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Original Article

Isoflavone and its metabolite equol inhibit development of azoxymethane-induced colorectal tumors and modulate proliferation of colon carcinoma cells

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Abstract: Although previous studies have demonstrated the inhibiting ability of isoflavones on the development of colorectal cancer, the effects and mechanisms of its metabolite equol on colorectal cancer remain unclear. To determine the effects of equol on the development of colorectal tumors, *in vivo* and *in vitro*, the present study induced colorectal tumors in bilaterally ovariectomized female Sprague-Dawley rats as postmenopausal rat models using azoxymethane, culturing the human colon carcinoma cell lines SW480 and HCT-15. RT-PCR and Western blotting were performed to examine mRNA and protein expression of two estrogen receptors (ERα and ERβ) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Moreover, concentrations of thiobarbituric acid reactive substance, 8-hydroxyguanosine, and superoxide dismutase in rat blood serum were examined. Equol treatment reduced the incidence of colon tumors and inhibited the proliferation of both colon carcinoma cells, in a dose-dependent manner. RT-PCR and Western blotting results showed that equol treatment significantly upregulated expression of Nrf2 and ERβ, *in vivo* and *in vitro*. Moreover, genistein and equol treatment groups significantly reduced concentrations of thiobarbituric acid reactive substances and increased concentrations of superoxide dismutase. Present results suggest that equol may significantly inhibit the development of colorectal tumors by Nrf2-induced antioxidant activity and upregulating expression of ERβ.

Keywords: Isoflavone, equol, colorectal tumor, antioxidant activity, estrogenic activity

Introduction

Colorectal cancer is the third highest occurring cancer, worldwide, accounting for 1.4 million new cases and 694,000 deaths in 2012 [1]. In China, 174,841 new cases of colorectal cancer were diagnosed in 2010 and the crude incidence for colorectal cancer was 20.90 per 100,000 people for the same year, ranking sixth in all cancer sites [2]. To prevent and treat colorectal cancer, more and more researchers have vigorously attempted to identify active materials, such as phytochemicals, that can be effective in preventing tumorigenesis.

Soy isoflavones, a cluster of phytoestrogens, have been reported to inhibit tumor development, especially estrogen-dependent types, such as breast and prostate cancers [3, 4]. Previous research has proven that isoflavone/equol intake significantly inhibited the development of postmenopausal mammary tumors through antioxidant activity and changing estrogen receptor expression profiles [5]. Since estrogen receptors, especially estrogen receptor-β, have been discovered in non-estrogen target organs, such as the colon or lung tissues, more and more researchers have suggested that isoflavones may be effective for prevention of non-estrogen dependent tumorigenesis [6]. Recently, epidemiological and animal studies have also demonstrated that dietary soy isoflavone intake is associated with reduced colorectal cancer risk [7-10]. Raju et al. found that both pre- and post-natal exposure to dietary soy isoflavones can inhibit the growth of colon tumors in azoxymethane-(AOM)-induced male rats [7]. Moreover, some studies have reported that isoflavone intake is inversely associated with colorectal cancer risk in post-

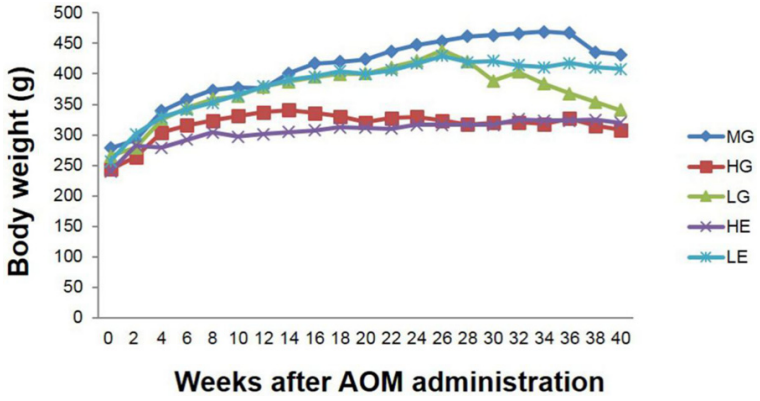

Figure 1. Per week body weights of rats in each group after AOM administration. MG, model group; HG, high genistein group; LG, low genistein group; HE, high equol group; LE, low equol group; AOM, azoxymethane.

menopausal women, but not in premenopausal women [9].

