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Research

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Abstract

Background: Tanshinone IIA (Tan IIA) is a major active ingredient extracted from *Salvia miltiorrhiza*, which has been proved to inhibit metastasis of various cancers including colorectal cancer (CRC). However, the detailed mechanisms of Tan IIA against CRC metastasis are not well explored. Epithelial-to-mesenchymal transition (EMT) exerts an important regulatory role in CRC metastasis, and our previous mechanism studies demonstrated that β -arrestin1 could regulate CRC EMT partly through β -catenin signaling pathway. Therefore, in this work we investigated whether Tan IIA could regulate CRC EMT through β -arrestin1-mediated β -catenin signaling pathway *in vivo* and *in vitro*.

Methods: The nude mice tail vein metastasis model was established to observe the effect of Tan IIA on CRC lung metastasis *in vivo*. The lung metastasis was evaluated by living animal imaging and hemaoylin-eosin staining. The migratory ability of CRC cells *in vitro* were measured by transwell and wound healing assays. The protein expression and cellular localization of β -arrestin1 and β -catenin were characterized by immunofluorescence staining and western blot. The β -catenin signaling pathway related proteins and EMT associated proteins in CRC cells were detected by western blot and immunohistochemistry.

Results: Our results showed that Tan IIA inhibited the lung metastases of CRC cells *in vivo* and extended the survival time of nude mice. *In vitro*, Tan IIA increased the expression of E-cadherin, decreased the secretion of Snail, N-cadherin and Vimentin, thus suppressed EMT and the migratory ability of CRC cells. Further study found the mechanism involving in Tan IIA regulating EMT and metastasis, referring to the suppression of β -arrestin1 expression, reduction of β -catenin nuclear localization, thereby the decreased activity of β -catenin signaling.

Conclusion: Our data revealed a new mechanism of Tan IIA on the suppression of EMT and metastasis in CRC via β -arrestin1-mediated β -catenin signaling pathway, and provided support for Tan IIA as anti-metastatic agents in CRC.

Background

Colorectal cancer (CRC) is the third most common cancer and the second cause of cancer-associated death in the world, consisting of over 1.7 million new cases and 861,663 deaths per year [1]. High mortality rate is due to that half of the CRC patients are diagnosed at advanced stages or recurrence and metastasis after tumor resection [2]. Hence, exploring the molecular mechanism of CRC metastasis, preventing recurrence and metastasis of CRC, and improving the survival rate of CRC patients have become the urgent problem to be solved.

Epithelial-to-mesenchymal transition (EMT) plays a key role in CRC metastasis [3]. Generally, epithelial cells lose cell polarity and the ability of cell adhesion, penetrate the basement membrane and enter the

circulatory system, then initiate and promote the invasion and metastasis of cancer cells [4]. During EMT, down-regulation of E-cadherin leads to the loss of cell adhesion, upregulation of Vimentin and N-cadherin causes the reorganization of the actin cytoskeleton and promotes the motility [5], and upregulation of transcription factors such as Snail, Slug and Twist induces EMT [6]. In addition, TGF- β /SMADs [7], PI3K/Akt [8] and other signaling pathways were participated in regulating EMT. In our previous study, we have found that β -arrestin1 had a higher expression in lung metastases than in primary CRC. Moreover, β -arrestin1 could promote EMT and metastasis through repressing the expression of GSK-3 β , inhibiting the degradation of β -catenin, thereby activating β -catenin signaling pathway (*Support information for review*).

Tanshinone is an ether or ethanol extract from the root of *Salvia miltiorrhiza*, and Tan IIA is major active constituent. Several studies have showed the pharmacological effects of Tan IIA on the apoptosis, metastasis, drug resistance, and angiogenesis in different cancers [9–11]. However, the mechanisms of Tan IIA on CRC EMT and metastasis are not well elucidated. In this study, we aim to explore the effect and mechanism of Tan IIA on CRC EMT and metastasis *in vivo* and *in vitro*, and provide a theoretical basis for further application of Tanshinone IIA in CRC therapy.

Materials And Methods

Cell culture and reagents

Human colorectal cancer cell line HCT-116 (ATCC, USA) was cultured in 1640 medium and LoVo (ATCC, USA) was cultured in F-12K medium, at 37°C in a 5% CO₂ incubator, containing 10% fetal bovine serum (FBS), 100U/mL penicillin, 100mg/mL streptomycin. Rabbit monoclonal antibodies against human E-cadherin, N-cadherin, Vimentin, Snail, β -arrestin1, GSK-3 β , β -catenin, c-Myc and CyclinD1 were purchased from Cell Signaling Technology (USA). Rabbit monoclonal antibodies against human MMP2 and MMP9 were purchased from Santa Cruz Biotechnology (USA). Mouse monoclonal antibodies against human GAPDH were purchased from Proteintech (China). Tanshinone IIA was purchased from Selleck (Houston, TX, USA).

Cell viability assays

Cell Counting Kit-8 (CCK-8) was used to detect cell proliferation. Firstly, the 96-well plate was covered with 1×10^4 cells/well. Secondly, when the cell density reached 60%, different concentrations of Tan IIA were added into the plate and incubated for 24 h, 48 h and 72 h. Thirdly, the medium containing CCK-8 reagent was used to culture the treated cells for 4 hours. Then, absorption was measured at 450 nm with a microplate reader (Biorad, USA). All experiments were carried out in 6 wells, and each experiment was repeated at least three times.

