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Daikenchuto (TU-100) Suppresses Tumor Development in the Azoxymethane and APC$^{min/+}$ Mouse Models of Experimental Colon Cancer

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Abstract

Chemopreventative properties of traditional medicines, Kampo and underlying mechanisms of action have not been well explored. This study demonstrates that daikenchuto (TU-100), comprised of ginger, ginseng, and Japanese pepper (50/30/20 by weight), effectively suppresses development and progression of intestinal tumors in the azoxymethane (AOM) and the APC$^{min/+}$ mouse models. TU-100 was included in the diet. TU-100 was provided after first of 6, biweekly AOM injections, mice sacrificed at 30 weeks. APC$^{min/+}$ mice were fed without or with TU-100 starting at 6 weeks, sacrificed at 24 weeks. In both models, dietary TU-100 decreased tumor size. In the APC $^{min/+}$ model, the number of small intestinal tumors was also significantly decreased, whereas in the AOM model, both TU-100 and Japanese ginseng decreased tumor numbers. TU-100 and ginseng decreased Ki67 and β-catenin immunostaining and activation of numerous transduction pathways involved in tumor initiation and progression. EGF receptor expression and

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stimulation/phosphorylation *in vitro* was investigated in Caco2BBE cells. TU-100, ginger, and 6-gingerol, but not ginseng, ginsenoside Rb1 and the bacterial metabolite compound K, nor Japanese pepper suppressed EGF receptor induced Akt activation. TU-100 and ginseng and to a lesser extent ginger or 6-gingerol, but not Japanese pepper, ginsenoside Rb1 or compound K inhibited EGF activation of ERK1/2. In conclusion, TU-100 and some of its components and metabolites inhibit tumor progression in two mouse models of colon cancer by blocking downstream pathways of EGF receptor activation.

Keywords

Adenomatous Polyposis Coli; β-catenin; Kampo; ginseng; AOM; EGF; ErbB2 receptor; cell proliferation; apoptosis; cancer treatment; alternative and complementary medicine; cell signaling; ginseng

Introduction

Colorectal cancer is the third most commonly diagnosed cancer in the world causing over half million deaths every year (1, SEER Database, http://seer.cancer.gov). Survival rates have increased due to the development of diagnostic and treatment interventions, such as surgery or chemotherapy (2, 3), however the need for preventive measures still exists. A number of agents have been tested and several are implicated to have efficacy in colon cancer prevention including most notably aspirin and sulindac, but also vitamin D and calcium (4–6). A number of natural products have also been investigated including curcumin, ellagiaic acid, polyphenols, isoflavones, and ginsenosides (7–11).

Daikenchuto (TU-100) is a Japanese traditional herbal medicine, also called Kampo medicine, that has been reported to have prokinetic and anti-inflammatory effects in the gut. Clinically, it has been used for the prevention of post-operative ileus and for maintenance of general health (12–14). TU-100 is an aqueous extract of a mixture of processed ginger root (*Zingiber officiale*), ginseng radix root (*Panax ginseng*) and Japanese green pepper (*Zanthoxylum piperitum*), mixed by ratio of 5: 3: 2 by weight. These components contain diverse compounds such as gingerols and shogaols in ginger, ginsenosides and polysaccharides in ginseng, and sanshools in Japanese green pepper. TU-100 has been shown to have anti-inflammatory effects in both chemically-induced and immune cell transfer models of murine colitis (15–17).

The ability of TU-100 to block colon cancer development has not been tested, although one TU-100 component, ginseng, has been shown by a number of studies to block the development of colon cancer (18–21). The chemopreventative properties of ginseng appear to be mediated by both its anti-inflammatory and growth suppressive effects (18, 19, 21), which in large part requires the conversion of saponin ginsenoside compounds, notably Rb1, to the bioactive factor, compound K. The conversion is dependent on intestinal microbial glycosidase activity that removes sugars of the backbone ring structure, leading to compound K generation (22–24). Compound K is more readily absorbed and has direct effects in inducing apoptosis and autophagy in cancer cells (25, 26). Therefore the chemopreventive or anti-inflammatory effects of ginseng are enhanced in mice that have

abundant ginseng-hydrolyzing bacteria represented in the gut microbiota (27). TU-100 also possesses microbe-independent anti-inflammatory effects that have been demonstrated in the small intestine in a model of T-cell activation. These effects, however. are due to the ginger component of TU-100 (17). Ginseng metabolites formed by bacterial glycosidases and glucosidases are important in its anti-cancer activity. Ginseng triterpene dammarane ginsenoside compounds are deglycosylated, converting of ginsenoside Rb1 to compound K (28, 29). Compound K stimulates apoptosis in colon cancer epithelial cells, which is believed to contribute to tumor suppression (30).

In the present study, we demonstrate that dietary TU-100 blocks the development of colon cancer in two different experimental mouse models. Chemical induction of colonic adenomas and cancer by DNA mutagenesis with azoxymethane (AOM)(31, 32) is highly dependent on genetic background - the Balb/C and A/J mouse strains being the most sensitive to this agent when used alone (33). The APC $^{min/+}$ mouse that possesses a spontaneous germline mutation of the APC gene, similar to human familial adenomatous polyposis coli (34, 35), is a genetic model of intestinal cancer. Truncation of the APC protein leads to β-catenin dysregulation and promotion of neoplastic growth (36, 37). In the present study, we examine the potential chemopreventative effects of dietary TU-100 in these two models of intestinal cancer. The study also explores individual constituents and some of the metabolites of TU-100 as well as the mechanisms of action underlying their chemopreventative actions.

Materials and methods

Animals

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC 72101). Male Balb/c mice were purchased from Jackson Laboratories and allowed free access to water and food. Housing conditions for the mice included a temperature setting of 31°C, relative humidity of 55%, and 12:12 light:dark cycle. APC $^{min/+}$ mice (C57BL/6J-APC $^{min/+}$ /J) were originally purchased from Jackson Labs but were bred in house for these studies.

