Ginsenoside Rg3 inhibits colorectal tumor growth through the down-regulation of Wnt/ß-catenin signaling

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Abstract. Colorectal cancer (CRC) is one of the most common and deadly malignancies in the world. Most CRCs are initiated by aberrant activation of the Wnt/ß-catenin signaling pathway. Despite the advances in its early diagnosis, optimized surgical approaches, and chemotherapies, the clinical management of advanced CRC requires effective adjuvant agents. Ginsenoside Rg3 is a single compound isolated from American ginseng (*Panax quinquefolius* L., Araliaceae) and Asian ginseng (*Panax ginseng* C. A. Meyer). We investigated the anticancer activity of Rg3 on colon cancer cells and its potential molecular mechanism behind Rg3's anticancer activity. We found that Rg3 inhibits cell proliferation and viability of cancer cells *in vitro*. This inhibitory effect of Rg3 is, at least in part, mediated by blocking nuclear translocation of the ß-catenin protein and hence inhibiting ß-catenin/Tcf transcriptional activity. Allelic deletion of the oncogenic ß-catenin in HCT116 cells renders the cells more sensitive to Rg3-induced growth inhibition. Using the xenograft tumor model of human colon cancer, we have demonstrated that Rg3 effectively inhibits the growth of tumors derived from the human colon cancer cell line HCT116. Histologic examination revealed that Rg3 inhibits cancer cell proliferation, decreases PNCA expression and diminishes nuclear staining intensity of ß-catenin. Taken together, our results strongly suggest that the anticancer activity of Rg3 may be in part caused by blocking the nuclear translocation of ß-catenin in colon cancer cells. This line of investigation may lead to the development of novel therapies in which Rg3 can be used as an effective adjuvant agent for the clinical management of colorectal cancers.

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Introduction

Cancers of the colon and rectum are the third most common type of cancer worldwide (1,2). Molecular genetics of colon cancer closely follows the paradigm of a single 'gatekeeper gene'. Mutations inactivating the APC (adenomatous polyposis coli) gene are found in approximately 80% of all human colon tumors, and heterozygosity for such mutations produces an autosomal dominant colon cancer predisposition in humans and in murine models. Of the remaining 20%, half of them harbor oncogenic mutations of ß-catenin. The common genetic alterations of colon cancer are the loss-of-function mutations of *APC* tumor suppressor or gain-of-function oncogenic mutations of ß-catenin (3-7). Therefore, aberrant activation of Wnt/ß-catenin signaling plays a critical role in colorectal tumorigenesis (3-7).

Owing to greater understanding of the genetic basis of inherited colorectal cancer and identification of patients at risk, clinical management of colon cancer has made substantial progress in the past decade (2). In recent years, adjuvant chemotherapy has been demonstrated to be effective, primarily in stage III patients, and several new target-oriented drugs are under evaluation and some of them (cetuximab and bevacizumab) have already exhibited a good activity/efficacy, mainly in combination with chemotherapy (1). Optimization of surgery for patients with localized disease has had a major effect on survival at 5 years and 10

years. Treatment with adjuvant fluoropyrimidine for colon and rectal cancers further improves survival, and oxaliplatin-based combination chemo-therapy is now routinely used for stage III disease. Thus, the integration of targeted treatments with conventional cytotoxic chemotherapy drugs can expand the treatment of metastatic disease resulting in survival gains.

Natural products have been one of the major sources for anticancer discoveries due to their tremendous chemical diversity (8,9). An analysis of the number of chemotherapeutic agents and their sources indicates that nearly 60% of approved drugs are derived from natural compounds (9,10). The treatment of human cancer owes much to the important drugs that have been derived from plants (8,9). In fact, plant-derived active principles and their semi-synthetic and synthetic analogs have served as one of the major sources to new anti-cancer drugs. Since 1961, nine plant-derived compounds have been approved for use as anticancer drugs in the US. These agents include vinblastine (Velba), vincristine (Oncovin), etoposide (VP-16), teniposide (VM-26), taxol (Paclitaxel), navelbine (Vinorelbine), taxotere (Docetaxel), topotecan (Hycamtin), and irinotecan (Camptosar) (8). *Panax* L. is a small genus of the family Arliaceae. Nearly all species in the genus *Panax*, such as Asian ginseng (*Panax ginseng* C. A. Meyer), American ginseng (*Panax quinquefolius* L.) and notoginseng (*Panax notoginseng* [Burk] F. H. Chen), are important herbs for different medical conditions (11). Ginseng has many reported health benefits (11,12).

It is generally believed that the active compounds in Asian ginseng, American ginseng and notoginseng are triterpene glycosides or dammarane saponins, commonly referred to as ginsenosides and notoginsenosides (11). The major active components of ginseng are ginsenosides (such as Rg3, Rh2, Rg5, Rk1 and Rp1), a diverse group of steroidal saponins (12,13). Although the anti-tumor activities of ginseng were initially attributed to its immunomodulation activities (12), it has been reported that ginseng or ginsenosides may exhibit direct anti-cancer properties (14-18). Several ginsenosides (e.g., Rh2, Rg3, and Rk1) exhibit anti-proliferative and anti-angiogenic activities *in vitro* and *in vivo* (19-21). Mechanistically, Rg3 and/or Rh2, Rk1, has been shown to inhibit the NF-κB signaling pathway (22) and cell proliferation, and to induce apoptosis in cancer cell lines (23-28). Nonetheless, ginsenosides exhibit a broad range of biological activities, including anticancer activities; and their molecular modes of actions may be diverse and remain largely undefined.

