Molecular Cancer Therapeutics

Nigericin Exerts Anticancer Effects on Human Colorectal Cancer Cells by Inhibiting Wnt/β-catenin Signaling Pathway

Fei Liu[1], Wei Li[2], Shangbo Hua[3], Ye Han[4], Zhihua Xu[4], Daiwei Wan[4], Yilin Wang[5], Weichang Chen[1], Yuting Kuang[4], Jianming Shi[6], and Qiaoming Zhi[4]

Abstract

Nigericin, an antibiotic derived from *Streptomyces hygroscopicus*, which works by acting as an H^+, K^+, and Pb^{2+} ionophore, has exhibited promising anticancer activity. The main purpose of this study is to investigate its inhibitory effects on Wnt/β-catenin signaling pathway in colorectal cancer cells and clarify the underlying mechanism. We exposed two colorectal cancer lines (SW620 and KM12) to increasing concentrations of nigericin for different time periods and the 50% inhibiting concentration (IC_{50}) values were evaluated. Our data showed that nigericin treatment significantly reduced tumor cell proliferation in dose- and time-dependent manners in colorectal cancer cells. The subsequent experiments *in vitro* and *in vivo* implied that nigericin could significantly suppress the tumor growth, migration, and invasion, and induce the apoptosis of colorectal cancer cells. Our results of Western blot and immunofluorescence assay showed that nigericin could suppress the Wnt/β-catenin signaling pathway in colorectal cancer cells with dose-dependent increased expressions of downstream effectors and target proteins. To further elucidate the inhibitory effects of nigericin via a β-catenin–dependent signaling mechanism, we established the stably β-catenin overexpression colorectal cancer cells. Western blot, SuperTOPFlash luciferase reporter, and immunoprecipitation assays all confirmed β-catenin as a critical intermediary and player in Wnt/β-catenin pathway, and nigericin exerted anticancer effects on colorectal cancer cells by directly targeting the β-catenin destruction complex. These results suggested that Wnt/β-catenin signaling might have an essential role in colorectal cancer progression. Nigericin targeting Wnt/β-catenin signaling might provide new insight into the molecular mechanism of nigericin toward cancer cells, and suggest possible clinical application in colorectal cancer. *Mol Cancer Ther; 17(5); 952–65. ©2018 AACR.*

Introduction

Colorectal cancer ranks the third most commonly diagnosed cancer in men and the second most commonly diagnosed cancer in women (1). Over 50% of patients are diagnosed with hepatic metastases, either at the time of initial presentation or as a result of disease recurrence. There have been no major breakthroughs in the treatment of metastatic colorectal cancer and the cure rate has remained low for decades (2). There is a constant demand for new therapies. Thus, additional new therapeutic agents or approaches that target colorectal cancer are urgently needed (3).

Nigericin is an antibiotic derived from *Streptomyces hygroscopicus* that acts as an antiporter of K^+/H^+ and raises the pH of acidic compartments (4). As early as in 1972, the effects of nigericin on intracellular pH, glycolysis, and K^+ concentration of Ehrlich ascites tumor cells were studied (5). Thereafter, reports from Rotin and Margolis had shown that nigericin inhibited DNA synthesis of cancer cells by increasing intracellular pH and causing acidification of cytoplasm (6, 7). Recently, nigericin was found to be involved in various types of cancer (8–11). For example, in 2010, the study of Mashima and colleagues showed that nigericin-like compounds suppressed androgen receptor expression at the mRNA level and could be applied as new-type therapeutic agents in hormone-refractory prostate cancer (12). Deng and colleagues found that nigericin could selectively target cancer stem cells (CSC) and sensitize CSCs in nasopharyngeal carcinoma to the widely used clinical drug cisplatin both *in vitro* and *in vivo* (13). A recent research from Yakisich had characterized the anticancer effects of nigericin in lung cancer cells, and demonstrated that nigericin potently inhibited the viability of cells growing under routine culture conditions, prolonged periods of serum starvation, and lung tumorspheres (14). In colorectal cancer, only one study by Zhou and colleagues reported that nigericin could partly reverse the epithelial–mesenchymal transition (EMT) process during cell invasion and metastasis, and suppress some of the CSC phenotypes generated by EMT (15).

[1]Department of Gastroenterology, The First Affiliated Hospital of Soochow University, Suzhou, China. [2]Department of Oncology, The First Affiliated Hospital of Soochow University, Suzhou, China. [3]Department of General Surgery, Kunshan Hospital of Traditional Chinese Medicine, Kunshan, China. [4]Department of General Surgery, The First Affiliated Hospital of Soochow University, Suzhou, China. [5]Department of Hepatic Surgery, Fudan University Shanghai Cancer Center; Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, China. [6]Department of Oncology, Suzhou Municipal Hospital, Nanjing Medical University Affiliated Suzhou Hospital, Suzhou, China.

F. Liu, W. Li, and S. Hua contributed equally to this article.

Corresponding Authors: Qiaoming Zhi, The First Affiliated Hospital of Soochow University, Suzhou 215006, China. Phone: 8615-2502-78285; Fax: +86 512 65225768; E-mail: strexboy@163.com; and Jianming Shi, 13815259001@163.com and Jianming Shi, Suzhou Municipal Hospital, Nanjing Medical University Affiliated Suzhou Hospital, Suzhou, 215002, China. Phone: 8613-8152-59001; Fax: +86 512 65334495; E-mail: 13815259001@163.com

doi: 10.1158/1535-7163.MCT-17-0906

The Wnt/β-catenin signaling pathway plays a pivotal role in regulating cellular processes of development, differentiation, and adult tissue homeostasis (16). Nowadays, it is well known that aberrant Wnt/β-catenin signaling is widely implicated in numerous malignancies and other disease states (17–19), including cancers of the gastrointestinal tract (20, 21). The main purpose of this study is to investigate the inhibitory effects of nigericin on Wnt/β-catenin signaling pathway in colorectal cancer cells and clarify the underlying mechanism.

Materials and Methods

Chemicals and cell culture

Nigericin was purchased from Sigma Aldrich. The stock solutions (100 μmol/L) were prepared with DMSO and stored at −20°C. Human colorectal cancer cell lines (SW620 and KM12) were obtained from ATCC and cultured in RPMI1640 medium (Invitrogen) with 10% FBS (Gibco, Invitrogen) in a humidified atmosphere of 5% CO_2 at 37°C. These two cell lines were authenticated by short tandem repeat (STR) profiling, and the latest verification was done in June 2016. Cells were tested monthly for mycoplasma contamination using the MycoAlert Plus Mycoplasma detection kit (Lonza), and were in the logarithmic phase of growth for all experiments.

Cell viability assay and IC_{50} values

A total of 3.5×10^3 cells were harvested and plated with 100 μL of culture medium in a 96-well plate. Then cells were exposed to 100 μL nigericin with increasing concentrations (final concentrations were 0, 0.25, 0.5, 1, 2, 5, 10, or 20 μmol/L) for different time periods (8, 24, or 48 hours), respectively. Twenty microliters Cell Counting Kit-8 (CCK-8, Dojindo) was added into each of the 96 wells. After 4-hour incubation at 37°C, the optical density (OD) values were detected at 450 nm using the scan reader (ELx800, BioTek Instruments). Finally, cell growth–inhibiting rates were described as the cell inhibiting curves and the 50% inhibiting concentration (IC_{50}) values were evaluated.

Colony formation assay and Hoechst33342 staining

For colony formation assay, 3×10^3 cells were trypsinized to single-cell suspensions and plated in 6-well plates in complete culture medium containing 0.3% agar on the top of 0.6% agar in the same medium. After the plates were incubated at 37°C in 5% CO_2 for 14 days, the colonies were fixed and stained with 50 μL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (5 mg/mL). Colonies containing at least 50 cells were scored. Data are presented as the mean ± SD of five randomly scored fields.

We used Hoechst33342 staining to evaluate the changes of cell apoptosis. In brief, cells (2×10^5/mL) were harvested and incubated with 10 μL of Hoechst33342 solution (Keygen Biotech) for 15 minutes at 37°C in the dark. Then, cells were centrifuged and washed with precold 1× PBS for three times, followed by incubating with 1 mL Buffer A solution (Keygen Biotech) at room temperature. Finally, a total of 20 μL cellular suspension buffer was dropped on a glass slide and the image was observed under an inverted phase-contrast fluorescence microscope IX71 (Olympus).

Migration and invasion assay

The migration and invasion assays were performed with transwell chambers (8-μm pore size, 24-well insert, Corning). Briefly, cells were harvested and suspended in the serum-free medium. Then, 1×10^4 cells in 200 μL serum-free medium were added to the top chamber, and 600 μL culture medium with 10% FBS was added to the bottom chamber as a chemoattractant. Cells were incubated for 48 hours at 37°C, and the noninvading cells were removed with cotton swabs. Finally, the insert membranes were fixed in 3.7% methanol for 10 minutes and stained with 0.1% crystal violet for 20 minutes. The permeating cells were counted and photographed under the inverted light System Microscope IX71 (Olympus). At least three randomly selected fields were counted, and the average number of cells was calculated per field. For cell invasion study, the inserts of the chamber were prepared by coating the top surfaces with Matrigel (100 μL, 1: 6 dilution in serum-free medium, BD Biosciences). Other procedures were the same with the migration assay.

Western blot analysis

Cells were seeded in 6-well plates with a density of 4×10^5 cells/well and treated with different concentrations of nigericin. Then cells were harvested and lysed in RIPA buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% NP-40, 1 mmol/L EDTA, and 1 mmol/L PMSF] supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche). Cell lysates including whole-cell extracts (WCE), cytoplasmic extracts (CE), and nuclear extracts (NE) were quantitated and denatured with 5× SDS-PAGE sample loading buffer by boiling for 10 minutes. Then, the protein extracts were fractionated on an SDS-PAGE gel and transferred onto nitrocellulose membranes (Millipore). Blots were incubated sequentially overnight with primary and secondary antibodies. Immunoreactive signals were developed with ECL kit (Thermo Scientific). The primary antibodies used in this study were raised against β-catenin (#8480), p-β-catenin (#4176), Axin1 (#3323), GSK-3β (#9315), and Survivin (#2808; Cell Signaling Technology); TCF-1 (#YT4577), Cyclin D1 (#YT1173), MMP-7 (#YT2663), and MMP-9 (#YT1892; ImmunoWay); c-Myc (ab32072) and Axin2 (ab32197; Abcam); β-actin (AP53385; Abgent); Lamin A/C (sc-20681; Santa Cruz Biotechnology). The band density was normalized to β-actin or Lamin A/C, and quantified by ImageJ software.

