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Anti-colorectal cancer activity of macrostemonoside A mediated by reactive oxygen species

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ABSTRACT

Macrostemonoside A (MSS.A), an active steroidal saponin from Allium macrostemon Bung has been shown to possess anti-coagulation and anti-obesity effects. However, the functional role of MSS.A on tumor growth has not been elucidated. We found that MSS.A significantly inhibited human colorectal cancer cell growth in Caco2 and SW480 cells. Incubation of SW480 cells with MSS.A for 48 h resulted in cell cycle arrest. Moreover, MSS.A dose-dependently induced apoptosis in SW480 cells as shown by increased AnnexinV positively stained cell population, caspase activation, increased pro-apoptotic and reduced anti-apoptotic Bcl-2 family protein levels. Treatment of SW480 cells with MSS.A resulted in increased reactive oxygen species (ROS) generation. However, pre-incubation of SW480 cells with anti-oxidant N-acetylcysteine (NAC) attenuated the ROS generation and anti-colorectal cancer activities of MSS.A. Lastly, intra-peritoneal injections of MSS.A significantly inhibited tumor formation in BALB/c nude mice carcinogenesis xenograft model by reduced tumor volume and tumor weight when treated at dosages of 10, 50 or 100 mg/kg daily for 35 days compared with PBS control. Taken together, our results indicate that MSS.A suppressed colorectal cancer growth and induced cell apoptosis by inducing ROS production, and that MSS.A may have therapeutic relevance in the treatment of human colorectal cancer.

1. Introduction

Colorectal cancer is the third most common malignant disease in the United States [1]. Colorectal cancer has also become a common malignancy in developing countries such as China because of environmental factors and increased proportion of older age classes [2,3]. The most common treatment of colorectal cancer is surgery. However, cancer involving the colon or rectum can reappear at a later time, even if the cancer was entirely removed during the initial treatment [4]. Besides surgical resection, treatment strategies for high risk patients are still mainly based on adjuvant use of 5-fluorouracil as chemotherapeutic agent alone or in combination with radiotherapy [5]. Unfortunately, use of the above standard therapeutic protocols results only in a moderate decline in mortality [6]. Therefore, significant effort has been exerted to identify novel drug targets to reduce incidence and malignancy of colorectal cancer cells.

Reactive oxygen species (ROS), which are the byproducts of cellular oxidative processes, act as second messengers in cell signaling and play critical roles in the regulation of diverse functions [7–10]. Excessive ROS stress could promote cell apoptosis by stimulating pro-apoptotic signaling molecules, such as ASK1, JNK and p38 or by directly accelerating mitochondrial depolarization and dysfunction [11]. Recently, inducing ROS generation has become a novel approach to treat cancer because its potential selectivity towards cancer cells. The cancer cells usually have a relatively high basal level of ROS, hence a small induction of ROS in tumor cells may push the level of ROS over the threshold that induces cell death, whereas normal cells are less vulnerable to the oxidative insults because of their lower ROS basal level [11]. Therefore, manipulating ROS levels by redox modulation is a way to selectively kill cancer cells without causing significant toxicity to normal cells [12]. Several compounds which exhibit potent ability to promote ROS generation or interfere with ROS metabolism have shown promising anti-cancer activity *in vitro* or *in vivo* [13–15].

Natural product-oriented synthetic derivatives have played an important role in anti-cancer drug discovery [16,17]. Allium macrostemon Bung (Chinese name, Xie bai), a traditional Chinese

Abbreviations: AFC, aminofluorocoumarin; MSS.A, macrostemonoside A; PI, propidium iodide; NAC, N-acetylcysteine; PCNA, proliferating cell nuclear antigen; ROS, reactive oxygen species.

