Tangeretin enhances radiosensitivity and inhibits the radiation-induced epithelial-mesenchymal transition of gastric cancer cells

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Abstract. Irradiation has been reported to increase radioresistance and epithelial-mesenchymal transition (EMT) in gastric cancer (GC) cells. The Notch pathway is critically implicated in cancer EMT and radioresistance. In the present study, we investigated the use of a Notch-1 inhibiting compound as a novel therapeutic candidate to regulate radiation-induced EMT in GC cells. According to previous screening, tangeretin, a polymethoxylated flavonoid from citrus fruits was selected as a Notch-1 inhibitor. Tangeretin enhanced the radiosensitivity of GC cells as demonstrated by MTT and colony formation assays. Tangeretin also attenuated radiation-induced EMT, invasion and migration in GC cells, accompanied by a decrease in Notch-1, Jagged1/2, Hey-1 and Hes-1 expressions. Tangeretin triggered the upregulation of miR-410, a tumor-suppressive microRNA. Furthermore, re-expression of miR-410 prevented radiation-induced EMT and cell invasion. An *in vivo* tumor xenograft model confirmed the antimetastasis effect of tangeretin as we observed *in vitro*. In nude mice, tumor size was considerably diminished by radiation plus tangeretin co-treatment. Tangeretin almost completely inhibited lung metastasis induced by irradiation. Tangeretin may be a novel antimetastatic agent for radiotherapy.

Introduction

Gastric cancer (GC) remains a severe public health problem worldwide. In China, GC is often diagnosed at an advanced clinical stage, characteristic of obvious lymphatic tumor dissemination (1). Radiotherapy is the main modality for unresectable GC (http://www.nccn.org/index.asp) (2).

However, radiation is also a double-edged sword; it not only kills tumor cells, yet also promotes radioresistance and induces distant metastases, and destroys normal tissues (3). The intricate process of metastasis includes a series of divergent steps. Epithelial-mesenchymal transition (EMT) is one of the main programs. The EMT process leads to acquisition of mesenchymal characteristics, including motility, invasiveness, chemoresistance and radioresistance (4). Uncovering the relationship between irradiation and EMT is essential. E-cadherin, which facilitates and ensures the continuous adhesive epithelium, is a biomarker of the EMT process (5). The loss of E-cadherin leads to the lack of stable intercellular junctions; therefore cell dispersion is accelerated due to the decrease in cellular adhesive forces. Jung *et al* reported that the morphology of cells changes after irradiation and cells appeared similar to fibroblasts corresponding to a mesenchymal phenotype (6).

The Notch axis is a key participant in EMT. Notch signaling is a conserved family of transmembrane receptors that determines cell fate (7). It includes 4 Notch family members (Notch-1-4), 5 Notch ligands, 3 δ-like ligands (Dll1/3/4) and 2 serrate-like ligands (Jagged1/2) (8). Notch is dysregulated in various types of cancers, accompanied by poor clinical outcomes (9). Notch signaling can be activated under radiation and there is accumulating evidence confirming the use of Notch inhibitors as promising radiosensitizers in cancer treatment. Notch has also been proven to mediate radioresistance in nasopharyngeal carcinoma and glioma cells (10-12).

MicroRNAs (miRNAs) are a class of small non-coding RNAs of 21-23 nucleotides that control specific mRNA translation or induce mRNA degradation (13). Dysregulated miRNA expression is well correlated with various malignancies. The deficiency in members of the miR-200 family (miR-200s), miR-124 and let-7, are observed in certain types of cancers, and are regarded as tumor suppressors (14). In contrast, various overexpressed miRNAs, such as miR-373 and miR-21, are regarded as oncogenes, involved in cell proliferation and metastasis (15). In GC tissues, the expression levels of miR-221, miR-222, miR 21 and miR-103 were found to be

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significantly higher than levels in normal samples. However, the expression of miR-143 and miR-195 in cancer samples was significantly lower than levels in normal tissues (16). Moreover, miR-200c deficiency promoted cancer metastasis, EMT and aggressiveness (17).

As a polymethoxylated flavonoid abundant in citrus fruits, tangeretin (5,6,7,8,4'-pentamethoxyflavone) has been reported to display apoptotic (18), antimetastatic (19) and antioxidant properties in various cancer models. It inhibited the proliferation and induced G1 phase arrest of MCF-7 cells after a 4-day treatment (20). Tangeretin induced the apoptosis of human leukemia cells (18) and enhanced the cytotoxic effect of doxorubicin on breast cancer cells (21). Importantly, tangeretin possessed little toxicity as there was no significant weight loss and no obvious change in the gross behavior of rats (22). In our previous studies (unpublished data), we screened for effective radiosensitizers of GC cells. Recently, we observed the potential radiosensitizing effects of tangeretin on GC cells, which led us to explore the mechanism involved.

Materials and methods

Materials. Tangeretin (98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Tangeretin was dissolved in dimethylsulfoxide (DMSO) for all *in vitro* experiments. DMSO, RPMI-1640 medium, fetal calf serum (FBS), Tween-20, sodium dodecyl sulfate (SDS), phenylmethylsulphonyl fluoride (PMSF), trypsin and carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Notch-1 siRNA and miR-410 mimics were obtained from Dharmacon (Chicago, IL, USA). The Notch-1 cDNA plasmid was purchased from OriBioGene Biological Inc. (Shanghai, China). Deionized water was purified by a Milli-Q water purification system (Millipore, Milford, MA, USA). All other reagents were of analytical grade and obtained from Nanjing Chemical Reagent Co. (Nanjing, China).

