NDRG4 sensitzes CRC cells to 5-FU by upregulating DDIT3 expression

RUIKAI LI1,2*, CHENXIANG HE3*, LIANGLIANG SHEN2,4, SHUAI WANG1,2, YAO SHEN2,4, FAN FENG1,2, JIAN ZHANG2,4 and JIANYONG ZHENG1,2

1Department of Gastrointestinal Surgery, Xijing Hospital; 2State Key Laboratory of Cancer Biology, Fourth Military Medical University, Xi’an, Shaanxi 710032; 3Department of General Surgery, Shanghai Fourth People’s Hospital Affiliated to Tongji University of Medicine, Shanghai 200080; 4Department of Biochemistry and Molecular Biology, Fourth Military Medical University, Xi’an, Shaanxi 710032, P.R. China

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Abstract. The incidence of colorectal cancer (CRC) has remained high in recent years, and 5-fluorouracil (5-FU) is a vital chemotherapeutic agent for its treatment. Our previous study reported that N-mycc downstream-regulated gene 4 (NDRG4) plays a tumor-suppressive role in CRC, but the mechanisms associated with NDRG4 and 5-FU chemosensitivity remain unclear. The results of the present study demonstrate that NDRG4 sensitized CRC cells to 5-FU by upregulating DNA damage inducible transcript 3 (DDIT3). NDRG4 inhibited the proliferation of CRC cells and the activation of PI3K/AKT and ERK signaling. Furthermore, NDRG4 promoted CRC cell apoptosis induced by 5-FU. Mechanistic analyses revealed that NDRG4 upregulated DDIT3 expression, and that the proapoptotic effect of NDRG4 under 5-FU treatment conditions was dependent on DDIT3. These findings support the biological value of the association between NDRG4, DDIT3 and 5-FU chemosensitivity in CRC, and may advance the clinical treatment of CRC in the future.

Introduction

Colorectal cancer (CRC) is the third most frequently occurring cancer type worldwide, and with a high mortality rate, accounted for ~930,000 deaths in 2020 (1). Surgery remains the principal CRC treatment method to achieve complete resection of the primary tumor and metastatic lesions (2). However, in a large number of cases, complete resection is difficult. As such, minimizing tumor size and inhibiting further growth and proliferation are the primary aims for patients whose tumors cannot be completely removed, or those who are unable to tolerate surgery, for which chemotherapy and radiotherapy are the key treatment options (3). In addition, adjuvant chemotherapy has been used to extend the lifespan of patients with CRC (4).

5-Fluorouracil (5-FU) is a thymidylate synthase inhibitor that prevents the methylation of deoxyuridine acid to deoxythymidine acid, and with notable anticancer properties, was one of the first therapeutic drugs developed for clinical cancer treatment (5,6). Since 5-FU is widely utilized as a first-line treatment, the incidence of CRC resistance to 5-FU is gradually increasing (5). Therefore, strategies for enhancing the chemosensitivity of CRC cells to 5-FU are urgently required.

N-myc downstream-regulated gene 4 (NDRG4) belongs to the NDRG family, the members of which are expressed in a variety of human organs, and are associated with a wide range of biological processes, such as organ development, tumor inhibition, angiogenesis and growth regulation (7). NDRG4 plays a tumor-suppressive role in various cancer types, including pancreatic ductal and esophageal adenocarcinoma (8,9). In addition, hypermethylation of the NDRG4 promoter is associated with gastric cancer tumorigenesis, and is a predictor of poor prognosis in patients with the disease (10). NDRG4 also plays an important role in CRC, as molecular analysis of the NDRG4 promoter region in stool samples can be used to screen for CRC (11).

The DNA damage inducible transcript 3 (DDIT3) gene encodes a member of the CCAAT/enhancer binding protein transcription factor family. DDIT3 is primarily involved in apoptosis associated with endoplasmic reticulum (ER)
stress, as it enhances the biological function of the BH3-only protein BCL2 interacting mediator of cell death and inhibits the antiapoptotic function of BCL2 (12). In addition, DDIT3 can inhibit CRC by promoting the cell apoptosis (13). In terms of chemotherapeutic resistance, low expression levels of DDIT3 have been associated with the chemoresistance of lung cancer cells to cisplatin (14). The aim of the present study was to investigate the tumor-suppressive effect of NDRG4 in the SW480 and SW620 CRC cell lines, as well as whether NDRG4 enhanced the sensitivity of CRC cells to 5-FU, and the associated molecular mechanism. The results of the present study may indicate a novel mechanism that reflects the important role of NDRG4 in CRC inhibition.

