



Amentoflavone inhibits colorectal cancer epithelial-mesenchymal transition via the miR-16-5p/*HMGA2*/β-catenin pathway

Kai Cai¹, Yang Yang¹, Zi-Jian Guo¹, Rui-Lin Cai², Hiroki Hashida³, Hong-Xia Li¹

¹Department of Oncology, the First Affiliated Hospital of Guangxi University of Chinese Medicine, Nanning, China; ²Department of Information Management and Information Systems, Xi'an Jiaotong-Liverpool University, Suzhou, China; ³Department of Surgery, Kobe City Medical Center General Hospital, Kobe, Japan

Contributions: (I) Conception and design: K Cai, Y Yang; (II) Administrative support: K Cai, Y Yang; (III) Provision of study materials or patients: K Cai, Y Yang, HX Li, ZJ Guo; (IV) Collection and assembly of data: K Cai, Y Yang, HX Li, ZJ Guo; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Professor Hong-Xia Li. Department of Oncology, the First Affiliated Hospital of Guangxi University of Chinese Medicine, 327 Xianhu Avenue, Qingxiu District, Nanning 530000, China. Email: 354935967@qq.com.

Background: Amentoflavone is a type of bioflavonoid that exists in many Chinese medicines and has anti-inflammatory, antioxidant, antiviral, and anticancer effects. However, the effect of amentoflavone on epithelial to mesenchymal transition (EMT) in human colorectal cancer (CRC) has not been studied. In this study, we aim to explore the effect of amentoflavone on EMT in CRC.

Methods: The effects of long noncoding RNA (lncRNA) miR-16-5p on proliferation, migration, and invasion were determined by *in vitro* and *in vivo* experiments. A luciferase reporter assay was carried out to reveal the interaction between miR-16-5p and targeted genes. Reverse transcription polymerase chain reaction (RT-PCR) was used to evaluate the expression of miR-16-5p. A western blot assay was used to detect the expression of targeted genes in CRC cells.

Results: The results showed that amentoflavone significantly inhibited CRC migration, invasion, and EMT by increasing miR-16-5p expression. Mechanistically, amentoflavone induced inactivation of the Wnt/β-catenin pathway via miR-16-5p, directly targeting 3'-UTR of *HMGA2* to suppress *HMGA2* expression in CRC. Clinically, combined miR-16-5p and *HMGA2* levels may serve as a predictor for poor prognosis in patients with CRC. Furthermore, an *in vivo* PDX model suggested that amentoflavone exhibited antitumor effects *in vivo* via the miR-16-5p/*HMGA2*/β-catenin pathway.

Conclusions: This is the first study to show that amentoflavone inhibits CRC EMT via the miR-16/*HMGA2*/β-catenin pathway. Amentoflavone may be beneficial in treating CRC patients in the clinic.

Keywords: Amentoflavone; colorectal cancer (CRC); miR-16; *HMGA2*; epithelial to mesenchymal transition (EMT)

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Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors in humans and is the main cause of cancer-related deaths worldwide (1). The Wnt/β-catenin signaling pathway plays a role in the invasion and metastasis of CRC (2-5). Epithelial to mesenchymal transition (EMT) is the key mechanism that drives most cancers, including

CRC invasion and metastasis (6). Dysregulation of the Wnt/β-catenin signaling pathway has been shown to play an important role in the EMT required for colorectal tumor metastasis (7,8).

Amentoflavone is a natural biflavone compound with many biological properties, including anti-inflammatory, anti-oxidative, and anti-apoptotic effects (9,10). Previous

studies have also found that amentoflavone exerts antitumor effects in solid tumors (11-13). However, the underlying mechanisms and functions of amentoflavone on CRC EMT have not been fully explored.

MicroRNAs (miRNAs) are a large class of small non-coding RNAs (14). The miR-16 family comprises six mature miRNAs (miR15a/b, miR-16-5p, miR-195, miR-424, and miR-497) widely explored in various cancers and considered tumor suppressors that inhibit tumor progression (15-20). MiR-16-5p has been reported to inhibit CRC progression (21). However, the potential role of miR-16-5p in amentoflavone-suppressed CRC tumor progression and its mediation of EMT in CRC remains unknown. In the present study, we demonstrated both *in vitro* and *in vivo* that amentoflavone increases miR-16-5p expression to inhibit CRC EMT and has an antitumor effect by blocking the *HMG2A2*/Wnt/ β -catenin pathway. We present the following article in accordance with the ARRIVE reporting checklist (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-3035/rc>).

Methods

Tissue specimens

Ninety-six CRC tissues and paired adjacent normal tissues were obtained from patients diagnosed with CRC who received surgery at the First Affiliated Hospital of Guangxi University of Chinese Medicine. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Medical Research Ethics Committee of the First Affiliated Hospital of Guangxi University of Chinese Medicine (No. UEXP00001325). Informed consent was taken from all the patients.

Cell culture and transient transfection

Human CRC cell lines (HCT116 and SW480) from the Chinese Type Culture Collection (Shanghai, China) were cultured in an Roswell Park Memorial Institute (RPMI) 1640 medium (RPMI 1640; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 2% penicillin/streptomycin (HyClone, Shanghai, China). All cell lines were authenticated by short tandem repeat (STR) profiling and all experiments were performed with mycoplasma-free cells. The cells were treated with amentoflavone (50 μ M) followed by Lipopolysaccharide (1 μ g/mL). The miRNA inhibitors and the miRNA negative control (NC) were synthesized

by the Shanghai Gene Pharma Co., Ltd. (Shanghai, China) and transiently transfected as previously described (22). The concentration of amentoflavone in the study was reference as previous (11-13).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from the CRC tissues and CRC cells. qRT-PCR was performed as previously described (22). The primers involved in this study were designed and provided by Sangon Biotech (Shanghai) Co. Ltd. The specific miRNA and mRNA expression levels were quantified using the $2^{-\Delta\Delta CT}$ method. U6 and ACTIN genes were used as normalization controls.

Cell invasion and migration assays

A 24-well chamber with an 8- μ m pore size (cat. no. 3422; Corning, Inc.) was used to detect the cell migratory and invasive abilities according to the manufacturer's protocol and performed as described previously (22). Use hole diameter of 8 μ M Transwell chamber (Corning Inc., Corning, NY, USA) to evaluate cell migration and invasion. 48 hours after transfection, 5 \times the transfected cells were put into 300 μ L medium without FBS and placed in the upper chamber, and 500 μ L medium with 20% FBS was placed in the lower chamber. For matrix invasion test, Transwell chamber is coated with matrix (BD Biosciences, San Jose, CA, USA). Inoculate 5 with 300 μ L culture medium without FBS \times 104 transfected cells were transferred to Transwell upper chamber, and 500 μ L culture medium with 20% FBS was placed in the lower chamber. The cells were incubated at 37 $^{\circ}$ C for 24 hours for migration and 48 hours for invasion. In the two tests, the cells were fixed with 100% methanol for 5 min (Baotai Biotechnology Research Institute, Haimen, China), and then stained with 0.5% crystal violet (Baotai Biotechnology Research Institute) for 5 min. Subsequently, the cells remaining on the upper surface of the membrane were carefully removed with a cotton swab. Then, in five randomly selected fields of view, an inverted microscope (magnification, \times 200) count migrating and invading cells. Each experiment was repeated 3 times.