Cell lines and cell viability. GC cell lines (MGC80-3, AGS, SGC7901 and MKN45) and normal gastric mucosa GES-1 cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and were cultured in RPMI-1640 supplemented with 10% FBS. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Radiosensitivity was examined by colony formation assay. Cells were exposed to increasing doses of irradiation (2, 4, 6 and 8 Gy) and tangeretin for 24 h. Irradiation was produced with a 4-MeV electron beam accelerator (Elekta, Sweden). After being washed with fresh medium, cells were allowed to grow for 14 days to form colonies, and cells were then fixed with 4% formaldehyde (Sigma). Fixed cells were then stained with 0.5% crystal violet (Sigma) for 15 min, and the colonies were counted. D0 values were calculated by a multitarget-single hit model.

In vivo study protocol. Athymic male nude mice weighing ~20 g (SLARC Laboratory Animal Center, Shanghai, China) were used and maintained under standard pathogen-free conditions. SGC7901 cells were harvested and injected subcutaneously into the nude mice. After the onset of tumor development, tangeretin [30 mg/kg dissolved in 1 ml of phosphate-buffered saline (PBS) containing 0.1% DMSO/nude mice] was administered intraperitoneally for 3 weeks. After the mice were anesthetized, the tumors were exposed to radiation with 2 Gy 5 times a week for 3 weeks. After 3 weeks of administration, all mice were sacrificed and the tumors were subjected to further analysis. The numbers of lung metastatic lesions were counted under a microscope (Zeiss Axio Observer A1; Zeiss, Germany).

Western blot analysis. Cells were extracted in extraction buffer. Equal amounts of protein extracts were separated on 10% polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride membranes (Invitrogen, Carlsband, CA, USA) with a semi-dry blot system. After blocking, the membranes were incubated with each primary antibody (Nanjing Bioworld Biotech Co., Nanjing, China) and then with IgG.

Cell invasion assays. After co-treatment with tangeretin (10 and 30 μM) and radiation (8 Gy) for 24 h, the cells were then placed in the upper chamber of a Transwell coated with BD Matrigel™. Medium with 10% FBS was placed in the lower chamber. After 18 h, the cells that had migrated to the lower surface were photographed and quantified in 5 fields at a magnification of x100 under a microscope (Zeiss Axio Observer A1).

Wound healing assay. Cells were seeded in 24-well plates and grown until reaching confluency. In each well, a scratch was made with a 1-ml pipette tip. The cells were washed thrice with PBS to remove detached cells. Then the cells were treated with irradiation (8 Gy) and tangeretin (10 and 30 μM), and images of the wound area were captured at 0 and 18 h under a microscope (Zeiss Axio Observer A1).

Immunofluorescence microscopy. After exposure to irradiation (8 Gy) for 24 h, immunofluorescence assay was performed to examine EMT biomarkers in the SGC7901 cells. After fixation and permeabilization, E-cadherin and N-cadherin were measured following staining with anti-E-cadherin and anti-N-cadherin antibodies. Nuclei were counterstained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) (Sigma Chemical Co.). The stained cells were observed with a fluorescence inverted microscope (Zeiss Axio Observer A1).

miRNA microarray analysis. Total RNA was harvested using miRNeasy Mini kit (Qiagen, Copenhagen, Denmark) according to the manufacturer's instructions (23,24). The profiles of samples were generated using Agilent Human miRNA Microarray V3 (Agilent Technologies, Inc., Santa Clara, CA, USA). The scanned images were imported into GenePix Pro 6.0 software (Axon Instruments, Molecular Devices Corp., Foster City, CA, USA) for data extraction. Real-time PCR was carried out to confirm the miRNA expression profiling with U6 as the internal reference gene.

