# Combining fisetin and ionizing radiation suppresses the growth of mammalian colorectal cancers in xenograft tumor models

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**Abstract.** Fisetin (3,7,3',4'-tetrahydroxyflavone), which belongs to the flavonoid group of polyphenols and is found in a wide range of plants, has been reported to exhibit a number of biological activities in human cancer cells, including anti-oxidant, anti-inflammatory, antiangiogenic, anti-invasive and antiproliferative effects. Although previous *in vitro* studies have shown that fisetin treatment increases the apoptotic rate and enhances the radiosensitivity of human colorectal cancer cells, the *in vivo* effects of fisetin on tumor growth remain unclear. In the present study a murine xenograft tumor model was employed to investigate the therapeutic effects of fisetin in combination with radiation on CT-26 colon cancer cells and human HCT116 colorectal cancer cells. This revealed that intratumoral injection of fisetin significantly suppressed the growth of CT-26 tumors compared with the untreated control group, but had little effect on the growth of HCT116 tumors. However, fisetin in combination with 2-Gy radiation enhanced tumor suppressor activity in murine colon and human colorectal xenograft tumors, as compared with 2-Gy fractionated radiation administered alone for 5 days and fisetin alone. Interestingly, fisetin downregulated the expression of the oncoprotein securin in a p53-independent manner. However, securin-null HCT116 tumors showed only moderate sensitivity to fisetin treatment, and the combination of fisetin and radiation did not significantly suppress securin-null HCT116 tumor growth compared with normal HCT116 tumors. Therefore, the role of securin in mediating the effect of fisetin on colorectal cancer growth warrants further investigation. In conclusion, the results of the current study provide important preclinical data for evaluating the efficacy of fisetin and radiation combination treatment as an adjuvant chemoradiotherapy for human colorectal cancers.

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## Introduction

Colorectal cancer is the third leading cause of mortality in the Western world (1) and has emerged as a common malignancy in the Asian population as a result of changes in diet and physical activity levels (2). Dietary habits have been related to the risk of colorectal cancer (3,4). Surgery and chemotherapy are the primary treatments for colorectal cancer. Radiotherapy is a typical adjuvant treatment after surgery or chemotherapy for high-stage colorectal cancers (5,6). However, colorectal carcinomas display a wide range of radiosensitivity (7,8). Therefore, new approaches are necessary to enhance the efficacy of radiation treatments for colorectal cancers.

Previous epidemiological studies have shown that the daily inclusion of fruit and vegetables in the diet decreases the risk of colon cancer (9). In addition, it has been reported that flavonoids, which are abundant in numerous plants, protect against a number of tumorigenic processes, including oxidative stress, inflammation, angiogenesis and cell invasion (10-12). Furthermore, flavonoids induce cell cycle arrest, apoptosis and radiosensitivity in cancer cells *in vitro* (13-15). The flavonoid fisetin (3,7,3',4'-tetrahydroxyflavone) is a polyphenol found in numerous plants. A number of previous reports have shown that fisetin activates p53 activity, and represses the cyclooxygenase-2 and Wnt/epidermal growth factor receptor/nuclear factor-B signaling pathways in human

cancer cells to promote apoptosis (14,16-18). In addition, fisetin inhibits the spindle checkpoint response that arrests cells in the radiosensitive G2/M phase (19,20). However, the *in vivo* effects of fisetin remain unclear. As fisetin is a natural and edible product with acceptable biosafety, the clinical potential of this compound is of particular interest and warrants further investigation.

Securin, which was originally isolated from rat pituitary tumor cells, is alternatively called the pituitary tumor transforming gene (21). Securin is a multi-functional protein that serves a number of biological roles, such as the regulation of cellular transformation, sister chromatid separation (22,23), gene transcription (24) and DNA damage repair (25,26). Notably, securin interacts with p53 and perturbs p53-mediated transcription and apoptosis in tumor cells (22). Thus, securin is regarded as an oncoprotein. The depletion of securin has been reported to sensitize human colorectal cancer cells to various types of treatment, including fisetin, butein and ionizing radiation (27-29). However, whether these effects can be repeated *in vivo* is unknown.