Reagents

TU-100 was provided by Tsumura & Co. (Ami, Ibaraki, Japan) in the form of powdered granules of an aqueous extract containing processed ginger, ginseng radix and Japanese green pepper combined in a ratio of 5:3:2 (w/w). The chemical composition of these materials were verified according to the standards of the Japanese Pharmacopoeia and Tsumura.

Induction of tumors

Mice were fed standard AIN-76A diet alone (Harlan, Madison WI, formulation number CA. 170481) or diet supplemented with 15 mg/kg TU-100 (Harlan, TD.110333). Azoxymethane (AOM, 10mg/kg)) was injected i.p. every 2 weeks for 6 injections to initiate tumors. Body weights were recorded weekly for the entire duration of treatment. Colons were opened

longitudinally and photos were taken. Tumor numbers and sizes were measured and ratios of tumor area to total colon area were calculated with using Image J.

Endoscopic procedures

Colonoscopy was performed on AOM-treated mice at 15 weeks to monitor tumor progression, using a miniature endoscope (30°, 1.9 mm, rigid) that had an examination sheath (9 French size), xenon light source, video monitor and camera with a capability to record video and still images (Karl Storz, Tuttlingen, Germany). Mice were not fasted and the procedure was conducted under anesthesia by isoflurane to minimize the pain. Colonic lavage was performed with distilled water using oral gavage needle (20 gauge) prior to sheath insertion.

Histological analysis

Tissues were fixed in 4% neutral buffered formalin overnight, washed with PBS and embedded in paraffin after dehydration with ethanol and xylene. Five μm sections were cut and stained with hematoxylin and eosin. For AOM- treated mice, Swiss rolls were prepared and sectioned as described (38). For APC $^{min/+}$ mice cross sections containing a tumor as well as adjacent normal appearing intestine region were taken.

Immunohistochemistry analysis

Immunohistochemistry of five μm sections was performed using antibodies to APC (1:200, Santa Cruz), p53 (1:500, Leica), β-catenin (1:200, Millipore) and Ki-67 (1:200, Thermo Scientific). Briefly, paraffin was removed with xylene after heating, sections were hydrated with graded ethanol and boiled in 10 mM citrate for 10 min, and blocked for peroxidase and proteins. For Ki-67 staining, sections were heated in 10mM Tris pH 9 for antigen retrieval. Each primary antibody was used in 0.1% Tween-TBS and incubated in 4°C overnight. Slides were washed with T-TBS and developed using the Dako Envision system. For both H&E and immunohistochemical staining, slides were scanned and imaged with Pannoramic View system (software version 1.15.1). For determination of nuclear staining, ImmunoRatio software was used (39).

Western blot analysis

Proteins collected from scraped colon mucosa were homogenized in protein lysis buffer with protease inhibitor. Ten to thirty μg of protein were used depending on the level of expression and quality of antibody. Standard procedures were used for Western blotting.

Studies of the expression, activation, and downstream effects of the EGF receptor

Caco2BBE cells were grown in high glucose DMEM with 10% fetal bovine serum, 2mM glutamine, and 0.1U/ml transferrin. For 24 hours prior to stimulation, cells were incubated in reduced serum (0.1% vol/vol) alone or in TU-100 (20μg/ml), ginseng extract (10μg/ml), ginger (6μg/ml), Japanese pepper extract (4μg/ml), ginsenoside Rb1 (30μM), compound K (10μM), or 6-gingerol (10μM). Cells were stimulated with 30ng/ml EGF and harvested at indicated times; 30 min for EGF receptor stimulation, 60 min for Akt phosphorylation and 120 min for ERK1/2 phosphorylation and proteins analyzed by Western blotting.

Statistical methods

Statistical differences for Western blot image analysis were calculated using GraphPad Prism Software using analysis of variance. For ImmunoRatio nuclear staining data, a Mann-Whitney nonparametric U test of Graph Pad Prism was used (http://153.1.200.58:8080/immunoratio; 39).

Results

Overall body weight change and tumor development in AOM treated mice

Male mice, 6 weeks of age and weighing 13–15 grams, were treated i.p. with AOM (10 mg/kg). One week after the first AOM injection, mice were placed on AIN76A diet without or with 1.5% (15 gm/kg diet) TU-100 or 0.45% (4.5 gm/kg diet) ginseng. These diets were started after the first AOM treatment to reduce potential effects of the dietary supplements to alter AOM metabolism and mutagenesis. Beginning two weeks after the first AOM treatment, mice received AOM every two weeks for an additional five injections (six total). Colonoscopy was performed on some mice at 15 weeks to assess tumor development. By this time small tumors were observed (Figure 1A). Mice were sacrificed at 30 weeks because some mice in each group were developing rectal prolapse and GI bleeding. Colons were cut longitudinally and opened flat on filter paper to count tumors and measure tumor size, the latter using digital calipers (shown in Figure 1B are two representative mice in each group). Colonic tumors, but not small intestinal tumors developed in AOM treated mice. The photos of Figure 1B are representative and were used for Image J analysis to assess tumor extent. Selected tumors in some of the colons are indicated by arrows. The average ratios of tumor to normal tissue in AIN-76A diet and AIN-76A with TU-100 diet fed mice were 23.7 ± 3.7% and 12.1 + 3.4% (average ± SEM, n=7 AIN and n=9 TU-100, * $p < 0.05$) (Figure 1D). The numbers of tumors were not statistically different among groups (totals from all sizes in Figure 1D), but the tumors were smaller in groups receiving dietary TU-100 or ginseng (Figure 1D).

