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CDKN3 regulates cisplatin resistance to colorectal cancer through TIPE1

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Abstract. – OBJECTIVE: The aim of this study was to investigate the level of cyclin-dependent kinase inhibitor 3 (CDKN3) in colorectal cancer (CRC), to explore the underlying mechanism of CDKN3 in modulating cisplatin resistance and promoting the malignant progression of CRC.

PATIENTS AND METHODS: 43 pairs of CRC tissues and para-cancerous tissues were collected from CRC patients. CDKN3 expression was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The relationship between CDKN3 expression and the prognosis of CRC patients was analyzed. Meanwhile, qRT-PCR was performed to verify CDKN3 level in CRC cell lines. Next, CDKN3 knockdown model was constructed in CRC cisplatin-resistant cell lines. The influence of CDKN3 on the biological function of CRC cells was analyzed by Cell Counting Kit-8 (CCK-8) and plate cloning assays. Furthermore, the mechanism of its regulation of TIPE1 affecting cisplatin resistance to CRC was explored.

RESULTS: QRT-PCR results showed that CDKN3 level in CRC tissues was remarkably higher than that of the adjacent tissues ($p<0.05$). Compared with patients with low expression of CDKN3, the prognosis of patients with high expression of CDKN3 was significantly worse ($p<0.05$). Similarly, the proliferation and colony formation ability of cells in CDKN3 knockdown group remarkably decreased when compared with the sh-NC group ($p<0.05$). In addition, CDKN3 level was remarkably elevated in CRC patients with cisplatin resistance. In cisplatin-resistant cell lines (including HT28 and HCT-116), the knockdown of CDKN3 remarkably reduced cell viability ($p<0.05$). Furthermore, TIPE1 expression was remarkably downregulated in CRC tissues ($p<0.05$). A negative correlation was observed between the expressions of TIPE1 and CDKN3. Cell reverse experiment demonstrated that TIPE1 could reverse the promoting effect of CDKN3 on the malignant progression of CRC. All these findings suggested that there might exist a mutual regulation between CDKN3 and TIPE1.

CONCLUSIONS: CDKN3 was highly expressed in CRC, which might be closely correlated with poor prognosis of CRC patients. In addition, CDKN3 regulated cisplatin resistance to CRC by modulating TIPE1, thereby promoting the proliferation of CRC.

Key Words:
CDKN3, TIPE1, Colorectal cancer (CRC), Cisplatin resistance.

Introduction

Colorectal cancer (CRC) is the third most common malignant tumor in the world, which ranks fourth among cancer-related deaths[1,2]. In recent years, significant breakthroughs have been made in the diagnosis and treatment of CRC. The improvement of surgery and the emergence of the targeted therapies have remarkably improved the 5-year survival rate of CRC patients. However, many patients still undergo local recurrence and distant metastasis[3-5]. Therefore, finding new prognostic markers is of great significance in determining CRC patients with poor prognosis[6,7].

Cisplatin is the first platinum drug and a non-specific cell cycle drug[8,9]. The main targets of cisplatin include DNA, protein, and RNA with nucleophilicity in cells[10,11]. Currently, cisplatin is a broad-spectrum anticancer drug for clinical treatment of multiple tumors. Meanwhile, it is one of the widely used anticancer drugs for solid tumors, such as breast cancer, ovarian cancer,

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testicular cancer, and lung cancer[12]. However, patients often experience resistance in the process of treatment. However, the specific reasons remain unclear[13,14]. One of the most important mechanisms affecting cisplatin resistance is the blockade or enhancement of cell cycle regulatory pathway[15]. Among them, cyclin-dependent kinase inhibitor 3 (CDKN3) is a member of the protein kinase family, which also plays an important role in cell cycle regulatory pathway[16,17]. Firstly, as a cyclin-dependent kinase, CDKN3 specifically binds to CDK2 and inhibits phosphorylation of Rb protein[17,18]. Secondly, CDKN3 can form a complex with MDM2 and p53 proteins, thereby inhibiting the induction of p21 protein. Ultimately, this may facilitate cell cycle progression[17,18]. Abnormal expression of CKDN3 has been reported in many tumors. However, its expression level and biological role in CRC are still unknown[16,18,19].

In this study, the plasmid-transfection technology was used to investigate the biological function of CKDN3 in CRC cell lines. CKDN3 was confirmed highly expressed in CRC. Meanwhile, its expression was correlated with CRC cell proliferation, providing a molecular basis for finding new and effective targets. Bioinformatics tools were used to mine and analyze biochip database data to construct a CKDN3 transcriptional regulatory network model. Furthermore, our findings elucidated the regulatory mechanism of CKDN3 in the development of cisplatin resistance in CRC at the genome level.

Patients and Methods

Patients and CRC Samples

43 pairs of CRC tissues and para-cancerous tissues were collected from patients undergoing radical resection in our hospital. No patient received any radiotherapy or chemotherapy before surgery. Pathological typing and staging criteria for CRC were performed in accordance with the International Union Against Cancer (UICC) CRC staging criteria. Informed consent was obtained from patients and their families before the study. Our study was approved by the Ethics Oversight Committee.