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medicine, has extensive pharmacological activities and was clinically used for the treatment of coronary heart disease. Macrostemonoside A (MSS.A) is the main steroidal saponin in Allium macrostemon Bung. Its structure is tigogenin-3-O-beta-D-glucopyranosyl(1-2)(beta-D-glucopyranosyl(1-3))-beta-D-glucopyranosyl(1-4)-beta-D-galactopyranoside (Fig. 1A). It was reported that MSS.A has anti-coagulation activity *in vitro* [18]. In addition, MSS.A markedly stimulated visfatin expression in 3T3-L1 adipocytes via p38 MAPK signaling pathway [19]. Most recent studies demonstrated the anti-obesity effect of MSS.A on high-fat diet-fed C57NL/6 mice via upregulating PPARγ2 expression [20]. In this study, for the first time, we investigated the potent therapeutic activity of MSS.A on colorectal cancer cells growth and apoptosis, examined its molecular targets and provided a basis for further development of this agent as human colorectal cancer therapy.

2. Materials and methods

2.1. Cell culture

Human Caco2 and SW480 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in DMEM/F-12 1:1 modified medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. MSS.A was purified from Allium macrostemon Bung with the purity >97% (Fig. 1A, supplemental Fig. 1).

2.2. Cell growth and proliferation assay

Cell growth kinetics was assessed by determining cellular confluence using trypan blue exclusion method. Briefly, cells (2×10^4/ml) were grown in 24-well culture plates in medium with 10% FBS. After 24 h, the medium was removed and fresh medium with 1% FBS containing MSS.A was added (0 h). On the subsequent 60 h, total cell counts were determined by trypan blue exclusion method using a hemocytometer. Data was presented as % of cell confluence. Cell proliferation assay was measured by CellTiter 96 AQueous One Solution Cell Proliferation (MTS) Assay. Briefly, cells (3000/well) were seeded in 96-well plates and treated with indicated reagents in media containing 1% FBS for 0, 1, 2 and 3 days. MTS (20 μl) solution was added for 1 h at 37 °C. Absorbance at 490 nm was recorded (Bio-Tek Instruments, Winooski, VT, USA).

2.3. Cell cycle analysis

Cultured cells were trypsinized and fixed with 70% ethanol at 4 °C overnight before being stained with propidium iodide (PI). DNA contents were detected by LSRII flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed by Flow Jo (Tree Star, Ashland, OR).

2.4. Cell apoptosis assay by flow cytometry

Cells were stained with FITC-conjugated Annexin V (Clontech, Beijing, China), an early marker for apoptotic cells and with PI for necrotic cells. Cells were measured by LSRII flow cytometer and analyzed by FACScan (BD Biosciences).

2.5. Assessment of caspase activity

Caspases 3 and 9 activities were determined by using aminofluorocoumarin (AFC)-conjugated substrates supplied by Bio-Rad Laboratories (Hercules, CA, USA). Briefly, cells (1×10^6) were lysed in 50 μl of chilled cell lysis buffer. 50 μl of 2× reaction buffer with 10 μM DTT and 5 μl of the conjugate substrate (DEVD-AFC for caspase-3 and LEHD-AFC for caspase-9) were added to each sample. Cells were incubated for 60 min at 37 °C. Caspase activity was determined by the relative fluorescence intensity at 505 nm following excitation at 400 nm with a spectrofluorometer (Shimadzu, Kyoto, Japan). Results are shown as fold increase in activity relative to control.