Despite the effectiveness of soy isoflavone as a chemo-preventive agent, it is the metabolite equol [7-hydroxy-3-(4-hydroxyphenyl)-chroman], instead of genistein, that becomes the major circulating isoflavone when soy foods are given [11]. Equol is a nonsteroidal estrogen and is a product of intestinal bacterial metabolism of dietary isoflavones [12]. Furthermore, equol possesses estrogenic activity and antioxidant effects like isoflavones. Previous studies have demonstrated that equol has antineoplastic effects on breast and prostate cancers [13, 14]. Choi et al. found that equol can induce anti-proliferation and apoptosis of human breast cancer MDA-MB-453 cells via cytochrome c and caspase-9 pathways [13]. However, few studies have investigated the antitumor effects of equol on colorectal cancer. Whether or not equol can explain the chemopreventive effects of soy on colorectal cancer remains unknown.

To determine the effects of equol on the development of colorectal tumors, *in vivo* and *in vitro*, the present study induced colorectal tumors in bilaterally ovariectomized female Sprague-Dawley rats as postmenopausal rat models using AOM, culturing the human colorectal adenocarcinoma-derived cell lines SW480 and HCT-15. RT-PCR and Western blotting were performed to examine mRNA and protein expression of two estrogen receptors (ERα and ERβ) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2), aiming to clarify underlying mechanisms. Moreover, this study examined the concentrations of thiobarbituric acid reactive substances (TBARS), 8-hydroxyguanosine (8-OHdG), and superoxide dismutase (SOD) in the blood serum.

Material and methods

Animals

A total of 75 female Sprague-Dawley rats of specific pathogen-free grades were obtained at 12 weeks of age from Peking University Laboratory Animal Centre (Beijing, China). Rats were housed in an air-conditioned room with an average temperature at 22°C, a relative humidity of 55 ± 10% and a light-dark cycle of 12 hours. Animal experimental procedures and care of laboratory animals followed the Guidelines for Animal Experiments of Peking University and this research was approved by the Peking University Biomedical Ethics Committee.

Study design

All rats were bilaterally ovariectomized under anesthesia to produce postmenopausal rat models after 2 weeks of acclimation to commercial powder chow, water, and the facility. Subsequently, all rats were randomly divided into five groups, with 15 rats in each group, via body weight four weeks after ovariectomy. These groups were given the corresponding diet: modified AIN-93G diet (model group, MG); modified AIN-93G diet with 100 mg/kg genistein (low genistein group, LG); modified AIN-93G diet with 400 mg/kg genistein (high genistein group, HG); modified AIN-93G diet with 100 mg/kg equol (low equol group, LE), and modified AIN-93G diet with 400 mg/kg equol (high equol group, HE). Additionally, all rats received a subcutaneous injection of AOM at 15 mg/kg body weight once a week for two successive weeks, as described in a previous study [15]. All rats were killed 40 weeks after azoxymethane administration. The modified AIN-93G diet, which contained 47.4% cornstarch, 25% flour, 10.5% bran, 9.1% cheese powder, 2.5% mineral mix, 2% bone meal, 2% corn oil, 1% fish meal, 0.5% barm, and 0.03% vitamin mix, was

Table 1. Colorectal tumor development of each group

Groups	Number of rats	Rats with tumors	Tumor incidence of rats (%)	Mean tumor number/rat	Mean size of the tumors (cm^3)
MG	15	12	80.0	3.5	3.85 ± 0.68
HG	15	3	20.0*	2.0*	3.27 ± 0.76
LG	15	5	33.3*	2.4*	3.32 ± 0.59
HE	15	7	46.7*	2.1*	3.49 ± 0.78
LE	15	9	60.0	3.2	3.39 ± 0.53

*P < 0.05 compared with MG. MG, model group; HG, high genistein group; LG, low genistein group; HE, high equol group; LE, low equol group.

obtained from The Chinese Academy of Preventive Medicine (Beijing, China). Soy oil was replaced by corn oil to minimize the amounts of extrinsic phytoestrogens in the modified AIN-93G diet. Genistein was obtained from North China Pharmaceutical Company (Beijing, China) and equol was obtained from Daicel Chiral Technologies (Shanghai, China).