Histological and immunohistochemical analysis of colon tumors

From the hematoxylin and eosin stained Swiss rolls (Figure 1E), tumors frequently appeared to arise near or over mucosal lymphoid aggregates. Tumors are indicated by arrows. Lymphoid aggregates were also noted in untreated (no AOM) Balb/c mice (images not shown). Epithelial cells over lymphoid aggregates also showed mild dysplasia characterized by hyperchromaticity and mild bleomorphism (changes in nuclear appearance).

At 30 weeks, tumors formed multi-layered glands with back-to-back glandular patterns. Many tumors contained necrosis within the tumor. The numbers of adenomas and carcinomas *in situ* were quantified from the Swiss rolls. For AIN76A diet alone, there were 7.2 ± 1.5 adenomas and 2.2 ± 0.6 carcinoma in situ, while for the TU-100 supplemented group (no ginseng group was included in this cohort), adenomas were 6.3±1.4 (not statistically different from AIN76A alone) and carcinoma in situ 0.9±0.5 (different from AIN76A alone $p < 0.05$). No metastases were found in liver or lung in any mice.

Tumor proliferation, assessed by Ki-67 staining was greater in AOM treated mice on AIN76A diet compared to tumors from those supplemented with TU-100 (Figure 2, lower panel). β-catenin and p53 immunostaining were greater and more extensive in tumors of mice that had been on unsupplmented diet (Figure 2). Levels of nuclear β-catenin, Ki-67, and p53 were quantified using ImmunoRatio software and are presented in the lower panel of Figure 2. Five adenomas each from Swiss rolls of three different mice stained for β-catenin, Ki-67, or p53 were selected and ImmunoRatio used to calculate DAB/total nuclear ratio. For β-catenin staining, AIN76A diet alone the ratio was 65.1 ± 7.4 and for diet with TU-100 47.6 ±8.7 ($p < 0.0001$ by Mann Whitney U test). For Ki-67 staining, the DAB/nuclear ratio of AIN76A 13.0 ± 2.4 and the diet including TU-100 8.2 ± 1.9 ($p < 0.0001$). For p52 AIN76A diet was 37.6 ± 15.0 and TU-100 containing diet 35.7 ± 12.4 (ns).

Western Blot analysis of colon tumors

To investigate potential pathways that might be targeted by TU-100 and its constituents, mRNA and protein expression of several tumor suppressors and proto-oncogenes along with number of key signal transduction components were examined. These measurements were performed on tumor tissue with a minimum of four tumors harvested from at least 4 individual mice and compared to those in colonic mucosa from control mice without AOM injection. Densitometric analysis of Western blots is presented in Table 1. Phospho-active EGFR and ErbB2 levels were significantly increased in tumors compared to tissues from control mice (Figure 3A). Dietary TU-100 and ginseng significantly decreased EGFR activation in the tissues, the densitometry values presented in Table 1. EGFR down-stream effectors pERK and pAkt were also significantly increased in AOM-induced tumors and these levels were reduced by dietary supplementation with TU-100 and ginseng (Figure 3B). In the AOM-induced tumors, there were increases in levels of several proto-oncogenes including cyclin D, Hes-1 (a Notch singaling regulated factor) and β-catenin and decreases in tumor suppressor E-cadherin (a marker of epithelial cell differentiation)(Figure 3C). Dietary TU-100 or ginseng partially blocked the tumor-associated changes in these proteins. Similarly, TU-100 reduced COX-2 (but not COX-1, data not shown) up-regulation in AOM-induced tumors (Figure 3C). The effects of ginseng on Cox-2 in AOM-induced tumors were more modest. No changes in apoptotic regulators bax, bad, or bcl-2 were noted (Figure 3D) despite induction of apoptosis within some tumors. On the other hand, both TU-100 and ginseng both increased apoptosis, as assessed by the activation of caspase 3 and polyADP ribose polymerase (PARP) using antibodies that recognized either intact and cleaved forms or only the cleaved, activated form (Figure 3D). Quantitative RT-qPCR analysis demonstrated changes in transcript abundance that paralleled protein changes for E-cadherin, COX-2 and Hes-1 in AOM-induced tumors and inhibitory effects of TU-100 and ginseng (Figure 3E).

Effects of TU-100 on APC $^{min/+}$ mouse intestinal adenoma development

Dietary supplementation with TU-100 in AIN-76A diet was initiated 6 weeks after birth and continued until sacrifice at 24 weeks. Colon tumors rarely developed in this model. In agreement with this, colonic tumors developed in only two mice - one on AIN-76A alone and one on the TU-100 diet. Small intestinal tumors were counted for each mouse and effects of dietary supplementation on size distribution analyzed (Figure 4A). Tumor

multiplicities were lower in the TU-100 group (determined at each size by Mann-Whitney U test) and of smaller size. At 24 weeks, only adenomas and no carcinomas were noted in mice. Adenomas were characterized by histology and protein and RNA extracted for target evaluation as described for AOM-induced tumors. Expression levels in tumors were compared to levels in adjacent normal-appearing intestinal mucosa.

Immunohistochemical analysis showed reduced nuclear β-catenin in adenomas from mice fed the TU-100 diet compared to adenomas from mice fed unsupplemented diet (Figure 4B). p53 staining was reduced to a lesser extent in tumors from unsupplemented AIN-76A diet-fed mice compared to adjacent mucosa. Dietary TU-100 had a modest effect in a direction to restore normal appearing p53 staining in both uninvolved mucosa and tumors (Fig 4). Ki-67 staining was limited to the base of the crypts in uninvolved areas but was detected throughout the tumors (Figure 4B). Compared to tumors from unsupplemented AIN-76A diet-fed mice, tumors from mice on diets supplemented with TU-100 demonstrated less Ki67 staining. ImmunoRatio analysis was used in $APC^{min/+}$ mice was performed for AOM-induced tumors and results are presented in the lower panel of Figure 4. For β-catenin % DAB/total nuclear ratio for AIN76A diet was 63.9 ± 8.5 compared to that for diet with TU-100 of 41.3 ± 5.8 ($p < 0.001$ by Mann Whitney U test). For Ki-67 AIN76A diet the % was 12.3 ± 12.5 compared to that for diet with TU-100 of 7.3 ± 12.0 ($p < 0.001$) and for p53 AIN76A diet the ratio was 40.8 ± 9.5 comparted to 32.3 ± 5.7 for TU-100 ($p=0.009$).

