Long Noncoding RNA LINC00958 Suppresses Apoptosis and Radiosensitivity of Colorectal Cancer Through Targeting miR-422a

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Primary research

Keywords: LINC00958, miR-422a, colorectal cancer, apoptosis, radiosensitivity

Posted Date: June 12th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-591977/v1

Abstract

Background: Long non-coding RNAs (lncRNAs) have been elucidated to participate in the development and progression of various cancers. In this study, we aim to explore the underlying functions and mechanisms of LINC00958 in colorectal cancer.

Methods: LINC00958 expression in colorectal cancer tissues was examined by qRT-PCR. The associations between LINC00958 expression with clinical characteristics and prognosis were evaluated. The biological functions of LINC00958 were detected by CCK-8, MTT, colony formation and Flow cytometric analyses. RNA-pull down, RIP and luciferase reporter assays were used to confirm the regulation of LINC00958 on miR-422a. Rescue experiments were performed to detect the effects of miR-422a on the roles of LINC00958.

Results: LINC00958 was upregulated in colorectal cancer tissues and cell lines; high LINC00958 level was significantly associated with tumor differentiation, T stage and TNM stage, and also predicted poor prognosis. Cell experiments showed that LINC00958 promoted cell proliferation and suppressed apoptosis and the sensitivity of radiotherapy in vitro, and promoted cell growth in vivo. Bioinformatics analysis predicted the binding site of miR-422a on LINC00958. Mechanistically, RNA-pull down, RIP and luciferase reporter assays demonstrated that LINC00958 specially targeted miR-422a. In addition, we provided evidence that miR-422a suppressed MAPK1 expression through directly binding to the 3'-UTR of MAPK1, thereby inhibiting cell proliferation and enhancing apoptosis and the radiosensitivity. Furthermore, miR-422a rescued the roles of LINC00958 on promoting MAPK1 expression and cell proliferation and decreasing apoptosis and the radiosensitivity.

Conclusions: LINC00958 promoted MAPK1 expression and cell proliferation and suppressed apoptosis and the radiosensitivity through targeting miR-422a, highlighting a potential biomarker for the prognosis and treatment of colorectal cancer.

Introduction

Colorectal cancer is one of the most serious malignancy across the world and the second main cause of cancer-related death[1, 2]. With the advancements in diagnoses and combination treatments, the morbidity and mortality of colorectal cancer patients with aged 65 years and older declined by 3.3% and 3.0% annually, respectively[3]. However, the incidence and death rates in aged younger than 50 years increased by 1% and 1.3% annually[3], respectively. Up to date, the potential molecular mechanisms underlying the development and progression of colorectal cancer are still ambiguous. Thus, it's urgent to explore the molecular mechanisms and identify more effective molecular targets for early diagnoses and treatments of colorectal cancer.

Human genomes produce a great quantity of noncoding RNAs with limited protein coding potential, many of which participates in diverse biological processes. Recently, larger than 200 nucleotides in length, long non-coding RNAs (lncRNAs) are attracting great attention[4, 5]. Dysregulation of lncRNAs has occurred in

various types of cancer. Increasing evidence has showed that lncRNAs play vital roles in the pathogenesis and progression, such as cell proliferation, apoptosis, angiogenesis, lymphangiogenesis, cell signaling transduction and distant metastasis[6–9]. Pan et al. revealed that lncRNA FOXC2-AS1 was upregulated in colorectal cancer tissues, and si-FOXC2-AS1 suppressed cell proliferation, invasion and metastasis in vitro and in vivo[10]. In addition, Tian et al. identified a novel lncRNA GCMA, which was highly expressed in gastric cancer tissues and predicted poor prognosis. Also, they demonstrated that GCMA promoted cell growth, induced epithelial-mesenchymal transition (EMT) and cell metastasis[11]. Adjuvant radiotherapy or palliative radiotherapy contributes to make stage down or delay tumor progression for many types of cancer[12]. Successful radiotherapy is based on a great understanding of the radiotherapy mechanisms. Recently, wang et al. revealed that lncRNA CCAT2 suppressed cell apoptosis and radiosensitivity of human esophageal carcinoma[13]. However, the detail and accurate mechanisms of lncRNAs in the progression of cancers haven't been elucidated. Currently, lncRNAs were concluded to exert their biological functions in four different ways, as signals, decoys, guides and scaffolds[14]. Wu et al. reported that lncRNA PVT1 acted as a miRNA sponge to relieve the inhibition of miR-16-5p on VEGFA, and activated the VEGFA/VEGFR/AKT pathway, thereby promoting tumorigenesis of colorectal cancer[15]. Hua et al. showed that hypoxia-induced lncRNA AC020978 promoted cell proliferation and glycolytic metabolism of non-small cell lung cancer through directly interacting with PKM2 and enhancing PKM2 protein stability[16]. As the most important mechanism, the miRNA sponge mechanism is the research hotspot of lncRNA in recent years.

In this study, we identified that lncRNA LINC00958 was upregulated in colorectal cancer tissues and cell lines, and significantly associated with clinicalpathological features and prognosis. As the most important member of mitogen-activated protein kinase family, MAPK1 and its downstream pathway are widely accepted to play oncogenic roles in various cancer types, such as cell proliferation, apoptosis and radiosensitivity[17–20]. We demonstrated that LINC00958 served as a miR-422a sponge and enhanced the MAPK1 expression, thereby promoting cell proliferation and suppressing apoptosis and radiosensitivity. Our data suggest that LINC00958/miR-422a/MAPK1 axis plays key roles in the cell growth and radiosensitivity of colorectal cancer, and it may be a promising candidate in diagnosis and treatment of colorectal cancer.

Materials And Methods

Tissues samples and cell culture

From 2013 to 2018, 63 pairs fresh frozen samples of colorectal cancer tissues and adjacent normal tissues were collected from colorectal cancer patients in Henan Provence People's Hospital. All the samples were snap-frozen into RNA keeper Tissue Stabilizer (Vazyme Biotech Co., Jiangsu, China), and kept in -80°C refrigerator after storing at 4°C overnight. All the patients received radical resection without radiotherapy or chemotherapy before surgery. Written informed consents were acquired from all the objects. The study was approved by Institutional Review Boards of Henan Provence People's Hospital.

Human colorectal cancer cells and human normal colorectal mucosa cell FHC cell were purchased from the Type Culture Collection of the Chinese Academy of Science (Shanghai, China). All the cells were cultured in DMEM medium (HyClone, USA) with 10% fetal bovine serum (Gibco, Australia) and 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C.

Transfection And Oligonucleotides And Plasmids

To regulate the expression of LINC00958 and miR-422a, siRNA targeting LINC00958 (si-LINC00958: 5'-GTGACTAGCTTAAACTAAATT-3') and overexpression plasmid were synthesized; the miR-422a mimics and inhibitor were purchased from RiBoBio (Guangzhou, China). When colorectal cancer cells become more 80% confluence, they were digested with 0.25% trypsin, resuspended and seeded in 6-well plates. According to the manufacturer's instructions, the plasmids or oligonucleotides were transfected into cells with LipofectamineTM 2000 (Invitrogen, USA) after culturing for 24 hours.

Quantitative Real-time Pcr (Qrt-pcr)

Total RNA was extracted from cells and tissues with TRIzol (TaKaRa, Shiga, Japan) according to manufacturer's instructions. RNAs were reversely transcribed into cDNA with PrimeScriptTM RT Master Mix reagent kit (TaKaRa, Shiga, Japan). Then, qRT-PCR was conducted to quantify RNA level with SYBR Premix Ex Taq™ (TaKaRa, Shiga, Japan). GAPDH or U6 were used as internal controls. The primers are as follows: LINC00958, F: 5'-CCATTGAAGATACCACGCTGC-3', R: 5'-GGTT GTTGCCCAGGGTAGTG-3'; MAPK1, F: 5'-CTGGACGTGCTCAGACATCG-3', R: 5'-GGTCAGCAGGGCATC ATGTAG-3'; GAPDH, F: 5'-CACCATTGGCAATGAGCGGTTC-3', R: 5'-AGGTC TTTGCGGATGTCCACGT-3'. The relative expression levels of RNA were assessed by ΔΔCt method.