Western blotting (WB)

Total protein from the CRC cell lines and tissues was

isolated, and the protein concentrations were measured using a bicinchoninic acid (BCA) protein assay kit (Pierce, IL, USA). The follow-up experiments were performed as described previously (22). E-Cadherin as the epithelial marker, and N-Cadherin and Vimentin as the mesenchymal marker were selected. The primary antibodies are listed in Table S1.

Luciferase assay

The pmirGLO-*HMGA2* 3'-UTR (WT) or pmirGLO-mutant *HMGA2* 3'-UTR (MUT) were co-transfected into cells with miR-16-5p or miR-NC by Lipofectamine 2000. The follow-up experiments were performed as described previously (22).

TOP/FOP flash assay

A TOP/FOP flash assay was performed as previously described (23). Luciferase activities were measured using the Dual-Glo Luciferase Assay System (Promega, Madison, Wisconsin, USA).

The patient-derived xenograft (PDX) model

To evaluate the therapeutic effect and molecular mechanism of Amentoflavone against CRC, the murine CRC model was used in this study. The female C57BL/6 mice were purchased from Changsha Tianqin BioTech Ltd. (China). All the mice were housed and bred under SPF condition, a 12-hour light/12-hour dark cycle was set under the animal facility, temperature was set 18–23 °C with 40–60% humidity.

For the PDX model, primary tumor samples were obtained for xenograft establishment as described previously (24). The mice were given oral doses of amentoflavone (50 mg/kg body weight) or vehicle control daily for 45 days (n=5 for each group). In this study, roughly 120 mice were used. The animals were euthanized when the diameter of the tumor exceeded 200 mm, to reduce any unnecessary pain, suffering and distress. No unexpected adverse events had been observed throughout this study. In this study, the endpoint refers the end stage of experimental period, and/or the tumor exceeded 200 mm. The mice with a CRC tumor were randomized into different groups.

Animal experiments were performed under a project license (No. ANIEXP00003106) granted by the Ethical Committee of the First Affiliated Hospital of Guangxi

University of Chinese Medicine, in compliance with national guidelines for the care and use of animals. A protocol was prepared before the study without registration.

Statistical analyses

To maintain the reliability of the conducted experiments, the performers were blinded, only the experiment designer(s) were aware of the group allocations. The data were analyzed using Prism 5 (GraphPad Software, San Diego, CA, USA) and SPSS 17.0 software (SPSS Inc., Cary, NC, USA). The values are presented as the means ± structural equation modeling (SEM). The details of the statistical methods are described in the Figure legends. All experiments were performed at least three times in duplicate.

Results

Amentoflavone inhibited CRC cell migration, invasion, and EMT

The Transwell assay indicated that compared with the control group, the migrative and invasive ability of HCT116 and SW480 cells treated with amentoflavone (50 μM) was significantly decreased (Figure 1A,1B). Therefore, migration and invasion of HCT116 and SW480 cells may be inhibited in response to amentoflavone treatment. The qRT-PCR and WB analyses indicated that the relative mRNA and protein expression of E-cadherin in HCT116 and SW480 treated with amentoflavone (50 μM) were significantly increased, while N-cadherin and vimentin were significantly decreased (Figure 1C,1D). The aforementioned results suggest that amentoflavone may inhibit CRC cell migration and invasion by EMT.

Amentoflavone increased miR-16-5p levels to inhibit EMT in CRC cells

The miR-16 family (miR-16-5p/miR-195-5p/miR-424-5p/miR-497-5p) exhibits a tumor suppressive potential, reducing EMT in many tumors. As shown in Figure 2A and Figure S1A, we found an increased level of miR-16-5p in HCT116 and SW480 cells treated with amentoflavone, while no changes were found in other family members. Additionally, we found that inhibited miR-16-5p enhanced EMT and reinstated the EMT ability in HCT116 and SW480 cells treated with amentoflavone (Figure 2B,2C

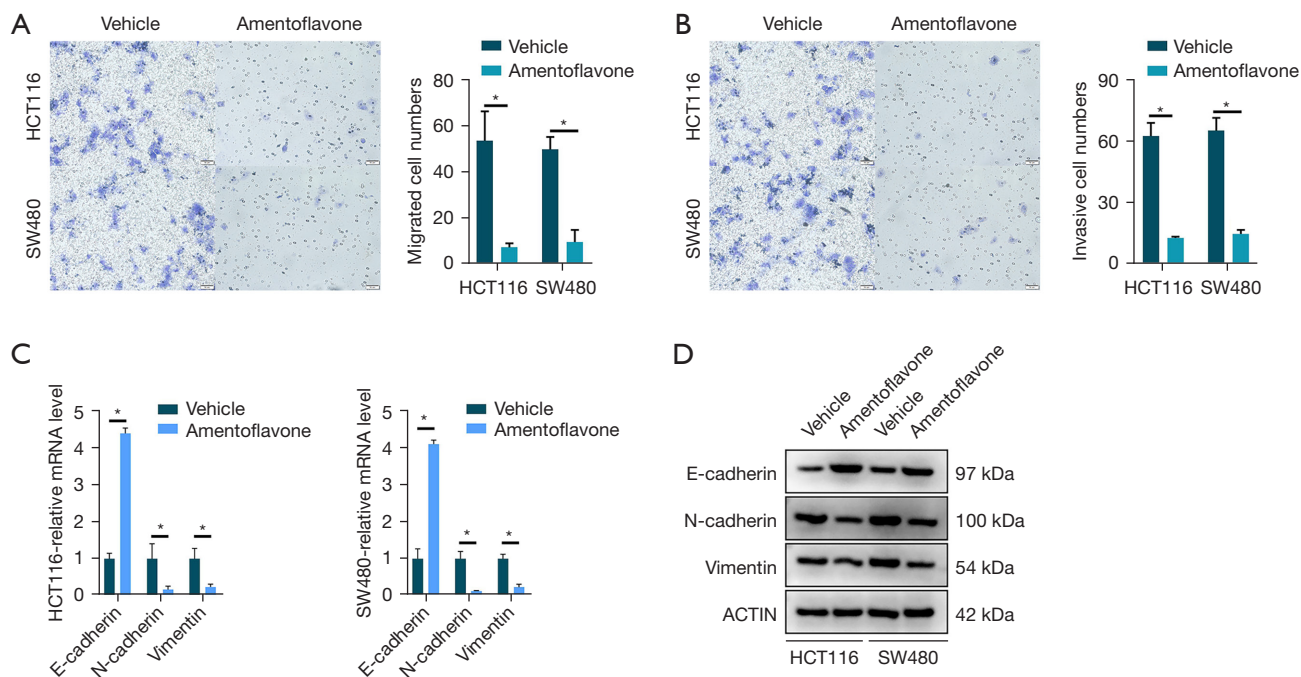


Figure 1 *In vitro* study showed Amentoflavone inhibits CRC cell migration, invasion, and EMT. (A,B) Transwell assays were performed to examine the potential migration (A) and invasion (B) of HCT116 and SW480 cells treated with amentoflavone (5 μ m) or vehicle (crystal violet staining, control group), where the scale bar =50 μ m. (C) qPCR validation of EMT marker mRNA expression in HCT116 and SW480 cells treated with amentoflavone (5 μ m) or vehicle. (D) WB validation of EMT marker protein expression in HCT116 and SW480 cells treated with amentoflavone (5 μ m) or vehicle. (A–C) N=3, mean \pm SEM, *P<0.05, by Student's *t*-test. Data represents similar results from 3 independent experiments. CRC, colorectal cancer; EMT, epithelial to mesenchymal transition; qPCR, quantitative polymerase chain reaction; SEM, structural equation modeling; WB, western blotting.

