Genistein, a soya isoflavone, prevents azoxymethane-induced up-regulation of WNT/β-catenin signalling and reduces colon pre-neoplasia in rats

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Abstract

The present study aimed to explore the role(s) of the soya isoflavone genistein (GEN) in preventing the development of colon pre-neoplasia, using Wingless/int (WNT)/β-catenin as a molecular marker of colon abnormality. Specifically, the effects on the WNT/β-catenin signalling pathway from GEN were examined by using an azoxymethane (AOM)-induced rat colon cancer model. Male Sprague–Dawley rats were fed a control (CTL), a soya protein isolate (SPI) or a GEN diet from gestation to 13 weeks of age. The first sampling was conducted at 7 weeks of age for pre-AOM analysis. The remaining rats were injected with AOM at 7 weeks of age. The descending colon was collected 6 weeks later for the evaluation of aberrant crypt foci (ACF), gene expression and nuclear protein accumulation. AOM injection induced aberrant nuclear accumulation of β-catenin in the CTL group but not in the SPI or GEN group. Moreover, the WNT target genes *Cyclin D1* and *c-Myc* were repressed by SPI and GEN. Meanwhile, SPI and GEN suppressed the expression of WNT signalling genes including *Wnt5a*, *Sfrp1*, *Sfrp2* and *Sfrp5* to the similar level to that of the pre-AOM period. Rats fed SPI and GEN had a decreased number of total aberrant crypts. GEN feeding also resulted in a reduced number of ACF with $N = 3$ per foci. The reduction of WNT/β-catenin signalling was correlated with the decrease in total aberrant crypts. By testing WNT/β-catenin signalling as a biomarker of colon carcinogenic potential, we showed the novel role of GEN as a suppressor of carcinogen-induced WNT/β-catenin signalling in preventing the development of early colon neoplasia.

Key words: Soya genistein: WNT signalling: Aberrant crypt foci: Colon cancer

Evidence from epidemiological studies has demonstrated that the intake of soya products reduces colon cancer risk[1–4], and this protective effect has been shown to be partially derived from genistein (GEN), a soya isoflavone[5]. Aberrant crypt foci (ACF), which are pre-neoplastic lesions in the colon, can be induced by carcinogens such as azoxymethane (AOM) in rat models[6,7]. To better understand the chemopreventive potential of dietary compounds, AOM-induced ACF development in rats was utilised to model colon carcinogenesis[8,9]. By analysing AOM-induced ACF in rats or mice, soya products containing isoflavones have been shown to reduce precancerous colon lesions[10,11]. Soya protein isolate (SPI) in the diet also reduced colon tumour incidence in male Sprague–Dawley rats[12]. Therefore, in the present study, we used a SPI diet containing GEN as a positive control for the GEN diet and investigated the effect of GEN on early stages of colon cancer development in an AOM-induced rat model.

Timing and exposure duration are critical for the effective prevention of colon cancer development by GEN. GEN diets (75 or 150 mg/kg) fed to F344 rats pre- and post-AOM induction contributed to decreased ACF[13]. In a more recent study, lifelong feeding (from gestation to 26 weeks of age) of a soya diet containing 182 mg/kg of GEN reduced AOM-induced colon tumour burden and size[14]. However, a soya diet containing 1200 mg/kg of GEN fed to 1, 11 and 22 months old female F344 rats 1 week before AOM injection showed no protection against the formation of ACF in all age groups[15]. Therefore, we used the rat model of lifelong GEN exposure, including the maternal period, with a moderate level of dietary GEN to investigate the protective role of GEN in colon cancer development.

Colon cancer is one of the most prevalent malignancies worldwide[16,17]. Increasing evidence has shown that Wingless/int (WNT)/β-catenin signalling is closely involved in normal colon development, as well as in the carcinogenic process[18–21]. Briefly, WNT signalling is initiated when a WNT protein, such as WNT1, binds to its membrane receptor[22]. The WNT signal is passed to β-catenin through a set of cytoplasmic components, including glycogen synthase kinase 3β (GSK3β), Axin1 and adenomatous polyposis coli (APC).

By forming a complex with T-cell factor/lymphoid enhancer factor (TCF/LEF) in the nucleus, β-catenin then triggers the expression of WNT target genes, such as *Cyclin D1* and *c-Myc*, which are critical for the proliferation of normal intestinal cells[23]. Once WNT signalling is disturbed, the intestinal cells may lose control of proliferation, leading to a greater chance of tumorigenesis[20], and blockage of WNT1 has been proposed to promote apoptosis in colon cancer cells[24]. WNT/β-catenin signalling promotes cell-cycle progression by activating its downstream genes *Cyclin D1* and *c-Myc*[22]. AOM induction enhances the level of β-catenin in the colon[9,25], and an elevated level of nuclear β-catenin has been observed in most cases of colorectal cancer[18,26]. Thus, we used nuclear β-catenin abundance as an indicator of colon abnormality. WNT/β-catenin signalling is regulated by several WNT antagonists, including the secreted frizzled-related proteins Sfrp1, Sfrp2 and Sfrp5[27]. Wnt5a has also been reported to be an inhibitor of WNT/β-catenin signalling[28], and the inactivation of WNT antagonists along with aberrant WNT signalling was observed in colon cancer[29].

In the present study, we focused on determining the effect of GEN on preventing the development of colon pre-neoplasia. In particular, we aimed to discover the mode of action of GEN on modulating the WNT/β-catenin signalling pathway both before and after exposure to a carcinogen. The present findings provided the first clue that lifelong exposure to GEN reduced the frequency and severity of pre-neoplastic lesions in the colon by suppressing carcinogen-induced up-regulation of WNT/β-catenin signalling. Therefore, dietary GEN may provide promising chemopreventive properties against the development of colon cancer. Furthermore, by using this model, we discovered that the expression of marker genes in the WNT/β-catenin signalling pathway was closely correlated with colon neoplasia and could serve as a tool for the early detection of colon abnormality.

