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SNHG20/miR-140-5p/NDRG3 axis contributes to 5-fluorouracil resistance in gastric cancer

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Abstract. 5-fluorouracil (5-FU)-based chemotherapy is the first line treatment for advanced gastric cancer. However, the effectiveness of 5-FU is limited by drug resistance. The N-myc downstream-regulated gene, family member 3 (NDRG3) is a member of the NDRG family and has been implicated in numerous types of cancer. However, the role of NDRG3 in gastric cancer remains unclear. In the present study, NDRG3 mRNA expression in gastric cancer and adjacent normal tissues was analyzed using the Gene Expression Profiling Interactive Analysis web tool. NDRG3 expression was silenced using short hairpin RNAs to examine the effect of NDRG3 on the growth of gastric cancer cells. Potential regulators of NDRG3 were identified using the TargetScan and MicroRNA tools and verified by a luciferase assay and reverse transcription-quantitative PCR analysis. The current study demonstrated that NDRG3 was upregulated in gastric cancer specimens and promoted cell proliferation in gastric cancer cell lines. Furthermore, the present study revealed that the small nucleolar RNA host gene 20 (SNHG20)/microRNA (miR)-140-5p signaling pathway may regulate the expression of NDRG3. SNHG20 was revealed to be involved in mediating resistance to 5-FU in gastric cancer cell lines via NDRG3. In conclusion, the results of the present study suggest that the SNHG20/miR-140-5p/NDRG3 axis may be involved in mediating resistance to 5-FU in gastric cancer.

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Introduction

Gastric cancer has the sixth highest incidence of cancer worldwide (1). Surgical resection can be effective for the treatment of patients with early gastric cancer (2). However, ~50% of patients are diagnosed with advanced-stage disease; at which point surgical treatment alone is not effective (2). Therefore, chemotherapy is one of the most common treatment options for these cancer patients (3). 5-Fluorouracil (5-FU)-based chemotherapy is the first line treatment for advanced gastric cancer; however, its effectiveness is limited by drug resistance (3). Therefore, there is a requirement for elucidating the molecular mechanisms underlying resistance to 5-FU, as well as the development of novel treatment strategies, for patients with advanced gastric cancer.

The N-myc downstream-regulated gene (NDRG) protein family includes 4 members, NDRG1, NDRG2, NDRG3 and NDRG4. NDRG proteins participate in multiple cellular processes, including cell proliferation, differentiation and the stress response (4). In addition, NDRG family members are tumor-associated proteins and their dysregulation may result in tumorigenesis (5). NDRG3 has been reported to promote colorectal cancer metastasis by activating Src phosphorylation (6), and is associated with poor survival in non-small cell lung cancer (7). However, the role of NDRG3 and regulatory mechanisms of NDRG3 in gastric cancer remain unclear.

The present study investigates the role of NDRG3 in gastric cancer. The clinical relevance of NDRG3 in gastric cancer was examined and it was revealed that NDRG3 was upregulated in tumor tissues obtained from patients with gastric cancer. The biological role of NDRG3 in gastric cancer cell lines was investigated and the results demonstrated that NDRG3 increased the proliferation of these cells. The long non-coding (lncRNA) small nucleolar RNA host gene 20 (SNHG20)/microRNA (miR)-140-5p signaling pathway was subsequently identified to regulate the expression of NDRG3. The SNHG20/miR-140-5p/NDRG3 axis was revealed to be implicated in resistance to 5-FU in gastric cancer cell lines, and may therefore present a potential therapeutic target for overcoming 5-FU-associated drug resistance in gastric cancer.

Materials and methods

Cell culture. The gastric cancer cell lines BGC-823 (cat. no. C023) and AGS (cat. no. C015) were purchased from Wuhan Fine Biotech Co., Ltd. All cells were cultured in RPMI-1640 medium (cat. no. 11875093; Thermo Fisher Scientific, Inc.) containing 5% fetal bovine serum (Thermo Fisher Scientific, Inc.). The cells were maintained in a humidified atmosphere at 37˚C and 5% CO_2.