and Figure S1B). Meanwhile, amentoflavone treatment inhibited the migration and invasion of HCT116 and SW480 cells, whereas inhibited miR-16-5p expression enhanced HCT116 and SW480 cell migration and invasion (Figure 2D,2E). Moreover, inhibited miR-16-5p reversed the migration and invasion ability in HCT116 and SW480 cells treated with amentoflavone (Figure 2D,2E). The above results indicated that amentoflavone inhibited EMT, migration, and invasion ability in CRC cells by increasing miR-16-5p levels.

miR-16-5p targets *HMGA2* in CRC

To explore the EMT function of miR-16-5p in CRC, we used the TargetScan database (http://www.targetscan.org/vert_71/) to identify the target genes of miR-16. Among the predicted targets, we focused on eight genes that had cumulative weighted context++ scores >0.50 and an aggregate PCT (percentage) >0.80 (Table S2). As shown in

Figure 3A, *HMGA2*, reported to be involved in inducing EMT in CRC (25), was increased in HCT116 and SW480 cells treated with miR-16-5p inhibitor, while there was no change in the other 21 genes. Therefore, we selected *HMGA2* for further validation in CRC. According to the prediction by the seed complementarity, a potential miR-16-5p-binding site was found at nt1302–1309 of *HMGA2* 3'-UTR (Figure 3B). Additionally, the results showed that co-transfection of miR-16-5p inhibitor significantly increased the firefly luciferase activity of the reporter with WT 3'-UTR of *HMGA2* but not that of the mutant reporter (Figure 3C), which indicates that miR-16-5p directly targets the 3'-UTR of *HMGA2*.

Amentoflavone decreased *HMGA2* via miR-16-5p

As shown in Figure 3D,3E, amentoflavone treatment suppressed the mRNA and protein of *HMGA2* in HCT116 and SW480 cells, whereas inhibited miR-16-5p expression

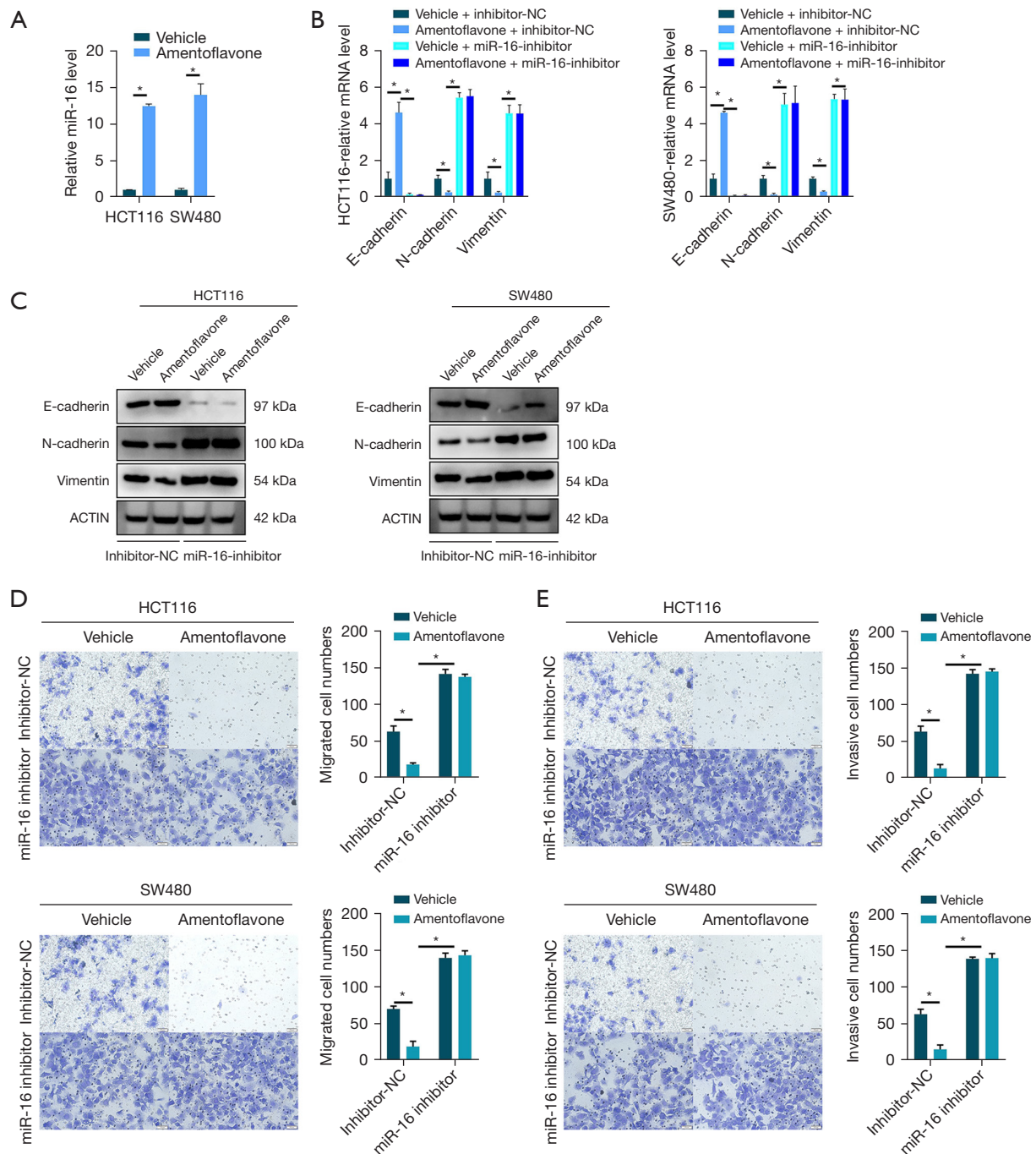


Figure 2 *In vitro* study amentoflavone inhibits EMT, migration, and invasion ability in CRC cells by increasing miR-16 levels. (A) qPCR validation of miR-16 expression in HCT116 and SW480 cells treated with amentoflavone (5 μ m) or vehicle. (B,C, control group) qPCR and WB validation of EMT marker mRNA (B) and protein (C) expression in HCT116 and SW480 cells treated with amentoflavone (5 μ m) or vehicle and transfected with inhibitor-NC or miR-16 inhibitor. (D,E) Transwell assays were performed to examine the potential migration (D) and invasion (E) of HCT116 and SW480 cells treated with amentoflavone (5 μ m) or vehicle and transfected with inhibitor-NC or miR-16 inhibitor (crystal violet staining), where the scale bar = 50 μ m. (A,B,D,E) N=3, mean \pm SEM, *P<0.05, by Student's *t*-test. Data represents similar results from 2 independent experiments. EMT, epithelial to mesenchymal transition; CRC, colorectal cancer; qPCR, quantitative polymerase chain reaction; NC, negative control; SEM, structural equation modeling.