Cck-8 And Mtt Assay

2000 cells were seeded into 96-well plates and cultured at 37°C. After culturing for 0 h, 24 h, 48 h, 72 h and 96 h, the optical density of each well was measured with CCK-8 kit or MTT kit (Beyotime Biotechnology, Jiangsu, China) at 450 nm or 570 nm. All experiments were executed in triplicate.

Flow Cytometric Analysis

The cell apoptosis assays were conducted with Annexin V Apoptosis Detection Kit (FITC) (eBioscience, USA). Cells were treated with trypsin without EDTA. Then, cells were washed with precooled PBS and diluted with 100 μl binding buffer. 5 μl fluorochrome-conjugated Annexin V was added and incubated at room temperature for 15 min. After resuspension in 200 μl binding buffer, 5 μl PI was added. Finally, the cell apoptosis percentages were detected and analyzed by flow cytometry. All experiments were executed in triplicate.

Radiant Exposure And Clone Formation Assay

A single dose of Ionizing radiation was delivered by Siemens 6 MV X-ray linear accelerator with a distance of 100 cm from source-skin at a dose rate of 2 Gy/min at room temperature. 200 transfected SW480 or HCT8 cells were seeded in six-well plates, respectively. Five dishes of cells were irradiated for 8h with different gradient of irradiation dose of 0, 2, 4, 6, 8 Gy, respectively. After incubation for 14 days at 37°C, the cells were fixed by 75% ethanol and stained by 0.1% crystalline purple. The number of colonies containing 50 cells or more was counted under an inverted microscope (40× magnification, Leica, Germany).The relative survival fraction is calculated as quotient of plating efficiency (treated) to PE (control): PE = number of colonies ÷ number of seeded cells; SF = PE (irradiated cells) ÷ PE (control cells). All experiments were executed in triplicate.

Western Blot

Western blot
Total protein was extracted from colorectal cancer cells using radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, Jiangsu, China) according to manufacturer's instructions. Protein concentrations were tested with BCA protein assay kit (Beyotime Biotechnology, Jiangsu, China). 30 μg total protein was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). The membranes were blocked with 5% non-fat milk for 1.5 h at room temperature, and next incubated with primary antibodies at 4°C overnight. After incubating with secondary antibodies at room temperature for 1.5 h, protein bands were detected by ECL chemiluminescent reagent (Millipore, MA, USA). The primary antibodies were as follows: Erk1/2 (1:1000; Cell Signaling Technology), pErk (1:1000; Cell Signaling Technology), BCL2 (1:1000; Cell Signaling Technology), Bax(1:2000; Abcam) and GAPDH (1:5000; Cell Signaling Technology).

Rna-fluorescence In Situ Hybridization (Fish) And Nuclear-cytoplasmic Fractionation

RNA-FISH assay was carried out to confirm the subcellular location of LINC00958 in colorectal cancer cell. Cy3-labeled LINC00958 probe was synthesized by GenePharma (Shanghai, China). The sequence of Cy3-labeled LINC00958 probe was as follows: 5'-TCCTCCCATGTTTTTGTCTTCCCTACCACC-3'. Hybridization was performed according to the manufacturer's instructions. The images were acquired on fluorescence microscope. Nuclear and cytoplasmic RNA was isolated by PARIS™ Kit (Invitrogen, USA) according to the manufacturer's instruction and then detected by qRT-PCR.

Rna-pull Down Assay

The biotinylated LINC00958 probe was produced by RiboBio (Guangzhou, China). Briefly, 1×10^7 SW480 cells were lysed with ultrasonic. Probe-coated beads were firstly incubated with oligo probe or LINC00958 probe using C-1 magnetic beads (Life Technologies) for 2 h at 25°C. Next, the cell lysates were incubated with C-1 magnetic beads combining with oligo probe or LINC00958 probe (Biotin-LINC00958 of wild type: 5'-TCCTCCCATGTTTTTGTCTTCCCTACCACC-3'; Bio- LINC00958 of mutation: 5'-AGGAGGGTACAAAAACAGAAGGGATGGTGG-3') at 4°C overnight. After washing with wash buffer for three times, the RNA complexes were extracted by RNeasy Mini Kit (QIAGEN, Germany) according to manufacturer's instructions. Finally, qRT-PCR was conducted to quantify the level of miR-422a.

Rna Immunoprecipitation (Rip) Assay

RIP assay was conducted in SW480 cells with magna RIPTM RNA-binding Protein Immunoprecipitation kit (Millipore, Billerica, MA). SW480 cells were transfected with miR-422a mimics or negative control. After transfection for 48 h, the cells were lysed with complete RNA lysis buffer. Magnetic beads were firstly conjugated with human anti-AGO2 antibody or negative control mouse IgG. Then, cell lysates were rotated in RIP immunoprecipitation buffer with above magnetic beads. Next day, immunoprecipitated RNA was incubated with Proteinase K for 30 min and extracted by TRIzol. Finally, qRT-PCR was performed to identify the level of LINC00958.

Luciferase Reporter Assay

The luciferase reporter plasmids (pGL3-LINC00958 sequence and pGL3-mutant LINC00958 sequence, pGL3-MAPK1 3'-UTR sequence and pGL3-mutant MAPK1 3'-UTR sequence) were produced by HarO Life Co. (Shanghai, China). The luciferase reporter plasmids were transfected into cells with miR-422a mimics or inhibitor. After 36 h, the activities of firefly luciferase and renilla luciferase were detected. The relative luciferase activity was calculated by the ratio of firefly luciferase/Renilla luciferase activity *100%.

Animal Experiments

To establish xenograft tumor models, the shRNA against LINC00958 (sh- LINC00958) and negative control (sh-NC) were generate and cloned into lentivirus. Then, lentiviruses were transfected into SW480 cells with 5mg/ml polybrene for 48 h. Finally, the stable SW480 cells were selected with puromycin (5μg/ml) for 2 weeks. The knockdown efficiency was confirmed by qRT-PCR. For in vivo tumorigenesis assay, 10 male BALB/c athymic nude mice of 4-week old were randomly divided into two groups (n = 5). 1.0×10^7 stable SW480 cells in 150μl PBS were subcutaneously injected into left inguinal region of mice. After 10 days, tumor volumes were measured every three days until 4 weeks. Tumor volume was calculated by the formula: tumor = (length×width^2)/2. Finally, the mice were sacrificed, and the volume and weight of tumors were detected. The animal experiments were approved by the Institutional Animal

Care and Use Committee of Zhengzhou University, and were performed according to the guidelines for the care and use of laboratory animals.

Statistical analysis

The SPSS 22.0 software was conducted for statistical analyses. Counting data were calculated by the χ^2 or Fisher's exact test. Paired and unpaired measurement data were compared by Student's t-test or Mann-Whitney U test. The survival curves were calculated by the Kaplan-Meier method and were analyzed by log-rank tests. A probability of 0.05 or less was considered statistically significant for all tests.