Tumors and non-involved nearby regions were analyzed for EGF singals and changes in proto-oncogenes, and apoptotic proteins by quantitative Western blotting (Figure 5A–D). Tumors demonstrated increased activation (phosphorylation) of EGFR and ErbB2 and activation of their downstream transduction pathways ERK1/2 and Akt. Tumors also demonstrated increased expression of Hes-1, PCNA, Myc, cyclin D, COX-2, VEGF and β-catenin and decreased expression of the epithelial marker E-cadherin. Tumors exhibited increased levels of apoptosis and activation of apoptosis, which were blocked by TU-100 to varying extents (densitometry presented in Table 2). Quantitative RT-qPCR analysis demonstrated tumore-related increases in transcript abundance that paralleled protein changes for E-cadherin, COX-2 and Hes-1 in APC tumors whereas TU-100 inhibited these effects (Figure 5E).

Effect of TU-100 on EGF receptor expression and activation and stimulation of Akt and ERK1/2 in human Caco2 colon cancer cells

To determine if TU-100 and its components had direct actions on human colon cancer cells, studies were performed on the human colonic adenocarcinoma cell line Caco2BBE. TU-100 and its components ginseng, ginger, and Japanese pepper extracts did not alter Caco2 cell EGF receptor expression (Figure 6). EGF receptor phosphorylation stimulated by EGF was also not decreased by TU-100 or its components (Figure 6). However, the activation (phosphorylation) states of the down-stream effectors Akt and ERK1/2 were decreased by TU-100. Akt activation was inhibited by the ginseng and component 6-gingerol, the latter being a major bioactive component of ginger. ERK1/2 activation was inhibited by TU-100 or ginseng extract and a lesser degree by the ginger extract and 6-gingerol.

Discussion

Despite significant progress in diagnosis and treatment of colon cancer (2,3) there remains a significant need for safe, effective, and well-tolerated measures to reduce the risk of this disease and its complications. Natural products have been used for centuries in many cultures and have served as the source of many anti-neoplastic compounds that are presently used. The current studies demonstrate that TU-100 or daikenchuto, blocks the development of colon cancer in both a chemically-induced model and a genetic model of colon cancer both of which mimic many gene regulatory changes present in sporadic human colon cancer.

Both the anti-inflammatory and anti-neoplastic effects of TU-100 could both be important in inhibiting colon cancer growth. Colon cancer induction by AOM involves a number of pivotal mediating steps including activating mutations of β-catenin and up-regulation of EGFR signals including activation down-stream effectors Akt and ERK1/2 activation (32, 40, 41, 42). Activation of the canonical Wnt pathway with increased nuclear β-catenin occurs in both the AOM and Min mouse model photocopying this major dysregulated pathway in sporadic human colon cancer (43). In the AOM model, β-catenin is frequently activated by gain-of-function mutations involving loss of negative regulatory sites in the amino terminus. This in turn induces Wnt signaling that mimics bi-allelic loss of tumor suppressor APC in humans (44). In the AOM model, tumors frequently develop over lymphoid aggregates that are believe to be a source of local inflammation. Cellular inflammation is also observed in sporadic human colon cancer as reflected by increases in NFκB signaling, and up-regulations of Cox-2, and iNOS (45, 46, 47).

TU-100 contains ginger and ginseng, both of which contain compounds that are anti-inflammatory (17–19, 48–50). Inflammation in mice fed diets supplemented with TU-100 showed reduced inflammation and developed smaller tumors. We therefore hypothesize that at least part the chemopreventive effects of TU-100 to block colon cancer growth are mediated by its anti-inflammatory effects. Additionally, ginseng and ginger have been shown to possess growth regulating compounds (18, 21, 30, 51, 52). TU-100 decreased expression of β-catenin, a central regulator of growth of colonic epithelial cells that plays a key role in early colon cancer development. Hes-1, a marker of Notch signaling was also elevated in AOM-induced tumors. Notch signals play an important role in colon cancer development (53). TU-100 blocked induction of Hes-1, indicating that the Notch pathway is also a target for TU-100.

The present studies also demonstrate that ginger compounds appear to inhibit EGF receptor activation of Akt while ginseng compounds are more active against ERK1/2 activation. TU-100 blocks EGF receptor activation in vivo in tumors, as assessed by activating phosphorylations in EGFr and ErbB2 in both AOM and APC $^{min/+}$ mouse models. Importantly using a cell culture system, TU-100 and the ginger and ginseng did not directly block EGF receptor activation/phosphorylation stimulated by EGF, but blocked subsequent Akt and ERK1/2 activation. This suggests actions mediated by paracrine signals from the stroma may be required for TU-100 to suppress activating phosphorylations in EGFR (54).

TU-100 decreased the number of tumors and more impressively limited the sizes of tumors in both models. This suggests that TU-100 targets growth promotion more than tumor initiation. These two events are mediated by different mechanisms. From a chemoprevention point of view, inhibition of tumor initiation and progression would both be beneficial. Since colon cancers are believed to require 15–20 years to develop, however, agents that inhibit promotion and progression theoreticaly have a wider window to interrupt the natural history of cancer development and could be used as an intervention even years after tumor initiation.