Cell transfection. The miR-140-5p mimic and miR-inhibitor (anti-miR-140-5p) used in this study were purchased from Shanghai Gene Pharma Co., Ltd. The sequences were as follows: miR mimic negative control (miR-NC), 5'-UUCUCCGAACGUGUCAACGUTT-3'; miR-140-5p mimic, 5'-CAGUGGUUUUACCCUAUGGUAGACCAUAGGGUAAAACCACUGUU-3; anti-miR-NC, 5'-CAGUACUUUGUGUAGUACAA-3'; anti-miR-140-5p, 5'-AACCCAUGGAAUUCAGUUCUCA-3'. The small interfering (si)RNA sequences used in this study were purchased from Shanghai Gene Pharma Co., Ltd., and their sequences were as follows: si-NC, 5'-CACTGACGGTGACCAGAACAAAGAT-3'; si-SNHG20, 5'-GAAUCGAUAGGUCGAGGGGTT-3'. AGS and BGC-823 cells were transfected with miR-140-5p mimic or inhibitor and their respective negative controls (100 nM), si-NC (50 nM) or si-SNHG20 (50 nM) sequences using a lipid-based method (Lipofectamine® 2000; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Gene and protein expression analyses were performed at 48 h following transfection.

Lentivirus-based negative control and gene-specific short hairpin (sh)RNAs were purchased from Sigma-Aldrich; Merck KGaA. Transfections were performed using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.). A total of 2 µg gene-specific shRNA or shControl were transfected into 293T cells ($5x10^5$ cells; cat. no. C004; Wuhan Fine Biotech Co., Ltd.). At 48 h following transfection, the culture medium of 293T cells was collected (5,000 viral particles/µl) and 10 µl viral medium was applied to 100,000 BGC-823 and AGS gastric cancer cells to get a multiplicity of infection of 0.5. Gastric cancer cells were cultured in 5% CO_2 at 37˚C for 48 h, before puromycin (0.75 µg/ml; Sigma-Aldrich; Merck KGaA) was added. Cells were collected at 72 h post-transfection. The knockdown efficiency was confirmed using western blotting analysis. The shRNA sequences used were as follows: shControl, 5'-CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG-3'; shNDRG3#1, 5'-CCGGCCACTCCATAATATAACATTTCTCGAGAAATGTTATATTATGGAGTGGTTTTTG-3'; shNDRG3#2, 5'-CCGGTTCCCGCCTGAACCCTATAAACTCGAGTTTATAGGGTTCAGGCGGGAATTTTTG-3'.

NDRG3 expression and correlation analysis using the Gene Expression Profiling Interactive Analysis (GEPIA) web tool. The GEPIA (gepia.cancerpku.cn/index.html) (8) online database was used to analyze RNA sequencing expression data downloaded from the The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov/) and the Genotype-Tissue Expression (GTEx; http://www.gtexportal.org/home/) projects. GEPIA performed NDRG3 expression analysis based on gene expression levels; NDRG3 expression was compared between gastric cancer (n=408) and normal stomach tissues (n=211). Tboxplot analysis used log2 (transcripts per million + 1) for the log-scale, and was conducted by the GEPIA web tool. In addition, GEPIA performed pairwise gene correlation analysis for gastric cancer tissue samples vs. normal gastric tissue samples of TCGA and/or GTEx expression data using Pearson's correlation test.