Immunofluorescence assay

Cells were cultured in RPMI1640 medium supplemented with 10% FBS on cover slides. After incubation overnight, cells were fixed with 4% paraformaldehyde for 30 minutes and followed by permeabilization in 0.5% Triton X-100 for 15 minutes and an additional 45 minutes for block in 5% BSA. Cells were sequentially incubated with indicated primary antibodies overnight at 4°C. Primary antibodies were diluted as follows: β-catenin (prediluted, ab15180, Abcam), c-Myc (1:200, ab32072, Abcam), MMP-9 (1:300, #YT1892, Immunoway). Then, the sections were incubated with Cy3-labeled anti-rabbit IgG (1:400, BS10007) secondary antibody at room temperature for 1 hour. Finally, the cells were stained with 4, 6-diamidino-2-phenylindole (DAPI) for nuclear staining at room temperature for 5 minutes. The fluorescence images were captured and then merged using an inverted phase-contrast fluorescence microscope IX71 (Olympus).

β-catenin overexpression in colorectal cancer cells

SW620 and KM12 cell lines were used for overexpression studies. β-catenin overexpression vector was constructed on the basis of the pMSCV-puro vector using primers 5′-CCGCTCGAGATGGCTACTCAAGCTG-3′ (F) and 5′-CCGGAATTCTTACAGGTCAGTATCAAAC-3′ (R). Lentivirus supernatants were used to

infect the target cells. Puromycin (5 ng/μL) was used to select cells stably overexpressing β-catenin and the empty vector cells for 6 weeks. The transfection efficiency was verified by the fluorescence microscopy (Olympus X71).

Luciferase reporter assay

Luciferase reporter assays of Wnt/β-catenin signaling were performed in 96-well plates with the TOP and FOP reporters (Upstate Biotechnology) that contained wild-type and mutated TCF/LEF DNA–binding sites, respectively. Cells were cultured in 96-well plate overnight and transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The amount of plasmids transfected per well were listed as follows: Wnt/β-catenin signaling responsive Firefly luciferase reporter plasmid SuperTOPFlash or negative control SuperFOPflash reporter plasmid, 100 ng; the overexpressing β-catenin plasmid or negative control empty vector, 100 ng; *Renilla* reporter plasmid, 10 ng. After 6-hour incubation, cells were exposed to different concentrations of nigericin for 24 hours, and then cells were lysed. Both the Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

Immunoprecipitation

Cells were pretreated with different concentration of nigericin for 48 hours, and harvested and lysed with lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, 1 mmol/L EDTA, 15 mmol/L $MgCl_2$, and 1 mmol/L PMSF] supplemented with protease and phosphatase inhibitor cocktail for 30 minutes on ice. After centrifugation for 10 minutes at 13,000 r/minute, the supernatant was collected and transferred into a new tube for the subsequent immunoprecipitation assay. Total protein (500 μg) in cell lysates were incubated with 2 μg anti-β-catenin antibody (#8480, Cell Signaling Technology) at 4°C for 2 hours, and the mixture was incubated with 20 μL A/G PLUSAgarose beads (Santa Cruz Biotechnology) at 4°C overnight. After incubation, the immunoprecipitated complex was washed five times with precold PBS with 0.1% NP-40 buffer at 4°C. Proteins bound to the beads were eluted with 5× SDS-loading buffer at 100°C for 10 minutes, followed by further Western blot analyses with β-catenin (#8480), Axin1 (#3323), and GSK-3β (#9315) antibodies (Cell Signaling Technology).

Tumor growth in nude mice

Four-week old male BALB/c-nude mice were purchased and maintained in a sterile environment. Different treated SW620 cells that stably expressed firefly luciferase were injected subcutaneously into the flanks of nude mice in a total volume of 200 μL. Nigericin (4 mg/kg) was injected intraperitoneally every 2 days as previous studies described *in vivo* (13, 22). Tumor volume (TV) was measured weekly using a digital caliper, and was calculated as follows: TV (mm^3) = length × $width^2$ × 0.5. Bioluminescent imaging was performed on day 28 after injection. At the end of the experiment, mice were anaesthetized and given D-luciferin potassium salt (150 mg/kg in PBS, Sciencelight). Twenty minutes after injection, these mice were anesthetized and the bioluminescence was imaged with a charge-coupled device camera (IVIS; Lumina II, PerkinElmer). Signal was displayed as photons/s/cm^2/sr. All mice were killed after the bioluminescent assay and the tumor mass were removed. Tumor weights were calculated and the tumors were immediately fixed in 10% neutralized formalin for Ki67 staining and terminal deoxynucleotidyl transferase–dUTP nick end labeling (TUNEL) analyses or stored at −80°C for qRT-PCR analysis. The Animal Care and Use Committee of the First Affiliated Hospital of Soochow University approved the experiments using mice.

IHC of Ki67 staining

Tumor tissue sections from mice (4 μm) were incubated in a dry oven at 60°C for 1 hour, and then deparaffinized in xylene and rehydrated with graded ethanol. Then, antigen retrieval was performed by pretreatment of the slides in 0.01 mol/L citrate buffer (pH 6.0) for 10 minutes using a microwave oven at high power (1,200 W). Subsequently, 3% hydrogen peroxide (H_2O_2) was applied to block endogenous peroxidase activity of the sections for 30 minutes at room temperature. After blocking with 5% BSA (Boster Bioengineering), the sections were incubated with the diluted Ki67 antibody (1: 400, #9449, Cell Signaling Technology) for 2 hours at room temperature. After rinsing, the sections were incubated with the secondary anti-mouse peroxidase–conjugated antibody (EnVision, DAKOA/S) for 45 minutes at room temperature according to the manufacturer's instructions. For each sample, the omission of primary antibody was used as a negative control. Finally, the slides were visualized with 3, 3-diaminobenzine (DAB, Vector Laboratories), and counterstained with hematoxylin for microscopic examination. The sections were dehydrated, cleared, and mounted under the light microscope.

TUNEL assay

Formalin-fixed paraffin-embedded tumor tissues were evaluated using the TUNEL assay with the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals) to identify cells undergoing apoptosis. According to the manufacturer's instructions, paraffin sections of the xenograft tumor tissues (5 μm) were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Then sections were rinsed in distilled water and incubated in 3% hydrogen peroxide (H_2O_2) in methanol for 5 minutes to block endogenous peroxidase activity. Tissue sections were incubated in 20 μg/mL proteinase K (DAKO Corporation) for 15 minutes, washed with 1× PBS, incubated in equilibration buffer and then in TdT enzyme solution in a humidified chamber at 37°C for 60 minutes. The sections were subsequently rinsed in PBS, and then incubated with streptavidin peroxidase (Vector Laboratories) conjugate for 30 minutes. Peroxidase activity was detected by application of DAB. Apoptotic activity was identified by an observed dark brown nuclear staining under the light microscope.

qRT-PCR

Tumor tissue sections from mice were homogenized and the total RNAs were extracted using TRIzol solution (Invitrogen) according to the manufacturer's instructions. Equivalent amounts of RNA (2 μg) were reverse-transcribed using the M-MLV reverse transcriptase with Oligo(dT). Triplicates were performed for all reactions by using FastStart Universal SYBR Green Master (Roche Diagnostics GmbH) on an Eppendorf Real-Time Detection System according to the manufacturer's protocol. Primer sequences were as follows: CTNNB1 (5′-CATCTACACAGTTTGATGCTGCT-3′ and 5′-GCAGTTTTGTCAGTTCAGGGA-3′), MYC (5′-GGCTCCTGGCAAAAGGTCA-3′ and 5′-CTGCGTAGTTGTGCTGATGT-3′), BIRC5 (5′-AGGACCACCGCATCTCTACAT-3′ and 5′-AAGTCTGGCTCGTTCTCAGTG-3′), MMP-9 (5′-GGGACGCAGACATCGTCATC-3′ and 5′-TCGTCATCGTCGAAATGGGC-3′), and GAPDH (5′-GGCACCACCATGTACCCTGGCAT-3′ and 5′-TCCTGCTTGCTGATCCACATCTGCT-3′). The PCR conditions involved preliminary denaturation at 95°C for 10 minutes to