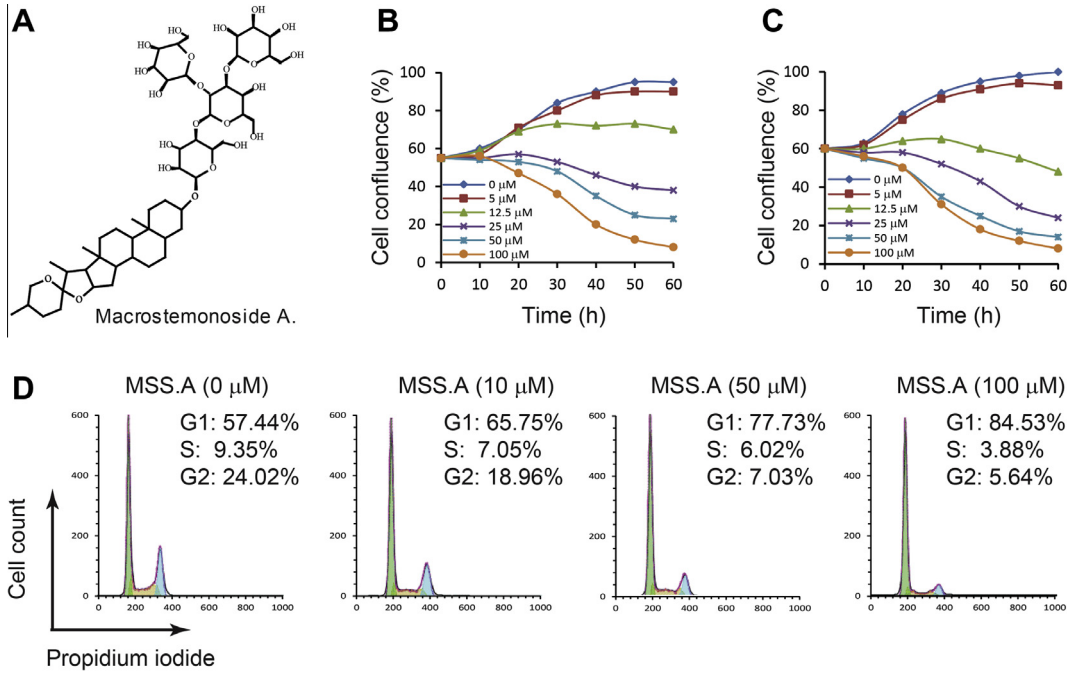

Fig. 1. Macrostemonoside A (MSS.A) inhibited human colorectal cancer cells growth. (A) Structure of MSS.A. (B and C) Cell growth assay in Caco2 (B) and SW480 cells (C). Cell numbers were determined by trypan blue exclusion method on the subsequent 60 h. (D) Cell cycle profiles in SW480 cells. Cells were starved in 1% FBS containing media overnight and then treated with MSS.A for 48 h. Cells were stained by propidium iodide (PI) and measured by LSRII. Data are shown as mean ± SEM of three independent experiments.

2.6. Western blot

Cells were lyzed in RIPA buffer supplemented with mixture of protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL, USA). The protein concentration was determined by the BCA protein assay (Thermo Scientific). The primary antibodies used in this study are anti-Bcl-2 (sc-492, Santa Cruz, CA), anti-Bcl-xL (sc-8392), anti-Bax (sc-493), and anti-GAPDH (sc-20357).

2.7. Measurement of intracellular reactive oxygen species (ROS) level

Flow cytometry analysis was carried out to study intracellular ROS. Intracellular ROS levels were measured by incubating cells with 10 mM 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA; Invitrogen) at 37 °C for 20 min.

2.8. Animals and subcutaneous tumor growth

Male athymic BALB/c nude mice (8 weeks old, 18–20 g) were purchased from Beijing Wei-tong Li-hua Laboratory Animals and Technology Ltd. All surgical and care procedures administered to the animals were in accordance with Harbin Medical University institutional guidelines. SW480 cells (1×10^6) in 0.1 ml of Hank's balanced salt solution were injected s.c. into the right scapular region of nude mice. In all studies, mice received either control (PBS) or MSS.A groups (10, 50 and 100 mg/kg) administered daily by i.p injection ($N = 8$ each group). Tumor size was determined every 5 days by caliper measurement of two perpendicular diameters of the implants. The animal body weights were recorded every 5 days. The tumor bearing mice were sacrificed on day 35.

2.9. Immunohistochemistry

Tumor sections were immunostained with anti-proliferating cell nuclear antigen (PCNA) antibody (Abcam). Briefly, paraffin embedded tissues were deparaffinized and rehydrated prior to antibody addition. Anti-PCNA antibody was used at a dilution of 1:1000. Goat anti-rabbit secondary antibody (Dako, Shanghai, China) were used at a dilution of 1:200. Photomicrographs were acquired using Olympus IX71 (Olympus).

2.10. Statistics

All values are expressed as mean ± SEM. Differences among groups were analyzed by ANOVA followed by Bonferroni post hoc analyses as appropriate. P value less than 0.05 was considered significant.