Experimental methods

Animals and diets

Timed-pregnant Sprague–Dawley rats (Charles River Laboratories) were obtained on day 2 of gestation and fed one of three isoenergetic diets throughout the 3-week gestation and 4-week lactation periods (see Fig. S1 of the supplementary material, available online at http://www.journals.cambridge.org/bjn). The diets were modified according to the AIN-93G diet formula[30] with maize oil as the fat source (see Table S1 of the supplementary material, available online). The diets were (1) control (CTL, casein protein), (2) SPI (NutroBio.com) and (3) GEN (CTL plus 140 mg/kg of GEN). The diets were balanced and matched for content of protein, carbohydrate, fat, amino acids, vitamins and minerals. The amount of GEN used in the SPI diet was analysed using HPLC and found to be 0·705 (SEM 0·025) mg/g (total aglycone equivalents; see Andrade *et al.*[31] for details of HPLC analysis). After formulating the SPI diet using the SPI as the only protein source at 20 % (w/w) level, the final GEN level in the SPI diet was confirmed by HPLC (131 (SEM 17) mg/g). When making the GEN diet, GEN content in the GEN diet was matched to the GEN level

in the SPI diet (aglycone equivalents). GEN was purified from soya, characterised and kindly provided by Dr William Helferich at the University of Illinois. All groups of rats had free access to food and water and were kept individually in standard polycarbonate cages in a humidity- and temperature-controlled room. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. At day 24, the male pups from each group were weaned and maintained on the same diet as their mother until they were killed at various time points. The first sampling occurred when the offspring were 7 weeks old (CTL, *n* 6, SPI and GEN, *n* 8) and the samples were used to examine the pre-induction gene profile. The remaining rats were injected with AOM (15 mg/kg body weight) at 7 and 8 weeks of age. At 6 weeks later, the AOM-induced rats were killed (*n* 8 animals per treatment group) by CO_2 asphyxiation and colon tissues were collected for all subsequent analyses.

Colon tissue sampling

At 13 weeks of age, all rats were killed by CO_2 asphyxiation. Descending colons were carefully removed, slit open longitudinally and washed twice with ice-cold PBS. The first one-third of the descending colon section was cut off and then stapled onto transparent film, and then subjected to 10 % formaldehyde fixation for 24 h followed by 10 % ethanol fixation. After staining by 0·1 % methylene blue, samples were subjected to ACF counting as described below. The epithelial cells were scraped from the remaining two-thirds of the section and immediately frozen in liquid N_2 and stored at −80°C for further gene and protein analysis.

Aberrant crypt foci identification and quantification

The frequency and multiplicity of ACF were determined under a dissection microscope. The identification criteria for ACF were as follows: two to three times larger than normal crypts; microscopically elevated; had slit-like openings; had thicker epithelial linings, which were stained darker than normal crypts; and had larger pericryptal zones[32]. The total number of aberrant crypts in the sampled descending colons was counted by a single-blind method, and then the average number of aberrant crypts of the colon, with no discrimination on the multiplicity, was determined as the mean of total aberrant crypts/length of section (frequency per cm). The crypt multiplicity was described as $N = 1$, $N = 2$ and $N = 3$ or above for the number of crypt(s) per foci (for examples, see Fig. 1(A)). The mean of ACF/cm with different multiplicity was determined accordingly.

Histology and immunofluorescent staining

Fixed colon samples were embedded in paraffin before sectioning. All sections were then stained with haematoxylin and eosin for histological analysis. Slides were deparaffinised and blocked with IT signal FX (Invitrogen), and then incubated with either anti-β-catenin (1:250; Cell Signaling
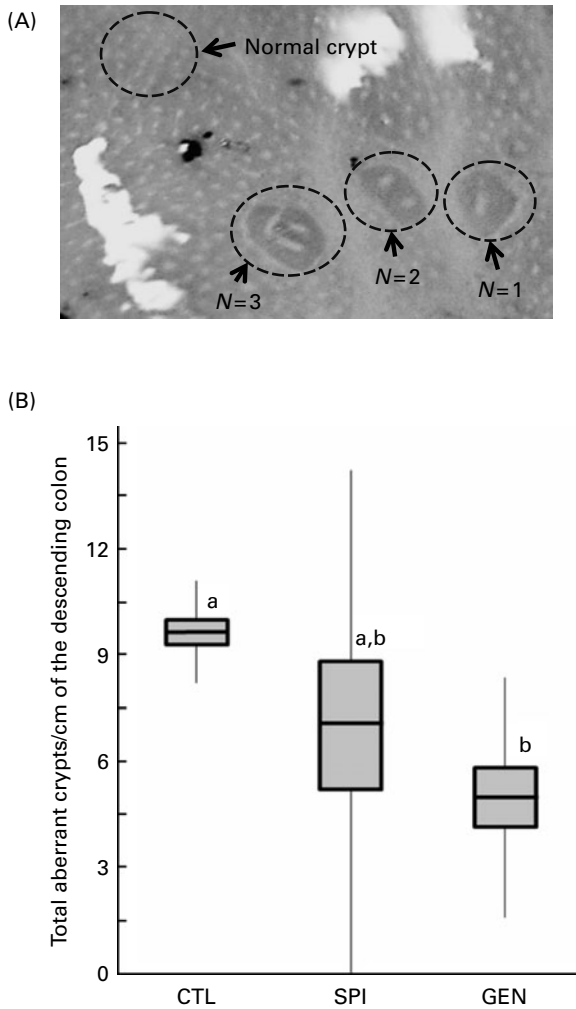
Fig. 1. (A) Representative image taken after methylene blue staining. The circled areas represent examples of normal crypts, and aberrant crypt foci with $N = 1$, $N = 2$ and $N = 3$ of crypt(s) per foci. (B) Box plot represents the number of total aberrant crypts per length (cm) of the descending colon in 13-week-old, azoxymethane-treated rats fed a control (CTL), soya protein isolate (SPI) or genistein (GEN) diet. [a,b] Mean values with unlike letters were significantly different ($P < 0.05$).