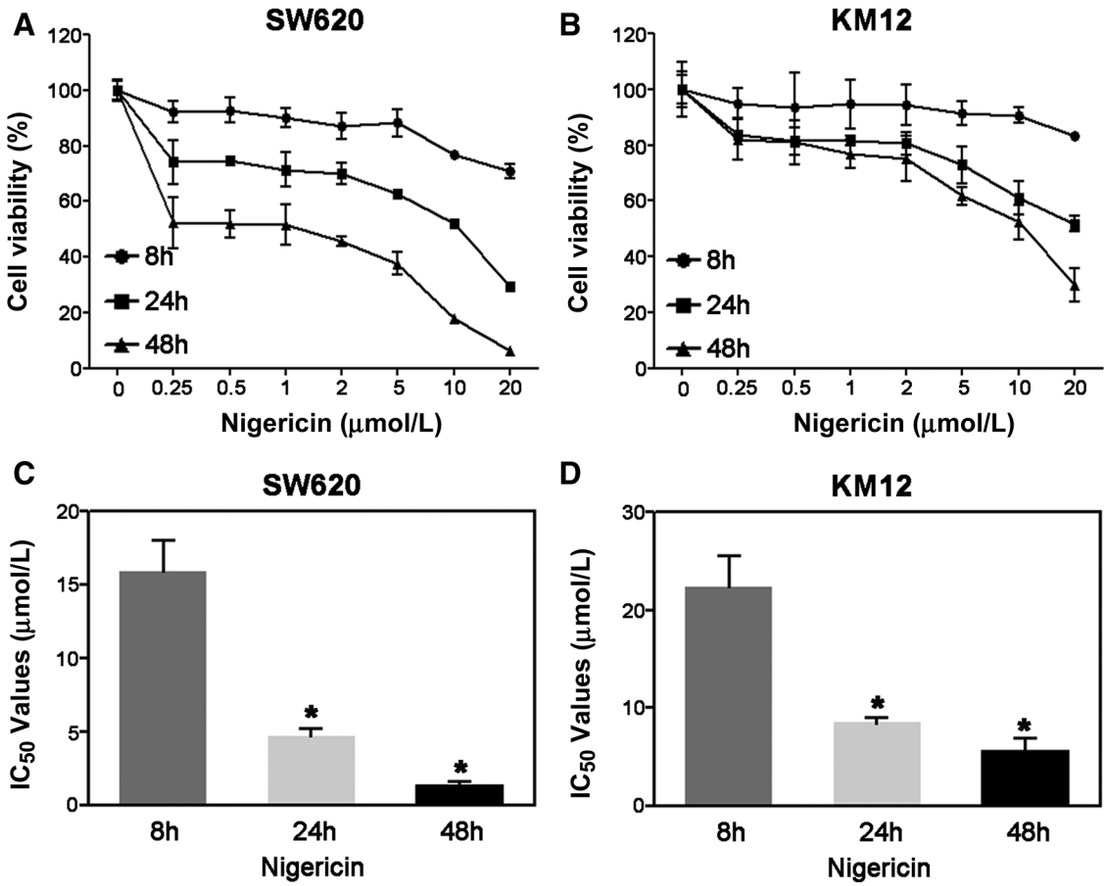

Figure 1.

Sensitivity of human colorectal cancer (CRC) cells to nigericin. **A** and **B,** SW620 and KM12 cells were exposed to increasing concentrations of nigericin for different time periods (8, 24, or 48 hours). The drug significantly reduced tumor cell proliferation in a concentration-dependent manner in SW620 and KM12 cells for 8, 24, or 48 hours. **C** and **D,** The IC_{50} values of nigericin in SW620 and KM12 cells at different time (8, 24, or 48 hours) were calculated. Nigericin inhibited the cancer cell viability time dependently (*, $P < 0.05$).

active Taq polymerase, and 40 cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, followed by a final elongation step at 60°C for 10 minutes. GAPDH was used as internal reference. All samples were processed in replicate and the gene expressions were quantified as $1{,}000 \times 2^{-\Delta C_t}$.

Tumor metastasis in nude mice

Four-week-old male BALB/c-nude mice were used for the mice colorectal cancer peritoneal disseminated model. In brief, 5×10^6 SW620 cells stably expressing firefly luciferase (β-catenin stably overexpressing or with empty vectors) were resuspended in 200-μL PBS and injected intraperitoneally into the nude mice. Then, nigericin (4 mg/kg) was injected intraperitoneally every two days. After one month, the peritoneal metastasis of SW620 cells in mice were observed by the bioluminescent assays as described. Finally, the abdominal metastasis nodules on the mesentery were dissected and calculated.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 software (SPSS Inc.). The data were presented as mean ± SD from experiments in replicate. Statistically significant differences were estimated using one-way ANOVA followed by Tukey test. Probability values (P) <0.05 were considered to indicate a statistically significant difference.

Results

Sensitivity of human colorectal cancer cells to nigericin

SW620 and KM12 cells were exposed to increasing concentrations of nigericin (0, 0.25, 0.5, 1, 2, 5, 10, and 20 μmol/L) for different time periods (8, 24, or 48 hours). As demonstrated in Fig. 1A and B, nigericin treatment significantly reduced tumor cell proliferation in a concentration-dependent manner in SW620 and KM12 cells. Meanwhile, we compared the IC_{50} values of nigericin in these two cell lines at different time (8, 24, or 48 hours). Our results manifested that nigericin also inhibited the cancer cell viability time dependently (15.90 ± 2.08, 4.72 ± 0.46, and 1.39 ± 0.21 μmol/L in SW620 cells; 22.31 ± 3.25, 8.48 ± 0.58, and 5.69 ± 1.30 μmol/L in KM12 cells) at 8, 24, and 48 hours (Fig. 1C and D).

Nigericin inhibited the tumorigenic behavior of colorectal cancer cells *in vitro*

As calculated above, we chose 1 and 4 μmol/L as the proper treated concentrations of nigericin on SW620 and KM12 cells,

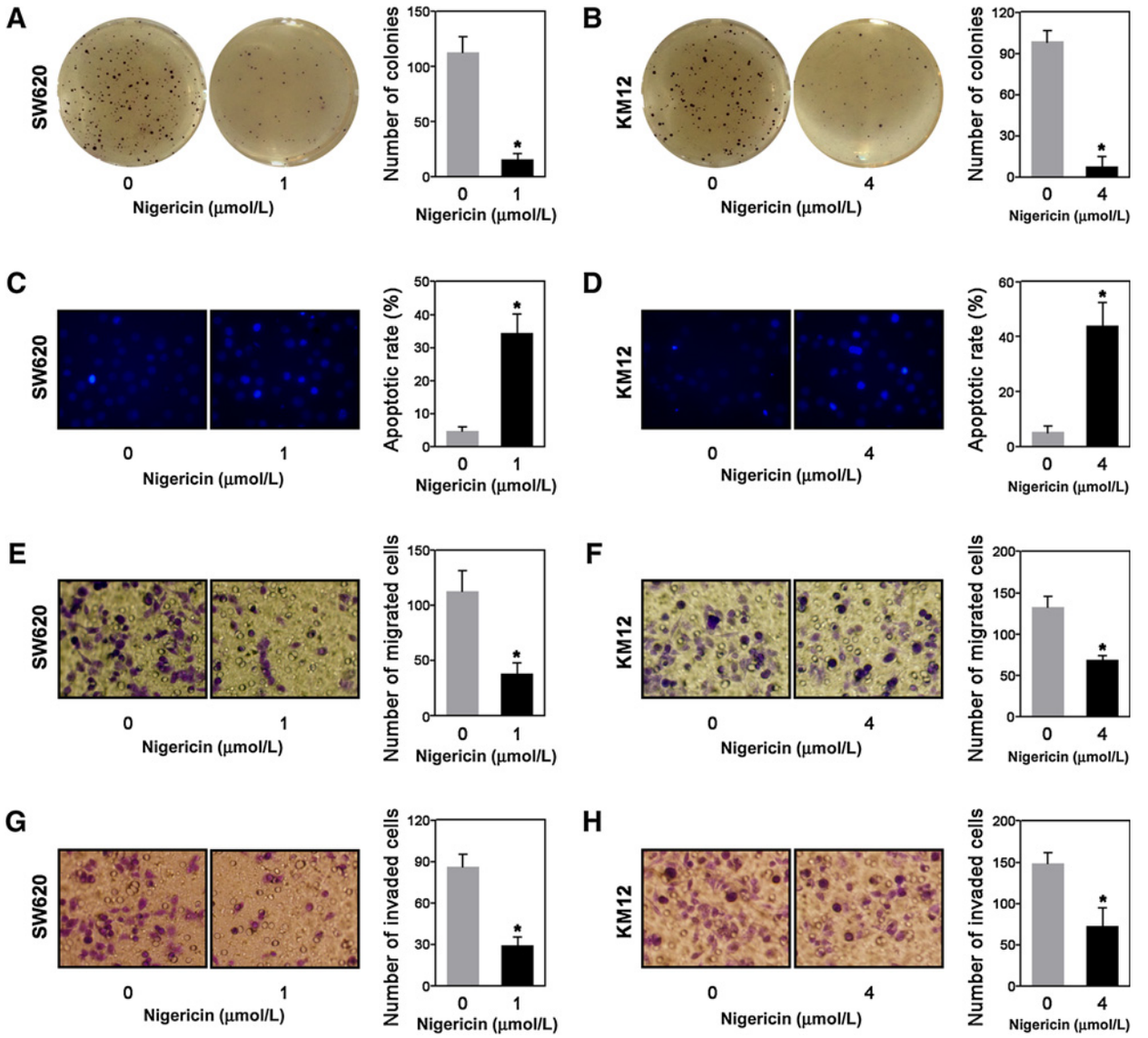

Figure 2.

Nigericin inhibited the tumorigenic behavior of colorectal cancer (CRC) cells *in vitro*. **A** and **B,** The ability of colony formation was significantly suppressed by nigericin in SW620 or KM12 cells. **C** and **D,** Hoechst33342 staining showed nigericin increased tumor apoptosis in SW620 or KM12 cells. **E** and **F,** The ability of migration was inhibited by nigericin in SW620 or KM12 cells. **G** and **H,** The ability of invasion was impaired by nigericin in SW620 or KM12 cells (*, $P < 0.05$).

respectively (less than the IC_{50} values for 48 hours) for our subsequent experiments *in vitro*. The effects of nigericin on the tumorigenic behavior of colorectal cancer cells were measured after treatment with respective concentrations of nigericin for 48 hours. Our results of the colony formation assay and Hoechst33342 staining demonstrated that nigericin could inhibit the cell proliferation and increased tumor apoptosis both in SW620 and KM12 cells (Fig. 2A–D). To detect the effect of nigericin on the cell metastatic capacity, transwell migration and invasion assays were performed. As shown in Fig. 2E and F, the number of migrated cells in the nigericin treatment SW620 or KM12 cells was significantly decreased, compared with the nondrug-treated cells. Similarly, colorectal cancer cell invasion through an artificial extracellular matrix using Matrigel-coated membranes was likewise significantly impaired in response to nigericin exposure (Fig. 2G and H). Taken together, these data implied that nigericin could obviously attenuate the tumorigenic potential of colorectal cancer cells *in vitro*.