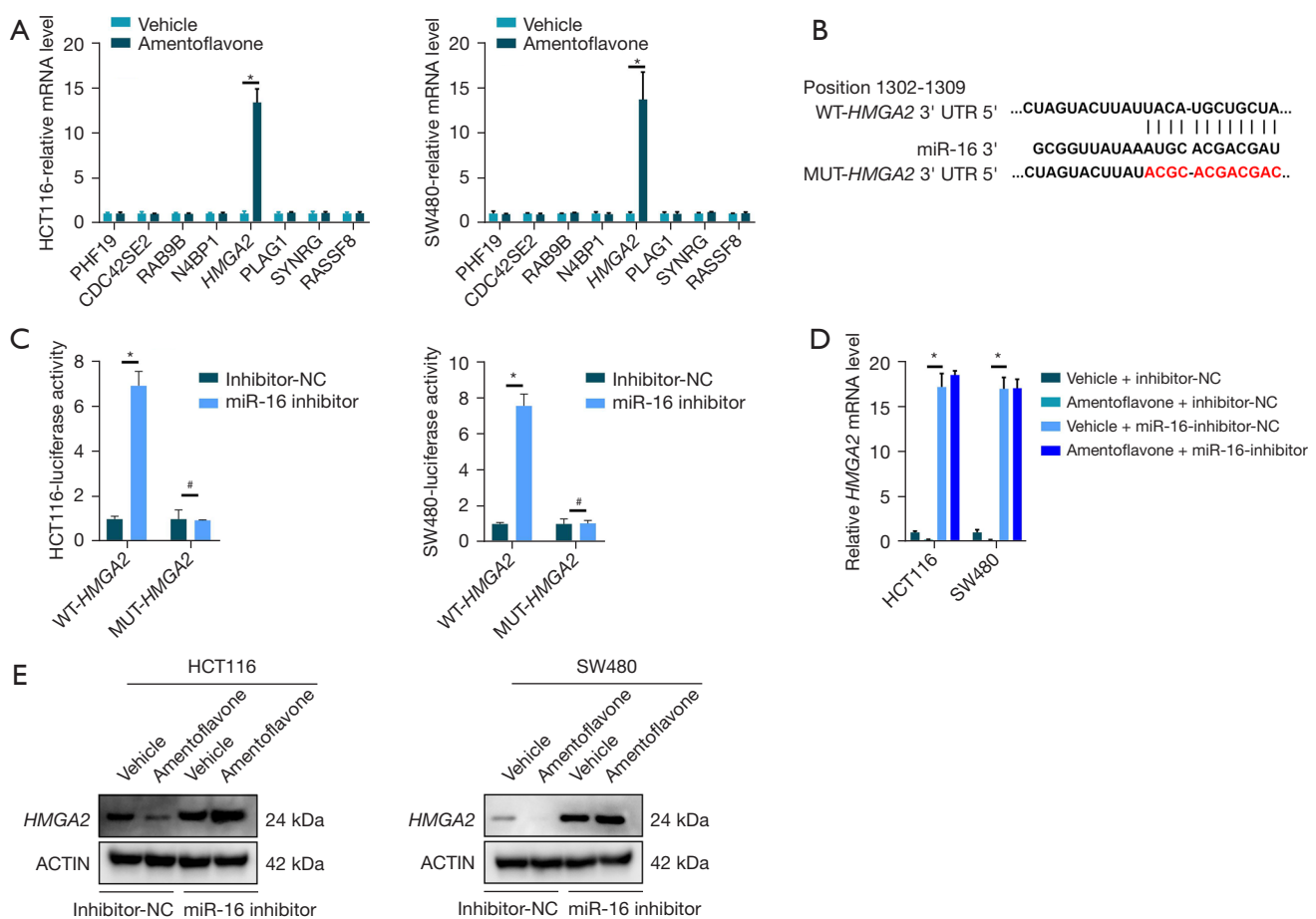


Figure 3 Amentoflavone decreases *HMGA2* via miR-16. (A) qPCR validation of 22 predicted miR-16 target gene expressions in HCT116 and SW480 cells treated with amentoflavone (5 μ m) or vehicle. (B) The putative miR-16-binding sites on the *HMGA2* 3'UTR and the mutated *HMGA2* 3'UTR sites generated by site-directed mutagenesis. (C) The luciferase activity of HCT116 and SW480 cells transfected with inhibitor-NC or miR-16 inhibitor. (D,E) qPCR and WB validation of *HMGA2* mRNA (C) and protein (D) expression in HCT116 and SW480 cells treated with amentoflavone (5 μ m) or vehicle and transfected with inhibitor-NC or miR-16 inhibitor. (A,C,D) N=3, mean \pm SEM, *P<0.05; #P<0.05, by Student's *t*-test. Data represents similar results from 3 independent experiments. qPCR, quantitative polymerase chain reaction; NC, negative control; WB, western blotting; SEM, structural equation modeling.

increased the mRNA and protein of *HMGA2* in HCT116 and SW480 cells. Moreover, inhibited miR-16-5p replenished the mRNA and protein of *HMGA2* in HCT116 and SW480 cells treated with amentoflavone (Figure 3D,3E). All the above results indicated that amentoflavone decreased *HMGA2* via miR-16.

Amentoflavone inactivated the Wnt/ β -catenin pathway via the miR-16/*HMGA2* axis

As *HMGA2* is a key regulator activating the Wnt/ β -catenin pathway and miR-16-5p targets *HMGA2* in CRC, we

wondered whether amentoflavone inactivates the Wnt/ β -catenin pathway via miR-16. As shown in Figure 4A, amentoflavone treatment suppressed the protein level of β -catenin in HCT116 and SW480 cells, whereas inhibited miR-16-5p expression increased the protein level of β -catenin in HCT116 and SW480 cells. Moreover, inhibited miR-16-5p replenished the protein level of β -catenin in HCT116 and SW480 cells treated with amentoflavone (Figure 4B). Additionally, amentoflavone treatment suppressed the Wnt/TCF luciferase reporter activity in HCT116 and SW480 cells, whereas inhibited miR-16-5p expression increased the Wnt/TCF luciferase reporter activity in HCT116 and

