Knockdown of circNRIP1 sensitizes colorectal cancer to 5-FU via sponging miR-532-3p

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Received May 4, 2021; Accepted July 29, 2021

DOI: 10.3892/or.2021.8169

Abstract. The present study aimed to investigate the influence of circular RNA nuclear receptor-interacting protein 1 (circNRIP1) on the chemotherapeutic effect of 5-fluorouracil (5-FU) in colorectal cancer (CRC) and reveal its potential molecular mechanisms. The effects of circNRIP1 on cell proliferation, migration and invasion, and apoptosis were evaluated using Cell Counting Kit-8, Transwell and flow cytometric assays, respectively. A dual-luciferase reporter assay was performed to verify the potential interaction between circNRIP1 and microRNA (miR)-532-3p. The results of the present study indicated that circNRIP1 was upregulated in CRC and its increased expression was associated with CRC progression. Furthermore, overexpression of circNRIP1 promoted CRC cell proliferation, invasion and migration, while it inhibited apoptosis. Knockdown of circNRIP1 significantly enhanced the 5-FU-induced inhibition of the viability of HCT116 and SW480 cells. Bioinformatics analysis predicted that miR-532-3p was a direct target of circNRIP1, which was further confirmed by a dual-luciferase reporter assay. miR-532-3p silencing reversed the effects of circNRIP1 knockdown on the sensitivity of 5-FU in the chemotherapy of CRC. The results suggested that circNRIP1 and miR-532-3p may be utilized to improve the diagnosis of CRC and serve as diagnostic markers. In conclusion, overexpression of circNRIP1 promoted the progression of CRC, while circNRIP1 silencing sensitized CRC cells to 5-FU via sponging miR-532-3p.

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Key words: circNRIP1, 5-fluorouracil, miR-532-3p, colorectal cancer

Introduction

Colorectal cancer (CRC) is the third most common type of cancer and its mortality rate has been increasing worldwide (1,2). CRC is a multifactorial disease with a high risk of metastasis and recurrence. Radical surgery is the most common treatment approach for CRC. However, the majority of patients with CRC are unable to undergo radical surgery, since they are already in an advanced stage of the disease and have metastases at the time of diagnosis (3). Therefore, identifying sensitive diagnostic biomarkers for the early detection of CRC and novel targets for CRC treatment are of great importance.

Circular RNAs (circRNAs/circ) are a novel class of non-coding RNAs, characterized by covalently closed circular structures, making them highly conserved and stable. circRNAs may act as therapeutic targets and biomarkers for several diseases (4,5). Increasing evidence has indicated that circRNAs are closely associated with the initiation and development of cancer (6-8). Furthermore, recent studies have confirmed that circRNAs may serve as pivotal targets for the diagnosis and treatment of CRC (9-11). It has been reported that circRNA nuclear receptor-interacting protein 1 (NRIP1), a novel circRNA, is upregulated in several tumor types, including gastric cancer (12), stomach adenocarcinoma (13) and esophageal squamous cell carcinoma (14). However, studies on the expression and biological function of circNRIP1 in CRC are still lacking.

'MicroRNA (miRNA/miR) sponging', referring to the interaction between circRNAs and miRNAs, is based on the competing endogenous RNA (ceRNA) theory. circRNAs most commonly exert their function via acting as ceRNAs to sequester miRNAs (15-17). Via this mechanism of acting as miRNA sponges, circRNAs have been confirmed to have roles in several types of cancer (18). For instance, circ-methionine adenosyltransferase 2B was reported to promote glycolysis and malignancy of hepatocellular carcinoma (HCC) via sponging miR-338-3p under hypoxic stress conditions (19). In addition, circ-protein arginine methyltransferase 5 promoted the metastasis of bladder urothelial carcinoma via sponging miR-30c to induce epithelial-mesenchymal transition (20). Of note, the ability of circRNAs to act as ceRNAs to sequester

miRNAs has also been indicated to have an important role in CRC (21). Similarly, circNRIP1 was able to promote tumor initiation and development via different miRNAs in several types of cancer (12,14,22).

5-Fluorouracil (5-FU), a chemotherapeutic drug, is commonly used in the treatment of CRC. It has been indicated that the combination of 5-FU and cisplatin was able to improve clinical efficacy (23). However, drug resistance, caused by numerous factors, may hamper the efficacy of 5-FU (24). Emerging evidence has also suggested that certain circRNAs have an important role in 5-FU resistance. For instance, upregulation of circ-cerebellar degeneration-related protein 1 (CDR1) was associated with 5-FU resistance and inhibited the circCDR1as/miR-7/cyclin E1 axis to enhance the sensitivity of breast cancer to 5-FU (25). In addition, another study suggested that circ-DEAD-box helicase 17 was significantly upregulated in CRC tissues and cells, while its silencing helped the cells overcome 5-FU resistance via regulating the miR-31-5p/KN motif and ankyrin repeat domains 1 axis (26).

The present study aimed to examine whether circNRIP1 affects the chemotherapeutic effects of 5-FU on CRC and further reveal its underlying potential molecular mechanisms of action. It was hypothesized that circNRIP1 has a significant role in the resistance of CRC to 5-FU. To verify this hypothesis, the effects of knockdown on 5-FU sensitivity and cell proliferation, migration, invasion and apoptosis of CRC cells were evaluated. Mechanistically, the present study aimed to predict whether circNRIP1 is able to act as a ceRNA to sponge miR-532-3p.

Materials and methods

Tissue specimens. A total of 72 pairs of tumor and adjacent normal tissues (collected >5 cm away from the tumor tissues and these specimens were histologically confirmed to be non-cancerous) were collected from patients with CRC (39 male and 33 female patients; age range, 43-77 years) who underwent surgical resection at the Zhengzhou Central Hospital Affiliated to Zhengzhou University (Zhengzhou, China) and the Shaanxi Provincial Cancer Hospital (Xi'an, China) between March 2015 and January 2016. None of the patients received any radiotherapy or chemotherapy prior to surgery. All subjects provided written informed consent prior to the collection and use of their specimens. The histopathological type of CRC was diagnosed by two clinical pathologists. The study protocol was approved by the Committee for the Protection of Human Subjects of Zhengzhou Central Hospital (Zhengzhou, China). The Cancer Genome Atlas (TCGA) (http://gepia.cancer-pku.cn/index.html) was used for analyzing miR-532-3p levels in CRC.

Cell culture and transfection. The human CRC cell lines SW620, HT-29, HCT116 and SW480, the human normal colon epithelial cell line NCM460 and 293T cells were obtained from the Cell Bank of the Chinese Academy of Sciences. Authentication of all cell lines was performed by short tandem repeat profiling. 293T cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) and the other cell lines in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) medium supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and penicillin-streptomycin solution (Gibco; Thermo Fisher Scientific, Inc.). Cells were incubated at 37˚C in a humidified atmosphere with 5% CO_2.