Nigericin suppressed Wnt/β-catenin signaling pathway in colorectal cancer cells

To examine the effects of nigericin on the Wnt/β-catenin pathway, the expressions of downstream effectors of the pathway were assessed. As the Western blot results showed, decreased protein expressions of β-catenin (including total, cytosolic and nuclear β-catenin) and TCF-1 were observed both in SW620 and KM12 cells. Whereas, the dose-dependent increased expressions

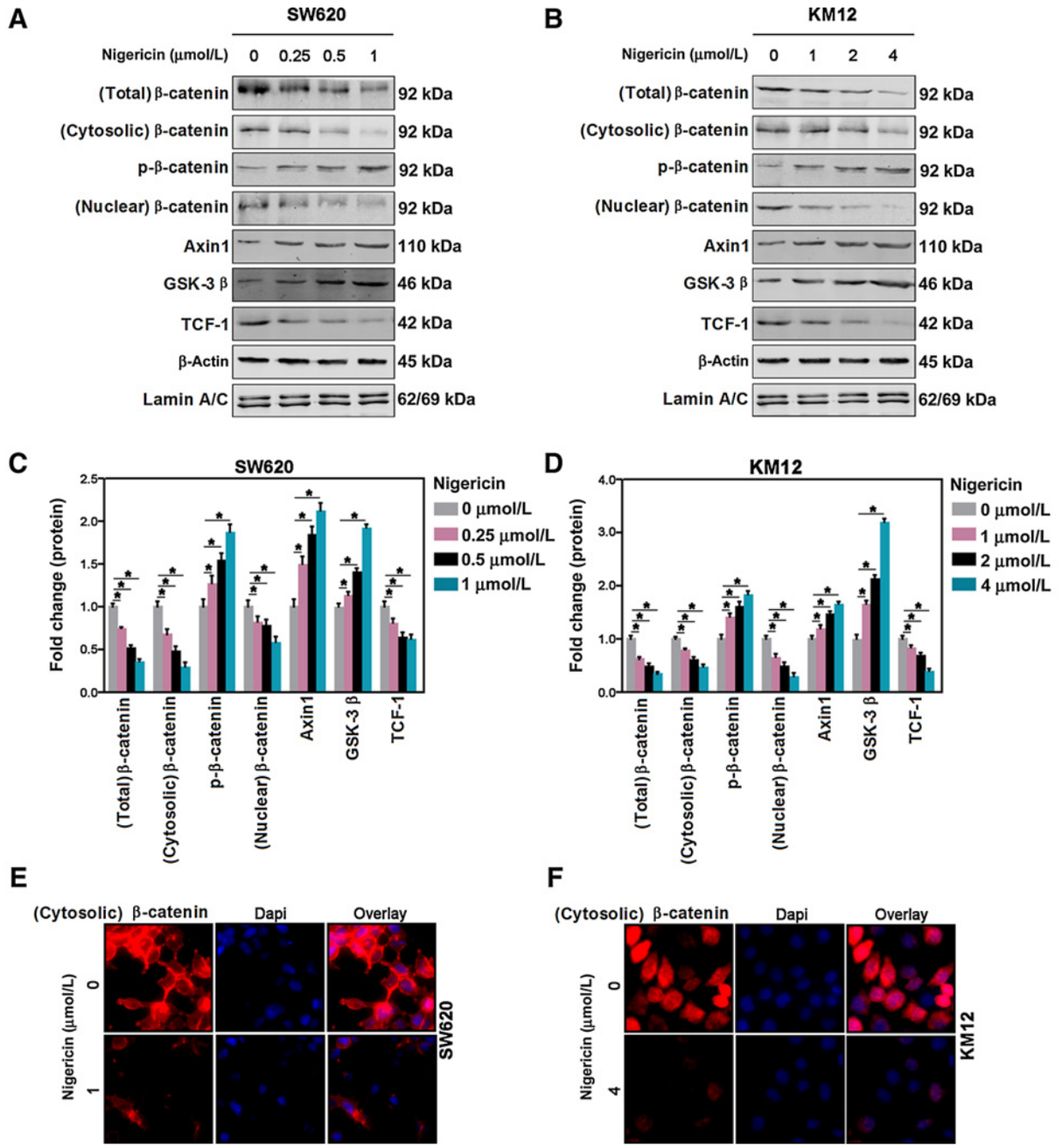

Figure 3.

Nigericin suppressed Wnt/β-catenin signaling pathway in colorectal cancer (CRC) cells. **A** and **B,** The inhibiting effects of Wnt/β-catenin pathway-related proteins by nigericin in SW620 or KM12 cells were observed using Western blot analysis. **C** and **D,** The gray levels of different Wnt/β-catenin pathway–related proteins. **E** and **F,** The immunofluorescence assay demonstrated that β-catenin in the cytoplasm was preferentially reduced by nigericin, compared with the control group (nonnigericin treatment; *, $P < 0.05$).

of p-β-catenin, Axin1 and GSK-3β proteins were also seen in these two cell lines after nigericin treatments at 48 hours (Fig. 3A–D). Using the immunofluorescence assay, we found that β-catenin in the cytoplasm was preferentially reduced in the nigericin-treated group compared with the control group (nonnigericin treatment) both in SW620 and KM12 cells (Fig. 3E and F).

Nigericin downregulated the expressions of Wnt/β-catenin target genes in colorectal cancer cells

Regarding the inhibiting effects of Wnt/β-catenin pathway–related proteins by nigericin, we also evaluated the changes of some Wnt target genes such as CyclinD1, c-Myc, Survivin, Axin2, MMP-7, and MMP-9 both in SW620 and KM12 cells. Consistent

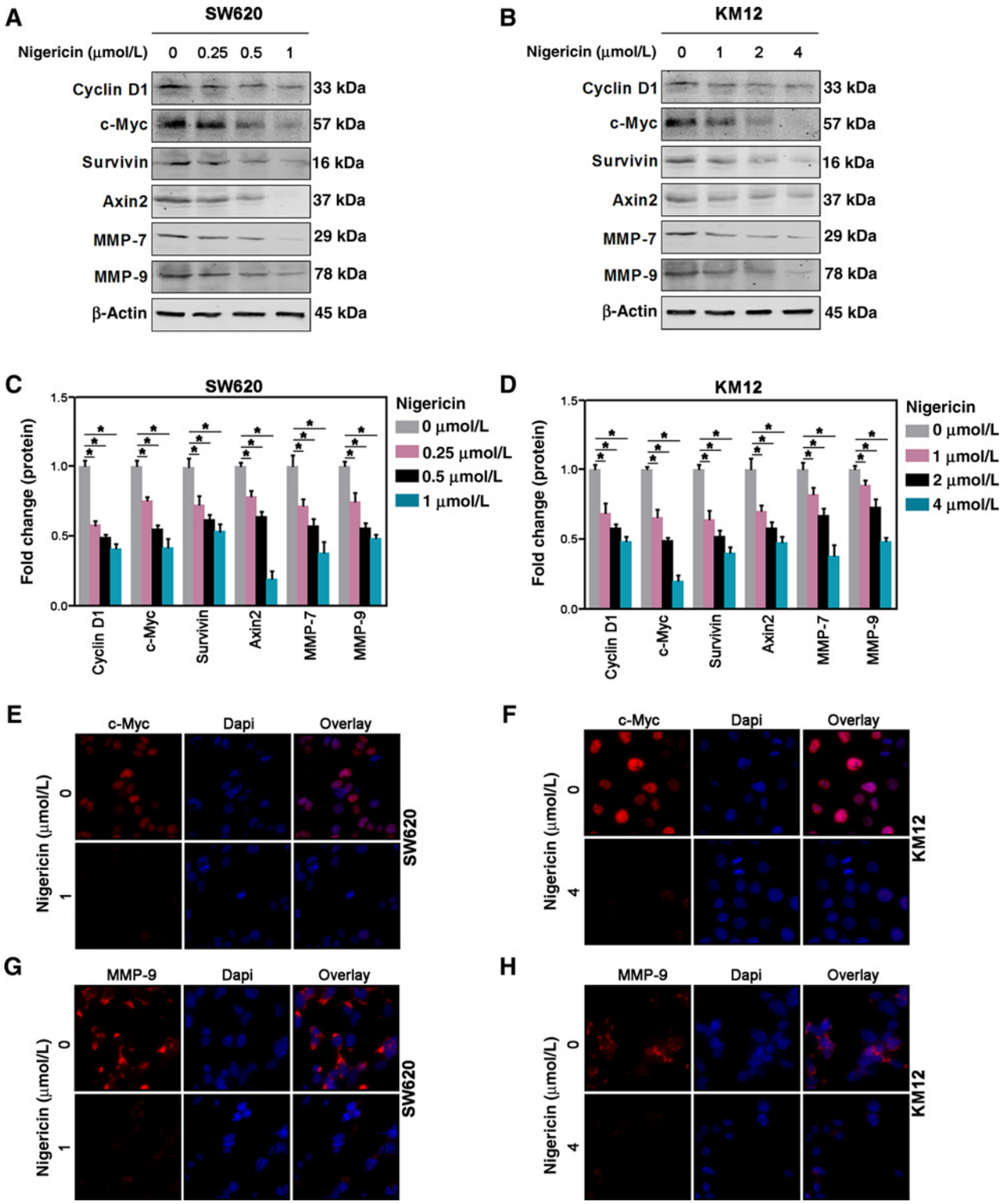

Figure 4.

Nigericin downregulated the expressions of Wnt/β-catenin target genes in colorectal cancer (CRC) cells. **A** and **B,** The Western blot analysis showed that Wnt target proteins such as CyclinD1, c-Myc, Survivin, Axin2, MMP-7, and MMP-9 were suppressed by nigericin in SW620 or KM12 cells. **C** and **D,** The gray levels of different Wnt target proteins. **E–H,** The immunofluorescence assay demonstrated that the protein expressions of c-Myc and MMP-9 were significantly reduced by nigericin, compared with the control group (nonnigericin treatment; *, $P < 0.05$).

