



Chemopreventive effects of standardized ethanol extract from the aerial parts of *Artemisia princeps* Pampanini cv. Sajabal via NF- κ B inactivation on colitis-associated colon tumorigenesis in mice



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ABSTRACT

Chronic inflammation is an underlying risk factor of colon cancer, and NF- κ B plays a critical role in the development of inflammation-associated colon cancer in an AOM/DSS mouse model. The aim of this study was to determine whether the standardized ethanol extract obtained from the aerial parts of *Artemisia princeps* Pampanini cv. Sajabal (EAPP) is effective at preventing inflammation-associated colon cancer, and if so, to identify the signaling pathways involved. In the present study, protective efficacy of EAPP on tumor formation and the infiltrations of monocytes and macrophages in colons of an AOM/DSS mouse model were evaluated. It was found that colitis and tumor burdens showed statistically meaningful improvements after EAPP administration. Furthermore, these improvements were accompanied by a reduction in NF- κ B activity and in the levels of NF- κ B-dependent pro-survival proteins, that is, survivin, cFLIP, XIAP, and Bcl-2. *In vitro*, EAPP significantly reduced NF- κ B activation and the levels of IL-1 β and IL-8 mRNA and pro-survival proteins in HT-29 and HCT-116 colon cancer cells. Furthermore, EAPP caused caspase-dependent apoptosis. Based on these results, the authors suggest EAPP suppresses inflammatory responses and induces apoptosis partly via NF- κ B inactivation, and that EAPP could be useful for the prevention of colitis-associated tumorigenesis.

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1. Introduction

More than 1 million new cases of colorectal cancer (CRC) are diagnosed worldwide each year (Tenesa and Dunlop, 2009). CRC is the third most common malignancy and the fourth most common cause of cancer mortality worldwide despite important advances in detection, surgery, and chemotherapy (Jemal et al., 2008, 2009). The largest fraction of CRC cases has been linked to environmental causes, including chronic intestinal inflammation, which precedes tumor development. Colitis-associated colon cancer is a CRC subtype associated with inflammatory bowel disease (IBD) and is difficult to treat and has high mortality (Feagins et al., 2009). More than 20%

of IBD patients develop colitis-associated cancer within 30 years of disease onset, and more than half of patients die of colitis-associated cancer (Lakatos and Lakatos, 2008).

Nuclear factor-kappaB (NF- κ B) plays an integral role in inflammation-induced carcinogenesis. Numerous studies have demonstrated the pivotal role played by NF- κ B in tumor initiation and progression in ulcerative colitis-associated colon cancer. Aberrant NF- κ B activation was detected in >50% of colorectal and colitis-associated tumors, and mouse studies have established a role for NF- κ B in colitis-associated colon cancer development (Karin and Greten, 2005). Furthermore, a recent study demonstrated that NF- κ B inhibition reduces tumor incidence in inflammation-induced models of colon cancer (Greten et al., 2004). In addition, mutations in inhibitor of kappa B (I κ B) lead to increased NF- κ B activity and result in larger tumors in xenograft models (Kisseleva et al., 2006), and the colon carcinogenesis induced by azoxymethane (AOM)/dextran sodium sulfate (DSS) were found to be significantly decreased in I κ B kinase (IKK) knockout mice (Clevers, 2004).

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Therefore, NF- κ B has emerged as an attracting target for drug discovery and for the development of colon cancer therapies (Baud and Karin, 2009).