Results

LINC00958 is overexpressed in colorectal cancer tissues

Previous studies showed that the level of LINC00958 was up-regulated in cancers and predicted poor prognosis, such as hepatocellularcarcinoma and oral squamous cell carcinoma[21, 22]. In this study, the level of LINC00958 was detected using qRT-PCR technology in 63 paired colorectal cancer tissues and matched adjacent normal tissues. We found that LINC00958 was upregulated in colorectal cancer tissues compared with that in matched normal tissues (82.54%, 52/63) (Fig. 1a, b). Next, the correlations between the level of LINC00958 and clinicopathological features were analyzed in these 63 paired colorectal cancer tissues. The results revealed that the level of LINC00958 was positively correlated with tumor differentiation, T stage and TNM stage (Table 1, $P < 0.05$). The level of LINC00958 was remarkably higher in colorectal cancer tissues at moderate and poor differentiation (G2 + G3) than these at well differentiation (G1) (Fig. 1c), higher in colorectal cancer tissues at T3 and T4 stages than these at T1 and T2 stages (Fig. 1d), and higher in colorectal cancer tissues at III and IV stages (TNM stage) than these at I and II stages (Fig. 1e). Kaplan-Meier survival analysis indicated that colorectal cancer patients with higher LINC00958 had a worse overall survival (Fig. 1f) and disease-free survival (Fig. 1g) than those with lower expression of LINC00958. Overall, these results show that LINC00958 is highly expressed in colorectal cancer tissues, and may act as a promising diagnostic, prognostic and therapeutic marker for colorectal cancer.

Table 1
Associations between LINC00958 expression and clinicopathological features in colorectal cancer (n = 63)

Parameters	Category	No.	LINC00958 expression		χ^2	P
			High(%)38	Low(%)25		
Age					0.607	0.436
	< 65	34	19	15		
	≥ 65	29	19	10		
Gender					0.613	0.434
	Male	39	25	14		
	Female	24	13	11		
Differentiation					4.565	**0.003**
	Well		3	7		
	Mederate + Poor		35	18		
T stage					6.091	**0.014**
	T1 + T2		6	11		
	T3 + T4		32	14		
N stage					3.770	0.052
	N0 + N1		8	11		
	N2 + N3		30	14		
TNM stage					4.552	**0.033**
	I + II		5	9		
	III + IV		33	16		
Nerve invasion					1.724	0.189
	Yes	39	26	13		
	No	24	12	12		
Vessel invasion					0.218	0.641
	Yes	40	25	15		
	No	23	13	10		
Tumore size, cm					0.138	0.710

Parameters	Category	No.	LINC00958 expression		χ^2	P
			High(%)38	Low(%)25		
	< 5	27	17	10		
	≥ 5	36	21	15		

LINC00958 promotes cell proliferation, suppresses cell apoptosis and radiosensitization of colorectal cancer in vitro

The expression of LINC00958 in colorectal cancer cell lines was detected using qRT-PCR. The results showed that the level of LINC00958 was higher in colorectal cancer cell lines than human normal colorectal mucosa cell FHC (Fig. 2a). According to the expression of LINC00958, we selected SW480 and HCT8 cells to investigate the function of LINC00958. Then, the siRNA targeting LINC00958 and an overexpression plasmid of LINC00958 were designed and synthesized. qRT-PCR results showed that si-LINC00958 markedly decreased the expression of LINC00958 in SW480 cells and the overexpression plasmid significantly increased its expression in HCT8 cells (Fig. 2b). To detect the role of LINC00958 in colorectal cancer cells, the flow cytometry, CCK-8, MTT and clone formation assays under different radiation dose were performed. The results of flow cytometry assay showed that knockdown of LINC00958 significantly increased the apoptosis percentage (Fig. 2c), while overexpression of LINC00958 remarkably decreased the apoptosis percentage (Fig. 2g). The CCK-8 and MTT results indicated that si-LINC00958 decreased the ability of cell proliferation (Fig. 2d and 2e), while overexpression of LINC00958 increased it (Fig. 2h and i). In addition, we found that downregulation of LINC00958 decreased the survival fraction under different irradiation dose (Fig. 2f), and upregulation of LINC00958 increased the survival fraction under different irradiation dose (Fig. 2j), indicating that LINC00958 decreased the radiosensitization of colorectal cancer cell. Taken together, these results demonstrated that LINC00958 promoted cell proliferation and suppressed apoptosis and the radiosensitivity of colorectal cancer cells.

Linc00958 Promotes Colorectal Cancer Cell Proliferation In Vivo

To explore the roles of LINC00958 in colorectal cancer cell growth *in vivo*, the stable SW480 cells (sh-LINC00958 and sh-NC) and a nude mouse xenograft model were constructed. The knockdown efficiency was detected by qRT-PCR. The results showed that sh-LINC00958 significantly decreased the expression of LINC00958 in SW480 cell (Fig. 3a). Represent pictures of tumor formation of SW480 cells were shown in Fig. 3b. The weight of the SW480/sh-LINC00958 tumors was significantly lower than those of the SW480/sh-NC tumors (Fig. 3c). In addition, the volume of the SW480/sh-LINC00958 tumors was remarkably lower than those of the SW480/sh-NC tumors (Fig. 3d). Collectively, our results demonstrate that LINC00958 promotes colorectal cancer cell proliferation in vivo.

LINC00958 acts as miRNA sponge of miR-422a in colorectal cancer cells

Since lncRNAs exert biological functions mainly through acting as miRNA sponges, we explore whether LINC00958 promotes colorectal cancer progression by sponging miRNAs. To explore the potential miRNAs of LINC00958, TargerScan (http://www.targetscan.org) and StarBase (http://starbase.sysu.edu.cn) databases were employed to predict the possible miRNAs and binding sites of LINC00958. The two databases both showed that LINC00958 contained the binding site of miR-422a (Fig. 4a). qRT-PCR results showed that si-LINC00958 significantly increased the expression of miR-422a in SW480 cell, and overexpression of LINC00958 decreased miR-422a expression (Fig. 4b), implying the potential correlation between LINC00958 and miR-422a. To explore the correlation between LINC00958 and miR-422a, the level of miR-422a in colorectal cancer tissues was detected. The results showed that miR-422a expression was downregulated in most of colorectal cancer tissues (74.60%, 47/63) (Fig. 4c). Further analysis indicated that the level of LINC00958 was negatively correlated with the level of miR-422a in colorectal cancer tissues ($R=-0.5122$, $P < 0.01$) (Fig. 4d). To identify the subcellular location of LINC00958 in colorectal cancer cells, RNA-FISH assays were conducted with cy3-labeled LINC00958 probe. The results showed that most of LINC00958 was located in cytoplasm in SW480 cell (Fig. 4e). In addition, nuclear-cytoplasmic fractionation assays were also performed. The results showed that most of LINC00958 was located in cytoplasm (Fig. 4f). To identify whether LINC00958 directly interacted with miR-422a, the biotinylated RNA-pull down and RNA immunoprecipitation (RIP) assays were performed. The RNA-pull down assay showed that miR-422a was captured by wild type of LINC00958, but not the mutation of LINC00958 (Fig. 4g). The RIP assay was used to immunoprecipitate LINC00958 with an anti-AGO2 antibody or control IgG. The results demonstrated that LINC00958 was enriched by miR-422a mimics compared with negative control (Fig. 4h). These data demonstrated that LINC00958 directly interacted with miR-422a. Furthermore, to investigate the regulation of LINC00958 on miR-422a, we constructed two luciferase reporter plasmids with wild type of LINC00958 (WT) and mutation of LINC00958 (MUT) in which the binding site of LINC00958 on miR-422a was mutant (Fig. 4a). Luciferase reporter assays showed that miR-422a mimics significantly decreased the luciferase activity of wild type of LINC00958, but not the mutation of LINC00958 (Fig. 4i); while miR-422a inhibitor evidently increased the luciferase activity of wild type of LINC00958 (Fig. 4j). In general, these data demonstrated that LINC00958 acted as a miR-422a sponge through directly binding to MREs.