Cells were transfected with the following small interfering RNAs (siRNAs) or miRNA: circNRIP1-specific si-RNA1 (si-cNRIP1-1), 5'-ACGCACAAAGAAAGAAGUGUU-3'; circNRIP1-specific si-RNA2 (si-cNRIP1-2), 5'-UUAAAUGCAAAUAUCAGUGUU-3', and siRNA negative control (si-NC), 5'-UUCUCCGAACGUGUCACGUTT-3'; miR-532-3p mimics, 5'-CCUCCCACACCCAAGGCUUGCA-3' and NC mimics, 5'-UUCUCCGAACGUGUCACGUTT-3'; miR-532-3p inhibitors, 5'-UGCAAGCCUUGGGUGUGGGAGG-3' and NC inhibitors, 5'-CAGUACUUUUGUGUAGUACAA-3' (all from Shanghai GenePharma Co., Ltd.). Between both circNRIP1-specific siRNAs, si-cNRIP1-1 was used for subsequent experiments and was then referred to as si-cNRIP1. Cells were transfected with 50 nM siRNAs and/or 50 nM miR-532-3p mimics or inhibitor using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. All transfection experiments were performed for a total of three times. The transfection efficiency was confirmed by reverse transcription-quantitative PCR (RT-qPCR) analysis.

RNA extraction and RT-qPCR. TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from tissues and cells. Subsequently, the RNA samples were treated with DNase I and their concentration was measured using a NanoDrop® 2000 spectrophotometer (Thermo Fisher. Scientific, Inc.). Total RNA was then reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher. Scientific, Inc.) according to the manufacturer's protocol. The target genes were amplified by qPCR using a SYBR Green reaction mix (Solarbio Life Science, Beijing, China) on the ABI 7500 Fast RT-qPCR system (Applied Biosystems; Thermo Fisher. Scientific, Inc.) according to the manufacturer's protocol. GAPDH and U6 were used as reference genes. The thermocycling conditions for qPCR were as follows: 95˚C for 2 min, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 34 sec. The primer sequences used for qPCR were as follows: circNRIP1 forward, 5'-GGATCAGGTACTGCCGTTGAC-3' and reverse, 5'-TGGACCATTACTTTGACAGGTG-3'; GAPDH, forward, 5'-GGGAGCCAAAAGGGTCAT-3' and reverse, 5'-GAGTCCTTCCACGATACCAA-3'; miR-532-3p forward, 5'-ACACTCCCCTCCCACACCCAAGG-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3'; and U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-CACAGCTTCTCTTTGATGTCAC-3'. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (27).

Cell viability assay. The proliferation rate of CRC cells was determined using a Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology). In brief, cells were seeded into 96-well plates at $5x10^3$ cells in 100 μl medium per well. Following cell incubation at 37˚C overnight, the medium was replaced with fresh medium containing different concentrations of 5-FU (1.25, 2.5, 5, 10, 20, 30 or 40 μM). Following incubation for 0, 24, 48 or 72 h, 10 μl CCK-8 solution was added to the designated wells and cells were incubated for

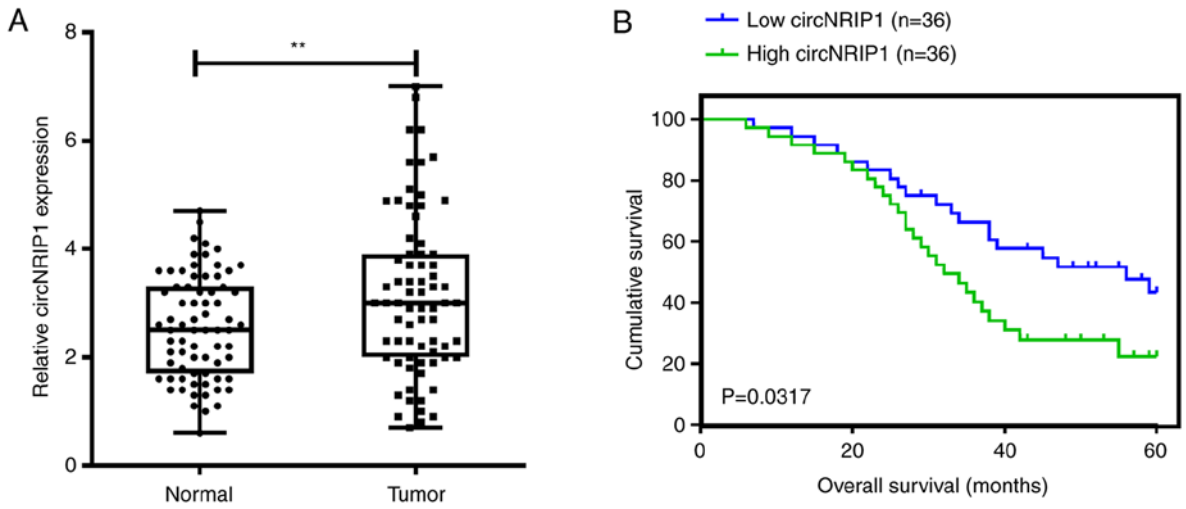

Figure 1. CircNRIP1 is upregulated in CRC. (A) The relative expression levels of circNRIP1 in paired CRC and adjacent normal tissues were measured by reverse transcription-quantitative PCR (n=72). **P<0.01. (B) Kaplan-Meier survival analysis revealed that the increased expression levels of circNRIP1 were associated with poor prognosis of patients with CRC. CircNRIP1, circular RNA nuclear receptor-interacting protein 1; CRC, colorectal cancer.

an additional 2 h. The optical density (OD) in each well was measured at a wavelength of 450 nm with an automatic microplate reader (SpectraMax M2; Molecular Devices, LLC). The cell viability was calculated using the following formula: Cell viability (%) = (average OD value of the experimental group - average OD value of the blank control group)/(average OD value of the control group - average OD value of the blank control group) x100%.

Cell apoptosis assay. Cell apoptosis was assessed using an Annexin V-FITC double staining assay and analyzed using the CytoFLEX flow cytometer (Beckman Coulter, Inc.; CytExpert 2.3). In brief, following treatment with 5-FU for 24 h, $2x10^6$ cells were harvested and washed with PBS. The cells were then resuspended in 400 μl 1X binding buffer and incubated with 5 μl Annexin V-FITC at room temperature for 15 min, followed by incubation with 10 μl propidium iodide in an ice bath for 5 min. Cell apoptosis was then determined with a flow cytometer and the percentage of early + late apoptotic cells was determined.