Materials and methods

Cell lines. The SW480 and SW620 cell lines were purchased from Procell Life Science & Technology Co., Ltd., (cat. no. CL-0223 and CL-0225, respectively) and cultured in Leibovitz's L15 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) at 37°C (100% air). The 293T cell line was purchased from Procell Life Science & Technology Co., Ltd. (cat. no. CL-0005) and cultured in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO2. All cell lines were authenticated by STR authentication.

Stable transfection. The NDRG4 lentivirus and its lentiviral vector GV358, were purchased from Shanghai GeneChem Co., Ltd. SW480 and SW620 cells were infected with lentivirus at a multiplicity of infection (MOI) of 10 and 80 for 24 h, respectively. And the stably transduced cells were selected with 3 µg/ml puromycin (Beyotime Institute of Biotechnology) for 2 weeks. In subsequent experiments, puromycin was maintained at 0.5 µg/ml. Short hairpin (sh)RNAs targeting the DDIT3 gene (shRNA-1, 5'-GATCCCCGTGACC AACGATGAAACATCTCGAATGTCTGGTGG TGCAAGTTTTG-3'; shRNA-2, 5'-GATCTCGAGGCTC AACGAGGAAATCTCGAGATTCTCGAGG-3'), and non-targeting shRNA-negative control (shRNA-NC) 5'-GATCCCAAGGATGAAGAGCACC AACTCGAGTTGTTGCTCTTCATCTTGTGTGTTTG-3') were cloned into the lentiviral vector PLVshRNA-EGFP(2A) Puro by Inogenen Biotechnology Pvt. Ltd. According to the manufacturer's protocol, 4 µg lentiviral shRNA plasmids were mixed with packaging vector (PAX2 plasmid) and envelope vector (PMD2G plasmid) at the mass ratio of 4:3:1, and subsequently transfected into 293T cells using 20 µl Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, Inc.). Following incubation at 37°C with serum-free DMEM for 12 h, the 293T cells were changed to be cultured in complete DMEM at 37°C for 48 h. The lentivirus particles were subsequently collected and purified from the cell supernatants through a 0.45 µm filter, and meanwhile, the titer of the lentivirus was determined using the qPCR Lentivirus Titer Kit (Applied Biological Materials, Inc.). Then SW480 cells in the logarithmic growth phase were added to the lentivirus suspension (MOI=10) and incubated at 37°C for 48 h, after which the medium was discarded and the SW480 cells were cultivated with screening Leibovitz's L15 medium containing 3 µg/ml puromycin for 2 weeks to select the positive infected cells. In subsequent experiments, they were maintained with 0.5 µg/ml puromycin.

MTT assay. Cells were seeded into a 96-well plate with five biological replicates per group. Before detection, 20 µl MTT solution (Beyotime Institute of Biotechnology) was added to each well. The medium was replaced with 150 µl DMSO (Amresco, LLC) after 4 h at 37°C, and the plates were then shaken for 10 min. The OD values were determined at 570 nm using a microplate reader. To investigate the effect of NDRG4 on the proliferative ability of CRC cell lines, activity was detected at 0, 1, 2, 3 and 4 days of cell culture. To determine the effects of 5-FU on cell viability, different concentrations of 5-FU (5, 10, 20, 40 and 50 µg/ml) were added to cells in the logarithmic growth phase, and the absorbance was measured 48 h after treatment.