In summary, we have show that TU-100 inhibits tumor development in two independent models of colon cancer: the AOM carcinogen model and the APc mutant Min mouse model of hereditary colon cancer. TU-100 is a widely used natural product with well controlled and characterized composition. Furthermore, its pharmacology, including safety and pharmacokinetics is established. The long safety record of TU-100 use for post-operative ileus and constipation (12, 14, 55, 56) together with our preclinical findings of chemopreventive efficacy of TU-100, suggest that this is a potentially promising agent to test in a clinical trial to prevent colonic polyp recurrence.

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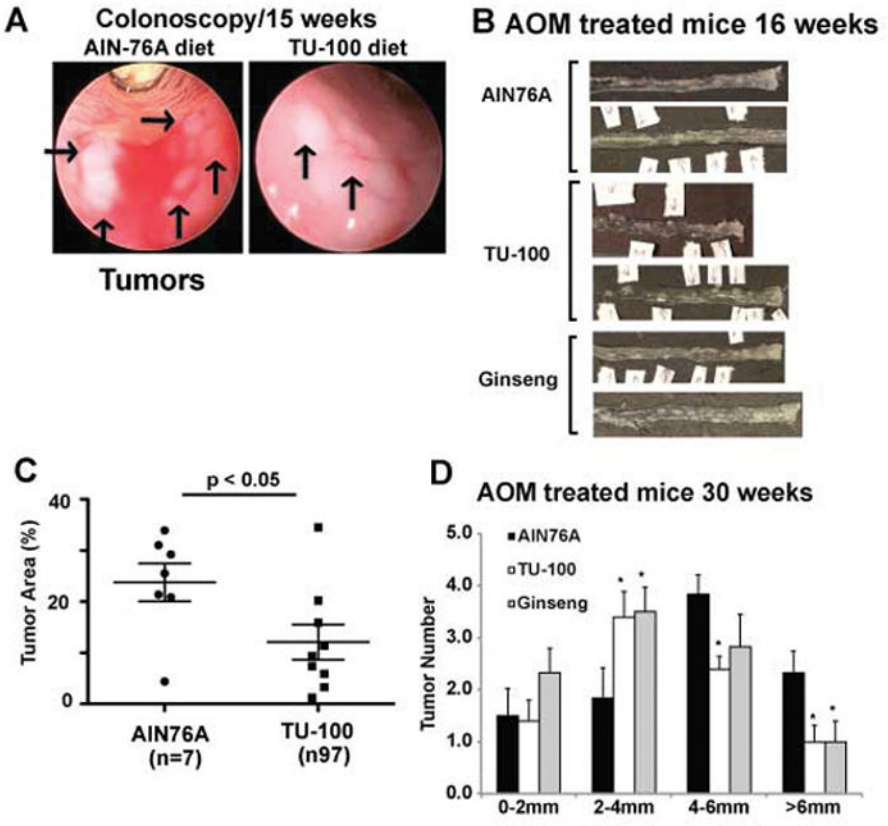

Figure 1.

Dietary TU-100 inhibits growth of AOM-induced colon tumors. AOM was given every two weeks for 6 injections and colonoscopy performed at 15 weeks after the first injection (A). Colonscopic images are representative of observations made in mice in each group at 15 weeks. Note there is more bleeding in mice given AIN76A alone. Mice were sacrificed at 15 and 30 weeks and tumor areas (designated by arrows) measured (B). Five mice per group were used for tumor size/area (C) and counts (D) at 15 weeks. Tumor multiplicity was similar in the AIN76A diet alone and AIN76A diet supplemented with TU-100, however, the tumors were smaller in mice fed TU-100 (D).