Western blot

In brief, all of the cells were lysed with RIPA Lysis, and the extracted protein was quantified by BCA protein assay (Beyotime Biotechnology, Shanghai, China). Approximately 50µg of proteins were added into the 10% SDS-PAGE gels for electrophoresis and then transferred onto a polyvinylidene difluoride (PVDF) membrane on ice. After that, PVDF was blocked with 5% BSA, and incubated with the primary antibodies following by the HRP-conjugated secondary antibodies. The results were examined with enhanced chemiluminescence (ECL, Millipore, CA, USA), and the target bands analysis by using the Scion Imaging application (Scion Corporation).

Immunofluorescence microscopy

HCT-116 and LoVo cells were fixed for 30 minutes with 4% paraformaldehyde at room temperature, permeabilized with Triton X-100 (0.5%) for 15 min, then blocked with 5% BSA solution for 1 h. The cells were incubated with primary antibodies for 1 h, following by incubating with secondary antibodies at room temperature for 1 h. After incubation, nuclear was labeled with DAPI for 5 minutes. Finally, cells were tested with a DMI3000B inverted microscope (Leica, Germany). **Transwell assay for migration** HCT-116 and LoVo cells were inoculated into the upper chamber of transwell plate and cultured in 600 µl 1640 or F12K medium with 10 µg/ml fibronectin, medium containing 15% FBS was added in the lower chamber of transwell plate. Then different concentrations of Tan IIA were added into the upper chamber and incubated for 48 h. Migrated cells were detected by crystal violet staining, and observed by using DMI3000B inverted microscope (Leica, Germany). Five random views were selected to count the migrated cells.

Wound-healing assay

HCT-116 or LoVo cells were seed into 6-well plates for 24 h, then were created an artificial scratch wound using a 20µl pipette tip and detached cells were removed by washing with PBS three times. After 48 h incubation, cell migration was photographed using inverted microscope and evaluated by measuring the difference in wound width.

Animal model

Male BALB/c nude mice (5 weeks old) were purchased from Department of Experimental Animals of Shanghai University of Traditional Chinese Medicine (Shanghai, China, license No. SCXK 2018-0006) and fed in specific pathogen-free conditions for 1 week. The mice were injected with 2×10^6 HCT-116/Luc cells by the tail vein. Two weeks later, the mice were randomly divided into five groups, 8 in each group. The mice were treated with different concentrations of Tan IIA (0.5, 1, 2 mg/kg) for 4 weeks by tail vein injection. For bioluminescence imaging, mice were anesthetized with 1% pentobarbital sodium

(200µl/per) by intraperitoneal injection, and injected intraperitoneally with D-luciferin (15mg/ml), and captured the data by using an IVIS Lumina system (Caliper, USA). After that, the lung organs were excised, fixed in 4% paraformaldehyde, and paraffin-embedded. The lung sections were fully cut, and paraffin-embedded tissues were cut into 5µm sections. All the lung sections were stained with hematoxylin-eosin (HE), and E-cadherin, Vimentin, Snail and β-arrestin1 were detected with immunohistochemistry (IHC). The animal experiments were performed under the approval of the animal ethics committee of Shuguang Hospital, Shanghai University of Traditional Chinese Medicine.

Statistical analysis

All the data were presented as the means ± SD of at least three independent experiments and analyzed with SPSS22.0 Software. The mean values of two groups were compared by Student's t test. $P < 0.05$ was considered as statistically significant.

Results

Tan IIA inhibited the lung metastasis of colorectal cancer *in vivo*

Tan IIA is a major active ingredient extracted from *Salvia miltiorrhiza*, which has been proved to inhibit metastasis of various cancers. In the present study, to assess the effect of Tan IIA on CRC metastasis *in vivo*, firstly, the lung metastasis model was established by tail vein injection, and then the mice were treated with different concentrations of Tan IIA. *In vivo* imaging results indicated that HCT-116/luc cells transferred to lung tissue after tail injection, and Tan IIA (0.5, 1, 2 mg/Kg) [9] inhibited the metastatic ability of HCT-116/luc cells in a concentration-dependent manner (Fig. 1A, 1B). We also found that Tan IIA could improve the mice survival. Compared with control group, the life prolongation rates of low, medium and high dose of Tan IIA in tumor-bearing mice were 8.73%, 19.09% and 34.63%, respectively (Fig. 1C). After resection of lung metastases, the numbers of lung metastases in Tan IIA groups were significantly fewer than control group. Especially in the high-dose of Tan IIA group, the number of lung metastases was the least (Fig. 1D). HE staining was used to observe lung metastases in nude mice, which was consistent with *in vivo* imaging results (Fig. 1E).

Tan IIA inhibited the EMT of CRC cells

Above studies have shown that Tan IIA could inhibit lung metastasis of colorectal cancer *in vivo*, and this ignites our interesting to explore the potential anti-metastatic mechanism of Tan IIA *in vitro*. EMT is mainly featured by the destruction of adherens junctions (AJs), and E-cadherin, N-cadherin, Vimentin and Snail were the characteristic proteins [12]. Firstly, we detected its anticancer activity of CRC HCT-116 cells for 24 h, 48 h, 72 h under different concentrations of Tan IIA (0, 1.25, 2.5, 5, 10, 20, 40, 80, 100 µM) by

using CCK assay. The data showed that Tan IIA inhibited HCT-116 cells (Fig. 2A) in a concentration- and time-dependent manner. Then, we calculated the IC₅₀ value (17.48 μ M for 48 h in HCT-116 cells) of Tan IIA, and took 0, 5, 10, 20 μ M and 48 h as the common dosage and treatment time in the next experiment.