Technology), anti-Wnt5a or anti-Sfrp2 antibodies (1:200; Santa Cruz Biotechnologies) at room temperature for 3·5 h. After washing with 1 × PBS, the slides were incubated with a goat anti-rabbit Alexa Fluor 647 labelled secondary antibody (1:200; Invitrogen) at room temperature for 45 min, and then rinsed again in 1 × PBS. All slides were incubated with Hoechst (10 μg/ml; Invitrogen) at room temperature for 15 min. After washing with 1 × PBS, the slides were mounted and dried in the dark for 24 h at room temperature. Images were taken using the Axiovert 200M microscope (Zeiss).

Quantitative RT-PCR

Scraped colon tissues were ground in liquid N_2 and treated with TRI Reagent (Sigma-Aldrich). Total RNA was isolated according to the manufacturer's instructions, and RNA concentrations were measured by a spectrophotometer (BioRad SmartSpec Plus). Complementary DNA (cDNA) was synthesised from RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a Thermal Cycler 2720 (Applied Biosystems). In each reaction system, 2 μg of total RNA were used in a 20 μl mixture containing 1 × RT buffer, 4 mM-dNTPs, 1 × RT random primers and 2·5 U/μl MultiScribe Reverse Transcriptase. cDNA synthesis was performed using the following programme: 25 °C for 10 min, 37 °C for 2 h and 85 °C for 5 s. Synthesised cDNA was then analysed by PCR in a 7300 thermal cycler (Applied Biosystems). In each reaction, 25 ng of synthesised cDNA were used in a 20 μl volume containing 10 μl of SYBR Green Master Mix (2 × ; Applied Biosystems) and 0·25 μmol/l of each primer. PCR was performed using the following programme: 95 °C for 10 min, followed by thirty-five cycles of 95 °C for 15 s and 60 °C for 1 min. After PCR, melting curves were acquired stepwise from 55 to 95 °C to ensure that a single product was amplified in the reaction. *L7a*, the gene encoding a ribosomal protein, was used as an internal control for normalisation of individual values. The primers used in the present study are listed in Table S2 of the supplementary material (available online at http://www.journals.cambridge.org/bjn).

Protein isolation and immunoblotting

For obtaining nuclear extracts, 100 mg of scraped colon tissues were pulverised in liquid N_2, washed and dislodged twice with ice-cold PBS, and collected by centrifugation at 500 ***g***. Cell pellets were lysed with fresh lysis buffer (20 mM−HEPES, pH 7·9, 10 mM-NaCl, 1·5 mM-magnesium chloride, 0·2 mM-EDTA, 20 % glycerol (v/v), 1 mM-dithiothreitol (DTT) (Roche), 1 mM-phenylmethylsulfonyl fluoride (PMSF) (Sigma), 1 × protease inhibitor cocktail (Roche) and 0·1 % Igepal CA-630 (v/v)) on ice for 15 min and centrifuged again. Lysis was repeated for the resuspended pellet, followed by centrifugation at 500 ***g***. The precipitates were then incubated with fresh nuclear extraction buffer (20 mM-HEPES, pH 7·9, 400 mM-NaCl, 1·5 mM-magnesium chloride, 0·2 mM-EDTA, 25 % glycerol (v/v), 1 mM-DTT, 1 mM-PMSF and 1 × protease inhibitor cocktail) at 4°C for 30 min. Nuclear protein extracts were then collected from the supernatants after centrifugation at 12 000 ***g***. Protein concentration was determined by the Lowry assay. For Western blot analysis, 30 mg of nuclear protein were size-fractionated on a 12 % Tris−HCl polyacrylamide gel. Immunoblotting was performed by using a 1:1000 rabbit polyclonal antibody against β-catenin (Cell Signaling Technology), 1:1000 rabbit polyclonal antibody to detect lamin A (Santa Cruz Biotechnology) and a 1:10 000 goat anti-rabbit horseradish peroxidase-conjugated secondary antibody. Nuclear protein lamin A was used as an internal loading control for nuclear extract[33,34]. The membranes were exposed to the enhanced chemiluminescence reagent SuperSignal West Dura (Pierce), and the signals were detected and quantified using the ChemiDoc XRS imaging system (Bio-Rad).

Statistical analysis

Differences in body weight and food intake between the groups were analysed by using repeated-measures one-way

Table 1. Effect of soya protein isolate (SPI) and genistein (GEN) on aberrant crypt foci (ACF) incidence (*n* 8)*

Diets	$N = 1$	$N = 2$	$N = 3$	P†
CTL	8	8	7	
SPI	8	6	4	0·03
GEN	8	7	5	0·13

N, number of ACF; CTL, control.
* ACF incidence indicates the number of rats that developed ACF with the indicated stage ($N = 1$, $N = 2$ and $N = 3$).
† P value is the difference in the slope of regression line compared with CTL.

