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ORIGINAL RESEARCH

RETRACTED ARTICLE: Downregulation of Circ_0071589 Suppresses Cisplatin Resistance in Colorectal Cancer by Regulating the MiR-526b-3p/KLF12 Axis

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Background: Chemoresistance is one key factor for the failure of cisplatin (CDDP)-based therapy in colorectal cancer (CRC). Although circular RNAs (circRNAs) are associated with chemoresistance development, the role and mechanism of hsa_circ_0071589 (circ_0071589) in the development of CDDP resistance in CRC remain unclear.

Methods: CDDP-resistant and sensitive CRC samples were collected. CDDP-resistant HCT116/CDDP and LOVO/CDDP cells were established. The levels of circ_0071589, microRNA (miR)-526b-3p and Kruppel-like factor 12 (KLF12) were detected via quantitative reverse transcription polymerase chain reaction, Western blot or immunohistochemistry. Cell viability, proliferation, cycle process, apoptosis, migration and invasion were examined via Cell Counting Kit-8, flow cytometry, transwell assay and Western blot. The association between miR-526b-3p and circ_0071589 or KLF12 was predicted by starBase, and explored via dual-luciferase reporter assay and RNA immunoprecipitation. The effect of circ_0071589 on CDDP resistance in CRC in vivo was investigated using a xenograft model.

Results: Circ_0071589 level was upregulated in CDDP-resistant CRC tissue samples and cell lines. Circ_0071589 knockdown inhibited CDDP resistance, proliferation, migration and invasion and promoted apoptosis in CDDP-resistant CRC cells. Circ_0071589 was a sponge for miR-526b-3p. MiR-526b-3p knockdown reversed the role of circ_0071589 inhibition in CDDP resistance. MiR-526b-3p suppressed CDDP resistance by directly targeting KLF12. Circ_0071589 regulated KLF12 expression through modulating miR-526b-3p. Circ_0071589 knockdown aggravated CDDP-induced reduction of xenograft tumor growth by upregulating miR-526b-3p and decreasing KLF12.

Conclusion: Knockdown of circ_0071589 repressed CDDP resistance in CDDP-resistant CRC cells by regulating the miR-526b-3p/KLF12 axis.

Keywords: colorectal cancer, circ_0071589, miR-526b-3p, KLF12, cisplatin resistance

Introduction

Colorectal cancer (CRC) is a common tumor malignancy worldwide.[1] Surgery is the cornerstone for the treatment options of CRC. However, there are many patients who are diagnosed at stage III or IV when patients' survival drops. Chemotherapy coupled with surgery improves the survival.[2] Cisplatin (CDDP) is involved in the DNA-damage response, and leads to cancer cell death.[3] CDDP-based chemotherapy is commonly employed for clinical option which is given intravenously as short-term infusion in physiological saline, but the adaptive response may induce the incidence of chemoresistance by reducing the anti-proliferative and cytotoxic effect of CDDP.[4]

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According to the treatment response, patients are sensitive or resistant to CDDP. The primary resistant patients do not respond to CDDP, and the acquired resistant patients develop chemoresistance, limiting the success of therapy and leading to treatment failure and tumor recurrence.[5] Hence, it is necessary to find novel targets for improving the cisplatin sensitivity in CRC.

Circular RNAs (circRNAs) are a type of noncoding RNAs with a closed continuous loop, and are widely expressed in mammalian cells.[6] CircRNAs can be implicated in cancer progression through regulating microRNAs (miRNAs) and mRNAs.[7] Moreover, circRNAs play possible roles in the development of CRC.[8] In addition, multiple circRNAs, such as hsa_circ_0000285, hsa_circ_0060060, hsa_circ_101505 and hsa_circ_0076305, have been reported to regulate CDDP resistance in human cancers.[9–12] Hsa_circ_0071589 (circ_0071589) is a circRNA derived from FAT atypical cadherin 1 gene, which promotes carcinogenesis in CRC.[13] Additionally, this circRNA is dysregulated in the resistant CRC tissues in our preliminary experiments. However, whether and how circ_0071589 regulates CDDP resistance in CRC remain unclear.

MiRNAs are 19-to-22-nucleotide noncoding RNA which are involved in the regulation of CRC development by modulating cell proliferation, apoptosis, migration and invasion.[14] MiR-526b-3p has been shown to play a tumor-suppressive role in many tumors, such as cervical cancer and glioma.[15,16] More importantly, miR-526b-3p suppresses proliferation, metastasis and glycolysis in CRC.[17] Nevertheless, whether miR-526b-3p can regulate CDDP resistance and whether miR-526b-3p is required for circ_0071589 in CRC are unknown. Furthermore, Krüppel-like factor 12 (KLF12) is an oncogene in human cancers.[18–20] Meanwhile, KLF12 enhances CDDP resistance in CRC.[20] Interestingly, starBase online predicted circ_0071589 might modulate KLF12 by regulating miR-526b-3p due to the complementary sequence. Thus, we assumed circ_0071589 might target KLF12 to regulate CDDP resistance in CRC by sponging miR-526b-3p.

In this study, we investigated the effect of the circ_0071589/miR-526b-3p/KLF1 axis on CDDP resistance in CDDP-resistant CRC cells. These findings might provide a new mechanism underlying CDDP resistance in CRC.

Materials and Methods

Patient Tissues

CRC tissue samples were obtained from 37 patients with CDDP-resistant and 19 with CDDP-sensitive from Hainan General Hospital (Haikou, China). All patients were subjected to surgical resection and CDDP therapy. Patients with CDDP-resistant CRC were defined as those with persistent disease or recurrent more than 2 months; patients with CDDP-sensitive CRC were defined as those without local residual lesions or recurrence at 2 months after CDDP therapy. The corresponding adjacent normal tissues (5-cm away from the CRC) were used as control (n=56). The tissue specimens were verified by histopathological examination. Tumor tissues were stored at −80°C until use. The written informed consents were provided for all patients. The clinical characters of CRC patients are shown in Table 1. This work was permitted via the Ethics Committee of Hainan General Hospital, and conducted in accordance with the Declaration of Helsinki.

Cell Culture and Treatment

CRC cell lines (HCT116 and LOVO) and normal human colorectal mucosa cells FHC were kindly provided by

Table 1 The Clinical Information of Patients

Clinical Features	Case Number	CRC Patients (N = 56)		P value
		CDDP-Resistant (N = 37)	CDDP-Sensitive (N = 19)	
Gender				
Male	29	18	11	0.512
Female	27	19	8	
Age (years old)				
<60	31	18	13	0.159
≥60	25	19	6	
Tumor size (cm)				
≤5	32	17	15	0.018*
>5	24	20	4	
Stage				
III	35	18	17	0.003*
II	21	19	2	

Note: *Statistical significance when P value was smaller than 0.05.

Bena Culture Collection (Beijing, China). All cells were grown at 37°C in 5% CO_2 in DMEM (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco) and 1% antibiotic (Thermo Fisher, Waltham, MA, USA). To establish the CDDP-resistant CRC cell lines (HCT116/CDDP and LOVO/CDDP), HCT116 and LOVO cells were incubated with the increasing concentrations of CDDP (Sigma-Aldrich, St. Louis, MO, USA) from 100 nM, until cells acquired the resistance to 2 μM CDDP. Before the experiments, HCT116/CDDP and LOVO/CDDP cells were cultured in medium without CDDP for 2 weeks.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

RNA extraction from tissues or cells was performed through TRIzol (Invitrogen, Carlsbad, CA, USA). For circRNA extraction, the isolated RNA was further treated with RNase R (Geneseed, Guangzhou, China) at 37°C. Reverse transcription was conducted to synthesize cDNA using a specific reverse transcription kit (Thermo Fisher). The cDNA was employed for qRT-PCR along with SYBR Green (TaKaRa, Dalian, China) and specific primers (Genscript, Nanjing, China). The primer sequences were shown in Table 2. GAPDH (for circ_0071589 and KLF12) and U6 (for miR-526b-3p) were used as references. Relative RNA expression was detected via delta-delta cycle threshold method.[21]

Cell Transfection

KLF12 overexpression vector (pcDNA-KLF12) was based on pcDNA3.1 vector. The empty vector (Thermo Fisher) was exploited as negative control (pcDNA-NC). siRNA for circ_0071589 (si-circ_0071589, 5′-UGACUAUGACAUGUCAGCUU-3′), siRNA negative control (si-NC, 5′-UUCUCCGAACGUGUCACGUUU-3′), miR-526b-3p mimic (5′-GAAAGUGCUUCCUUUUAGAGGC-3′), mimic negative control (miR-NC, 5′-CGAUCGCAUCAGCAUCGAUUGC-3′), miR-526b-3p inhibitor (anti-miR-526b-3p, 5′-GCCUCUAAAAGGAAGCACUUUC-3′), and inhibitor negative control (anti-miR-NC, 5′-UGAGCUGCAUAGAGUAGUGAUUA-3′) were synthesized via RiBoBio (Guangzhou, China). These constructed vectors or oligonucleotides were transfected into HCT116/CDDP and LOVO/CDDP cells using Lipofectamine 3000 reagent (Thermo Fisher) for 24 h.