In the present study, tumor-bearing mice were used to examine the effect of fisetin alone and in combination with radiation on the growth of colorectal tumors *in vivo*. In addition, tumors with defective securin expression were assessed to investigate if securin depletion would enhance sensitivity to these treatments.

## Materials and methods

*Cell culture*. Murine CT-26 colon cancer cells, and human $HCT116^{WT}$, $HCT116^{p53-/-}$, $HCT116^{securin-/-}$ and p53-R273H mutant HT-29 colorectal cancer cell lines (30) were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc.), 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 1 mM sodium pyruvate. Cultures were maintained at 37˚C in a 95% humidified incubator (Thermo Fisher Scientific, Inc.) with 5% $CO_2$ and passaged at 1:3 every 2 days.

*Mouse xenograft models*. A total of 62 male BALB/c nude mice (weight, 20 g; age, 6 weeks) were purchased from the National Laboratory Animal Center (NLAC, Nankang, Taipei, Taiwan). The mice were maintained at 22-24˚C and 70% humidity under a 12-h light/dark cycle. Food and water were available *ad libitum*. Five mice were kept in each 77.4x77.4-cm cage. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of National Yang-Ming University (Taipei, Taiwan; approval no. 1001270). Prior to tumor xenografting, mice were anesthetized with ketamine (50 mg/kg; IMALGENE®; Merial Laboratoire de Toulouse, Lyon, France) and xylazine (15 mg/kg; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). Subsequently, CT-26 ($1x10^6$) or HCT116 ($2x10^6$) cells were subcutaneously injected into the hind legs of the mice (n=5 for injection of each cell line). Prior to further treatment, mice were maintained until tumors reached 100 $mm^3$. Tumor volume was measured using a caliper and calculated as the following: (Length x $width^2$) / 2. Tumor volume was measured every 2-3 days to draw tumor growth curves followed by measurement of body weight.

*Reagents and radiation treatments*. Fisetin was purchased from Sigma-Aldrich (Sigma-Aldrich) and intratumorally injected at a dose of 5 mg/kg on days 0 and 7. Tumors were irradiated with 2 Gy/day for 5 days using an X-ray machine (RS 2000 Biological Research X-ray Irradiator; Rad Source Technologies, Inc., Suwanee, GA, USA) operating at 160 kVp and 25 mA. The dose rate at a source to subject distance of 38 cm was 1.83 Gy/min. For combined fisetin and radiation treatment, mice were treated with 5 mg/kg fisetin followed by 2 Gy irradiation on days 0 and 7. For the control group, the mice were injected with DMSO at the same volume of dissolved fisetin on days 0 and 7. Five mice were used in each group. These treatments are illustrated on a timeline in Fig. 1. Excluding the tumor site, all areas of the mice were masked using a lead radiation protector.

*Western blotting and antibodies*. Western blot analysis was performed as described previously (29). The following primary antibodies were used: Anti-p53 (Cat. no. GEX70214; 1:2,000; GeneTex, Inc., Irvine, CA, USA), anti-securin (Cat. no. ab3305; 1:1,000; Abcam, Cambridge, UK) and anti-GAPDH (Cat. no. PB197650; 1:5,000; Thermo Fisher Scientific Inc.). The secondary antibodies included a goat anti-mouse antibody (Cat. no. AP124P; 1:10,000; EMD Millipore, Billerica, MA, USA) for detecting the anti-securin and anti-GAPDH primary antibodies, and a goat anti-rabbit antibody (Cat. no. AP132P; 1:10,000; EMD Millipore) for detecting the anti-p53 primary antibody. Band intensities were measured by densitometry using ImageJ 1.x software (National Institutes of Health, Bethesda, MD, USA) (31).

*Cell proliferation measurement*. Cells ($1x10^5$) were seeded into 6-cm culture dishes and collected for hemocytometric calculation every day for 7 days. For each time point, the mean number of cells was calculated from three independent cell cultures. The results were plotted as cell proliferation curves.