3. Results

3.1. MSS.A inhibited cell growth in human colorectal cancer cells

To investigate whether MSS.A (Fig. 1A) affects the growth of colorectal cancer cells, we measured cell growth in two human colorectal cancer cell lines (Caco2 and SW480). First, exponentially growing Caco2 and SW480 cells were treated with 0, 5, 12.5, 25, 50 or 100 μM MSS.A and live cell numbers were counted on the subsequent 60 h to determine the cell confluency. As shown in Fig. 1B and C, increasing dose of MSS.A dramatically inhibited cell growth in both cells and at 60 h less than 10% cells alive in 100 μM MSS.A treatment groups. Next we assessed cell cycle arrests. We chose SW480 cells, for which the growth inhibition by MSS.A was more sensitive than Caco2 cells. At 48 h after treatment, MSS.A (10, 50 and 100 μM) treated cells showed a higher average proportion of cells in G1 phase (65.75%, 77.73% and 84.53%) compared with the control cells (57.44%), and concomitant decreases in the proportions of cells in S and G2 phase (Fig. 1D). These results suggest that MSS.A significantly inhibited human colorectal cancer cell growth.

3.2. MSS.A induced apoptosis in human colorectal cancer cells

To determine whether MSS.A induces apoptosis in colorectal cancer cells, we performed flow cytometry analysis on MSS.A treated SW480 cells (Fig. 2A). At 24 h, the exposure of SW480 cells to 10 μM MSS.A resulted in about 7.3% of the cells going into early apoptotic phase. With the increase in the dose up to 50 and 100 μM, an significant increase in both early apoptotic cells and late apoptotic cells was observed in MSS.A treated cells, respectively ($P < 0.01$). To determine whether the mitochondrial apoptotic signaling pathway was involved in MSS.A induced apoptosis, expression patterns of pro-apoptotic and anti-apoptotic Bcl-2 family proteins were investigated. As shown in Fig. 2B, MSS.A up-regulated the expression of pro-apoptotic protein Bax, whereas downregulated the expression of anti-apoptotic protein Bcl-2 and Bcl-xL in a dose-dependent manner. To further confirm these findings, we determined the effects the MSS.A treatment on the activation of various caspases. The results (Fig. 2C) showed that MSS.A treatment significantly induced caspase-3 and 9 activities. Taken together, these results suggest that MSS.A triggered apoptosis in human colorectal cancer cells.

3.3. ROS was involved in apoptosis induced by MSS.A

A growing body of evidence indicate that ROS affects cell sensitivity and leads to the activation of cell death pathways [11,21]. Herein, we tested whether the anti-cancer activity of MSS.A is associated with the change in intracellular ROS levels. SW480 cells were treated different doses of MSS.A for 48 h and then loaded with the fluorescent probes carboxy-H2DCFDA for flow cytometry analysis. Compared with the control group, intracellular ROS levels significantly increased in MSS.A treatment group (Fig. 3A). Furthermore, to determine whether ROS was involved in MSS.A induced apoptotic pathway of SW480 cells, ROS scavenger N-acetyl-L-cysteine (NAC) were pretreated on SW480 cells. As expected, the presence of 1 mM antioxidant NAC diminished the accumulation of intracellular ROS caused by MSS.A (Fig. 3B). Meanwhile, the antiproliferative effect of MSS.A was largely reversed by pretreating cells with NAC at 2 and 3 days (Fig. 3C). Annexin V-FITC and PI double staining assay also revealed that the pretreatment with NAC protected SW480 cells from MSS.A induced apoptosis (Fig. 3D). These results indicate that intracellular ROS had an essential role in the anti-colorectal cancer activity of MSS.A.

3.4. MSS.A significantly inhibited the growth of colorectal cancer in vivo

We observed that MSS.A inhibited growth and induced apoptosis in human colorectal cells. Then, we evaluated the ability of MSS.A to inhibit tumor growth in a mouse s.c. injection model. SW480 cells injected s.c. to nude mice gave rise to exponentially growing tumors with an average tumor volume of 964.3 mm^3 at day 35 (Fig. 4A). As can be seen in Fig. 4A and B, daily administration of MSS.A by i.p. injection inhibited SW480 cells growth in a dose-dependent manner with 42.4%, 56.9% and 77.8% inhibition, respectively, at the dose of 10, 50 and 100 mg/kg at day 35. Consistent with tumor volumes, the average tumor weights at 35 days was 2.49 ± 0.66 g and inhibited by 30.4%, 55.2% and 74.4%, respective, at the dose of 10, 50 and 100 mg/kg (Table 1). Cell proliferation marker PCNA was further examined *in situ* in tumor samples from the four groups. As shown in Fig. 4C, MSS.A treated groups