In this study, we sought to investigate the anticancer activity of Rg3 and its potential molecular mechanism in colon cancer cells. We found that Rg3 can inhibit cell proliferation and viability of cancer cells *in vitro*. This inhibitory effect of Rg3 on colon cancer cells is at least in part mediated by blocking nuclear translocation of ß-catenin protein and hence inhibiting ß-catenin/Tcf transcriptional activity. Allelic deletion of the oncogenic ß-catenin in HCT116 cells renders the cells more sensitive to Rg3-induced growth inhibition. Using the xenograft tumor model of human colon cancer, we have demonstrated that Rg3 effectively inhibits the growth of tumors derived from human colon cancer line HCT116 cells. Histologic examination has revealed that Rg3 inhibits cancer cell proliferation, decreases PNCA expression and diminishes nuclear staining intensity of ß-catenin in Rg3 treatment group. Therefore, our results strongly suggest that the anticancer activity of Rg3 may be in part caused by blocking the nuclear translocation of ß-catenin in colon cancer cells. This line of investigation may lead to the development of novel therapies in which Rg3 can be used as an effective adjuvant agent for the clinical management of colorectal cancers.

Materials and methods

Cell culture. Human colorectal cancer cells HCT116 and SW480 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in the McCoy's 5A medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA) and 50 U penicillin/streptomycin in 5% CO_2 at 37˚C. HEK-293 cells were also obtained from ATCC and maintained in complete DMEM (Invitrogen).

Chemicals and drug preparations. Chemical ginsenoside Rg3 was purchased from the Delta Information Center for Natural Organic Compounds (Xuancheng, Anhui, China). The compound was dissolved in DMSO to make stock solution (15 mM) and was kept at -80˚C as aliquots. Unless otherwise indicated, other chemicals were from Fisher Scientific (Pittsburgh, PA, USA) or Sigma-Aldrich (St. Louis, MO, USA).

Establishment of stable HCT116-Luc cell line. HCT116-Luc cell line that stably expresses firefly luciferase was generated by using a retroviral vector expressing firefly luciferase as described (29-31). Briefly, recombinant retrovirus was packaged in HEK-293 cells by co-transfecting cells with pSEB-Luc and pAmpho packaging plasmid using Lipofectamine (Invitrogen). Pooled stable cells (designated HCT116-Luc) were selected with blasticidin S (0.6 μg/ml) for 7 days. The firefly luciferase activity was confirmed by using Promega's Luciferase Assay kit (Promega, Madison, WI, USA).

MTT proliferation assay. A modified MTT assay was used to examine the cell proliferation effect of ginsenoside Rg3 as described (29-33). Briefly, cells were seeded in 96-well plates ($1x10^4$ cells/well, 50-70% density). Ginsenoside Rg3 was added to the cells in variable concentrations or solvent control without Rg3. At 48 h after treatment, 15 μl MTT dye solution were added to each well and incubated for additional 4 h. Subsequently, 100 μl/well Solubilization/Stop Solution were added to terminate the reactions and to dissolve formazan crystals in a humidified atmosphere overnight. Finally, absorbance at 570 nm was measured using a 96-well micro-plate reader.

Colony formation assay. The colony formation assay was carried out as described (31,34,35). Briefly, cells were treated with ginsenoside Rg3 (100 μM) for 18 h and then replated into 12-well plates with two cell densities (2,000 and 200 cells/well). Colonies were formed in two weeks. Cells were washed with PBS and fixed with methanol and stained was with methylene blue (0.04%) (36).

Crystal violet viability assay. Crystal violet assay was conducted as described (29-35,37,38). Experimentally, HCT116

cells were treated with indicated concentrations of Rg3. At 48 h or 72 h after treatment, cells were carefully washed with PBS and stained with 0.5% crystal violet formalin solution at room temperature for 20-30 min. The stained cells were washed with tape water and air dried for taking macrographic images. For quantitative measurement, the stained cells were dissolved in 10% acetic acid (1 ml per well for 12-well plate) at room temperature for 20 min with shaking. Five hundred microliters were taken and added to 2 ml ddH_2O. Absorbance at 570-590 nm was measured (39-41).

Luciferase reporter assay. Firefly luciferase reporter assay was carried out as described previously (29,34,37,38,42-49). Briefly, HCT116 cells were seeded in 25 cm^2 culture flasks and transfected with 3.0 μg per flask of pTOP-Luc luciferase reporter (42,43,48,49) using Lipofectamine (Invitrogen). At 16 h post transfection, cells were replated in 12-well plates and treated with various concentrations of Rg3 or solvent control. At 36 h, cells were lysed and subjected to luciferase activity assays using Luciferase Assay kit (Promega). Each assay condition was done in triplicate.