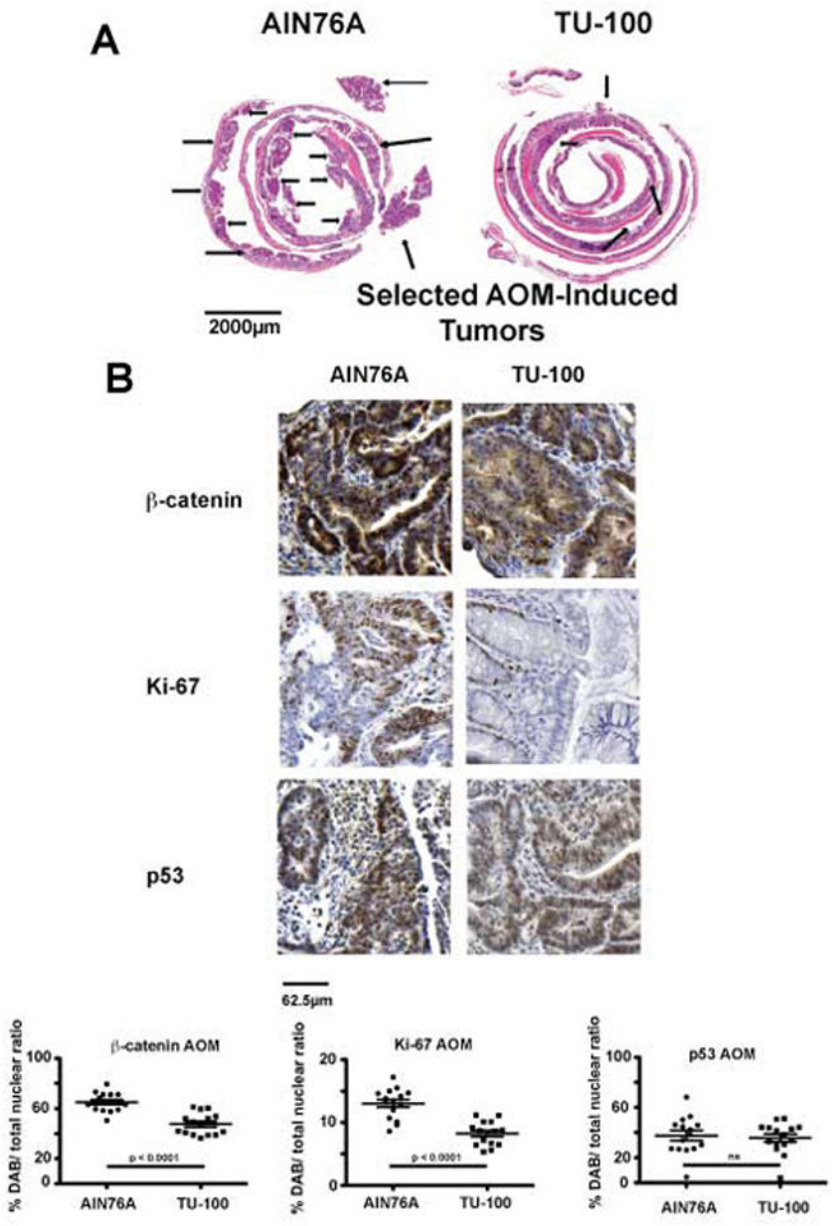

Figure 2.

Dietary TU-100 decreases Ki-67, β-catenin staining in AOM-induced tumors. AOM-induced tumors from mice on the AIN76A diet without and with TU-100 for 30 weeks were fixed in formalin and paraffin sections. Sections were stained first with hematoxylin and eosin (A) and then for Ki-67, β-catenin, or p53 and imaged as described in Methods using Pannoramic View software (B). Images presented are from 40X magnification and length bar of 62.5μm is presented. Images shown are representative of those of tumors from four different mice. ImmunoRatio analysis of nuclear staining is presented at the bottom from three mice, five adenomas in each mouse. Significance values indicated were calculated using a Mann-Whitney U test in Graph Pad Prism software.

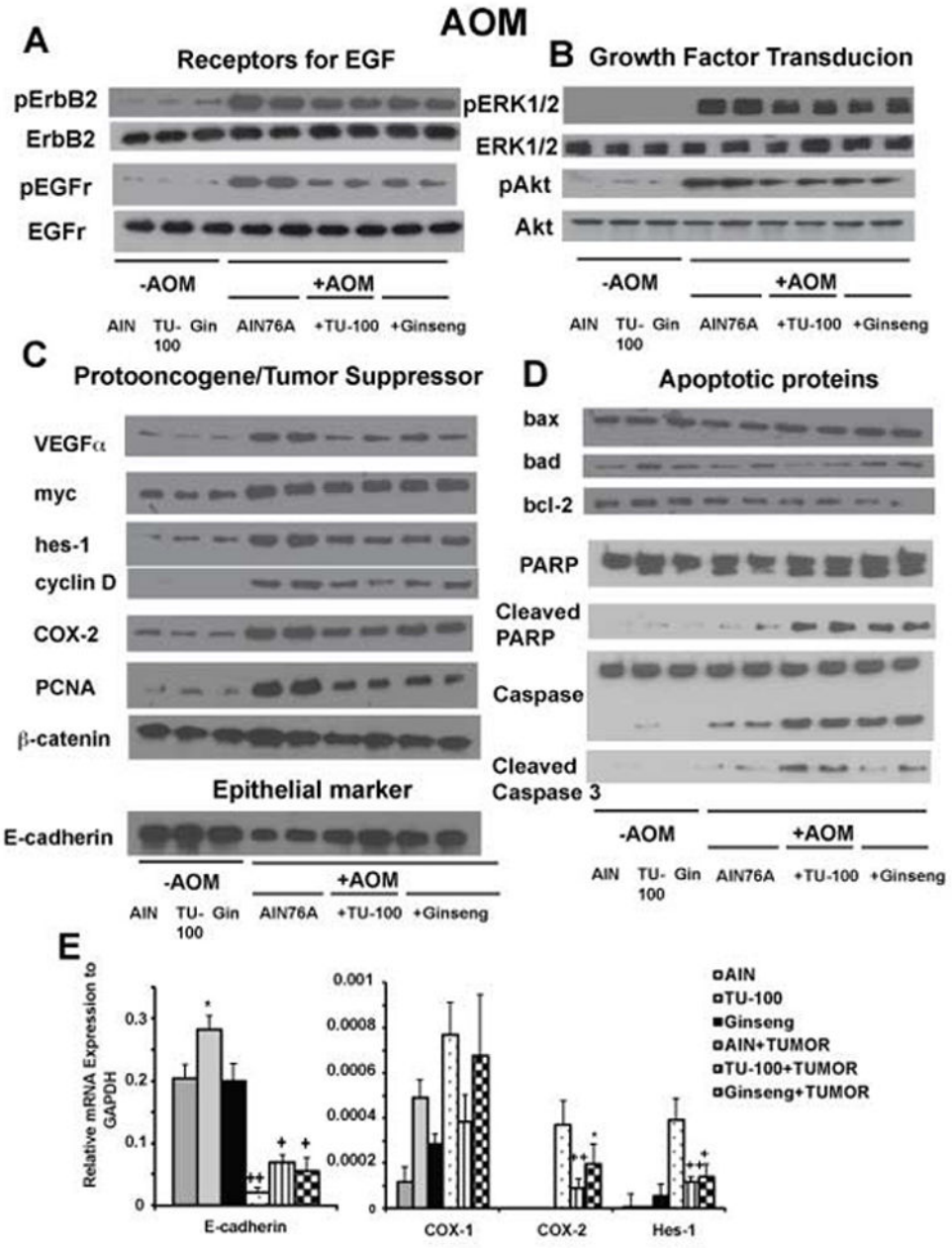

Figure 3.

