Chemopreventive effect of 4'-hydroxychalcone on intestinal tumorigenesis in *Apc*^{Min} mice

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Abstract. Chalcones and its derivatives are reported to exhibit anti-cancer effects in several cancer cell lines, including colon cancer cells. However, the *in vivo* anticancer effects and associated mechanisms of chalcones against intestinal tumorigenesis currently remain unclear. The aim of the present study was to investigate the chemopreventive effect of a chalcone derivative, 4'-hydroxychalcone (4-HC), in a transgenic adenomatous polyposis coli multiple intestinal neoplasia mouse model (*Apc*^{Min}) of spontaneous intestinal adenomas. *Apc*^{Min} mice were fed 4-HC (10 mg/kg/day) or the vehicle control by oral gavage starting at 8 weeks of age, and were sacrificed at 20 weeks. The administration of 4-HC significantly decreased the number of colon adenomas by 45% and the size of colon adenomas by 35% compared with the respective controls. Similarly, the number of adenomas in the distal small intestine (DSI) and proximal small intestine also decreased by 35 and 33%, respectively, in 4-HC-treated mice, and adenoma size in the DSI decreased by 39% compared with the respective controls. Treatment with 4-HC strongly decreased proliferation in colon and DSI adenomas, as detected by immunofluorescence staining with the proliferation marker protein Ki-67, and promoted apoptosis in colon adenomas, as detected by TUNEL immunofluorescence staining. In addition, decreased mRNA expression of β-catenin target genes, including *c-Myc*, *Axin2* and *CD44,* in colon adenomas of 4-HC-treated animals demonstrated the involvement of the Wnt/β-catenin signaling pathway in the initiation and progression of colon neoplasms. Treatment with 4-HC also decreased the protein levels of β-catenin in colon adenomas, as demonstrated by immunofluorescence staining. The results suggested that 4-HC may be a promising candidate for the chemoprevention of intestinal tumorigenesis, and further investigations are required to evaluate its clinical utility.

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Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-associated mortality in the USA, with an estimated 145,600 new cases and 51,020 deaths in both men and women in 2019 (1). Although the death rate is decreasing as a result of increased use of colonoscopy in early screening, the decline in incidence has tapered in recent years (2). The pathogenesis of CRC is a multistep process that originates from premalignant neoplastic lesions, known as adenomas, and progresses to invasion and further metastasis (3). Early adenoma formation is accompanied by the mutation of the tumor suppressor gene adenomatous polyposis coli (*APC*), which is present in ~80% of sporadic CRCs and all familial adenomatous polyposis cases (4). Most somatic *APC* mutations result in a truncated Apc protein and contribute in CRC development (5). Mechanistically, dysregulation of the Wnt signaling pathway as a result of the *APC* mutation leads to dysfunction of the multiprotein 'destruction complex' and translocation of β-catenin into the nucleus to activate transcription factors that belong to the T-cell factor (TCF)/lymphoid enhancer factor family (6,7). This gives rise to increased expression of genes that regulate cell proliferation and apoptosis, such as *c-Myc* and *Axin2*. Mutated *APC* multiple intestinal neoplasia mouse model (*Apc*^{Min}) possess a nonsense mutation at codon 850 of *APC*, increasing its predisposition to intestinal adenoma formation, which is similar to the human somatic mutation that is associated with the progression of human CRC (8). Therefore, *Apc*^{Min} mice have been widely used as a model for the study of intestinal tumorigenesis. This model is particularly

advantageous for assessing the effect of anticancer agents in the early stages of cancer formation, since Apc^{Min} mice spontaneously grow detectable intestinal adenomas within several months (9).

It has been well established that diets high in fruit and vegetables decrease the risk of developing various types of cancer, including CRC (10-12). A class of natural products abundant in these types of foods are flavonoids, which have been widely reported to have preventive effects against CRC, as demonstrated in animal studies (13,14) and population-based studies (15-17). The chemistry of chalcones attracts much attention owing to its simple structure, easy synthesis, variable construct derivates and promising biological functions, including its activity against inflammation, angiogenesis, bacterial infection, diabetes and cancer, as well as its role in immunomodulation (18-21). Chalcones are reported to possess chemopreventive activities in a variety of solid tumors, such as prostate cancer, melanoma and colon cancer (22,23). Chalcones serve as precursors for flavonoid synthesis and are considered promising candidates for various disease treatments, including dietary cancer prevention (24,25). Previous *in vitro* studies have indicated that chalcones and their derivatives selectively induce apoptosis and restrain proliferation in human cancer cell lines (26), such as Caco-2 cells, particularly when the hydroxyl groups are present in chalcone molecules (27), and these are thought to be potent modulators of angiogenesis (19). However, the *in vivo* anticancer effects of these chalcone derivatives remain to be fully elucidated. In the present study, the chemopreventive effects of 4'-hydroxychalcone (4-HC; an α,β-unsaturated ketone with the chalcone backbone and one hydroxyl-substituent at the 4' position of the A ring; Fig. 1A), was evaluated for the first time on the spontaneous intestinal tumorigenesis in Apc^{Min} mice. The results of the present study provide scientific evidence that supports 4-HC as a potential chemopreventive regimen for intestinal neoplasms originating from *APC* mutations.