Western blot analysis. Western blotting experiments were carried out essentially the same as described (45-47,50-52). Briefly, HCT116 cells were seeded in 6-well plates ($2x10^5$ cells/well) and treated with 60 μM, or 100 μM Rg3 or solvent control for 24 h. Cells were first washed with ice-cold PBS and lysed in 300 μl cell lysis buffer. Cell lysates were boiled for 10 min. The total cellular protein concentration of each supernatant was determined by using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Hercules, CA, USA). The supernatants were subjected to SDS-PAGE and electrical transfer onto PVDF membranes (GE Healthcare Life Sciences, Piscataway, NJ, USA). The membranes were immunoblotted with various primary antibodies against ß-catenin, c-Myc, and ß-actin (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with biotinylated second antibodies. The immunoblots were subsequently incubated with streptavidin-HRP. The proteins of interest were visualized by using the SuperSignal West Pico Substrate (Pierce, Rockford, IL, USA).

Immunofluorescence staining of ß-catenin in cultured cells. Immunofluorescence staining procedure was conducted as described (29,31,37,38,47,50-53). Briefly, SW480 cells were seeded in 24-well plates and treated with different concentrations of Rg3, and solvent control for 1 h. Cells were fixed with ice-cold methanol in -20˚C for 15 min, washed twice with cold PBS and permeablized with 20% IGEPAL (prepared in PBS) at room temperature for 10 min. Cells were blocked with 3% BSA at room temperature for 60 min, followed by incubation with ß-catenin antibody or isotype mouse IgG (at room temperature x 1 h) and then with anti-mouse-FITC secondary antibody (at room temperature x 30 min). Finally, cells were stained with a DNA specific dye, Hoechst 33258. Fluorescence images were recorded under an inverted microscope.

Xenograft tumor model of human colon cancer. The use and care of animals was approved by the Institutional Animal Care and Use Committee. Female athymic nude mice, age of 4-6 weeks, 5 mice per group (Harlan Sprague Dawley, Indianapolis, IN, USA) were used. Subconfluent HCT116-Luc cells were harvested and resuspended in PBS to a final density of $4x10^7$ cells/ml. Prior to injection, cells were resuspended in PBS and analyzed by 0.4% trypan blue exclusion assay (viable cells >90%). For subcutaneous injection, approximately $2x10^6$ HCT116-Luc cells in 50 μl PBS were injected into the flanks of each mouse using a 27 G needle. At 1 week after tumor cell injection, Rg3 was administered at 20 mg/kg body weight to mice daily for 3 weeks via i.p. injection.

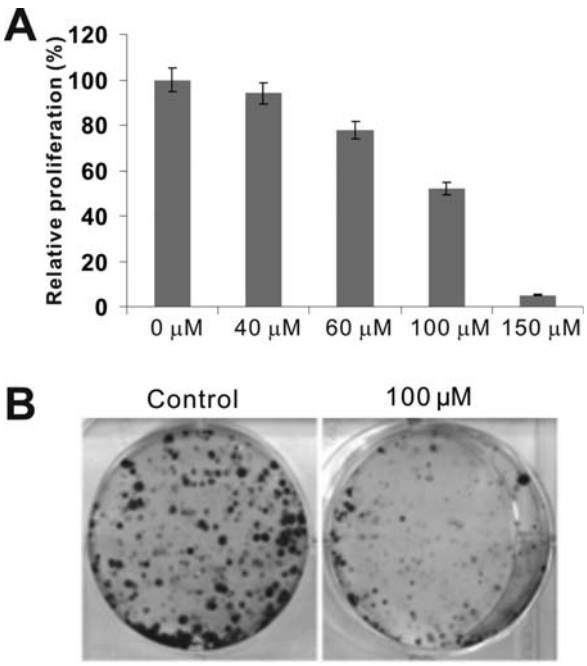

Figure 1. Effect of Rg3 on proliferation activity of human colorectal cancer cells. (A) MTT proliferation assay. HCT116 cells were plated at $1x10^4$ cells/well and treated with indicated concentrations of ginsenoside Rg3 or solvent control for 48 h. MTT assays were carried out as described in Materials and methods. (B) Colony growth assay. HCT116 cells were first seeded at a low density on 100 mm culture dishes, followed by treatment with Rg3 (100 μM) or solvent for 18 h. The treated cells were then replated in 12-well plates and grown for 2 weeks. Macrographic images were taken to document the results.

Xenogen bioluminescence imaging. Small animal whole body optical imaging was carried out as described (29-31). Briefly, mice were anesthetized with isoflurane attached to a nose-cone mask equipped with Xenogen IVIS 200 imaging system (Caliper Life Sciences, Hopkinton, MA, USA) and subjected to imaging weekly after subcutaneous injection. For imaging, mice were injected (i.p.) with D-Luciferin sodium salt (Gold Biotechnology, St. Louis, MO, USA) at 100 mg/kg bw in 0.1 ml sterile PBS. Acquired pseudo images were obtained by superimposing the emitted light over the grayscale photographs of the animal. Quantitative analysis was done with Xenogen's Living Image V2.50.1 software as described (29-31). Animals were sacrificed after 3 weeks, and tumor samples were retrieved for histologic examination.

