β-arrestin2 promotes 5-FU-induced apoptosis via the NF-κB pathway in colorectal cancer

WEIXIA REN[1,2], TINGTING WANG[2], XIANGJUN HE[1], QI ZHANG[1], JIANHUA ZHOU[1], FANGFANG LIU[3], FANGFANG GAO[1], YUJUN ZHANG[1] and YULAN LIU[1,2]

[1]Institute of Clinical Molecular Biology and Central Laboratory, and Departments of [2]Gastroenterology and [3]Pathology, Peking University, People's Hospital, Beijing 100044, P.R. China

Received May 9, 2017; Accepted March 12, 2018

DOI: 10.3892/or.2018.6340

Abstract. It has been demonstrated that β-arrestin2 is involved in the initiation and development of many types of cancers. However, its role in colorectal cancer (CRC) remains poorly understood. The present study investigated the role of β-arrestin2 in CRC using CRC patient tissues as well as the LoVo and HCT116 CRC cell lines. Briefly, significantly higher expression of β-arrestin2 was observed in CRC tissues compared with normal colon tissues. In addition, the downregulation of β-arrestin2 reduced 5-FU-induced apoptosis in the LoVo cells, while the overexpression of β-arrestin2 increased the apoptosis of HCT116 cells *in vitro*. Furthermore, the downregulation of β-arrestin2 reduced the expression of the pro-apoptotic proteins cleaved-caspase-3 and Bax, and increased the expression of the anti-apoptotic protein Bcl-2 after 5-FU treatment. In addition, the expression of p-p65 was increased after the β-arrestin2 downregulation and was decreased after the β-arrestin2 overexpression. However, β-arrestin2 downregulation had no effect on the proliferation, migration and invasion capacity of the LoVo cells. In conclusion, these results indicated that β-arrestin2 promoted 5-FU-induced CRC cell apoptosis via the NF-κB pathway and may be used as a prognosis marker for CRC.

Correspondence to: Dr Yujun Zhang, Institute of Clinical Molecular Biology and Central Laboratory, Peking University, People's Hospital, 11 South Avenue, Xi Zhi Men, Xicheng, Beijing 100044, P.R. China
E-mail: zhangyujun@pkuph.edu.cn

Dr Yulan Liu, Department of Gastroenterology, Peking University, People's Hospital, 11 South Avenue, Xi Zhi Men, Xicheng, Beijing 100044, P.R. China
E-mail: liuyulan@pkuph.edu.cn

Abbreviations: CRC, colorectal cancer; siRNA, small interfering RNA; NC, negative control; GPCR, G protein-coupled receptor; PCR, polymerase chain reaction; NSCLC, non-small cell lung cancer

Key words: colorectal cancer, β-arrestin2, 5-FU, apoptosis, NF-κB

Introduction

Colorectal cancer (CRC) is the third most common malignancy and one of the leading causes of cancer-related deaths worldwide (1-3). The global burden of CRC is increasing and is likely to persist until the year 2035 and beyond (4). With the introduction of CRC screening, more and more patients benefit from the early detection of precancerous lesions. However, despite the developments in colonoscopy as well as in treatment, the therapeutic effect of CRC remains unsatisfactory. The main obstacles in CRC therapy are metastasis and drug resistance. Therefore, understanding the molecular mechanism of CRC is important for the development of an effective therapy.

β-arrestin1 and β-arrestin2 belong to the nonvisual β-arrestins and are ubiquitous proteins. β-arrestins are multifunctional proteins and are well-known for their classical role in the G protein-coupled receptor (GPCR) desensitization, sequestration and internalization (5,6). Furthermore, β-arrestins are scaffold proteins that can interact with many other signaling molecules and regulate cellular responses, such as proliferation, migration and invasion as well as apoptosis (7,8). β-arrestins play an important role in physiological and pathological conditions.

Numerous studies have demonstrated that β-arrestin2 is abnormally expressed in many types of cancer, including breast, lung, castration-resistant prostate and hepatocellular cancer (9,10). β-arrestin2 is essential for the tumorigenesis of chronic myelogenous leukemia and colon cancer (11,12). It contributes to the proliferation of castration-resistant prostate cancer but inhibits lung cancer growth (13,14). It also decreases metastasis of hepatocellular cancer, but promotes breast cancer migration and invasion (15,16). Above all, β-arrestin2 is involved in the tumorigenesis and progression of cancer in multifunctional ways.

Bonnans *et al* (12) demonstrated that β-arrestin2 is required for the initiation of colon cancer through the elevated Wnt pathway *in vivo* and *in vitro* (12). Nonetheless, the expression and clinicopathological significance of β-arrestin2 in CRC have not been reported. Liu *et al* (17) demonstrated that β-arrestin2 deficiency protracts the activation of the NF-κB pathway and suppresses radiation-induced intestinal crypt progenitor cell apoptosis (17). However, the role of β-arrestin2

in chemo-induced colon epithelial cell apoptosis remains to be explored. The aim of this study was to investigate the role of β-arrestin2 in CRC and CRC cell apoptosis. To assess the expression and clinical significance of β-arrestin2 in CRC, CRC tissues were analyzed. Immunohistochemistry assay demonstrated that β-arrestin2 was overexpressed in CRC tissues compared with normal tissues, although its high expression was not related with the clinicopathological features. Furthermore, β-arrestin2 downregulation did not alter the cell proliferation rate, migration and invasion capacity *in vitro*, although, the data indicated that β-arrestin2 downregulation inhibited the 5-FU-induced CRC cell apoptosis, reduced the expression of cleaved-caspase-3 and Bax and increased the expression of Bcl-2. In addition, β-arrestin2 overexpression increased the apoptosis rate of CRC cells stimulated by 5-FU. The p-p65 expression increased following β-arrestin2 downregulation and decreased following β-arrestin2 overexpression. Collectively, these data indicated that β-arrestin2 played a critical role in CRC and contributed to CRC cell apoptosis via the NF-κB signaling pathway.

Materials and methods

Patient tissue. From April 2009 to February 2016, 59 primary CRC samples (41 cases) and normal colon tissues (18 cases) were collected at the Peking University People's Hospital (Beijing, China). The CRC group consisted of 20 women and 21 men aged between 48-88 years. The control group consisted of adjacent non-cancerous mucosa tissue from 12 CRC patients and adjacent non-inflammation mucosa tissue from 6 inflammation bowel disease patients. CRC diagnosis was confirmed by 2 pathologists. Patients who received chemotherapy and radiotherapy prior to surgery were not enrolled in this study. The present study was approved by the Human Ethics Review Board of the Peking University People's Hospital (Beijing, China). All patients obtained informed consent to donate their tissue samples and clinical information for research, and written consent was given from all the patients.

Cell lines. The human colon cancer cell lines LoVo and HCT116 were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) in a humidified atmosphere containing 5% CO_2 and 95% air at 37˚C.

Immunohistochemistry assay. Paraffin sections were deparaffinized in xylene and hydrated in alcohol gradient. The slides were incubated with 3% H_2O_2 for 10 min. Antigen retrieval was performed at 95˚C for 20 min in sodium citrate solution (Solarbio Science and Technology, Beijing, China). The slides were blocked with 5% bovine serum albumin (BSA) for 1 h and incubated with rabbit anti-β-arrestin2 monoclonal antibody (1:200; cat. no. 3857; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4˚C overnight. After being washed with PBS, the slides were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:1,000; cat. no. ZB2301; ZSGB-BIO, Beijing, China) for 1 h at room temperature. After washing with PBS, the DAB solution was used to visualize β-arrestin2 expression and then the nuclei were stained with hematoxylin. PBS were used as a negative control. Data were analyzed on an Olympus microscope (Olympus Inc., Tokyo, Japan) by two independent single-blinded pathologists.