Cell migration and invasion assays. Cell migration and invasion were assessed using Transwell assays and matching Transwell upper chamber inserts (pore size, 8 μm; Corning, Inc.). In brief, cells were harvested and resuspended in serum-free medium. Only the Transwell inserts in the upper chamber for the invasion assays, but not those for the migration assays, were pre-coated with Matrigel® (BD Biosciences). Subsequently, $5x10^4$ cells were seeded into the upper chamber with FBS-free RPMI-1640, while the lower one was filled with 600 μl RPMI-1640 supplemented with 10% FBS. Cells were incubated for 24 h at 37˚C. Migrated or invaded cells were fixed in 4% paraformaldehyde, stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA) and observed under a microscope (Olympus Corporation).

Luciferase reporter assay. The interaction between circNRIP1 and miR-532-3p was predicted using the StarBase database (http://starbase.sysu.edu.cn/). The sequences of circNRIP1 encompassing the wild-type (WT) or mutant (MUT) binding sites of miR-532-3p were inserted into the pmirGLO luciferase reporter vector (Promega Corporation) to generate the corresponding luciferase reporter plasmids, namely WT-cNRIP1 and MUT-cNRIP1, respectively. The above reporter plasmids were co-transfected with miR-532-3p or miR-NC mimics into 293T cells. Following incubation for 48 h, the luciferase activity was measured using a luciferase reporter assay kit (Promega Corporation) according to the manufacturer's protocol.

Statistical analysis. All statistical analyses were performed using SPSS 19.0 (IBM Corp.) The differences between two groups were compared using paired or unpaired Student's t-test, while those among ≥3 groups were compared using one-way ANOVA followed by Tukey's post hoc test. The association between circNRIP1 expression and clinicopathological features was analyzed using a χ^2 test. The correlation between two variables was determined using the Pearson correlation test. The overall survival of patients was assessed by the Kaplan-Meier method and a log-rank test. The diagnostic value for CRC were assessed by receiver operating characteristic analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

circNRIP1 is upregulated in CRC and is associated with CRC progression. In the present study, the expression levels of circNRIP1 were determined in 72 paired CRC and normal tissues samples. circNRIP1 was significantly upregulated in CRC tissues compared with normal samples (Fig. 1A). Furthermore, the prognostic value of circNRIP1 was evaluated. Kaplan-Meier survival analysis indicated that CRC cases with high circNRIP1 expression levels exhibited poor overall survival compared with those with low expression (median survival, 31 vs. 46 months; log-rank=4.614; P=0.0317; Fig. 1B). To investigate the association between the expression levels

of circNRIP1 and the clinicopathological features of patients, patients with CRC were divided into the high (n=36) and low (n=36) expression groups, based on the median circNRIP1 expression levels. A significant association was observed between the expression of circNRIP1 and lymph node metastasis, distant metastasis and TNM stage (P=0.029, 0.012 and 0.001, respectively; Table I). No other significant associations were obtained between the expression of circNRIP1 and other clinicopathological parameters such as tumor size, sex, age, smoking history and alcohol consumption.

Overexpression of circNRIP1 attenuates the 5-FU-mediated inhibition of viability of HCT116 and SW480 cells. To investigate the biological effect of circNRIP1 on CRC, the expression of circNRIP1 was first determined in CRC cell lines and normal NCM460 colon epithelial cells. The results indicated that circNRIP1 was markedly upregulated in both CRC cell lines compared with NCM460A cells (Fig. 2A). To further investigate the roles of circNIP1 in CRC, two circNRIP1 siRNAs were synthesized to knockdown circNRIP1 expression. RT-qPCR results demonstrated that the expression of circNRIP1 was significantly decreased in HCT116 and SW480 cells transfected with si-cNRIP1 (Fig. 2B).

Subsequently, the effects of circNRIP1 on the biological function of CRC cells were explored. CCK-8 assays revealed that circNRIP1 knockdown markedly attenuated HCT116 and SW480 cell proliferation (Fig. 2C and D). However, circNRIP1 knockdown significantly promoted HCT116 and SW480 cell apoptosis (Fig. 2E). In addition, Transwell assays indicated that circNRIP1 knockdown significantly decreased the invasive and migratory abilities of HCT116 and SW480 cells (Fig. 2F and G). All of these results suggested that circNRIP1 acts as an oncogene in CRC.

To determine whether circNRIP1 affects the 5-FU-mediated inhibition of HCT116 and SW480 cell viability, cells transfected with si-cNRIP1 or si-NC were treated with different concentrations of 5-FU Compared with that of si-NC-transfected cells, the 5-FU-mediated inhibition of the cell viability of HCT116 and SW480 was significantly enhanced following transfection with si-cNRIP1. Furthermore, the present study also evaluated the effects of si-cNRIP1 silencing on the IC_{50} of 5-FU in HCT116 and SW480 cells. Compared with those in the si-NC group, the 5-FU IC_{50} values were markedly reduced in HCT116 and SW480 cells transfected with si-cNRIP1 (decreased by 33.74 and 34.47% in HCT116 cells; and 37.91 and 40.79% in SW480 cells; Fig. 2H and I).

circNRIP1 sponges miR-532-3p in CRC cells. To explore the mechanisms underlying the effect of circ-NRIP1 to regulate the resistance of CRC cells to 5-FU, the potential miRNA targets of circNRIP1 were predicted using the StarBase database. The bioinformatics analysis predicted that miR-532-3p may be directly targeted by circNRIP1 (Fig. 3A). In addition, the expression profile of miR-532-3p in CRC was extracted from the TCGA database. The analysis indicated that miR-532-3p was downregulated in tumor tissues compared with normal ones (Fig. 3B). The expression of miR-532-3p was also evaluated in the 72 paired CRC and normal tissue samples. RT-qPCR analysis demonstrated that the expression of miR-532-3p was reduced in CRC tissues compared with that in adjacent normal tissues (Fig. 3C). Subsequently, the correlation between miR-532-3p and circNRIP1 was analyzed and the results suggested that their expression levels were negatively correlated in CRC tissues (Fig. 3D). The dual-luciferase reporter assay demonstrated that co-transfection of cells with WT-circNRIP1 and miR-532-3p mimics significantly decreased the luciferase activity in 293T cells (Fig. 3E). The expression levels of miR-532-3p were also detected in cell lines. Consistent with the results obtained in tumor tissues, miR-532-3p was significantly downregulated in CRC cell lines compared with NCM460 cells (Fig. 3F). Cells transfected with miR-532-3p mimics exhibited significantly upregulated miR-532-3p expression compared with the NC mimics group and those transfected with miR-532-3p inhibitor exhibited significantly downregulated miR-532-3p expression compared with the NC inhibitor group (Fig. 3G), while circNRIP1 silencing markedly enhanced the expression of miR-532-3p compared with the control group (Fig. 3H).