*Statistical analysis*. Results are presented as the mean ± standard deviation. A Student's t-test was performed to determine if differences between groups were statistically significant. For survival analysis, the Kaplan-Meier estimator was used and the results were analyzed using the log-rank test. Statistical analyses were performed using GraphPad Prism 3.0 software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Fisetin inhibits tumor growth in a mouse CT-26 xenograft model*. To examine the effect of fisetin on colorectal cancer growth *in vivo*, a mouse tumor xenograft model generated by injection of CT-26 colon cancer cells was treated with fisetin. Compared with the untreated control group, a single intratumoral injection of 5 mg/kg fisetin significantly reduced tumor volume for the following 10 days (Fig. 2A). As tumors appeared to re-grow 6 days after the first injection, a second dose of fisetin was administered on day 11 to examine if tumor growth could be inhibited. The results demonstrated that tumor growth was significantly suppressed for 3 days following the second administration of fisetin, as compared with the control

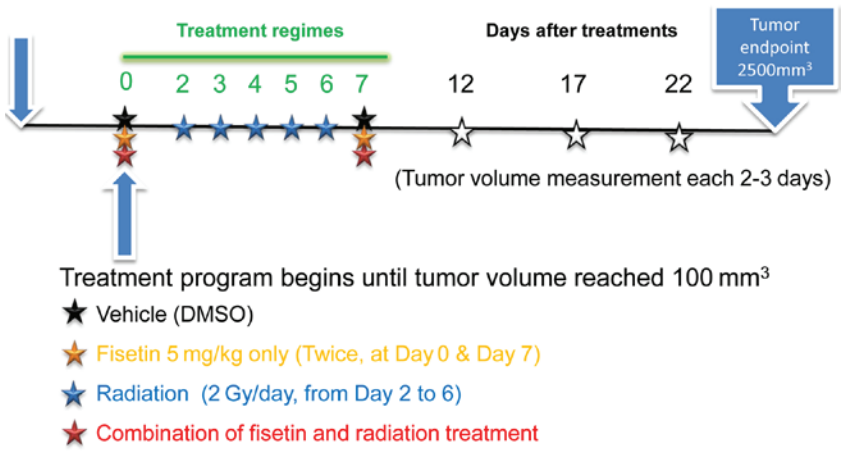


Figure 1. Timeline of treatments of tumor-bearing mice (n=5 per group). Treatments began when tumor volume reached 100 $mm^3$.

group (Fig. 2A), although tumor growth was not completely inhibited. Fluctuations in body weight between the control and fisetin-treated group were similar (Fig. 2B), indicating that the concentration of fisetin used in this study was not cytotoxic. In addition, the survival rate of fisetin-treated tumor-bearing mice was increased compared with the untreated control group (Fig. 2C). These results demonstrate that administration of fisetin suppresses *in vivo* tumor growth in a mouse CT-26 xenograft model.

*Combining fisetin and radiation for the treatment of mammalian colorectal cancers*. Treatment with a combination of fisetin and radiation treatment was investigated in mouse CT-26 and $HCT116^{WT}$ xenograft tumor models. The timeline of fisetin and radiation treatment is illustrated in Fig. 1; 2-Gy X-rays were administered to the tumor site five times between the first and second fisetin treatments to mimic a clinical regime. The results showed that CT-26 tumor growth was suppressed by fisetin and radiation alone; however, this effect was enhanced by combining the two treatments at 16 to 29 days following treatment (Fig. 3A). Body weight was not significantly different between any of the groups, suggesting that combined fisetin/radiation treatment did not cause systemic toxicity (Fig. 3B). In addition, HCT116 tumor growth was completely and significantly inhibited by combined fisetin/radiation treatment ($P<0.05$ vs. the control group; Fig. 3C), without significant loss of body weight (Fig. 3D). These results suggest that combined fisetin and radiation treatment is highly effective in suppressing colorectal cancer.