Five random fields were selected for scoring under x200 magnification. The scoring was performed based on staining scope: 1 (0-25%); 2 (25-50%); 3 (50-75%); and 4 (75-100%). The staining intensity was also divided into 4 levels: 0, negative; 1, weakly positive (light yellow); 2, moderately positive (yellow brown); 3, strongly positive (dark brown). The expression score was calculated as follows: Staining scope x intensity. Scores ≥4 reflected positive expression, while those below 4 represented negative expression (18).

Cell transfection. Small interfering RNA (siRNA) was synthesized by GenePharm Co. (Suzhou, Jiangsu, China). The target sequences for β-arrestin2 were as follows: 5'-CGUAGACUU UGAGAUUCGATT-3', β-arrestin2-siRNA-1; 5'-CUCAAC UCGAACAAGAUGATT-3', β-arrestin2-siRNA-2; 5'-CCA ACCUCAUUGAAUUUGATT-3', β-arrestin2-siRNA-3. The sequence of unrelated siRNA was 5'-UUCUCCGAACGUGUC ACGUTT-3' (NC-siRNA). The full length of β-arrestin2 were cloned into pCMV vector in frame with GFP (Sino Biological, Beijing, China). The cells were seeded in proper dishes and transfected at 70% confluency. In 6-cell culture cluster, the transfection was conducted with siRNAs (100 nmol/well) or plasmids (2,500 ng/well) using Lipofectamine 3000 (5 μl/well) according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Quantitative real-time PCR. Real-time PCR was used to detect gene silencing expression of β-arrestin2. Total RNA was isolated using the RNA Isolation kit (Omega Bio-Tek, Inc., Norcross, GA, USA) and complementary DNA (cDNA) was synthesized using a ReverTra Ace qPCR RT kit (Toyobo Life Science, Osaka, Japan), according to the manufacturer's instructions. Real-time PCR was performed using SYBR-Green Mix kit (cat. no. 4385612; Applied Biosystems; Thermo Fisher Scientific, Inc.) and Applied Biosystems ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermal cycling conditions for RT-PCR were as follows: Denaturation at 95˚C for 2 min, followed by 40 cycles of denaturation 95˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 45 sec. The prime sequences for each gene were as follows: β-arrestin2 sense, TCCATG CTCCGTCACACTG and antisense, ACAGAAGGCTCG AATCTCAAAG (length=82 bp); GAPDH sense, GTCTCC TCTGACTTCAACAGCG and antisense, ACCACCCTGTTG CTGTAGCCAA (length=131 bp). The data were calculated using the $2^{-\Delta\Delta Ct}$ method and GAPDH was the reference gene.

Western blot analysis. Total proteins were prepared from the cell lines after 48 h transfection and stimulation with 5-FU. Briefly, cells were washed twice with ice-cold PBS and lysed in RIPA lysis buffer (containing 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride and 1% protease inhibitor cocktails) for 20 min on ice. Samples were then centrifuged at 13,400 x g for 20 min. The supernatant was

collected and the protein concentration was determined by BCA protein assay (Pierce Chemical, Rockford, IL, USA). The supernatants were added appropriate volume 5X SDS-PAGE loading buffer (Applygen Technologies Inc., Beijing, China) and incubated at 100˚C for 5 min. Equal amounts of protein were separated by 10% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore; Merck KGaA, Darmstadt, Germany). The membranes were blocked with 5% non-fat dry milk in TBS for 2 h and then incubated with mouse anti-β-arrestin2 monoclonal antibody (1:500; cat. no. ab54790; Abcam, Cambridge, MA, USA), rabbit anti-Bax polyclonal antibody (1:500; cat. no. sc-526; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) and mouse anti-Bcl-2 monoclonal antibody (1:500; cat. no. sc-7382; Santa Cruz Biotechnology), rabbit anti-cleaved-caspase-3 monoclonal antibody (cat. no. 5A1E; Cell Signaling Technology, Inc.) and rabbit anti-p-p65 monoclonal antibody (cat. no. 3033P; Cell Signaling Technology, Inc.) and rabbit anti-GAPDH polyclonal antibody (1:1,000; cat. no. AF0911; Abmart, Shanghai, China) at 4˚C overnight. The membranes were washed with TBST and incubated with the appropriate horseradish peroxidase-conjugated second antibodies (cat. nos. ZB2305 and ZB2301; ZSGB-BIO) for 1 h at room temperature. Proteins were visualized by enhanced ECL detection kit (Pierce Chemical). Band intensities were analyzed using the ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA).

Cell proliferation assay. Cell proliferation was conducted using the Cell Counting Kit-8 (CCK-8; Dojindo, Tokyo, Japan). Forty-eight hours after transfection with siRNA, equal amounts of cells ($3x10^3$ cells/well) were seeded in a 96-well plate and cultured in the medium supplemented with 10% FBS at indicated time-points. Every 24 h, 10 μl of CCK-8 were added and the cells were incubated for 3 h in the humidified incubator that contained 5% CO_2 at 37˚C. Relative proliferation was obtained by scanning with an ELISA reader with a 450-nm filter.

Migration and invasion assay. Cell migration and invasion were analyzed using a Boyden chamber (Corning Costar, Rochester, NY, USA) with a gelatin-coated polycarbonate membrane filter (6.5 mm diameter, 8 μm pore size). For the invasion assay the upper surface of the filter was coated with 20 μl Matrigel (BD Biosciences, Bedford, MA, USA) at 37˚C for 1 h. After being transfected with siRNA for 48 h, the cells were trypsinized and resuspended with 1% FBS culture medium at a final density of $5x10^5$ cells/ml. Cell suspension (200 μl) was added to the upper chamber, and 10% FBS culture medium was added to the lower chamber as a chemoattractant. The cells were incubated in the humidified incubator that contained 5% CO_2 at 37˚C. After 24 h, the upper surface of the filter was scrubbed with a cotton swab and then the non-migrated or non-invaded cells were removed. The cells at the lower surface of the chamber were fixed with 4% paraformaldehyde for 30 min. After being washed twice with PBS, migration or invasion cells were stained with 0.5% (w/v) crystal violet for 15 min. Cells were then counted using a light microscope (Olympus Inc.). Five random fields were selected for cell counting under x100 magnification.

Table I. The expression of β-arrestin2 in colon epithelial cells of normal colon mucosal tissue and colorectal cancer tissue.