Cell Counting Kit-8 (CCK-8)

IC50 of CDDP and proliferative ability were measured utilizing CCK-8 (Solarbio, Beijing, China). To analyze the IC50 of CDDP, cells (2×10^4 cells/well) were dispersed into 96-well plates overnight, and incubated with different doses of CDDP (0–30 μM) for 48 h. Next, cells were incubated with 10 μL CCK-8 for 4 h. The absorbance at 450 nm was detected with a microplate reader (BioGene Technology, Guangzhou, China). Cell viability was normalized to the non-CDDP group × 100%, and IC50 of CDDP was analyzed according to the viability curve. The experiments were performed 3 times.

For proliferative ability analysis, 1×10^4 HCT116/CDDP and LOVO/CDDP cells were added in 96-well plates, and nurtured for different times (0, 24, 48, or 72 h). Then, cells were incubated with 10 μL CCK-8 for 4 h. The optical density (OD) value at 450 nm was examined using a microplate reader.

Flow Cytometry

For cycle distribution assay, HCT116/CDDP and LOVO/CDDP cells (2×10^5 cells/well) were added in 12-well plates for 72 h. Next, cells were fixed, and stained using propidium iodide (PI). Cells in different cycle processes

Table 2 The Primer Sequences for qRT-PCR in this Study

Gene	Sequence	
	Forward (5'-3')	Reverse (5'-3')
miR-526b-3p	GAAAGTGCTTCCTTTT	GAACATGTCTGCGTATCTC
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
circ_0071589 (Divergent)	CAAACTCCCCTTCTGACAGC	CCGAATCACACTGACAAACG
circ_0071589 (Convergent)	GGCGGAACATGCTTTGACAG	TGGCTGCAGTTGCAGTGATA
KLF12	GCAGCTTCTGTTCAGGATCAAT	AAGTCCACTGGCTCAGTTTGT
GAPDH	ACAGTCAGCCGCATCTTCTT	TTCCCGTTCTCAGCCTTGAC

Abbreviations: qRT-PCR, quantitative reverse transcription polymerase chain reaction; miR, microRNA; circ_0071589, hsa_circ_0071589; KLF12, Krüppel-like factor 12; GAPDH, glyceraldehyde-3phosphate dehydrogenase.

were analyzed through a flow cytometer (Agilent, Hangzhou, China).

Annexin V-FITC apoptosis kit (Solarbio) was used for cell apoptosis assay by flow cytometry. HCT116/CDDP and LOVO/CDDP cells (2×10^5 cells/well) were added into 12-well plates, and cultured for 72 h. Then, cells were collected by trypsin, and resuspended in the binding buffer. Next, cells were dyed with 5 μL Annexin V-FITC and PI for 10 min. Apoptotic cells were examined with a flow cytometer, and apoptotic rate was presented as the percentage of cells (Annexin V-FITC^{+} and PI$^{-/+}$). This experiment was performed 3 times.

Transwell Assay

Cell migration and invasion were measured via transwell assay. The transwell chambers (Corning Costar, Corning, NY, USA) were coated with Matrigel for invasion assay, and uncoated for migration assay. HCT116/CDDP and LOVO/CDDP cells (1×10^4 cells/well for migration assay; and 5×10^4 cells/well for invasion assay) in serum-free medium were dispersed into upper chambers, while lower chambers were infused with complete medium containing 10% serum. Following culture for 24 h, migratory or invasive cells were dyed with 0.5% crystal violet (Beyotime). The stained cells were imaged via a 100× magnification microscope (Nikon, Tokyo, Japan). The number of migratory or invasive cells was counted with ImageJ software (NIH, Bethesda, MD, USA). Three replicates were conducted.

Western Blot

Protein was isolated from tissues or cells using RIPA lysis buffer (Boster, Wuhan, China) and quantified via a BCA assay kit (Beyotime). Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes (Solarbio). After blocking in 5% fat-free milk, the membranes were incubated with primary and secondary antibodies, followed via incubation of ECL Western Blotting Substrate (Solarbio). The antibodies were provided by Abcam (Cambridge, MA, USA), including: anti-CyclinD1 (ab226977, 1:1000 dilution), anti-B-cell lymphoma-2 (Bcl-2) (ab196495, 1:2000 dilution), anti-Cleaved-caspase-3 (Cleaved-casp-3) (ab2302, 1:2000 dilution), anti-KLF12 (ab129459, 1:5000 dilution), and anti-GAPDH (ab22555, 1:5000 dilution), and horseradish peroxidase (HRP)-conjugated IgG (ab97051, 1:5000 dilution). GAPDH was used as a reference control. The bolts were visualized via film, and analyzed by Image J software. Relative protein level was normalized to the control group. The experiments were carried out 3 times.

Dual-Luciferase Reporter Analysis

starBase (http://starbase.sysu.edu.cn/) was used to search the complementary sites between miR-526b-3p and circ_0071589 or KLF12. The wild-type sequence of circ_0071589 or KLF12 3′UTR containing binding sites of miR-526b-3p was cloned into psiCHECK-2 (Promega, Madison, WI, USA) to form the corresponding luciferase reporter vectors (WT-circ_0071589 and KLF12 3′UTR-WT). The mutant-type MUT-circ_0071589 and KLF12 3′UTR-MUT were obtained via mutating the seed sites. These constructed luciferase reporter vectors were co-transfected with miR-526b-3p mimic or miR-NC into HCT116/CDDP and LOVO/CDDP cells for detection of luciferase activity using a dual-luciferase assay kit (Promega).

RNA Immunoprecipitation (RIP)

A Magna RIP kit (Sigma-Aldrich) was utilized for RIP assay. HCT116/CDDP and LOVO/CDDP cells were lysed, and incubated with Ago2- or IgG-coated magnetic beads overnight. The levels of circ_0071589 and miR-526b-3p were detected by qRT-PCR.

Xenograft Model

BALB/c nude mice (male, 5-week-old) were obtained from Vital River (Beijing, China). The lentiviral vector carrying short hairpin RNA for circ_0071589 (sh-circ_0071589) or negative control (sh-NC) was synthesized via RiBoBio. HCT116/CDDP cells (5×10^6 cells/mouse) stably transfected with sh-circ_0071589 or sh-NC were inoculated into mice in the flanks via subcutaneous injection. After 7 days, mice were intraperitoneally injected with CDDP (5 mg/kg twice a week). Tumor volume was detected every 4 days and calculated as: length (mm) × width^2 (mm^2)/2. After cell injection for 27 days, mice were killed by cervical dislocation. Tumor samples were weighed, and harvested to examine the levels of circ_0071589, miR-526b-3p and KLF12. The animal experiments were conducted in line with the Guide for the Care and Use of Laboratory Animals (NIH Publications), and performed under the approval of the Ethics Committee of Hainan General Hospital.

Immunohistochemistry

Xenograft tissues were fixed with 4% paraformaldehyde (Solarbio), embedded in paraffin, and cut in 4-μm sections, followed by blocking using 3% H_2O_2 (Thermo Fisher). The sections were nurtured with anti-KLF12 (ABP52850, 1:100 dilution; Abbkine, Wuhan, China) for 6 h, and HRP-conjugated IgG (ab97051, 1:1000 dilution) for 2 h, and then dyed with diaminobenzidine (DAB; Beyotime). The section was observed with a microscope.

Statistical Analysis

GraphPad Prism 7 (GraphPad, La Jolla, CA, USA) was used for statistical analysis. The experiments were repeated 3 times. Data were normally distributed and exhibited as mean ± standard error of the mean. The linear correlation among circ_0071589, miR-526b-3p and KLF12 was analyzed via Pearson coefficient analysis. The difference was analyzed by Student's *t*-test or ANOVA followed via Tukey post hoc test as appropriate. $P<0.05$ was considered as statistically significant.