Table I. Associations between circNRIP1 expression and clinicopathological characteristics of patients with colorectal cancer.

		circNRIP1 expression		
Variables	Cases	Low	High	P-value
Sex				0.637
Male	39	21	18	
Female	33	15	18	
Age, years				0.155
<60	33	20	13	
≥60	39	16	23	
Smoking history				>0.999
Yes	37	18	19	
No	35	18	17	
Alcohol consumption				0.809
Yes	44	21	23	
No	28	15	13	
Tumor size, cm				0.153
≥5	41	17	24	
<5	31	19	12	
Lymph node metastasis				0.029
Yes	46	19	27	
No	26	18	8	
Distant metastasis				0.012
Yes	13	2	11	
No	59	34	25	
TNM stage				0.001
I/II	44	29	15	
III/IV	28	21	7	

Circ-NRIP1, circRNA nuclear receptor-interacting protein 1.

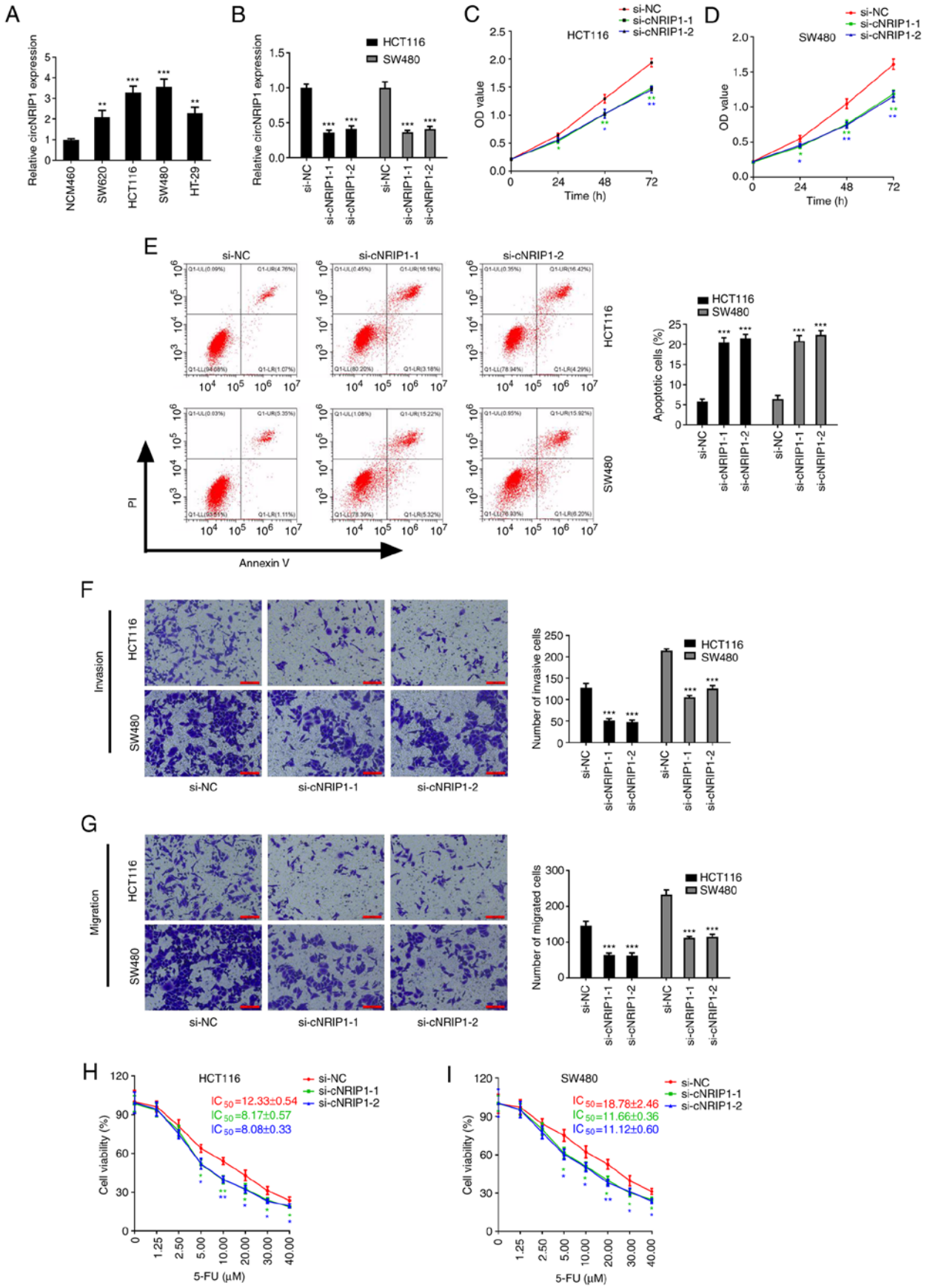

Figure 2. Overexpression of circNRIP1 in CRC attenuates 5-FU-mediated inhibition of HCT116 and SW480 cell viability. (A) Expression levels of circNRIP1 in normal and CRC cell lines. (B) circNRIP1 expression in CRC cells transfected with si-cNRIP1 or si-NC. (C-G) Effects of circNRIP1 knockdown on the proliferation of (C) HCT116 and (D) SW480 cells, and on (E) apoptosis, (F) invasion and (G) migration of CRC cells (scale bars, 100 μm). (H and I) Effect of circNRIP1 knockdown on the sensitivity of (H) HCT116 and (I) SW480 cells to 5-Fu. *P<0.05, **P<0.01 and ***P<0.001 vs. the NCM460 group or the si-NC group. circNRIP1, circRNA nuclear receptor-interacting protein 1; CRC, colorectal cancer; 5-FU, 5-fluorouracil; si, small interfering RNA; NC, negative control; OD, optical density; PI, propidium iodide; Q, quadrant; LR, lower right; LL, lower left; UL, upper left; UR, upper right.