Group	Positive	Negative	P-value
Normal colon mucosal tissue	5	13	0.043
Colorectal cancer tissue	25	16	

Caspase-3 activity assay. Caspase-3 activity kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) was used to estimate the caspase-3 activity according to the manufacturer's instructions. After being transfected with siRNA, LoVo cells were stimulated with 5-FU for 48 h and then the cells were collected for the caspase-3 activity assay. Briefly, the cells were trypsinized and washed with cold PBS. Subsequently, the cells were resuspended in lysis buffer ($2x10^6$ cells/100 μl) and were shaken on ice for 15 min. Cell lysis was then centrifuged at 13,400 x g and 4˚C for 20 min. The supernatants were collected and the protein concentrations were determined by Bradford protein assay (Beyotime Institute of Biotechnology). Protein supernatants (50 μl), 40 μl reaction buffer and 10 μl caspase-3 substrate (Ac-DEVD-pNA, 2 mM) were added to the 96-well microtiter plates and then incubated at 37˚C for 4 h. Caspase-3 activity was quantified using a microplate reader at an absorbance of 405 nm and then was demonstrated as a percentage of enzyme activity compared to the negative control group.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay. Nucleosomal DNA fragmentation was determined by TUNEL assay using an *in situ* Apoptosis Detection kit (KeyGen Biotech. Co., Ltd., Nanjing, Jiangsu, China) according to the manufacturer's instructions. After being transfected with β-arrestin2 overexpression plasmid, the HCT116 cells were stimulated with 5-FU for 48 h and then cells were collected for the TUNEL assay. The cells were fixed with 4% paraformaldehyde for 30 min at 4˚C and washed three times with PBS. The fixed cells were then incubated in PBS containing 1% Triton X-100 for 15 min at room temperature. Subsequently, cells were incubated with 3% H_2O_2-methanol for 15 min at room temperature. TdT enzyme solution (10 μl) was added into the samples and incubated for 1 h at 37˚Cin the dark, and then with 10 μl streptavidin-HRP for 30 min, at 37˚C in the dark. DAB solution was used to visualize DNA fragmentation. The nucleus was stained with hematoxylin. Data were analyzed on an Olympus microscope. Five random fields were selected for counting the apoptosis rate under x100 magnification.

Statistical analysis. Statistical analyses were performed using the SPSS software version 19.0 (IBM Corp., Armonk, NY, USA). Chi-square test was used to analyze the correlation between β-arrestin2 expression and clinicopathological characteristics. Data were presented as the mean ± SE. Group differences were determined by one-way ANOVA and Student's t-test. P-values <0.05 were considered to indicate statistically significant differences.

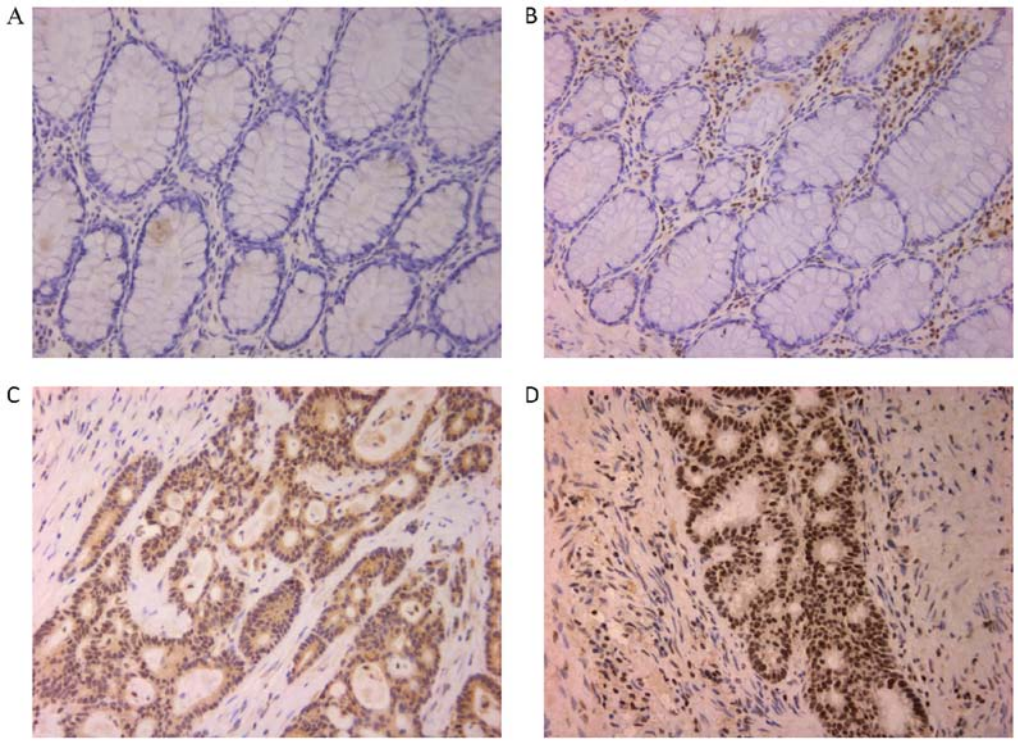

Figure 1. The expression of β-arrestin2 in colon epithelial cells of normal colon and CRC tissue (magnification, x200). (A) Negative expression of β-arrestin2 in colon epithelial cells of normal colon tissue. (B) Stromal expression of β-arrestin2 in normal colon tissue. (C) Positive cytoplasmic expression of β-arrestin2 in CRC tissue. (D) Positive nucleus expression of β-arrestin2 in CRC tissue.

Results

Increased expression of β-arrestin2 is observed in CRC tissues compared to healthy colon tissues. Immunohistochemistry was used to detect the expression of β-arrestin2 in CRC and normal colon tissues. Forty-one CRC and 18 healthy tissues were isolated. Significantly higher expression of β-arrestin2 protein was observed in CRC tissues compared to healthy tissues (P<0.05; Fig. 1; Table I). The positive rate of β-arrestin2 expression in CRC was 60.98% (25/41), while it was 27.78% (5/18) in the healthy tissues. Furthermore, β-arrestin2 was mainly expressed in the cytoplasm and nucleus (Fig. 1C and D). In summary, these data indicated that β-arrestin2 has an important role in the initiation and development of CRC. However, no correlation between β-arrestin2 expression in CRC and clinicopathological characteristics, including TNM stage, tumor volume and CEA was found (Table II).

β-arrestin2 downregulation has no effect on LoVo cell proliferation, migration and invasion. To explore the role of β-arrestin2 in colon cancer biological behavior (proliferation, invasion and migration) *in vitro*, β-arrestin2 was downregulated in LoVo cells (β-arrestin2-siRNA group). Reduced β-arrestin2 expression was confirmed by RT-PCR and western blot analysis (Fig. 2). Notably, no significant difference in cell viability was observed between the NC group and β-arrestin2-siRNA group (Fig. 3), indicating that β-arrestin2 downregulation was not associated with cell proliferation.

To determine the effect of β-arrestin2 on cell migration and invasion, Transwell assay was performed. No significant

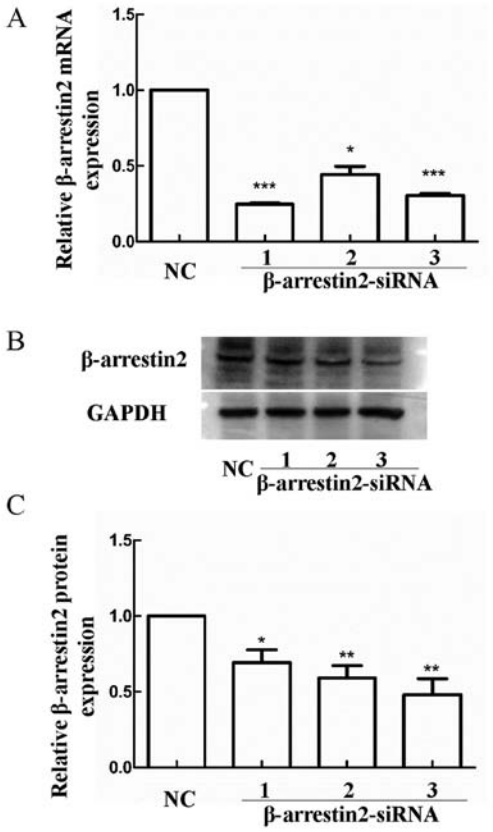

Figure 2. The examination of β-arrestin2 of human colon cancer LoVo cells following transfection with β-arrestin2-siRNA. (A) The impact of β-arrestin2 siRNA on β-arrestin2 mRNA expression (***P<0.001, *P<0.05). (B) Reduced β-arrestin2 expression was confirmed by western blot analysis. (C) The impact of β-arrestin2 siRNA on β-arrestin2 protein expression (**P<0.01, *P<0.05). Data are expressed as the mean ± standard deviation (n=3).