Western blotting. Cells were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology), and the protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). A total of 40 µg protein per lane was separated by 10% or 15% SDS-PAGE and transferred onto PVDF membranes. The PVDF membrane was then blocked with TBST (Twee-20 at 0.1%) containing 5% skim milk at room temperature for 1.5 h. The following primary antibodies were diluted 1:1,000 in Primary Antibody Dilution Buffer (Beyotime Institute of Biotechnology): anti-NDRG4 (monoclonal, rabbit anti-human; cat. no. 9039; Cell Signaling Technology, Inc.), anti-DDIT3 (monoclonal, mouse anti-human; cat. no. 2895; Cell Signaling Technology, Inc.), anti-p-AKT (monoclonal, rabbit anti-human; cat. no. 4060; Cell Signaling Technology, Inc.), anti-AKT (monoclonal, rabbit anti-human; cat. no. 4685; Cell Signaling Technology, Inc.), anti-p-ERK (monoclonal, rabbit anti-human; cat. no. ab4819; Abcam), anti-ERK (monoclonal, rabbit anti-human; cat. no. ab32053; Abcam), anti-cleaved caspase-3 (monoclonal, rabbit anti-human; cat. no. 9661; Cell Signaling Technology, Inc.), anti-PARP (monoclonal, rabbit anti-human; cat. no. 9532; Cell Signaling Technology, Inc.), and anti-β-actin (monoclonal, mouse anti-human; cat. no. D191047; Sangon Biotech, Co., Ltd.). After incubated by the primary antibody overnight at 4°C, the membrane was washed with TBST (Twee-20 at 0.1%) at room temperature three times for 10 min each. Appropriate secondary antibodies derived from the same species as the primary antibodies (anti-rabbit IgG, HRP-linked; cat. no. 7074; Cell Signaling Technology, Inc.; and anti-mouse IgG, HRP-linked; cat. no. 7076; Cell Signaling Technology, Inc.) were diluted at 1:5,000 and added to the membranes, incubated at room temperature for 1 h. After that, the membrane was washed with TBST three times for 10 min each, and then ECL bio-dura ECL Kit (Hangzhou Fude Biological Technology Co., Ltd.) was added for visualization. The blots were detected using the Tanon 550 Imaging System (Tanon Science and Technology Co., Ltd.).

Reverse transcription-quantitative (RT-q) PCR and PCR array. The RNeasy Mini kit (Qiagen, Inc.) was used to extract total cellular RNA, from which cDNA was then synthesized using
the PrimeScript RT-PCR kit (Takara Bio, Inc.) according to the manufacturers’ protocols. qPCR was performed using qPCR SYBR-Green Master Mix (Shanghai Yeasen Biotechnology Co., Ltd.) per the manufacturer’s protocol. The thermocycling conditions were as follows: Pre-denaturation at 95˚C for 5 min, then 40 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 20 sec and extension at 72˚C for 20 sec. Relative mRNA expression levels were determined using the 2−ΔΔCq method (15). The primer sequences were as follows: DDIT3 forward, 5’-GGAAACAGAGTGCTATCC-3’ and reverse, 5’-CTGCTTGAGGCGCTATCC-3’; CASP7 forward, 5’-AGTGACAGTATGCGCGTC-3’ and reverse, 5’-CGTCTTATCGTGCTCTT-3’; and β-actin forward, 5’-GGGATGGAGTCCG-3’ and reverse, 5’-TCTTGATGGGCTGCC-3’. For PCR array analysis, the extracted cDNA was used for with the real-time RT2 Profiler PCR Array (Qiagen, Inc.) according to the manufacturer’s protocol.

TdT-UTP nick end labeling (TUNEL) assay. TUNEL assays were performed using the One Step TUNEL Apoptosis Assay Kit (Beyotime Institute of Biotechnology) according to the manufacturer’s protocol. TUNEL regent was added to cells after 48 h of treatment with PBS or 5-FU. Each sample was observed by microscopy in five visual fields. Images of the cells were acquired using a fluorescence microscope.

Flow cytometry. Apoptosis assays were performed with cell lines using an Annexin V-FITC/PI apoptosis detection kit (Shanghai Yeasen Biotechnology Co., Ltd.) according to the manufacturer’s protocol. The experiments were conducted 48 h after treatment with PBS or 5-FU. Flow cytometry was performed using a FC500 Flow Cytometer (Beckman Coulter Co., Ltd.), and the data were analyzed using FlowJo 10 software (FlowJo LLC). Colony formation assay. Single cells (~200 per dish) were seeded into cell culture dishes with a diameter of 6 cm. After 18 days of culture at 37˚C, visible colonies (>50 cells per colony) were formed and the culture was terminated. The medium was replaced every 3 days during the culture period. The colonies were then washed, fixed with 4% paraformaldehyde (Beyotime Institute of Biotechnology) at room temperature for 15 min and stained with crystal violet (Sigma-Aldrich; Merck KGaA) at room temperature for 20 min. Finally, images of the stained colonies were captured, and the colonies were manually counted.