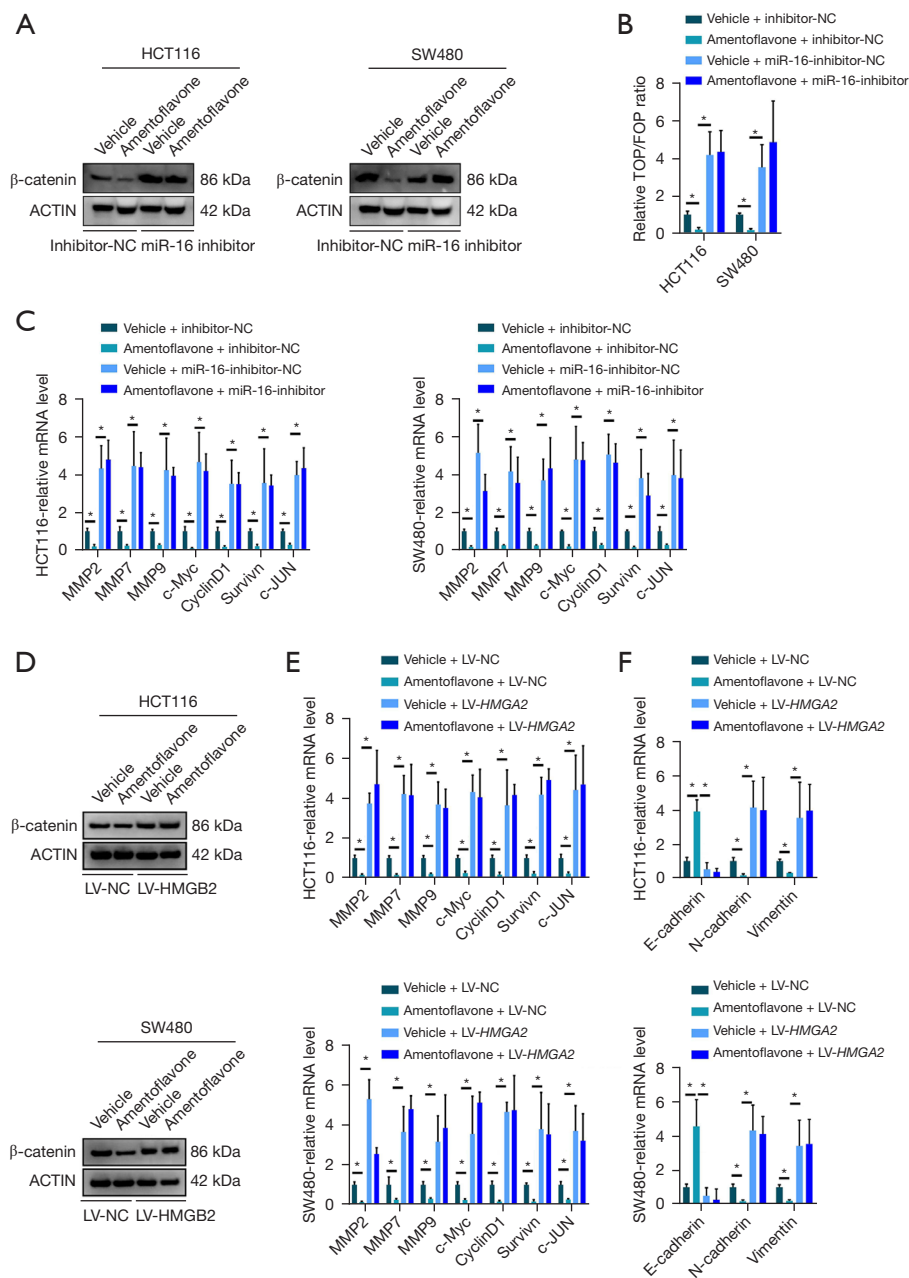


Figure 4 Amentoflavone inactivates the Wnt/ β -catenin pathway via the miR-16/*HMGA2* axis. (A) WB validation of β -catenin protein expression in HCT116 and SW480 cells treated with amentoflavone (5 μ m) or vehicle and transfected with inhibitor-NC or miR-16 inhibitor. (B) The TOP/FOP ratio of HCT116 and SW480 cells treated with amentoflavone (5 μ m) or vehicle and transfected with inhibitor-NC or miR-16 inhibitor. (C) qPCR validation of genes related to the Wnt/ β -catenin pathway protein expression in HCT116 and SW480 cells treated with amentoflavone (5 μ m) or vehicle and transfected with inhibitor-NC or miR-16 inhibitor. (D) WB validation of β -catenin protein expression in HCT116 and SW480 cells treated with amentoflavone (5 μ m) or vehicle and transfected with LV-NC or LV-*HMGA2*. (E) qPCR validation of genes related to the Wnt/ β -catenin pathway protein expression in HCT116 and SW480 cells treated with amentoflavone (5 μ m) or vehicle and transfected with LV-NC or LV-*HMGA2*. (F) qPCR validation of genes related to the EMT in HCT116 and SW480 cells treated with amentoflavone (5 μ m) or vehicle and transfected with LV-NC or LV-*HMGA2*. (B,C,E,F) N=3, mean \pm SEM, *P<0.05, by Student's *t*-test. Data represents similar results from 3 independent experiments. WB, western blotting; NC, negative control; qPCR, quantitative polymerase chain reaction; SEM, structural equation modeling; MT, epithelial-mesenchymal transition.

SW480 cells. Moreover, inhibited miR-16-5p replenished the Wnt/TCF luciferase reporter activity in HCT116 and SW480 cells treated with amentoflavone (Figure 4B). As shown in Figure 4C, amentoflavone treatment suppressed the mRNA expression of Wnt targets in HCT116 and SW480 cells, whereas inhibited miR-16-5p expression increased the mRNA expression of Wnt targets in HCT116 and SW480 cells. Moreover, inhibited miR-16-5p replenished the mRNA expression of Wnt targets in HCT116 and SW480 cells treated with amentoflavone (Figure 4C). Meanwhile, amentoflavone treatment suppressed the protein level of β -catenin in HCT116 and SW480 cells, whereas overexpression of *HMGA2* expression increased the protein level of β -catenin in HCT116 and SW480 cells. Moreover, overexpression of *HMGA2* expression replenished the protein level of β -catenin in HCT116 and SW480 cells treated with amentoflavone (Figure 4D). Additionally, amentoflavone treatment suppressed the Wnt/TCF luciferase reporter activity in HCT116 and SW480 cells, whereas overexpression of *HMGA2* expression increased the Wnt/TCF luciferase reporter activity in HCT116 and SW480 cells. Moreover, overexpression of *HMGA2* expression replenished the Wnt/TCF luciferase reporter activity in HCT116 and SW480 cells treated with amentoflavone (Figure 4E). As shown in Figure 4F, amentoflavone treatment suppressed the mRNA expression of Wnt targets in HCT116 and SW480 cells, whereas overexpression of *HMGA2* expression increased the mRNA expression of Wnt targets in HCT116 and SW480 cells. Moreover, overexpression of *HMGA2* expression replenished the mRNA expression of Wnt targets in HCT116 and SW480 cells treated with amentoflavone (Figure 4F). All the above results indicated that amentoflavone inactivated the Wnt/ β -catenin pathway via the miR-16/*HMGA2* axis.

The combination of miR-16-5p and HMGA2 predicts prognosis in CRC patients

To assess the clinical significance of the miR-16-5p/*HMGA2* axis in CRC, we examined the expression of miR-16 and *HMGA2* mRNA in 96 CRC patients. As shown in Figure 5A,5B, miR-16-5p was decreased in CRC tumors compared to adjacent normal tissues, while *HMGA2* mRNA was increased in CRC tumors compared to adjacent normal tissues. The expression of miR-16-5p was negatively correlated with the expression of *HMGA2* mRNA in the 96 patients with CRC (Figure 5C and Table S3). Kaplan-Meier analysis revealed the survival benefits in CRC

patients with low miR-16-5p or high *HMGA2* mRNA levels (Figure 5D,5E). Of note, the combination of low miR-16-5p and high *HMGA2* mRNA predicted a better overall survival of CRC patients (Figure 5F). Collectively, these results suggest the prognostic value of the combination of miR-16-5p and *HMGA2* in predicting the outcome of CRC patients.

Amentoflavone exhibited antitumor effects in the PDX model

Next, by virtue of PDXs from CRC tumors, we found that, compared with the vehicle control, amentoflavone partially blocked the growth of PDXs derived from CRC tumors (Figure 6A,6B). Additionally, as shown in Figure 6C,6D, amentoflavone treatment suppressed the mRNA of *HMGA2*, whereas it increased miR-16-5p expression in PDXs. Meanwhile, amentoflavone treatment suppressed *HMGA2* and β -catenin protein in the PDXs (Figure 6E,6F), which demonstrates that amentoflavone inhibited CRC tumor growth *in vivo* via the miR-16-5p/*HMGA2*/ β -catenin pathway.