MiR-422a suppresses cell proliferation, increases cell apoptosis and radiosensitization of colorectal cancer through MAPK1

To explore the target gene of miR-422a, miRDB (http://mirdb.org), TargerScan and miRanda (http://www.miranda.org) databases were employed to predict potential target genes. All the three databases predicted that MAPK1 3'-untranslated region (3'-UTR) contained the binding site of miR-422a (Fig. 5a). Next, we explored whether MAPK1 is a target gene of miR-422a. qRT-PCR results showed that miR-422a mimics markedly decreased the expression of MAPK1, while miR-422a inhibitor significantly increased its expression (Fig. 5b). Western blot results indicated that overexpression of miR-422a decreased the expression of both ERK1/2 and p-ERK1/2 (MAPK1 protein), also decreased the expression of Bcl-2 and increased the expression of Bax, the downstream protein of ERK pathway; while knockdown of miR-422a increased the expression of ERK1/2, p-ERK1/2 and Bcl-2, but decreased the expression of

Bax (Fig. 5c). To explore the regulation mechanisms of miR-422a on MAPK1, luciferase reporter plasmids with wild type of MAPK1 mRNA 3'-UTR (WT) or mutant type of MAPK1 mRNA 3'-UTR (MUT) with mutant binding sites of MAPK1 on miR-422a were constructed (Fig. 5a). Luciferase reporter assays implied that overexpression of miR-422a decreased the luciferase activity of WT, but did not decrease that of MUT, while knockdown of miR-422a remarkably increased the luciferase activity of WT, but not that of MUT (Fig. 5d). In summary, these data proved that miR-422a negatively regulate MAPK1 expression by directly binding to MAPK1 3'-UTR.

To test effects of miR-422a on colorectal cancer cells, the flow cytometry, CCK-8, MTT and clone formation assays under different radiation dose were performed. The flow cytometry assay showed that miR-422a mimics significantly increased the apoptosis percentage (Fig. 5e), while miR-422a inhibitor remarkably decreased the apoptosis percentage (Fig. 5i). The CCK-8 and MTT results indicated that miR-422a mimics decreased the ability of cell proliferation (Fig. 5f and 5g), and miR-422a inhibitor increased it (Fig. 5j and 5k). In addition, overexpression of miR-422a enhanced the radiosensitization (Fig. 5h) of colorectal cancer cell, while down-regulation of miR-422a decreased the radiosensitization (Fig. 5l). In conclusion, these data confirmed that miR-422a suppressed cell proliferation, increased cell apoptosis and radiosensitization of colorectal cancer through targeting MAPK1.

LINC00958 promoted the progression of colorectal cancer through targeting miR-422a

It has been revealed that lncRNAs weaken miRNAs activity by competing shared MREs, thereby regulating expression of miRNAs' target genes. Therefore, we next explored whether LINC00958 could regulate the expression of MAPK1. Pearson's correlation analysis showed that the level of LINC00958 positively correlated with the level of MAPK1 in 63 paired colorectal cancer tissues (Fig. 6a). Knockdown of LINC00958 down-regulated the expression of ERK1/2 and pERK1/2 protein and MAPK1 mRNA, also down-regulated the expression of Bcl-2 protein and up-regulated the expression of Bax protein (Fig. 6b and 6c). Furthermore, si-LINC00958 decreased the luciferase activity of wild type of MAPK1 (Fig. 6d). These results implied that LINC00958 promoted MAPK1 expression.

To test the effects of miR-422a on the roles of LINC00958 in colorectal cancer, rescue experiments were performed. Western blot and qRT-PCR results showed that miR-422a inhibitor relieved the suppression of si-LINC00958 on the level of ERK1/2 and pERK1/2 (MAPK1 protein), also the level of Bcl-2 and Bax (Fig. 6b), and MAPK1 mRNA (Fig. 6c),while the si-LINC00958 + inhibitor group is not significantly different from the si-NC group (Fig. 6b and 6c), implying that the rescue experiments prove without doubt that the effects elicited by the LINC00958 on the MAPK1 pathway were purely via miR-422a. Moreover, miR-422a inhibitor rescued the suppression of si-LINC00958 on the luciferase activity of wild type of MAPK1 (Fig. 6d). Functionally, the flow cytometry assay showed that miR-422a inhibitor reversed the ability of si-LINC00958 to promote cell apoptosis, while the si-LINC00958 + inhibitor group is not significantly different from the si-NC group, implying that knockdown of LINC00958 increased the apoptosis percentage through miR-422a (Fig. 6e). The CCK-8 and MTT results indicated that si-LINC00958 decreased the ability of cell proliferation, while miR-422a inhibitor relieved the ability of si-LINC00958 to

inhibit cell proliferation (Fig. 6f and 6g). In addition, miR-422a inhibitor increased the survival fraction under different irradiation dose downregulated by si-LINC00958, while the si-LINC00958 + inhibitor group is not significantly different from the si-NC group (Fig. 6h). Overall, these data demonstrated that si-LINC00958 suppressed cell proliferation, increased cell apoptosis and radiosensitization of colorectal cancer through targeting miR-422a.

Discussion

Up to now, large numbers of lncRNAs have been revealed to play vital roles in the development and progression of various cancers. However, the functions and mechanisms of lncRNAs in cell proliferation, apoptosis and radiosensitization of colorectal cancer have not been identified. In this study, we found that LINC00958 promoted cell proliferation, suppressed cell apoptosis and radiosensitization in colorectal cancer. Mechanistically, LINC00958 served as a ceRNA to sponge miR-422a, thereby relieving its ability to suppress the expression of MAPK1.

In recent years, noncoding RNAs were attracting researchers' attentions in the exploration of biological process. As a class of endogenous noncoding RNAs, lncRNA has been being the hotspot of gene regulation, especially in tumor research. Abundant lncRNAs have been successfully confirmed in various cancer cell lines and different cancers[23–25]. Importantly, many lncRNAs have been identified to participate in various biological functions, such as cell proliferation, EMT, migration, invasion and radiosensitization. Tang et al. found that lncRNA AATBC was upregulated in nasopharyngeal carcinoma, positively correlated with poor prognosis, and promoted cell migration and invasion[26]. LncRNA HOTAIRM1 was reported to be downregulated in papillary thyroid cancer, significantly associated with lymph node metastasis and TNM stage and better prognosis, and suppress cell growth, invasion and migration[27]. In addition, Brownmiller et al. found significant difference in the expression of Y chromosome lncRNAs linc-SPRY3-2/3/4 between radiation sensitive and radiation resistant non-small cell lung cancer cells, and confirmed that knockdown of linc-SPRY3-2/3/4 promoted cell viability and resistance to apoptosis after treated with 8Gy[28]. In this study, we found that LINC00958 was upregulated in colorectal cancer tissues. Further analysis showed that the high level of LINC00958 was positively correlated with tumor differentiation, T stage and TNM stage, and predicted poor overall survival and disease-free survival. Functional experiments indicated that LINC00958 remarkably enhanced the cell growth and decreased the apoptosis percentage and radiosensitization of colorectal cancer cell in vitro, and promoted cell growth in vivo. Taken together, these results demonstrated that LINC00958 promoted cell proliferation and suppressed apoptosis and the radiosensitivity of colorectal cancer cells. Our data suggest that LINC00958 plays an oncogenic role in the progression of colorectal cancer and would serve as a promising diagnostic, prognostic and therapeutic marker for colorectal cancer.

Accumulating evidences demonstrated that lncRNAs are able to act as a ceRNA to sponge miRNAs, thereby protecting target genes from being suppressed or degraded[29–31]. The ceRNA role has been widely accepted to be the main mechanism for lncRNAs in the biological process. Wei et al. demonstrated

that lncRNA HOTAIR promoted cell growth through sponging miR-1277-5p and upregulating COL5A1 expression in gastric cancer[32]. Also, lncRNA-SOX2OT serves as a miRNA sponge of miR-122-5p, thereby enhancing the expression of PKM2[33]. However, the mechanisms of LINC00958 in the progression of colorectal cancer are still unidentified. Bioinformatics analysis showed that LINC00958 contained a binding site of miR-422a. Pearson's correlation showed the negative correlation between the level of LINC00958 and miR-422a. Next, biotinylated-RNA pull-down, RIP and luciferase reporter assays confirmed that LINC00958 could directly bind to miR-422a. Further rescued experiments identified that miR-422a reversed the roles of LINC00958 in colorectal cancer progression. These data suggested that LINC00958 may exert its biological function by sponging miR-422a in colorectal cancer.