Dietary TU-100 modulates AOM-induced epidermal growth factor receptor activation (A), growth factor signal transduction (B), and proto-oncogene or epithelial marker (C) and apoptosis induction (D). Protein were extracted from mucosal scrapings from mice fed AIN76A diet (AIN or AIN76A) or supplemented with TU-100 or ginseng (Gin or Ginseng) and where designated treated with AOM (+AOM) were analyzed by Western blotting for designated target protein. Western blotting densitometry was analyzed for the indicated proteins by Image J. One control (no AOM) for each dietary condition and two samples from AOM treated mice for each dietary group are shown. (E) RNA was analyzed by RT-qPCR for designated targets. Representative images are shown for analysis from four mice

on each diet without and with AOM. Densitometric values used Image J (Rasband NIH) and significance comparisons are presented in table 1. * $p < 0.05$ + $p < 0.01$ ++ $p < 0.001$ compared by analysis of variance.

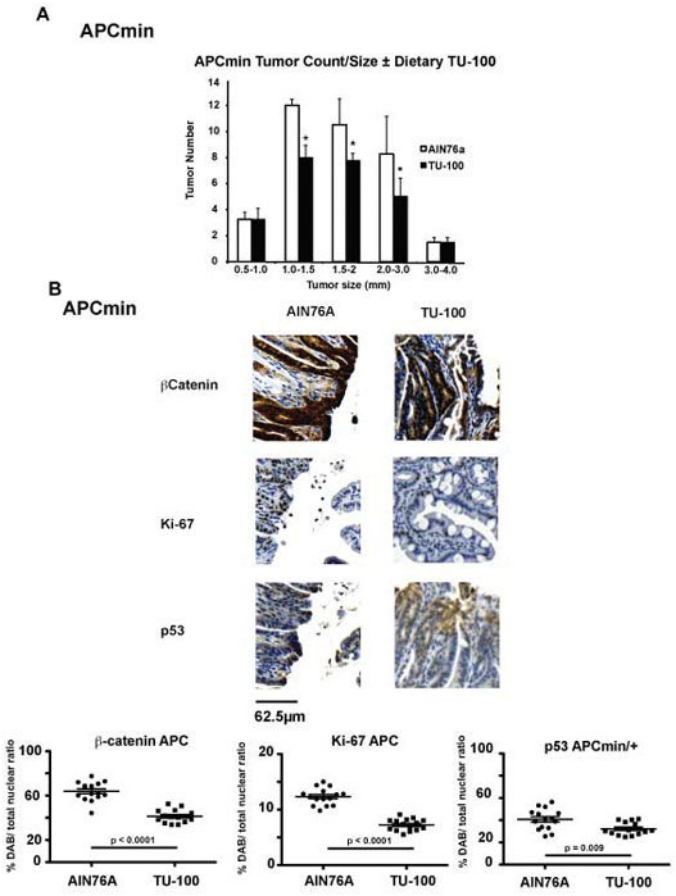

Figure 4.

Dietary TU-100 decreases tumor size and decreases Ki-67 and β-catenin expression in $APC^{min/+}$ mice. (A) Small intestinal tumors were counted and size recorded. Four $APC^{min/+}$ mice were in each diet group. * $p < 0.05$ + $p < 0.01$ compared by Mann-Whitney U test of Graph Pad Prism. (B) Tumors were fixed in formalin and paraffin sections stained for Ki-67, β-catenin, and APC. Images shown are representative of those of tumors from four different mice. ImmunoRatio analysis of nuclear staining is presented at the bottom from three mice, five adenomas in each mouse. Significance values indicated were calculated using a Mann-Whitney U test in Graph Pad Prism.

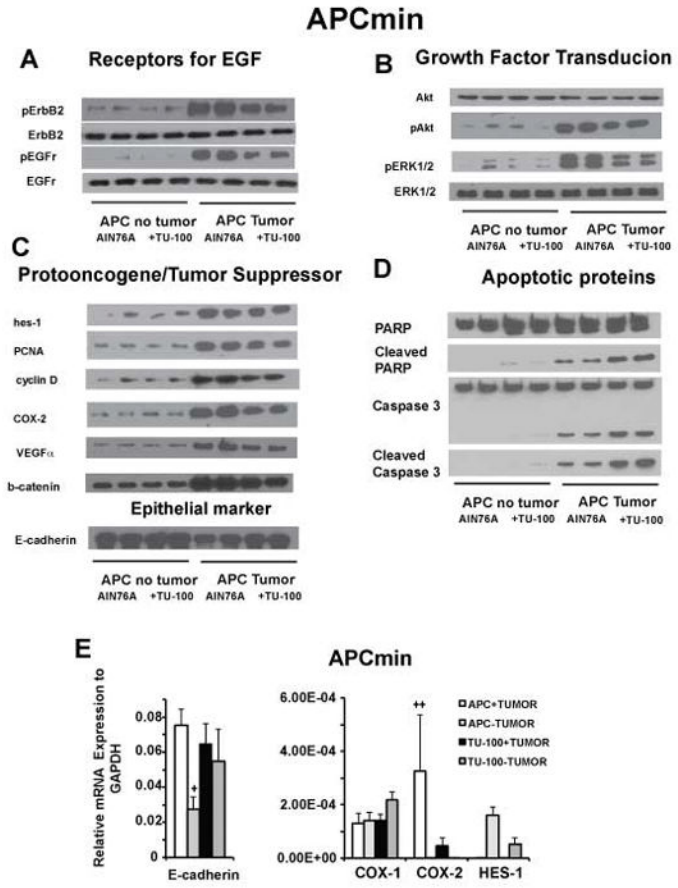

Figure 5.

Dietary TU-100 suppresses activation of growth stimulatory and proto-oncogene pathways in APC $^{min/+}$ mice. APC $^{min/+}$ mice were fed TU-100 for 25 weeks starting 5 weeks after birth and sacrificed at 30 weeks. For Western blot analysis, scrapings from mucosa with tumors and adjacent normal appearing mucosa were compared using two mice in each dietary group as shown. Images shown are representative of four mice in each group. Densitometric values using Image J (Rasband NIH) and significance comparisons are presented in table 2.