Statistical analysis. Data are expressed as the mean ± SD from 3 independent experiments. Group results were analyzed using
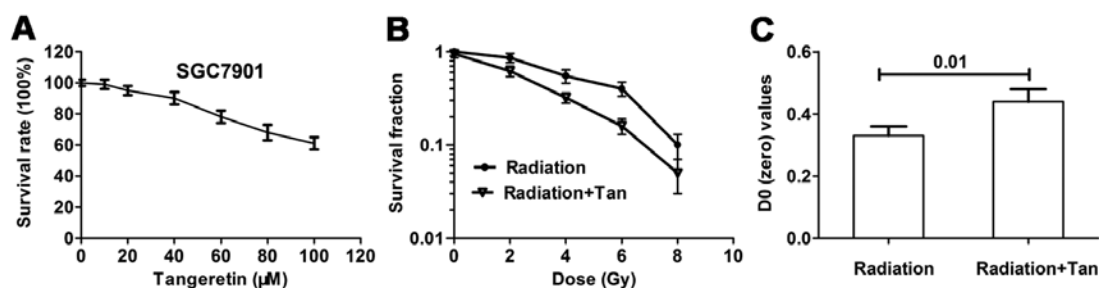
Figure 1. Tangeretin increases the radiosensitivity of SGC7901 cells. (A) MTT assay. Survival rates after incubation with tangeretin (Tan) (0-100 μM) for 24 h. (B) Clonogenic survival assay. Cells were exposed to radiation (2, 4, 6 and 8 Gy) with or without a subtoxic concentration of tangeretin (30 μM) for 24 h. After being washed with fresh medium, the cells were allowed to grow for 14 days to form colonies. (C) D0 value.
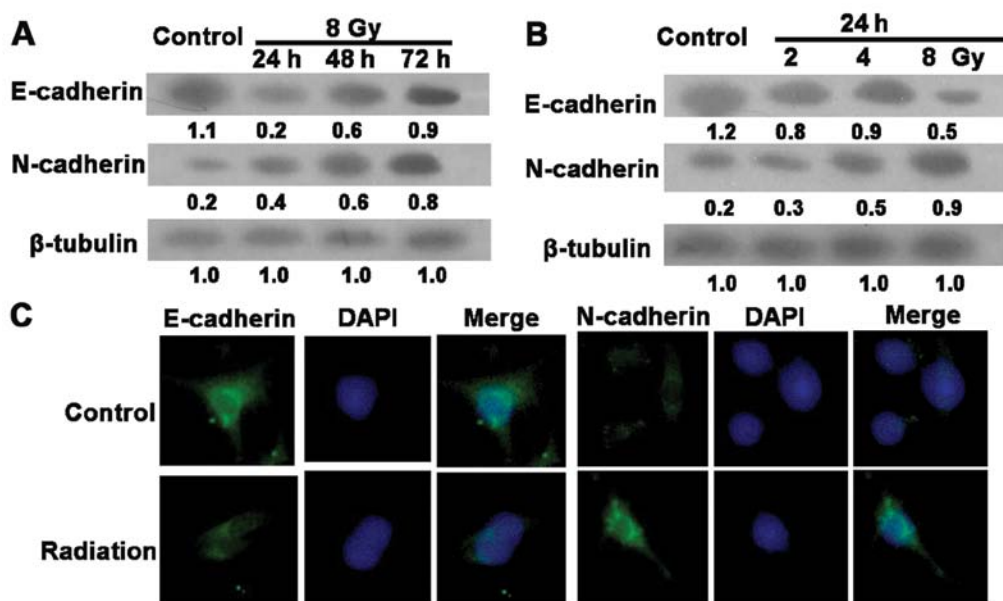

Figure 2. Irradiation modulates EMT markers in SGC7901 cells in a time- and dose-dependent manner. (A and B) SGC7901 cells were irradiated with increasing doses of irradiation (2, 4 and 8 Gy) and were harvested at 24, 48 and 72 h post-irradiation. Biomarkers for EMT were analyzed by western blot analysis. β-tubulin was used as a loading control. (C) After irradiation (8 Gy) for 24 h, SGC7901 cells were harvested, and an immunofluorescence assay was carried out to determine EMT biomarker expression. EMT, epithelial-mesenchymal transition.

one-way analysis of variance (ANOVA). Two groups were analyzed by the Student's t-test. P-value <0.05 was considered to indicate a statistically significant result.

Results

Tangeretin decreases cell viability and increase radiosensitivity in the SGC7901 cells. Cytotoxicity was detected by MTT assay. Cells were treated with tangeretin (0-100 μM) for 24 h. Tangeretin caused a cytotoxicity effect on the SGC7901 cells in a dose-dependent manner. Tangeretin did not affect cell viability until reaching concentrations of 30 μM (Fig. 1A). The non-cytotoxic concentrations used for the present study were identified, to ensure that the radiosensitizing effect of tangeretin was not caused by a direct cytotoxic effect.

A colony forming assay was carried out to explore whether tangeretin increased radiosensitivity in the SGC7901 cells. Cell survival curves measured by clonogenic survival assay are illustrated in Fig. 1B. Tangeretin considerably increased radiation-induced cell clonogenic death. D0 of the tangeretin

plus radiation group was significantly increased (P=0.01), compared with the radiation alone group (Fig. 1C).

Radiation induces EMT in the gastric cancer cells. The loss of the epithelial phenotype marker, E-cadherin, is obvious in the EMT process. Features of the mesenchymal phenotype, such as augmented formation of pseudopodia and the appearance of spindle-shaped cells, were observed at 24 h post-irradiation (data not shown). To determine whether this alteration correlates with EMT, we examined EMT-specific markers by immunofluorescence assay and western blotting. As shown in Fig. 2A and B, radiation induced a decrease in E-cadherin and promoted the expression of the mesenchymal marker N-cadherin in the SGC7901 cells. Similar results were observed in the immunofluorescence assay (Fig. 2C). These data suggest that radiation induced EMT in the human GC SGC7901 cells.