Western blotting. Total protein was extracted from BGC-823 and AGS gastric cancer cells using Cell Lysis Buffer for Western and Immunoprecipitation (cat. no. P0013; Beyotime Institute of Biotechnology) and centrifuged at 10,000 x g for 5 min at 4˚C. Protein concentrations were determined using a bicinchoninic acid assay. Proteins were denatured at 100˚C for 5 min in sample buffer. An equal amount of protein (60 µg/lane) for each sample was separated by 6-10% SDS-PAGE and transferred to nitrocellulose membranes. Subsequently, membranes were incubated with primary antibody overnight at 4˚C. The membranes were then washed with 1X Tris-buffered saline and Tween 20 and incubated with anti-rabbit immunoglobulin G (IgG; cat. no. MR-R100; 1:3,000 dilution; Shanghai MRbiotech, Co., Ltd.) and anti-mouse IgG (cat. no. MR-M100; 1:3,000 dilution; Shanghai MRbiotech, Co., Ltd.) horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The protein bands were visualized by chemiluminescence using SuperSignal West Pico Stable Peroxide solution (Thermo Fisher Scientific, Inc.). Image J software (ImageJ bundled with Java version 1.8.0_112; National Institutes of Health) was used for semi-quantification of protein expression levels. The primary antibodies used were purchased from Santa Cruz Biotechnology, Inc., and were as follows: Anti-NDRG3 (dilution, 1:1,000; cat. no. sc-514561) and anti-GAPDH (dilution, 1:5,000; cat. no. sc-47724).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cultured cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.). A total of 2 µg RNA was reverse transcribed into cDNA using a cDNA reverse transcription kit (PrimeScript™ RT reagent kit; cat. no. RR037A; Takara Bio, Inc.) according to the manufacturer's protocol. qPCR analysis was performed using a PCR kit (TB Green™ Fast qPCR Mix; cat. no. RR430A; Takara Bio, Inc.) according to the manufacturer's protocol. The two kits were purchased from Takara Bio Inc. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 60 sec; denaturation at 95˚C for 20 sec, annealing at 58˚C for 30 sec and extension at 72˚C for 30 sec (43 cycles); and melting curve at 65-95˚C with increments of 0.5˚C for 5 sec. The following primer pairs were used for qPCR: NDRG3 forward, 5'-GCAGCTTCCAAACTCTCTGG-3' and reverse, 5'-AGCTGCAGGTTGTCTTGGTT-3'; and GAPDH forward, 5'-TGTGGTCATGAGTCCTTCCA-3' and reverse, 5'-CGAGATCCCTCCAAAATCAA-3'. NDRG3 mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method (9) and normalized to the internal reference gene GAPDH.

Luciferase assay. Potential regulators of NDRG3 were identified using the TargetScan (version 7.2; http://www.targetscan.org/vert_72/) and MicroRNA (August 2010 Release; http://www.microrna.org/microrna/home.do) tools according