Table II. The correlation between the expression of β-arrestin2 and patient clinicopathological characteristics.

Clinicopathological characteristics	Cases	β-arrestin2		P-value
		Positive	Positive ratio	
Sex				
Male	21	14	0.67	>0.05
Female	20	11	0.55	
Age (years)				
≥60	31	20	0.65	>0.05
<60	10	5	0.5	
Clinical stage				
I+II	20	12	0.6	>0.05
III+IV	21	15	0.71	
Histopathological type				
Mucoid adenocarcinoma	6	5	0.83	>0.05
Non-mucoid adenocarcinoma	35	22	0.63	
pT				
pT1-3	20	15	0.75	>0.05
pT4	21	12	0.57	
pN				
pN0	20	12	0.6	>0.05
pN1-2	21	15	0.71	
pM				
pM0	33	22	0.67	>0.05
pM1-2	8	5	0.63	
Lymph node metastasis				
Negative	22	12	0.55	>0.05
Positive	19	13	0.68	
Liver metastasis				
Negative	36	24	0.67	>0.05
Positive	5	3	0.6	
Peritoneal dissemination				
Negative	37	25	0.68	>0.05
Positive	4	2	0.5	
Grade				
Well-moderate	28	15	0.54	>0.05
Poor	13	10	0.77	
Tumor volume (mm)				
<50	20	12	0.6	>0.05
≥50	21	13	0.62	
General type				
Non-ulcerative type	15	10	0.67	>0.05
Ulcerative type	26	17	0.65	
CEA				
≥10	13	8	0.62	>0.05
<10	28	17	0.61	

difference in cell migration and invasion between the NC group and β-arrestin2-siRNA group was observed (migration, 137.86±44.29 vs. 140.78±39.06, P>0.05; invasion, 184.34±79.26 vs. 183.25±76.61, P>0.05) (Fig. 4). These results demonstrated that β-arrestin2 downregulation was not associated with cell invasion and migration.

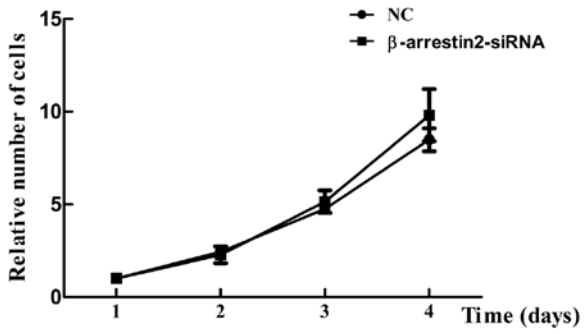

Figure 3. The proliferation rate of human colon cancer LoVo cells following transfection with β-arrestin2-siRNA. Data are expressed as the mean ± standard deviation (n=3).

β-arrestin2 downregulation inhibits the 5-FU-induced CRC cell apoptosis. 5-FU was selected to induce apoptosis of LoVo colon cancer cells *in vitro*. CCK-8 assay was used to determine the cell viability pre- and post- treatment. In the NC group, no increase in caspase-3 activity was observed by increasing 5-FU concentration, while, after treatment with 0.02 μmol/ml 5-FU, decreased caspase-3 expression was observed in the β-arrestin2-siRNA group compared with the NC group (15.614±3.781 vs. 8.133±1.173, P<0.05) (Fig. 5).

To further confirm our results, we detected the apoptotic protein by western blot analysis. In the NC group, the pro-apoptotic protein Bax and cleaved-caspase-3 were increased, while the anti-apoptotic protein Bcl-2 was decreased after stimulation with 0.02 μmol/ml 5-FU compared with the untreated group (0 μmol/ml 5-FU) (Fig. 6, P<0.05). Furthermore, the opposite effect was observed in the β-arrestin2-siRNA group after stimulation with 0.02 μmol/ml 5-FU. Cleaved-caspase-3 and Bax were significantly decreased, while Bcl-2 was increased in the β-arrestin2-siRNA group compared to NC group (Fig. 6, P<0.05).

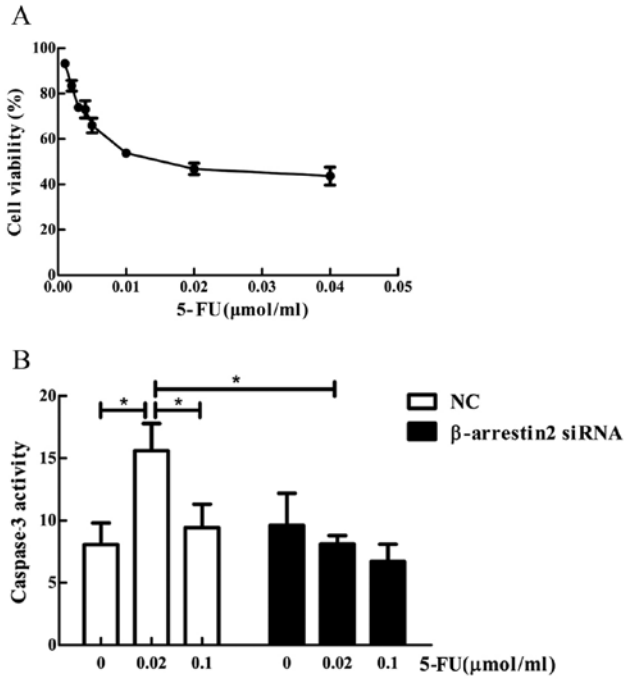

Figure 5. The 5-FU induced apoptosis of human colon cancer LoVo cells following transfection with siRNA. (A) The IC_{50} of 5-FU for LoVo cells as determined by CCK-8 assay. (B) Cell apoptosis as determined by caspase-3 activity (*P<0.05). Data are expressed as the mean ± standard deviation (n=3).

Altogether, the above mentioned data indicated that β-arrestin2 downregulation inhibited the 5-FU-induced CRC cell apoptosis, by reducing the expression of the pro-apoptotic proteins cleaved-caspase-3 and Bax.

β-arrestin2 overexpression enhances apoptosis after 5-FU treatment. In the present study we explored the effect of β-arrestin2 overexpression on apoptosis after 5-FU treatment. The human