Results

Circ_0071589 Level is Enhanced in CDDP-Resistant CRC Tissues and Cells

To explore whether circ_0071589 was involved in CDDP resistance in CRC, circ_0071589 level was detected in CDDP-resistant CRC tissues and cells. By analyzing circ_0071589 expression level using divergent or convergent primers on cDNA or gDNA, we found circ_0071589 was amplified by the divergent primers on cDNA and not on gDNA, indicating the circular structure of circ_0071589 (Supplementary Figure S1). Table 1 displays CDDP resistance might be associated with the tumor size and stage ($P<0.05$). Circ_0071589 level was evidently increased in CDDP-resistant (n=37) or sensitive patients (n=19) compared with control samples (n=56), and circ_0071589 expression was higher in resistant tissues than sensitive samples (Figure 1A). Moreover, the CDDP-resistant cells (HCT116/CDDP and LOVO/CDDP) were established. As shown in Figure 1B and C, circ_0071589 expression was enhanced in HCT116 and LOVO cells compared with FHC cells, and it was higher in HCT116/CDDP and LOVO/CDDP cells. Furthermore, the IC50 of CDDP was clearly increased in HCT116/CDDP and LOVO/CDDP cells compared with HCT116 and LOVO cells, suggesting HCT116/CDDP and LOVO/CDDP cells had higher resistance to CDDP (Figure 1D and E). These results suggested that increased expression of circ_0071589 might be associated with CDDP resistance in CRC.

Circ_0071589 Knockdown Inhibits CDDP Resistance, Proliferation, Migration and Invasion, and Promotes Apoptosis in CDDP-Resistant CRC Cells

To explore the role of circ_0071589 in CDDP-resistant cell development, circ_0071589 level was knocked known by transfection of si-circ_0071589 in HCT116/CDDP and LOVO/CDDP cells. The knockdown efficacy of si-circ_0071589 was validated in Figure 2A. Moreover, circ_0071589 knockdown suppressed CDDP resistance by decreasing the IC50 of CDDP to HCT116/CDDP and LOVO/CDDP cells (Figure 2B and C). In addition, we measured cell proliferation, apoptosis, migration and invasion in the two cell lines. Silencing circ_0071589 evidently decreased cell proliferation by inducing cycle arrest at G0/G1 phase, and promoted apoptosis of the two resistant cells (Figure 2D–H). Furthermore, downregulation of circ_0071589 obviously restrained the abilities of migration and invasion in HCT116/CDDP and LOVO/CDDP cells (Figure 2I and J). Additionally, the apoptotic-related proteins were measured by Western blot. Results exhibited that reduction of CycinD1 and Bcl-2, and elevation of Cleaved-casp-3 were induced by circ_0071589 interference in HCT116/CDDP and LOVO/CDDP cells (Figure 2K and L). These data indicated that circ_0071589 silence suppressed CDDP resistance and development in CDDP-resistant CRC cells.

Circ_0071589 is a Sponge for MiR-526b-3p

To explore the mechanism of circ_0071589 in CRC resistance, the target of circ_0071589 was predicted via starBase. Five predicted targets were selected, and miR-526b-3p expression was upregulated most by circ_0071589 knockdown (Supplementary Figure S2A and B). Hence, miR-526b-3p was selected for further studies. The complementary sequence between circ_0071589 and miR-526b-3p is shown in Figure 3A. To confirm the target correlation between circ_0071589 and miR-526b-3p, we constructed WT-circ_0071589 and

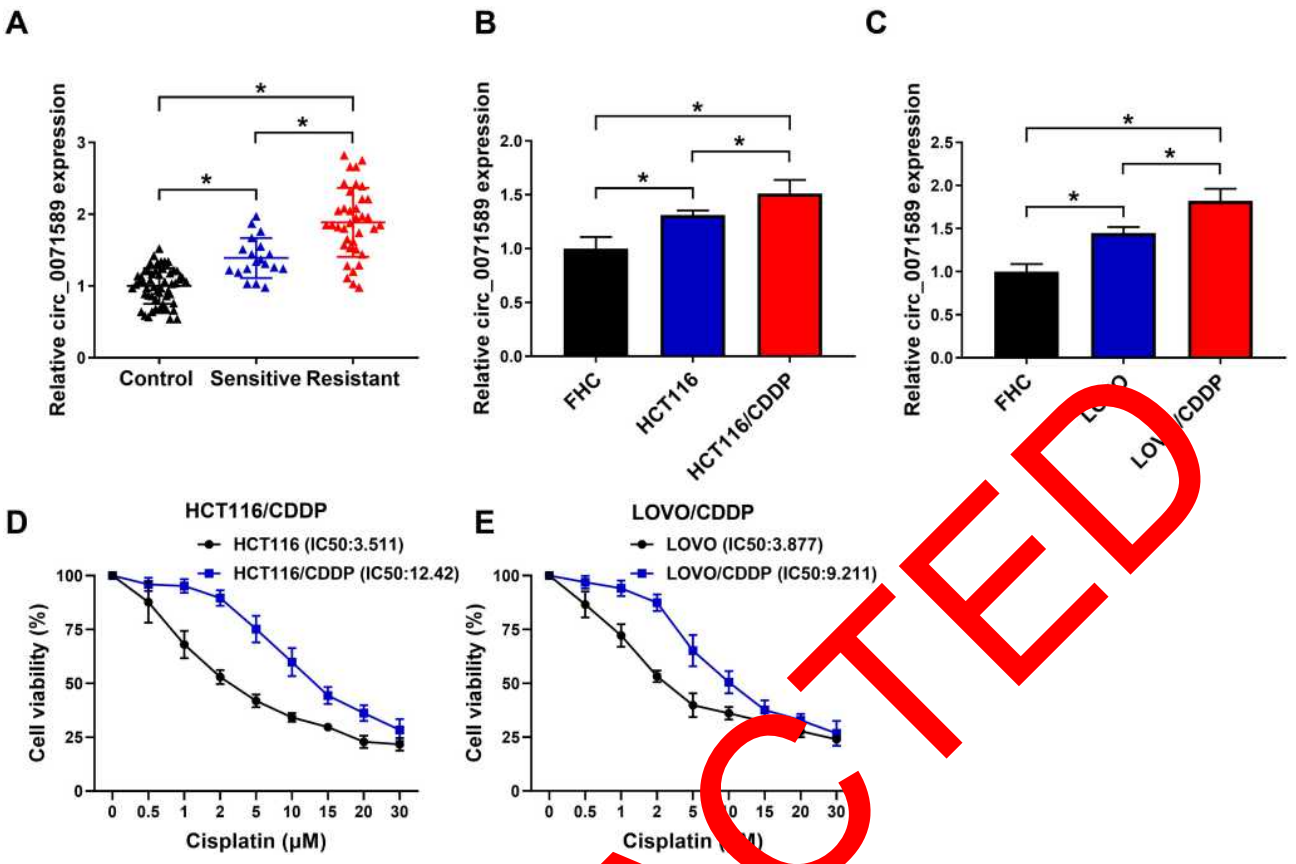

Figure 1 Circ_0071589 expression in CRC tissues and cells. (**A**) Circ_0071589 level was measured in CRC tissues from CDDP-resistant (n=37) or sensitive patients (n=19) and control samples (n=56) via qRT-PCR. (**B** and **C**) Circ_0071589 level was measured in normal human colorectal mucosa cells FHC, CRC cell lines (HCT116 and LOVO cells) and corresponding resistant cell lines (HCT116/CDDP and LOVO/CDDP cells). (**D** and **E**) Cell viability and IC50 of CDDP were examined in HCT116 and LOVO cells and corresponding resistant HCT116/CDDP and LOVO/CDDP cells. *P < 0.05, versus the indicated group.

Abbreviations: circ_0071589, hsa_circ_0071589; CRC, colorectal cancer; CDDP, cisplatin; qRT-PCR, quantitative reverse transcription polymerase chain reaction; IC50, half maximal inhibitory concentration

MUT-circ_0071589. MiR-526b-3p overexpression markedly decreased the luciferase activity of WT-circ_0071589 in HCT116/CDDP and LOVO/CDDP cells, while this effect was abrogated when the binding sites were mutated in MUT-circ_0071589 group (Figure 3B and C). In addition, RIP assay showed circ_0071589 and miR-526b-3p could be enriched by Ago2 (Figure 3D and E). Furthermore, miR-526b-3p level was reduced in CDDP-resistant or sensitive tissues, and the resistant tissues had lower miR-526b-3p level (Figure 3F). Additionally, miR-526b-3p level was reduced in HCT116 and LOVO cells in comparison to FHC cells, and lower level of miR-526b-3p was shown in HCT116/CDDP and LOVO/CDDP cells (Figure 3G and H). Moreover, miR-526b-3p level was increased by circ_0071589 knockdown (Figure 3I). These findings indicated that circ_0071589 could directly target and regulate miR-526b-3p.