ANOVA (SAS Institute, Inc.). Results are reported as means with their standard errors. Differences were considered significant at $P < 0.05$. By performing regression analysis (Data Analysis for Excel 2010), the possible relationships between the total number of ACF/cm and the expression level of *Wnt* genes, including *Wnt5a*, *Sfrp1*, *Sfrp2* and *Sfrp5* were tested, as well as the relationship between the nuclear β-catenin level and the total number of ACF/cm. A linear relationship was considered significant at $P < 0.05$. A box plot was used to analyse the frequency of total ACF/cm. The lower line in the box represents Q1, the top point of the lower quartile; the middle line in the box represents Q2, the median point; and the upper line in the box represents Q3, the lowest point of the upper quartile. The smallest value was calculated as $Q1 - 1.5 \times (Q3 - Q1)$ and is represented as the endpoint of the lower whisker; the largest value was calculated as $Q3 + 1.5 \times (Q3 - Q1)$ and is represented as the endpoint of the upper whisker. The slope of regression lines of ACF incidence on the stage of ACF (*N*) between either SPI or GEN and CTL was calculated by ANCOVA (Table 1). The ACF counting results were analysed by one-way ANOVA. Results from immunoblotting and quantitative real-time PCR analysis were analysed using two-way ANOVA with interactions with diet and AOM induction as main effects. Letters were assigned by the *post hoc* Tukey test. Bars with unlike letters were significantly different at $P < 0.05$.

Results

General observations

All rats survived the full treatment period. There was no difference in body weight among the dietary groups during the trial ($P = 0.30$), but rats fed the SPI diet gained more weight following AOM injection than the other two groups (see Fig. S2 of the supplementary material, available online at http://www.journals.cambridge.org/bjn). After AOM injection, rats fed SPI had higher food intake compared with the CTL or GEN group, while there was no difference between the CTL and GEN groups. No rats were observed to have behavioural changes or stress responses before or after AOM induction. All rats developed aberrant crypts after the AOM treatment, and the highest number of aberrant crypts/aberrant foci was $N = 3$. There were no morphological differences observed in descending colons among the three dietary groups after AOM induction (see Fig. S3 of the supplementary material, available online).

Genistein inhibited aberrant crypt foci in the descending colon

At the end of the study period (6 weeks after AOM induction), eight rats from each dietary group were killed for ACF identification and quantification (see a representative image of ACF in Fig. 1(A)). Compared with the CTL diet, the GEN diet significantly reduced the total number of aberrant crypts/cm ($P = 0.02$; Fig. 1(B)). Rats fed the SPI diet also tended to have a decreased number of aberrant crypts/cm. Additionally, the GEN and SPI groups did not differ in total aberrant crypts/cm.

The total aberrant crypts were grouped into three subcategories according to the number of crypts (*N*) per foci: $N = 1$; $N = 2$; $N = 3$. A higher *N* value represents a more severe crypt abnormality. All rats in all the three diet groups were induced with ACF with $N = 1$ (100 %). The slope of the regression line of ACF incidence on *N* was significantly different between the CTL and SPI groups, suggesting that compared with the CTL group, fewer rats in the SPI group developed ACF with $N = 2$ and $N = 3$. The slope of the regression line of the GEN diet was not significantly different from that of the CTL diet, but there was a significant effect from *N* ($P = 0.02$; Table 1). Further analysis of multiplicity showed that there was a significant diet effect on the number of ACF with $N = 3$/cm of the descending colon ($P = 0.038$, Table 2). In particular, the GEN diet significantly decreased the number of ACF with $N = 3$/cm of the colon compared with the CTL diet ($P = 0.019$; Table 2).

Genistein prevented azoxymethane-induced nuclear β-catenin accumulation and suppressed Cyclin D1 and c-Myc expression in the descending colon

The role of WNT/β-catenin signalling was examined by Western blot and immunofluorescent staining. The mRNA levels of WNT/β-catenin downstream genes were evaluated by real-time RT-PCR. Overall, there was a significant diet effect on the abundance of nuclear β-catenin ($P = 0.008$) and the expression of *c-Myc* ($P = 0.02$). Additionally, AOM induction had a significant impact on nuclear β-catenin and the expression of *Cyclin D1* ($P < 0.0001$; Fig. 2(C)). There was a significant interaction ($P < 0.05$ for nuclear β-catenin and *c-Myc* and $P = 0.06$ for *Cyclin D1*) between diet and AOM induction because during normal development at 7 weeks, before

Table 2. Effect of soya protein isolate (SPI) and genistein (GEN) on aberrant crypt foci (ACF) frequency (frequency, number of ACF (*N*)/cm of colon (*n* 8))*

(Mean values with their standard errors)

Diets	$N = 1$		$N = 2$		$N = 3$	
	Mean	SE	Mean	SE	Mean	SE
CTL	1·93ᵃ	0·58	1·72ᵃ	0·34	0·85ᵃ	0·25
SPI	2·92ᵃ	0·58	1·25ᵃ	0·41	0·38ᵃ'ᵇ	0·21
GEN	1·85ᵃ	0·37	1·69ᵃ	0·84	0·28ᵇ	0·11

CTL, control.
[a,b] Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).
* Letter assignments were done by Tukey's test.

the AOM injection, the nuclear accumulation of β-catenin and the expression of *Cyclin D1* and *c-Myc* in the descending colon did not differ among the diet groups (pre-AOM; Fig. 2(A)–(C)) but post-AOM, nuclear β-catenin and *c-Myc* expression were significantly repressed in the SPI and GEN groups. In particular, the nuclear accumulation of β-catenin was reduced to the pre-AOM level. *Cyclin D1* was significantly lower in the GEN group when compared with CTL. Moreover, by using immunofluorescent staining, we confirmed the result from the Western blot analysis, showing that the level of β-catenin protein in the descending colon of CTL rats was higher than that in either SPI or GEN rats (Fig. 3). Additionally, regression analysis indicated that the decrease in total aberrant

crypts/cm in all animals was correlated with the attenuation of nuclear β-catenin accumulation following the AOM injection (R^2 0·74, $P=0·003$).