Food intake and body weights were measured weekly. Complete autopsies were performed 40 weeks after AOM was subcutaneous injected. All organs were examined for gross abnormalities. Visible colorectal tumors were rapidly excised and weighed. Several colorectal tumor characteristics, such as number of rats with tumors, mean number of tumors in each rat, and size of tumor issues with calipers (Mitsutoyo CD-15 CP, Kanagama, Japan), were recorded. The two largest perpendicular diameters of each tumor were measured and the mean of these two diameters was used to estimate tumor size. In the histological examination, tumors with adjacent normal glands were fixed in 10% neutral buffered formalin and embedded in paraffin. Tumor tissues were cut at 2 μm and stained with hematoxylin-eosin to make histological diagnoses.

Cell lines and cell culture

Human colorectal adenocarcinoma-derived cell lines SW480 and HCT-15 cells (Cell Resource Center for Biomedical Research, Union Medical College, Beijing, China) were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, streptomycin sulfate (100 μg/mL), and penicillin (100 U/mL). Cells were cultured in a standard humidified incubator at 37°C in a 5% carbon dioxide atmosphere.

Cell proliferation assay

Cells were plated at a density of 2000 cells/well (HCT-15 cell) or at a density of 1500 cells/well (SW480 cells) in a 96-well plate. Cells were then incubated with controls and 4 different concentrations of equol (LC Laboratories, USA): 0.5, 1, 5, and 10 μmol/L. After incubating overnight, cells were labeled with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) to measure cell proliferation. Absorbance was measured with an OPTI max microplate reader (Molecular Devices, Sunnyvale, CA) at 570 nm and reference wavelength of 650 nm every 24 hours for 4 days.

RT-PCR

Total RNA was isolated from frozen rat colon tissue or HCT-15 cells treated by different equol concentrations for 8 hours using TRIzol (Invitrogen). Complementary DNA (cDNA) was generated using the TaqMan RT reagent kit (Applied Biosystems, Branchburg, NJ, USA). Specific PCR primers targeted for ERα, ERβ, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, as an internal control) were designed as follows: left primer 5'-GGTCCAATTCTGACAATCGAGC-3' and right primer 5'-TTTCGTATCCCGCCTTTCATC-3' for ERα; left primer 5'-AACACTTGCGAAGTCGGCAG-3' and right primer 5'-AACCTCAAAAGAGTCCTTGGTGTG-3' for ERβ; left primer 5'-CCACCACCATCTTCCAGGAG-3' and right primer 5'-CCTGCTTCACCACCTTCTTG-3' for GAPDH. Amplification was performed using HotStarTaq DNA polymerase kit (Qiagen, Tokyo, Japan). PCR conditions were as follows: (1) 95°C for 15 minutes; (2) 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute; (3) 72°C for 10 minutes; and (4) 4°C hold.

Western blotting

Rat colon tissues or HCT-15 cells treated by different equol concentrations for 8 hours were homogenized by using a polytron homogenizer in a radioimmunoprecipitation assay lysis buffer with proteinase inhibitors. Equal amounts of