*Fisetin induces p53 and suppresses securin protein expression in human colorectal cancer cells*. p53 is a tumor suppressor protein that is regulated by a number of different proteins. A previous study demonstrated that the securin oncoprotein binds to p53 and modulates its activity (32). Although securin protein levels have been reported to be elevated by radiation independently of p53 activity (27), it is unknown whether fisetin influences the expression of p53 or securin. In the present study, an experiment with a series of fisetin doses was performed to investigate whether fisetin influences the expression of p53 and securin in different human colorectal cancer cell lines. The results identified that in $HCT116^{WT}$ cells, p53

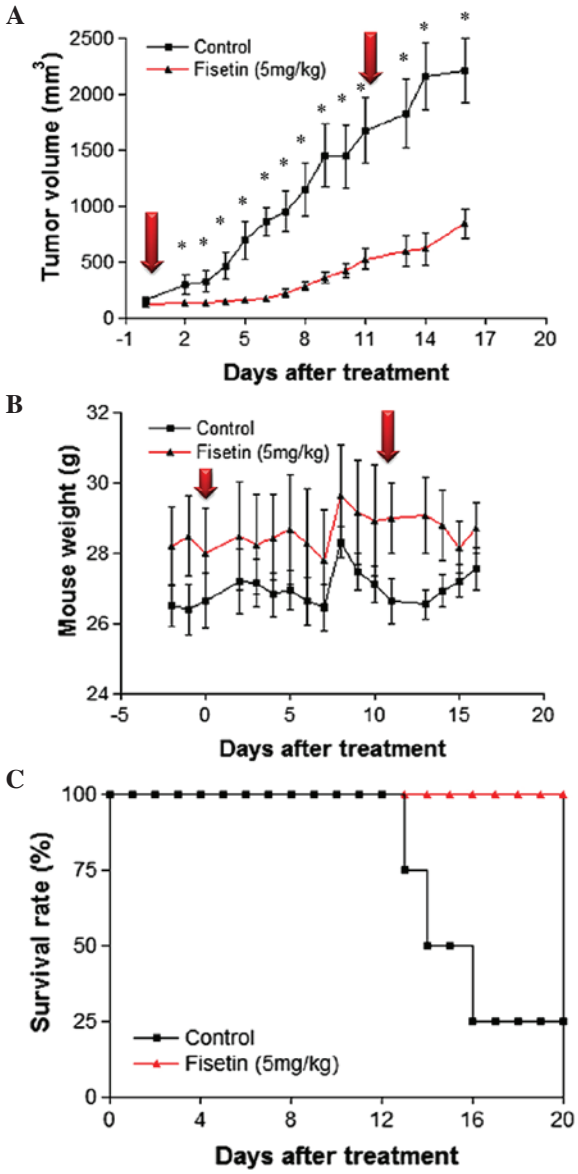


Figure 2. Effect of fisetin on CT-26 colorectal tumors *in vivo*. (A) Tumor growth curves of tumor-bearing mice with or without intratumoral injection of fisetin (n=5 for each group). *P<0.05. (B) Body weights of mice following measurement of tumor size. Arrows represent when fisetin injections occurred. (C) Kaplan-Meier estimator survival curves for tumor-bearing mice with or without intratumoral injection of fisetin.