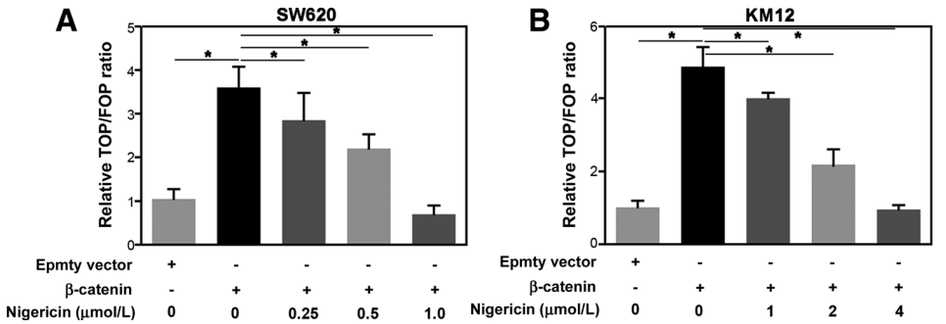

Figure 5.
The inhibitory effects of nigericin via a Wnt//β-catenin-dependent signaling mechanism in colorectal cancer (CRC) cells were confirmed by the TOPFlash cell reporter system. **A,** SW620 cells. **B,** KM12 cells; (*, $P < 0.05$).

with our expectation, our data of Western blot analysis showed that the protein expression levels of CyclinD1, c-Myc, Survivin, Axin2, MMP-7, and MMP-9 were downregulated after nigericin treatment in a concentration-dependent manner (Fig. 4A–D). Similarly, we also performed the immunofluorescence assay to observe the influence of nigericin on Wnt-related target proteins (c-Myc and MMP-9). As predicted, the drug significantly reduced the c-Myc and MMP-9 levels both in SW620 and KM12 cells (Fig. 4E–H).

Nigericin suppressed Wnt/β-catenin pathway that was activated by β-catenin overexpression *in vitro*

To further confirm the inhibitory effects of nigericin via a Wnt//β-catenin–dependent signaling mechanism in colorectal cancer, we established the stably β-catenin overexpression colorectal cancer cells and discussed the suppressing effects of nigericin by the TOPFlash cell reporter system. As demonstrated in Fig. 5A and B, the transcriptional activities of β-catenin/TCF/LEF affected by nigericin were evaluated. Our data implied that the relative TOP/FOP ratios of β-catenin overexpressing cells were increased 3.58 and 4.42-fold changes in SW620 and KM12, respectively, compared with the control cells. When treated with nigericin, the relative TOP/FOP ratios were significantly downregulated in a dose-dependent manner, compared with the β-catenin overexpressing SW620 or KM12 cells. This was the preliminary evidence that nigericin could also suppress the Wnt/β-catenin pathway that was activated by β-catenin overexpression *in vitro*.

Our subsequent experiments redetected the expressions of Wnt//β-catenin downstream effectors and target proteins, and the results (Fig. 6A–D) showed that upregulation of β-catenin could significantly increase the levels of TCF-1, CyclinD1, c-Myc, Survivin, Axin2, MMP-7, and MMP-9, while Axin1 and GSK-3β protein expressions were decreased in β-catenin overexpressing cells. The results strongly confirmed β-catenin as a critical intermediary and player in Wnt/β-catenin pathway. Moreover, our data also found that nigericin exhibited obvious inhibitory effects of Wnt//β-catenin downstream proteins between nonnigericin and nigericin-treated β-catenin overexpressing cells. To better understand the nigericin effects on Wnt signaling, we next assessed the changes of TOP/FOP ratios when β-catenin overexpressing cells were exposed to much high concentrations of drug (1 and 4 μmol/L in SW620 and KM12, respectively). The results (Fig. 6E and F) showed that overexpression of β-catenin could activate the Wnt-induced signaling pathway with relative high TOP/FOP ratios both in SW620 and KM12 cells. However, when treated with nigericin, the TOP/FOP ratios were significantly downregulated compared with the β-catenin-overexpressing cells. These were evidence that nigericin could effectively inhibit Wnt/β-catenin signaling pathway, and that the activated pathway induced by β-catenin overexpression could also be partly reversed by nigericin *in vitro*.

Nigericin antagonized β-catenin through inducing phosphorylation and subsequent proteasomal degradation of β-catenin

β-catenin destruction complex GSK-3β/Axin1/β-catenin played an important role in the stabilization of β-catenin, and induced its phosphorylation and subsequent proteasomal degradation. To further elucidate the role of GSK-3β and Axin1 in nigericin-induced downregulation of β-catenin, the lysates were immunoprecipitated with β-catenin and then Western blotted for β-catenin, GSK3β, and Axin. Our data in Fig. 7A–D showed that nigericin treatment for 48 hours significantly decreased the expression of β-catenin and enhanced the expression of GSK3β and Axin1 both in SW620 and KM12 cells. Thus, these results suggested that GSK3β and Axin1 might be involved in the nigericin-induced degradation of β-catenin in colorectal cancer cells.

Nigericin inhibited the tumor growth and metastasis of colorectal cancer cells *in vivo*

Given the observed effects of nigericin on colorectal cancer cell growth and metastasis *in vitro*, we subsequently determined whether nigericin suppressed tumorigenicity *in vivo*. As demonstrated in Fig. 8A and B, subcutaneous injection of mice with β-catenin–overexpressing SW620 cells resulted in larger tumors than injection of control SW620 cells, whereas nigericin could significantly reduce the tumor mass of SW620-β-catenin cells. Our results of tumor weights at 4 week also confirmed the inhibitory effects of nigericin in the tumor formation *in vivo* (Fig. 8C). Next, we obtained the tissue sections from the mice xenograft tumors, and used IHC and TUNEL assay to detect the expressions of Ki67 and cell apoptosis, respectively. Our results showed that β-catenin overexpression could increase the percentage of Ki67-positive staining and decrease the cell apoptotic rate (Fig. 8D–F), while nigericin treatment could antagonize the prompting effects of tumor growth induced by β-catenin. Furthermore, the mRNA expressions of CTNNB1, MYC, BIRC5, and MMP-9 were obviously upregulated by β-catenin *in vivo* that were in accordance to our previous reports *in vitro*, and nigericin could significantly inactivate the expressions of these Wnt-related genes (Fig. 8G–J).

Besides, we built the mice tumor metastasis model and injected different treated cells into the peritoneal cavity. As shown in Fig. 9A, we could see that β-catenin significantly increased the peritoneal spreading of SW620 cells at day 35. Nigericin in SW620

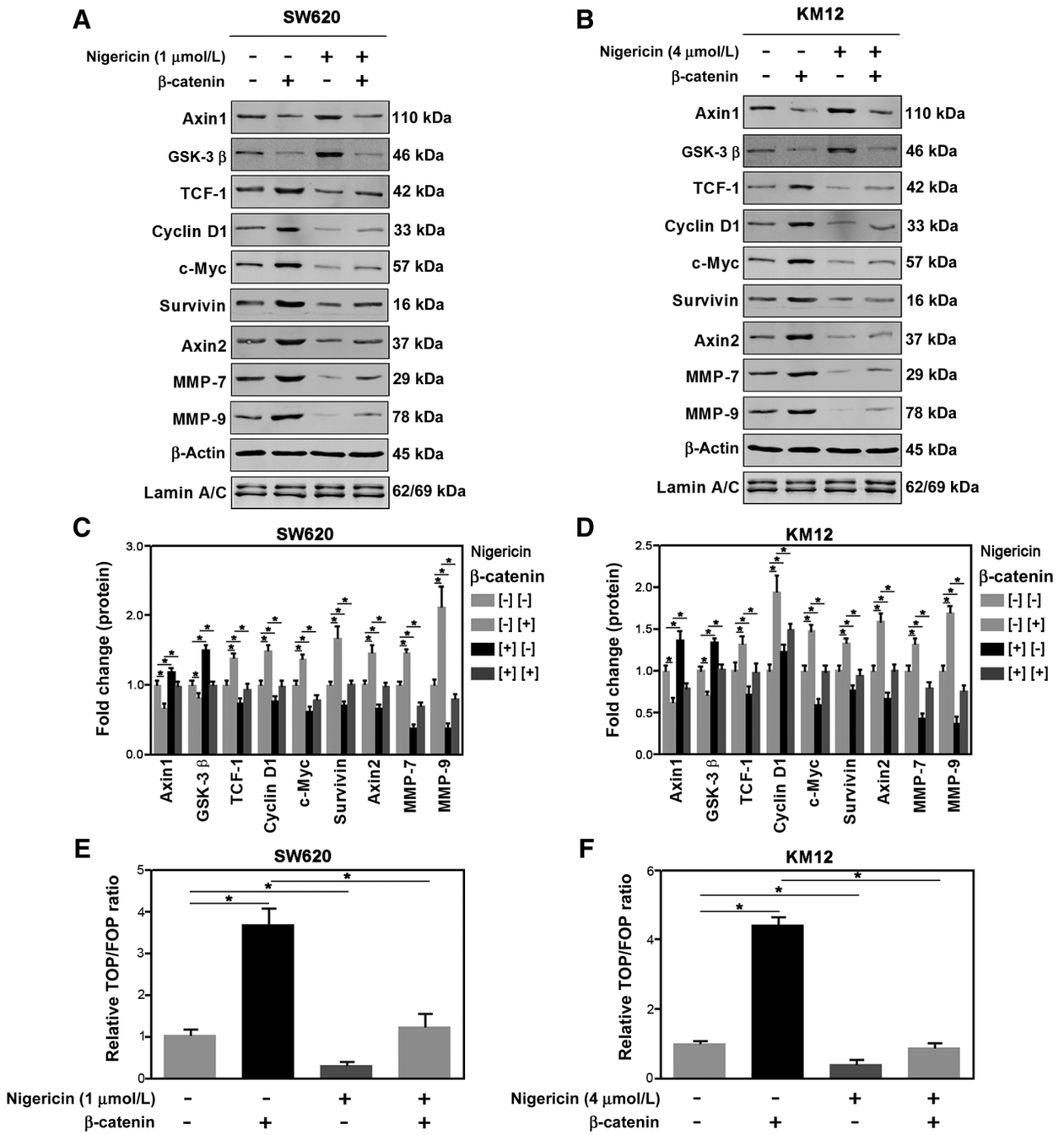

Figure 6.