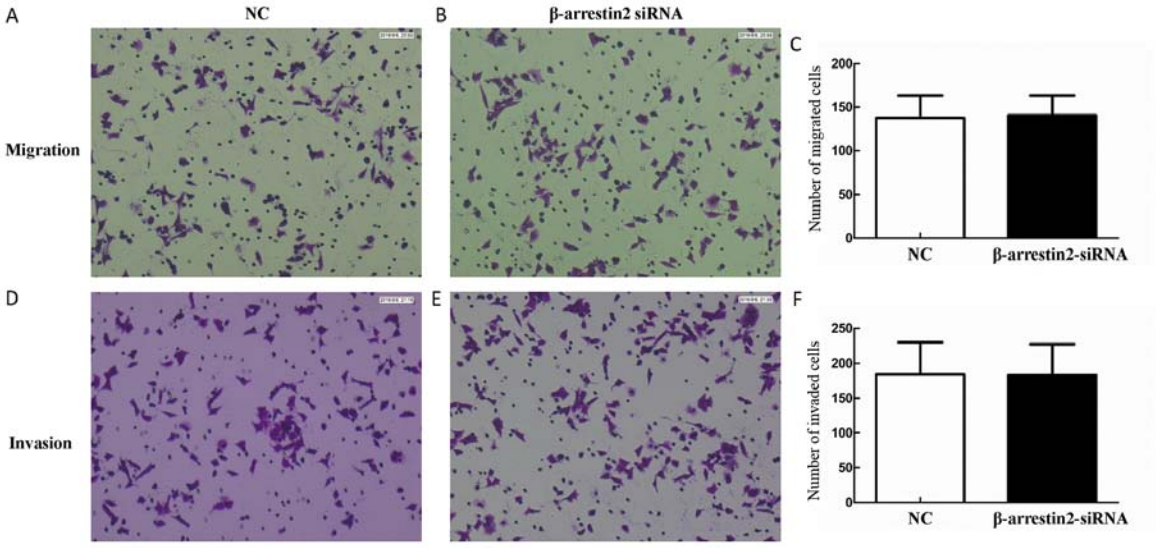

Figure 4. The migration and invasion cell numbers of human colon cancer LoVo cells following transfection with siRNA (magnification, x100). (A) The migration of the LoVo human colon cancer cell line after transfection with negative control siRNA. (B) The migration of LoVo cells after transfection with β-arrestin2 siRNA. (C) The number of migrated cells was quantified and is shown in a column graph. (D) Invasion image of the LoVo cells following transfection with negative control siRNA. (E) Invasion image of human colon cancer LoVo cells after transfection with β-arrestin2 siRNA. (F) The number of invaded cells was quantified and is shown in a column graph. Data are expressed as the mean ± standard deviation (n=3).

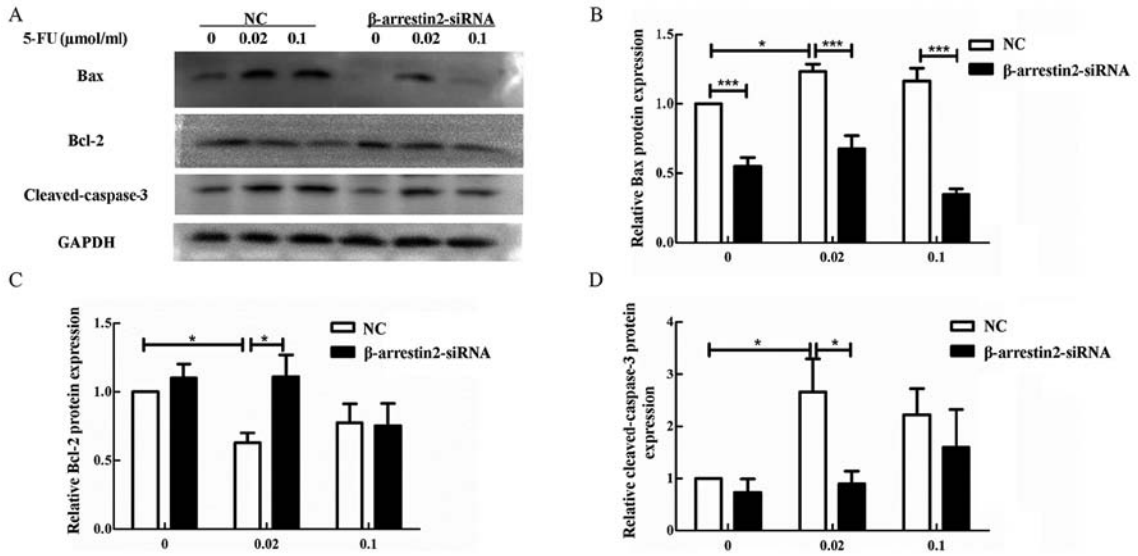

Figure 6. The pro-apoptotic protein Bax, anti-apoptotic protein Bcl-2 and cleaved-caspase-3 protein expression in the NC group and β-arrestin2-siRNA group following stimulation with 5-FU. (A) Pro-apoptotic protein Bax, anti-apoptotic protein Bcl-2 and cleaved-caspase-3 protein expression as determined by western blot analysis in the NC group and β-arrestin2-siRNA group after stimulation with 5-FU. (B) The expression of the pro-apoptotic protein Bax was quantified and displayed in a column graph. (C) The expression of the anti-apoptotic protein Bcl-2 was quantified and displayed in a column graph. (D) The expression of cleaved-caspase-3 protein was quantified and displayed in a column graph (*P<0.05, ***P<0.001). Data are expressed as the mean ± standard deviation (n=3).

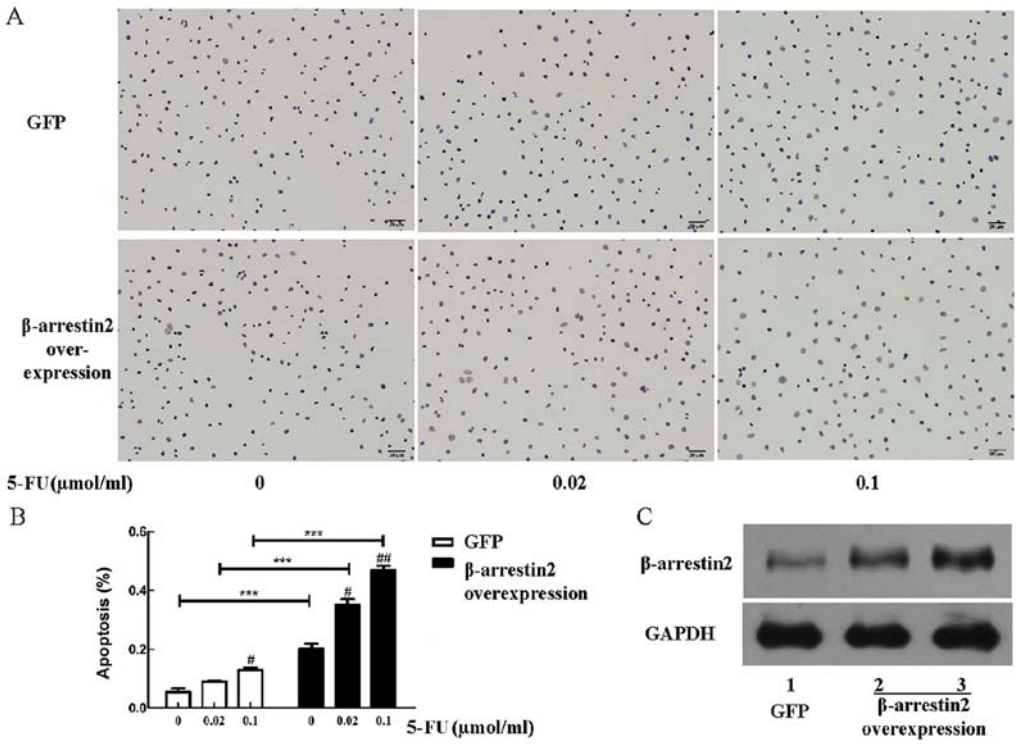

Figure 7. Apoptosis of human colon cancer HCT116 cells following transfection with the β-arrestin2 overexpression plasmid. (A) The 5-FU-induced apoptotic image as determined by TUNEL assay between the GFP control group and the β-arrestin2 overexpression group (magnification, x100). (B) The apoptotic proportion was quantified and displayed in a column graph. (C) The protein examination of β-arrestin2 overexpression: 1 represents the GFP control group; 2 represents the 24 h protein expression after transfection with the β-arrestin2 overexpression plasmid; 3 represents the 48 h protein expression after transfection with the β-arrestin2 overexpression plasmid (***P<0.001; β-arrestin2 overexpression group, 0 vs. 0.02 μmol/ml, $^{\#}$P<0.001; 0.02 vs. 0.1 μmol/ml, $^{\#\#}$P<0.001). Data are expressed as the mean ± standard deviation (n=3).

colon cancer cell line HCT116 was selected for the β-arrestin2 overexpression experiment. Briefly, apoptosis of HCT116 cells increased with the concentration of 5-FU and enhanced significantly after β-arrestin2 overexpression (Fig. 7A and B), indicating that the overexpression of β-arrestin2 plays an important part in cell apoptosis.