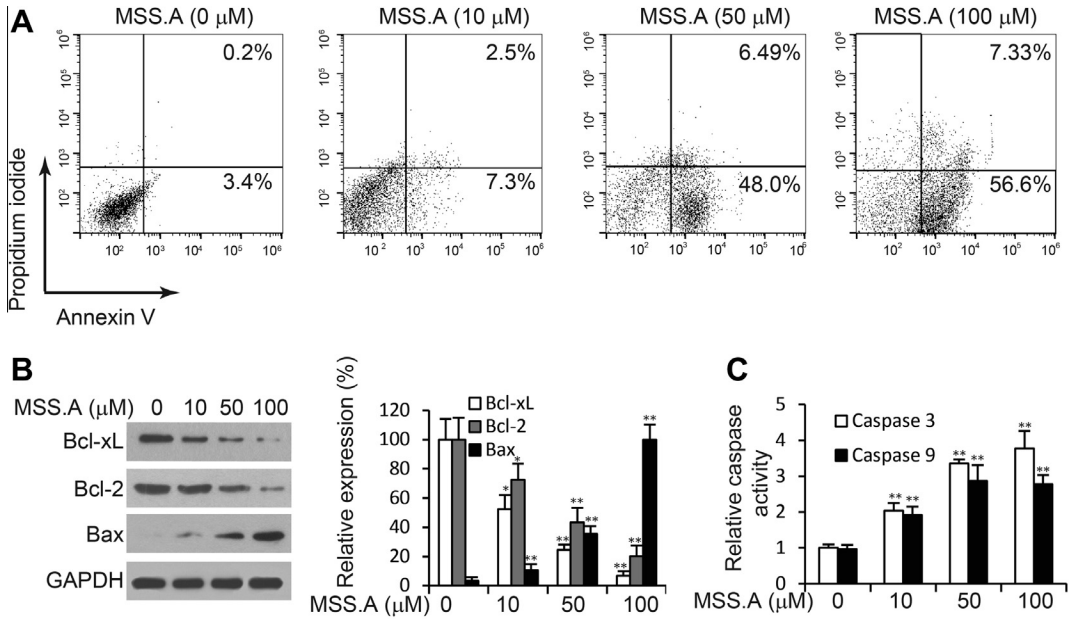

Fig. 2. MSS.A induced human colorectal cancer cells apoptosis. (A) Cell apoptosis profiles in SW480 cells. SW480 cells were treated with MSS.A for 48 h and stained with Annexin V-FITC and PI. (B) Western blot depicting the level of Bcl-2 family members, Bcl-xL, Bcl-2 and Bax in SW480 cells treated with MSS.A for 48 h. The Western blots showed representative of three independent experiments. The intensity of bands from Western blot was quantified using Image J software and normalized to those of GAPDH. (C) Activity assay of caspase3 and 9 in SW480 cells after treated with MSS.A for 48 h. Data are shown as mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control.