Nigericin could effectively inhibit Wnt/β-catenin signaling pathway, and the activated pathway by β-catenin overexpression could also be partly reversed by nigericin *in vitro*. **A** and **B,** The inhibiting effects of Wnt/β-catenin pathway-related and target proteins by nigericin in SW620 or KM12 cells were observed using Western blot analysis. Meanwhile, our data also implied β-catenin as a critical intermediary in Wnt/β-catenin pathway, and nigericin also exhibited obvious inhibitory effects of Wnt//β-catenin downstream proteins between nonnigericin and nigericin-treated β-catenin–overexpressing cells. **C** and **D,** The gray levels of different Wnt/β-catenin downstream proteins. **E** and **F,** The changes of TOP/FOP ratios also confirmed the inhibiting effects of nigericin in β-catenin overexpressing SW620 or KM12 cells (*, $P < 0.05$).

control cells had the opposite effect, suppressing tumorigenicity and peritoneal spreading. More importantly, we found that nigericin also had strong inhibitory effects on β-catenin stably expressing cells. Meanwhile, the abdominal metastasis nodules on the mesentery were dissected. We calculated the number of abdominal metastasis nodules on the mesentery among different groups, and the results supported our observed bioluminescent imaging of tumors *in vivo* (Fig. 9B and C).

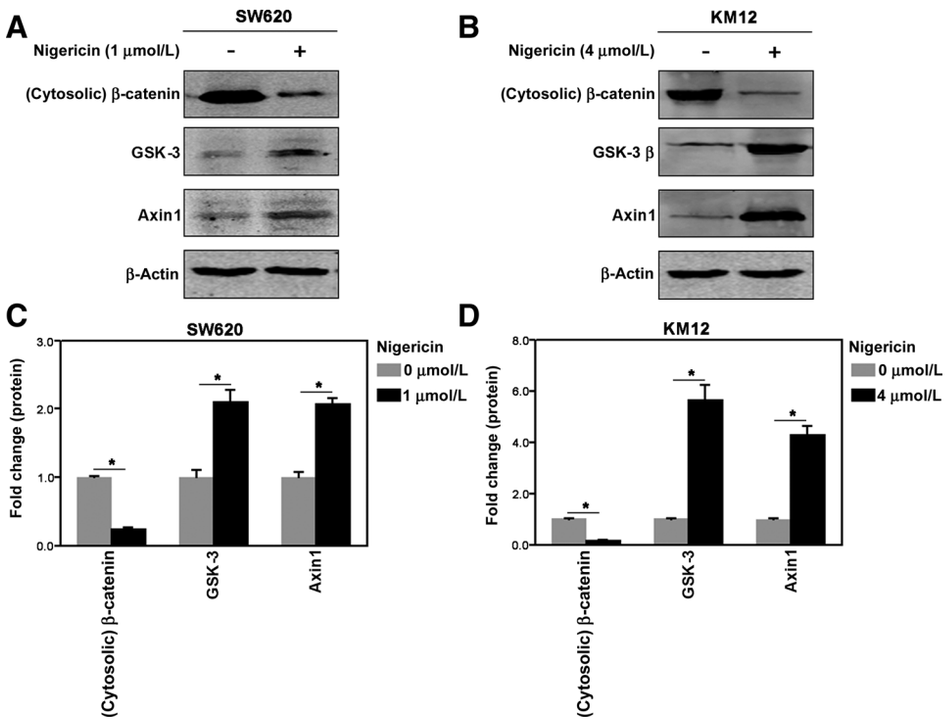

Figure 7.
Immunoprecipitation assay demonstrated that nigericin could inhibit the Wnt/β-catenin signaling by directly targeting the β-catenin destruction complex GSK-3β/Axin1/β-catenin in SW620 or KM12 cells. **A** and **B,** A decreased expression of β-catenin and increased levels of GSK-3β and Axin1 were observed in β-catenin immunoprecipitated lysates. **C** and **D,** The gray levels of β-catenin, GSK-3β, and Axin1 proteins (*, $P < 0.05$).

Discussion

Nigericin is a monocarboxylic polyether antibiotic potassium ionophore that is widely used as a coccidiostatic agent in chickens. It tends to lower the intracellular pH (pHi) by allowing exchange of intracellular K^+ for extracellular H^+, which probably leads to cell death in an acidic environment. As revealed by previous studies, nigericin was reported to inhibit cancer cells, and the underlying biochemical mechanisms for its anticancer effects had been comprehensive studied. Nigericin at relatively high concentration might trigger apoptosis or necrosis by depletion of intracellular potassium due to its ability to increase K+ efflux (8, 23, 24). It was also shown to overcome multidrug resistance of cancer cells and to target CSCs (13, 25–27). Besides, nigericin inhibited cellular processes associated with increased stemness such as metastasis and epithelial–mesenchymal transition (EMT) in colorectal cancer, breast cancer, and ovarian cancer cells (15, 28, 29). More interestingly, nigericin might be exploited to enhance drug sensitivity when used in combination with other drugs, such as adriamycin, amiloride, a-Sarcin, and melphalan (8, 24, 30, 31).

Colorectal cancer is regarded as a heterogeneous and multipathway disease, the development of which is mainly associated with genetic background and environmental factors, including diet, lifestyle, and medication use (32, 33). Apart from these surrogate risk factors, activation of the Wnt pathway and its effects on *in vitro* and *in vivo* tumorigenesis offer the most compelling evidence of its importance in colorectal cancer progression (21, 34, 35). The Wnt pathway is commonly divided into β-catenin dependent (canonical) and independent (noncanonical) signaling (36). In canonical Wnt signaling, absence of Wnt ligands (Wnt signaling inactive state) leads to phosphorylation of β-catenin by the destruction complex, which contains the scaffold protein Axin, APC, and the kinases GSK3β and casein kinase (CK1α). In this state, β-catenin is phosphorylated by GSK3β, and targeted for proteasomal degradation. Absence of nuclear β-catenin, a repressive complex containing TCF/LEF and transducing-like enhancer protein (TLE/Groucho), recruits HDACs to repress target genes. Once the Wnt pathway activated, β-catenin is stabilizated, accumulated, and translocated into the nucleus, which finally amplifies the expressions of downstream target genes such as Cyclin D1, c-Myc, Survivin, Axin2, MMP-7, and MMP-9, and leads to a change of multiple cellular processes (17, 37).

In this study, we firstly exposed colorectal cancer cell lines to increasing concentrations of nigericin for different time periods and the IC_{50} values were evaluated. The data showed that nigericin significantly reduced colorectal cancer cell proliferation both in dose- and time-dependent manners. The results were partly similar to the study of Zhou and colleagues in 2013, in which nigericin showed more toxicity for the two colorectal cancer cell lines (HT29 and SW1116), compared with oxaliplatin (15). Next, the effects of nigericin on the tumorigenic behaviors of colorectal cancer cells including the abilities of colony formation, apoptosis, migration, and invasion after respective concentrations of nigericin for 48 hours were applied. Our results implied that nigericin could significantly attenuate the tumorigenic potential of colorectal cancer cells *in vitro*, and downregulate the expressions of Wnt-related and target proteins in colorectal cancer cells. Nigericin was found to downregulate the Wnt signaling pathway not only in noncancer cells, such as human foreskin fibroblast (38) and human embryonic kidney cells (39), but also in cancers. In 2011, Lu and colleagues identified salinomycin and nigericin as potent inhibitors of the Wnt signaling cascade by interfering with LPR6 phosphorylation in chronic lymphocytic leukemia cells (39). Similarly, nigericin was also reported to inhibit cytomegalovirus replication by targets Wnt