Under normal cellular conditions, NF- κ B binds to and is negatively regulated by I κ B in cytoplasm, but after an inflammatory stimulus, I κ B is phosphorylated and undergoes proteasomal degradation (Schetter et al., 2010). This allows activated NF- κ B to translocate to the nucleus and activate the transcriptions of target genes, including inflammation-related genes, such as, those of cytokines and chemokines, inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2 (Aggarwal et al., 2009). By increasing the expressions of several cell cycle genes, activated NF- κ B leads to increased cell proliferation, i.e. a pro-tumorigenic environment. NF- κ B activation also leads to other pro-tumorigenic changes, including the stimulation of angiogenesis via the activations of vascular endothelial growth factor (VEGF) and angiopoietin, and makes cells more resistant to necrosis and apoptosis via the direct and indirect regulations of a variety of genes, such as, c-Jun-N-terminal kinase (JNK), inhibitors of apoptosis proteins 1 and 2 (IAP-1 and -2), TNF receptor-associated factors 1 and 2 (TRAF-1 and -2), p53, and Bcl-2 family members (Schetter et al., 2010).

Artemisia princeps is cultivated in East Asia and its leaves are commonly used as teas, spices, and cooking ingredients. Traditionally, the decoction of this herb has been used as health food and for the treatment of inflammation, carbuncle, diarrhea, and circulatory disorders and a hepatoprotective and antibacterial agent in Korea, China, and Japan (Jung et al., 2007). *A. princeps* extract have been reported to have anti-tumor promotion effects (Seo et al., 2002) and anti-cancer effects against several cancers via the induction of apoptosis (Cho et al., 2011; Sarath et al., 2007). We previously found that standardized flavonoid-rich fraction of *A. princeps* Pampanini cv. Sajabal induces apoptosis via mitochondrial pathway in human cervical cancer HeLa cells (Ju et al., 2012) and *Artemisia* leaf extract also induces apoptosis in human endometrial cells through regulation of the p38 and NF- κ B pathways (Kim et al., 2013). Its flavonoid components, such as, eupatilin, jaceosidin, and eupafolin, induce G₂/M cell cycle arrest and apoptosis via the caspase- and mitochondrial-dependent pathway and the inactivation of NF- κ B pathways in human endometrial and cervical cancer cells (Cho et al., 2011; Chung et al., 2010; Lee et al., 2013). However, no report has been issued on the detailed *in vivo* and *in vitro* biological efficacies and involved the underlying molecular mechanism of EAPP in human colon cancer. For a comprehensive evaluation of the cancer chemopreventive/chemotherapeutic properties of EAPP, it is required that a standardized ethanol extract of *A. princeps* (EAPP) be tested in a cancer model before recommending its evaluation in humans. In the present study, we evaluated the ability of oral administration of EAPP to inhibit colitis-associated colorectal neoplasia in a DSS/AOM model and then followed this with an investigation of the molecular mechanism responsible for the effect of EAPP on apoptosis in colon cancer cells.

2. Materials and methods

2.1. Chemicals

RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. Dimethyl sulfoxide (DMSO), RNase A, phenylmethylsulfonylfluoride (PMSF), Triton X-100, and propidium iodide (PI), Nonidet P-40 (NP-40), protein inhibitor cocktail (PIC), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), camptothecin (CPT) acetylsalicylic acid (ASA) and hematoxylin and eosin (H&E) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies for anti-poly (ADP-ribose) polymerase-1 (PARP-1), anti-p65 (A), anti-survivin, anti-cFLIP, anti-Bcl-2, anti-Bcl-xL, anti-Mcl-1, anti-cIAP-1, anti-nucleolin, anti- α -tubulin and anti- β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for anti-p-p65 (S536) was purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies for anti-X-linked inhibitor of apoptosis

protein (XIAP, (48/hILP)) was purchased from BD Biosciences, Pharmingen (San Diego, CA, USA).

2.2. Preparation and chemical characterization of ethanol extracts from the aerial parts of *A. princeps* Pampanini cv. Sajabal (EAPP)

A. princeps Pampanini cv. Sajabal was obtained from the Gang Hwa Agricultural R&D Center (Incheon, Korea), and its identity was confirmed by Dr. Hae-Gon Chung (Gang Hwa Agricultural R&D Center). A voucher specimen (NPCL05063) has been deposited at the Laboratory of Natural Product Chemistry, Kyung Hee University (Suwon, Korea). EAPP was prepared as described previously (Park et al., 2008). Flavonoids, sesquiterpenoids and steroids in Supplementary Table S1 were previously isolated and identified from the aerial parts of *A. princeps* Pampanini cv. Sajabal (Bang et al., 2005, 2008a, 2008b).