Materials and methods

Animals and chemicals. A total of 4 male Apc^{Min} (8 weeks old, average starting weight, 22 g) and 8 female C57BL/6 mice (8 weeks old, average starting weight, 19 g) were obtained Beijing Vital River Laboratory Animal Technology Co., Ltd. Animals were housed under optimal conditions (21˚C, 60% relative humidity, 12-h light/dark cycle, free access to food and water) in the barrier facility of the Laboratory Animal Center, Sichuan University. The animal experiments were reviewed and approved by the Animal Investigation Committee of the West China Second University Hospital, Sichuan University (Chengdu, China). The 4-HC was purchased from Selleck Chemicals and was dissolved in DMSO to a final concentration of 10 mg/ml for storage, and further dissolved in corn oil prior to administration.

Adenoma burden assessment. Male Apc^{Min} mice were bred with C57BL/6 mice and the offspring were genotyped by polymerase chain reaction (PCR) using the following primers according to the manufacturer's instructions (Sigma-Aldrich: Merck KGaA): IMR0033, 5'-GCCATCCCTTCACGT TAG-3'; IMR0034, 5'-TTCCACTTTGGCATAAGGC-3'; and IMR0758, 5'-TTCTGAGAAAGACAGAAGTTA-3'. Male 8-week-old Apc^{Min} mice were treated with vehicle (DMSO in corn oil; n=11) or 10 mg/kg/day 4-HC (n=12) by oral gavage every day for 12 weeks. The dose of 4-HC used was considered based on a previous study (28) and the final concentration was tested across 3 different concentrations (4-week-old mice were treated with 5, 10 or 20 mg/kg of 4-HC for 8 weeks; Fig. S1). At the end of the experimental period, 20-week-old mice were euthanized by CO_2 asphyxiation, with a flow rate of 30% displacement of the cage volume per min, followed by cervical dislocation. Small intestines and colons were removed and opened longitudinally along the mesenchymal side. A stereoscopic dissection microscope (Stemi 2000-c; Carl Zeiss AG) was utilized to assess the tumor burden by counting the number of adenomas and determining their dimensions (AxioVision Application, version 4.6, Cal Zeiss Microscopy). Cardiac puncture was performed as soon as mice were euthanized and mouse blood was collected. Complete blood cell counts were performed by the clinical laboratory at West China Second University Hospital.

Tissue processing, immunofluorescence staining and TUNEL. Adenoma-containing mouse intestines were prepared using the Swiss-roll technique and fixed in 4% paraformaldehyde overnight at 4˚C. Tissues were then embedded in paraffin and subsequently cut into 5-μm-thick sections. Paraffin-embedded slides were deparaffinized in room temperature with xylene and rehydrated with a graded ethanol series (100, 100, 95, 95 and 50%). Histological analysis was performed using hematoxylin and eosin staining (5 min each in room temperature). For tissue immunofluorescence staining, antigen retrieval was performed in citrate buffer (Sigma-Aldrich; Merck KGaA) with heating for 13.5 min in a microwave oven at 95˚C. The samples were then incubated in blocking buffer consisting of PBS supplemented with 3.5% normal goat serum (Thermo Fisher Scientific, Inc.) at room temperature for 30 min. Samples were then incubated in a humidified chamber overnight at 4˚C with primary antibodies diluted in blocking buffer. The primary antibodies included: β-catenin (1:200; cat. no. ab32572) and proliferation marker protein Ki-67 (1:500; cat. no. ab15580) (both from Abcam). Following three washes in PBS and one wash in blocking buffer, tissue samples were incubated with goat anti-rabbit Alexa Fluor 488-conjugated secondary antibody in room temperature for 20-30 min (1:400; cat. no. A11008; Thermo Fisher Scientific, Inc.) and then washed three times in PBS, followed by the addition of Hoechst 33342 nuclear stain (15 min at room temperature) and coverslip mounting with SlowFade Gold Antifade reagent (Thermo Fisher Scientific, Inc.).

To determine if 4-HC affects apoptosis of intestinal adenomas, TUNEL staining was performed using the DeadEnd™ Fluorometric TUNEL System (Promega Corporation) according to manufacturer's instructions. A total of 12 polyps from 4 mice in each treatment group were selected for TUNEL staining, and five fields per slide were examined to quantify the TUNEL-positive cells.