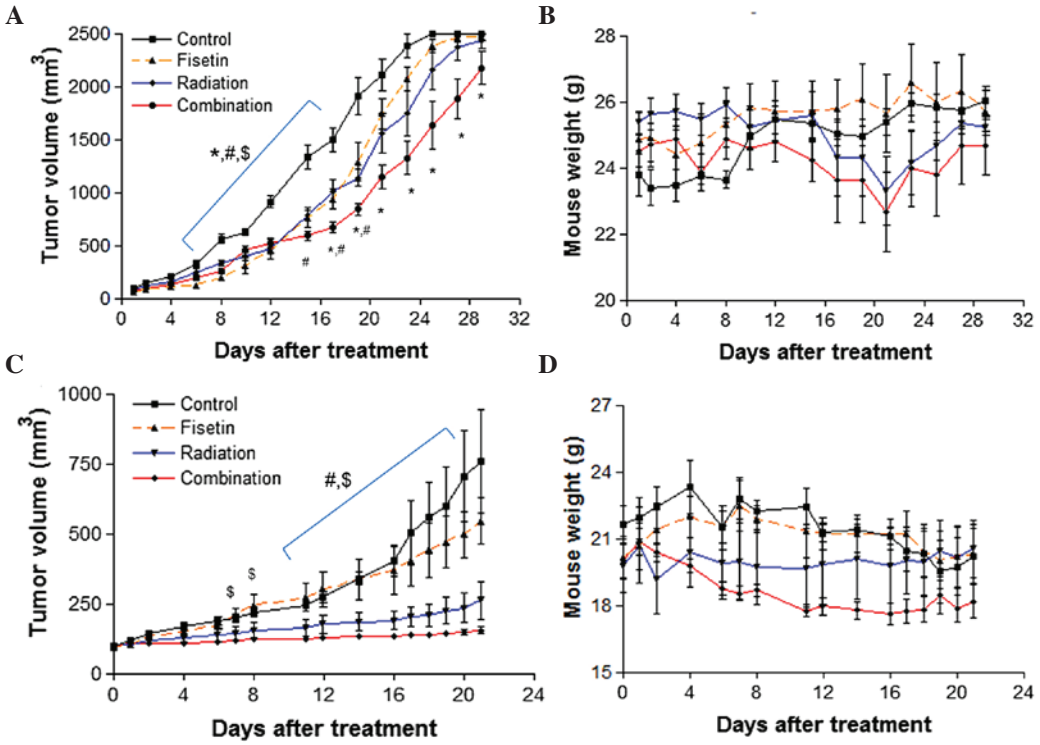


Figure 3. Effect of fisetin and radiation combination treatment on colorectal tumor growth *in vivo*. (A) Growth curves for CT-26 xenograft tumors. (B) Body weights of mice with CT-26 xenograft tumors. (C) Growth curves for HCT116 xenograft tumors. (D) Body weights of mice with HCT116 xenograft tumors. The symbols $^{*}$, $^{\#}$ and $^{\$}$ on the top of the black line are P<0.05 for fisetin, radiation and combined treatment vs. the untreated control, respectively; the symbols $^{*}$ and $^{\#}$ beneath the red lines are P<0.05 for fisetin and radiation treatment alone vs. the combined treatment, respectively.

protein levels were increased and securin protein levels were decreased following fisetin treatment (Fig. 4A). Interestingly, fisetin increased the expression of p53 in HCT116$^{securin-/-}$ cells and decreased the expression of securin in HCT116$^{p53-/-}$ cells (Fig. 4A). In p53 mutant HT-29 cells, fisetin downregulated the expression of securin protein (Fig. 4A). Quantification of the protein bands on the Western Blot analysis revealed the same results (Fig. 4B). These results indicate that fisetin increases the expression of p53 and decreases the expression of securin, which suppresses tumor growth. Furthermore, fisetin-mediated expression of p53 and securin was independent of the expressive status of p53/securin (null or wild-type).

*Effect of fisetin and radiation combination treatment on securin-null colorectal cell-formed xenograft tumors*. As fisetin could induce the expression of the p53 protein in the absence of securin, the ability of fisetin and radiation treatment to suppress tumor growth *in vivo* was investigated. Firstly, the growth curves of HCT116$^{WT}$ and HCT116$^{securin-/-}$ cells were compared, which showed that the proliferation of HCT116$^{securin-/-}$ cells was significantly slower than that of HCT116$^{WT}$ cells after 3 days of proliferation (Fig. 5A). Tumor growth was moderately suppressed by fisetin treatment in HCT116$^{securin-/-}$ cells; however, no significant advantage was observed following fisetin and radiation combination treatment (Fig. 5B). The body weight of the mice following fisetin, radiation and fisetin/radiation showed no significant difference compared with the control group (Fig. 5C). Therefore, depletion of securin does not enhance the effects of fisetin and radiation combination treatment on colorectal tumors *in vivo*. However, a larger sample size is necessary to validate this result.

