred in the larger polyps >3 mm (Liu et al. 2013; Rajamanickam et al. 2010). We analyzed the inhibitory effects of naringin on the intestinal polyps
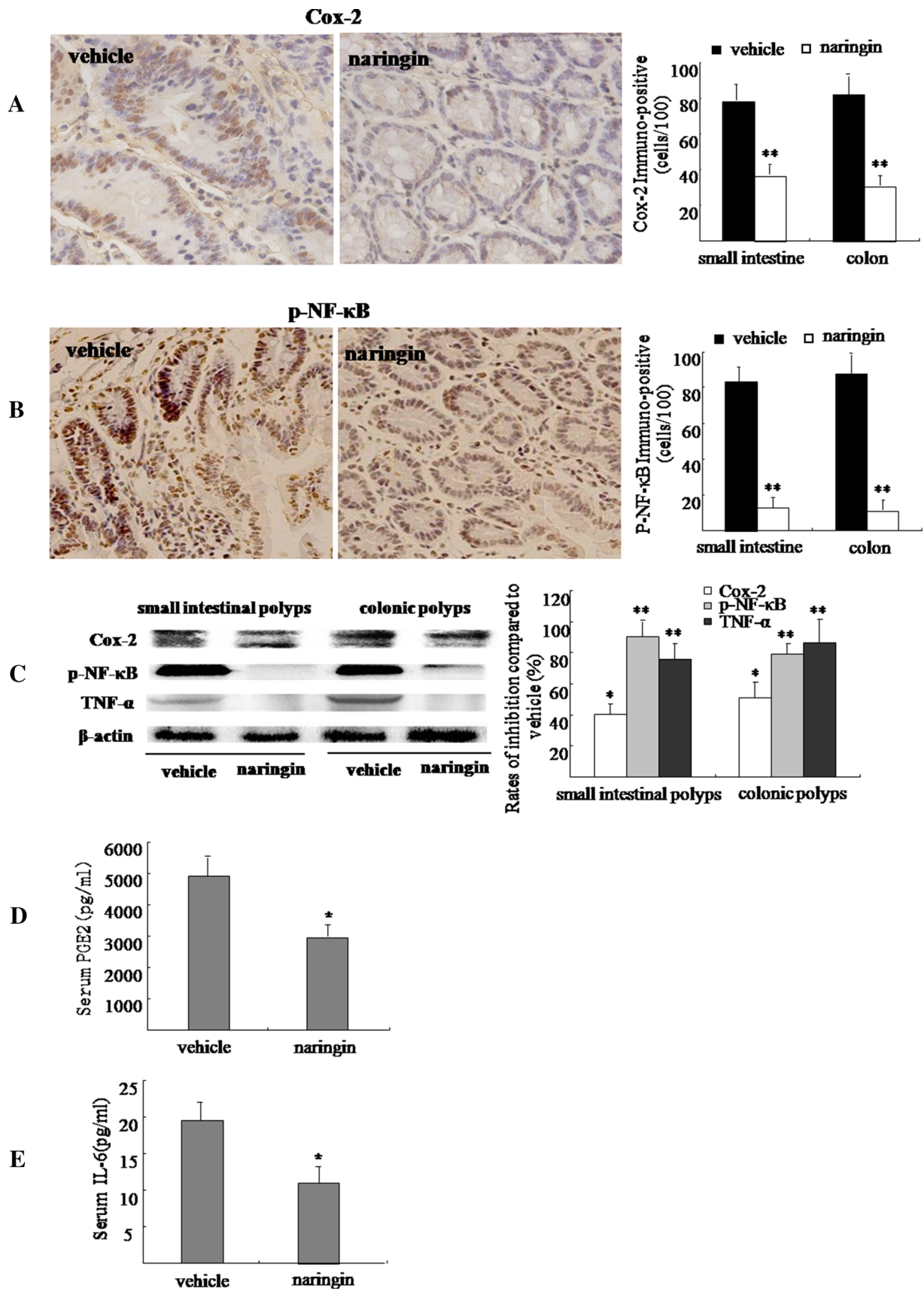
of different sizes. Naringin was found to reduce larger polyps (>2 mm) more significantly than smaller-sized polyps. Pathology analysis showed the reduction of dysplastic cells and dysplasia degree in intestinal polyps. These effects of naringin might be associated with its selective anti-proliferation and induction of apoptosis in adenomatous cells, because naringin did not significantly inhibit the proliferation of normal cells or induce adjacent normal tissue to apoptosis (data not shown).