Discussion

In this study, we revealed that amentoflavone reduced the migration, invasion, and EMT in CRC cell lines by increasing miR-16-5p levels. Mechanistically, amentoflavone treatment inactivated the Wnt/ β -catenin pathway via miR-16-5p, directly targeting 3'-UTR of *HMGA2* to suppress *HMGA2* expression in CRC. In addition, our clinical investigations revealed a close correlation between miR-16-5p and *HMGA2* levels and CRC progression and patient prognosis. Moreover, the *in vivo* PDX model suggested that amentoflavone exhibited antitumor effects *in vivo* via the miR-16-5p/*HMGA2*/ β -catenin pathway. Hence, these findings indicate that amentoflavone's ability to inhibit migration, invasion, and EMT and exert antitumor effects might be attributable to the inhibition of the *HMGA2*/Wnt/ β -catenin pathway activation by the increase in miR-16-5p expression (Figure 7).

In our previous study, amentoflavone exhibited antitumor effects in several solid tumors (19-21). However, the anticancer effect and the mechanism of action of amentoflavone in CRC are unknown. In this study, we demonstrated the anti-CRC cell migration, invasion, and EMT effect of amentoflavone and the role of increased miR-16-5p expression on amentoflavone-induced inhibition

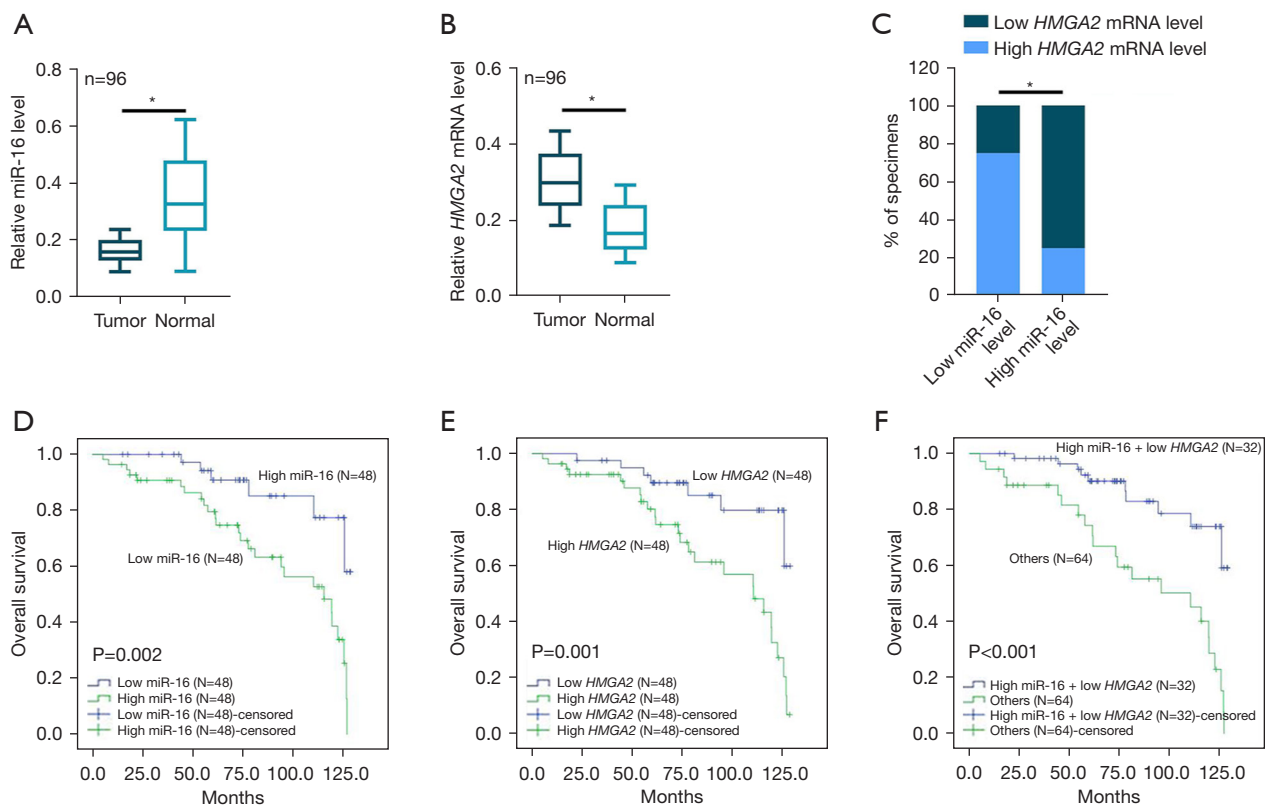


Figure 5 The combination of miR-16 and *HMG2* predicts prognosis in CRC patients. (A,B) qPCR validation of miR-16 and mRNA of *HMG2* expression in 96 CRC patients. (C) The percentages of specimens showing low or high miR-16 expression relative to the levels of *HMG2* are shown. (D) The comparison of overall survival between 96 CRC patients exhibiting low or high miR-16 levels. (E) The comparison of overall survival between 96 CRC patients exhibiting low or high *HMG2* mRNA levels. (F) The comparison of overall survival between patients exhibiting high miR-16 and low *HMG2* levels with the other patients in (D). (A,B) $N=3$, mean \pm SEM, $*P<0.05$, by Student's *t*-test. (C) The *P* value was evaluated by variance analysis. (D-F) The *P* value was evaluated by the log-rank test. A high miR-16 or *HMG2* mRNA expression in each of the 96 CRC patients was defined as a value above the 50th percentile. Data represents similar results from 2 independent experiments. CRC, colorectal cancer; qPCR, quantitative polymerase chain reaction; SEM, structural equation modeling.

of tumor progression in CRC. miR-16-5p is a member of the miR-15/-16/-195/-424/-497/-503 family. A remarkably inhibitory effect of miR-16-5p on the progression of several types of cancer has been reported previously (10-15). However, the function of miR-16-5p in the progression of CRC remains unknown. In this study, we found that amentoflavone reduced migration, invasion, and EMT in CRC cell lines dependent on an increased miR-16-5p expression, suggesting that miR-16-5p is a crucial tumor suppressor in CRC. In addition, our clinical investigations revealed a close correlation between miR-16-5p levels and CRC patient prognosis, thus making miR-16-5p an optimal target for CRC therapy.

MicroRNAs can function as tumor suppressors or oncogenes by targeting their respectively associated genes (23). Using TargetScan bioinformatics, the *HMG2* gene was potentially identified as a direct target for miR-16-5p. *HMG2* is a nonhistone and architectural transcription factor, which exerts an oncogenic effect on numerous cancers. Recently, overexpression of *HMG2* in CRC patients has been shown to play an important role in metastasis development and is a potential indicator of poor survivability (26-29). In this study, we demonstrated a negative regulation of *HMG2* by miR-16-5p using a luciferase reporter assay. Following amentoflavone treatment, *HMG2* RNA and protein levels decreased