Image acquisition and quantification. Bright-field microscopy images were acquired with an Optronics MicroFire

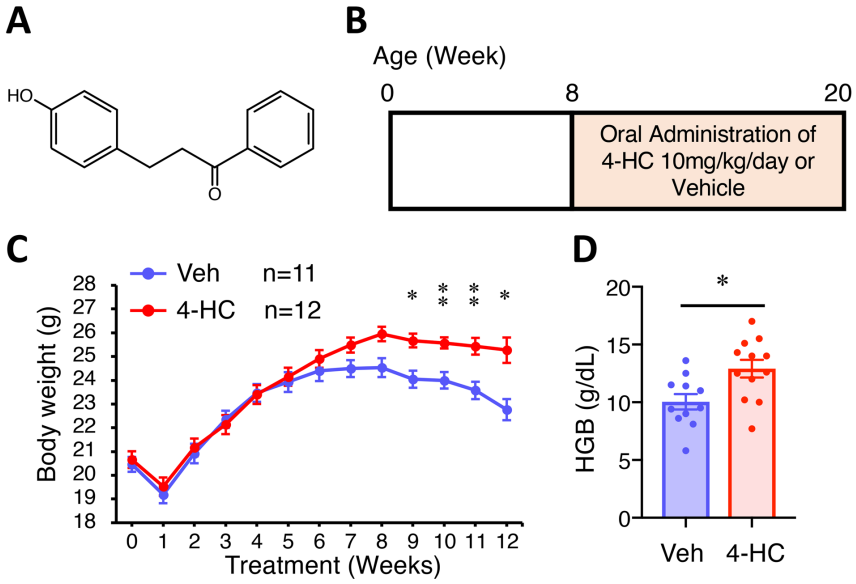

Figure 1. Experimental design and general *in vivo* observations. (A) The chemical structure of 4-HC. (B) Experimental design for the evaluation of the chemopreventive effect of 4-HC in male Apc^{Min} mice. Mice were randomly allocated into groups for oral administration with 10 mg/kg/day 4-HC (n=12) or Veh control (n=11) from 8 to 20 weeks of age. Changes in (C) body weight over time and (D) HBG levels at 20 weeks for Apc^{Min} mice orally treated with 4-HC (n=12) or Veh (n=11). Data are presented as the mean ± SEM. *P<0.05, **P<0.01 vs. Veh. 4-HC, 4'-hydroxychalcone; Apc^{Min}, adenomatous polyposis coli multiple intestinal neoplasia mouse model; HGB, hemoglobin; Veh, vehicle.

charge-coupled device camera on a Leica DM2000 Upright Compound Microscope (Leica Microsystems, Inc.). Fluorescence microscopy images were acquired using a Nikon A1R confocal microscope (x20 magnification used; p to x60/1.4 oil immersion objective lens; Nikon Corporation). Images were analyzed using ImageJ software (v2.0.0-rc-69/1.52p; National Institutes of Health) (29). Quantification of Ki-67-, TUNEL- and β-catenin-positive cells was performed by measuring the area of target fluorescence and normalizing it to the area of nuclear fluorescence, and was expressed as fold change over the vehicle treated group.

Tissue mRNA expression analysis by reverse transcription-quantitative PCR (RT-qPCR). Adenoma tissue samples were collected and stored in RNAlater (Qiagen GmbH) overnight, and total RNA was extracted using the RNeasy Mini kit (Qiagen GmbH) according to the manufacturer's instructions. For cDNA synthesis, 2-5 μg of the total RNA was reverse transcribed using a SuperScript III Reverse Transcriptase kit according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR was performed using SYBR Green-based detection (cat. no. 4364346, Invitrogen; Thermo Fisher Scientific, Inc.) and a Mastercycler (Eppendorf) with the following thermocycling conditions: 2 min of initial denaturation at 94˚C, followed by 25-30 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec and elongation at 72˚C for 60 sec, and then 10 min of final extension at 94˚C. The gene targets and corresponding primers used in this experiment included: Mouse *c-Myc*, forward, 5'-ATG CCCCTCAACGTGAACTTC-3', and reverse, 5'-CGCAAC ATAGGATGGAGAGCA-3'; mouse *Axin2*, forward, 5'-TGA CTCTCCTTCCAGATCCCA-3', and reverse, 5'-TGCCCA CACTAGGCTGACA-3'); and mouse *CD44*, forward, 5'-TCG ATTTGAATGTAACCTGCCG-3', and reverse, 5'-CAG TCCGGGAGATACTGTAGC-3'. Technical triplicates were used, and data were normalized to the housekeeping gene *GAPDH* (forward, 5'-AGGTCGGTGTGAACGGATTTG-3' and reverse, 5'-TGTAGACCATGTAGTTGAGGTCA-3'), and the relative abundance of transcripts was calculated by the comparative $2^{-\Delta\Delta Cq}$ method (30).