The following data revealed that, treatment with Tan IIA reduced the expression of N-cadherin, Vimentin and Snail, while E-cadherin had an opposite result in a concentration-dependent manner (Fig. 2B-2E). Immunofluorescence images demonstrated that, the levels of E-cadherin in the membrane increased, but the levels of Vimentin in the cytoplasm decreased after adding Tan IIA (Fig. 2F-2I), which was similar to the results of western blot.

Tan IIA inhibited the migration of CRC cells

Since the effect of Tan IIA on EMT has been confirmed, next we investigated the role of Tan IIA on CRC metastasis *in vitro*. Transwell assay showed that, compared with control group, Tan IIA could inhibit the migration of colorectal cancer cells in a concentration-dependent manner (Fig. 3A-3D). Similar to the results of Transwell assay, Wound-healing assay suppressed the migratory ability of CRC cells after treating with Tan IIA 48 h (Fig. 3E-3H). Extracellular matrix (ECM) is the physical barrier in the process of tumor metastasis. The change of ECM structure is closely related to the invasion and metastasis of tumor. Abnormal expression of matrix metalloproteinases (MMPs) accounts for degradation of ECM, especially for MMP-2 and MMP-9 [13-14]. The results indicated that, the expression levels of MMP-2 and MMP-9 were obviously decreased (Fig. 3I-3L), which was consistent with previous analysis.

Tan IIA inhibited the migration of CRC cells via β -arrestin1-mediated β -catenin signaling pathway

To study whether the anti-metastasis effect of Tan IIA was associated with β -arrestin1/ β -catenin signaling pathway in HCT-116 and LoVo cells, GSK-3 β , β -catenin, the downstream genes of β -catenin signaling including c-Myc and CyclinD1 were detected by western blot. As shown in the following figure, the level of β -arrestin1 was reduced, while the level of GSK-3 β was increased when treatment with Tan IIA. Further studies discovered that Tan IIA inhibited β -catenin translocating into nucleus, and decreased the accumulation of β -catenin in the nucleus, with a corresponding reduction in c-Myc and CyclinD1 (Fig. 4A-4D).

Tan IIA inhibited CRC metastasis via β -arrestin1/ β -catenin signaling pathway *in vivo*

Next, we investigated metastasis-related proteins in each group of lung metastases by using immunohistochemistry and western blot. Immunohistochemistry discovered that Tan IIA obviously decreased β -arrestin1, Vimentin and Snail protein levels, while increased E-cadherin protein level in a

concentration-dependent manner (Fig. 5A). Consistent with the previous results, lung metastases treated with Tan IIA had a higher expression of E-cadherin and lower expression of N-cadherin, Vimentin and Snail (Fig. 5B-5C). Further detection of metastasis-related proteins found that the levels of MMP-2 and MMP-9 were reduced after Tan IIA treatment (Fig. 5D-5E). The Wnt/ β -catenin signaling activity was partly blocked by Tan IIA. The result was shown that Tan IIA down-regulated the expression levels of β -arrestin1, β -catenin and the downstream gene of β -catenin, such as c-Myc and CyclinD1, while up-regulated the protein expression of GSK-3 β in a concentration-dependent manner (Fig. 5F-5G)

Discussion

Tan IIA, which is derived from *Salvia miltiorrhiza*, has been proved to have antioxidant, coronary artery dilatation and anti-inflammatory effects [15–16]. Recent studies have shown that Tan IIA has anti-cancer effect in many malignant tumors, such as colon cancer, breast cancer, ovarian cancer [17–19]. Our previous studies demonstrated that Tan IIA had an effect on the invasion and metastasis of CRC [9]. EMT is characterized by loss of polarity of epithelial cells and acquisition of mesenchymal properties, which is closely related to invasion and distant metastasis of tumor cells [20]. When EMT was triggered, the epithelial marker E-cadherin and mesenchymal marks Vimentin had significant changes [21]. *In vivo*, our present results suggested that Tan IIA could inhibit lung metastasis of CRC and improve the survival of tumor-bearing mice via suppressing the EMT process. *In vitro*, the data indicated that Tan IIA upregulated E-cadherin levels and downregulated Vimentin levels through inhibiting the expression of Snail transcription factors in a concentration-dependent manner. As EMT is one of the most key steps for metastasis of CRC, and likewise, we found the expression of MMP-2 and MMP-9 were decreased after treating with Tan IIA.

A series of studies have found that Wnt/ β -catenin acts as a major way to be involved in EMT and invasion and metastasis of tumors [22–24]. When canonical Wnt signaling pathway is abnormally activated, the function of Axin, APC, GSK-3 β and CK1 α complex is greatly restricted, accumulated β -catenin enters into the nucleus from cytoplasm, then the β -catenin/TCF/LEF complex transcriptional activates Snail directly and triggers the EMT [25–26]. Our previous data discovered that, β -arrestin1 promoted EMT through β -catenin signaling pathway in CRC progression *in vivo* and *in vitro*. The mechanism of β -arrestin1 regulating EMT and metastasis partly through decreasing the expression of GSK-3 β , leading to the accumulation of β -catenin in the cytoplasm and promoting β -catenin to get into the nucleus. In present study, firstly, *in vitro* experimental results demonstrated that Tan IIA inhibited the expression of β -arrestin1 while promoted the expression of GSK-3 β , thus blocking β -catenin entry into the nucleus. Subsequently, the expression of c-Myc and CyclinD1 were reduced as a result of the inhibition of β -catenin. Secondly, *in vivo* experiments obtained a similar result of Tan IIA, demonstrated the effects of Tan IIA on EMT, β -catenin signaling pathway and its related proteins.

Conclusion

In summary, our results found that Tan IIA could inhibit EMT and metastasis in CRC through β -arrestin1-mediated β -catenin signaling pathway, which partly accounting for its potential anti-metastatic mechanism for CRC (Fig. 6).