EdU staining. EdU staining was performed using the BeyoClick EdU Cell Proliferation Kit with Alexa Fluor 594 (cat. no. C0078S; Beyotime Institute of Biotechnology) according to the manufacturer’s protocol. Cells in the logarithmic growth phase (~24-48 h in culture) were used for detection. Images of the cells were captured using a fluorescence microscope.

Statistical analysis. All data are expressed as the mean ± SD. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc.). Student’s t-test was used to analyze two independent groups. For comparisons between multiple groups, ANOVA was applied; Sidak’s multiple comparisons test was used following two-way ANOVA, and Dunnett’s multiple comparisons test was used following one-way ANOVA. P<0.05 was considered to indicate a statistically significant difference.

Results

**NDRG4 inhibits the proliferation of CRC cells.** SW480 and SW620 CRC cells overexpressing NDRG4 were successfully constructed through lentiviral infection. The NDRG4 protein levels of the overexpression cells were notably higher than those of their control counterparts (Fig. 1A). The MTT assay results indicated that the proliferative capacity of NDRG4-overexpressing cells was significantly lower than that of the control cells on days 1-4 of cell culture (Fig. 1B). The colony formation assay revealed that the NDRG4-overexpressing cells formed fewer visible clones than the control cells (Fig. 1C). Furthermore, EdU analysis indicated that the proliferation of NDRG4-overexpressing cells was inhibited relative to that of the control cells (Fig. 1D). These results indicate that NDRG4 inhibited CRC cell proliferation.

**NDRG4 inhibits the activation of PI3K/AKT and ERK signaling.** Since both the PI3K/AKT and ERK signaling pathways are associated with cellular proliferation (16), the levels of AKT and ERK phosphorylation can be measured to reflect their degrees of activation. Western blotting revealed decreased levels of p-AKT and p-ERK in NDRG4-overexpressing cells (Fig. 1E), indicating that NDRG4 inhibited the activation of the PI3K/AKT and ERK signaling pathways.

**NDRG4 promotes 5-FU-induced CRC cell apoptosis.** MITT assays were used to assess cell viability after 48 h of culture with different concentrations of 5-FU. The overexpression of NDRG4 significantly decreased the viability of cells treated with 5-FU at five different concentrations (Fig. 2A). Moreover, the survival rates of NDRG4-overexpressing SW480 and SW620 cells were decreased most significantly following treatment with 40 and 10 µg/ml 5-FU, respectively. Therefore, subsequent experiments were carried out using these two concentrations of 5-FU. Flow cytometry was performed to detect apoptosis, which showed that NDRG4 overexpression increased the rates of SW480 and SW620 apoptosis induced by 5-FU, compared with those of the control cells; measurements were based on the percentage of annexin-V-positive cells (Q2 + Q3), and two-way ANOVA revealed that the interaction between NDRG4 and 5-FU was statistically significant (Fig. 2B). Subsequently, a TUNEL apoptosis assay was conducted, and the experimental results confirmed that NDRG4 overexpression increased the apoptotic rate induced by 5-FU, compared with that of control cells (Fig. 2C and D). Furthermore, expression of the apoptosis-associated molecules cleaved caspase-3 (C-caspase-3) and poly-ADP-ribose polymerase (PARP, the cleaved substrate of caspase) was also examined. The expression level of C-caspase-3 was increased in NDRG4-overexpressing cells compared with the control cells, indicating an increase in cellular apoptosis. After adding 5-FU, the expression of C-caspase-3 was notably increased, and the difference between NDRG4-overexpressing cells and control cells was
still apparent. Meanwhile, NDRG4 overexpression and 5-FU treatment also increased PARP expression (Fig. 2E). These results suggest that NDRG4 promoted the apoptosis of CRC cells induced by 5-FU.