Histologic evaluation and immunohistochemical staining. Retrieved tumor tissues were fixed in 10% formalin and

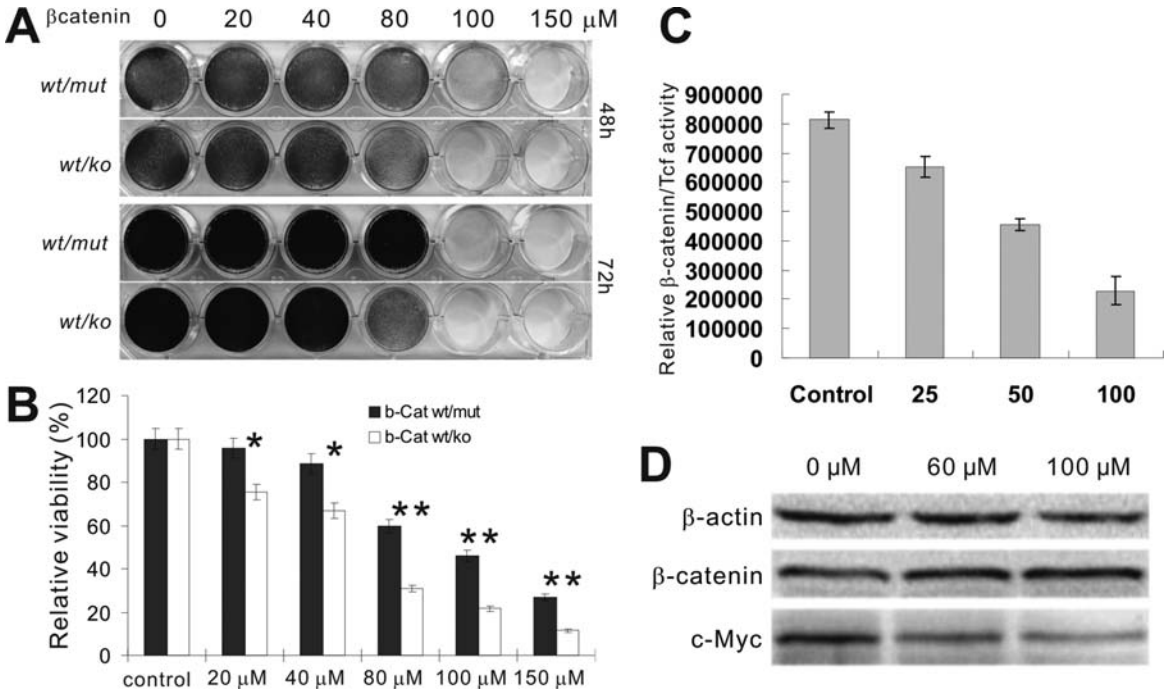

Figure 2. Rg3-mediated inhibition of Wnt/ß-catenin signaling in human colorectal cancer cells. (A) Crystal violet cell viability assay. HCT116 parental cells (HCT116 ß-catenin$^{wt/mut}$) cells and the oncogenic ß-catenin knockout subline (HCT116 ß-catenin$^{wt/ko}$) were seeded in 24-well plates and treated with indicated concentrations of Rg3 for 48 h. Cells were fixed and subjected to crystal violet staining as described in Materials and methods. Each assay condition was done in duplicate. (B) Quantitative analysis of crystal violet staining assays. Crystal violet stained cells (72 h treatment) were dissolved in 10% acetic acid solution. Absorbance at 570 nm was measured. $^{*}p<0.05$; $^{**}p<0.01$. (C) Inhibition of Wnt/ß-catenin-mediated transcriptional activity by Rg3 in human colon cancer cells. HCT116 cells were seeded in 25 cm^2 culture flasks and transfected with the ß-catenin/Tcf reporter pTOP-Luc for 16 h. The transfected cells were trypsinized, replated into 12-well plates and treated with indicated concentrations of Rg3 or solvent control. Each assay condition was done in triplicate. (D) Effect of Rg3 on protein expression of ß-catenin and c-Myc in human colon cancer cells. Subconfluent HCT116 cells were treated with indicated concentrations of Rg3 or solvent control for 24 h. Cells were lyzed and subjected to SDS-PAGE Western blot analyses using ß-catenin, c-Myc, and ß-actin (loading control) antibodies (Santa Cruz Biotechnology). The presence of the proteins of interest was detected by biotinylated second antibodies Streptavidin-HRP (Pierce), and visualized by using SuperSignal West Pico Chemiluminescent Substrate (Fisher-Thermo) as described in Materials and methods.

embedded in paraffin. Serial sections of the embedded specimens were stained with hematoxylin and eosin (H&E). For immunohistochemical staining, slides were deparaffinized and then rehydrated in a graduated fashion (29-31,33,35,44). The deparaffinized slides were subjected to antigen retrieval and probed with an anti-proliferating cell nuclear antigen (PCNA) antibody or anti-ß-catenin antibody (both from Santa Cruz Biotechnology), or isotype IgG control, followed by incubation with biotin secondary antibodies and streptavidin-HRP. The proteins of interest were visualized by 3,3'-diaminobenzidine staining (29-31,33,35,44).

Statistical analysis. All quantitative experiments were performed in triplicate and/or repeated 3 times. Data were expressed as mean ± standard deviation (SD). Statistical significances between vehicle treatment vs. drug-treatment were determined by one-way ANOVA and the Student's t-test. A value of $p<0.05$ was considered statistically significant.

Results

Ginsenoside Rg3 inhibits the proliferation of human colon cancer cells in vitro. In order to determine if Rg3 can function as a novel chemotherapeutic and/or chemopreventive agent for human colon cancer, we first examined the effect of Rg3 on the proliferative activity of human colon cancer cells. As shown in Fig. 1A, the proliferation of human colon cancer HCT116 cells was inhibited by Rg3 in a dose-dependent fashion *in vitro*, although the most significant inhibition was found between 100 to 150 μM as assessed by MTT assays. Similar results were found when another human colon cancer line SW480 was used (data not shown). We also tested the effect of Rg3 on colony formation and found that treatment of Rg3 at 100 μM effectively inhibited colony formation of HCT116 cells (Fig. 1B). These results indicate that Rg3 has potential to inhibit the proliferation of human colon cancer cells.