2.3. Experimental animals and sample treatment

All animal care and experimental procedures complied with the Guidelines of the Committee for Animal Care and Use of Laboratory Animals, College of Pharmacy, Kyung Hee University according to an animal protocol (Approval number # KHP-2009-12-2). Male ICR mice weighing 28–30 g were purchased from Daehan Biolink (Eumseong, Korea). All mice were housed 4/cage and fed standard laboratory chow in the animal room with 12 h dark/light cycles and constant temperature (temperature: 20 $\pm$ 5 $^{\circ}$ C, humidity: 40–60%, light/dark cycle: 12 h) for 2 weeks or more. For experiment the animals were divided into five groups: Group 1 – control group (normal colon treated with vehicle solution: 5% EtOH and 5% Cremophor in saline); Group 2 – vehicle group (colitis-associated colon cancer treated with vehicle solution: 5% EtOH and 5% Cremophor in saline); Group 3 – positive control (colitis-associated colon cancer treated with ASA75 mg/kg/day; p.o.); Group 4 – positive control (colitis-associated colon cancer treated with CPT 1.0 mg/kg/day; i.p.); Group 5 – colitis-associated colon cancer treated with EAPP (25 mg/kg/day, p.o.). From day 0 of colitis-associated colon cancer induction, mice were treated three times a week after the cessation of DSS administration (Fig. 1A) with vehicle solution, ASA, CPT, or EAPP for experiment periods. CPT and ASA were used as a positive control.

2.4. Induction of colitis-associated colon cancer model

To induce colon cancer, mice were injected intraperitoneally with 12.5 mg/kg body weight of AOM dissolved in PBS. Seven days later, the mice were given a course of 2% DSS in drinking water for 7 days followed by drinking water for 14 days. Body weight was measured every week, and animals were sacrificed at 63 days. The entire mice colons were removed from the cecum to the anus and slit open longitudinally, and fixed flat between wet filter papers for 48 h in 10% neutral buffered formalin prior to 30 s staining with 0.2% methylene blue dissolved in the same formalin solution. All multiple nodular were counted as a tumor in each colon and calculated the group mean value. Tumor counts were performed in a blinded fashion. The experiments were conducted two times with ten mice in each group.

2.5. Monocytes and macrophages (MOMA) staining

The colons of AOM/DSS-treated mice were fixed in 4% paraformaldehyde, embedded in paraffin block and collected in 7 μ m sections on each glass. Monocytes and macrophages were stained with rat monoclonal MOMA-2 antibody (Biomedicals AG, Switzerland) in 2.5% normal goat serum, followed by HRP-coupled rabbit anti-rat IgG (Jackson ImmunoResearch Laboratories) in 2.5% normal goat serum as secondary antibody, and detected with Vectastain Elite ABC Kit (Vector Laboratories) and peroxidase substrate kit DAB (Vector Laboratories). The sections were counterstained with H&E (Sigma Chemical Co.).

2.6. Western blot analysis

Protein extracts were isolated from the tumors of the various AOM/DSS-treated mice and colon cancer cell lines using the protein lysis buffer (Intron, Seoul, Republic of Korea). Protein samples were mixed with an equal volume of 5X SDS sample buffer, boiled for 4 min, and then separated by 8–12% SDS-PAGE gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked in 5% non-fat dry milk for 1 h, rinsed, and incubated with specific antibodies in Tris-buffered saline (TBS) containing Tween-20 (0.1%) overnight at 4 $^{\circ}$ C. Primary antibody was removed by washing the membranes 3 times in TBS-T, and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:1000–2000). Following 3 times of washing in TBS-T, immuno-positive bands were visualized by ECL and exposed to X-ray film (Amersham, Piscataway, NJ, USA).

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