Statistical analysis. All data are presented as the mean ± SEM analyzed using GraphPad Prism v7 (GraphPad Software, Inc.). Data for continuous variables involving two groups were analyzed by unpaired Student's t-test. For multiple-time-point comparisons (Figs. 1C and S1A), two-way ANOVA followed by Sidak post hoc test was performed using built-in functions in GraphPad Prism v7 (GraphPad Software, Inc.). For multigroup comparisons (Fig. S1B), one-way ANOVA followed by Tukey's post hoc test was performed. P<0.05 was considered to indicate a statistically significant difference.

Results

General in vivo observations. To determine the dose of 4-HC used in the present study, a toxicity test was performed. The 4-week-old C57BL/6 mice were orally treated with vehicle or gradient doses of 4-HC (5, 10 or 20 mg/kg/day) for 8 weeks. The body weight change was similar among mice treated with vehicle, 5 mg/kg/day 4-HC and 10 mg/kg/day, but the body weight of mice treated with 20 mg/kg/day 4-HC was significantly lower than that of mice treated with vehicle over time (Fig. S1A and B). Therefore, 10 mg/kg/day of 4-HC administration was considered to exert no marked toxicity and was selected for further treatment for the tumor burden assessment of Apc^{Min} animals.

A total of 24 male Apc^{Min} animals were randomly allocated for treatment with vehicle or 10 mg/kg/day of 4-HC by oral administration from 8 to 20 weeks (Fig. 1B). Apc^{Min} mice treated with 4-HC exhibited significantly less body weight loss than