MiR-526b-3p Knockdown Attenuates the Effect of Circ_0071589 Silence on CDDP Resistance, Proliferation, Migration, Invasion and Apoptosis in CDDP-Resistant CRC Cells

To probe if miR-526b-3p was responsible for circ_0071589-mediated regulation of CDDP-resistant CRC cell development, HCT116/CDDP and LOVO/CDDP cells were transfected with si-NC, si-circ_0071589, si-circ_0071589 + anti-miR-NC or anti-miR-526b-3p. MiR-526b-3p expression was remarkably enhanced by circ_0071589 knockdown, which was weakened via transfection of anti-miR-526b-3p (Figure 4A). Moreover, downregulation of miR-526b-3p alleviated silencing circ_0071589-mediated inhibition of CDDP resistance in HCT116/CDDP and LOVO/CDDP cells (Figure 4B and C). In addition, knockdown

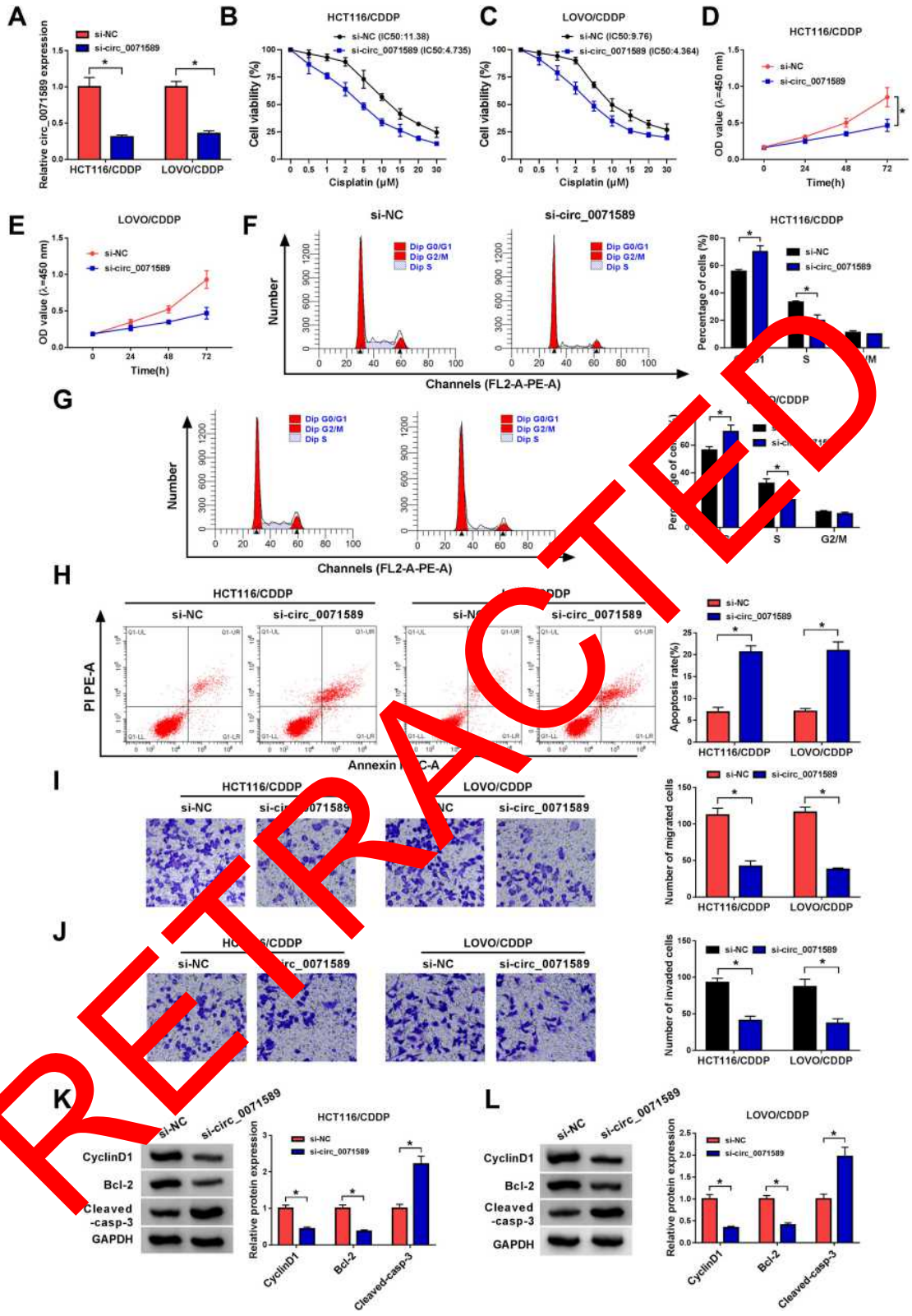

Figure 2 The effect of circ_0071589 silence on CDDP-resistant CRC cell development. (**A**) Circ_0071589 expression was detected in HCT116/CDDP and LOVO/CDDP cells transfected with si-circ_0071589 or si-NC. (**B** and **C**) Cell viability and IC50 of CDDP were measured in HCT116/CDDP and LOVO/CDDP cells transfected with si-circ_0071589 or si-NC after treatment of different concentrations of CDDP for 48 h. Cell proliferation (**D** and **E**), cycle distribution (**F** and **G**), apoptosis (**H**), migration and invasion (**I** and **J**), and related protein levels (**K** and **L**) were examined in HCT116/CDDP and LOVO/CDDP cells transfected with si-circ_0071589 or si-NC. **P*<0.05, versus the indicated group.

Abbreviations: circ_0071589, hsa_circ_0071589; CRC, colorectal cancer; CDDP, cisplatin; si-circ_0071589, siRNA for circ_0071589; si-NC, siRNA negative control; OD, optical density; IC50, half maximal inhibitory concentration; Bcl-2, B-cell lymphoma-2; Cleaved-casp-3, Cleaved-caspase-3; GAPDH, glyceraldehyde-3phosphate dehydrogenase.