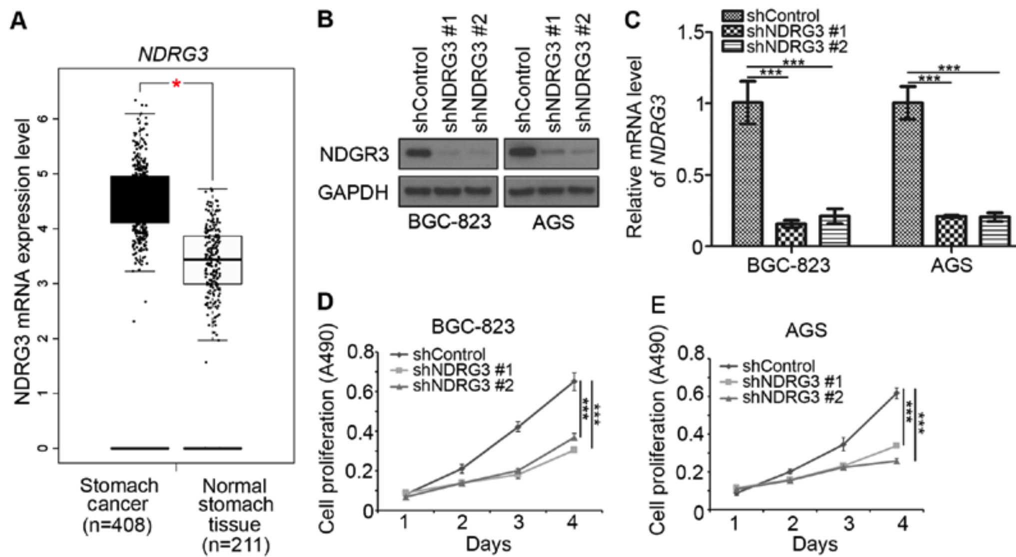

Figure 1. Knockdown of NDRG3 inhibits gastric cancer proliferation. (A) The Gene Expression Profiling Interactive Analysis database revealed that NDRG3 expression was significantly increased in gastric cancer tissues (n=408) compared with normal stomach tissue (n=211). Tboxplot analysis used log2 (transcripts per million + 1) for log-scale. BGC-823 and AGS cells were infected with lentivirus expressing control and NDRG3-specific shRNAs. A total of 48 h following infection, cells were harvested for (B) western blot analysis and (C) reverse transcription-quantitative polymerase chain reaction analysis. A cell proliferation assay for (D) BGC-823 and (E) AGS cells was performed. Data are presented as the mean ± standard deviation of three replicates. *P<0.05 and ***P<0.001, as indicated. NDRG3, N-myc downstream regulated gene, family member 3; sh, short hairpin; A, absorbance.

to the protocols of these web tools. A miR-140-5p recognition site in the 3'-untranslated region (UTR) of NDRG3 was identified The NDRG3 3'-UTR containing the putative miR-140-5p binding site was cloned into a pMIR-REPORT plasmid (Promega Corporation) to construct the reporter vector, pMIR-NDRG3-WT. The GeneArt™ Site-Directed Mutagenesis system (Thermo Fisher Scientific, Inc.) was used to the produce mutant-type NDRG3 reporter (NDRG3-Mut). The NDRG3-WT (50 nM) and Mut (50 nM) reporters were co-transfected with miR-140-5p mimics (100 nM) into the BGC-823 and AGS gastric cancer cells using a lipid-based method (Lipofectamine® 2000; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. At 24 h post-infection, luciferase activities were determined using a dual-luciferase assay system (Promega Corporation) according to the manufacturer's protocol. The red firefly luciferase signal was used as a normalization control.

MTS assay. Cell viability was determined using a colorimetric MTS assay kit (Abcam) according to the manufacturer's protocol. Briefly, 10,000 cells/wells were plated into the 96-well plates. At 48 h after infection, cells were treated with increasing concentrations (0, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80 and 160 μg/ml) of 5-FU (cat. no. HY-90006; MedChemExpress) for a further 24 h at 37˚C. MTS reagent was added to each well of the 96-well plate [cells not treated with 5-FU were treated with a vehicle (dimethylsulfoxide)]. After 60 min of incubation at 37˚C in a cell incubator, the absorbance at a wavelength of 490 nm was measured using a plate reader.

Statistical analysis. Microsoft Excel software (Microsoft Excel 2013 version 15.19.1; Microsoft Corporation) was used for statistical analysis. A two-sided paired Student's t-test was used for comparisons between two groups, and one-way analysis of variance followed by a Tukey's multiple comparisons test was used for comparing multiple groups. All values were expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Knockdown of NDRG3 inhibits gastric cancer proliferation. Since aberrant expression of NDRG3 promotes colorectal cancer metastasis and is associated with poor survival in non-small cell lung cancer (7), the role of NDRG3 in gastric cancer was investigated in the current study. The expression of NDRG3 mRNA in gastric cancer and adjacent normal gastric tissues was analyzed using the GEPIA web tool (8). The mRNA level of NDRG3 in gastric cancer was significantly increased when compared with adjacent normal gastric tissues (Fig. 1A). Therefore, NDRG3 may serve a role in gastric cancer. To examine the effect of NDRG3 on the growth of gastric cancer cells, NDRG3 expression was silenced using two different shRNAs (shNDRG3-1 and shNDRG3-2) in BGC-823 and AGS gastric cancer cells (Fig. 1B and C). Knockdown of NDRG3 significantly decreased the cell growth rate of BGC-823 and AGS cells when compared with controls (Fig. 1D and E). The two different shRNAs (shNDRG3-1 and shNDRG3-2) could knockdown NDRG3 effectively, and shNDRG3-1 was used in subsequent experiments. Together, these data suggest that NDRG3 may be an important protein in gastric cancer.

miR-140-5p inhibits NDRG3 expression in gastric cancer cells. Potential regulators of NDRG3 were identified using the TargetScan and MicroRNA tools according to the protocols of these web tools. A miR-140-5p recognition site in the 3'-UTR of NDRG3 was identified (Fig. 2A). Overexpression and knockdown of miR-140-5p in BGC-823 and AGS cells were examined by RT-qPCR analysis (Fig. 2B). The interaction between NDRG3 and miR-140-5p was examined using