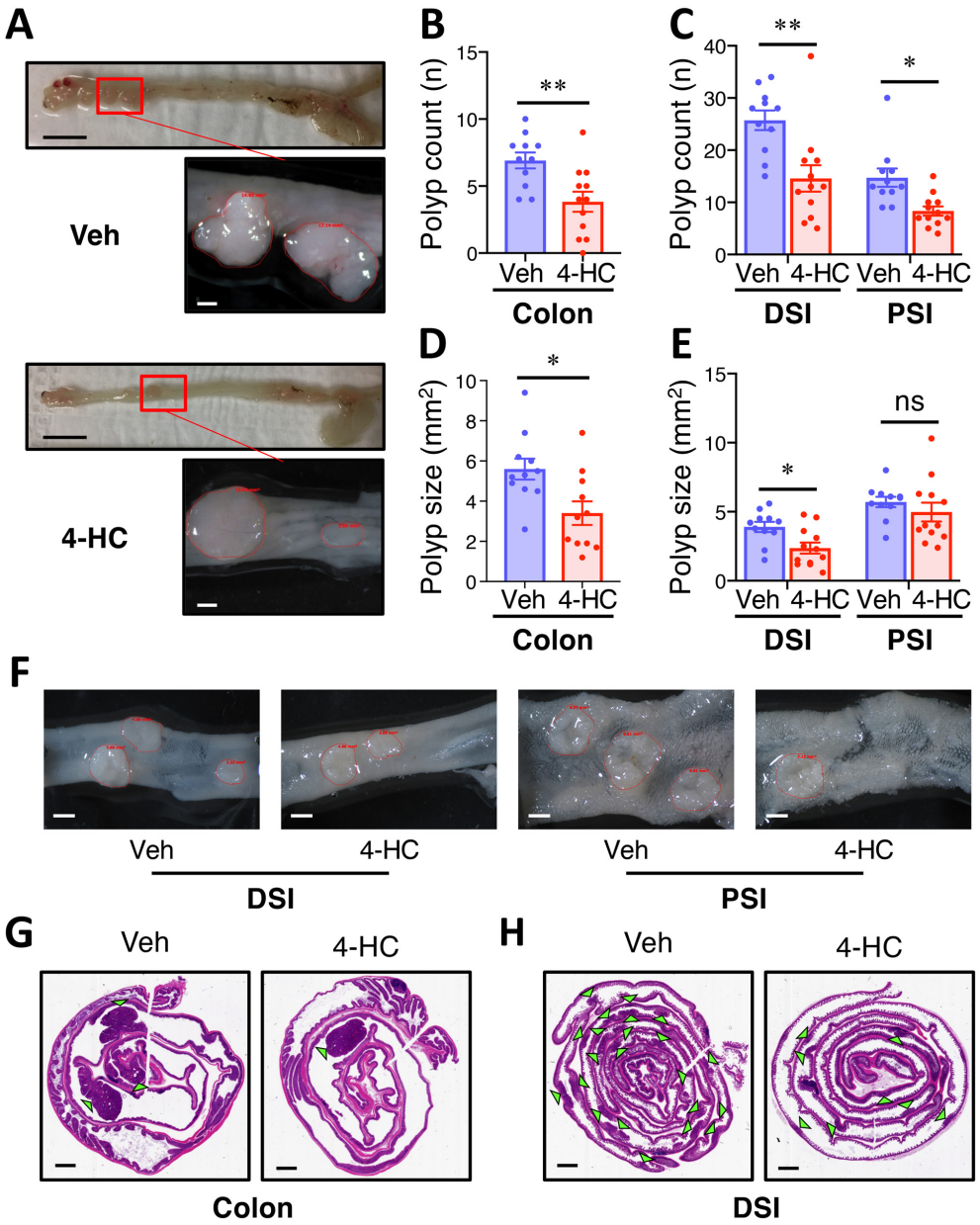

Figure 2. Oral administration of 4-HC prevents spontaneous intestinal polyposis in *Apc*^{Min} mice. (A) Representative images of colon polyps in *Apc*^{Min} mice treated with 4-HC or Veh for 12 weeks. Images were depicted macroscopically (scale bar, 10 mm; top images) and under a stereoscopic dissection microscope (scale bar, 1 mm; bottom images). *Apc*^{Min} mice treated with 10 mg/kg/day 4-HC (n=12) and Veh (n=11) for 12 weeks and (B) colon polyp number, (C) DSI/PSI polyp number, (D) colon polyp size, and (E) DSI/PSI polyp size were analyzed. Polyp size is the tumor surface area observed under the microscope. (F) Representative images of polyps in the small intestine of *Apc*^{Min} mice under a stereoscopic dissection microscope. Scale bar, 1 mm. Representative images of adenoma burden in the (G) colon and (H) DSI from mice treated with Veh or 4-HC. Green triangles indicate microscopic intestinal adenomas. Scale bar, 2 mm. Data are presented as the mean ± SEM. *P<0.05, **P<0.01. 4-HC, 4'-hydroxychalcone; *Apc*^{Min}, adenomatous polyposis coli multiple intestinal neoplasia mouse model; DSI, distal small intestine; ns, not significant; PSI, proximal small intestine; Veh, vehicle.

those treated with vehicle (Fig. 1C). Mice treated with 4-HC weighed 4% more at 7 weeks of treatment (P=0.049) and 11% more at 12 weeks (P<0.01). Body weight loss in both groups occurred at the first week of treatment due to inadaptation to oral gavage handling, and also at a late timepoint (8-12 weeks of treatment) owing to the increased intestinal adenoma burden and subsequent anemia. Similarly, the endpoint hemoglobin level in 4-HC-treated mice was significantly higher compared with that in control mice (P=0.01; Fig. 1D).

Oral administration of 4-HC prevents spontaneous intestinal polyposis in Apc^{Min} *mice*. Most adenomas in *Apc*^{Min} mice developed in the small intestine, with fewer in the colon, and were identified histologically as adenomatous polyps using H-E stained slides. At the age of 20 weeks, mice in the control group developed an average of 6.9, 25.7 and 12.5 polyps in the colon, distal small intestine (DSI) and proximal small intestine (PSI), respectively; whereas 4-HC treatment led to a significant reduction (45%) in the number of colon adenomas (P<0.01) (Fig. 2A and B), and also a decrease in colon adenoma size (35% reduction) compared with the control treatment (P<0.05; Fig. 2D). Similarly, 4-HC strongly decreased the number of polyps by 35% (P<0.01) and 33% (P=0.03) in the DSI and PSI (Fig. 2C), respectively. A prominent decrease in polyp size was