Abbreviations

Tan IIA: Tanshinone IIA; CRC: colorectal cancer; EMT: Epithelial-to-mesenchymal transition; FBS: fetal bovine serum; CCK-8: Cell Counting Kit-8; PVDF: polyvinylidene difluoride; ECL: enhanced chemiluminescence; HE: hematoxylin-eosin; IHC: immunohistochemistry; AJs: adherens junctions; MMPs: matrix metalloproteinases

Declarations

Ethics approval and consent to participate

All the animal protocols were approved by the Animal Care Commission of Shanghai University of Traditional Chinese Medicine.

Consent for publication

Not applicable.

Availability of data and materials

All of the data and materials in this paper are available when requested.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Q.J. and Q.L. conceived and designed the study and the experimental setup and wrote the manuscript. Q.S., L.Y., Z.H., X.W., R.L., L.Z., N.L. and H.S. performed the experiments. Q.J., Q.L., J.C. and Y.W analyzed the data. All authors read and approved the final version of the manuscript.

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Figures

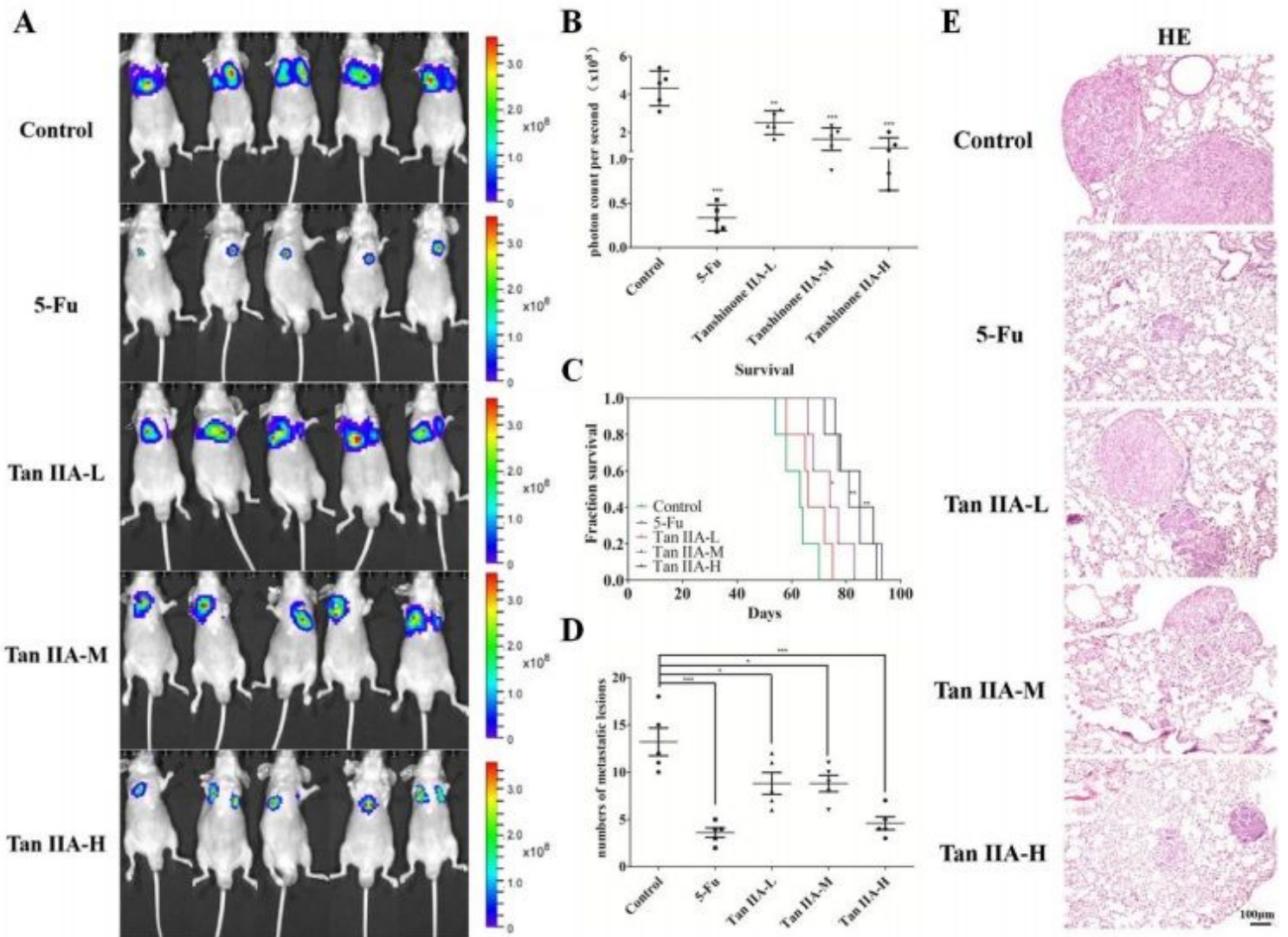


Figure 1

Tan IIA inhibited the metastasis of colorectal cancer in vivo. A-B Each group of mice were injected with HCT-116/luc cells by the tail vein. After treatment with Tan IIA drug concentration (0.5, 1, 2 mg/Kg) for 4 weeks, Luciferase imaging data was collected by IVIS Lumina system. *** $P \leq 0.001$, compared with control group. C The survival of tumor-bearing mice were evaluated, * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$, compared with control group. D-E The lung metastasis were excised, hemaoylin-eosin (H&E) staining of tumor cytostructure and cytomorphology analysis in tumors, and the numbers of metastatic lesions were counted, * $P \leq 0.05$; *** $P \leq 0.001$, compared with control group.