The growth inhibitory effect of Rg3 may be mediated by ß-catenin function in human colon cancer cells. Aberrant activation of Wnt/ß-catenin signaling plays a critical role in colorectal tumorigenesis (3-7). The common genetic alterations of colon cancer are mostly loss-of-function mutations of APC tumor suppressor or gain-of-function oncogenic mutations of ß-catenin (3-7). Human colon cancer line HCT116 harbors an oncogenic mutation of one allele of ß-catenin (designated as the parental line, or HCT116$^{wt/mut}$) (54-56). To determine if Rg3 targets Wnt/ß-catenin signaling pathway, we took advantage of a previously generated isogenic knockout line of HCT116 that contains the deletion of oncogenic ß-catenin (designated as HCT116$^{wt/ko}$) (57). We first examined the cell

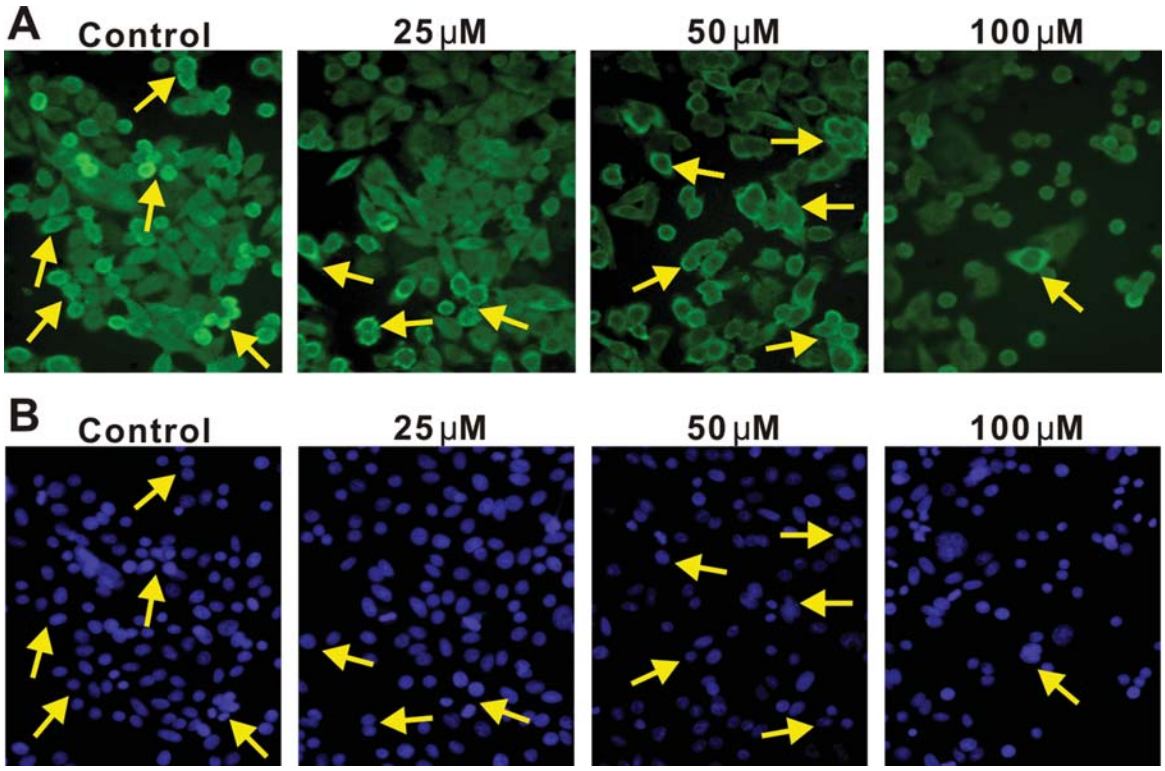

Figure 3. Effect of Rg3 on the cellular localization of ß-catenin in human colorectal cancer cells. (A) Cellular localization of ß-catenin after Rg3 treatment. Subconfluent SW480 human colon cancer cells were subjected to treatment for 1 h with the indicated concentrations. The cells were fixed, permeabilized and stained with ß-catenin antibody or control IgG (green). Arrows indicate nuclear and perinuclear staining of ß-catenin. Representative images are shown. (B) Nuclear staining with Hoechst 33258. The SW480 cells were treated in the same fashion as that in (A), and subjected to Hoechst 33258 staining (blue). Arrows indicate the same cells highlighted in (A). Representative images are shown.

viability upon Rg3 treatment of the parental HCT116 (i.e., wt/mut form) and the ß-catenin knockout subline (i.e., wt/ko form). Using the crystal violet viability assay, we found that cell viability decreased in both HCT116$^{wt/mut}$ and HCT116$^{wt/ko}$ cell lines in a dose-dependent fashion at 48 h and 72 h post treatment (Fig. 2A), although the oncogenic ß-catenin-deficient line (HCT116$^{wt/ko}$) was more sensitive to Rg3 treatment.