MAPK1 is the main member of mitogen-activated protein kinase family, which is an important signal transmitter from cell surface to nucleus. MAPK1 pathway has been confirmed to participate in many biological processes, such as cell proliferation, apoptosis, migration and invasion[34]. The radiosensitivity is one of the main factors affecting the efficacy of radiotherapy for various cancers. Soma et al. revealed that MAPK1 pathway involved in radiosensitivity of multiple of tumors[35]. Bioinformatics analysis identified that MAPK1 mRNA 3'-UTR contained the binding site of miR-422a. In our study, a series of molecular experiments demonstrated that miR-422a suppressed the expression of MAPK1 through directly binding to 3'-UTR of MAPK1 mRNA, thereby suppressing cell proliferation, increasing cell apoptosis and radiosensitization of colorectal cancer. Additionally, we found that LINC00958 promoted the expression of MAPK1. Furthermore, rescue experiments demonstrated miR-422a reversed the regulation of LINC00958 on MAPK1 expression. These data revealed that LINC00958 served as a miR-422a sponge to relieve its suppression on MAPK1 expression and enhanced MAPK1 gene expression.

Conclusion

In conclusion, we identify that LINC00958 is upregulated in colorectal cancer tissues and positively correlated with clinical pathological features and poor prognosis. LINC00958 served as a miR-422a sponge and enhanced MAPK1 gene expression, thereby promoting cell proliferation and suppressing apoptosis and radiosensitivity. Our results provide an insight into the understanding of progression and radiosensitivity of colorectal cancer and suggest that LINC00958 may serve as a promising diagnostic, prognostic and therapeutic marker for colorectal cancer.

Declarations

Ethics approval and consent to participate

The studies involving human participants were approved by Institutional Review Boards of Henan Provence People's Hospital. Written informed consents were acquired from all the objects.

Consent for publication

Not applicable.

Competing interests

The authors confirm that there are no conflicts of interest.

Funding

This work was supported by Henan Province 2019 overseas Research Project of Health Science and Technology Talents (HWYX2019116), Henan Medical Science and Technology Research Project (No.201303132, No.201303163, No.2018020432), Construction project of medical service capacity improvement project of medical institutions directly under Henan Province (NO.CYQ20190073).

Authors' contributions

HL: Project administration; Methodology; Roles/Writing - original draft; Software; Visualization. QZ: Investigation; Formal analysis; Resources. ZZ: Data curation; Writing - review & editing. CZ: Project administration; Validation; Supervision. HZ: Conceptualization; Project administration; Validation.

Acknowledgements

None.

Availability of data and materials

Please contact the correspondence author for the data request.

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Figures

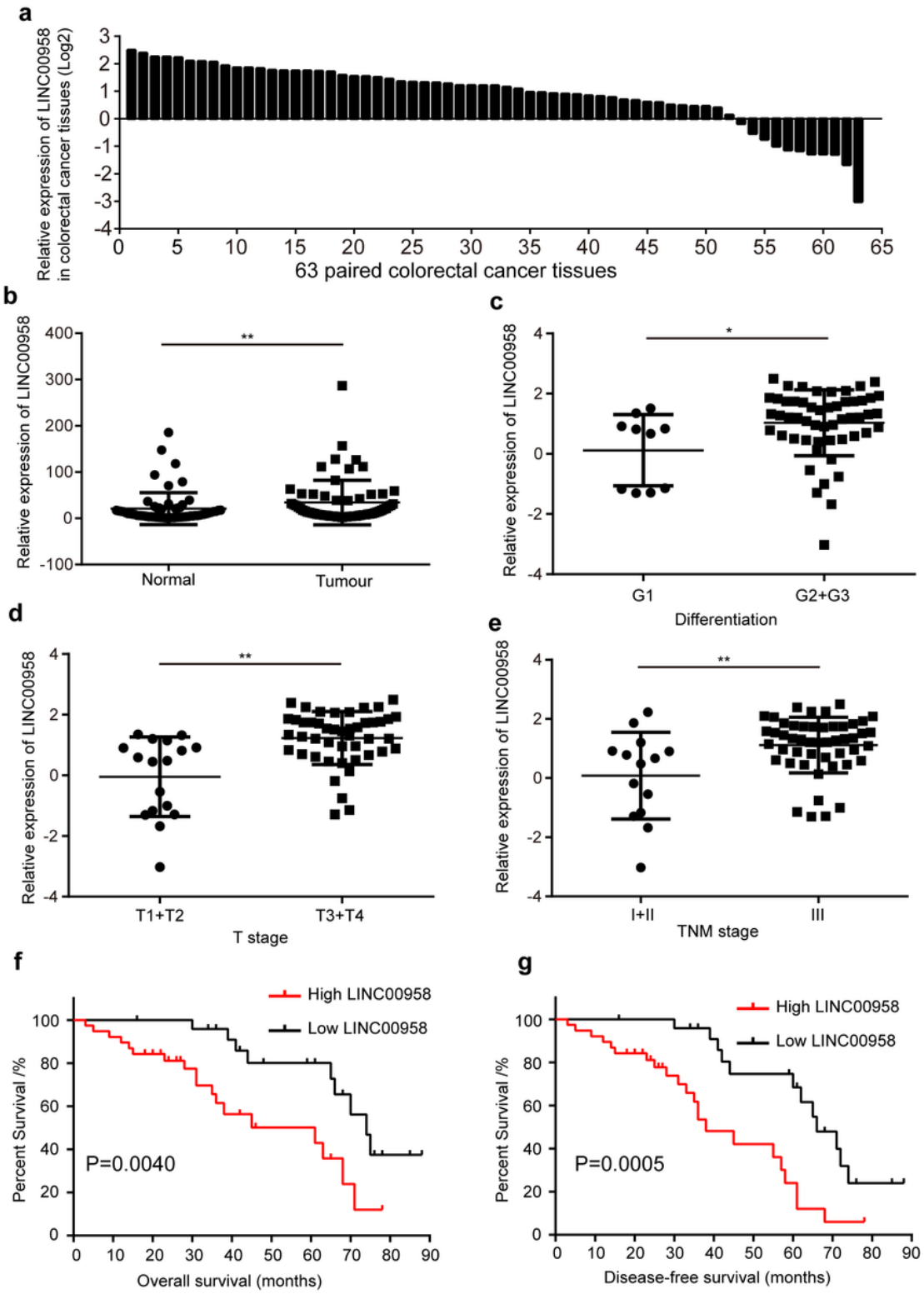

Figure 1

Features of LINC00958 in colorectal cancer tissues. (a) LINC00958 expression was upregulated in most (82.54%, 52/63) of colorectal cancer tissues. (b) LINC00958 expression was significantly higher in 63 colorectal cancer tissues than adjacent normal colorectal tissues. (c) The level of LINC00958 was remarkably higher in colorectal cancer tissues at moderate and poor differentiation (G2 and G3) than tissues at well differentitaion (G1). (d) The level of LINC00958 was significantly higher in colorectal cancer tissues at T3 and T4 stages than these at T1 and T2 stages. (e) LINC00958 was higher in colorectal cancer tissues at III and IV stages (TNM stage) than these at I and II stages. (f) and (g) Kaplan-Meier Plotter analysis revealed that high LINC00958 level predicted a poorer overall survival (f) and disease-free survival (g) for colorectal cancer patients. **p<0.01.