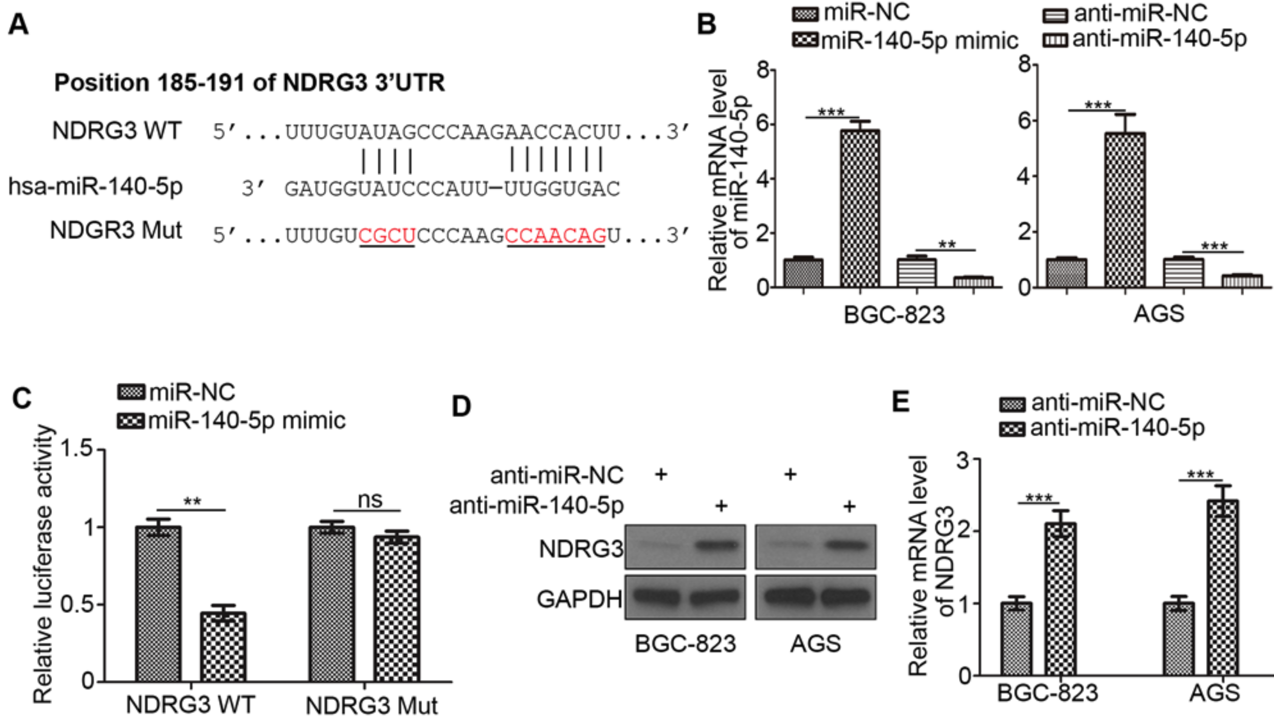

Figure 2. miR-140-5p inhibits NDRG3 expression in gastric cancer cells. (A) The predicted position of miR-140-5p binding sites on the NDRG3 3'-UTR. BGC-823 cells were transfected with NDRG3 (WT) or NDRG3 (Mut) reporters and miR-NC or miR-140-5p mimics. (B) BGC-823 and AGS cells were transfected with miR-NC, miR-140-5p mimic, anti-miR-NC or anti-miR-140-5p. At 48 h post-transfection, cells were harvested for RT-qPCR analysis. (C) A total of 48 h following transfection of BGC-823 cells, the relative luciferase activity was detected using a dual-luciferase reporter assay. BGC-823 and AGS cells were transfected with the indicated constructs. A total of 48 h following transfection, cells were harvested for (D) western blotting and (E) reverse transcription-quantitative polymerase chain reaction analysis. Data are presented as the mean ± standard deviation of three replicates. **P<0.01 and ***P<0.001, as indicated. miR, microRNA; NDRG3, N-myc downstream regulated gene, family member 3; UTR, untranslated region; WT, wild type; Mut, mutant; NC, negative control; ns, not significant.

a luciferase assay in BGC-823 cells (Fig. 2C). The expression of NDRG3 was increased following treatment with anti-miR-140-5p in BGC-823 and AGS gastric cancer cells when compared with the controls (Fig. 2D and E). These results suggest that the expression of NDRG3 in gastric cancer may be regulated by miR-140-5p.