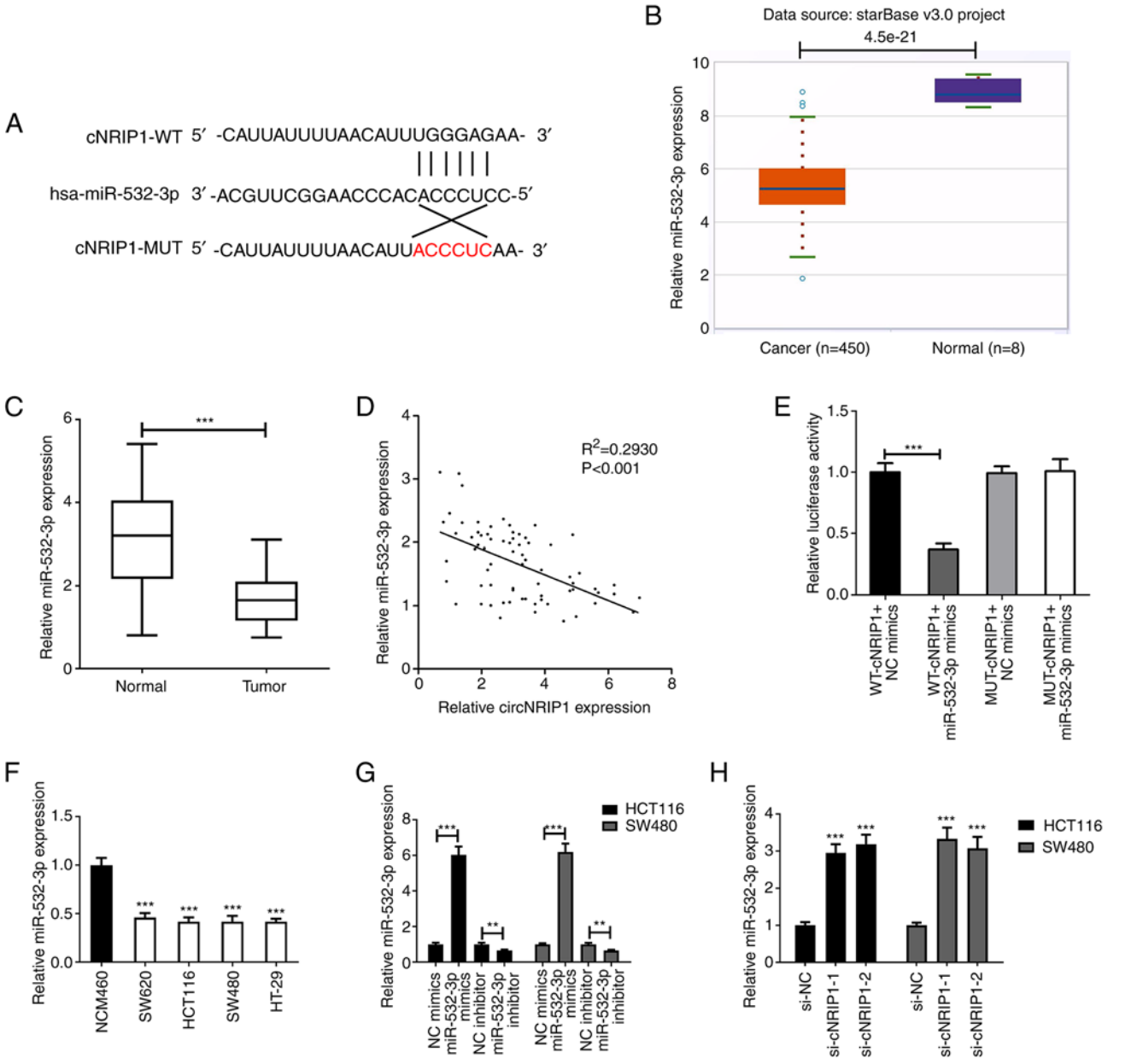

Figure 3. circNRIP1 acts as a sponge for miR-532-3p in CRC cells. (A) The complementary binding site between miR-532-3p and circNRIP1 is presented. (B) miR-532-3p expression in CRC in The Cancer Genome Atlas database. (C) The relative expression levels of miR-532-3p in paired CRC and adjacent normal tissues were measured by reverse transcription-quantitative PCR (n=72). (D) miR-532-3p expression is negatively associated with the expression of circNRIP1 in CRC tissues. (E) The interaction between miR-532-3p and circNRIP1 was confirmed by a dual-luciferase reporter assay. (F) The expression levels of miR-532-3p in normal and CRC cell lines are presented. (G) Effect of cell transfection of miR-532-3p mimics or its inhibitor on miR-532-3p expression. (H) The effect of cell transfection with si-cNRIP1 on miR-532-3p expression is shown. **P<0.01 and ***P<0.001 vs. NCM460, si-NC group or as indicated. c/circNRIP1, circRNA nuclear receptor-interacting protein 1; CRC, colorectal cancer; si, small interfering RNA; NC, negative control; hsa, *Homo sapiens*; MUT, mutant; WT, wild-type; miR, microRNA.

Roles of miR-532-3p in CRC cells. To further investigate the effects of miR-532-3p expression on CRC, cell proliferation and apoptosis in CRC cells were evaluated. The results suggested that overexpression of miR-532-3p significantly inhibited proliferation and promoted apoptosis in HCT116 and SW480 cells (Fig. 4A-C). The 5-FU chemosensitivity was also determined in CRC cells overexpressing miR-532-3p. Compared with the miR-NC group, miR-532-3p overexpression significantly enhanced the 5-FU-induced inhibition of cell viability and decreased the IC_{50} values of 5-FU in HCT116 and SW480 cells (Fig. 4D and E).

circNRIP1 reduces the sensitivity of CRC cells to 5-FU via regulating the expression of miR-532-3p. The aforementioned results suggested that circNRIP1 knockdown and miR-532-3p overexpression significantly enhanced the sensitivity of CRC cells to 5-FU at least in part through the binding of circNRIP1 to miR-532-3p to inhibit its expression. Therefore, it was hypothesized that circNRIP1 reduces the sensitivity of CRC cells to 5-FU via regulating the expression of miR-532-3p. To verify this hypothesis, CRC cells were co-transfected with si-cNRIP1 and miR-532-3p inhibitor and treated with 5-FU. The results demonstrated that cell co-transfection with