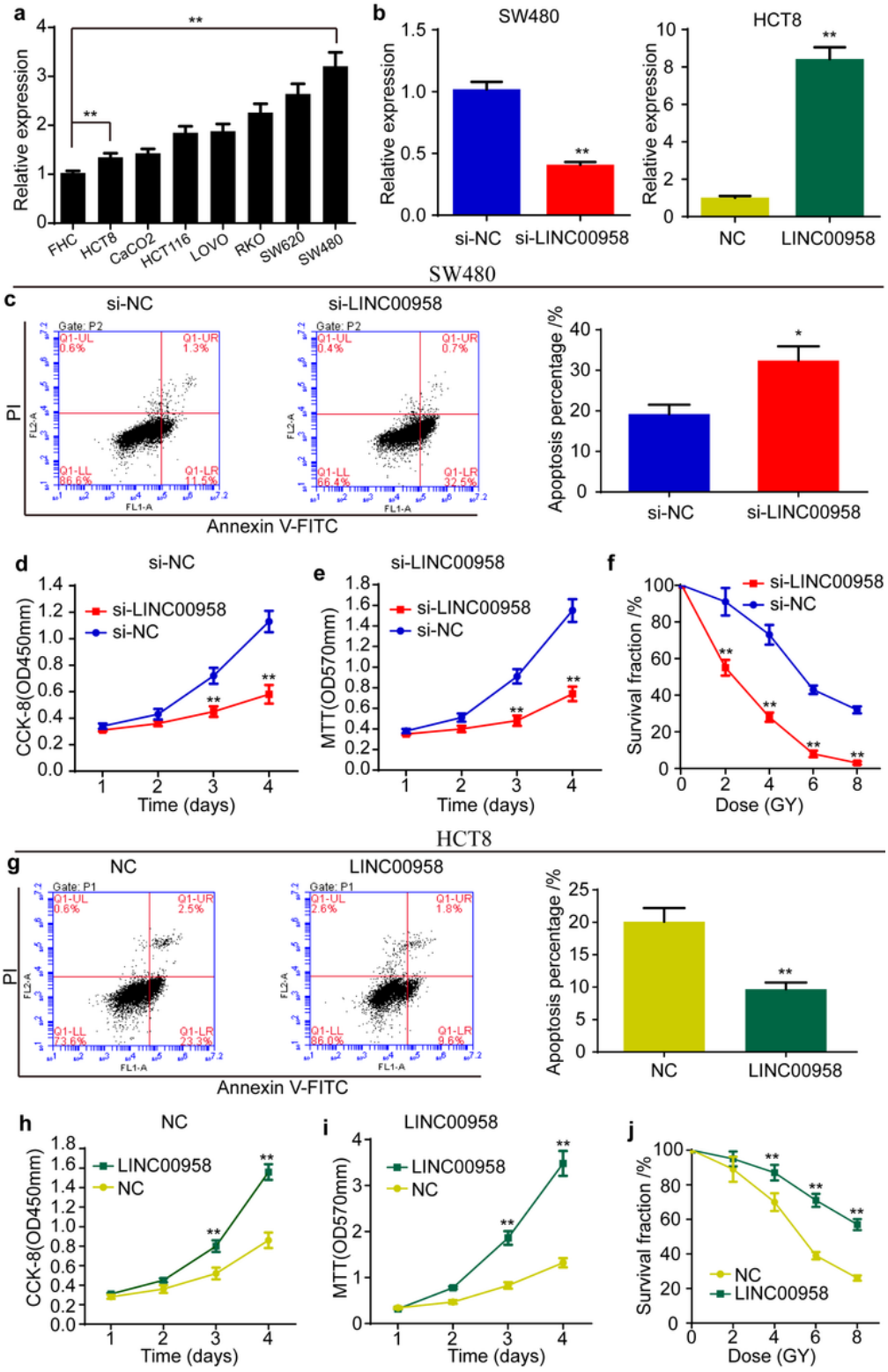

Figure 2

LINC00958 promotes cell proliferation, suppresses cell apoptosis and radiosensitization of colorectal cancer. (a) The expression of LINC00958 in colorectal cancer cell lines. (b) The efficacies of siRNA targeting LINC00958 and an overexpression plasmid of LINC00958 were detected. (c) The flow cytometry assay showed that knockdown of LINC00958 significantly increased the apoptosis percentage. (d) and (e) The CCK-8 (d) and MTT (e) results indicated that si-LINC00958 decreased the ability of cell

proliferation. (f) si-LINC00958 decreased the survival fraction under different irradiation dose. (g) The flow cytometry assay showed that overexpression of LINC00958 remarkably decreased the apoptosis percentage. (h) and (i) The CCK-8 (h) and MTT (i) results indicated that overexpression of LINC00958 increased the ability of cell proliferation. (j) Overexpression of LINC00958 increased the survival fraction under different irradiation dose. Data are reported as means ± standard deviation of three independent experiments. *p<0.05; **p<0.01.

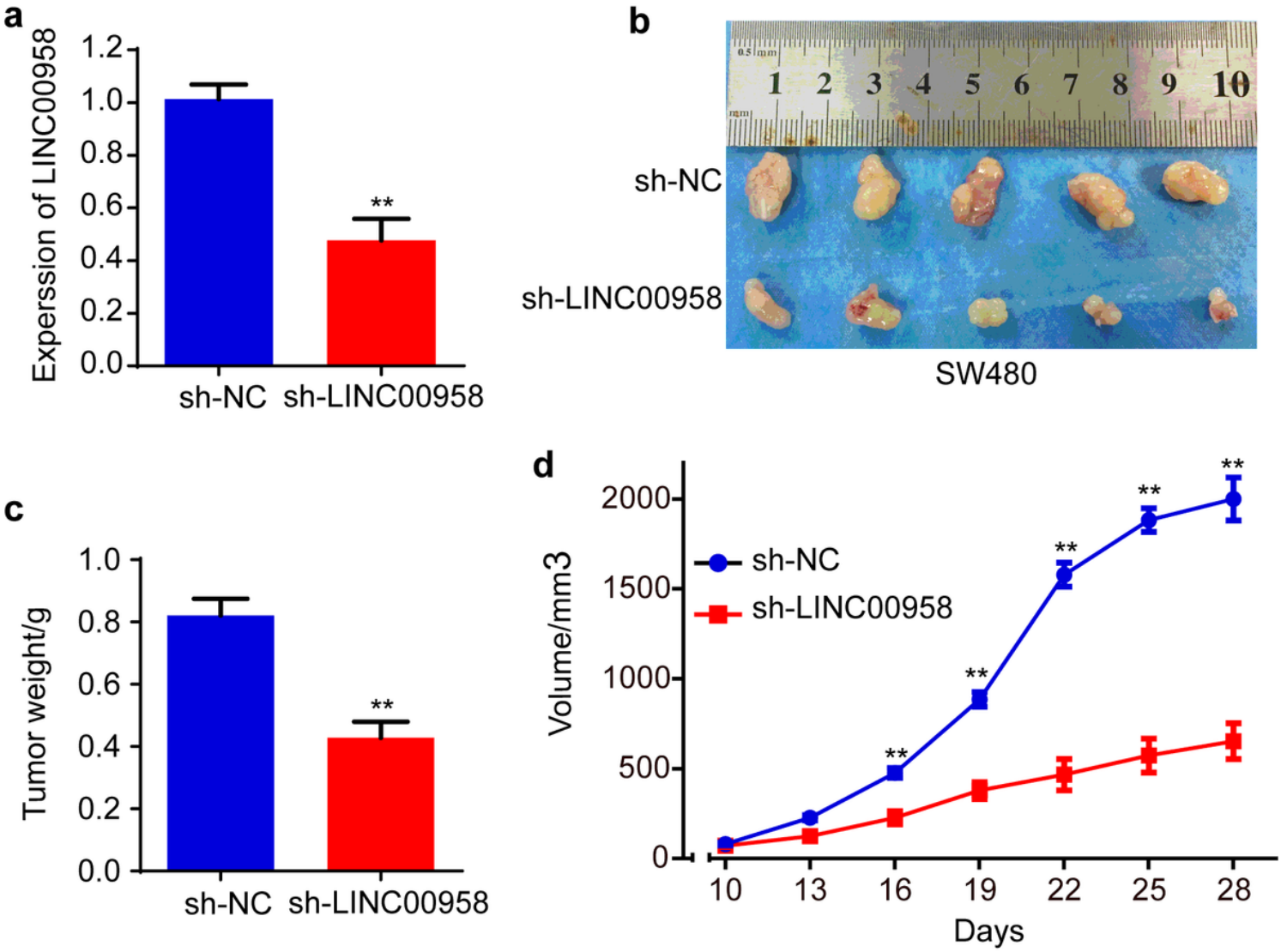

Figure 3

LINC00958 promotes colorectal cancer cell proliferation in vivo. (a) The knockdown efficiency of stable SW480/sh-LINC00958 cells was detected by qRT-PCR. (b) Represent pictures of tumor formation of SW480 cells. (c) The final tumor weight of SW480 cells was shown. (d) Tumor volumes of SW480 cells were measured every three days. **p<0.01.