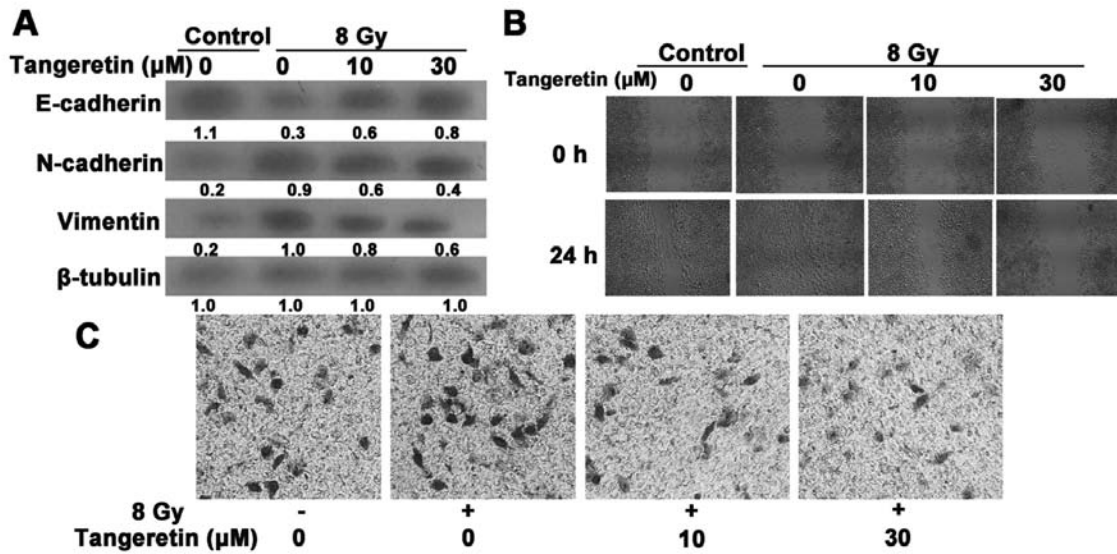*Tangeretin reduces radiation-induced EMT and invasion and migration in the SGC7901 cells.* Tangeretin suppressed

Figure 3. Tangeretin suppressed EMT, invasion and migration induced by radiation. Cells were co-treated with tangeretin (10 and 30 µM) and radiation (8 Gy) for 24 h. (A) Biomarkers for EMT were evaluated by western blot analysis. (B) Wound closure assays. Cells were co-treated with radiation and tangeretin (10 and 30 µM) after wound scratches were made. Images were captured at 0 and 18 h after wounding. (C) Invasion assays. After co-treatment with radiation and tangeretin for 24 h, the invasive ability was measured by Transwells. The number of invaded SGC7901 cells in 5 fields at a magnification of x100 was counted under a microscope. EMT, epithelial-mesenchymal transition.
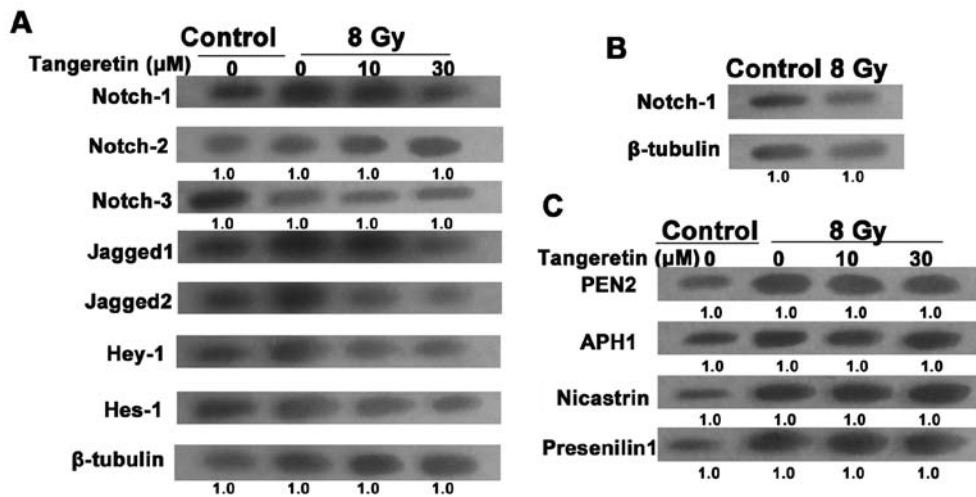

Figure 4. Tangeretin inhibits the Notch-1 pathway in irradiated SGC7901 cells. Cells were co-treated with radiation and tangeretin for 24 h, and the cells were harvested 24 h post-radiation. (A) Expression levels of Notch-1, Notch-2, Notch-3, Hes-1, Hey-1, Jagged1 and Jagged2 were detected by western blotting. (B) Notch-1 expression in the GES-1 cells. (C) γ-secretase complex proteins.

radiation-induced EMT, as revealed by a reduced reduction in expression of vimentin and N-cadherin and an increase in expression of E-cadherin (Fig. 3A). The EMT process is also accompanied with enhanced cell invasion and migration. We further evaluated the invasive and migratory properties of SGC7901 cells via Matrigel Transwell and wound-healing assays. Radiation accelerated the rate of wound closure (Fig. 3B). Likewise, the acquisition of increased invasive ability induced by irradiation was prohibited by tangeretin treatment (Fig. 3C). These data indicate that tangeretin diminished the radiation-induced responses in SGC7901 cells.

Tangeretin inhibits the Notch-1 pathway in the irradiated SGC7901 cells. Evidence has proven the essential role of

the Notch-1 pathway in the EMT process (25). To uncover the molecular mechanism responsible for radiation-induced EMT, the expression of the Notch pathway in irradiated cells was examined. After treatment with tangeretin for 24 h, the upregulation of Notch-1, Jagged1/2, Hes-1 and Hey-1 expression levels in the irradiated cells was almost blocked. However, radiation or/and tangeretin co-incubation failed to influence the expression of Notch-2/3 (Fig. 4A). Moreover, western blot assays showed that there was no significant change in Notch-1 expression in the gastric mucosa GES-1 cells after exposure to irradiation (Fig. 4B).