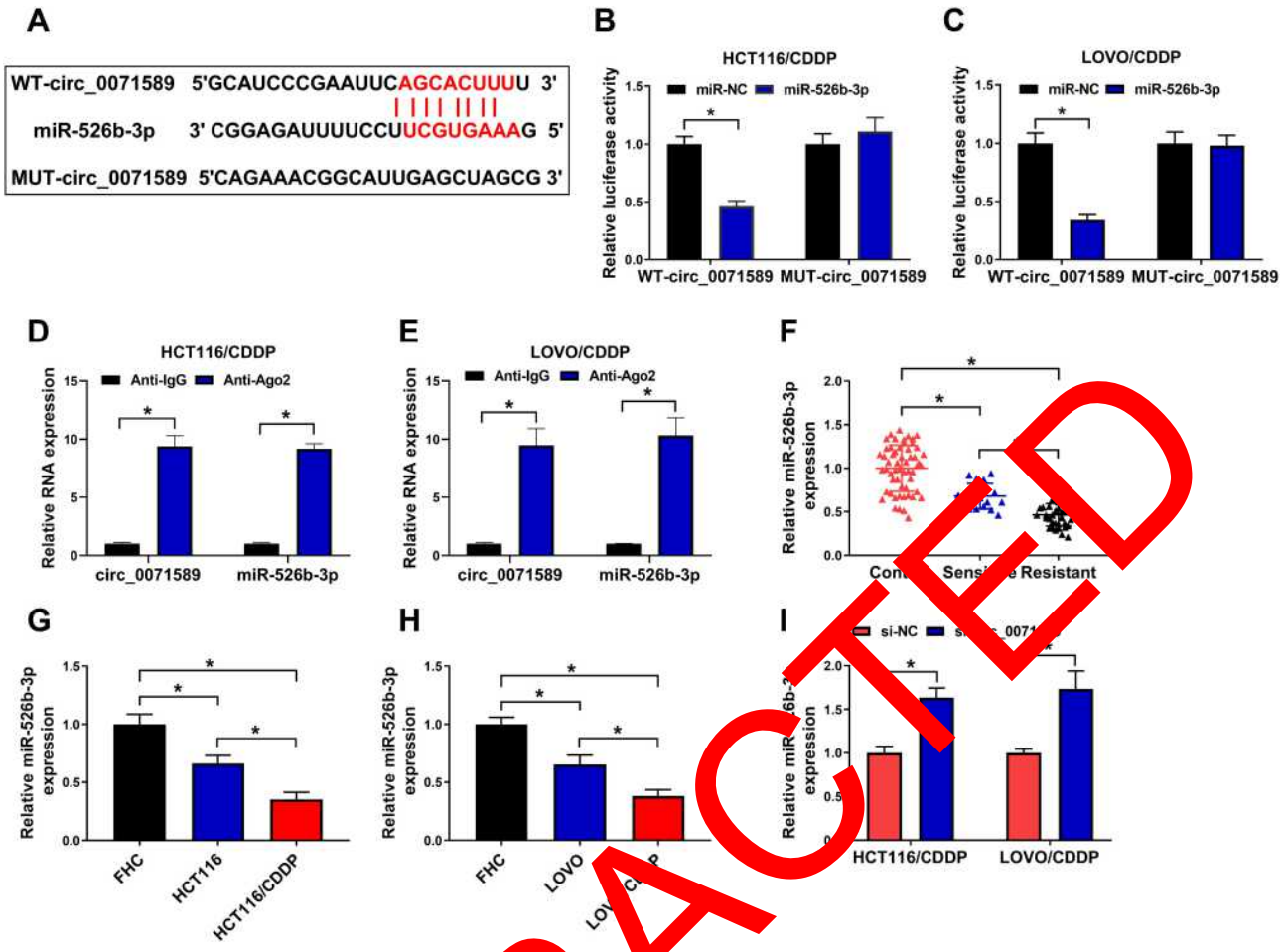

Figure 3 The association between circ_0071589 and miR-526b-3p. The target correlation between circ_0071589 and miR-526b-3p was explored via starBase (**A**), and confirmed via dual-luciferase reporter assay (**B** and **C**), and RIP (**D** and **E**). (**F**) MiR-526b-3p expression was measured in CDDP-resistant (n=37) or sensitive (n=19) CRC tissues and control samples (n=56). (**G** and **H**) MiR-526b-3p level was detected in CDDP-resistant or sensitive CRC cells. (**I**) MiR-526b-3p expression was detected in HCT116/CDDP and LOVO/CDDP cells transfected with si-circ_0071589 or si-NC. *P<0.05, versus the indicated group.

Abbreviations: circ_0071589, hsa_circ_0071589; miR, microRNA; CDDP, cisplatin; CRC, colorectal cancer; si-circ_0071589, siRNA for circ_0071589; si-NC, siRNA negative control; miR-NC, mimic negative control.

of miR-526b-3p weakened interference of circ_0071589-induced proliferation reduction, cycle arrest and apoptosis induction in the two cell lines (Figure 4D–H). Furthermore, deficiency of miR-526b-3p mitigated knockdown of circ_0071589-mediated suppression of migration and invasion in HCT116/CDDP and LOVO/CDDP cells (Figure 4I and J). Additionally, knockdown of miR-526b-3p attenuated the regulatory effect of silencing circ_0071589 on protein expression of CycinD1, Bcl-2 and Cleaved-casp-3 (Figure 4K and L). These data suggested that circ_0071589 regulated CDDP resistance and development in CDDP-resistant CRC cells by sponging miR-526b-3p.

KLF12 is Targeted by MiR-526b-3p

The target of miR-526b-3p was searched via starBase. We selected 5 candidates, and KLF12 expression was decreased most by miR-526b-3p mimic (Supplementary Figure S3A and B). Therefore, KLF12 was selected for subsequent experiments. The binding sites of miR-526b-3p and KLF12 are displayed in Figure 5A. To confirm the target relationship between miR-526b-3p and KLF12, we constructed KLF12 3′UTR-WT and KLF12 3′UTR-MUT, and co-transfected them with miR-526b-3p mimic or miR-NC into HCT116/CDDP and LOVO/CDDP cells. MiR-526b-3p addition resulted in great decrease of luciferase activity in KLF12 3′UTR-WT group, but it did not alter the activity in KLF12 3′UTR-MUT group (Figure 5B and C). Moreover, KLF12

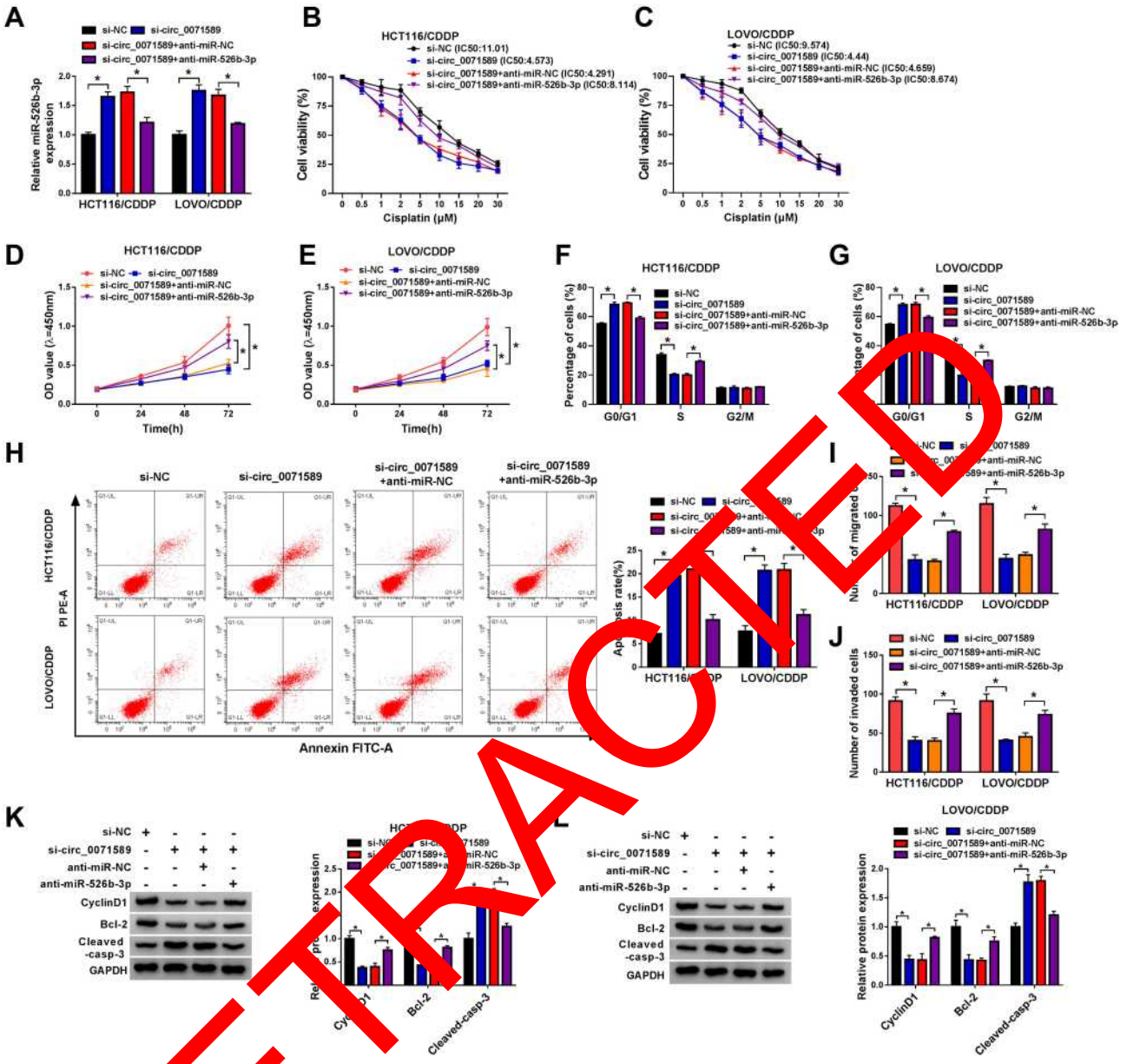

Figure 4 The effect of miR-526b-3p knockdown on silence of circ_0071589-mediated CDDP-resistant CRC cell development. MiR-526b-3p expression (**A**), cell viability and IC50 of CDDP (**B** and **C**), cell proliferation (**D** and **E**), cycle distribution (**F** and **G**), apoptosis (**H**), migration (**I**), invasion (**J**), and related protein levels (**K** and **L**) were detected in HCT116/CDDP and LOVO/CDDP cells transfected with si-NC, si-circ_0071589, si-circ_0071589 + anti-miR-NC or anti-miR-526b-3p. *P<0.05, versus the indicated group.

Abbreviations: circ_0071589, hsa_circ_0071589; miR, microRNA; CRC, colorectal cancer; CDDP, cisplatin; si-circ_0071589, siRNA for circ_0071589; si-NC, siRNA negative control; anti-miR-526b-3p, miR-526b-3p inhibitor; anti-miR-NC, inhibitor negative control; OD, optical density; IC50, half maximal inhibitory concentration; Bcl-2, B-cell lymphoma-2; Cleaved-casp-3, Cleaved-caspase-3; GAPDH, glyceraldehyde-3phosphate dehydrogenase.

levels were significantly elevated in resistant or sensitive tissues compared with control samples, and KLF12 expression was higher in resistant group than sensitive group (Figure 5D and E). Additionally, KLF12 expression was evidently elevated in HCT116 and LOVO cells compared with FHC cells, and it was higher in HCT116/CDDP and LOVO/CDDP cells (Figure 5F and G). These results indicated that KLF12 was directly targeted by miR-526b-3p in CDDP-resistant CRC cells.