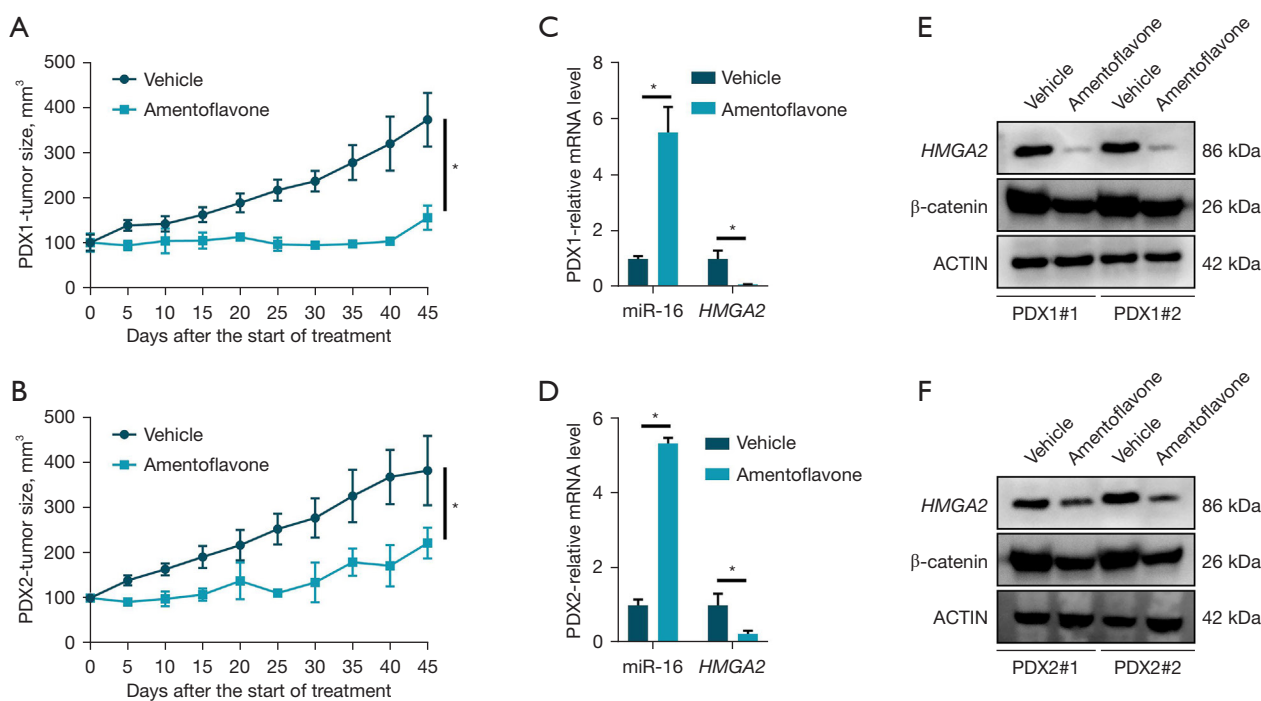


Figure 6 Animal experiments showed Amentoflavone exhibits antitumor effects in the PDX model. (A,B) PDX1 and 2 treated with amentoflavone (50 mg/kg body weight) or vehicle (control group) for 45 days (n=5 for each group). The xenograft growth was monitored. (C,D) qPCR validation of miR-16 and mRNA of *HMG2* expression from the tumors of PDX-1 and -2. (E,F) qPCR validation of *HMG2* and β-catenin protein expression from the tumors of PDX-1 and -2. (A-D) N=3, mean±SEM, *P<0.05, by Student's *t*-test. Data represents similar results from 3 independent experiments. PDX, patient-derived xenograft; qPCR, quantitative polymerase chain reaction; SEM, structural equation modeling.

and were recovered by inhibiting miR-16-5p expression. In addition, we observed that miR-95-3p expression was negatively correlated with *HMG2* transcription in CRC. This finding implies that the miR-16-5p/*HMG2* axis might serve as a novel therapeutic target in patients with CRC.

There are multiple reports of Wnt/β-catenin pathway activation by *HMG2* to enhance migration, invasion, and EMT (30-32). In this study, we found that amentoflavone inhibited the Wnt/β-catenin pathway, and inhibiting miR-16-5p could reverse this inhibitory effect. Wnt/β-catenin signaling has been confirmed to promote tumor metastasis by inducing EMT (33). EMT is a process in which cancer cells lose their epithelial characteristics, obtain mesenchymal phenotypes, and develop motility and invasiveness (34). In the current study, we found that amentoflavone played an antitumor role in CRC by increasing miR-16-5p expression and suppressing *HMG2* and β-catenin levels, which suggests that the miR-16-5p/*HMG2*/β-catenin axis

determines the amentoflavone response.

There are some limitations associated with the current study. Future studies need to establish a metastatic model, such as a tail vein injection model, to validate the effects of amentoflavone on CRC cell metastasis *in vivo*. In this study, only the TargetScan database was explored to identify miR-16 target genes. Therefore, future studies should use additional online databases to define the potential targets of miR-16 in CRC cells. Our PDX model showed amentoflavone partially blocked the growth of PDXs derived from CRC tumors, but the *in vitro* data failed to show that amentoflavone inhibited the proliferation of CRC cells. Further research should use more PDX models to define the effect of amentoflavone in inhibiting CRC cell growth; to explore the novel lncRNA regulating the miR-16/*HMG2*/β-catenin pathway in CRC; to explore the effect of amentoflavone on T cell infiltration and tumor microenvironment during EMT in CRC; supplemented results of morphological experiments *in vivo* experiments.