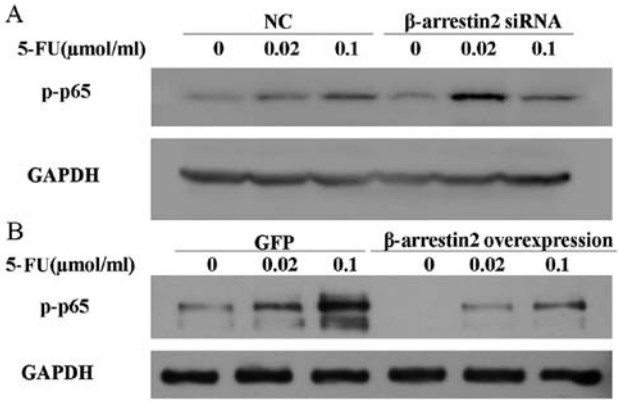

Figure 8. The p-p65 expression of human colon cancer cells following stimulation with 5-FU. (A) The 5-FU-induced p-p65 expression of NC group and β-arrestin2 siRNA group demonstrated by western blot analysis. (B) The 5-FU-induced p-p65 expression of GFP group and β-arrestin2 overexpression group demonstrated by western blot analysis.

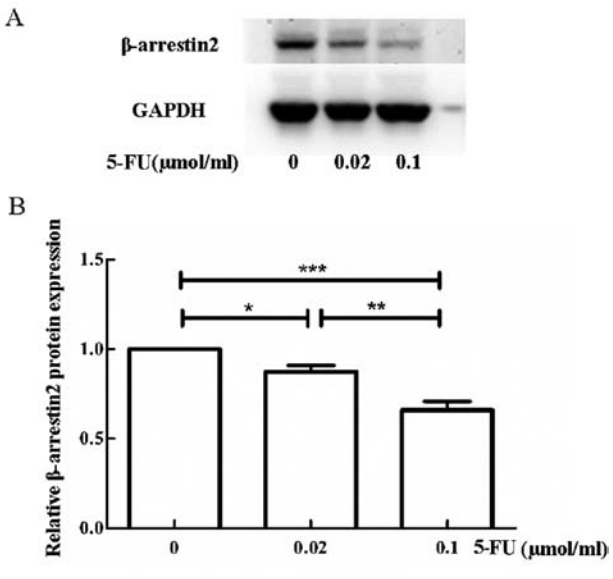

Figure 9. The expression of β-arrestin2 of human colon cancer LoVo cells stimulated with 5-FU. (A) The β-arrestin2 expression of human colon cancer LoVo cells stimulated with 5-FU demonstrated by western blot analysis. (B) The β-arrestin2 expression was quantified and displayed in a column graph (*P<0.05, **P<0.01, ***P<0.001). Data are expressed as the mean ± standard deviation (n=3).

β-arrestin2 inhibits the expression of p-p65. It has been proved that the NF-κB activation is associated with the development of chemoresistance to 5-FU in colon and breast cancer cells. In addition, β-arrestin2 is a negative element for the NF-κB activity. Therefore, we questioned whether the effect of β-arrestin2 on apoptosis depends on NF-κB activity. The expression of p-p65 was determined by western blot analysis after the downregulation and the overexpression of β-arrestin2. As displayed in Fig. 8, in the cells were β-arrestin2 was downregulated, the level of p-p65 increased after stimulation with 0.02 μmol/ml 5-FU, while it decreased in the cells were β-arrestin2 was overexpressed. Collectively, these data demonstrated that β-arrestin2 was involved in 5-FU-induced cell apoptosis in an NF-κB-dependent manner.

β-arrestin2 expression decreases with 5-FU stimulation. Since β-arrestin2 has a pro-apoptotic effect, we explored the β-arrestin2 expression of the LoVo cells after stimulation with 5-FU. As displayed in Fig. 9, β-arrestin2 expression of LoVo cells decreased with 5-FU stimulation.

Discussion

CRC is a common cancer with a high mortality rate especially in metastatic CRC (1,3). Although many different methods have been used for the treatment of CRC, 5-FU is considered the first line treatment of CRC. However, 5-FU has been shown to be effective only in 31% of cases when used as a sole drug for CRC. Understanding the mechanisms that lead to the unsatisfactory effect of CRC could be extremely beneficial in identifying effective therapies for CRC. The present study revealed that β-arrestin2 was essential in 5-FU-stimulated apoptotic responses, and it delineated the downstream biochemical pathways responsible for 5-FU-stimulated, β-arrestin2-mediated pro-apoptotic effect. Thereby, it revealed a novel potential mechanism for 5-FU effect mediated by β-arrestin2.

β-arrestin2, also known as arrestin3 and ARRB2, has been initially known for its ability to mediate the desensitization of GPCR signaling (19). Accordingly, it can interact with GPCR acting as a scaffold protein for the recruitment of many cytoplasmic signaling proteins, such as c-Src (5). β-arrestin2 can mediate agonist-induced signaling leading to the signaling pathway activation of ERK, MAPK and PI3K, and the inhibition of NF-κB (7,8). Thus, β-arrestin2 is involved in many cellular processes associated with cell proliferation, migration, invasion and apoptosis. Growing evidence indicates that by affecting cellular responses, β-arrestin2 has many additional effects that are related to cancer initiation and progression (9,10).

Many studies have confirmed that β-arrestin2 is abnormally expressed in cancer. Compared with normal tissues, β-arrestin2 is overexpressed in many cancers, such as breast cancer (20). However, the expression of β-arrestin2 decreases in hepatocellular cancer and non-small cell lung cancer (15,21). To confirm the role of β-arrestin2 in CRC, we first examined the expression of β-arrestin2 in CRC tissues and normal colon tissues. The obtained results demonstrated that β-arrestin2 expression in CRC was significantly increased compared with normal colon tissues, which means that β-arrestin2 may exert a different regulatory function in CRC. It has been established that low β-arrestin2 expression in hepatocellular cancer was correlated with aggressive pathological features, including advanced tumor stage, metastasis, poor cell differentiation and large tumor size (15). Nonetheless, its role in CRC is still not well understood. In contrast, β-arrestin2 overexpression is not associated with CRC clinicopathological features, such as tumor volume, TNM stage and CEA level. Furthermore, β-arrestin2 expression may serve as a prognosis indication. Lower β-arrestin2 expression indicates poor prognosis in NSCLC and hepatocellular cancer (15,21). We will follow the survival time of CRC patients in our next study. It will clarify the relation between the survival rate of CRC patients and β-arrestin2 expression. The role of β-arrestin2 in cancer is very distinct which could mean it is tissue-specific.