Wnt5a and Sfrp expression profiles were affected by genistein

The possible relationship between the decrease in total ACF and the regulation of WNT gene expression by SPI and GEN was tested by RT-PCR and immunofluorescent staining of the colon sections. In general, AOM induction and diet affected the expression of *Wnt1* (AOM, $P=0·007$, diet, $P=0·84$), *Sfrp1* (AOM, $P=0·002$, diet, $P=0·07$), *Sfrp2* (AOM,
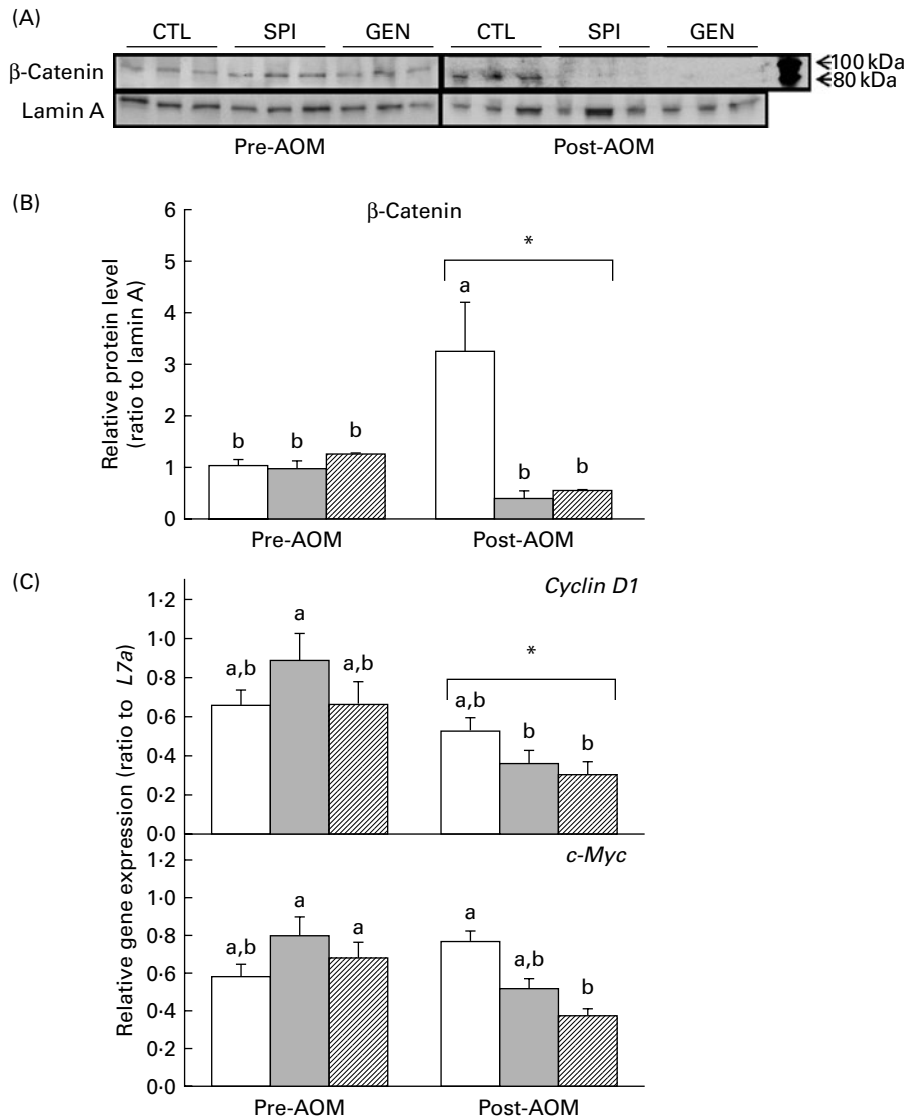
Fig. 2. Nuclear β-catenin abundance and WNT/β-catenin downstream gene expression. Nuclei were extracted from the scraped descending colons of rats, and protein levels were assessed by immunoblotting. (A) Representative blots of β-catenin and lamin A from Western blot analysis. (B) Quantification of Western blot analysis. Lamin A served as the loading control for nuclear extracts. Samples from three rats in each dietary group were analysed. Values are means, with their standard errors represented by vertical bars. (C) The expression levels of *Cyclin D1* and *c-Myc* were analysed by quantitative real-time PCR. Samples were collected from the control (CTL, □; $n_{pre-AOM}=6$; $n_{post-AOM}=8$), soya protein isolate (SPI, ▨; $n_{pre-AOM}=8$; $n_{post-AOM}=8$) and genistein (GEN, ▧; $n_{pre-AOM}=8$; $n_{post-AOM}=8$) diets. *Mean values were significantly different for the effect of azoxymethane (AOM) induction ($P<0·05$). [a,b] Mean values with unlike letters were significantly different ($P<0·05$).
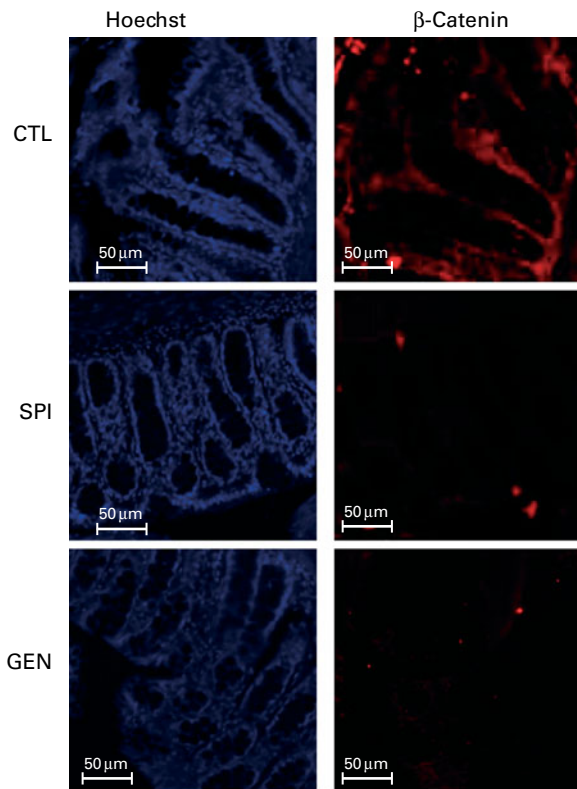
Fig. 3. β-Catenin distribution in the descending colon after azoxymethane induction. An anti-β-catenin antibody was used for the immunofluorescent staining of β-catenin in colon sections (red). Hoechst was used for the staining of nuclei (blue). Scale bar: 50 μm. CTL, control; SPI, soya protein isolate; GEN, genistein.