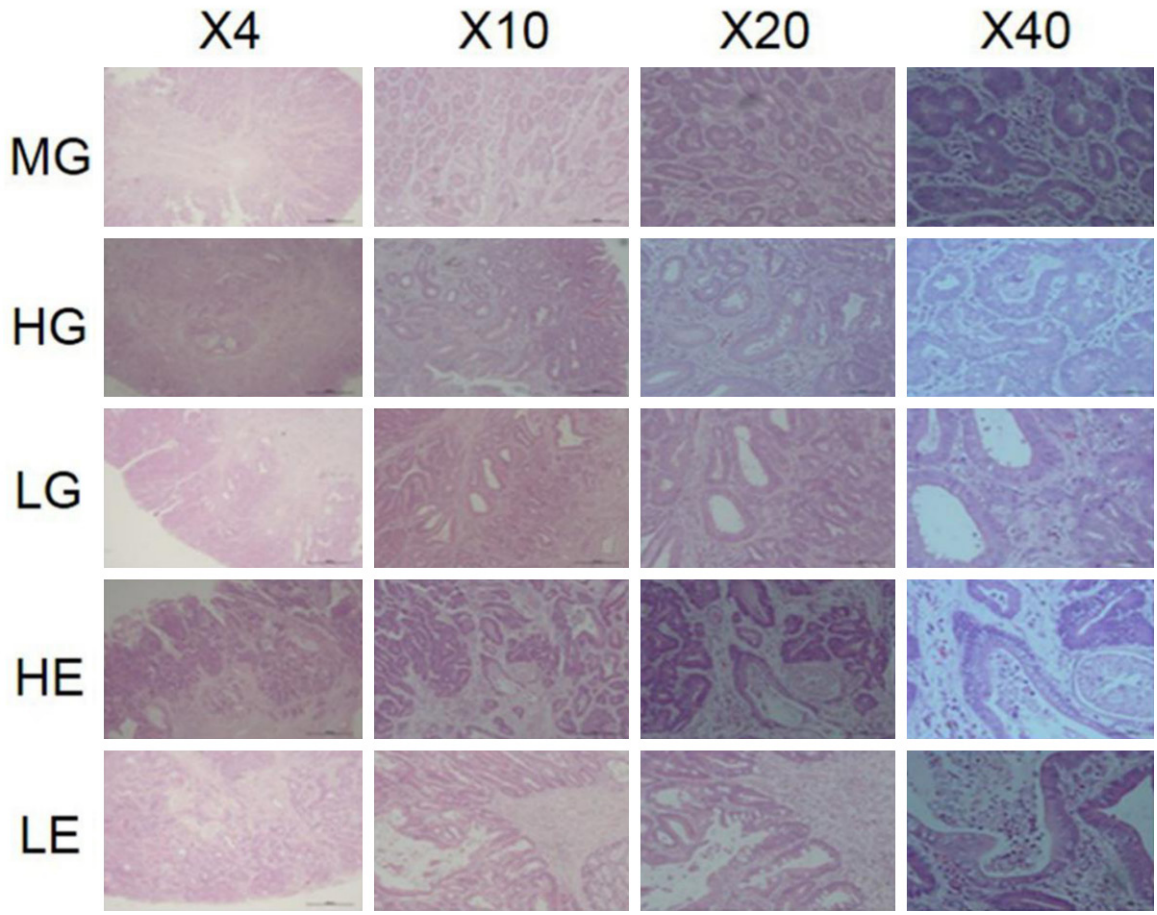

Figure 2. Histological findings of colorectal tumor tissues under hematoxylin-eosin staining. MG, model group; HG, high genistein group; LG, low genistein group; HE, high equol group; LE, low equol group.

protein (30 μg) solubilized in a sample buffer were separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred electrophoretically to polyvinylidene difluoride membranes. Membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 plus 5% nonfat dried milk for 1 hour at room temperature, then probed with primary antibodies at 4°C overnight. Primary antibodies of mouse anti-β actin monoclonal antibody, rabbit anti-ERα polyclonal antibody, and rabbit anti-ERβ polyclonal antibody were used at 1:200 dilutions (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Primary antibody of rabbit anti-Nrf2 polyclonal antibody was used at 1:1000 dilutions (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were washed 3 times for 10 minutes in Tris-buffered saline containing 0.05% Tween 20 and incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse-secondary antibody at 1:10000 dilutions (Rockland, Montgomery, PA, USA) for 1 hour at room temperature. Antibody complexes were visualized by using Odyssey Imaging System (Li-Cor Biosciences Company, Nebraska, USA).