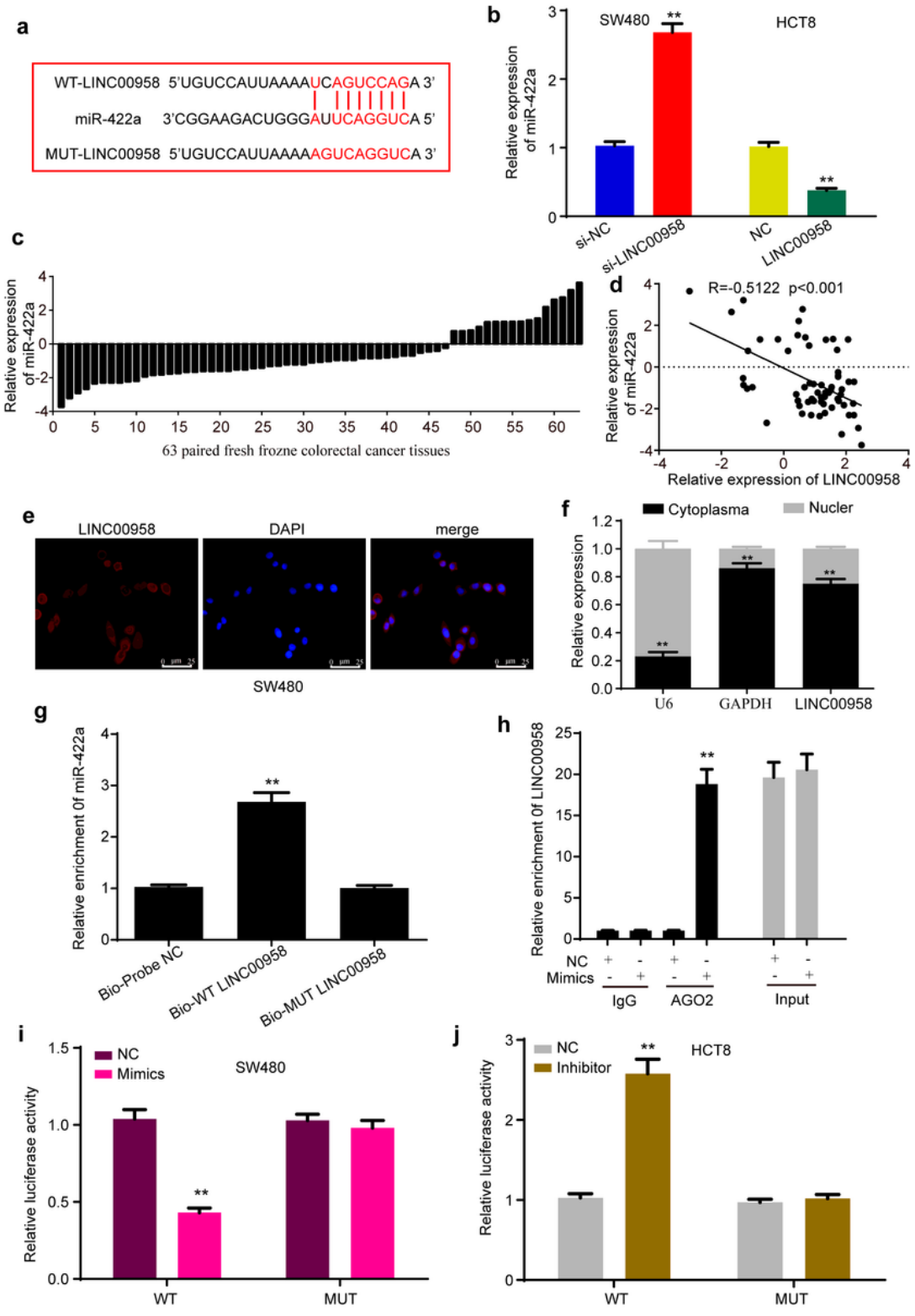

Figure 4

LINC00958 acts as a miRNA sponge of miR-422a. (a) Bioinformatics databases predicted that LINC00958 contained the binding site of miR-422a. (b) Expression of miR-422a after knockdown or overexpression of LINC00958 in colorectal cancer cells. (c) The expression of miR-422a in 63 paired colorectal cancer tissues. (d) Pearson's correlation showed that miR-422a level negatively correlated with LINC00958 level in 63 paired colorectal cancer tissues (R=-0.5122, p<0.001). (e) RNA-FISH assays

showed that most of LINC00958 was located in cytoplasm in SW480 cell. (f) qRT-PCR results of U6, GAPDH and LINC00958 expressions in cell nucler and cytoplasm. (g) The biotinylated RNA pull-down showed that miR-422a was pulled down by LINC00958 probe, but not by mutant LINC00958 probe, in SW480 cells. (h) RIP assay was performed with AGO2 antibody in SW480 cells transfected with miR-422a mimics or NC, and the enrichment of LINC00958 was detected. (i) miR-422a mimics significantly decreased the luciferase activity of wild type of LINC00958, but not the activity of mutation of LINC00958. (j) miR-422a inhibitor evidently increased the luciferase activity of wild type of LINC00958, but not it of mutation of LINC00958. Three independent experiments were performed for each group. All data are reported as the mean ± SD. **P < 0.01.