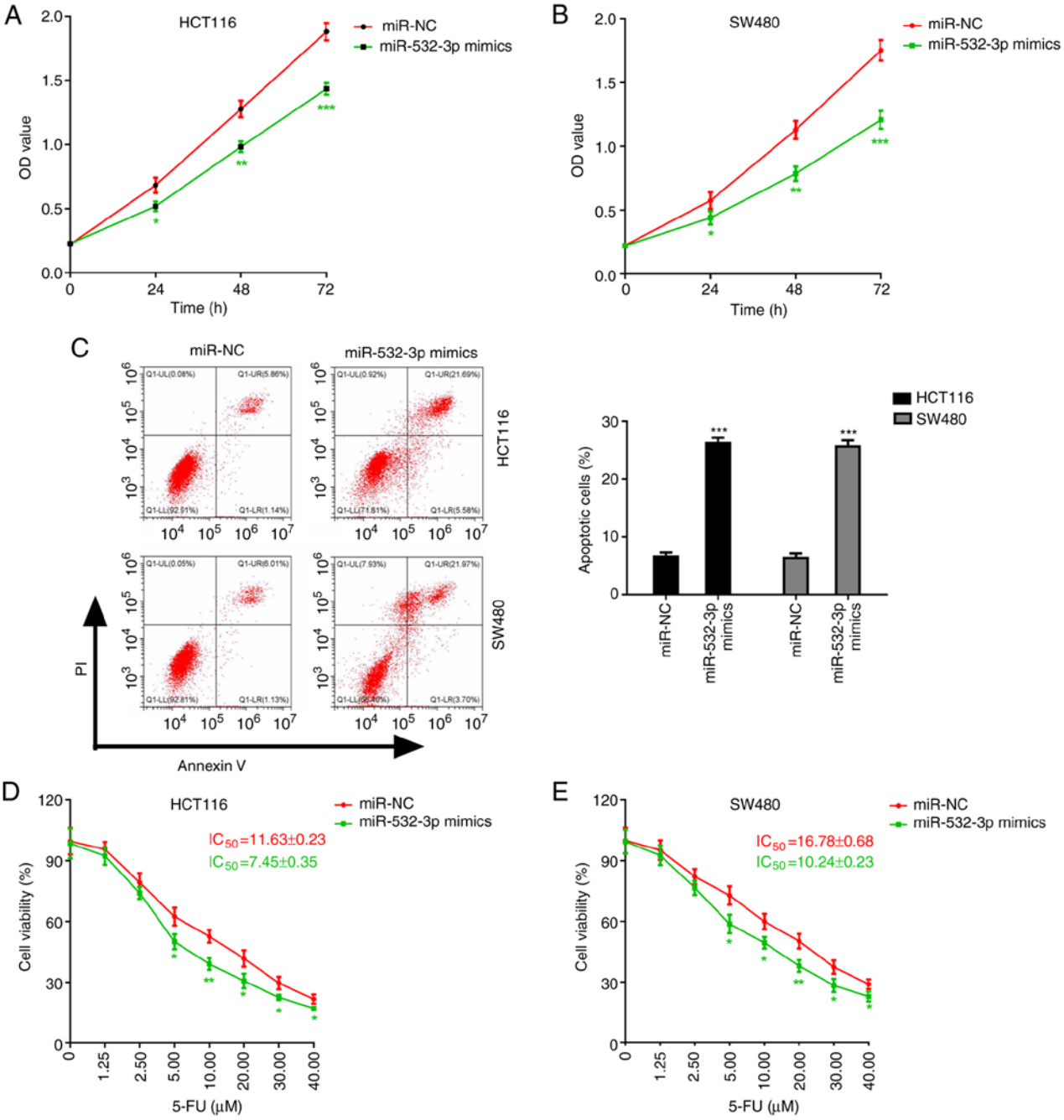

Figure 4. Roles of miR-532-3p in CRC cells. (A and B) Effect of miR-532-3p overexpression on the proliferation of (A) HCT116 and (B) SW480 cells. (C-E) Influence of miR-532-3p on the toxicity of 5-FU on CRC cells. (C) Apoptosis of CRC cells and viability of (D) HCT116 and (E) SW480 cells treated with increasing concentrations of 5-FU. *P<0.05, **P<0.01 and ***P<0.001 vs. the miR-NC group. miR-532-3p, microRNA-532-3p; CRC, colorectal cancer; IC_{50}, half maximal inhibitory concentration; NC, negative control; 5-FU, 5-fluorouracil; OD, optical density; PI, propidium iodide; Q, quadrant; LR, lower right; LL, lower left; UL, upper left; UR, upper right.

si-cNRIP1 and miR-532-3p inhibitor significantly promoted cell proliferation and inhibited apoptosis compared with the si-cNRIP1 + miR-NC group (Fig. 5A-C). In addition, compared with the si-cNRIP1 + miR-NC group, co-transfection with si-cNRIP1 and miR-532-3p inhibitor significantly attenuated the 5-FU-mediated inhibition of cell viability and increased the IC_{50} values of 5-FU in HCT116 and SW480 cells (Fig. 5D and E).

Diagnostic value of circNRIP1 and miR-532-3p in CRC. Subsequently, the association between circNRIP1 and miR-532-3p with the clinicopathological features of patients and their diagnostic value for CRC were investigated using receiver operating characteristic curves (Fig. 6). The results indicated that both circNRIP1 and miR-532-3p exhibited good predictive value regarding lymph node metastasis [area under the curve (AUC), 0.814 and 0.701, respectively; P<0.001 and P=0.009, respectively; Fig. 6A and B], distant metastasis (AUC, 0.863 and 0.768, respectively; P<0.001 and P=0.002, respectively, Fig. 6D and E) and TNM stage (AUC, 0.747 and 0.760, respectively; P<0.001 for each; Fig. 6G and H). In addition, the combination of the two indicators also exhibited a