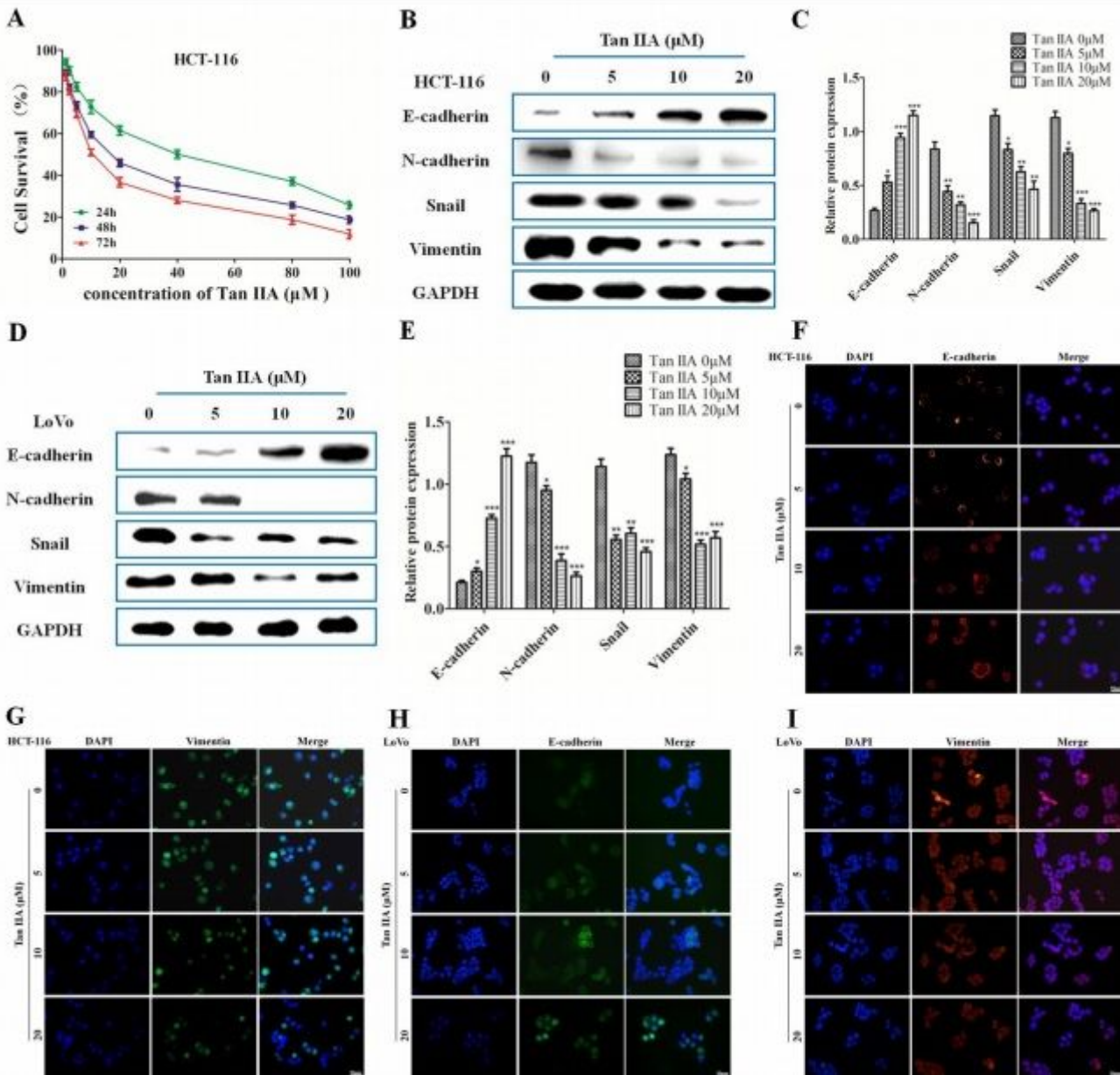


Figure 2

Tan IIA inhibited EMT in colorectal cancer cells. A CCK-8 assay was used to detect the cellular inhibition of Tan IIA (0, 1.25, 2.5, 5, 10, 20, 40, 80, 100 μM) in HCT116 cells for 24, 48, and 72 h. B-E HCT-116 cells and LoVo cells were treated with Tan IIA at 0 μM , 5 μM , 10 μM , and 20 μM , western blot was used to detect the expression of E-cadherin, N-cadherin, Snail, Vimentin, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with group without treatment of Tan IIA. F-I The effect of Tan IIA on epithelial phenotype marker E-cadherin and mesenchymal phenotype marker Vimentin were detected by immunofluorescence staining.