Analysis of blood serum

Blood collected from the femoral arteries of the rats was transferred to two tubes with 5 mL in each. Blood was immediately centrifuged at a revolving speed of 4000 rpm for 15 minutes. The blood serum was stored at -20°C until analysis. Examination methods of TBARS, 8-OHdG, and SOD activity were described previously [5]. Briefly, TBARS was assayed using an assay kit (Nanjing Jiancheng Company, Nanjing, China) by the thiobarbituric acid method. 8-OHdG concentrations were measured using a commercial enzyme-linked immune sorbent assay kit (Da An Gene Company, Guangzhou, China). SOD

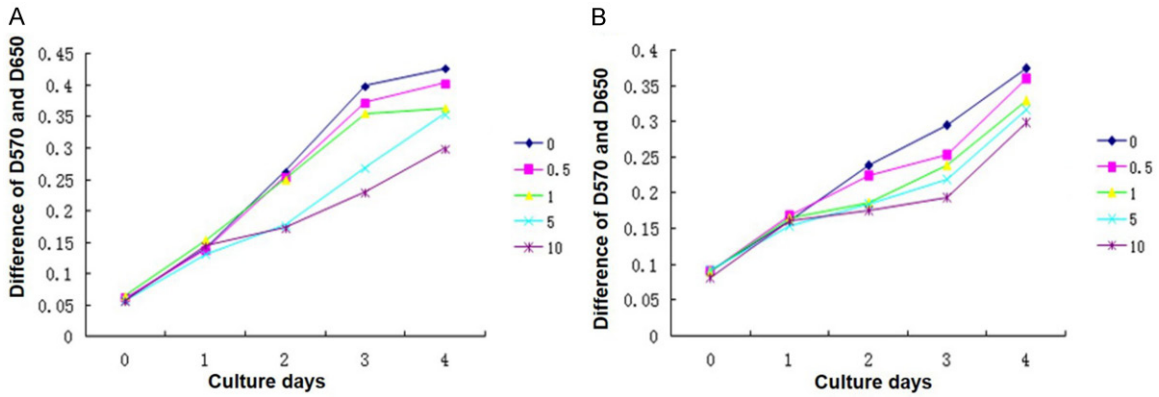

Figure 3. Equol impedes colon cancer cell proliferation (A, SW480; B, HCT-15) in a dose-dependent manner.

activity was measured immediately by the xanthine oxidase method using a colorimetric assay kit of SOD (Nanjing Jiancheng Company, Nanjing, China). Independent experiments were performed three times and the mean of the three measurements was used to estimate TBARS, 8-OHdG, and SOD concentrations in blood serum, respectively.

Statistical analysis

Fisher's exact probability test was used to compare tumor incidence in each group. Mean number of tumors in each rat, mean size of tumor tissue, and concentrations of TBARS, 8-OHdG, and SOD among different groups were analyzed using one-way ANOVA, followed by least significant difference multiple comparison tests. Data analyses were performed using SPSS ver. 20.0 for Windows (SPSS Inc., Beijing, China) and differences are considered significant at $P < 0.05$ (two tailed).

Results

Body weight and food intake of rats

All rats were killed at 40 weeks after AOM administration. Body weights differed significantly in each group during the experiment period (**Figure 1**). As shown in **Figure 1**, body weights of rats in MG were significantly higher than that of HE and HG. No significant differences were found in body weights between genistein and equol treatment groups. Moreover, no significant differences were observed concerning food intake among all groups (data not shown).