After binding to Jagged or δ ligands, the Notch receptor is activated and goes through a succession of cleavages. The final cleavage depends on the γ-secretase protease complex, which
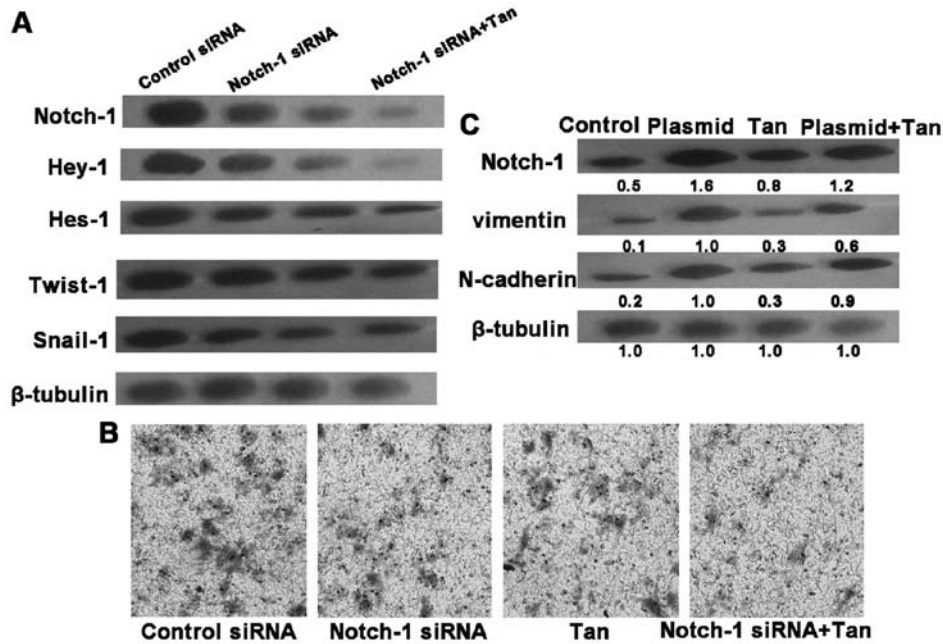
Figure 5. Knockdown of Notch-1 alleviates EMT in SGC7901 cells. (A) Cells were transfected with a control or a Notch-1-specific siRNA. After 24 h post-trans-fection, cells were irradiated at 8 Gy with or without tangeretin (Tan) (10 μM) co-treatment and cell lysates were collected to measure EMT-related protein levels. (B) Invasion assays. After 24 h post-transfection, cells were irradiated at 8 Gy with or without tangeretin (10 μM) co-treatment, and the invasive ability was measured by Transwells. (C) Overexpression of Notch-1. Cells were transfected with empty vector or plasmid of Notch-1. After 24 h, cells were irradiated at 8 Gy with or without tangeretin (30 μM) co-treatment. EMT biomarkers were determined by western blot assay. EMT, epithelial-mesenchymal transition.
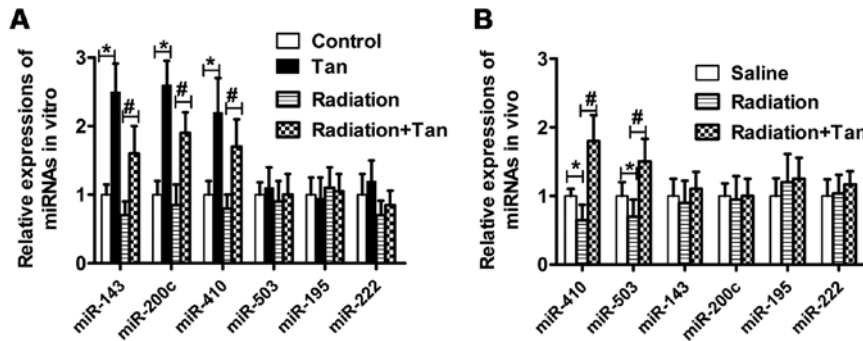

Figure 6. Tangeretin elevates miR-410 expression both in vitro and in vivo. (A) miRNA levels in vitro. SGC7901 cells were exposed to radiation and tangeretin (Tan) (30 μM) for 24 or 48 h. *P<0.05, compared with medium control; #P<0.05, compared with radiation alone. (B) miRNA levels in vivo. After the onset of tumors, tangeretin (30 mg/kg BW) was administered intraperitoneally for another 3 weeks, and then the tumor tissues were removed to determine miRNA levels (n=5). *P<0.05, compared with the saline group; #P<0.05, compared with the radiation alone group.

contains 4 catalytic subunits such as PEN2, presenilin 1, APH1 and nicastrin (26), causing the translocation of Notch into the nucleus (27). We then tested the effect of tangeretin on the activation of Notch-1 signaling. Tangeretin did not influ-ence the protein level of the 4 catalytic subunits, suggesting that tangeretin only suppressed the expression rather than the activation of Notch-1 (Fig. 4C).

Knockdown of Notch-1 alleviates EMT in SGC7901 cells. To further demonstrate the essential role of Notch-1, the Notch-1 gene was knocked down by siRNA. Notch-1 siRNA transfection downregulated the protein levels of Notch-1, Hey-1, Hes-1, Snail1 and Twist1 in the irradiated SGC7901 cells, accompanied with a decline in invasive ability. After 24 h post-transfection, tangeretin treatment inhibited both Notch-1 activity and cell invasion to a more significant degree

in the irradiated SGC7901 cells than tangeretin treatment alone (Fig. 5A and B). We then explored the relationship between tangeretin and Notch-1 with the overexpression plasmid of Notch-1. Overexpression of Notch-1 overrode the inhibitory effect of tangeretin on EMT (Fig. 5C). Accordingly, we hypothesized that tangeretin prevented EMT probably via suppressing Notch-1 expression.