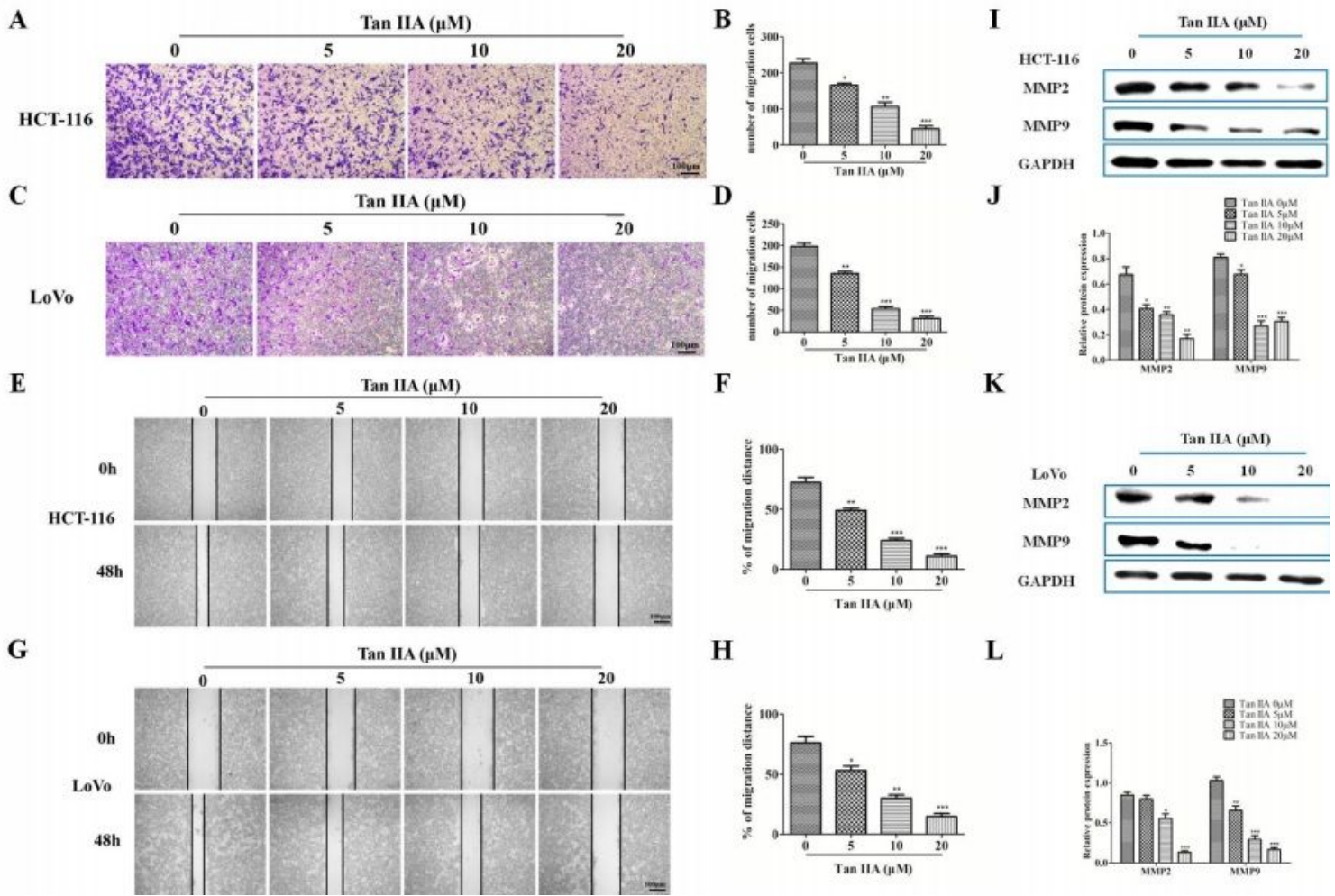


Figure 3

Tan IIA inhibited the migration of CRC cells. A-D HCT-116 and LoVo cells treated with different concentration of Tan IIA, transwell assay was used to detect the migratory cells and count from five random microscopic fields, * $P \leq 0.05$; ** $P \leq 0.01$, ** $P \leq 0.01$, compared with group without treatment of Tan IIA. E-H HCT-116 and LoVo cells treated with or without Tan IIA for 48 h, collection of the wound-healing assay data are shown. The black line was used to mark the ranges of the scratches, * $P \leq 0.05$; ** $P \leq 0.01$, ** $P \leq 0.01$, compared with group without treatment of Tan IIA. I-L The expression of MMP-2 and MMP-9 were examined by western blot, * $P \leq 0.05$; ** $P \leq 0.01$, ** $P \leq 0.01$, compared with group without treatment of Tan IIA.