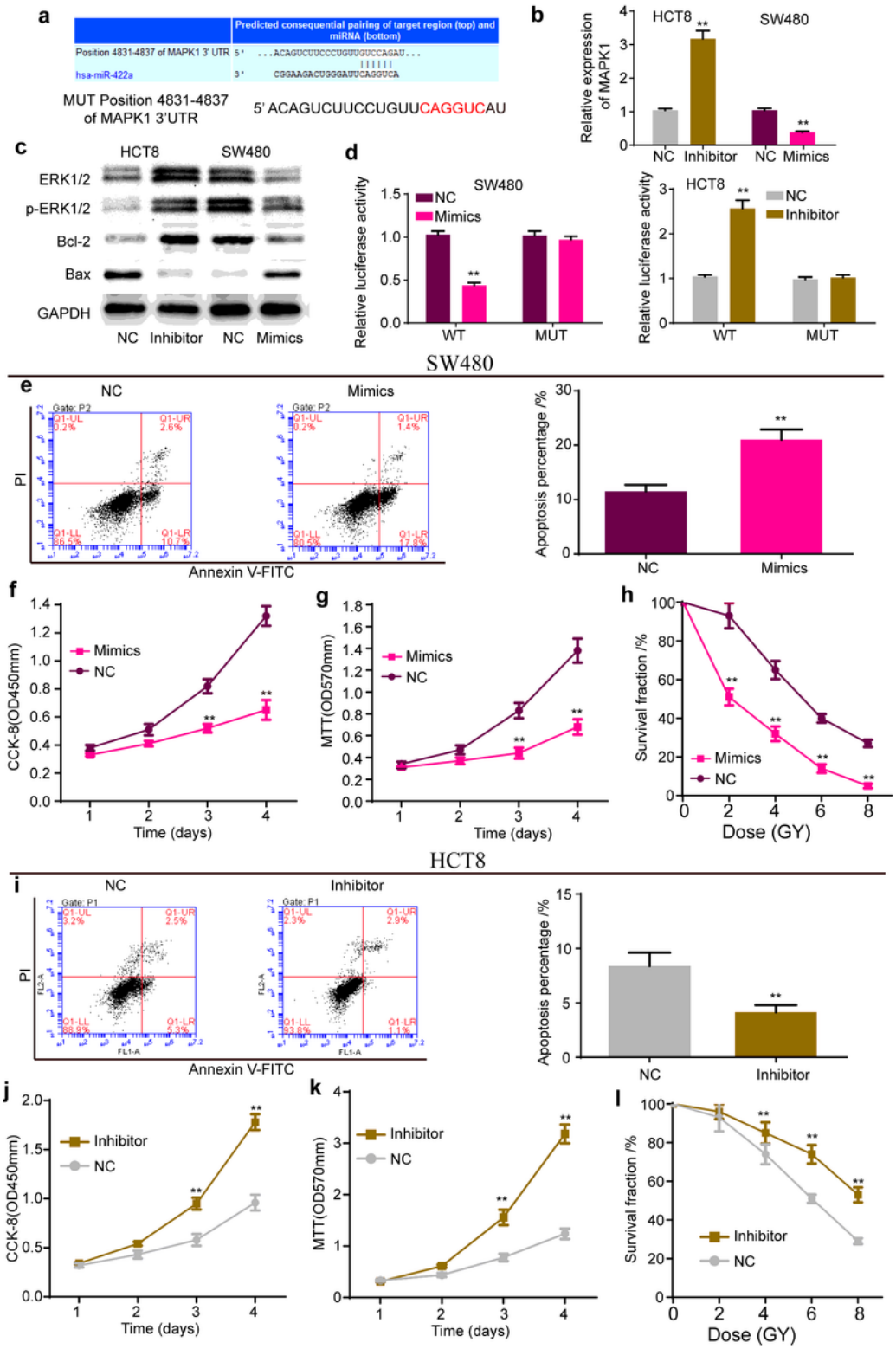

Figure 5

miR-422a suppresses cell proliferation, increases cell apoptosis and radiosensitization of colorectal cancer through MAPK1. (a) Bioinformatics databases predicted that MAPK1 was a target of miR-422a. (b) and (c) mRNA and protein expression of MAPK1 after knockdown or overexpression of miR-422a in colorectal cancer cells. (d) miR-422a mimics significantly decreased the luciferase activity of wild type of MAPK1, but not the activity of mutation of MAPK1. In addition, miR-422a inhibitor evidently increased the

luciferase activity of wild type of MAPK1, but not it of mutation of MAPK1. (e) and (i) The flow cytometry assay showed that miR-422a mimics significantly increased the apoptosis percentage (e), while miR-422a inhibitor remarkably decreased the apoptosis percentage (i). (f-g) and (j-k) The CCK-8 and MTT results indicated that miR-422a mimics decreased the ability of cell proliferation, and miR-422a inhibitor increased it. (h) and (l) miR-422a mimics enhanced the radiosensitization of colorectal cancer cell (h), while miR-422a inhibitor decreased the radiosensitization (l). Data are reported as means ± standard deviation of three independent experiments. **P<0.01.

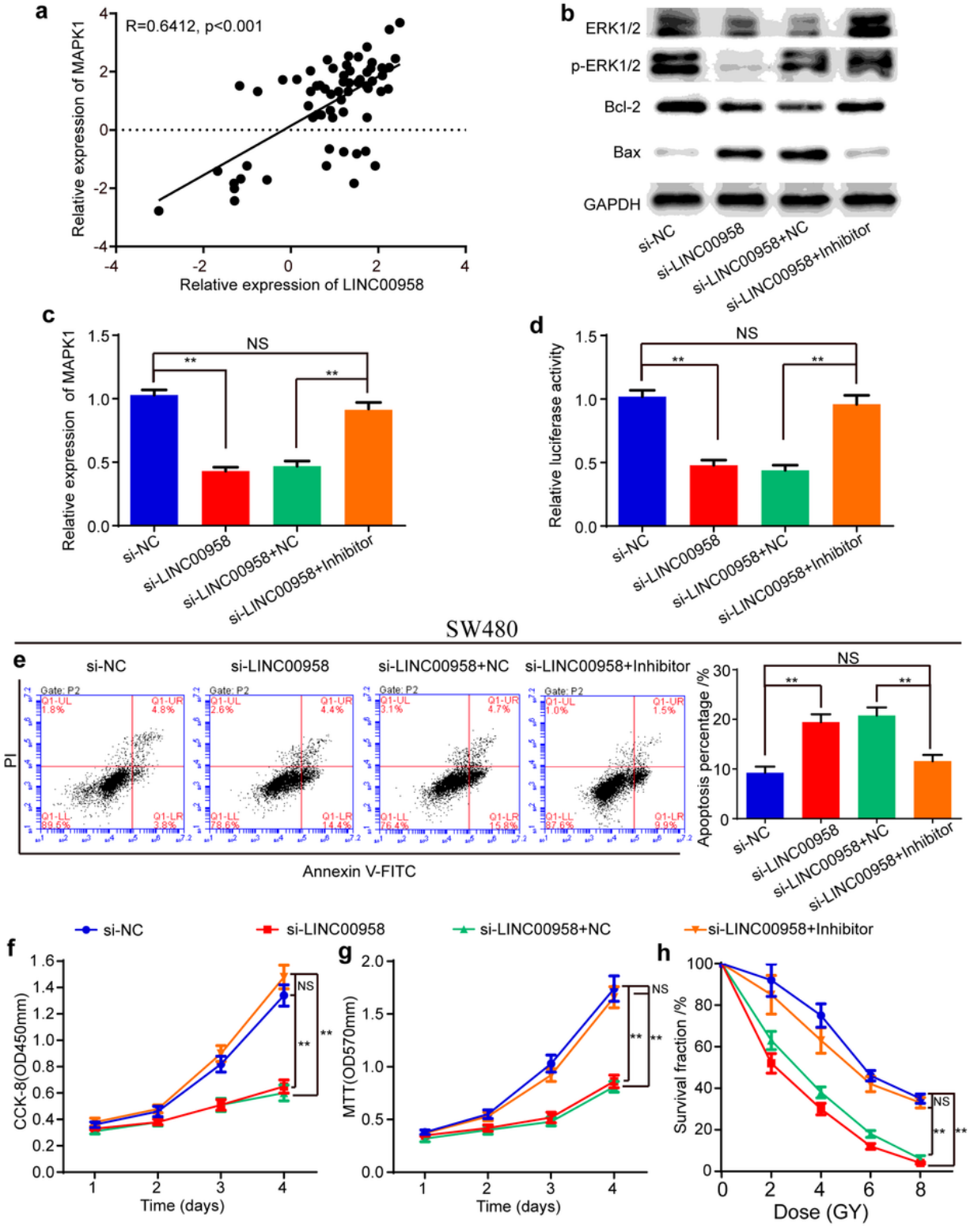

Figure 6

LINC00958 promotes MAPK1 expression and progression of colorectal cancer through miR-422a. (a) Pearson's correlation showed that LINC00958 level positively correlated with MAPK1 level in 63 paired colorectal cancer tissues (R=0.6412, p<0.001). (b) The expression of MAPK1 protein ERK1/2 pERK1/2, Bcl-2 and Bax after knockdown of LINC00958 and miR-422a in SW480 cells. (c) The mRNA expression of MAPK1 after knockdown of LINC00958 and miR-422a in SW480 cells. (d) The relative luciferase activity of wild type of MAPK1 mRNA 3'-UTR after knockdown of LINC00958 and miR-422a in SW480 cells. (e) The results of flow cytometry assay after knockdown of LINC00958 and miR-422a in SW480 cells. (f) and (g) The CCK-8 (f) and MTT (g) results after knockdown of LINC00958 and miR-422a in SW480 cells. (h) The survival fraction under different irradiation dose after knockdown of LINC00958 and miR-422a in SW480 cells. Data are reported as means ± standard deviation of three independent experiments. **p<0.01.