SNHG20 regulates NDRG3 expression via miR-140-5p in gastric cancer cells. The SNHG20 lncRNA has been demonstrated to interact with miR-140-5p and decrease its expression in cervical cancer (10). In addition, SNHG20 promotes gastric cancer progression by inhibiting the expression of cyclin dependent kinase inhibitor 1A (CDKN1A) and by regulating the glycogen synthase kinase-3β/β-catenin signaling pathway (11). To investigate the role of SNHG20 in gastric cancer further, SNHG20 expression was silenced in BGC-823 and AGS gastric cancer cell lines in the current study. The expression of NDRG3 was decreased following SNHG20 knockdown when compared with the controls (Fig. 3A and B). In addition, the correlation between NDRG3 and SNHG20 mRNA levels in clinical specimens derived from patients with gastric cancer was analyzed using the GEPIA web tool. Consistent with the results observed in BGC-823 and AGS cells, NDRG3 was positively correlated with SNHG20 ($P=4\times10^{-10}$, r=0.3; Fig. 3C). SNHG20 knockdown downregulated the expression of NDRG3 in BGC-823 and AGS cells and this effect was reduced following transfection of anti-miR-140-5p (Fig. 3D). Taken together, these results suggest that the SNHG20/miR-140-5p axis may regulate the expression of NDRG3 in gastric cancer.

SNHG20 contributes to 5-FU resistance in gastric cancer cells by regulating the expression of NDRG3. The present study investigated whether the SNHG20/NDRG3 axis may be responsible for mediating resistance to 5-FU in gastric cancer. The cell proliferation rate following exposure to different concentrations of 5-FU following NDRG3 knockdown in BGC-823 and AGS cells was first measured (Fig. 4A and B). The half maximal inhibitory concentration of 5-FU was decreased following knockdown of NDRG3 in BGC-823 and AGS gastric cancer cells, and the viability of cells in the shNDRG3 group was significantly decreased when compared with that of the shControl group at a concentration of 5 μg/ml (Fig. 4A and B). Since Shen *et al* (12) revealed that after 3 days of treatment with 5-FU the viability of BGC-823 and AGS cells is significantly decreased in the 5-FU group compared with the control group, the difference in viability of cells transfected with shControl and shNDRG3 was then compared at the same concentration of 5-FU on day 3 (Fig. 4C and D). Knockdown of NDRG3 significantly enhanced the sensitivity of gastric cancer cell lines to 5-FU treatment. Knockdown of SNHG20 increased the sensitivity of the gastric cancer cell lines to 5-FU treatment (Fig. 4C and D). Co-knockdown of SNHG20 and NDRG3 did not further increase the sensitivity to 5-FU compared with knockdown of either SNHG20 or