**NDRG4 upregulates DDIT3 expression.** To investigate the molecular mechanism by which NDRG4 inhibits CRC cells, a PCR array experiment was conducted using SW480 cells to identify genes with considerable fold changes between NDRG4-overexpressing cells and control cells (Fig. 3A). Then, apoptosis-related genes, such as CASP7 and DDIT3, were selected for qPCR verification. DDIT3 exhibited the greatest differential expression between NDRG4-overexpressing cells and control cells (Fig. 3B). The western blot results also confirmed that DDIT3 was expressed at higher levels in NDRG4-overexpressing SW480 and SW620 cells than in their control counterparts (Fig. 3C). These results indicate that NDRG4 upregulated DDIT3 expression in CRC cells.

**Proapoptotic effect of NDRG4 under 5-FU treatment is dependent on DDIT3.** To further confirm whether the promotion effect of NDRG4 on 5-FU-induced CRC cell apoptosis was associated with the increase in DDIT3 expression, two shRNAs were designed to target the DDIT3 gene, and transfected into SW480 cells, which showed a successful decrease in DDIT3 mRNA expression (Fig. S1), and then into NDRG4-overexpressing SW480 cells to verify the gene silencing effect (Fig. 4A); further experiments were performed with shRNA-2, which exhibited the most prominent gene-silencing effect. The expression levels of the apoptosis-related proteins C-caspase-3 and PARP were decreased following DDIT3-knockdown in SW480 NDRG4-overexpressing cells (Fig. 4B). Subsequently, SW480 NDRG4-overexpressing cells were analyzed by flow cytometry, revealing that DDIT3-knockdown resulted in a decreased apoptotic rate compared with that of control cells, and that the
A decreasing trend was more apparent after the addition of 5-FU (Fig. 4C and D). These results indicate that DDIT3 plays an important role in the NDRG4-mediated promotion of CRC cell apoptosis induced by 5-FU.

Discussion

The incidence and morbidity rates of CRC are high worldwide (1), and surgery remains the primary and most effective treatment type (17). In addition, adjuvant chemotherapy has been widely utilized to improve the survival of patients with both International Union Against Cancer (UICC) stage III CRC and high-risk UICC stage II CRC (18). Despite progress in cancer treatment, with the implementation of novel chemotherapeutic agents such as afibercept, ramucirumab and bevacizumab, 5-FU remains one of the most effective and commonly used therapeutic drugs for CRC (19). Chemoresistance to anticancer agents is a major obstacle to attaining anticancer therapies with sufficient benefits (5), and the resistance of CRC to 5-FU is becoming increasingly prevalent.

Chemoresistance to 5-FU may be due to the disruption of 5-FU metabolic enzymes, drug transporters or crucial cellular activities, such as apoptosis and the cell cycle (20). For instance, Uppada et al. (21) revealed that MASTL induces chemoresistance in colon cancer by promoting Wnt/β-catenin signaling. Correspondingly, 5-FU sensitivity is influenced...
by a variety of genes and chemical substances, and CDGSH iron-sulfur domain-containing protein 2 reportedly augments the chemosensitivity of gastric cancer by enhancing 5-FU-induced apoptosis (22). Another study indicated that dichloroacetate enhanced the chemosensitivity of CRC to 5-FU through vital metabolic pathways mediated by miRNAs (23).

The NDRG family contains four members, NDRG1-4, which share 57-65% identity at the amino acid level, and contain an α/β hydrolase-fold region (24). The four members have multiple biological functions (7). NDRG4 was reported to be expressed primarily in cells of the nervous system, including enteric neurons, suggesting its involvement in CRC through the enteric neuron system, and its potential as an early detection marker for CRC (25). However, the expression profile of NDRG4 has not been unified. Since human stool contains exfoliated intestinal epithelial cells (26), detecting the abnormal expression of certain molecules in stool (including NDRG4) has been used to screen for CRC, suggesting that NDRG4 may not be exclusively expressed in the enteric neurons in the colorectum. Although NDRG4 plays a tumor-suppressive role in various cancer types, the mechanism is rarely studied. Our previous study identified NDRG4 as a prognostic predictor for patients with CRC, and as a novel candidate tumor suppressor (27,28). The present study revealed that NDRG4 inhibited the proliferation of SW480 and SW620 cells, which further confirmed the tumor-suppressive effect of NDRG4 in CRC. Interestingly, the effect of NDRG4 on chemosensitivity to 5-FU has not been previously reported.