MiR-526b-3p Overexpression Suppresses CDDP Resistance, Proliferation, Migration and Invasion, and Induces Apoptosis by Targeting KLF12 in CDDP-Resistant CRC Cells

To probe the function of miR-526b-3p, and explore if it required KLF12 in CDDP-resistant CRC cell development, HCT116/CDDP and LOVO/CDDP cells were

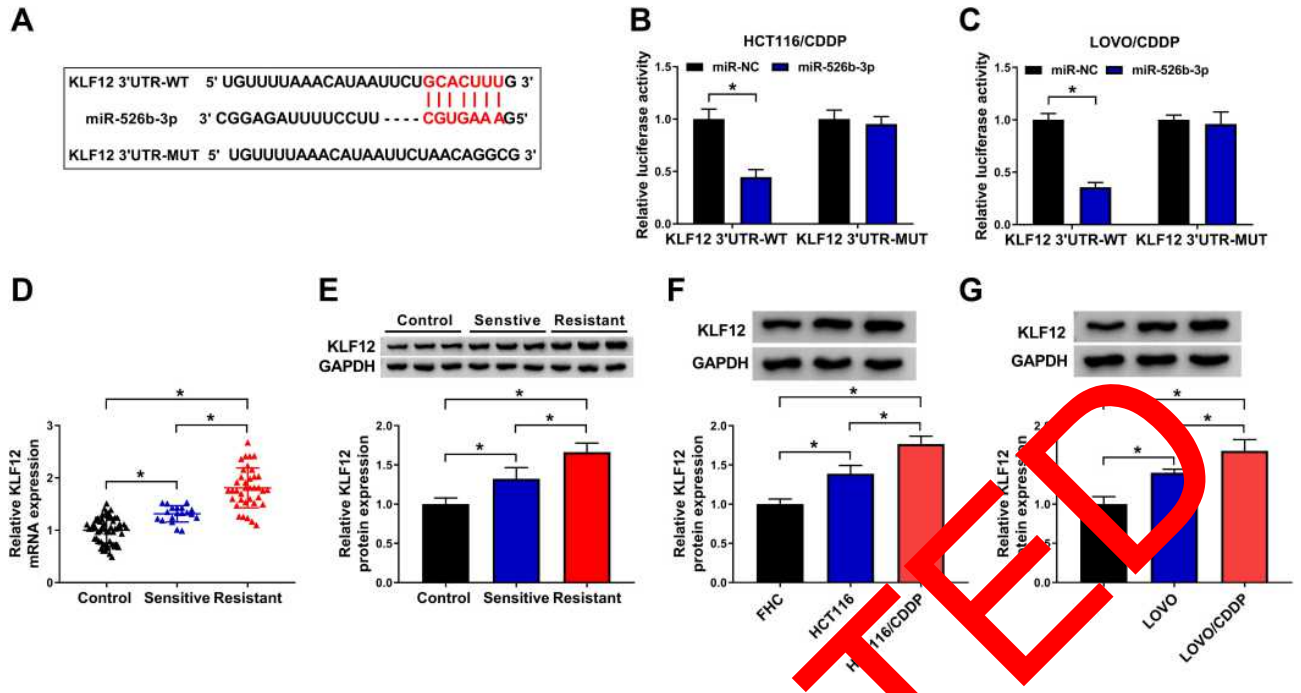

Figure 5 The association between miR-526b-3p and KLF12. The target correlation between miR-526b-3p and KLF12 was explored via starBase (**A**), and validated by dual-luciferase reporter assay (**B** and **C**). (**D** and **E**) KLF12 expression was measured in CDDP-resistant or sensitive CRC tissues and control samples. (**F** and **G**) KLF12 level was detected in CDDP-resistant or sensitive CRC cells. *P<0.05, versus the indicated group.

Abbreviations: miR, microRNA; CRC, colorectal cancer; CDDP, cisplatin; miR-NC, mimic negative control; KLF12, Krüppel-like factor 12; GAPDH, glyceraldehyde-3phosphate dehydrogenase.

transfected with miR-NC, miR-526b-3p mimic, miR-526b-3p mimic + pcDNA-NC or pcDNA-KLF12. As displayed in Figure 6A and B, KLF12 expression was evidently declined via miR-526b-3p overexpression, which were restored by introduction of pcDNA-KLF12. Furthermore, miR-526b-3p overexpression evidently decreased IC50 of CDDP (Figure 6C and D), suppressed cell proliferation (Figure 6E–H), promoted apoptosis (Figure 6I), inhibited migration and invasion (Figure 6J and K), decreased levels of CyclinD1 and Bcl-2, and increased Cleaved-Caspase-3 protein expression (Figure 6L and M) in HCT116/CDDP and LOVO/CDDP cells. Meanwhile, upregulation of KLF12 mitigated these effects (Figure 6C–M). These data showed that miR-526b-3p overexpression repressed CDDP resistance and development by decreasing KLF12 in CDDP-resistant CRC cells.

Circ_0071589 Regulates KLF12 Expression by MiR-526b-3p

To further explore the potential regulatory network of circ_0071589/miR-526b-3p/KLF12, the linear correlation among their levels in CDDP-resistant CRC tissues was analyzed. As displayed in Figure 7A–C, miR-526b-3p expression was negatively correlated with circ_0071589 (P<0.0001, r=−0.7474) and KLF12 (P<0.0001, r=−0.644), while KLF12 expression was positively associated with circ_0071589 (P<0.0001, r=0.7794). Moreover, the effect of circ_0071589 on KLF12 expression was assessed in HCT116/CDDP and LOVO/CDDP cells. Knockdown of circ_0071589 evidently reduced KLF12 expression, and this effect was weakened via miR-526b-3p downregulation (Figure 7D). These results displayed that circ_0071589 could regulate KLF12 by competitively binding with miR-526b-3p.

Knockdown of Circ_0071589 Enhances the Anti-Cancer Role of CDDP in CRC in vivo

To explore the effect of circ_0071589 on CDDP resistance in CRC in vivo, murine xenograft model was established using nude mice by injecting with HCT116/CDDP cells stably transfected with sh-circ_0071589 or sh-NC, followed by treatment with CDDP (n=6/group). As shown in Figure 8A–C, the sensitivity of CDDP to CRC xenograft tumor was enhanced via circ_0071589 knockdown, revealed by the aggravated reduction of tumor volume and