$P < 0.0001$, diet, $P = 0.06$) and *Sfrp5* (AOM, $P = 0.005$, diet, $P = 0.08$). The interaction between AOM induction and diet for *Wnt5a* expression ($P = 0.02$) was due to the fact that while there was no diet effect pre-AOM, the post-AOM SPI and GEN groups were significantly lower than the CTL. The expression levels of *Wnt1*, *Wnt5a*, *Sfrp1*, *Sfrp2* and *Sfrp5* in the descending colon were not different among the dietary groups at 7 weeks, before the AOM injection (Fig. 4). However, at 13 weeks of age, AOM induction up-regulated the expression levels of *Wnt1*, *Wnt5a*, *Sfrp1* and *Sfrp5* significantly in the CTL group when compared with the other dietary groups. Notably, the expression level of *Wnt5a*, *Sfrp2* and *Sfrp5* returned to be similar to or even below that of the pre-AOM values in both SPI and GEN groups, while *Sfrp1* was significantly lower in the GEN group only. Moreover, the repression of WNT-associated gene expression was correlated with the decrease in the total number of aberrant crypts/cm (*Wnt5a*: R^2 0·31, $P = 0.017$; *Sfrp1*: R^2 0·46, $P = 0.002$; *Sfrp2*: R^2 0·65, $P < 0.001$; *Sfrp5*: R^2 0·50, $P = 0.001$). However, *Wnt1* expression did not respond to either the SPI or GEN diet (Fig. 4(A)). By conducting immunofluorescent staining using currently available antibodies, we further showed that the protein levels of Wnt5a and Sfrp2 were also repressed by the SPI and GEN diets (Fig. 4(B) and (C)). The overexpression of Wnt5a and Sfrp2 proteins after AOM induction was clearly visible within the colon crypts in the CTL

animals, while both SPI and GEN diets considerably reduced the signals of the two proteins (Fig. 4(B) and (C)).

Discussion

In the present study, using an *in vivo* model, we report that a lifetime exposure to GEN reduces the incidence and frequency of colon pre-neoplasia in the descending colon of AOM-induced rats. We suggest that this suppression occurs through the minimised level of WNT/β-catenin signalling similar to or lower than that before AOM injection.

Previous studies have revealed conflicting effects of the intake of soya isoflavones on AOM-induced ACF formation in the rat colon. Various factors contribute to the differences, including the duration of exposure. In the present study, we analysed the effect of a lifelong dietary exposure to GEN in both pre- and post-carcinogen periods, which included both maternal periods of gestation and lactation. We showed that dietary GEN decreased the frequency and severity of ACF when examined at the early stage of colon pre-neoplastic development. Specifically, GEN reduced the number of ACF with $N = 3$, suggesting that GEN prevented colon crypts from becoming abnormal. Although rats in the present study and in the report by Raju *et al.*[14] had similar exposure times to diets before AOM induction, we aimed to define the effect of GEN on the relatively early stage of colon cancer development. Raju *et al.* analysed ACF at 26 weeks after carcinogen exposure and observed colon tumour formation, which may explain why they observed a repressive effect of soya isoflavones on colon tumour formation, but not on ACF.