The differential responsiveness to Rg3 by HCT116$^{wt/mut}$ and HCT116$^{wt/ko}$ cell lines was more pronounced by a quantitative analysis. As shown in Fig. 2B, cell viability upon Rg3 treatment was significantly decreased in the absence of oncogenic ß-catenin at a concentration as low as 20 μM ($p<0.05$). The calculated IC_{50} for Rg3 from the crystal violet assay was 76.4 μM and 57.7 μM for HCT116$^{wt/mut}$ and HCT116$^{wt/ko}$ lines, respectively. These data suggest that Rg3 may inhibit the proliferative and survival abilities of oncogenic ß-catenin in parental HCT116 cells.

To further examine the potential point of action for Rg3 on Wnt/ß-catenin signaling, we analyzed the effect of Rg3 on Wnt/ß-catenin-mediated transcriptional activity by employing a commonly used pTOP-Luc reporter (42,43,48,54,55,58). We transfected this reporter into HCT116 cells and treated the cells with Rg3. Our results revealed that the pTOP-Luc reporter activity was inhibited by Rg3 in a dose-dependent fashion (Fig. 2C). Similar results were obtained from SW480 cells (data not shown). We further examined the expression levels of one of the known targets of Wnt/ß-catenin signaling, c-Myc (43,59), in response to Rg3 treatment. We found that c-Myc expression decreased in Rg3-treated HCT116 cells, while the protein level of ß-catenin remained relatively unchanged (Fig. 2D). Therefore, the above results suggest that Rg3 may directly target ß-catenin/Tcf transcriptional activity without significantly affecting ß-catenin protein level in colon cancer cells.

Decreased nuclear localization of the ß-catenin in response to Rg3 treatment. To further explore the mechanism of Rg3-mediated inhibition of Tcf/ß-catenin signaling activity, we conducted the immunofluorescence staining assay in human colon cancer line SW480 cells. It has been reported that SW480 cells harbor an APC truncation mutation at amino acid 1338 (56,60). Therefore, these cells exhibit an uncontrolled, aberrantly activated Tcf/ß-catenin function, as demonstrated by a strong nuclear ß-catenin staining (Fig. 3A, control panel). When SW480 cells were treated with various concentrations of Rg3, nuclear staining of ß-catenin decreased significantly in the treated cells (Fig. 3A vs. B). Interestingly, the nuclear localization of ß-catenin in the control group significantly shifted to perinuclear staining pattern in Rg3-treated cells. These results are consistent with the findings shown in Fig. 2C and D, in which ß-catenin/Tcf-mediated transcriptional activity and the expression of the Wnt target gene c-Myc were inhibited by Rg3. Taken together, these results suggest that Rg3-mediated inhibition of ß-catenin/Tcf activity may be at least in part caused by the blockade of nuclear translocation of ß-catenin protein in colon cancer cells.

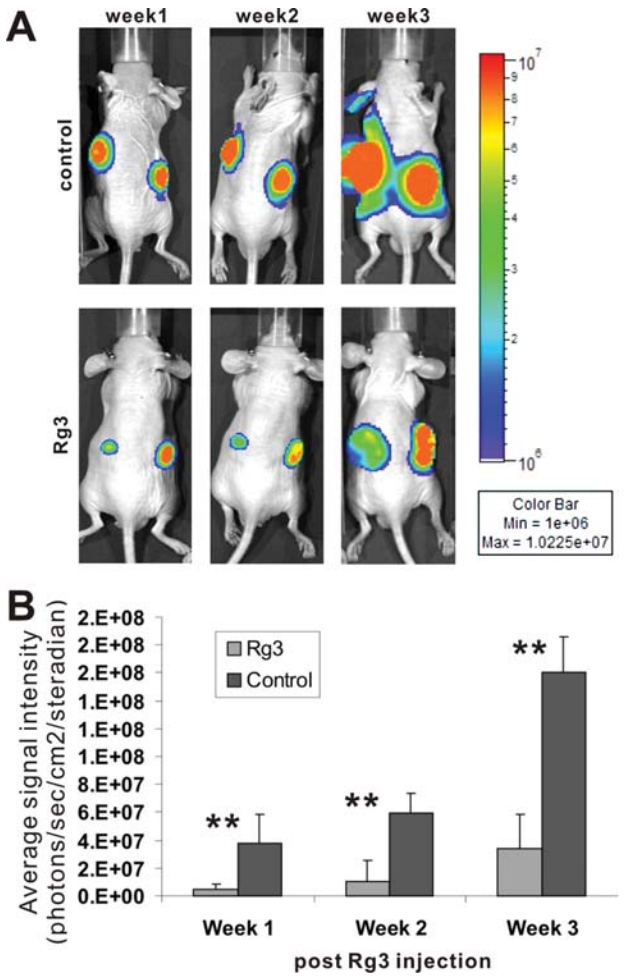

Figure 4. Inhibition of xenograft tumor growth of human colon cancer cells by Rg3. (A) Representative bioluminescence images of tumor-bearing athymic nude mice. Firefly luciferase-tagged HCT116 cells were collected and injected into the flanks of athymic mice subcutaneously ($2x10^6$ cells/injection; five animals per assay condition). After 1 week, mice were treated with Rg3 (20 mg/kg body weight, i.p., 5 times/week) or solvent control. The animals were subjected to weekly Xenogen bioluminescence imaging for additional 3 weeks. Representative Xenogen images of the athymic mice are shown. (B) Quantitative analysis of Xenogen bioluminescence imaging. The signal intensity of tumors derived from HCT116 cells (in photons per second per square centimeter per steradian) were calculated by using Xenogen's Living Image software. Data are expressed as mean ± SD. $^{**}p<0.001$.