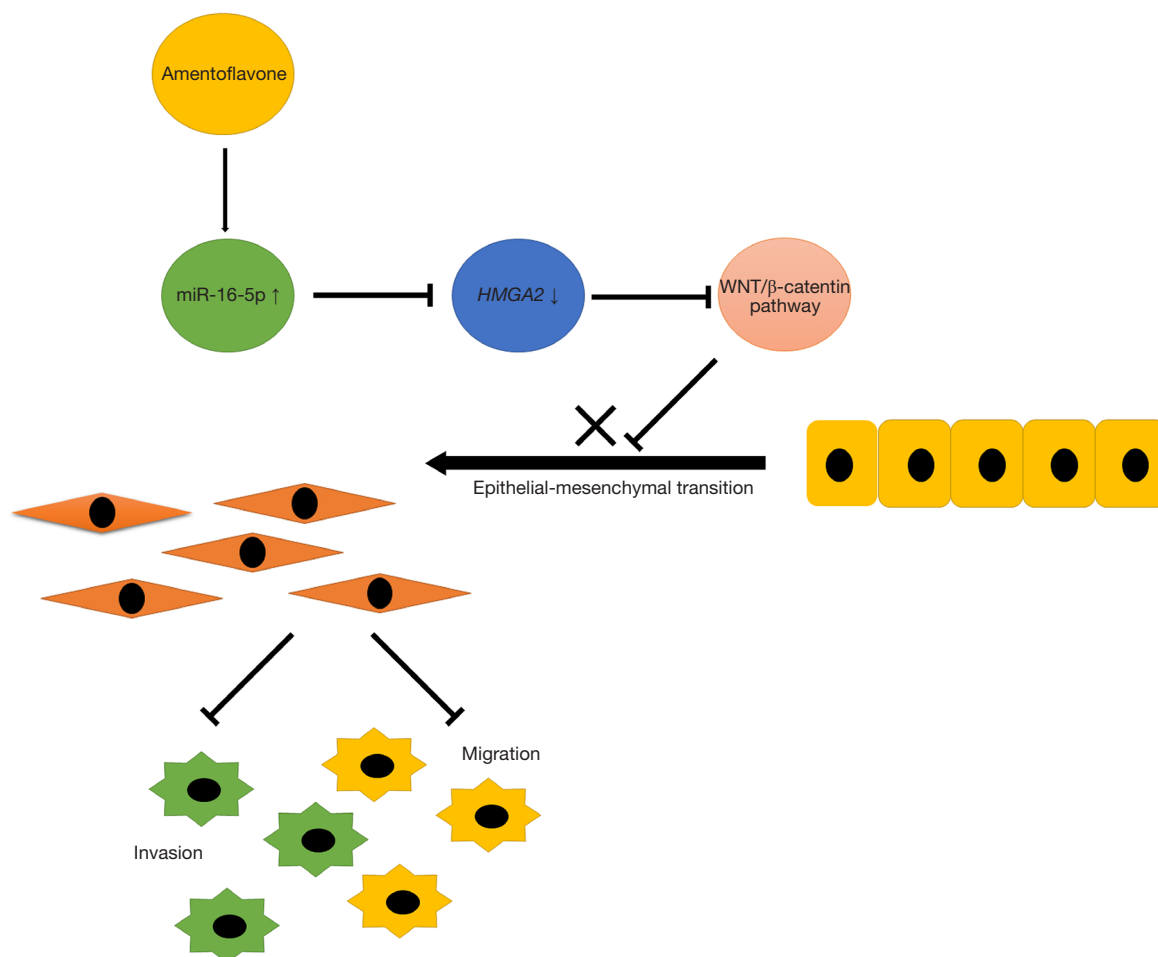


Figure 7 The graphic illustration of amentoflavone inhibiting CRC EMT via the miR-16/*HMGA2*/β-catenin pathway. CRC, colorectal cancer; EMT, epithelial to mesenchymal transition.

Till now, the achievements from this study are still in preclinical stage, though it's still need to accomplish comprehensive studies to evaluate its therapeutic effect on patients, it shed lights on discovery of new biomarkers for clinical purposes.

Taken together, our results demonstrated that amentoflavone has the potential to inhibit CRC progression through suppression of *HMGA2*/Wnt/β-catenin activation by increasing miR-16-5p levels.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <https://atm.amegroups.com/article/view/10.21037/atm-22-3035/rc>

Data Sharing Statement: Available at <https://atm.amegroups.com/article/view/10.21037/atm-22-3035/dss>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-3035/icoi>)

amegroups.com/article/view/10.21037/atm-22-3035/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Medical Research Ethics Committee of the First Affiliated Hospital of Guangxi University of Chinese Medicine (No. UEXP00001325). Informed consent was taken from all the patients. Animal experiments were performed under a project license (No. ANIEXP00003106) granted by the Ethical Committee of the First Affiliated Hospital of Guangxi University of Chinese Medicine, in compliance with national guidelines for the care and use of animals. A protocol was prepared before the study without registration.

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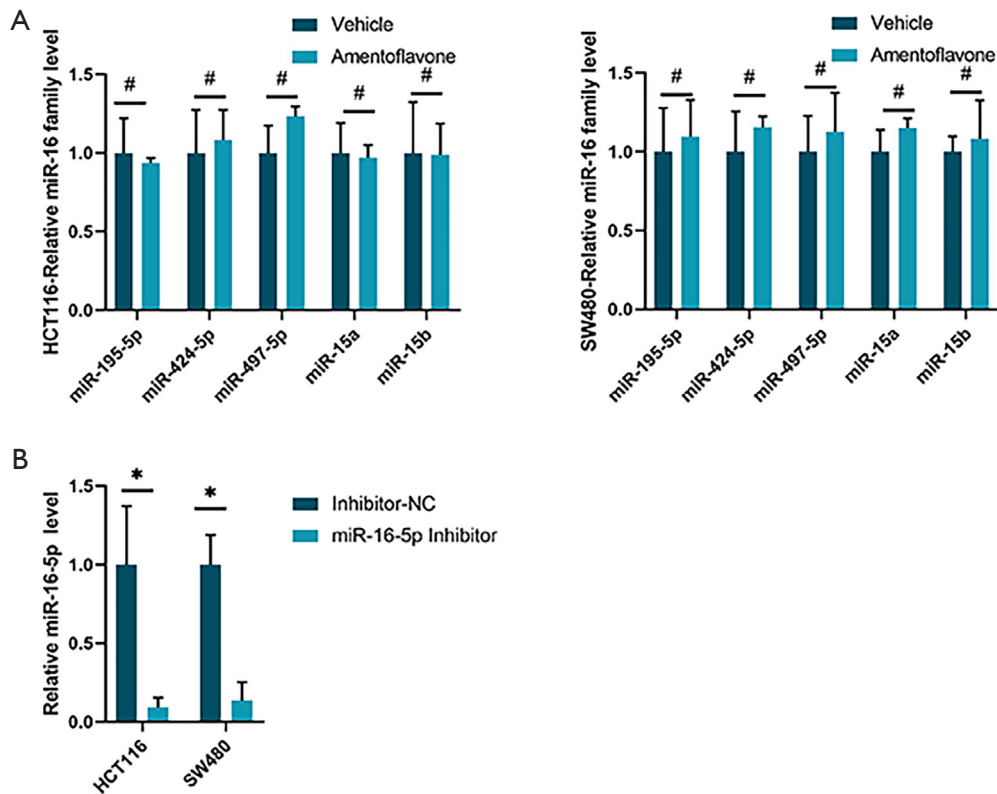


Figure S1 Amentoflavone increases miR-16-5p levels to inhibit EMT in CRC cells. (A) qPCR validation of the miR-16 family miRNAs expression in HCT116 and SW480 cells treated with amentoflavone (5 nM) or vehicle. (B) qPCR validation of miR-16-5p expression in HCT116 and SW480 cells treated with inhibitor-NC or miR-16 inhibitor. (A, B) N=3, mean \pm SEM, *P<0.05 and [#]P>0.05, by Student's *t*-test. EMT, epithelial to mesenchymal transition; CRC, colorectal cancer; qPCR, quantitative polymerase chain reaction; SEM, Structural Equation Modeling.

Table S1 Antibody list

Antigens	Manufacturer	Application
ACTIN	abcam (ab8226)	1:10,000 for WB
HMGA2	abcam (ab207301)	1:1,000 for WB
E-Cadherin	abcam (ab40772)	1:1,000 for WB
N-Cadherin	abcam (ab76011)	1:1,000 for WB
Vimentin	abcam (ab92547)	1:1,000 for WB
β -Catenin	abcam (ab32572)	1:1,000 for WB

Table S2 The predicted target genes of miR-16-5p

Target gene	Representative transcript	Representative miRNA	Cumulative weighted context++ score	Aggregate P _{CT}
PHF19	ENST00000373896.3	hsa-miR-16-5p	-0.83	0.97
CDC42SE2	ENST00000360515.3	hsa-miR-16-5p	-0.78	0.85
RAB9B	ENST00000243298.2	hsa-miR-16-5p	-0.69	0.93
N4BP1	ENST00000262384.3	hsa-miR-16-5p	-0.58	0.9
HMGA2	ENST00000403681.2	hsa-miR-16-5p	-0.55	0.85
PLAG1	ENST00000316981.3	hsa-miR-16-5p	-0.52	0.98
SYNRG	ENST00000339208.6	hsa-miR-16-5p	-0.51	0.89
RASSF8	ENST00000541490.1	hsa-miR-16-5p	-0.51	0.9

Table S3 Association between miR-16-5p expression and HMGA2 mRNA levels in 96 CRC patients

HMGA2 mRNA level	miR-16-5p level		χ^2	P value [§]
	High (N=48)	Low (N=48)		
High (N=48)	12	36	24.000	<0.001
Low (N=48)	36	12		

[§], Pearson chi-square test was used for comparison between subgroups. CRC, colorectal cancer.