The effect of GEN on colon cancer development differs with the dosage and timing of exposure. In the present study, we focused on testing the consequences of a maximal GEN exposure time. Therefore, we defined the lifelong period as occurring from day 2 of gestation until after carcinogen induction. Meanwhile, we used a relevant dosage of GEN, which is achievable by soya consumption. Application of the above-mentioned approach may be the reason for the protective effects observed in the present study compared with those published previously. For instance, Gee *et al.*[35] showed that when soya isoflavones were administered only immediately before AOM induction, with a comparatively shorter exposure time than ours, both pure GEN (250 mg/kg) and SPI (250 mg GEN/kg) triggered a 3- or 2-fold increase in ACF, respectively, in the distal colon. Moreover, Rao *et al.*[36] reported that a postnatal-only intake of 250 mg GEN/kg diet promoted both non-invasive and total adenocarcinoma multiplicity. Another study reported that a high dose feeding of 2 g soya isoflavone/kg diet (supplemental dosage of 1200 parts per million GEN) enhanced sensitivity to AOM in older female rats[15]. Compared with the administration of 250 or 1200 parts per million GEN in these studies, the SPI and GEN diets used in the present study contained less GEN (140 parts per million), therefore making it a model more relevant to dietary intake level[37]. Additionally, we fed rats with the same diet throughout their lifetime. A similar animal model was previously used by another group[38], who reported
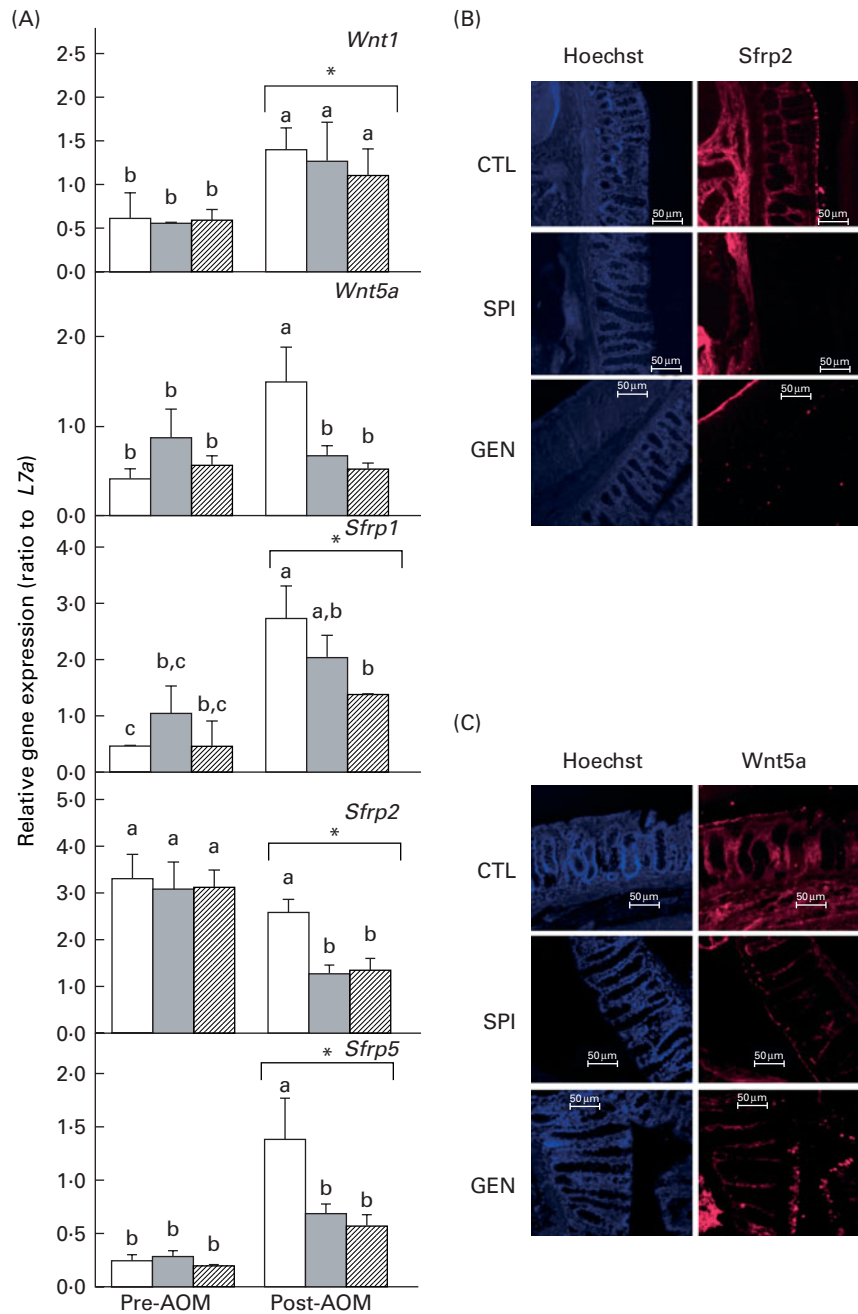
Fig. 4. Expression profiles of WNT-related genes in the descending colon. Samples were collected from rats fed the control (CTL, □; $n_{pre-AOM} = 6$; $n_{post-AOM} = 8$), soya protein isolate (SPI, ▨; $n_{pre-AOM} = 8$; $n_{post-AOM} = 8$) and genistein (GEN, ▨; $n_{pre-AOM} = 8$; $n_{post-AOM} = 8$) diets. (A) The mRNA expression levels of Wnt1, Wnt5a, Sfrp1, Sfrp2 and Sfrp5 were analysed by quantitative real-time RT-PCR. Values are means, with their standard errors represented by vertical bars. *Mean values were significantly different for the effect of azoxymethane (AOM) induction ($P<0.05$). [a,b,c] Mean values with unlike letters were significantly different ($P<0.05$). (B, C) Immunofluorescent staining of Wnt5a and Sfrp2 in the colon sections of post-AOM rats (red). Hoechst was used for the staining of nuclei (blue). Anti-Wnt5a and anti-Sfrp2 antibodies were used for the staining of Wnt5a and Sfrp2 proteins. Scale bar: 50 μm.

inhibitory effects of lifelong soya protein consumption on tumour incidence. In the present study, in addition to confirming the protective effect of SPI, we intended to use the SPI diet as a positive control to specifically investigate the effects of GEN as a major component of soya isoflavones. The present findings highlighted that lifelong intake of GEN reduced the frequency of ACF with N = 3. The SPI diet, which contained a mixture of other dietary isoflavones and soya proteins, showed less of a protective effect than the GEN diet alone.