In the present study, a series of experiments was conducted to determine whether NDRG4 enhanced the sensitivity of CRC cells to 5-FU. The inhibitory effect of 5-FU on CRC cells has been reported to be positively correlated with its concentration (29). In order to determine the concentration used in subsequent experiments, five different concentrations of 5-FU were initially evaluated for their effects on the viability of NDRG4-overexpressing cells and control cells. The optimal concentration of 5-FU in SW480 and SW620 cells was 40 and 10 µg/ml, respectively. The expression of apoptosis-related proteins was significantly increased in NDRG4-overexpressing cells compared with non-overexpressing cells when treated with 5-FU. However, the levels of NDRG4 were similar in NDRG4-overexpressing cells in the presence and absence of 5-FU, though the expression of apoptosis-related proteins was significantly higher in 5-FU treated NDRG4-overexpressing cells compared with untreated NDRG4-overexpressing cells (Fig. 2E). This suggests that 5-FU treatment did not affect the expression of NDRG4, but increased the expression of apoptosis-related proteins.
Lu et al (30) demonstrated that treatment with 5-FU significantly increased the expression levels of C-caspase-3 and PARP, which is consistent with the results of the present study. The reason for the increased expression of apoptotic proteins may be that 5-FU drives the expression of apoptosis pathway genes by inducing conformational changes in the chromatin regions containing binding motifs for activator protein-1 family transcription factors (31).

To the best of our knowledge, no other studies have explored the relationship between NDRG4 and DDIT3 in CRC. However, there are reports supporting the association between DDIT3 and chemosensitivity. For example, Tan et al (14) noted that increasing the expression of DDIT3 enhanced the sensitivity of lung cancer cells to cisplatin. Another study reported that decreased expression of DDIT3 was an important factor underlying the 5-FU resistance of rectal cancer resulting from the high expression of rhomboid domain containing 2 (32). These reports are consistent with the findings of the present study; apoptosis experiments showed that NDRG4 overexpression enhanced the 5-FU-induced apoptosis of CRC cells, which was significantly weakened by DDIT3-knockdown in NDRG4-overexpressing SW480 cells, indicating the importance of DDIT3 in the apoptosis pathway.

DDIT3 is principally involved in ER stress-related apoptosis (33), and NDRG4 promotes the expression of DDIT3, suggesting that ER stress is associated with the tumor-suppressive effect of NDRG4. In addition, Zhang et al (34) observed that low DDIT3 expression was associated with the poor prognosis of patients with advanced gastric cancer, for whom it was suggested as a potential prognostic marker. Furthermore, as our previous studies showed that NDRG4 was associated with the prognosis of CRC (27,28), DDIT3 may also be a prognostic biomarker for CRC.

Since SW480 cells are a classic CRC cell line with proliferative, invasive, migratory and tumorigenic characteristics, they are a commonly used model for the study of CRC in vitro. SW480 cells were primarily used in the present study, with partial verification studies conducted using the SW620 cell line. Therefore, PCR-array and DDIT3-knockdown experiments were not performed in SW620 cells, which is a study limitation. Organoids can simulate various real-organ characteristics, and are important models for studying disease (35). Considering that the enteric nervous system may play an important role in the function of NDRG4 (25), the use of intestinal organoids co-cultured with enteric neurons may be a future research prospect, along with the relevant molecular biological experiments, so as to further investigate the upstream and downstream molecular pathways of NDRG4. Of note, cell function and animal experiments were also not performed, and additional experiments, such as transwell assays and subcutaneous tumor-bearing experiments in athymic mice and tumor-specific patient-derived xenograft (PDX) models, will be a future consideration to further elucidate how NDRG4/DDIT3 regulates CRC cell responses to 5-FU treatment in vivo and in vitro.

In conclusion, the present study revealed that NDRG4 increased the chemosensitivity of CRC cells to 5-FU by increasing the expression of DDIT3, though the underlying mechanisms require further study in the future.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

Authors’ contributions
JYZ and JZ conceived and designed the study and revised the manuscript. RKL and CXH performed most of the experiments and analyzed the data. RKL drafted the initial manuscript. LLS, SW and YS performed the MTT and TUNEL assays and analyzed the data. PF designed part of the study, analyzed the data and revised the manuscript. JZ and CXH confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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