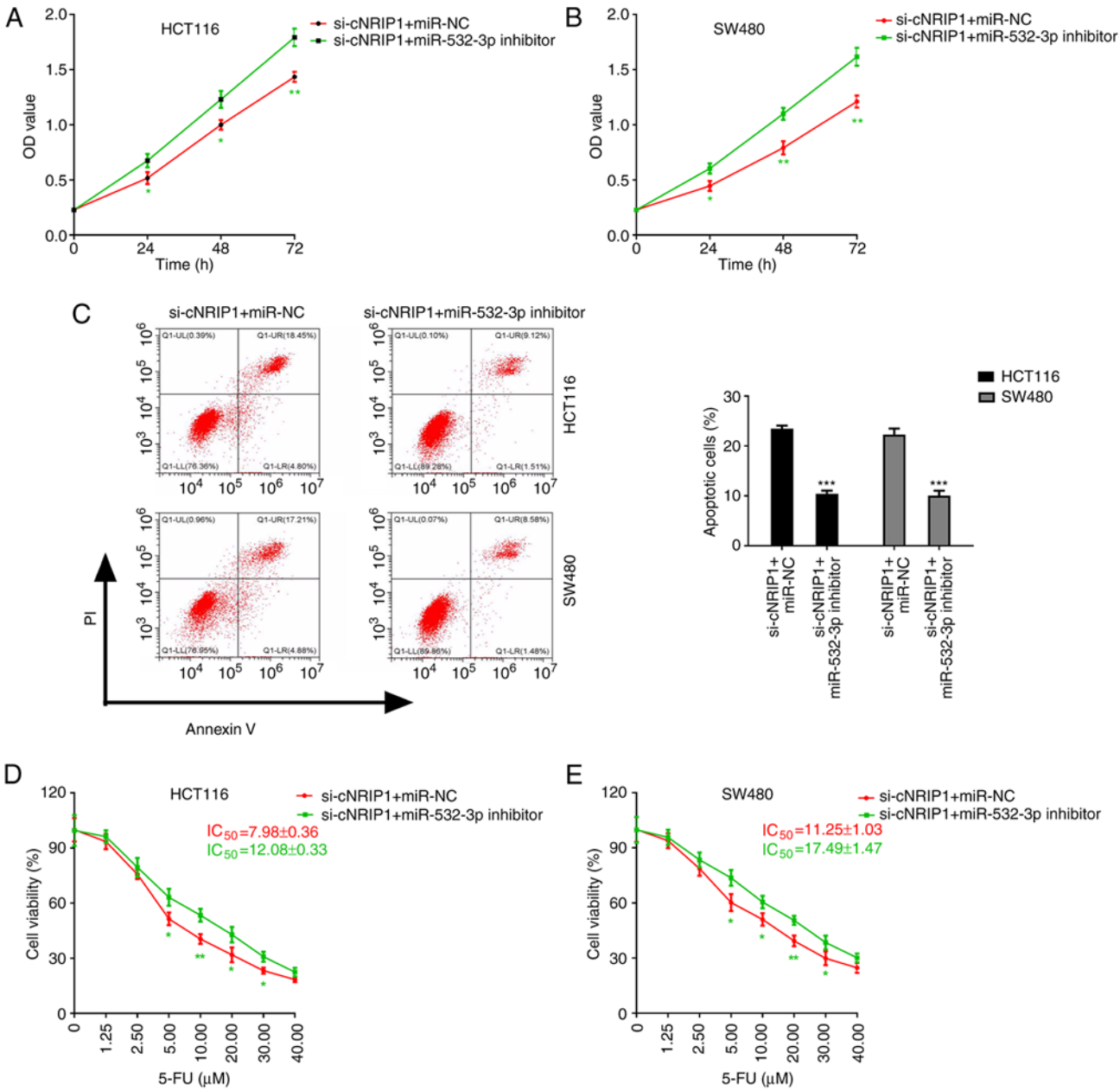

Figure 5. circNRIP1 attenuates the sensitivity of CRC cells to 5-FU via regulating the expression of miR-532-3p. (A and B) Effect of cell co-transfection with si-cNRIP1 and miR-532-3p inhibitor on the proliferation of (A) HCT116 and (B) SW480 cells. (C-E) Influence of co-transfection with si-cNRIP1 and miR-532-3p inhibitor on the effect of 5-FU on CRC cells. (C) Apoptosis of CRC cells and viability of (D) HCT116 and (E) SW480 cells treated with increasing concentrations of 5-FU. *P<0.05, **P<0.01 and ***P<0.001 vs. the si-cNRIP1 + miR-NC group. circ-NRIP1, circRNA nuclear receptor-interacting protein 1; CRC, colorectal cancer; miR-532-3p, microRNA-532-3p; 5-FU, 5-fluorouracil; si, small interfering RNA; IC_{50}, half maximal inhibitory concentration; NC, negative control; OD, optical density; PI, propidium iodide; Q, quadrant; LR, lower right; LL, lower left; UL, upper left; UR, upper right.

good predictive value for lymph node metastasis (AUC=0.849; P<0.001; Fig. 6C), distant metastasis (AUC=0.906; P<0.001; Fig. 6F) and TNM stage (AUC=0.802; P<0.001; Fig. 6I). These results indicated the combination of circNRIP1 and miR-532-3p may be used as a biomarker for the diagnosis of CRC.

Discussion

Emerging evidence suggested that circRNAs have a crucial role in resistance to chemotherapeutics and serve as key targets for the diagnosis and treatment of CRC (9-11). circNRIP1 was reported to be abnormally expressed in numerous tumor types and may have an important role in promoting malignant features (12-14). However, the expression and biological function of circNRIP1 in CRC has remained elusive. The present study also demonstrated that circNRIP1 was abnormally upregulated in CRC specimens and cells. In terms of clinical value, the expression of circNRIP1 was associated with the clinical progression of CRC. Increased expression of circNRIP1 was indicated to promote tumor metastasis and was associated with TNM stage and poor prognosis of patients with CRC. Hence, circNRIP1 may be used as a biomarker for the diagnosis of CRC.