The present study provides the first clue for the potential mechanism by which dietary soya prevents colon neoplasia, which is proposed to occur through the regulation of WNT/β-catenin signalling. WNT/β-catenin signalling plays an important role in maintaining intestinal homeostasis, as well as promoting colon cancer development[21,26]. WNT signalling is a major driving force in the renewal process of the epithelium, which is critical for intestinal development[20,21,39,40]. On the other hand, its hyper-activation, marked by abnormal accumulation

of nuclear β-catenin, disrupts normal cellular events and leads to carcinogenesis[41]. The present results demonstrate that during colon development, before AOM injection, the nuclear accumulation of β-catenin was not different among the dietary groups, indicating normal WNT signalling activity, which was also confirmed by the fact that the expression levels of *Cyclin D1*, *c-Myc* and the WNT-associated genes tested were not different among the dietary groups. Therefore, stabilisation of β-catenin is critical for colon tumorigenesis[19,22]. Data from the present study showed that AOM induction resulted in tissue-wide abnormality in WNT/β-catenin signalling before actual tumour formation. Therefore, in the present study, we validated the use of nuclear β-catenin to predict the carcinogenic potential of the rat colon by AOM in different groups, and as a marker to evaluate the protective effect of GEN-containing diets against colon carcinogenesis.

To investigate the involvement of WNT/β-catenin at the early stage of colon cancer development, we conducted an AOM-induced animal study with a non-hyperplasia endpoint, which was confirmed by histological analysis. Using structurally intact colon samples, we examined the nuclear distribution of β-catenin protein and its localisation within the colon crypt, as well as the expression of WNT-related genes in the descending colon. Instead of only investigating the abnormal areas of the colon by limiting our analysis to ACF, we considered the abnormality of β-catenin in the entire colon epithelia as a potential marker for colon carcinogenesis. After AOM induction, we observed that β-catenin was induced throughout the descending colon epithelia in the CTL group but returned to the same level as the pre-AOM values in the SPI and GEN groups, suggesting that the hyper-activation of WNT signalling induced by AOM was prevented by the SPI and GEN diets. Moreover, the expression of the oncogenic WNT/β-catenin downstream genes *Cyclin D1* and *c-Myc* was also inhibited, confirming that SPI and GEN protected rats against AOM-induced aberrant WNT signalling. Interestingly, the expression of *Wnt1* was up-regulated in the CTL, SPI and GEN groups when compared with the pre-AOM period, and without differences among the three diets. The up-regulation of *Wnt1* expression indicates that a subsequent activation of WNT signalling was potentially induced by AOM. However, the critical marker of WNT signalling, nuclear β-catenin, was reduced in the SPI and GEN groups, indicating the overall protective effect of SPI and GEN against AOM-induced carcinogenesis.

Using colon cancer cells, we previously identified that GEN treatment suppressed the overactivated WNT signalling pathway by up-regulating the expression of WNT antagonists, including Sfrp2 and Wnt5a, thus inhibiting cancer cell growth[34,42]. The mechanism may be that when Wnt is up-regulated in cancer cells, GEN treatment induces the down-regulation of this malignant signalling pathway under this situation by up-regulating the WNT antagonists including Wnt5a and Sfrp2. In the present study, we showed that at the early stage of colon cancer development, the repressed WNT/β-catenin signalling by SPI and GEN did not result from the up-regulation of the WNT antagonists tested, including *Sfrp1*, *Sfrp2*, *Sfrp5* and *Wnt5a*. A possible explanation for our observations from the *in vivo* animal model is that when administered before tumorigenesis, as opposed to the

in vitro cancer cell model, SPI and GEN can keep WNT/β-catenin signalling low, thus rats fed with SPI and GEN do not require the same amount of the gene expression of WNT antagonists as those fed a CTL diet to counteract the carcinogen-induced aberrant WNT/β-catenin activity. Further studies need to be conducted to reveal which precise steps and components of WNT/β-catenin signalling are directly affected by GEN.

As a predominantly expressed oestrogen receptor (ER) in the colon[43], ER-β plays an important role in colon tumorigenesis[44]. Soya isoflavones have been reported as a preferred ligand to ER-β[45,46]. However, the interactions between soya isoflavones and ER-β during colon carcinogenesis are less studied. In the established colon cancer cell line DLD-1, ER-β has been shown to be responsible for soya isoflavone-mediated growth suppression[47]. Similarly, rats fed diets containing soya isoflavones had a higher level of ER-β in developed tumours[14]. On the other hand, a study in which the relationship between the expression of ERβ and ACF formation was examined has demonstrated that feeding of soya isoflavones did not affect the expression of ERβ in the colon among all age groups[15]. Similarly, we examined the expression of ERβ in the descending colon epithelium where most of the tissues were still in normal condition without developing ACF, thus we did not observe any dietary or AOM effect on the expression of ERβ. Moreover, we conducted the present study at the ACF stage of tumorigenesis without any tumour formation, whereas others showed most change in ERβ in colon tumour or colon cancer cells. It is possible that ERβ would be altered by the diet at a later stage of carcinogenesis with tumour formation. In a report by Bises *et al.*[48], a difference has been observed in response to a soya diet between male and female mice. Only female mice fed the soya diet showed an approximately 20 % increase in ERα mRNA expression in the ascending colon and, moreover, the soya diet did not affect the expression of ERβ in either sex[48]. The possibility of a sex-specific response to GEN and/or soya diets will be further explored by examining both female and male rats fed soya diets after AOM induction.

Overall, the present results demonstrate that the attenuation of nuclear β-catenin accumulation and WNT-related gene expression was strongly correlated with the decrease in the number of total ACF/cm of the colon, indicating that GEN produced a protective effect and kept WNT/β-catenin signalling low when rats were exposed to a carcinogen. Taken together, the present findings indicate that a lifelong intake of GEN may be protective against a carcinogenic load by preventing the overactivation of WNT/β-catenin signalling, and this leads to a decreased risk of colon tumorigenesis.

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