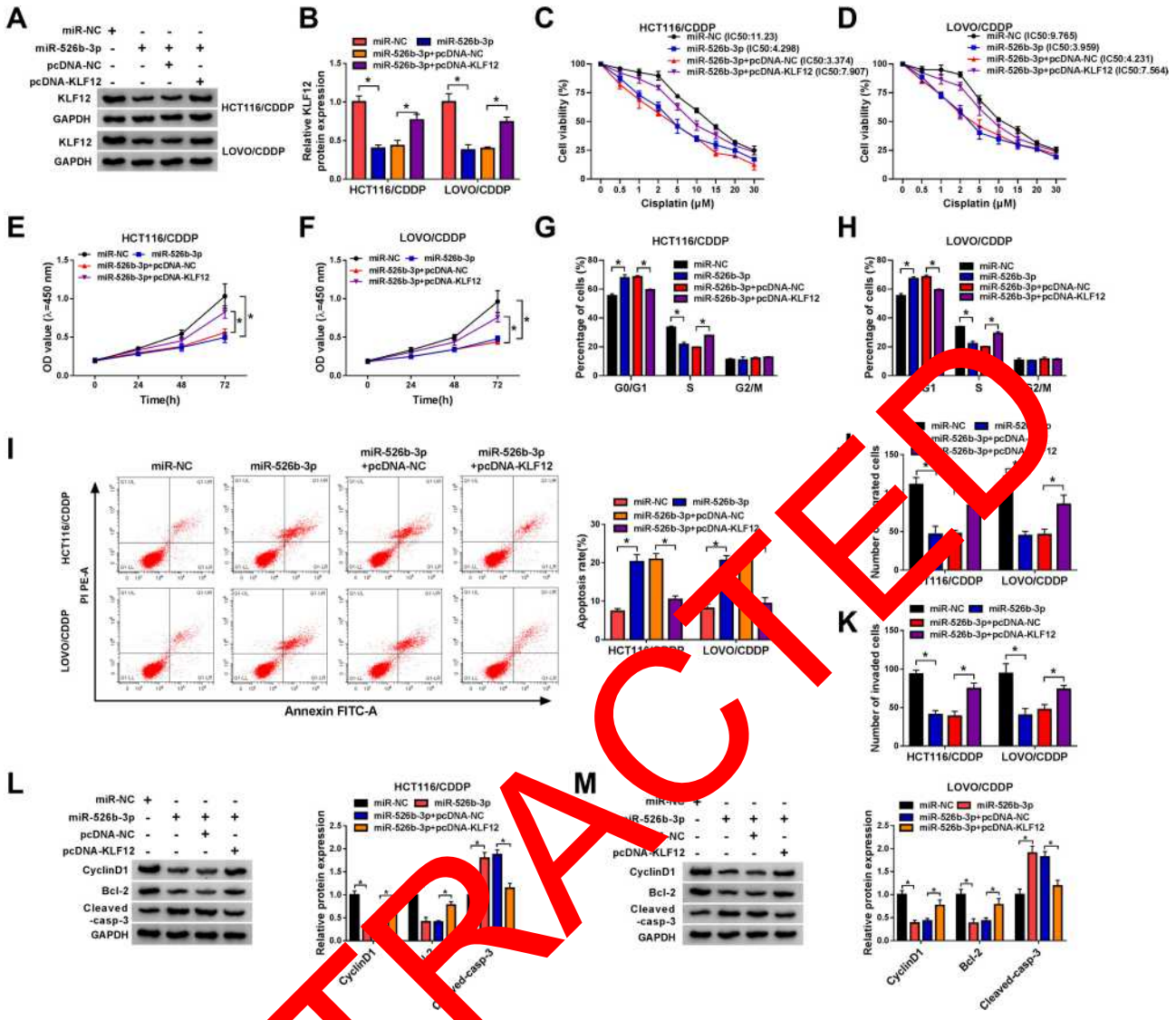

Figure 6 The effects of miR-526b-3p and KLF12 on CDDP-resistant CRC cell development. KLF12 expression (**A** and **B**), cell viability and IC50 of CDDP (**C** and **D**), cell proliferation (**E** and **F**), cycle distribution (**G** and **H**), apoptosis (**I**), migration (**J**), invasion (**K**), and related protein levels (**L** and **M**) were measured in HCT116/CDDP and LOVO/CDDP cells transfected with miR-NC, miR-526b-3p mimic, miR-526b-3p mimic + pcDNA-NC or pcDNA-KLF12. *P<0.05, versus the indicated group.

Abbreviations: miR, microRNA; CRC, colorectal cancer; CDDP, cisplatin; miR-NC, mimic negative control; KLF12, Krüppel-like factor 12; pcDNA-KLF12, KLF12 overexpression vector; pcDNA-NC, pcDNA negative control; OD, optical density; IC50, half maximal inhibitory concentration; Bcl-2, B-cell lymphoma-2; Cleaved-casp-3, Cleaved-caspase-3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

weight. Moreover, tumor tissues were collected, and circ_0071589, miR-526b-3p and KLF12 levels were detected. As displayed in Figure 8D–G, circ_0071589 and KLF12 levels were markedly declined, while miR-526b-3p expression was elevated in sh-circ_0071589 group compared with sh-NC group in the presence or absence of CDDP. These data indicated that circ_0071589 knockdown decreased CDDP resistance in CRC in vivo. Circ_0071589 expression was increased in CRC. Circ_0071589 could target KLF12 via modulating miR-526b-3p, thus to regulate the proliferation, apoptosis, migration and invasion of CDDP-resistant CRC cells. Collectively, knockdown of circ_0071589 repressed CDDP resistance in CDDP-resistant CRC cells by regulating the miR-526b-3p/KLF12 axis (Figure 9).

Discussion

Globally, CRC is a deadly cancer with high incidence.[22] Chemoresistance is a public problem for the chemotherapy of patients with CRC. CircRNAs are associated with the regulation of drug resistance in human cancers.[23] In the present research, we found that circ_0071589 knockdown

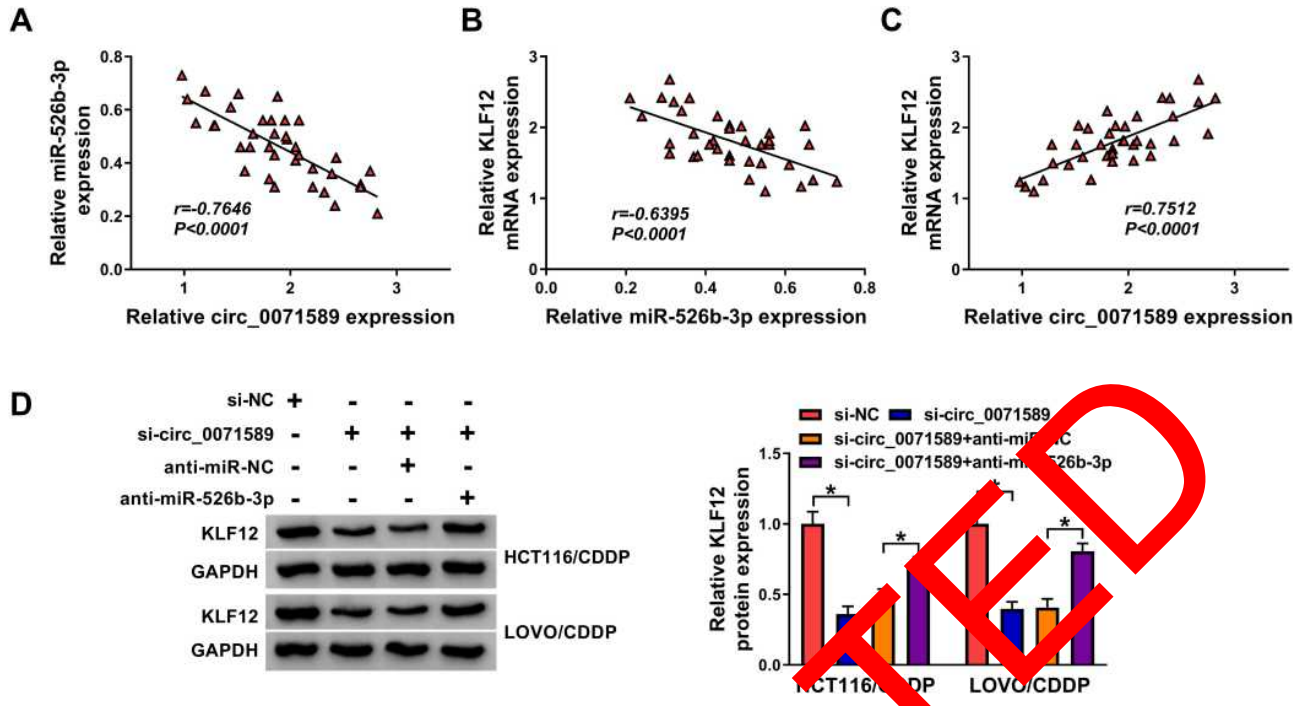

Figure 7 The correlation among circ_0071589, miR-526b-3p and KLF12. (**A**–**C**) The linear correlation among circ_0071589, miR-526b-3p and KLF12 levels in CDDP resistant CRC tissues was analyzed. (**D**) KLF12 expression was detected in HCT116/CDDP and LOVO/CDDP cells transfected with si-NC, si-circ_0071589, si-circ_0071589 + anti-miR-NC or anti-miR-526b-3p. *P<0.05, versus the indicated group.

Abbreviations: miR, microRNA; circ_0071589, hsa_circ_0071589; KLF12, Krüppel-like factor 12; CRC, colorectal cancer; CDDP, cisplatin; si-circ_0071589, siRNA for circ_0071589; si-NC, siRNA negative control; anti-miR-526b-3p, miR-526b-3p inhibitor; anti-miR-NC, inhibitor negative control; GAPDH, glyceraldehyde-3phosphate dehydrogenase.