Tangeretin elevates miR-410 expression both in vitro and in vivo. Radiation regulates miRNA expression, and in turn affects tumor progression. As demonstrated here, in SGC7901 cells exposed to tangeretin, enhanced expression of miR-143, miR-200c and miR-410 was noted when compared to the control cells. Moreover, the relative change in miR-410 expres-sion was greater in the tangeretin plus irradiation group, than the radiation alone group (Fig. 6A). In addition, tangeretin
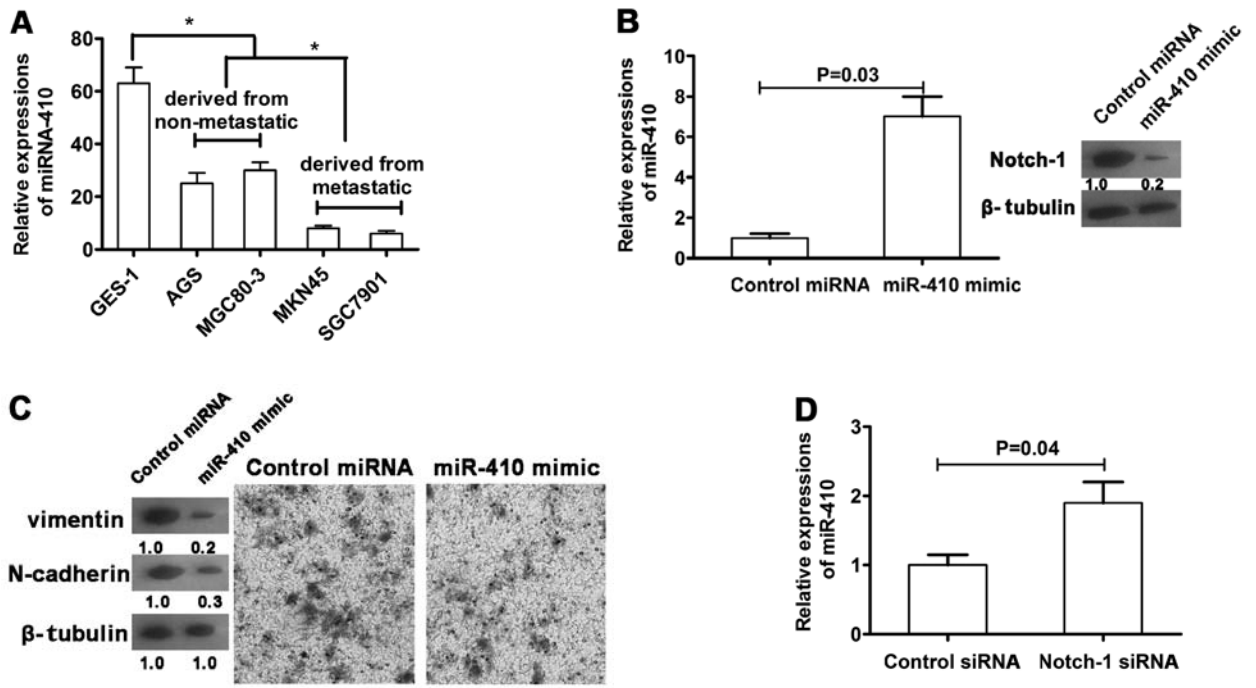
Figure 7. Overexpression of miR-410 inhibits EMT. (A) qRT-PCR analysis of miR-410 expression in 4 GC cell lines and GES-1 cells. U6 was used as the internal reference gene. (B) qRT-PCR analysis of miR-410 expression and western blot analysis of Notch-1 expression in irradiated-SGC7901 cells transfected with miR-410 mimics. U6 was used as the internal reference gene. (C) Western blotting and Transwell analysis in the irradiated-SGC7901 cells transfected with miR-410 mimics. (D) qRT-PCR analysis of miR-410 expression after knockdown of Notch-1 in the irradiated SGC7901 cells. EMT, epithelial-mesenchymal transition.
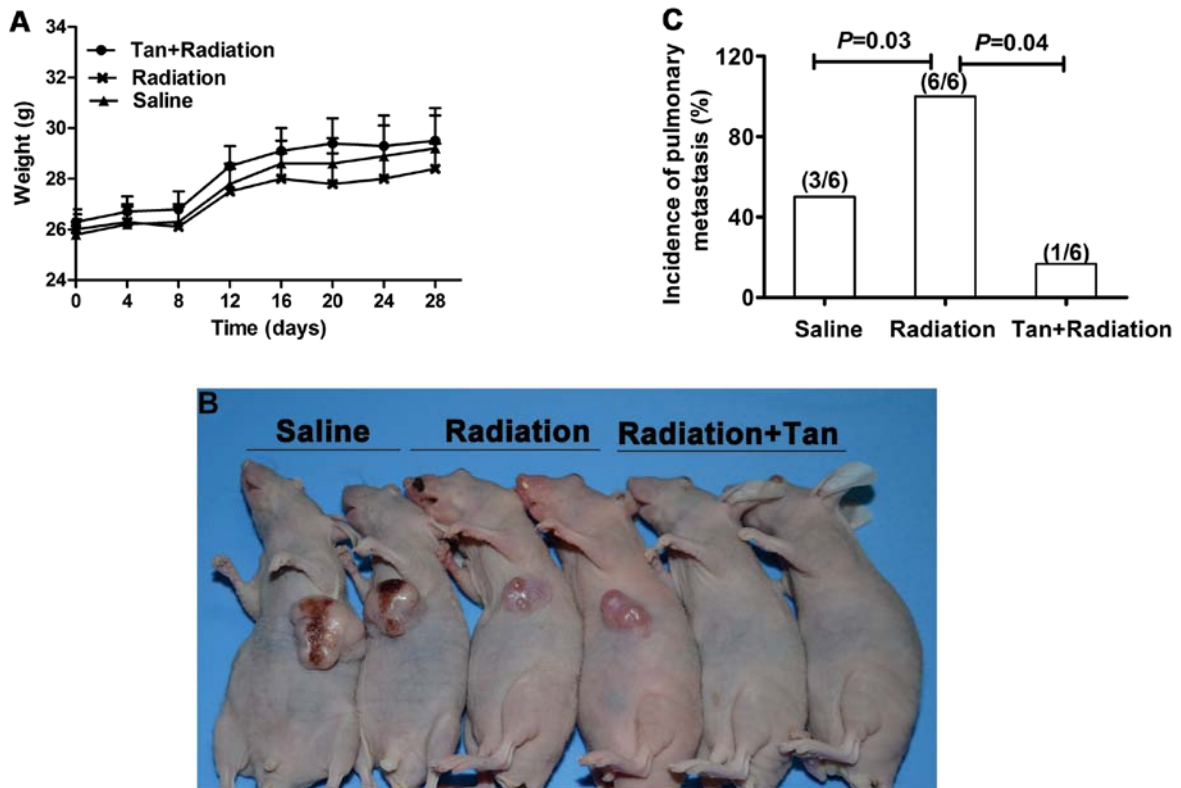