Equol and genistein significantly reduce incidence of colorectal tumors

At the end of the study, almost half of the rats (48%) developed colorectal tumors (**Table 1**). Multiple tumors, with varying sizes, were frequently found in one rat. Colorectal tumor incidence of rats in MG (80%) was significantly higher than that in HG (20%), LG (33.3%), and HE (46.7%). Moreover, the mean number of tumors per rat in MG was 3.5. This result was significantly larger than that in HG (2.0), LG (2.4), and HE (2.1). However, no significant differences were found in mean sizes of the tumors. Moreover, there were no significant differences in colorectal tumor incidence and the mean number of tumors per rat between genistein treatment groups and equol treatment groups. Two pathologists classified colorectal tumor tissues, independently, based on the histological findings after necropsy. Results showed that all tumors in different groups were adenocarcinomas (**Figure 2**).

Equol treatment impedes cell proliferation of colon cancer cells SW480 and HCT-15

To further substantiate the effects of equol on colon tumorigenesis, equol was used to treat colon cancer cells SW480 and HCT-15. Effects on cell proliferation were examined using the MTT cell proliferation assay (**Figure 3**). It was found that equol impedes colon carcinoma cell proliferation in a dose-dependent manner ($P < 0.01$). Cell proliferation of SW480 and HCT-15 cells was significantly lower at 4 days when drug concentrations exceeded 0.5 μmol/L.

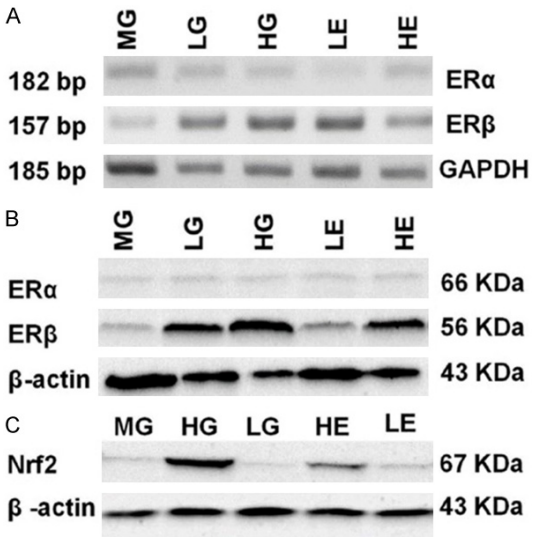

Figure 4. Expression of ER and Nrf2 in colon tissues. A. mRNA expression of ERα and ERβ in colon tissue in all groups; B. Protein expression of ERα and ERβ in colon tissue in all groups. C. Protein expression of Nrf2 in colon tissue in all groups. MG, model group; HG, high genistein group; LG, low genistein group; HE, high equol group; LE, low equol group; Nrf2, nuclear factor (erythroid-derived 2)-like 2.

Equol increases expression of ERβ not ERα in vivo and in vitro

To clarify underlying mechanisms, the present study examined mRNA and protein expression of two estrogen receptors (ERα and ERβ) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) in rat colon tissues of different groups or HCT-15 cells treated by different equol concentrations. As shown in **Figure 4**, both genistein (HG and LG) and equol (HE and LE) treatment groups significantly increased mRNA and protein expression of ERβ not ERα. Afterward, HCT-15 cells were treated by different equol concentrations for 8 hours. RT-PCR and Western blotting showed that equol treatments significantly increased ERβ, but not ERα, expression in a dose-dependent manner. HCT-15 cells treated with higher concentrations of equol (1, 5, and 10 μmol/L) presented significantly higher ERβ expression, compared with HCT-15 cells without equol treatment (**Figure 5**).

Equol increases expression of Nrf2 and reduces oxidative stress

To clarify the effects of isoflavone/equol antioxidant activity, this study examined the concentrations of TBARS, 8-OHdG, and SOD in blood serum of different rat groups (**Table 2**). Based on the least significant difference multiple comparison test results, TBARS concentrations of rats in MG were significantly higher than that of HG, LG, and HE. Moreover, SOD