## Discussion

Previous studies have shown that fisetin possesses a wide range of activities to suppress the growth of human cancer cells, including breast, prostate, bladder, lung, melanoma and colorectal cancer cells (16,33-39). Among these types of cancer, colorectal cancer is particularly interesting with regards to fisetin, since fisetin is a nutrient supplement that can be administrated orally (40). It is thought that fisetin is absorbed through the digestive tract without the need for intravascular injection (41). Although *in vitro* studies have demonstrated the efficacy of fisetin in the treatment of colorectal cancer (15,16), few *in vivo* studies have been reported. Since preclinical studies are essential for potent therapeutic agents to be accepted for clinical trials, the experimental animal data in the present study is important for evaluating the application of fisetin to colorectal cancer therapy. In addition, the current study investigated whether fisetin treatment combined with ionizing radiation exerted synergistic effects. A previous study reported that a combination of cisplatin and fisetin exerted anticancer activity in embryonic carcinoma cells *in vitro* and *in vivo* (42). Although radiotherapy is not typically used in the treatment of colorectal cancer, it is frequently applied to adjuvant chemotherapy in rectal cancer (43,44). The combination of fisetin and radiotherapy is an interesting alternative adjuvant therapy, since it would likely avoid the side effects associated with chemotherapy.

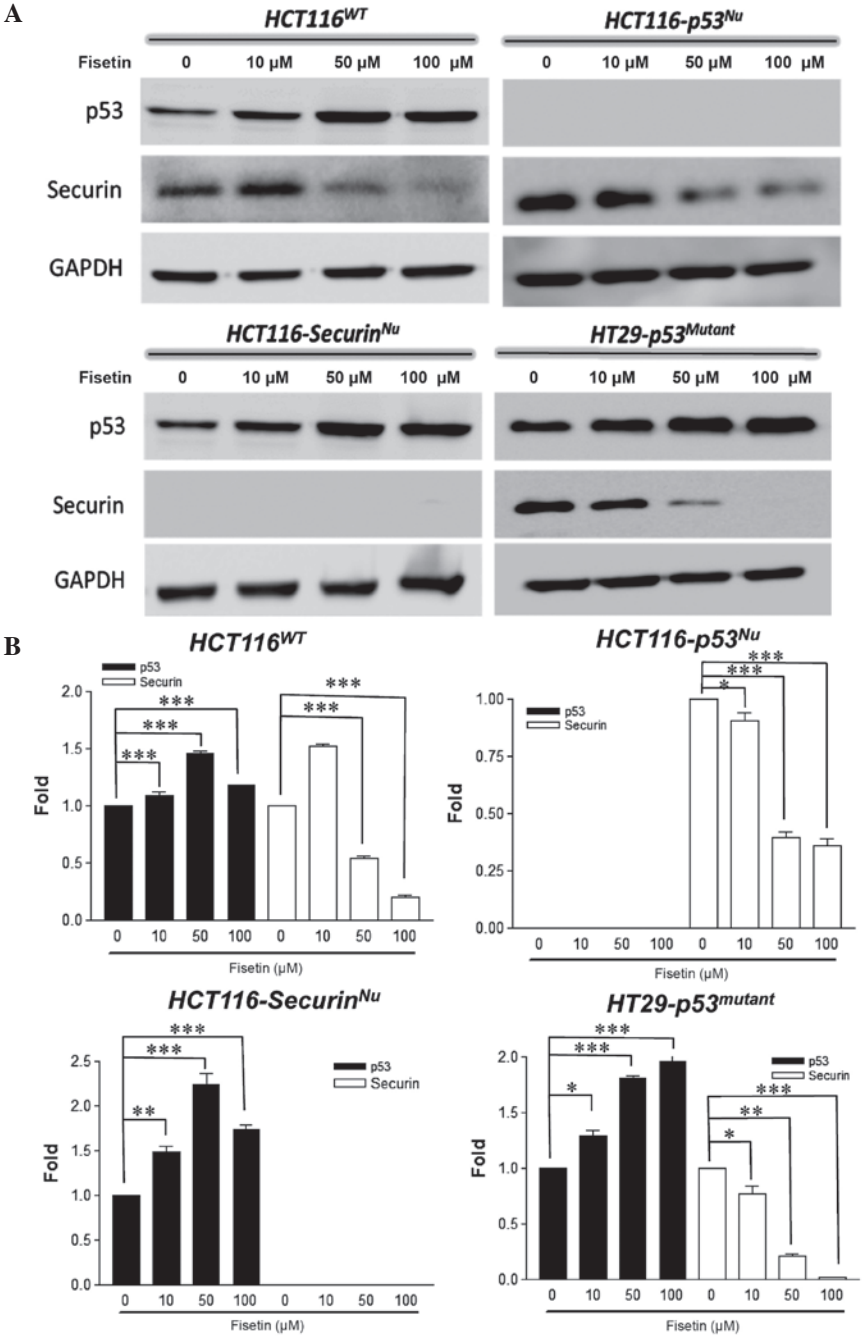