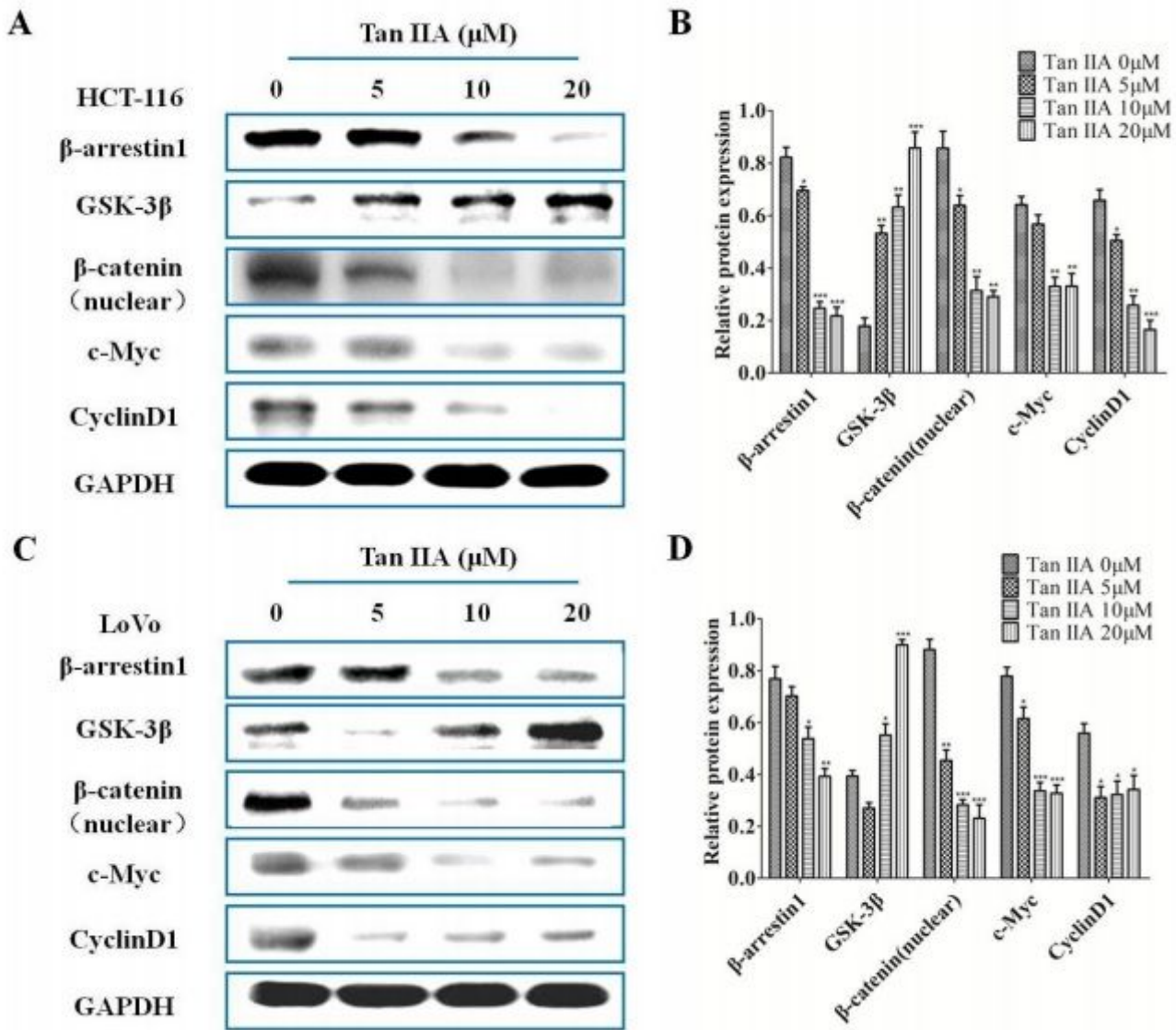


Figure 4

Tan IIA inhibited β -arrestin1-mediated β -catenin signaling pathway. A-B Western blot analysis the expression of β -arrestin1, GSK-3 β , β -catenin, c-Myc, CyclinD1 in HCT-116 cells with or without Tan IIA treatment, * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$, compared with group with Tan IIA at 0 μM . C-D After using Tan IIA at 0 μM , 5 μM , 10 μM , 20 μM treatment in LoVo cells, the level of β -arrestin1, GSK-3 β , β -catenin, and downstream genes including c-Myc, CyclinD1 were detected by western blot, * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$, compared with group without treatment of Tan IIA.