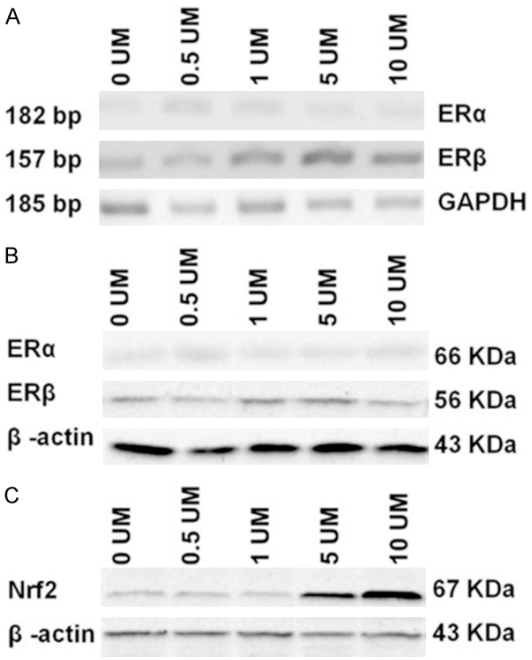

Figure 5. Expression of ER and Nrf2 in HCT-15 cells. A. mRNA expression of ERα and ERβ in HCT-15 cell treated by different equol concentrations; B. Protein expression of ERα and ERβ in HCT-15 cell treated by different equol concentrations. C. Protein expression of Nrf2 in HCT-15 cell treated by different equol concentrations. MG, model group; HG, high genistein group; LG, low genistein group; HE, high equol group; LE, low equol group; Nrf2, nuclear factor (erythroid-derived 2)-like 2.

Table 2. Concentrations of TBARS, 8-OHdG, and SOD in blood serum in different groups

Groups	TBARS (nmol/ml)	8-OHdG (ng/L)	SOD (U/ml)
MG	25.8 ± 5.21	5.34 ± 0.56	136 ± 5.08
HG	16.2 ± 3.45*	4.68 ± 0.42	153 ± 4.36*
LG	20.4 ± 3.34*	4.96 ± 0.46	148 ± 3.34*
HE	22.9 ± 6.17*	4.86 ± 0.54	157 ± 3.67*
LE	24.3 ± 2.14	5.04 ± 0.56	145 ± 2.87*

*$P < 0.05$ compared with MG. MG, model group; HG, high genistein group; LG, low genistein group; HE, high equol group; LE, low equol group; TBARS, thiobarbituric acid reactive substances; 8-OHdG, 8-hydroxyguanosine; SOD, superoxide dismutase.

concentrations of rats in MG were significantly lower than that of HG, LG, and HE. No significant differences were found in the concentrations of TBARS and SOD between genistein treatment groups and equol treatment groups. 8-OHdG concentrations of rats in MG were higher than those in other groups. However, the differences were not significant. Moreover, this study examined the expression of antioxidant gene Nrf2. Protein expression of Nrf2 in colon tissues were analyzed in all groups by Western blotting (**Figure 4**). It was found that HG and HE groups significantly increased protein expression of Nrf2. In addition, HCT-15 cells treated with higher concentrations of equol (5 and 10 μmol/L) presented significantly higher Nrf2 protein expression, compared with HCT-15 cells without equol treatment (**Figure 5**).

Discussion

Results of the present animal study showed that colorectal tumor incidence of rats and mean number of tumors per rat in genistein and equol treatment groups were significantly lower than those in MG. These results suggest that genistein and equol possess antineoplastic effects on the development of colorectal tumors in ovariectomized rats. In addition, results of MTT assay demonstrated that equol could impede cell proliferation of SW480 and HCT-15 cells in a dose-dependent manner. To the best of our knowledge, the present study demonstrated the inhibiting effects of equol on the development of postmenopausal colorectal tumors in rats for the first time.

Epidemiological studies have shown an inverse association between soy food intake and colorectal cancer risks [9, 10]. Yu et al. performed a meta-analysis of 13 case-control and 4 prospective cohort studies, finding that soy isoflavone consumption can reduce colorectal cancer risks by 22% [16]. Moreover, some experimental studies have also reported the anti-tumor activity of soy isoflavones. Zhang et al. found that isoflavones could inhibit cell growth and facilitate apoptosis and cell cycle arrest in the G2/M phase via ATM/p53-dependent pathways in HCT-116 and SW480 cells [17]. Zhang et al. reported that genistein could prevent the development of early colon neoplasia by suppressing WNT/β-catenin signaling pathways [18]. Consistent with previous studies, present results demonstrated the inhibiting effects of equol on colorectal tumors *in vitro* and *in vivo*.