In normal cells, GSK-3β phosphorylates β-catenin and therefore induces its ubiquitination and degradation by proteasome system (Schneikert et al. 2013). GSK-3β destabilizes β-catenin by phosphorylating it at Ser33, 37 and Thr41 (Boo et al. 2009). However, in intestinal adenomatous cells, because of *Apc* mutation, these phosphorylation sites are mutated and therefore fail to phosphorylate β-catenin, thereby leading to its accumulation in the cytoplasm. It then translocates to the nucleus and engages the Tcf/Lef transcription factor complex to activate the transcription of a large number of target genes, such as cyclinD1, PCNA, c-Myc and CRDBP (van Noort et al. 2002; Fujisawa et al. 2008; Tarapore et al. 2012). Intestinal adenomatous cells in *Apc$^{min/+}$* mice have low level of active non-phosphorylated GSK-3β and high level of phosphorylated GSK-3β (Kumar et al. 2012; Lee et al. 2012). Our results showed an increase in phosphorylated β-catenin at the site of Ser37. Because naringin increased the active non-phosphorylated GSK-3β and decreased its non-function phosphorylated form, we accordingly suggested that naringin might inhibit intestinal tumorigenesis through modulation of GSK-3β to block the accumulation of β-catenin in the nuclei of intestinal adenomatous cells.

Chronic inflammation contributes to amplifying and sustaining ongoing tumorigenesis in *Apc$^{min/+}$* mice. Increased inflammatory stress stimulates the growth of adenomatous cells (Pierre et al. 2015; Jamieson et al. 2012). Important inflammatory stimuli include cytokines COX-2, NF-κB, PGEs, IL-6, TNF-α, lipoxygenase (LOX) and lipopolysaccharide (LPS) (Liu et al. 2013). In the progression of tumorigenesis, NF-κB-COX-2 interaction might play a pivotal role in the stimulation of intestinal adenomatous polyps (Diab et al. 2015). NF-κB acts as 'first responder' to various cellular stresses, such as free radicals, proinflammatory cytokines and bacterial components or LPS, and thereafter intercellular communication takes place leading to tumorigenesis. COX-2 is an immediate-early growth response gene product (Greene et al. 2011). TNF-α induces COX-2 and cyclin D1 in adenomatous cells in which these molecules have the NF-κB binding site in their promoters. The activated NF-κB further induces COX-2 expression in response to cellular stresses (Liu et al. 2013). Accumulation of these cellular stresses further stimulates the APC/β-catenin pathway, thereby leading to the expansion of intestinal polyps.

Mountains of studies showed that high level of PGE$_2$, IL-6, IL-1β and TNF-α is associated with the expansion of intestinal polyps size (Khare et al. 2015). PGE$_2$ is a potential stimulator to accelerate the growth of adenomatous cells through activation of prostaglandin receptor EP2-mediated cellular events (O'Callaghan et al. 2008). In *Apc$^{Min/+}$* mice, high level of IL-6, TNF-α and PGE$_2$ was found to respond with rapid increase in intestinal polyps at earlier age (O'Callaghan et al. 2008; Khare et al. 2015). Further increase in these cytokines with age was found to associate with increased polyp size (O'Callaghan et al. 2008; Khare et al. 2015). These proinflammatory cytokines were also found to provoke the phosphorylated GSK3β and activate nuclear β-catenin, thereby stimulating the expansion of intestinal polyps (Klampfer 2011; Kumar et al. 2012). Naringin possessed the activity of reducing chronic inflammation factors IL-6 and PGE$_2$ both in the adenomatous polyps and in the plasma. We therefore suggested that the naringin's effect was also associated with its biological activity of anti-chronic inflammation.

Conclusion

Naringin possessed the activity of chemoprevention malignant progression of intestinal adenomatous polyps in *Apc$^{Min/+}$* mice. The underlying mechanisms of chemopreventive effects were associated with its multiple biological activities including anti-proliferation, induction of apoptosis and anti-inflammation. Naringin feeding did not produce considerable adverse effects to mice. Naringin is a potential chemopreventive agent for reducing the risk of colonic cancers.

Compliance with ethical standards

Conflict of interest We confirm that there is no potential conflict of interest or financial dependence regarding this paper.

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