Figure 8. Radioenhancement efficacy of tangeretin *in vivo*. (A) Changes in body weight. (B) Tumor volumes. (C) Pulmonary metastasis.

promoted the expression of miR-410 and miR-503 in tumor tissue lysates from nude mice (Fig. 6B). These results demonstrated that tangeretin elevated miR-410 expression both *in vitro* and *in vivo*.

miR-410 expression is reduced in gastric camcer cells. We then compared miR-410 expression between GC cell lines (MGC80-3, AGS, SGC7901 and MKN45) and the normal gastric mucosa GES-1 cells. miR-410 expression was reduced in the GC cell lines compared to the GES-1 cells. MGC80-3 and AGS cell lines were derived from non-metastatic tissues, and SGC7901 and MKN45 cell lines were from metastatic tissues. Expression of miR-410 was higher in the MGC80-3 and AGS cells than those derived from metastatic tissues (P<0.05, Fig. 7A).

miR-410 plays a role in the biologic function of tangeretin in EMT. Since miRNAs are crucial modulators of EMT and Notch-1 signaling, we further investigated the effect of miR-410 on the EMT of GC cells subjected to radiation. Enhanced miR-410 expression and weakened Notch-1 expression were observed in the irradiated-SGC7901 cells after miR-410 mimics were transfected into the cells (Fig. 7B). Overexpression of miR-410 also reduced EMT-specific marker expression and invasion in the irradiated cells, indicating that miR-410 is closely associated with EMT (Fig. 7C). In addition, miR-410 expression was upregulated after the knockdown of Notch-1 by siRNA in the irradiated SGC7901 cells, and these functions were similar to the effect by tangeretin (Fig. 7D). In summary, miR-410 plays an essential role in the biologic function of tangeretin in EMT.

Effects of tangeretin in vivo. Body weight partly reflects a healthy condition. A decrease in body weight was observed in the group exposed to radiation for 3 weeks. Tangeretin considerably alleviated radiation-induced weight loss in nude mice after 3 weeks of administration. During the period of administration, mice in the tangeretin + radiation group were in better physical condition and exhibited higher weight gain, compared with the radiation group (Fig. 8A).

Following treatment with tangeretin + radiation for 3 weeks, the tumor sizes were smallest among all the groups at the observation endpoint (Fig. 8B). The incidence of pulmonary metastasis in the radiotherapy and control groups was 100 and 50% (6/6 vs. 3/6, P<0.05; Fig. 8C). Compared with the radiation group, the tangeretin + radiation group had considerably attenuated lung metastases, with metastatic rates of 16.67% (1/6 vs. 6/6, P<0.05).

Discussion

The National Comprehensive Cancer Network guidelines recommend radiotherapy as a standard treatment for gastric cancer (GC) patients with a high-risk of recurrence (28). However, radiotherapy has shown the tendency to induce metastasis. Moreover, increased irradiation has toxic effects on the skin and other normal tissues (29). Identification of radiosensitizers that maximize radiotherapeutic efficacy and minimize toxicity has attracted worldwide attention. In the present study, we showed that tangeretin enhanced the radiosensitivity of SGC7901 cells and suppressed irradiation-induced epithelial-mesenchymal transition (EMT) and metastasis both *in vitro* and *in vivo*, probably via inhibition of Notch-1 signaling transduction and the 'switching on' of miR-410. This opens perspectives for the development of novel

radiosensitizers in GC therapy. Moreover, the absorption and pharmacokinetics of tangeretin have been studied both *in vivo* and *in vitro*. In hamsters administered 1% tangeretin flavone for 35 days, total tangeretin and tangeretin metabolites reached levels equivalent to 21 μM intact tangeretin in serum and 16-67 μM in liver (30). In rats intraperitoneally administered tangeretin (50 mg/kg), the concentration of tangeretin in serum reached a peak of 12.1 μM at 0.5 h (31). In the present study, the concentrations of tangeretin (10 and 30 μM) used *in vitro* were comparable to the levels achievable *in vivo*. Nevertheless, the detailed metabolic features of tangeretin in nude mice bearing tumor xenografts warrant more profound study.