Rg3 inhibits tumor growth in xenograft model of human colon cancer. We further examined the anticancer activity of Rg3 *in vivo*. Using a xenograft tumor model, we first injected the firefly luciferase-tagged colon cancer line HCT116 cells subcutaneously into the flanks of athymic nude mice. At one week after tumor cell injection, mice were treated with Rg3 (20 mg/kg body weight, i.p., 5 times/week) or solvent control. The animals were subjected to weekly Xenogen bioluminescence imaging on a weekly base for 3 weeks. Whole body bioluminescence imaging results indicated that tumor signals were much weaker in Rg3 treatment group at each time point (Fig. 4A), although the tumors seemed to grow slowly even in the Rg3 treatment group. Quantitative analysis of the Xenogen imaging signals revealed that the reduction of signal intensity in Rg3 treatment group vs. that in control group was statistically significant at each time point ($p<0.001$) (Fig. 4B).

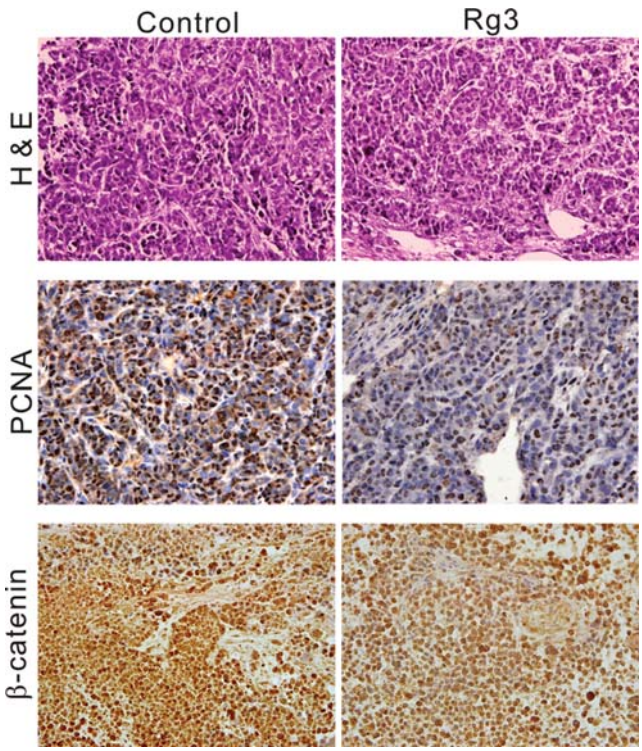

Figure 5. Histologic and immunohistochemical staining of Rg3 treated colorectal tumors. Tumor samples derived from HCT116 xenografts were retrieved, fixed and paraffin embedded. Sections were used for hematoxylin and eosin (H&E) staining. Deparaffinized slides were stained with antibodies against ß-catenin (Santa Cruz Biotechnology) and PCNA (Santa Cruz Biotechnology). Isotype IgG was used as a negative control (not shown). Representative images are shown.

These results suggest that Rg3 may exert strong anticancer activity in colon cancer cells both *in vitro* and *in vivo*.

Rg3 inhibits the proliferative activity of colon tumor cells. We subsequently conducted histologic evaluation of the HCT116 xenograft tumor samples. Hematoxylin and eosin (H&E) staining indicated that tumor cells were highly proliferative in the control group while slightly less proliferative in Rg3 treatment group (Fig. 5, top panel). The difference in proliferative activity was more pronounced when the tumor sections were immunostained with an anti-PCNA antibody (Fig. 5, middle panel). PCNA-positively stained cells were significantly decreased in the Rg3 treatment group, compared with that in the vehicle control group (Fig. 5, middle panel). We further analyzed the expression pattern of ß-catenin protein and found that the signal intensity of ß-catenin staining was significantly decreased in Rg3 treatment group, compared to that of the control group (Fig. 5, bottom panel). Consistent with the results shown in Fig. 3, the reduced ß-catenin staining in Rg3 treatment group was seemingly caused by the decrease in nuclear staining intensity while the overall numbers of positively stained cells were comparable to that of the control group. Thus, these results strongly suggest that the anticancer activity of Rg3 may be at least in part caused by blocking the nuclear translocation of ß-catenin in colon cancer cells. However, the exact mechanism through which Rg3 inhibits tumor growth and down-regulates ß-catenin/Tcf activity in cancer cells is currently unknown and needs to be thoroughly investigated.

Discussion

More than 1 million individuals worldwide will develop colorectal cancer annually and the disease-specific mortality rate is nearly 33% in the developed world (1,2,5,61). The classic description of colorectal carcinogenesis is the adenoma to carcinoma sequence and multistep tumorigenesis that is determined by gatekeeper and caretaker molecular pathways, which takes years to decades (5,62). However, colorectal cancer is increasingly classified into specific phenotypes on the basis of molecular profiles, two of which represent genetic instability classes (2,63). Most sporadic cases (about 85%) have chromosomal instability, an allelic imbalance at several chromosomal loci and chromosome amplification and translocation (62,63). By contrast, the remaining cases (about 15%) have high-frequency microsatellite instability phenotypes, i.e., frameshift mutations and base-pair substitutions that commonly arise in short tandem repeat nucleotide sequences (microsatellites) (64,65).