Figure 4. Effect of fisetin on p53 and securin expression in human colorectal cancer cells. (A) Western blots detecting the expression of p53 and securin in wild-type, p53-null and securin-null HCT116 cells, and p53 mutant HT-29 cells, following treatment with different concentrations of fisetin. (B) Quantification of western blots. $^{*}$P<0.05, $^{**}$P<0.0005 and $^{***}$P<0.0001 vs. 0 $\mu$M fisetin.

In the present study, a mouse xenograft model was used to examine the tumor-suppressive efficacy of fisetin *in vivo* on colon and colorectal cancers. Animal (CT-26) and human (HCT116) colon and colorectal cancer cells, respectively, were used to establish xenograft tumor models. Fisetin treatment was shown to suppress the growth of tumors formed by CT-26 cells, but not HCT116 cells. However, treatment with fisetin combined with radiation exhibited enhanced tumor suppressive effects on CT-26 and HCT116 xenograft tumors. This finding supports the potential application of fisetin to adjuvant radiotherapy for colorectal cancers. As fisetin did not exert toxicity in the current study, or in other reports (45,46), its use as an adjuvant treatment should be feasible.

In the present study, CT-26 and HCT116 cell lines, which express wild-type p53, formed tumors that were sensitive to radiation treatment. Since p53 is known to be upregulated by radiation (47), it is reasonable that this phenomenon was observed *in vivo* in the present study. Importantly, the current study used clinically comparable fractionated radiation of 2-Gy/day/fraction for 5 days (48). Compared with this regime, the fisetin-combined radiation treatments only irradiated tumors at 2 Gy twice in one week, yet remained more efficient than the fractionated radiation on tumor suppression, suggesting that fisetin enhances the tumor response to radiation *in vivo*. Thus, the use of fisetin in adjuvant radiotherapy may reduce total radiation exposure and improve the quality of life of patients during