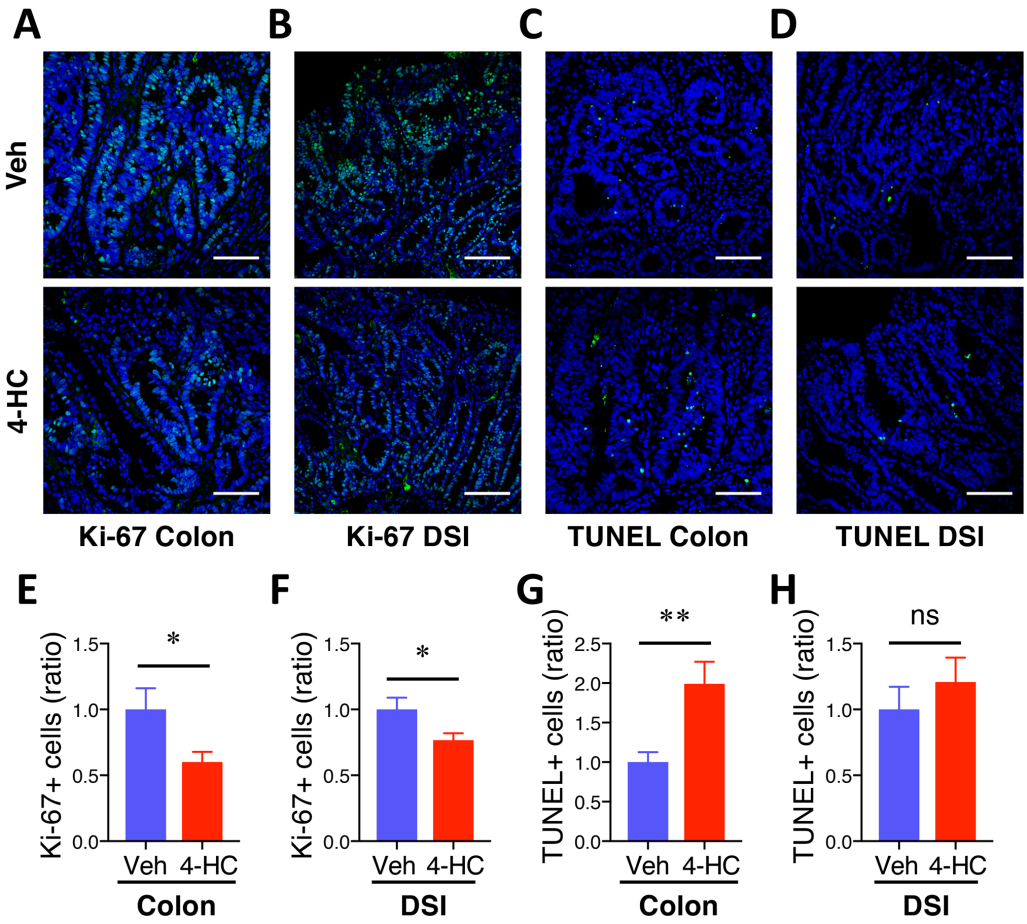

Figure 3. 4-HC prevents proliferation and induces apoptosis during intestinal adenoma formation. DSI and colon adenomas of 20-week-old Apc^{Min} mice were sectioned for immunofluorescent (IF) staining and corresponding analysis. 12 polyps from 4 individual mice in each treatment group were included in the analysis. (A) Representative images of Ki-67 IF staining of colon adenomas. (B) Representative images of Ki-67 IF staining of DSI adenomas. (C) Representative images of TUNEL IF staining of colon adenomas. (D) Representative images of TUNEL IF staining of DSI adenomas. (E) Relative quantification of Ki-67+ cells in colon adenomas. (F) Relative quantification of Ki-67+ cells in DSI adenomas. (G) Relative quantification of TUNEL+ cells in colon adenomas. (H) Relative quantification of TUNEL+ cells in DSI adenomas. Data are presented as the mean ± SEM. Scale bar, 100 μm. ns, not significant; *P<0.05, **P<0.01. 4-HC, 4'-hydroxychalcone; DSI, distal small intestine; Apc^{Min}, adenomatous polyposis coli multiple intestinal neoplasia mouse model; ns, not significant; Veh, vehicle.

also observed in the DSI, with a 39% reduction in adenoma surface area (P=0.01; Fig. 2E). Hematoxylin and eosin staining of Swiss-rolled intestines exhibited similar phenotypes, and histological analysis showed comparable dysplasia and invasion in the polyps in both groups (Fig. 2F and G).

4-HC treatment prevents proliferation and induces apoptosis during intestinal adenoma formation. To assess whether 4-HC efficacy is associated with antiproliferative and proapoptotic properties, Ki-67 and TUNEL immunofluorescence staining were performed on colon and DSI adenomas. Qualitative microscopic examination of Ki-67-stained sections showed a decrease in Ki-67-positive cells in both colon and DSI adenomas from mice treated with 4-HC (Fig. 3A and B) compared with the vehicle control. The quantification of Ki-67 immunofluorescence staining showed 40% (P=0.03) and 23% (P=0.03) decreases in Ki-67 positive cells from polyps from the colon and DSI, respectively, compared with the vehicle control (Fig. 3E and F). Fig. 3C, D, G and H summarizes the effects of 4-HC on adenoma cell apoptosis. Qualitative microscopic examination of TUNEL-stained sections showed an increase in TUNEL-positive cells selectively in colon adenomas from mice treated with 4-HC compared with the vehicle. The quantification of TUNEL staining showed a 99% increase in TUNEL-positive cells in colon polyps with 4-HC treatment compared with those that received control treatment (P<0.01; Fig. 3G). No significant difference in the percentage of TUNEL-positive cells was identified in DSI polyps between the treatment groups (P>0.05; Fig. 3H).