Although clinical data have not revealed a clear role of β-arrestin2 in CRC, we obtained colon cancer cells for *in vitro* experiments. β-arrestin2 has progressive and restrictive double functions in cell proliferation (11,13,14). β-arrestin2 promotes the proliferation of chronic myelogenous leukemia and castration-resistant prostate cancer, but inhibits lung cancer growth (13,14). However, in the present study the proliferation rate did not change following β-arrestin2 downregulation in CRC, which was in accordance with results previously reported by Bonnans *et al* (12), where β-arrestin2 depletion caused only 33% of the tumors in $Apc^{\Delta 14/+}$ mice while the tumor size revealed no alterations compared to the WT $Apc^{\Delta 14/+}$ mice (12). As for the effect of β-arrestin2 on metastasis, it suppresses the migration and invasion of hepatocellular cancer and lung cancer, but promotes breast cancer (14-16,20,22). In the present study, there were no differences in cell migration and invasion capacity after the β-arrestin2 downregulation, indicating that β-arrestin2 did not affect the proliferation, migration and invasion capacity of colon cancer cells. Each cancer has its specific mechanisms of initiation and development. β-arrestin2 is a multifunctional protein that is involved in many cellular responses and signal pathways (7,8). Accordingly, we postulated that the role of β-arrestin2 in cancer depends on the organizational specificity.

Accumulating evidence has uncovered the role of β-arrestin2 in apoptosis (23,24) and the dual effect of β-arrestin2 in apoptosis (24-27). Under different conditions, β-arrestin2 can be pro-apoptotic or anti-apoptotic (28-30). In the present study, 5-FU, as a core drug for CRC, was selected as a drug-interfering factor. Caspase-3 activation is a hallmark for apoptosis. We examined the activity of caspase-3 using caspase-3 activity assay kit and determined the cleaved-caspase-3, Bax and Bcl-2 expression by western blot analysis. Our results revealed that the activity of caspase-3 was suppressed, the expression of pro-apoptotic protein Bax and cleaved-caspase-3 was decreased and the anti-apoptotic protein Bcl-2 was increased after β-arrestin2 downregulation. Thus, these results indicated that β-arrestin2 downregulation prevented colon cancer cells from 5-FU-induced apoptosis. To obtain a better understanding of β-arrestin2 function on apoptosis, we overexpressed the expression of β-arrestin2 in human colon cancer cell line HCT116 and found that the cell apoptosis induced by 5-FU increased after β-arrestin2 overexpression. This indicated that β-arrestin2 promoted 5-FU-induced apoptosis in colon cancer cells. In a previous study by Zeng *et al* (29) it has been proved that β-arrestin2 promotes inflammation-induced epithelial apoptosis through ER stress/PUMA in colitis. Liu *et al* (17) demonstrated that β-arrestin2 deficiency was associated with radiation-induced intestinal crypt progenitor cell apoptosis through protracted NF-κB activation and suppression of PUMA. Consequently, we surmised that β-arrestin2 can be pro-apoptotic in the intestinal crypt cells. However, additional studies need to be performed in order to confirm this hypothesis.

We have proved that β-arrestin2 promoted 5-FU-induced apoptosis in colon cancer cells but the underlying mechanisms remain to be investigated. The activation of the NF-κB pathway has shown to be enhanced in a variety of cancers, including renal cancer, CRC and prostate cancer (31-33). It has been proved that NF-κB activation is associated with the development of chemoresistance to 5-FU in CRC and gastric cancer (34,35). Many chemotherapy drugs have been demonstrated to be more effective when combining the inhibition of the NF-κB pathway (36-39). β-arrestin2 has been proved to inhibit the NF-κB activation through its direct interaction with IκBα or by inhibiting TRAF6 self-ubiquitination (40,41). Accordingly, we postulated that β-arrestin2 promoted apoptosis by inhibiting the NF-κB activation. The increase of p-p65 following 5-FU stimulation in the LoVo and HCT116 cells is a firm proof of chemoresistance. As we expected, the level of p-p65 increased by 0.02 μmol/ml 5-FU stimulation after the β-arrestin2 downregulation in LoVo cells, and decreased after β-arrestin2 overexpression in HCT116 cells. Accordingly, we concluded that β-arrestin2 promoted apoptosis by inhibiting the NF-κB activation.

Furthermore, we noticed that the caspase-3 activity was not enhanced upon stimulation with 0.1 μmol/ml 5-FU compared with the 0 μmol/ml 5-FU stimulation. We also demonstrated that β-arrestin2 decreased with 5-fu stimulation. Based on these findings, we postulated that the expression of β-arrestin2 decreased to a low level following stimulation by 5-FU. Furthermore, β-arrestin2 in CRC can be indicative of sensitivity to chemotherapy and can even serve as a marker of prognosis, which could be proved by further studies.

In conclusion, our results demonstrated the role of β-arrestin2 in CRC. β-arrestin2 promoted 5-FU-induced apoptosis via the NF-κB pathway and could serve as a favorable prognostic biomarker for CRC.

Acknowledgements

We would like to thank Dr Tiezheng Sun for his technical support.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 812246) and the Peking University People's Hospital Research and Development Funds (no. 2118000542).

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YZ and YL conceived and designed the study. WR, TW, QZ, FL and FG performed the experiments. XH and JZ offered the technical support. WR wrote the manuscript. YZ reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were approved by the Human Ethics Review Board of the Peking University People's Hospital (Beijing, China).