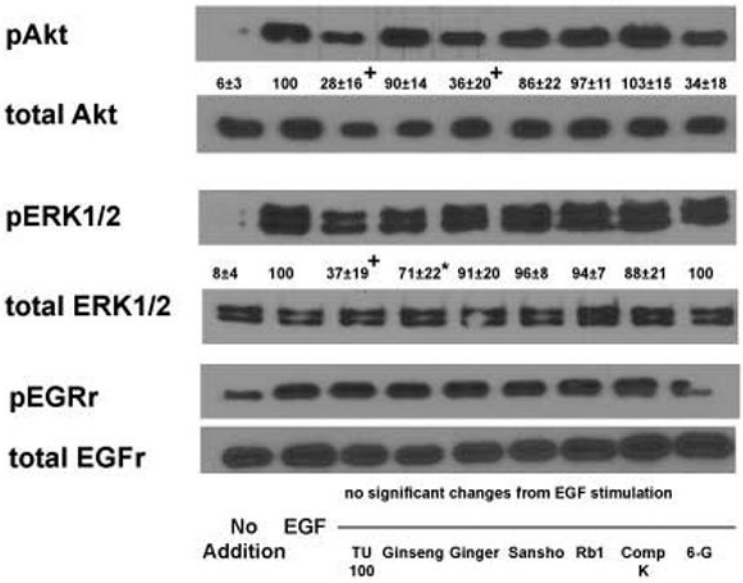

Figure 6.

TU-100 does not alter EGFR expression or activation, but blocks downstream effectors Akt and ERK1/2 activation in human colon cancer Caco2BBE cells that were serum-deprived and stimulated with EGF as described in Methods. Cells were pre-treated with TU-100, ginseng, ginger or Japanese pepper (sansho) extracts, ginsenoside Rb1(Rb1), the bacterial Rb1 metabolite compound K (Comp K), or 6-gingerol (6-G) as described in Methods. Cells were harvested at 30 min to assess EGFR phosphorylation, 60 min to assessd Akt and 120 min to assess ERK1/2 activation. Protein lysates were analyzed for the indicated proteins by Western blotting and images shown are representative of 4 separate experiments. Densitometric values using Image J (Rasband NIH). * $p < 0.05$ + $p < 0.01$ ++ $p < 0.001$ compared by analysis of variance.

Table 1

Densitometry AOM-induced colon cancer

Protein	AIN	TU-100	Ginseng	AOM/AIN	AOM/TU-100	AOM/Ginseng
pErbB2	100	112±14	129±23	1046±127	721±102 *	812±86 *
pEGFr	100	102±7	97±11	462±85	196±44 +	204±59 +
pAkt	100	10094±7	104±11	407±62	208±42 *	214±51 *
pERK1/2	100	100±4	95±7	511±89	320±62 *	275±105 *
VEGFa	100	102±6	109±12	406±89	188±62 +	209±75 *
Myc	100	102±11	108±11	379±52	261±45 *	270±68 *
Hes-1	100	125±35	128±26	510±83	213±41 +	234±59 +
cyclin D	100	100±12	106±14	409±46	181±66 ++	208±75 *
COX-2	100	97±16	92±14	344±55	149±31 ++	161±38 ++
PCNA	100	102±4	107±11	1355±204	296±144 ++	329±147 ++
β-catenin	100	109±14	95±18	179±16	129±18	127±21
E-cadherin	100	103±7	106±9	64±12	82±8 *	85±9 *
bax	100	105±10	106±14	110±17	98±19	97±21
bad	100	109±14	95±12	113±20	102±22	117±22
bcl-2	100	111±16	105±14	87±11	89±14	82±16
cleaved PARP	100	102±8	106±11	127±21	225±27 *	214±46 *
cleaved caspase 3	100	101±11	109±15	145±28	209±32 *	196±30 *

Densitometry was performed using Image J software, setting AIN diet to 100% for each protein. Values are means ± SEM for four mice in each group, one tumor from non-treated mice on each diet (4 tumors total), two tumors each from mice on each diet treated with AOM (8 tumors total).

* $p < 0.05$

+ $p < 0.01$

++ $p < 0.001$ compared with AIN/tumor by analysis of variance.

Table 2

Densitometry APCmin colon cancer

Protein	AIN/no tumor	TU-100/no tumor	AIN/tumor	TU-100/tumor
pErbB2	100	107±11	1209±141	594±85 $^{+}$
pEGFr	100	98±12	403±89	229±86 *
pAkt	100	95±11	774±201	416±115 *
pERK1/2	100	102±6	911±165	382±109 $^{+}$
Hes-1	100	103±15	360±49	284±27 *
PCNA	100	98±7	436±75	240±62 *
cyclin D	100	108±12	494±65	217±109 *
COX-2	100	113±16	814±201	311±115 $^{+}$
VEGFa	100	95±18	242±35	206±22ns
β-catenin	100	99±10	677±107	329±91 $^{++}$
E-cadherin	100	106±14	76±9	93±6 *
cleaved PARP	100	100±14	344±52	709±68 $^{+}$
cleaved caspase 3	100	100±7	306±59	916±115 $^{++}$

Densitometry was performed using Image J software, using adenomas from two different mice for none treated AIN without and with TU-100 setting AIN diet to 100% for each protein and averaging adenomas from two different mice for each diet but treated with AOM. Four mice were analyzed in each group, a total of 8 adenomas for each group.

* $p < 0.05$

$^{+}$ $p < 0.01$

$^{++}$ $p < 0.001$ compared with AIN/tumor by analysis of variance.