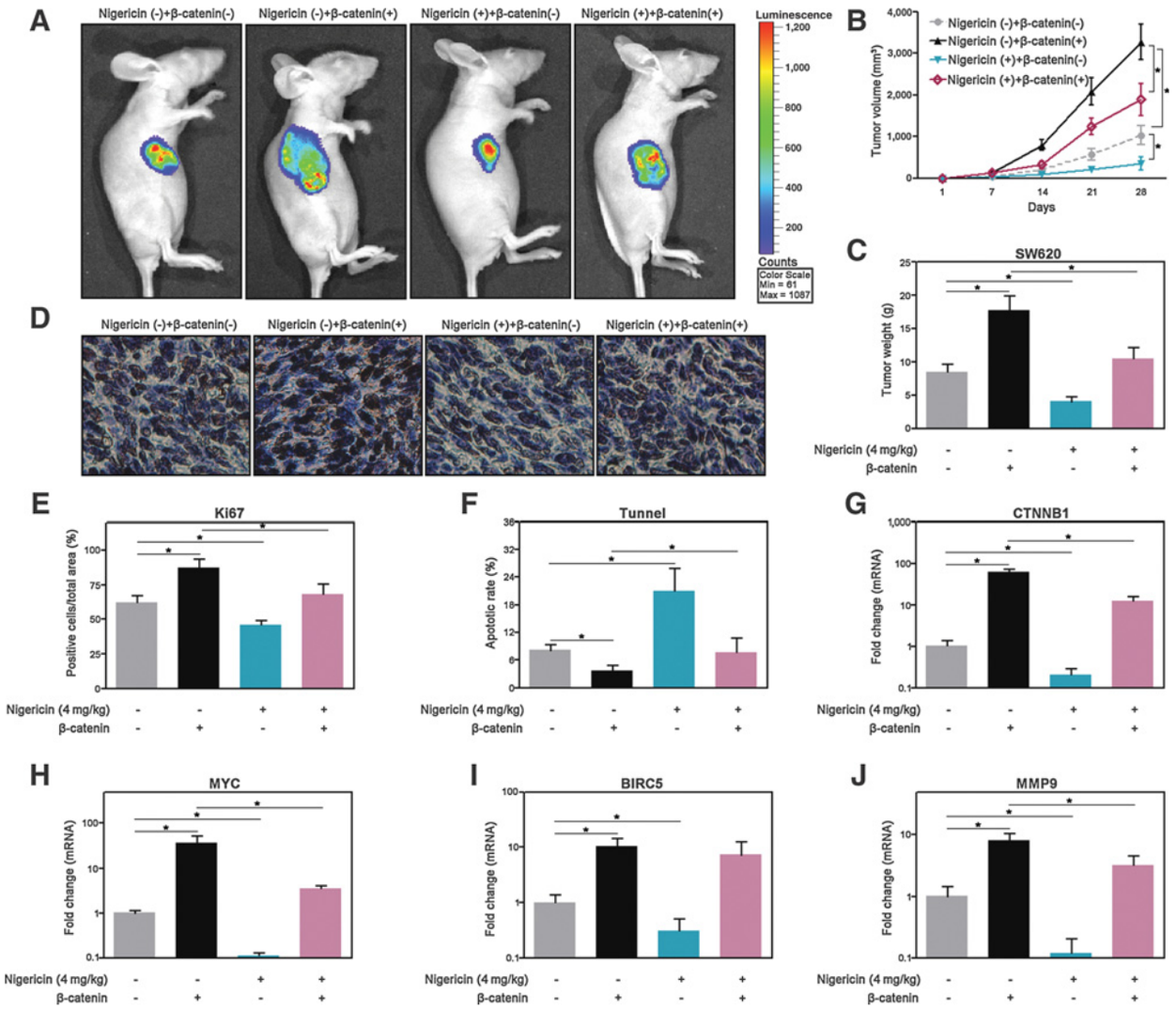

Figure 8.

Nigericin inhibited the tumor growth of colorectal cancer (CRC) cells *in vivo*. **A,** Cells were subcutaneously injected into mice and the tumor mass at 4 week were observed by bioluminescent assays. **B,** The inhibitory effects of nigericin on the tumor growth in mice were compared in different groups. **C,** The tumor weights were compared in different groups. **D** and **E,** The tissue sections from the mice were obtained and the IHC method was used to detect the Ki67 staining. **F,** Cell apoptosis *in vivo* were detected by the TUNEL assay. **G–J,** The mRNA expressions of CTNNB1, MYC, BIRC5, and MMP9 were determined by qRT-PCR. (*, $P < 0.05$).

signaling (38). A recent study by Yakisich demonstrated that nigericin decreased the viability of multidrug-resistant cancer cells and lung tumorspheres in lung cancer (13). In their study, nigericin at micromolar concentration decreased intracellular (K^+) concentration by its ability to increase K^+ efflux and at nanomolar concentrations inhibited the expression of key proteins of the Wnt signaling pathway and promoted translocation of β-catenin into the nucleus. The results obtained in this study contribute to the previously proposed mechanism of action of nigericin as a Wnt inhibitor in cancer cells.

In the absence of activated Wnt/β-catenin signaling, cytosolic β-catenin is rapidly phosphorylated by a complex of proteins collectively termed the "destruction complex," comprised of the core proteins AXIN, APC (adenomatous polyposis coli), GSK3 (glycogen synthase kinase) and CK1 (casein kinase1). The destruction complex phosphorylates the N-terminus of β-catenin targeting the protein for proteasomal degradation and thereby maintaining low baseline cytosolic levels (40, 41). Therefore, stabilization of cellular β-catenin has been supposed as an important step in many human cancers (17, 42, 43). To further elucidate the inhibitory effects of nigericin via a Wnt//β-catenin–dependent signaling mechanism in colorectal cancer, we established the stably β-catenin overexpression colorectal cancer cells and discussed the antagonizing effects of nigericin on β-catenin. Our data confirmed that nigericin also had anticancer effects on β-catenin overexpressing cells by effectively inhibiting Wnt/β-catenin signaling pathway.

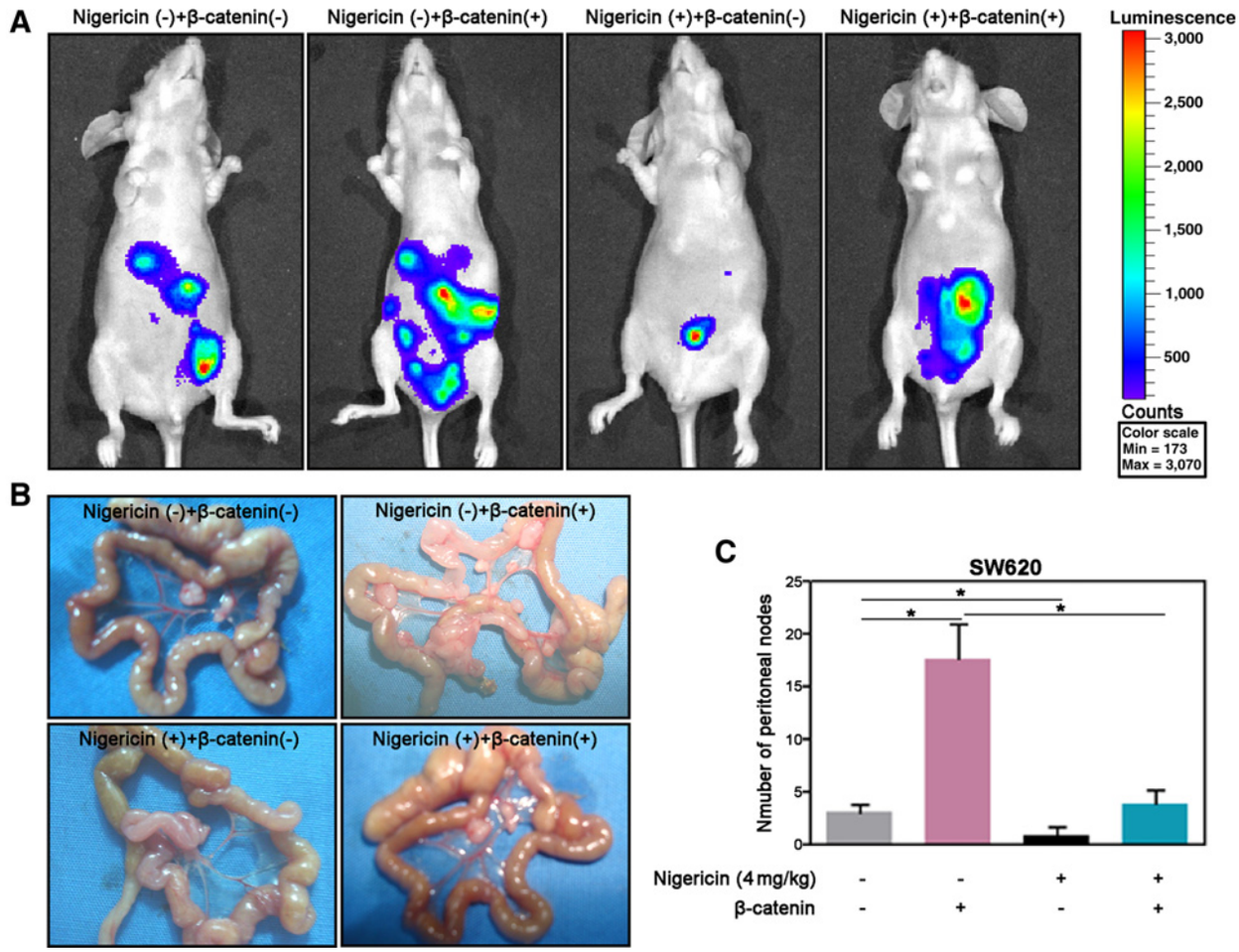

Figure 9.

Nigericin inhibited the tumor metastasis of colorectal cancer (CRC) cells *in vivo*. **A,** The mice tumor metastasis models were built and different treated cells were injected into the peritoneal cavity. The peritoneal spreading was observed by bioluminescent assays. **B** and **C,** The abdominal metastasis nodules on the mesentery were dissected and calculated (*, $P < 0.05$).

Moreover, the immunoprecipitation assay demonstrated that nigericin could inhibit the Wnt signaling by directly targeting the β-catenin destruction complex GSK-3β/Axin1/β-catenin, which accompanied with a decreased expression of pull-downed β-catenin and increased levels of GSK-3β and Axin1. Taken together, these results strongly confirmed β-catenin as a critical intermediary and player in Wnt/β-catenin pathway, and nigericin exerted anticancer effects on colorectal cancer cells by directly targeting the β-catenin destruction complex. As we know, using subcutaneous tumor model can help researchers observe the tumor growth in mice directly. But it cannot accurately reflect the actual environment of tumor growth and metastasis in animals. The mice tumor metastasis model by injecting cells intraperitoneally into animals may simulate the tumor metastatic process in the peritoneal cavity. Given the observed effects of nigericin on colorectal cancer cell growth and metastasis *in vitro*, we simultaneously built the colorectal cancer subcutaneous and metastasis models to determine whether nigericin suppressed tumorigenicity *in vivo*. Fortunately, our experiments *in vivo* identified nigericin's anticancer effects on colorectal cancer cells with the decreased mRNA expressions of Wnt-related genes such as *CTNNB1*, *MYC*, *BIRC5*, and *MMP9*, and more peritoneal spreading.

Taken together, we had shown that nigericin reduced the tumor cell proliferation in a dose- and time-dependent manner in colorectal cancer cells. *In vitro and vivo*, nigericin significantly suppressed the tumor growth and metastasis by inhibiting Wnt/β-catenin signaling pathway and directly targeting the β-catenin destruction complex. These results suggested that Wnt/β-catenin signaling might have an essential role in colorectal cancer progression, and nigericin acted as an effective inhibitor of Wnt/β-catenin signaling. To the best of our knowledge, there are no studies discussing the existing or potential use of nigericin in the clinic. Our data provided new insight into the molecular mechanism of nigericin toward cancer cells, and suggested possible clinical application in colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: F. Liu, Y. Han, Q. Zhi
Development of methodology: Y. Wang, Y. Kuang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Liu, W. Li, S. Hua, Y. Kuang, J. Shi, Q. Zhi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Liu, W. Li, S. Hua, Z. Xu, D. Wan, Y. Wang, W. Chen, Y. Kuang, J. Shi
Writing, review, and/or revision of the manuscript: F. Liu, W. Li, Y. Han, J. Shi, Q. Zhi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Liu, S. Hua, Y. Han, Z. Xu, D. Wan, Y. Wang, W. Chen, Q. Zhi
Study supervision: Y. Han, W. Chen, Y. Kuang

Acknowledgments

This study was supported by grants from the Special Subject of Diagnosis Treatment of Key Clinical Diseases of Suzhou City Sci-tech Bureau (#LCZX201401; to Y.T. Kuang) and the Project of Invigorating Health Care through Science, Technology and Education, Jiangsu Provincial Medical Youth Talent (#QNRC2016723; to Q.M. Zhi).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 19, 2017; revised November 20, 2017; accepted February 7, 2018; published first February 26, 2018.