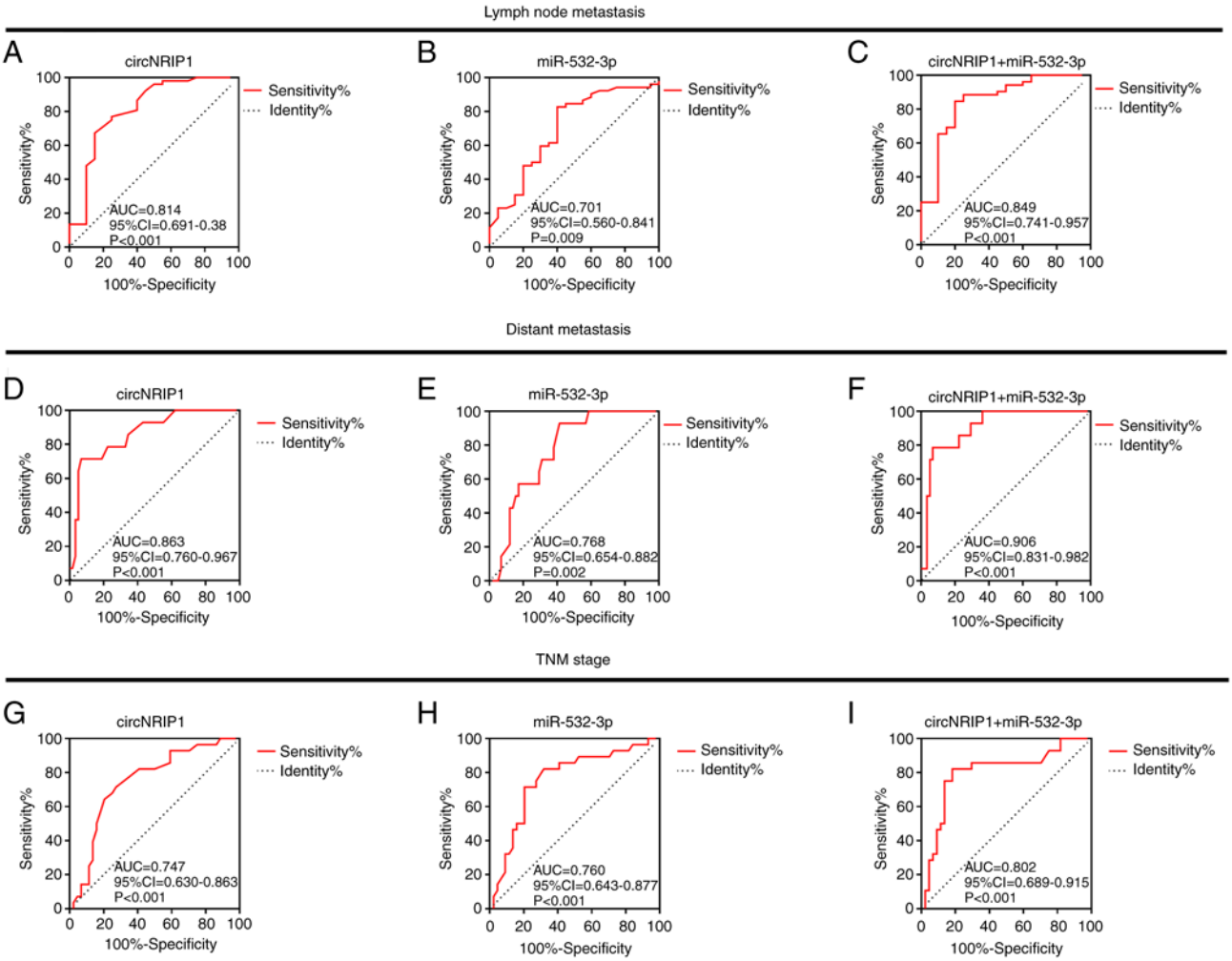

Figure 6. Diagnostic value of circNRIP1 and miR-532-3p. Predictive value of (A) circNRIP1, (B) miR-532-3p and (C) their combination for lymph node metastasis in patients with CRC. Predictive value of (D) circNRIP1, (E) miR-532-3p and (F) their combination for distant metastasis in patients with CRC. Predictive value of (G) circNRIP1, (H) miR-532-3p and (I) their combination for TNM stage in patients with CRC. circ-NRIP1, circRNA nuclear receptor-interacting protein 1; AUC, area under the receiver operating characteristic curve; miR-532-3p, microRNA-532-3p; CRC, colorectal cancer.

As a type of circRNA, circNRIP1 may affect the biological properties of tumor cells. Li *et al* (22) reported that circNRIP1 promoted cell migration and invasion in cervical cancer. In addition, Dong *et al* (28) demonstrated that circNRIP1 silencing attenuated cell viability, migration and colony formation and promoted apoptosis in renal carcinoma cell lines. Furthermore, Zhang *et al* (29) revealed that circNRIP1 knockdown inhibited cell proliferation, migration and invasion and downregulate AKT1 in gastric cancer cells. The functional experiments of the present study also demonstrated that circNRIP1 promoted CRC cell proliferation, migration and invasion, while inhibiting apoptosis. These results suggested that circNRIP1 may also exert an oncogenic role in CRC.

Of note, the biological functions of circRNAs are triggered by miRNAs. Recent studies have suggested that circNRIP1 promotes the progression of tumors via sponging miRNAs. For instance, a study indicated that circNRIP1 has an oncogenic role in a renal carcinoma cell line via targeting miR-505 (28). Another study revealed that circNRIP1 acts as an miR-149-5p sponge to promote gastric cancer progression via the AKT1/mTOR signaling pathway (29). Hence, the present study aimed to investigate whether circNRIP1 is able to promote the progression of CRC via sponging miRNAs. miR-532-3p was predicted as a target miRNA of circNRIP1, which was confirmed by dual-luciferase reporter assays. Of note, miR-532-3p has been recognized as a tumor suppressor gene in several types of human cancer (30-32). Analysis of the TCGA dataset and *in vitro* results of the present study indicated that miR-532-3p was significantly downregulated in CRC tumor tissues and cell lines. Clinical value analysis and functional experiments also demonstrated that miR-532-3p acts as a tumor suppressor in CRC. In addition, the results of co-transfection experiments of CRC cells with si-cNRIP1 and miR-532-3p inhibitor suggested that miR-532-3p silencing abrogated the defects caused by circNRIP1 depletion. These findings indicated that knockdown of miR-532-3p reversed the circNRIP1 depletion-mediated effects in CRC cells. Consistent with the results of the present study, previous studies suggested that certain circRNAs affect tumor progression via acting on miR-532-3p. Furthermore, Ouyang *et al* (30) demonstrated that loss of androgen receptors promoted HCC invasion and metastasis via activating the circ-leucyl and cystinyl aminopeptidase/miR-532-3p/ras-related protein rab-9a signaling pathway under hypoxia. In addition, Dai *et al* (33) reported

that circ-FYVE, RhoGEF and PH domain containing 4 attenuated gastric cancer progression via modulating the miR-532-3p/adenomatous polyposis coli/β-catenin signaling pathway.

It has also been reported that the differential expression of circRNAs, including that of circNRIP1, may affect the sensitivity of cancers to chemotherapy with 5-FU. Lin *et al* (34) reported that circNRIP1 is able to regulate the resistance of nasopharyngeal carcinoma cells to 5-FU and cisplatin via the miR-515-5p/IL-25 axis. In addition, Xu *et al* (35) demonstrated that circNRIP1 was able to maintain the hypoxia-induced resistance of gastric carcinoma cells to 5-FU through hypoxia-inducible factor-1α-dependent glucose metabolism via sponging miR-138-5p. The present study indicated that circNRIP1 knockdown significantly enhanced the sensitivity of CRC cells to 5-FU. Furthermore, the results confirmed that circNRIP1 promoted the progression of CRC via sponging miR-532-3p. Hence, in the present study, it was hypothesized that circNRIP1 promoted the resistance of CRC cells to 5-FU via sponging miR-532-3p. First, the present study verified that increased expression of circNRIP1 significantly suppressed the expression of miR-532-3p. In addition, co-transfection experiments revealed that miR-532-3p silencing reversed the effects of circNRIP1-depletion on the chemotherapy sensitivity of CRC cells to 5-FU. However, the absence of an experiment of overexpression of circNRIP1 is a limitation of the present study.

In conclusion, the present study demonstrated that circ-NRIP upregulation promoted the progression of CRC, while circNRIP1 silencing sensitized CRC cells to 5-FU via sponging miR-532-3p. These results suggested that circ-NRIP1 and miR-532-3p may be considered as potential sensitizing targets and biomarkers for the diagnosis of CRC.

Acknowledgements

Not applicable.

Funding

This work was supported by the Science and Technology Project of Henan Province (grant no. 212102310121) and the Medical Science Research Project of Henan Province (grant no. LHGJ20191039).

Availability of data and materials

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

Authors' contributions

FL and RL performed the majority of the experiments in the study. RZ collected clinical specimens. YZ and MH contributed to the analysis of experimental data. FL and YZ contributed to the study design, manuscript writing and provided experimental funding support. All authors have read and approved the final manuscript. FL and YZ checked and confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

All aspects of experimental design and protocols were reviewed and approved by the Committee for the Protection of Human Subjects at Zhengzhou Central Hospital (Zhengzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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