β-catenin and related gene expression levels are selectively suppressed by 4-HC in colon adenomas. The aforementioned data demonstrated the antiproliferative and apoptosis-promoting role of 4-HC in spontaneous intestinal tumorigenesis, which is associated with the properties of the β-catenin signaling pathway in human CRC progression (31). Furthermore, chalcone and a number of its analogues have been described to possess the ability to inhibit β-catenin signaling pathways (25,32-35). To examine this hypothesis, β-catenin target gene expression levels were analyzed in adenomas by RT-qPCR. A marked suppression of *c-Myc*, *Axin2* and *CD44* gene expression was observed in colon adenomas treated with 10 mg/kg/day 4-HC compared with those treated with the vehicle (Fig. 4A). Notably, the suppressive effect of 4-HC

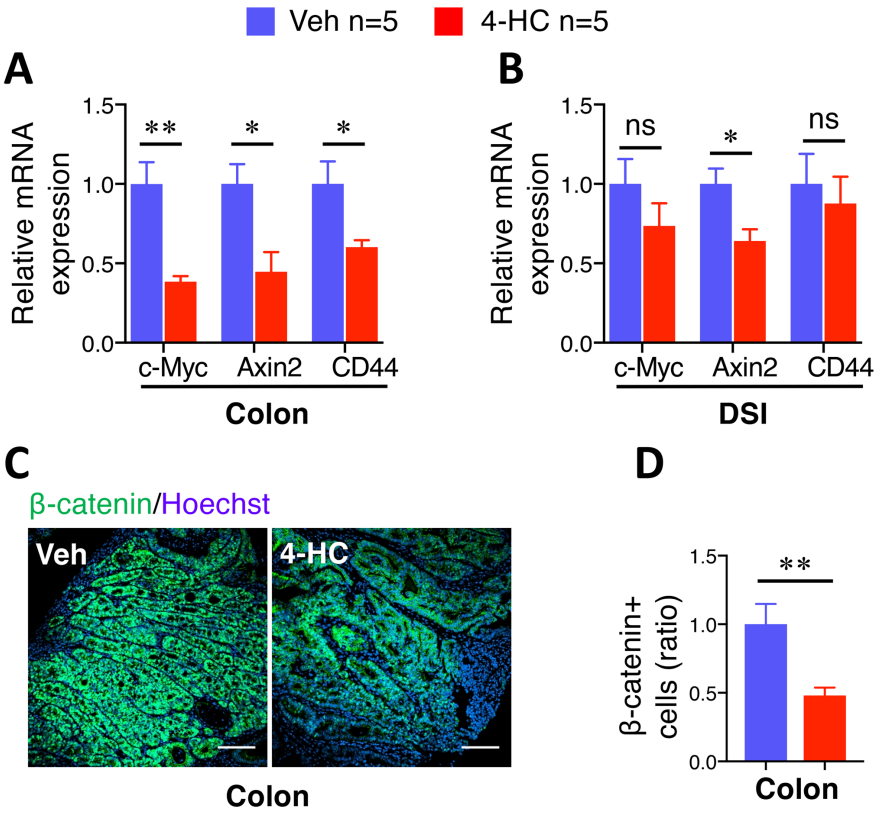

Figure 4. β-catenin and related gene expression level are selectively suppressed by 4-HC in colon adenomas. mRNA expression levels of β-catenin-related genes *c-Myc*, *Axin2* and *CD44*, were determined using reverse transcription-quantitative PCR analysis in (A) colon and (B) DSI adenomas from *Apc*^{Min} mice treated with 4-HC and Veh (n=5 mice/group). (C) Representative images of β-catenin immunofluorescence staining in colon polyps from *Apc*^{Min} mice following oral administration of 4-HC or Veh. (D) Relative quantitative analysis of β-catenin staining from (C) (n=5 per group). Data are presented as the mean ± SEM. *P<0.05, **P<0.01. 4-HC, 4'-hydroxychalcone; *Apc*^{Min}, adenomatous polyposis coli multiple intestinal neoplasia mouse model; DSI, distal small intestine; ns, not significant; Veh, vehicle.

was demonstrated to occur selectively in adenomas from the colon, but not in adenomas from the DSI (Fig. 4B). This effect was confirmed by immunofluorescence staining of β-catenin. Microscopic examination of β-catenin staining images depicted a decrease in the accumulation of cellular β-catenin in the colon adenomas of 4-HC-treated mice (Fig. 4C). The quantification of β-catenin staining showed a significant 52% reduction in colon adenomas (P<0.01; Fig. 4D), whereas no significant difference was observed in the DSI adenomas (data not shown).

Discussion

To the best of our knowledge, the present study demonstrated the chemopreventive effects of 4-HC on spontaneous intestinal adenoma formation in *Apc*^{Min} mice for the first time, which is a model that mimics numerous gene regulatory changes present in sporadic human CRC (8). Examination of intestinal adenoma number and size under a dissection microscope revealed that 10 mg/kg/day 4-HC led to a significant suppression of both colon and small intestinal adenoma formation. Treatment with 4-HC decreased both the number and size of adenomas, particularly those from the colon, suggesting its ability to affect tumor initiation and also tumor progression. Although different biological changes are involved in these two events, inhibition of tumor initiation and progression could both be beneficial for the treatment of CRC. Generally, intestinal polyps are often found in middle-aged patients during colonoscopy examinations, and their progression to cancerous lesions occurs within 10-15 years (36). Therefore, agents with the ability to inhibit tumor initiation and progression have a wider window of time to intervene with cancer development and could be used effectively even years after cancer initiation (37).