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015;65:87–108.
2. Fiorentini G, Sarti D, Aliberti C, Carandina R, Mambrini A, Guadagni S. Multidisciplinary approach of colorectal cancer liver metastases. World J Clin Oncol 2017;8:190–202.
3. Bignucolo A, De Mattia E, Cecchin E, Roncato R, Toffoli G. Pharmacogenomics of targeted agents for personalization of colorectal cancer treatment. Int J Mol Sci 2017;18:1522.
4. Steinrauf LK, Pinkerton M, Chamberlin JW. The structure of nigericin. Biochem Biophys Res Commun 1968;33:29–31.
5. Poole DT, Butler TC, Williams ME. The effects of nigericin, valinomycin, and 2,4-dinitrophenol on intracellular pH, glycolysis, and K + concentration of Ehrlich ascites tumor cells. Biochim Biophys Acta 1972;266:463–70.
6. Rotin D, Wan P, Grinstein S, Tannock I. Cytotoxicity of compounds that interfere with the regulation of intracellular pH: a potential new class of anticancer drugs. Cancer Res 1987;47:1497–504.
7. Margolis LB, Rozovskaja IA, Skulachev VP. Acidification of the interior of Ehrlich ascites tumor cells by nigericin inhibits DNA synthesis. FEBS Lett 1987;220:288–90.
8. Schindler M, Grabski S, Hoff E, Simon SM. Defective pH regulation of acidic compartments in human breast cancer cells (MCF-7) is normalized in adriamycin-resistant cells (MCF-7adr). Biochemistry 1996;35:2811–7.
9. Kuin A, Aalders M, Lamfers M, van Zuidam DJ, Essers M, Beijnen JH, et al. Potentiation of anti-cancer drug activity at low intratumoral pH induced by the mitochondrial inhibitor m-iodobenzylguanidine (MIBG) and its analogue benzylguanidine (BG). Br J Cancer 1999;79:793–801.
10. Andersson B, Behnam-Motlagh P, Henriksson R, Grankvist K. Pharmacological modulation of lung cancer cells for potassium ion depletion. Anticancer Res 2005;25:2609–16.
11. Boesch M, Zeimet AG, Rumpold H, Gastl G, Sopper S, Wolf D. Drug transporter-mediated protection of cancer stem cells from ionophore antibiotics. Stem Cells Transl Med 2015;4:1028–32.
12. Mashima T, Okabe S, Seimiya H. Pharmacological targeting of constitutively active truncated androgen receptor by nigericin and suppression of hormone-refractory prostate cancer cell growth. Mol Pharmacol 2010;78:846–54.
13. Deng CC, Liang Y, Wu MS, Feng FT, Hu WR, Chen LZ, et al. Nigericin selectively targets cancer stem cells in nasopharyngeal carcinoma. Int J Biochem Cell Biol 2013;45:1997–2006.
14. Yakisich JS, Azad N, Kaushik V, O'Doherty GA, Iyer AK. Nigericin decreases the viability of multidrug-resistant cancer cells and lung tumorspheres and potentiates the effects of cardiac glycosides. Tumour Biol 2017;39:1010428317694310.
15. Zhou HM, Dong TT, Wang LL, Feng B, Zhao HC, Fan XK, et al. Suppression of colorectal cancer metastasis by nigericin through inhibition of epithelial-mesenchymal transition. World J Gastroenterol 2012;18:2640–8.
16. Katoh M. Molecular genetics and targeted therapy of WNT-related human diseases (Review). Int J Mol Med 2017;40:587–606.
17. Paul S, Dey A. Wnt signaling and cancer development: therapeutic implication. Neoplasma 2008;55:165–76.
18. Arend RC, Londono-Joshi AI, Straughn JM Jr, Buchsbaum DJ. The Wnt/beta-catenin pathway in ovarian cancer: a review. Gynecol Oncol 2013;131:772–9.
19. Noguti J, DE Moura CF, Hossaka TA, Franco M, Oshima CT, Dedivitis RA, et al. The role of canonical WNT signaling pathway in oral carcinogenesis: a comprehensive review. Anticancer Res 2012;32:873–8.
20. Chiurillo MA. Role of the Wnt/beta-catenin pathway in gastric cancer: an in-depth literature review. World J Exp Med 2015;5:84–102.
21. Novellasdemunt L, Antas P, Li VS. Targeting Wnt signaling in colorectal cancer. A Review in the Theme: Cell Signaling: Proteins, Pathways and Mechanisms. Am J Physiol Cell Physiol 2015;309:19.
22. Hegazy AM, Yamada D, Kobayashi M, Kohno S, Ueno M, Ali MA, et al. Therapeutic strategy for targeting aggressive malignant gliomas by disrupting their energy balance. J Biol Chem 2016;291:21496–509.
23. Jahde E, Glusenkamp KH, Rajewsky MF. Nigericin enhances mafosfamide cytotoxicity at low extracellular pH. Cancer Chemother Pharmacol 1991;27:440–4.
24. Maidorn RP, Cragoe EJ Jr, Tannock IF. Therapeutic potential of analogues of amiloride: inhibition of the regulation of intracellular pH as a possible mechanism of tumour selective therapy. Br J Cancer 1993;67:297–303.
25. Yoon MJ, Kang YJ, Kim IY, Kim EH, Lee JA, Lim JH, et al. Monensin, a polyether ionophore antibiotic, overcomes TRAIL resistance in glioma cells via endoplasmic reticulum stress, DR5 upregulation and c-FLIP down-regulation. Carcinogenesis 2013;34:1918–28.
26. Riccioni R, Dupuis ML, Bernabei M, Petrucci E, Pasquini L, Mariani G, et al. The cancer stem cell selective inhibitor salinomycin is a p-glycoprotein inhibitor. Blood Cells Mol Dis 2010;45:86–92.
27. Manago A, Leanza L, Carraretto L, Sassi N, Grancara S, Quintana-Cabrera R, et al. Early effects of the antineoplastic agent salinomycin on mitochondrial function. Cell Death Dis 2015;22:263.
28. Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell 2009;138:645–59.
29. Wang W, Zhao Y, Yao S, Cui X, Pan W, Huang W, et al. Nigericin inhibits epithelial ovarian cancer metastasis by suppressing the cell cycle and epithelial-mesenchymal transition. Biochemistry 2017;82:933–41.
30. Olmo N, Turnay J, Gonzalez de Buitrago G, Lopez de Silanes I, Gavilanes JG, Lizarbe MA. Cytotoxic mechanism of the ribotoxin alpha-sarcin. Induction of cell death via apoptosis. Eur J Biochem 2001;268:2113–23.
31. Wood PJ, Sansom JM, Newell K, Tannock IF, Stratford IJ. Reduction of tumour intracellular pH and enhancement of melphalan cytotoxicity by the ionophore Nigericin. Int J Cancer 1995;60:264–8.
32. Peters U, Hutter CM, Hsu L, Schumacher FR, Conti DV, Carlson CS, et al. Meta-analysis of new genome-wide association studies of colorectal cancer risk. Hum Genet 2012;131:217–34.
33. Chan AT, Giovannucci EL. Primary prevention of colorectal cancer. Gastroenterology 2010;138:2029–43.
34. White BD, Chien AJ, Dawson DW. Dysregulation of Wnt/beta-catenin signaling in gastrointestinal cancers. Gastroenterology 2012;142:219–32.
35. Basu S, Haase G, Ben-Ze'ev A. Wnt signaling in cancer stem cells and colon cancer metastasis. F1000Res 1000:19.
36. Zhong ZA, Zahatnansky J, Snider J, Van Wieren E, Diegel CR, Williams BO. Wntless spatially regulates bone development through beta-catenin-dependent and independent mechanisms. Dev Dyn 2015;244:1347–55.
37. Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. Oncogene 2017;36:1461–73.

38. Kapoor A, He R, Venkatadri R, Forman M, Arav-Boger R. Wnt modulating agents inhibit human cytomegalovirus replication. Antimicrob Agents Chemother 2013;57:2761–7.
39. Lu D, Choi MY, Yu J, Castro JE, Kipps TJ, Carson DA. Salinomycin inhibits Wnt signaling and selectively induces apoptosis in chronic lymphocytic leukemia cells. Proc Natl Acad Sci U S A 2011;108:13253–7.
40. Kitazawa M, Hatta T, Ogawa K, Fukuda E, Goshima N, Natsume T. Determination of rate-limiting factor for formation of beta-catenin destruction complexes using absolute protein quantification. J Proteome Res 2017;16:3576–84.
41. Pronobis MI, Deuitch N, Posham V, Mimori-Kiyosue Y, Peifer M. Reconstituting regulation of the canonical Wnt pathway by engineering a minimal beta-catenin destruction machine. Mol Biol Cell 2017;28: 41–53.
42. Vergara D, Stanca E, Guerra F, Priore P, Gaballo A, Franck J, et al. β-catenin knockdown affects mitochondrial biogenesis and lipid metabolism in breast cancer cells. Front Physiol 2017;8:544.
43. Gao F, Xu T, Wang X, Zhong S, Chen S, Zhang M, et al. CIP2A mediates fibronectin-induced bladder cancer cell proliferation by stabilizing beta-catenin. J Exp Clin Cancer Res 2017;36:70.