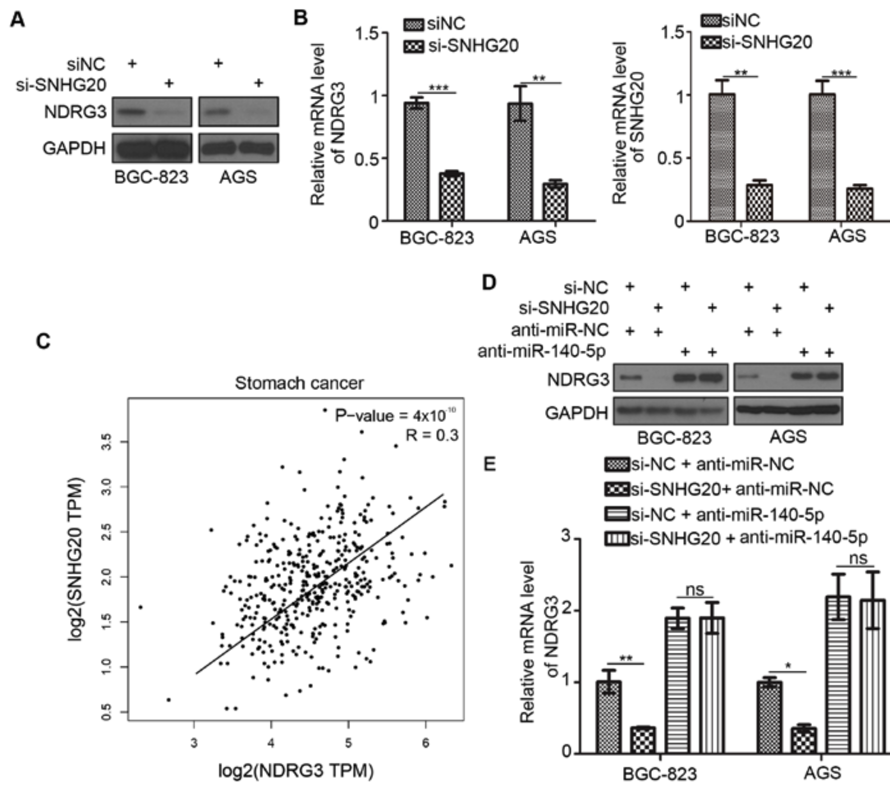

Figure 3. SNHG20 regulates NDRG3 expression via miR-140-5p in gastric cancer cells. BGC-823 and AGS cells were transfected with the indicated constructs. A total of 48 h following transfection, cells were harvested for (A) western blotting and (B) RT-qPCR analyses. (C) The Gene Expression Profiling Interactive Analysis web tool was used to determine the correlation between the mRNA expression levels of NDRG3 and SNHG20 among patients with gastric cancer. BGC-823 and AGS cells were transfected with indicated constructs. 48 h post transfection, cells were harvested for (D) western blotting and (E) RT-qPCR analyses. Data are presented as the mean ± standard deviation of three replicates. *P<0.05, **P<0.01 and ***P<0.001, as indicated. SNHG20; small nucleolar RNA host gene 20; NDRG3, N-myc downstream regulated gene, family member 3; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR, microRNA; ns, not significant; NC, negative control; TPM, transcripts per million.