Although the association between flavonoid intake and CRC risk remains to be fully elucidated, mechanistic studies have revealed the anti-CRC properties of various flavonoids, such as anthocyanidins, apigenin and quercetin. Anthocyanidins have been reported to decrease CRC risk (38,39), largely due to their ability to negatively regulate inflammatory signaling pathways, including NF-κB, MAPK, JNK and STAT. Apigenin can induce G_2/M cell cycle arrest in multiple colon cancer cell lines with decreased expression of cyclin B1 proteins and the cyclin-dependent kinase p34 (40), and possesses proapoptotic features that are associated with its pro-oxidative effect, leading to the increased production of reactive oxygen species and oxidative stress (41). A chalcone derivative, L2H17, was previously reported to have cytotoxic effects on colon cancer cell lines through various biological processes, including induction of G_0/G_1 cell cycle arrest and apoptosis, attenuation of cell migration and invasion, and inactivation of the NF-κB signaling pathway (42). L2H17 also possesses *in vivo* antitumor activity, as determined using a xenograft mouse model of colon cancer (42).

Wnt/β-catenin signaling pathways are abnormally activated in the early stages of CRC (43). A crucial and heavily studied Wnt pathway is canonical Wnt signaling, which functions by regulating the transcriptional coactivator β-catenin and promotes the expression of key developmental genes (44). For example, *c-Myc*, which is a key component of the Wnt signaling pathway and an important transcription factor, is often constitutively expressed in CRC and leads to increased expression of numerous genes that are involved in cell proliferation, differentiation and apoptosis, including c-MYC, CDKN1A, LGR5, CD44, AXIN2 and CCND1 (45-47). Several studies have demonstrated the chemopreventive effect of flavonoids that act as inhibitors of components in the Wnt/β-catenin signaling pathways. For example, apigenin can inhibit Wnt signaling in CRC cells *in vitro*, potentially through lysosomal degradation of β-catenin and downregulation of Wnt target genes such as cyclin D1 and *c-Myc* (48). Quercetin has been reported to disrupt the TCF/β-catenin interaction by suppressing the binding of the Tcf complexes to its specific DNA-binding sites (49). Chalcone lonchocarpin is reported to be a potent inhibitor of the Wnt/β-catenin pathway that acts downstream to stabilize β-catenin expression and impair TCF-mediated transcription (34). Cardamonin, a natural chalcone, was recently described to increase 5-fluorouracil chemosensitivity in gastric cancer cells (BGC-823 cells) by targeting Wnt/β-catenin signaling pathways and blocking β-catenin/TCF4 complex formation (33).

To the best of our knowledge, the present study is the first to identify 4-HC as a negative modulator of the Wnt/β-catenin signaling pathway exclusively in colon adenoma formation in Apc^{Min} mice. Treatment with 4-HC attenuated β-catenin expression at the post-transcriptional level and also affected the expression of downstream genes, including *c-Myc*, *Axin2* and *CD44*. Further studies are required to ascertain why 4-HC inhibits the Wnt/β-catenin pathway selectively in adenomas from the colon, but not in those from the small intestine. Interactions between dietary flavonoids and intestinal microbiota have been proven to be important in the metabolism of dietary flavonoids, and to influence their efficacy on human disease, which may contribute to the phenotypical difference between small intestine and colon adenoma formation (50,51).

There are a number of limitations to the present study. The experimental design in the present study is not optimal. Using other drugs as positive controls may improve the ability to demonstrate chemopreventive effects of 4-HC. Furthermore, additional studies are needed to investigate the dose-dependent effect of 4-HC in intestinal tumorigenesis. Such studies are currently under investigation in our laboratory, awaiting results. Last, the assessment of toxicity in the present study relies on the change of body weight. However, the result would be biased if there is a significant difference in the food consumption, which is lacking in the present study. We will include this in future studies. Overall, the results of the present study provided compelling evidence that 4-HC may prevent the development of small intestinal and colon adenomas in Apc^{Min} mice by inhibiting cancer cell proliferation (Ki-67), increasing cancer cell apoptosis (TUNEL), and in colon adenomas, by attenuating β-catenin activation and limiting downstream gene expression. 4-HC treatment may therefore be a promising natural preventive agent against intestinal tumorigenesis.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

QC, QH and BG designed and supervised the project. QC, JL, JZ and SM performed the experiments. QC, JL, JZ and SM performed the data analysis and figure preparation. QC drafted the manuscript. QH and BG edited the manuscript and oversaw the project and made intellectual efforts in paper revision. All authors have read and approved the final manuscript.

Ethics approval and consent for participate

All animal experiments were approved by and performed in accordance with the recommendations of the Animal Investigation Committee of the West China Second University Hospital, Sichuan University (Chengdu, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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