Consent for publication

Written informed consents for publication of their clinical details were obtained from the patient or their parents.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD, Fedewa SA, Ahnen DJ, Meester RGS, Barzi A and Jemal A: Colorectal cancer statistics, 2017. CA Cancer J Clin 67: 177-193, 2017.
2. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2017. CA Cancer J Clin 67: 7-30, 2017.
3. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J: Cancer statistics in China, 2015. CA Cancer J Clin 66: 115-132, 2016.
4. Douaiher J, Ravipati A, Grams B, Chowdhury S, Alatise O and Are C: Colorectal cancer-global burden, trends, and geographical variations. J Surg Oncol 115: 619-630, 2017.
5. Lefkowitz RJ and Shenoy SK: Transduction of receptor signals by beta-arrestins. Science 308: 512-517, 2005.
6. Luttrell LM and Lefkowitz RJ: The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci 115: 455-465, 2002.
7. Lefkowitz RJ, Rajagopal K and Whalen EJ: New roles for beta-arrestins in cell signaling: Not just for seven-transmembrane receptors. Mol Cell 24: 643-652, 2006.
8. Barki-Harrington L and Rockman HA: Beta-arrestins: Multifunctional cellular mediators. Physiology 23: 17-22, 2008.
9. Sobolesky PM and Moussa O: The role of β-arrestins in cancer. Prog Mol Biol Transl Sci 118: 395-411, 2013.
10. Hu S, Wang D, Wu J, Jin J, Wei W and Sun W: Involvement of β-arrestins in cancer progression. Mol Biol Rep 40: 1065-1071, 2013.
11. Fereshteh M, Ito T, Kovacs JJ, Zhao C, Kwon HY, Tornini V, Konuma T, Chen M, Lefkowitz RJ and Reya T: β-Arrestin2 mediates the initiation and progression of myeloid leukemia. Proc Natl Acad Sci USA 109: 12532-12537, 2012.
12. Bonnans C, Flacelière M, Grillet F, Dantec C, Desvignes JP, Pannequin J, Severac D, Dubois E, Bibeau F, Escriou V, *et al*: Essential requirement for β-arrestin2 in mouse intestinal tumors with elevated Wnt signaling. Proc Natl Acad Sci USA 109: 3047-3052, 2012.
13. Duan X, Kong Z, Liu Y, Zeng Z, Li S, Wu W, Ji W, Yang B, Zhao Z and Zeng G: β-Arrestin2 contributes to cell viability and proliferation via the down-regulation of FOXO1 in castration-resistant prostate cancer. J Cell Physiol 230: 2371-2381, 2015.
14. Raghuwanshi SK, Nasser MW, Chen X, Strieter RM and Richardson RM: Depletion of beta-arrestin-2 promotes tumor growth and angiogenesis in a murine model of lung cancer. J Immunol 180: 5699-5706, 2008.
15. Sun WY, Hu SS, Wu JJ, Huang Q, Ma Y, Wang QT, Chen JY and Wei W: Down-regulation of β-arrestin2 promotes tumour invasion and indicates poor prognosis of hepatocellular carcinoma. Sci Rep 6: 35609, 2016.
16. Goertzen CG, Dragan M, Turley E, Babwah AV and Bhattacharya M: KISS1R signaling promotes invadopodia formation in human breast cancer cell via β-arrestin2/ERK. Cell Signal 28: 165-176, 2016.
17. Liu Z, Tian H, Jiang J, Yang Y, Tan S, Lin X, Liu H and Wu B: β-Arrestin-2 modulates radiation-induced intestinal crypt progenitor/stem cell injury. Cell Death Differ 23: 1529-1541, 2016.
18. Jing X, Zhang H, Hu J, Su P, Zhang W, Jia M, Cheng H, Li W and Zhou G: β-arrestin 2 is associated with multidrug resistance in breast cancer cells through regulating MDR1 gene expression. Int J Clin Exp Pathol 8: 1354-1363, 2015.
19. DeWire SM, Ahn S, Lefkowitz RJ and Shenoy SK: Beta-arrestins and cell signaling. Annu Rev Physiol 69: 483-510, 2007.
20. Li TT, Alemayehu M, Aziziyeh AI, Pape C, Pampillo M, Postovit LM, Mills GB, Babwah AV and Bhattacharya M: Beta-arrestin/Ral signaling regulates lysophosphatidic acid-mediated migration and invasion of human breast tumor cells. Mol Cancer Res 7: 1064-1077, 2009.
21. Wu Z, Tong W, Tan Z, Wang S and Lin P: The clinical significance of β-arrestin 2 expression in the serum of non-small cell lung cancer patients. Zhongguo Fei Ai Za Zhi 14: 497-501, 2011 (In Chinese).
22. Alemayehu M, Dragan M, Pape C, Siddiqui I, Sacks DB, Di Guglielmo GM, Babwah AV and Bhattacharya M: β-Arrestin2 regulates lysophosphatidic acid-induced human breast tumor cell migration and invasion via Rap1 and IQGAP1. PLoS One 8: e56174, 2013.
23. Sharma D and Parameswaran N: Multifaceted role of β-arrestins in inflammation and disease. Genes Immun 16: 499-513, 2015.
24. Revankar CM, Vines CM, Cimino DF and Prossnitz ER: Arrestins block G protein-coupled receptor-mediated apoptosis. J Biol Chem 279: 24578-24584, 2004.
25. Ahn S, Kim J, Hara MR, Ren XR and Lefkowitz RJ: {beta}-Arrestin-2 mediates anti-apoptotic signaling through regulation of BAD phosphorylation. J Biol Chem 284: 8855-8865, 2009.
26. Wang P, Gao H, Ni Y, Wang B, Wu Y, Ji L, Qin L, Ma L and Pei G: Beta-arrestin 2 functions as a G-protein-coupled receptor-activated regulator of oncoprotein Mdm2. J Biol Chem 278: 6363-6370, 2003.
27. Luan B, Zhang Z, Wu Y, Kang J and Pei G: Beta-arrestin2 functions as a phosphorylation-regulated suppressor of UV-induced NF-kappaB activation. EMBO J 24: 4237-4246, 2005.
28. Sun X, Zhang Y, Wang J, Wei L, Li H, Hanley G, Zhao M, Li Y and Yin D: Beta-arrestin 2 modulates resveratrol-induced apoptosis and regulation of Akt/GSK3β pathways. Biochim Biophys Acta 1800: 912-918, 2010.
29. Zeng LX, Tao J, Liu HL, Tan SW, Yang YD, Peng XJ, Liu ZH, Jiang J and Wu B: β-Arrestin2 encourages inflammation-induced epithelial apoptosis through ER stress/PUMA in colitis. Mucosal Immunol 8: 683-695, 2015.
30. Chang F, Liu J, Fu H, Wang J, Li F, Yue H, Li W, Zhao J and Yin D: GSK-3β promotes PA-induced apoptosis through changing β-arrestin 2 nucleus location in H9c2 cardiomyocytes. Apoptosis 21: 1045-1055, 2016.
31. Oya M, Takayanagi A, Horiguchi A, Mizuno R, Ohtsubo M, Marumo K, Shimizu N and Murai M: Increased nuclear factor-kappa B activation is related to the tumor development of renal cell carcinoma. Carcinogenesis 24: 377-384, 2003.
32. Lind DS, Hochwald SN, Malaty J, Rekkas S, Hebig P, Mishra G, Moldawer LL, Copeland EM III and Mackay S: Nuclear factor-kappa B is upregulated in colorectal cancer. Surgery 130: 363-369, 2001.
33. Lessard L, Mes-Masson AM, Lamarre L, Wall L, Lattouf JB and Saad F: NF-kappa B nuclear localization and its prognostic significance in prostate cancer. BJU Int 91: 417-420, 2003.
34. Camp ER, Li J, Minnich DJ, Brank A, Moldawer LL, MacKay SL and Hochwald SN: Inducible nuclear factor-kappaB activation contributes to chemotherapy resistance in gastric cancer. J Am Coll Surg 199: 249-258, 2004.
35. Körber MI, Klingenbrunner S, Bartsch R, Steger GG and Mader RM: NF-κB addiction and resistance to 5-fluorouracil in a multi-stage colon carcinoma model. Int J Clin Pharmacol Ther 51: 35-37, 2013.
36. Endo F, Nishizuka SS, Kume K, Ishida K, Katagiri H, Ishida K, Sato K, Iwaya T, Koeda K and Wakabayashi G: A compensatory role of NF-κB to p53 in response to 5-FU-based chemotherapy for gastric cancer cell lines. PLoS One 9: e90155, 2014.
37. Kwon OH, Kim JH, Kim SY and Kim YS: TWEAK/Fn14 signaling mediates gastric cancer cell resistance to 5-fluorouracil via NF-κB activation. Int J Oncol 44: 583-590, 2014.
38. Shakibaei M, Mobasheri A, Lueders C, Busch F, Shayan P and Goel A: Curcumin enhances the effect of chemotherapy against colorectal cancer cells by inhibition of NF-κB and Src protein kinase signaling pathways. PLoS One 8: e57218, 2013.
39. Xu B, Guo X, Mathew S, Armesilla AL, Cassidy J, Darling JL and Wang W: Triptolide simultaneously induces reactive oxygen species, inhibits NF-kappaB activity and sensitizes 5-fluorouracil in colorectal cancer cell lines. Cancer Lett 291: 200-208, 2010.
40. Gao H, Sun Y, Wu Y, Luan B, Wang Y, Qu B and Pei G: Identification of beta-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-kappaB pathways. Mol Cell 14: 303-317, 2004.
41. Wang Y, Tang Y, Teng L, Wu Y, Zhao X and Pei G: Association of beta-arrestin and TRAF6 negatively regulates Toll-like receptor-interleukin 1 receptor signaling. Nat Immunol 7: 139-147, 2006.