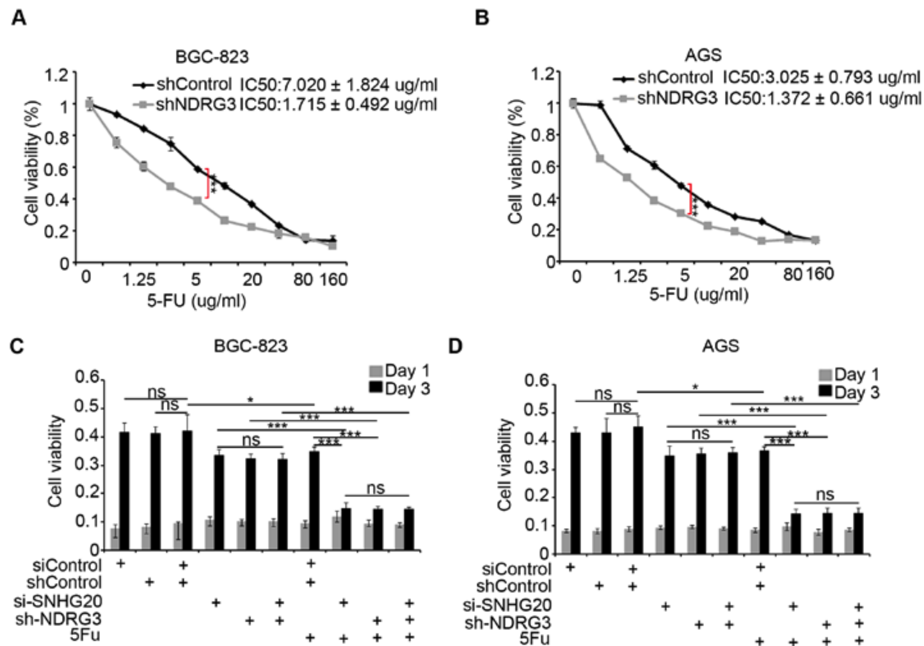

Figure 4. SNHG20 may be involved in mediating resistance to 5-FU in gastric cancer cells via NDRG3. (A) BGC-823 and (B) AGS cells were infected with lentivirus expressing control, NDRG3-specific shRNAs. A total of 48 h following infection, cells were treated with increasing concentrations of 5-FU for a further 24 h. Cells were subsequently harvested for the MTS assay. The cell viability was compared at the concentration of 5 μg/ml in the two gastric cancer cell lines. (C) BGC-823 and (D) AGS cells were transfected with indicated constructs. A total of 48 h following transfection, cells were treated with or without 2.5 μg/ml of 5-FU. Cell viability was measured using the MTS assay. Data presented as the mean ± standard deviation from five replicates; *P<0.05 and ***P<0.001, as indicated. SNHG20; small nucleolar RNA host gene 20; 5-FU, 5-fluorouracil; NDRG3, N-myc downstream regulated gene, family member 3; shRNA, short hairpin RNA; IC_{50}, half maximal inhibitory concentration; ns, not significant.

NDRG3 alone in the BGC-823 and AGS cells (Fig. 4C and D). The viability of cells in the co-knockdown group was decreased when compared with the control group in the two gastric cancer cell lines (Fig. 4C and D). Together, these data indicated that SNHG20/NDRG3 axis may contribute to 5-FU resistance in gastric cancer.

Discussion

The NDRG family serves important roles the proliferation, invasion and metastasis of cells in solid tumors, including breast, colon and prostate cancer (13,14). However, the specific role of NDRG3 in gastric cancer remains unclear. The results of the present study revealed that NDRG3 may function as a tumor growth promoting protein in gastric cancer. NDRG3 was previously reported to mediate drug resistance in hepatocellular cancer (15). Consistent with this aforementioned study, the results of the present study suggest that NDRG3 may be involved in the development of 5-FU resistance in gastric cancer.

lncRNAs are non-coding transcripts consisting of >200 nucleotides (16). Aberrant expression of lncRNAs is common in several types of cancer, including gastric cancer, breast cancer and osteosarcoma, and may be involved in chromatin remodeling and transcriptional and post-transcriptional regulation (16-18). As such, lncRNAs serve important roles in regulating the tumor cell growth, metastasis and drug resistance of tumors (19). SNHG20 has been identified as an oncogenic RNA in numerous types of cancer, including cervical (10), breast (18) and colorectal cancer (20). SNHG20 decreased the expression of CDKN1A and increased the proliferation and decreased apoptosis of non-small cell lung cancer (21) and gastric cancer cells (22). The present study demonstrated that SNHG20 increased the expression level of NDRG3 and that it may be involved in mediating resistance to 5-FU in gastric cancer cell lines, indicating a novel role of SNHG20 in tumorigenesis.

In conclusion, the present study revealed that NDRG3 was upregulated in gastric cancer samples compared with normal healthy controls, and promoted the proliferation of gastric cancer cell lines. Furthermore, the current study demonstrated that the SNHG20/miR-140-5p signaling pathway may regulate the expression of NDRG3. As different types of cancer may develop resistance to traditional chemotherapies (23), future studies investigating the mechanisms underlying drug resistance may promote the development of novel treatment strategies. The current study also revealed that the SNHG20/miR-140-5p/NDRG3 axis may serve an important role in mediating resistance to 5-FU in gastric cancer. Therefore, therapeutic targeting of this axis may present a novel treatment strategy to overcome 5-FU resistance in gastric cancer.

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Availability of data and materials

The datasets generated/analyzed in the present study are available upon reasonable request from the corresponding author.

Authors' contributions

JY, JS and XQ performed the experiments and wrote the manuscript. LC, ZY, HY, CX, QZ and PW collected data. ZG wrote the manuscript and analyzed the data.

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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