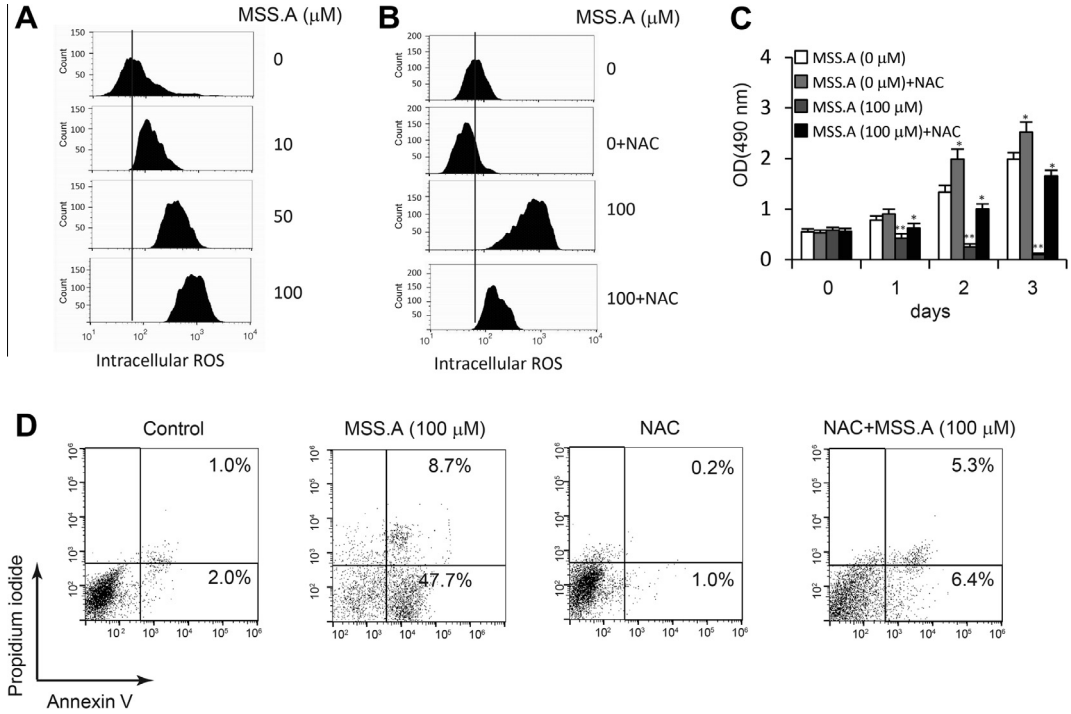

Fig. 3. MSS.A anti-colorectal cancer activity was mediated by intracellular Reactive oxygen species (ROS). (A) Intracellular ROS level was determined by flow cytometry. SW480 cells were treated with MSS.A for 48 h and incubated with carboxy-H2DCFDA for 30 min at 37 °C before flow cytometry analysis. (B) Intracellular ROS level. SW480 cells were pretreated with or without 1 mM N-acetylcysteine (NAC) for 1 h and then co-incubated with 100 μM MSS.A for 48 h. (C) Cell proliferation was determined by MTS assay. SW480 cells were pre-treated with 1 mM NAC for 1 h when co-incubated with 100 μM MSS.A for another 1, 2 and 3 days. (D) Apoptosis assay by Annexin V-FITC/PI staining followed by flow cytometry analysis. SW480 cells were pre-treated with 1 mM NAC for 1 h when co-incubated with 100 μM MSS.A for another 48 h. Data are shown as mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control.

had reduced expression of PCNA compared to control group. Moreover, we found no loss in body weights (Fig. 4D). Taken together, MSS.A inhibited tumor cell growth *in vivo*. The compound shows therapeutic implications in tumor therapy.

4. Discussion

The steroidal saponins isolated from herbs have attracted scientific attention because of their structural diversity and significant