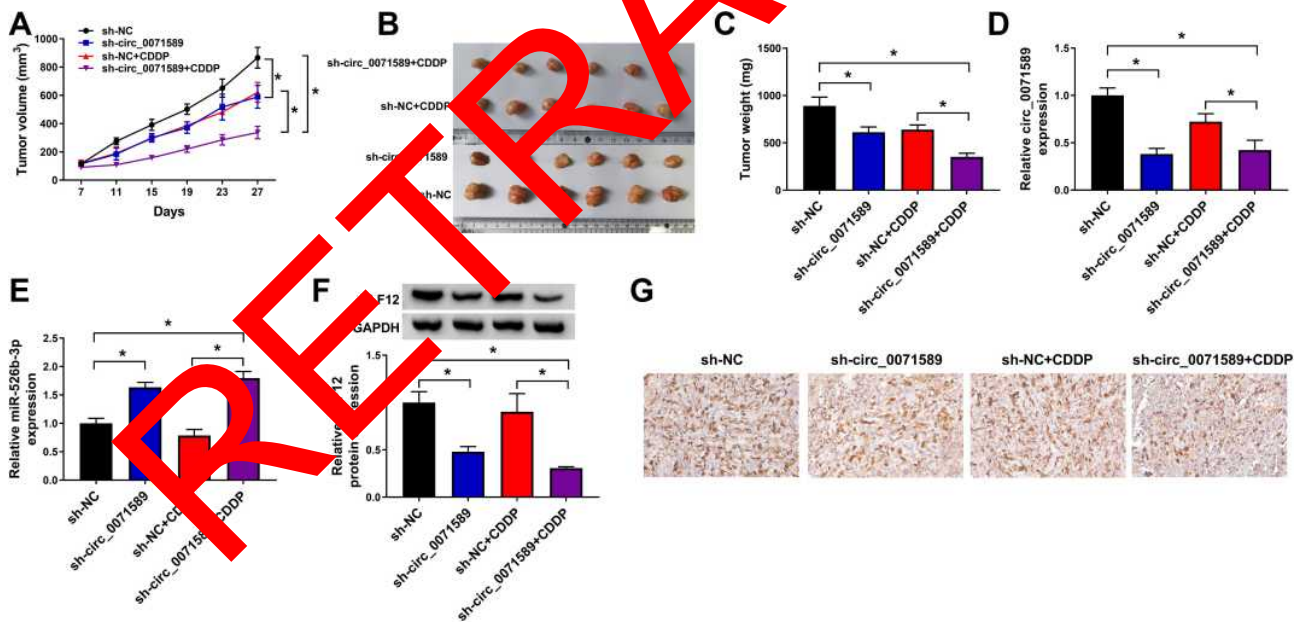

Figure 8 The effects of circ_0071589 and CDDP on CRC xenograft tumor growth. (**A**) Tumor volume was measured every four days from CDDP treatment at 7th day (n=6). (**B**) The representative tumor images in each group. (**C**) Tumor weight was detected in each group. (**D**–**G**) circ_0071589, miR-526b-3p and KLF12 levels were measured in each group. *P<0.05, versus the indicated group.

Abbreviations: circ_0071589, hsa_circ_0071589; CRC, colorectal cancer; CDDP, cisplatin; sh-circ_0071589, shRNA for circ_0071589; sh-NC, shRNA negative control; miR, microRNA; KLF12, Krüppel-like factor 12; GAPDH, glyceraldehyde-3phosphate dehydrogenase.

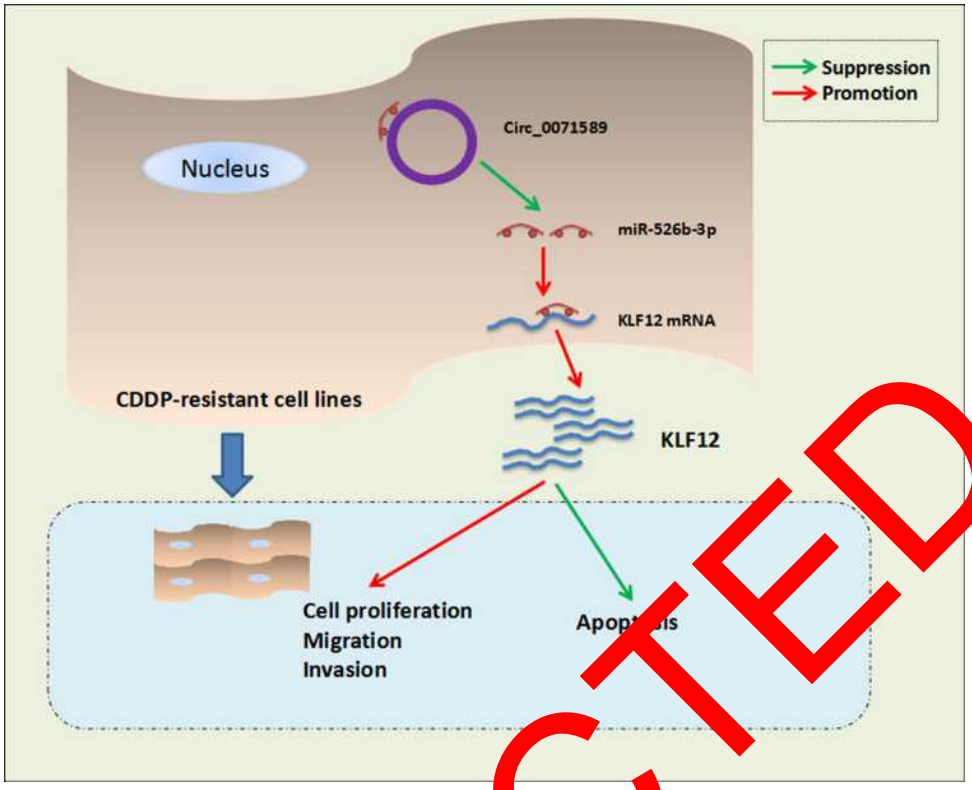

Figure 9 The schematic diagram of this study. Circ_0071589 could target KLF12 via m[illegible]lating miR-526[illegible]3p, thus to regulate the proliferation, apoptosis, migration and invasion of CDDP-resistant CRC cells.
Abbreviations: circ_0071589, hsa_circ_0071589; miR, microRNA; KLF[illegible]pel-like facto[illegible]DDP, cisplatin.

could reduce CDDP resistance in CRC. Moreover, this study firstly validated the regulatory network of circ_0071589/miR-526b-3p/KLF1[illegible].

A previous study indicated that circ_0071589 contributed to CRC cell proliferation, migration and invasion via sponging miR-600.[13] However, there is no direct evidence supported the association between circ_0071589 and chemoresistance in CRC. In this study, high expression of circ_0071589 was measured in CDDP-resistant CRC tissues and cell lines, implying that circ_0071589 might be involved in CDDP resistance development in CRC. By knocking down circ_0071589, we found that cytotoxicity of CDDP to resistant cells was enhanced. Moreover, through measuring cell proliferation and apoptotic rate, and analyzing CyclinD1, Bcl-2 and Cleaved-casp-3 which were the important factors associated with CDDP resistance,[24,25] we found that knockdown of circ_0071589 suppressed cell proliferation, and increased apoptosis. These results further indicated that silence of circ_0071589 reduced CDDP resistance. Besides, migration and invasion are two key processes in resistant cells, which contributed to CDDP resistance.[26,27] Here we measured cell migration and invasion using transwell assay, and found that knockdown of circ_0071589 repressed the migratory and invasive abilities of CDDP-resistant CRC cells. Collectively, inhibition of circ_0071589 decreased CDDP resistance in CRC, indicating the potential of circ_0071589 as a target for improving drug sensitivity in cancers.

CircRNAs play major roles in cancer progression by regulating mRNA expression via sponging miRNAs.[7] A previous study confirmed that circ_0071589 could regulate the miR-600/enhancer of zeste homolog 2 axis in CRC.[13] However, the mechanism addressed by circ_0071589 is complex, and this study aimed to explore an additional regulatory network. In this research, we identified circ_0071589 could target and inhibit miR-526b-3p expression. A previous work suggested that miR-526b-3p played as a tumor suppressor by targeting hypoxia inducible factor 1-alpha in CRC.[17] Moreover, miR-526b-3p could reduce proliferation of CRC cells by decreasing E2F1.[28] Additionally, miR-526b-3p repressed proliferation, migration and invasion of CRC cells via decreasing cyclin D1.[29] These reports indicated the anti-

cancer role of miR-526b-3p in CRC. However, the effect of miR-526b-3p on CDDP resistance is undetermined. Here we firstly found that miR-526b-3p could suppress CDDP resistance in CDDP-resistant CRC cells, which indicated miR-526b-3p as a sensitizer of CDDP in CRC. Moreover, we confirmed that circ_0071589 modulated CDDP resistance via miR-526b-3p. Furthermore, the targets of miR-526b-3p were analyzed. Our study identified miR-526b-3p could directly target KLF12, which was reported as an oncogene in CRC.[30] In addition, previous studies suggested KLF12 could promote CDDP resistance in human cancers, especially in CRC.[20,31] Here we found that KLF12 reversed the effect of miR-526b-3p on CDDP resistance. Furthermore, by analyzing the linear association and measuring the effect of circ_0071589 on KLF12 expression, we confirmed that circ_0071589 targeted and regulated KLF12 expression through binding with miR-526b-3p in vitro. Besides, the effect of circ_0071589 on CDDP resistance was also validated in vivo using a xenograft model.

Conclusion

In conclusion, circ_0071589 knockdown restrained CDDP resistance to the resistant CRC cells, possibly via increasing miR-526b-3p and decreasing KLF12. This study indicated a new insight in drug resistance, and provided a promising target for improving chemotherapy of CRC.

Data Sharing Statement

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

The present study was approved by the ethical review committee of Hainan General Hospital. Written informed consent was obtained from all enrolled patients.

Patient Consent for Publication

Not applicable.

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Disclosure

The authors declare that they have no conflicts of interest for this work.

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