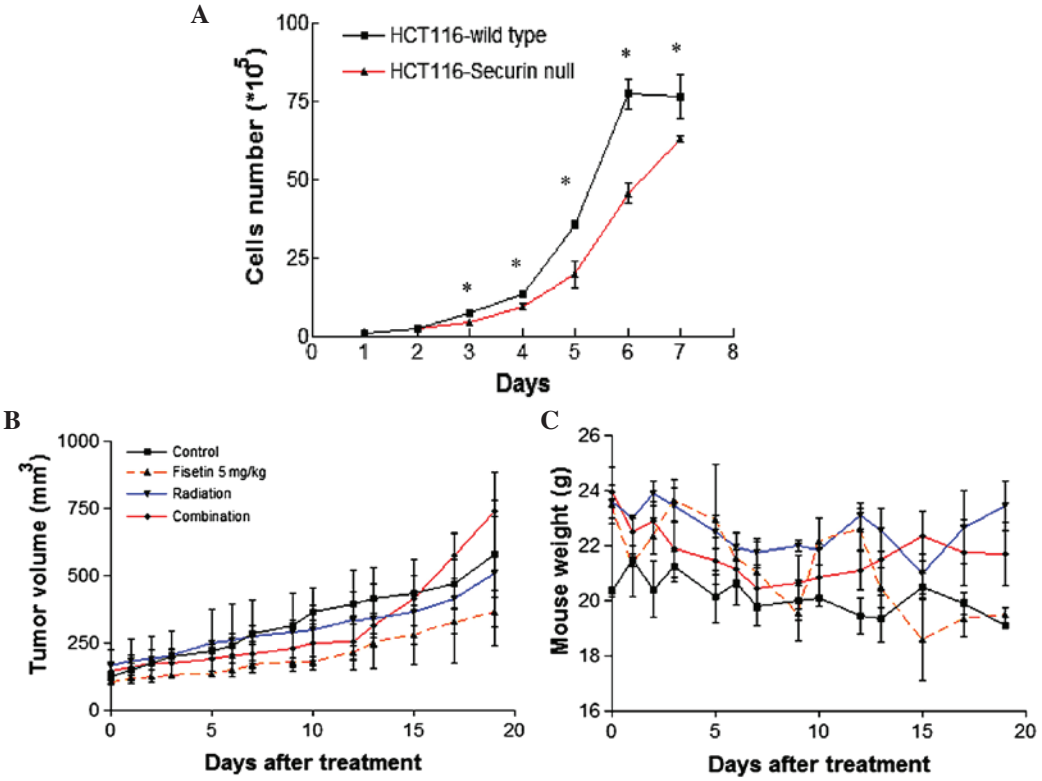


Figure 5. Effect of fisetin and radiation combination treatment on the growth of securin-null HCT116 xenograft tumors *in vivo*. (A) Cell growth curves of $HCT116^{WT}$ cells and $HCT116^{securin-/-}$ cells. The cell number of each time point was compared. $^{*}P<0.05$ vs. the same time point in the $HCT116^{securin-/-}$ cells. (B) Growth curves of $HCT116^{securin-/-}$ xenograft tumors following treatment with fisetin, radiation, or fisetin and radiation combined (n=3 per group). (C) Body weights of mice with $HCT116^{securin-/-}$ xenograft tumors.

and following treatment. Further investigations into the optimal combination for colorectal cancer therapy are warranted.

The genetic background of a tumor is known to influence the prognosis (49,50). The authors of the present study were interested in p53 and securin because of the results of our previous studies (15,27). Briefly, fisetin was shown to enhance the radiosensitivity of p53-mutant human HT-29 colorectal cancer cells, and promote apoptosis in securin-depleted human HCT-116 colorectal cancer cells. Consistent with a previous report (14), in the present study, we also showed that fisetin could induce p53 protein expression in wild-type and securin-null HCT116 cells. Furthermore, expression of the securin protein was downregulated by fisetin regardless of the p53 expression status. In addition, in the current study xenograft securin-null colorectal tumors exhibited increased sensitivity to fisetin compared with the untreated control. However, individual variance in the tumor-bearing mice may have reduced the extent of tumor suppression resulting from fisetin treatment. Therefore, an increased sample size is required to validate the effect of fisetin on securin-null colorectal cancers. In addition, this limitation should be considered for the investigation of fisetin and radiation combination treatment. In the current study, p53 protein expression was still induced in securin-null HCT116 cells by fisetin, indicating that p53 and securin protein expression should be modulated by fisetin and radiation combination treatment in order to achieve optimal tumor suppression.

In conclusion, the present study used mouse xenograft tumor models to investigate the effect of fisetin alone or in combination with radiation on colon and colorectal tumor growth. The results showed that fisetin treatment alone was sufficient to suppress murine CT-26 colon tumors, but not human HCT116 colorectal tumors. Interestingly, a combination of fisetin and radiation treatment enhanced the tumor suppression compared with fractionated irradiation alone. In addition, the results of the current study revealed that securin-null HCT116 tumors exhibited increased sensitivity to fisetin treatment, although this observation needs to be validated in a larger sample set. Investigating whether securin is the key molecule in mediating the effects of fisetin and radiation combination treatment on colon and colorectal cancers warrants future exploration. To the best of our knowledge, this is the first report demonstrating the therapeutic efficacy of fisetin and radiation combination treatment on colon and colorectal cancer *in vivo*. The results of the present study provide important preclinical information for evaluating the potential use of fisetin in adjuvant cancer radiotherapy.

## Acknowledgements

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