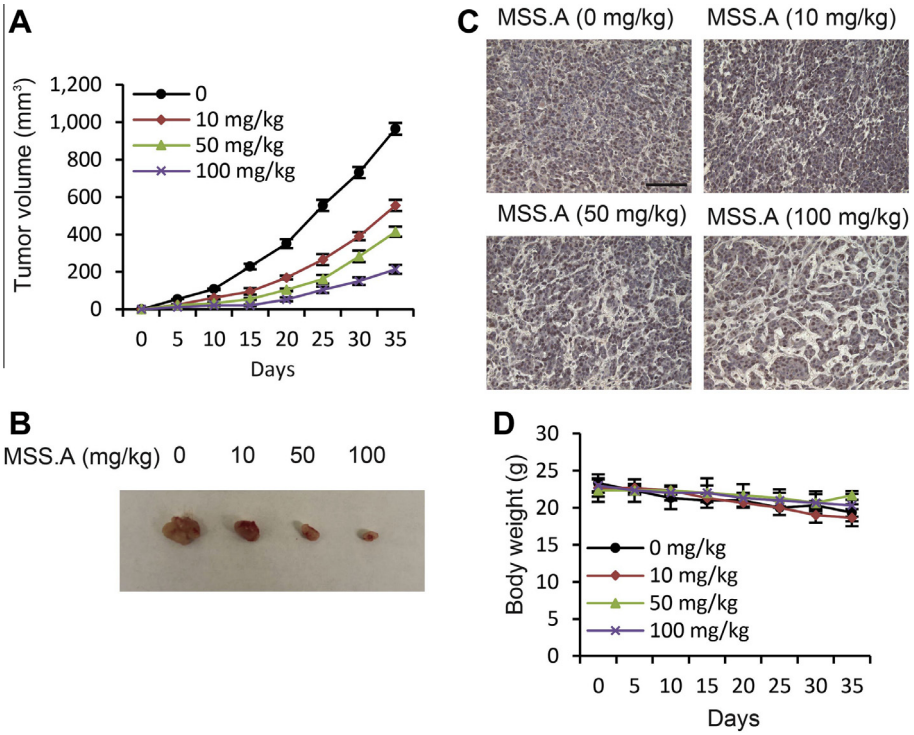

Fig. 4. MSS.A inhibited tumorigenesis in the *in vivo* xenograft model. SW480 cells were injected s.c. into nude mice. Mice were treated with control (PBS) or MSS.A (10, 50 and 100 mg/kg) daily by i.p. injection for 35 days ($N = 8$ mice per group). (A) Mean tumor volumes measured every 5 days by caliper measurement of two perpendicular diameters of the tumors. (B) Representative pictures of tumor tissues from different treatment groups at 35 days after injection. (C) Proliferation analysis of tumor tissues by PCNA immunohistochemistry staining. Scale bar: 0.4 mm. (D) Average mice body weight measured every 5 days. Data are shown as mean ± SEM.

Table 1
Tumor weight.

Groups (μM)	Tumor weight (g)	Inhibition rate (%)
MSS.A (0)	1.25 ± 0.33	
MSS.A (10)	0.87 ± 0.12	30.4
MSS.A (50)	0.56 ± 0.05	55.2
MSS.A (100)	0.32 ± 0.16	74.4

anti-cancer activities [22–24]. In this study, we reported that a newly found steroidal saponin MSS.A negatively regulated colorectal cancer cell growth and induced cell apoptosis, and *in vivo* MSS.A inhibited tumor growth in a xenograft murine model. Mechanistically, the anti-colorectal cancer activity of MSS.A occurred mainly through inducing ROS generation.

The cellular redox homeostasis is maintained by a tight balance between the rates of ROS production and the ability of the various antioxidant defense systems to modulate intracellular redox status. It is now well established that the cellular redox status has an important role in a variety of cellular processes including cell proliferation and apoptosis. Our results showed the anti-cancer activity of MSS.A on colorectal cells along with the elevated level of intracellular ROS. Moreover, antioxidants NAC inhibited the accumulation of MSS.A-stimulated ROS and could block MSS.A activities, indicating that MSS.A functions by inducing ROS generation. However, it is not clear if the effects of MSS.A are selectively on cancer cells with little toxicity on normal cells. Therefore, it is necessary to obtain further safety and efficacy data of MSS.A.

JNK, a member of MAPK family, is a major signaling molecule mediating ROS-induced mitochondrial apoptosis. After activation by ROS, JNK would translocate close to mitochondria membrane to activate the release of Bax and inhibit the activation of Bcl-2 and Bcl-xL. Zhou et al. reported that MSS.A activated the phosphorylation of p38 kinase, another member in MPAK signaling family, in 3T3-L1 adipocytes [19]. Thus, MSS.A might also activate MAPK signal cascade in colorectal cancer cells.

Dietary fat intake is a major risk factor for colorectal cancer. About 30–50% of incidence of colorectal cancer were estimated to be associated with diet and nutrition problems [25]. Insulin resistance is certainly a key biologic mechanisms underlying the relationship between adiposity and tumor development [26]. The anti-diabetic drug, metformin, in addition to reduction of insulin resistance has also shown anti-tumor properties, and is increasingly being considered as a drug to prevent and treat obesity-related cancers such as colorectal cancer [27,28]. Xie et al. reported the anti-diabetic effect of MSS.A by increasing cell insulin sensitivity [20]. Therefore, MSS.A might also benefit colorectal tumor patients with diabetes by regulating excess body weight and insulin resistance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.148.

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