Mounting evidence indicates that radiation is one of the inducers of EMT, and EMT directly induces radioresistance (32). Irregular EMT activation in the stomach is closely related with gastric carcinogenesis (33). EMT activation endows gastric epithelial cells with augmented characteristics of mesenchymal cells and decreases their epithelial features. Radiation-induced metastasis is also closely related to a mesenchymal phenotype. E-cadherin, an epithelial biomarker, is a member of a large superfamily of cell-cell adhesion molecules (34). During EMT, cadherin changes from E-cadherin to N-cadherin which is expressed in mesenchymal cells. Thus, decreased E-cadherin and increased N-cadherin are noted in the EMT process, and both can be identified as EMT biomarkers. Tangeretin has been found to inhibit invasion of cancer cells in an E-cadherin-dependent manner (34). Tangeretin also reduced the number of metastatic nodules in mice bearing B16F10 cell xenografts (35). In the present study, a decrease in E-cadherin as well as a simultaneous increase in N-cadherin and vimentin were observed after exposure to radiation, indicating that the epithelial cells acquired a mesenchymal-like morphology. Tangeretin successfully inhibited E-cadherin expression, invasion and migration induced by irradiation.

The Notch-1 pathway plays an important role in EMT progression (25). Blocking Notch-1 signaling by Hey-1 or Jagged1 knockdown mitigated EMT (36). As an oncogene in various solid malignancies, Notch-1 is expressed in most GC cell lines and normal gastric mucosa. The γ-secretase inhibitor DAPT (one of the GSIs) successfully inhibited the EMT and metastasis of GC cells. There is crosstalk between the EMT transcription factors and Notch such as Slug and Snail (37). Notably, Du *et al* showed that increased expression of Notch-1 is associated with non-cardia location, positive lymphovascular invasion, diffuse type, tumor size >5 cm and distal metastasis in GC cancer patients (38); Notch-1 may be regarded as a poor prognostic predictor in GC. Consistently, we found that Notch-1 was expressed in both SGC7901 cells and normal mucosa GES-1 cells, but a higher level was observed in SGC7901 cells than in GES-1 cells after exposure to irradiation, suggesting that Notch-1 is activated under irradiation. Tangeretin exhibited potent Notch-1 inhibiting capacity. However, tangeretin only suppressed the expression of Notch-1, not the activation of Notch-1. Furthermore, the overexpression of Notch-1 overrode the inhibitory effect of tangeretin on EMT. Consequently, Notch-1 may be a target of tangeretin in the inhibitory effect on EMT.

Recently, a mount of evidence strongly supports the multifactorial role of miRNAs in critical cellular processes. In particular, miRNAs may function as tumor-suppressor genes

or oncogenes. In the present study, we compared the expression of 6 miRNAs (miR-410, miR-143, miR-195, miR-222, miR-200c and miR-503) between GC cell lines/tissues and normal gastric cells/tissue. Studies indicate that miR-143 and 200c play an important role in blocking cancer progression (13,39). Our data revealed that tangeretin promoted miR-143 and miR-200c expression only *in vitro*, without a significant effect *in vivo*. The miR-503 level has been reported to be considerably reduced in GC tissues compared to normal mucosa tissues in 76 patients who experienced gastric surgery between 2012 and 2013 (16). miR-503 expression levels also negatively correlated with metastases in patients. The present study demonstrated that tangeretin only promoted miR-503 in tumor tissue from nude mice. Zhang *el al* reported that upregulation of miR-222 induced the malignant phenotype of SGC7901 cells, whereas knockdown of miR-222 reversed this phenotype (40). In the present study, tangeretin displayed no effect on miR-222 expression in the SGC7901 cells. In the present study, in SGC7901 cells, as well as in tumor tissues, combined treatment of tangeretin and irradiation upregulated the expression of miR-410. Gattolliat *et al* showed that the miR-410 level was considerably related with disease-free survival of the non-amplified neuroblastoma (41). Shen *et al* suggested that miR-410 acts as a tumor suppressor by targeting the MDM2 gene and inhibiting GC cell proliferation, migration and invasion (42). Similarly, we found that miR-410 expression was lower in the SGC7901 cells than that in the normal gastric mucosa cells. Moreover, the miR-410 level was higher in cells derived from non-metastatic tissues than those derived from metastatic tissues.

We further investigated the correlation between miR-410 and Notch-1. Overexpression of miR-410 significantly weakened Notch-1 expression and inhibited the EMT process in irradiated GC cells. In addition, Notch-1 siRNA led to the enhancement of miR-410 expression in the irradiated SGC7901 cells. Tangeretin treatment also promoted miR-410 expression in the irradiated SGC7901 cells.

Taken together, we conclude that both Notch-1 and miR-410 are key effectors in the biologic function of tangeretin. Inactivation of Notch-1 signaling leads to the reversal of EMT, along with less invasive characteristics. Tangeretin plus radiation has noteworthy potential as an effective anti-GC strategy.

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