Although the mechanisms on how soy isoflavones alter colorectal tumorigenesis remain unclear, several hypotheses have been presented to interpret the inverse association between soy isoflavone intake and decreased colorectal cancer risks. Swiatkowska et al. found that oxidative stress and increased production of reactive oxygen species may act as a signal transduction messenger in various processes of tumorigenesis [19]. Oxidative damage is common in tumors and soy isoflavones possess antioxidant activity. Erba et al. reported that plasma isoflavones can increase membrane fluidity and O-β-N-acetyl-D-glucosaminidase activity, reducing DNA oxidative damage [20]. Kawakami et al. found that isoflavone supplementation can reduce oxidative stress by activating the hepatic cholesterol 7α-hydroxylase in cholesterol oxidation products induced rats [21]. These findings indicate that the health effects ascribed to soy consumption may be partially related to the antioxidant potential of isoflavones. Considering the importance of lipid peroxidation in the expression of reactive oxygen species induced by oxidative stress, this study examined TBARS, 8-OHdG, and SOD to access oxidative stress. Results showed that blood serums of rats in genistein and equol treatment groups were lower in TBARS concentrations and higher in SOD concentrations than those in MG. These results suggest that genistein and equol treatment groups expressed significantly lower oxidative stress than MG. Moreover, this study assessed the protein expression of Nrf2 in rat colon tissues and HCT-15 cells. Investigators found that Nrf2 plays an essential role in fighting oxidative stress in the liver and lungs, as well as macrophages and neurons via regulating the inducible expression of detoxifying or antioxidant enzymes [22-25]. In this study, both genistein and equol treatment groups significantly increased the protein expression of Nrf2 in rat colon tissues, in agreement with the concentrations of SOD and TBARS. Furthermore, HCT-15 cells treated with equol (5 and 10 μmol/L) presented significantly higher Nrf2 protein expression than that of HCT-15 cells without equol treatment. Results indicate that genistein or equol may inhibit the development of colorectal tumors via Nrf2-induced antioxidant activity.

Isoflavones in soybeans possess estrogenic activity and may function as a dietary estrogen by binding unoccupied ERs under low circulating endogenous estrogen conditions [26]. Rat, mouse, and human ERs exist mainly in two subtypes, ERα and ERβ. ERα mediates the proliferative activities of estrogens, while ERβ may inhibit cellular proliferation by antagonizing the activities of ERα [27]. Some studies have reported the crucial roles of ERβ in the progression of colon cancer. Rudolph et al. carried out a population-based case-control study, in which 1,262 colorectal cancer patients were recruited between 2003 and 2007. They found that lack of ERβ expression was associated with bad prognosis and poor survival [28]. Edvardsson et al. conducted genome-wide expression studies in combination with gene-pathway analyses and cross-correlation to ERβ-chromatin-binding sites in three colorectal cancer cell lines (SW480, HT29, and HCT-116). They found that ERβ expression was associated with tumorigenesis, including apoptosis, cell differentiation, and cell cycle, in all three cell lines [29]. In the present study, animal experiment results showed that both genistein and equol treatment groups significantly increased mRNA and protein expression of ERβ. Moreover, cell experiment results showed that equol significantly increased ERβ expression in a dose-dependent manner. Present results are in line with the inhibiting effects of equol on colorectal tumors *in vitro* and *in vivo*, suggesting that equol may inhibit colon cancer via upregulating expression of ERβ.

Conclusion

The present established postmenopausal colorectal tumor models successfully. Results suggest that genistein or equol may significantly inhibit the development of postmenopausal colorectal tumors by Nrf2-induced antioxidant activity and upregulating expression of ERβ.

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Disclosure of conflict of interest

None.

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