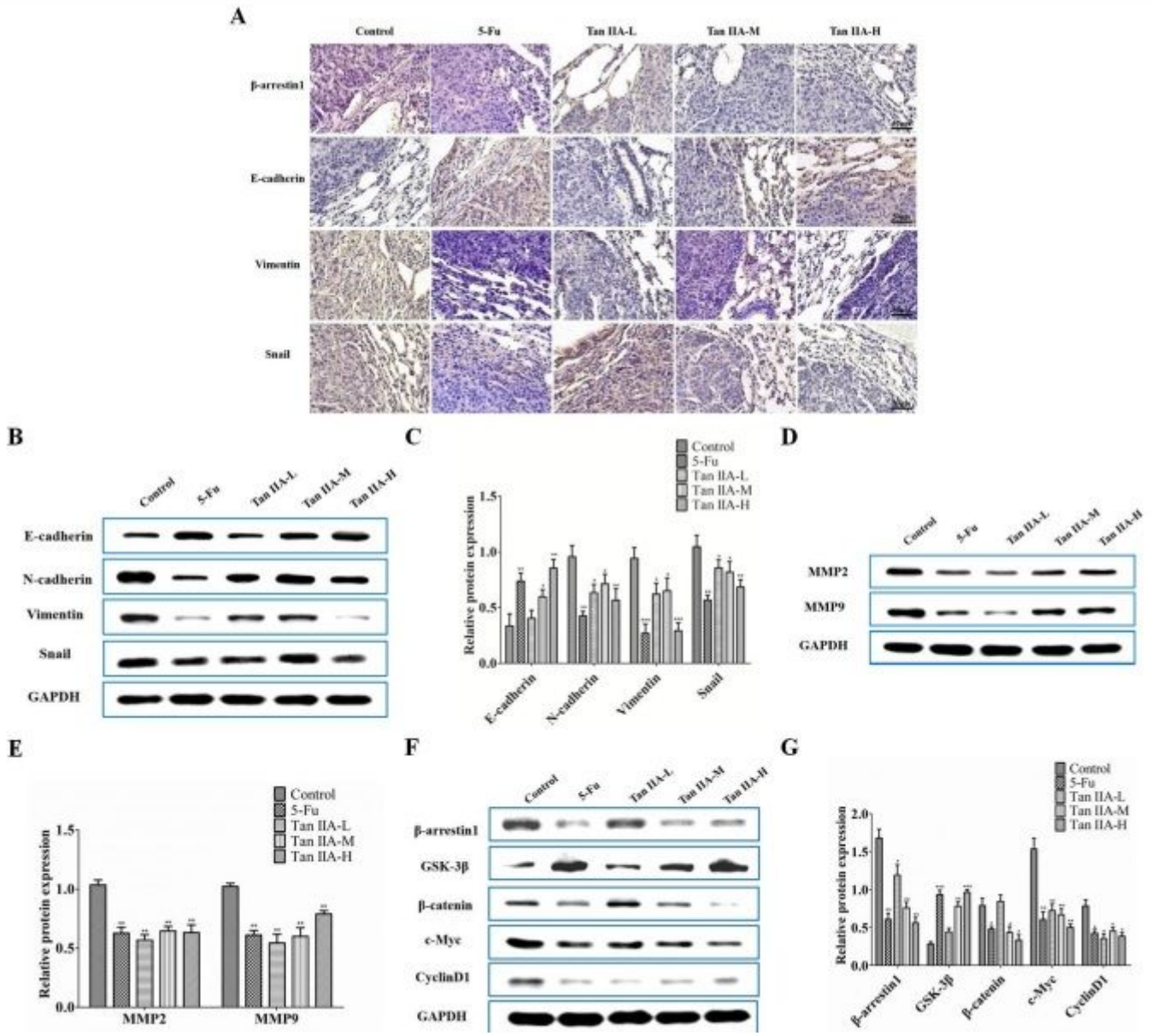


Figure 5

Tan IIA inhibited metastasis of CRC via β -arrestin1/ β -catenin signaling pathway in vivo. A Immunohistochemistry detected the expression of β -arrestin1, E-cadherin, Vimentin and Snail in each groups. B-C Western blot was applied to assess the expression of E-cadherin, N-cadherin, Snail and Vimentin, * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$, compared with control group. D-E The levels of MMP-2 and MMP-9 were checked, ** $P \leq 0.01$, compared with control group. F-G The protein expression of β -arrestin1, GSK-3 β , β -catenin, c-Myc and CyclinD1 were examined by western blot, * $P \leq 0.05$; ** $P \leq 0.01$, *** $P \leq 0.001$, compared with control group.

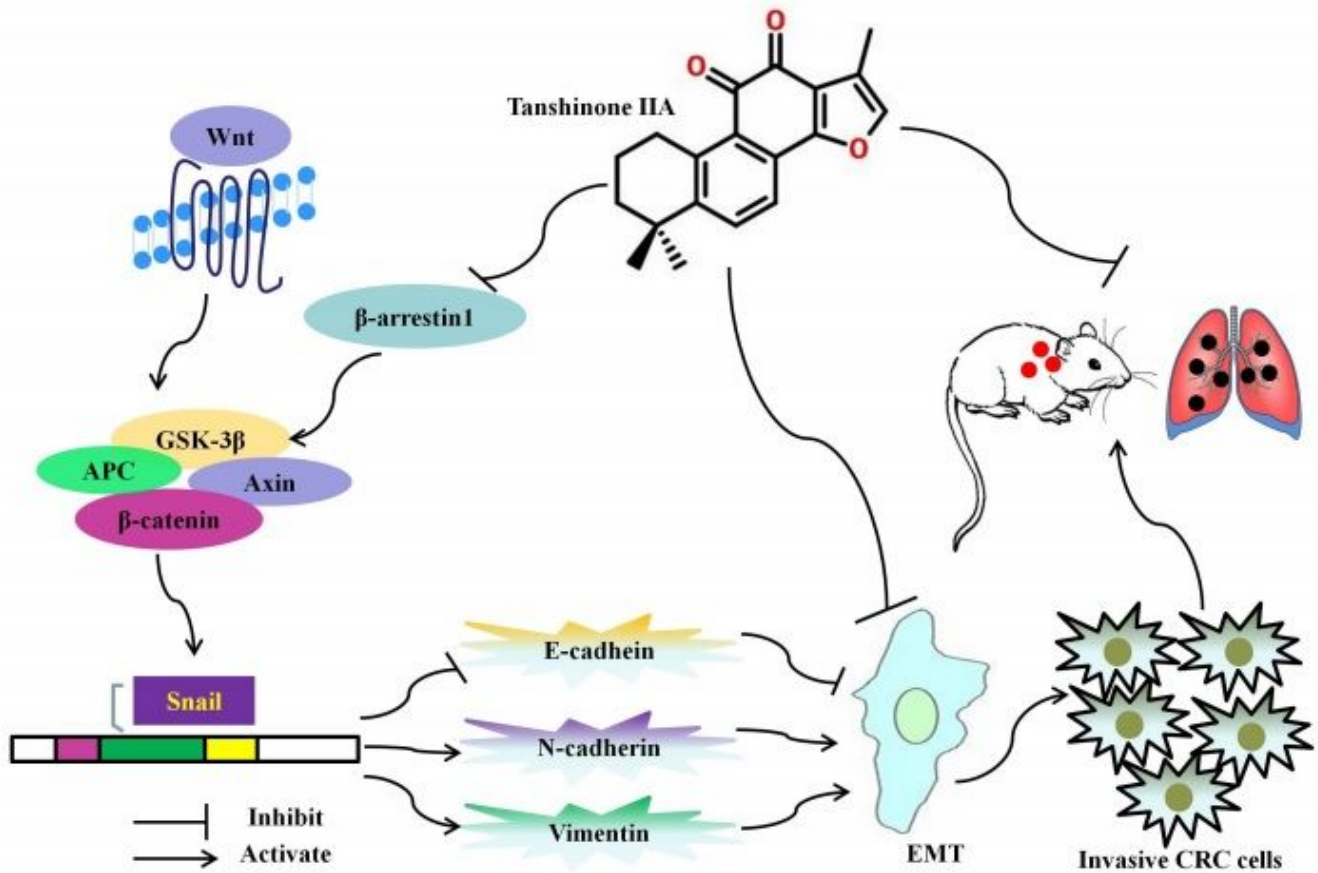


Figure 6

Hypothetical diagram of the mechanism of Tanshinone IIA on EMT and metastasis in colorectal cancer.

Supplementary Files

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