There have been considerable advances in understanding the molecular pathogenesis, in diagnosis (both hereditary and sporadic), and in treatment of colorectal cancer (2). The integration of targeted treatments with conventional cytotoxic drugs has expanded the treatment of metastatic disease resulting in incremental survival gains. Despite the use of active targeted drugs for treatment of metastatic colorectal cancer in the past decade, cure rates remain low. Therefore, clinical and translational research on new therapies is needed to provide the much promised hope of personalized medicine in the management of this cancer.

In this study, we have investigated the anticancer activity of ginsenoside Rg3 and its potential mechanism in colon cancer cells. We have found that Rg3 can inhibit cell proliferation and viability of cancer cells *in vitro*. This inhibitory effect of Rg3 on colon cancer cells is at least in part mediated by blocking nuclear translocation of ß-catenin protein and hence inhibiting ß-catenin/Tcf transcriptional activity. Using a xenograft tumor model of human colon cancer, we have demonstrated that Rg3 effectively inhibits the growth of tumors derived from human colon cancer line HCT116 cells. Histologic examination has revealed that Rg3 inhibits cancer cell proliferation, decreases PNCA expression and diminishes nuclear staining intensity of ß-catenin in Rg3 treatment group. Therefore, our *in vitro* and *in vivo* results strongly suggest that the anticancer activity of Rg3 may be in part mediated by blocking the nuclear translocation of ß-catenin in colon cancer cells. However, the exact mechanism through which Rg3 inhibits tumor growth and down-regulates ß-catenin/Tcf activity in cancer cells is currently unknown and needs to be further investigated.

Ginsenoside Rg3 is a single compound isolated from both American ginseng (*Panax quinquefolius* L., Araliaceae) and Asian ginseng (*Panax ginseng* C. A. Meyer) (66-69). Ginseng has been used herbal medicine for many therapeutic purposes for thousands of years (12,66,70). It is generally accepted that the anti-tumor activities of ginseng are mediated by ginseng's immunomodulation (67-69,71). However, for the past decade investigations have demonstrated the direct anticancer properties possessed by ginseng and its active anticancer compounds ginsenosides (14-18,72). We previously evaluated the cancer chemopreventive activities of American ginseng root extract (AGE and S-AGE) and fraction (S2h) on human colorectal and breast cancer (27). Our microarray data indicated that the expression levels of 76 genes were changed significantly after treatment with S2h or Rg3 in HCT116 cells (28). Gene functions involved in cell morphology, gene expression, cell proliferation, and cell cycle, are among the most significantly affected functions by both Rg3 and S2h in human cancer line HCT116 cells (28).

As the major compounds isolated from ginseng, ginsenosides (such as Rg3, Rh2, Rg5, Rk1 and Rp1) exhibit anti-proliferative activity and anti-angiogenesis effects. Kim *et al* studied 11 ginsenosides and determined that Rg3 and Rh2 inhibited proliferation of prostate cancer cells (19). Rg3 has been shown to function as an angiogenic inhibitor alone or in combination with other chemotherapeutic agents (20,73-75). Iishi *et al* used a rat AOM-induced tumor model to determine the effects of Rg3 in inhibiting cell proliferation of colon cancer cells (21). Rg3 has been shown to inhibit NF-κB signaling (22,76-81) and enhances the susceptibility of prostate cancer cells to docetaxel and other chemotherapeutics (22). Jia *et al* reported that Rh2 inhibited proliferation, induced apoptosis in cancer cell lines, and sensitized drug-resistant breast cancer cells to paclitaxel (23). Rk1 can inhibit the proliferation of human hepatocellular carcinoma cells by reducing the activity of telomerase (24). Rg5 also exhibits anti-proliferative activity by suppressing cyclin E-dependent protein kinase activity (25). Rp1 may act as an inhibitor of IL-1ß production by inhibiting the NF-κB pathway (26). Thus, it is conceivable that different ginsenosides may exhibit distinct biological activities.

In summary, although ginsenosides may exhibit a broad range of biological activities, including anticancer activities, their molecular modes of actions may be diverse and remain largely undefined. The reported studies have been designed to examine the anticancer activity of Rg3 *in vivo*, and more importantly to uncover its molecular mechanism through which Rg3 exerts its anticancer activity in human colon cancer. We have found that Rg3 can inhibit cell proliferation and viability of cancer cells *in vitro*. This inhibitory effect of Rg3 on colon cancer cells is at least in part mediated by blocking nuclear translocation of ß-catenin protein and hence inhibiting ß-catenin/Tcf transcriptional activity. We have further demonstrated that Rg3 effectively inhibits the growth of tumors *in vivo*. Rg3 has been shown to inhibit cancer cell proliferation, decrease PNCA expression and diminish nuclear staining intensity of ß-catenin in colon tumors. Therefore, our results strongly suggest that the anticancer activity of Rg3 may be in part mediated by blocking the nuclear translocation of ß-catenin in colon cancer cells. Future studies should be directed towards dissecting the exact mechanism through which Rg3 inhibits tumor growth and down-regulates ß-catenin/Tcf activity in cancer cells. Ultimately, this line of investigation may lead to the development of novel therapies in which Rg3 can be used as an effective adjuvant agent for the clinical management of colorectal cancers.

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