Cell Lines and Reagents

Human colon cancer cell lines (including HT29, HCT-8, HCT-116) and normal human intestinal epithelial cell line (FHC) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). HT29 and HCT-8 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL), while HCT-116 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% FBS and penicillin (100 U/mL) and streptomycin (100 μg/mL). All cells were maintained in a 37°C, 5% CO_2 incubator. When the cells reached 80%-90% of confluence, they were digested with 1×trypsin+EDTA (ethylenediaminetetraacetic acid).

Cell Transfection

HT28 and HCT-116 CRC cells were first plated into 6-well plates and cultured to a cell density of 43-70%. Cell transfection was performed in the sh-NC group and sh-CDKN3 group by adding 10 μL of sh-NC and sh-CDKN3 plasmids. Subsequently, CRC cells in the sh-CDKN3+si-NC group and sh-CDKN3+si-TIPE1 group were transfected with si-NC and si-TIPE1, respectively. After culture for 48 hours, the transfected cells were collected for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis and cell function experiments.

Cell Counting Kit-8 (CCK-8) Assay

After 48 h of transfection, the cells were harvested and seeded into 96-well plates at 2000 cells per well. After culture for 24, 48, 72, and 96 h, respectively, CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent was added to each well, followed by incubation for 2 h in the dark. Optical density (OD) value of each well at the absorption wavelength of 490 nm was measured by a microplate reader. For drug sensitivity test, cisplatin (Solarbio, Beijing, China) was dissolved in physiological saline at 1 mg/ml and diluted by the gradient (0, 1, 2, 4, 8, 16, 32, 64, 128 μm) for 48 h. CRC cell viability was then detected. After incubation, the OD value of each well at 450 nm was measured by a microplate reader. Finally, experimental data was analyzed.

Colony Formation Assay

After the proportion of cells in the bottom of the chamber increased over 90%, a horizontal line perpendicular to the ground was scratched with 100 μL tip. The backline of the plate was used as a reference line. After scraping, the cells

were washed with prepared phosphate-buffered saline (PBS) solution and removed, and the necrotic cells were taken out. Then, complete medium containing 10% serum was added, and the cells were cultured again in a 37°C incubator. After the operation, the formed colonies in each group were observed under a microscope and photographed.

QRT-PCR

Real-Time quantitative PCR was used to detect the mRNA expressions of CDKN3, TIPE1, and β-actin in CRC tissues and cell lines. The total RNA was extracted in one step by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Later, the extracted RNA was reverse transcribed into the first strand of the complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan) reverse transcription kit. The primers were designed using Primer 5.0 software. QRT-PCR reaction was performed using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) and StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Three replicate wells were set for each sample, and the experiment was repeated for three times. The Bio-Rad PCR instrument (Bio-Rad, Hercules, CA, USA) was used to analyze and process the data. β-actin and U6 were used as internal references for mRNA and miRNA, respectively. The gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study were as follows: TIPE1, F: 5'-CTTGACGAGTGCCGTTAGCT-3', R: 5'-CGTAGCCTGTCTCCCAAGAGA-3'; CDKN3, F: 5'-GAGGAGAAGCGTTGCCAA-3', R: 5'-ACCGACGACTAGCCACCGTATA-3'; U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'; β-actin: F: 5'-CCTGGCACCCAGCACAAT-3', R: 5'-GCTGATCCACATCTGCTGGAA-3'.

Western Blot

The transfected cells were lysed using PROPREP™ lysis buffer and shaken on ice for 30 minutes, followed by centrifugation at 14,000 g for 15 minutes at 4°C. The concentration of the extracted protein was calculated by the CRCA Protein Assay Kit (Pierce, Rockford, IL, USA). Anti-CDKN3, TIPE1 rabbit anti-human monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); horseradish peroxidase-labeled goat anti-rabbit secondary antibody was purchased from Genscript. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk powder for 1 h at room temperature, the membranes were incubated with primary antibodies overnight at 4°C. On the next day, the membranes were rinsed 3 times with Tris Buffered Saline and Tween-20 (TBST) and incubated with the corresponding secondary antibody for 1 h at room temperature. The immunoreactive bands were finally developed and analyzed with enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) was used for all statistical analysis. The *t*-test was used to compare the measurement data, and the categorical variables were analyzed by the χ^2-test or Fisher's exact probability method. The survival analysis was performed using the Kaplan-Meier method, and the survival curves were plotted. Experimental data were expressed as mean ± standard deviation ($\bar{x} \pm s$). $p<0.05$ was considered statistically significant.

Results

CDKN3 Was Highly Expressed in CRC Tissues and Cell Lines

QRT-PCR showed that CDKN3 expression in CRC tissues was remarkably higher than that of adjacent tissues, and the difference was statistically significant ($p<0.05$; Figure 1A). Similarly, the same results were observed by Western blotting (Figure 1B). Compared with FHC, CDKN3 was highly expressed in CRC cell lines, especially in HT28 and HCT-116 cells. Therefore, these two cells were selected for subsequent experiments (Figure 1C). According to CDKN3 level in 43 pairs of CRC tumors and para-cancerous tissues, the relationship between CDKN3 expression and prognosis of CRC patients was analyzed. As shown in Figure 1D, the prognosis of patients with high expression of CDKN3 was remarkably worse than that of patients with low CDKN3

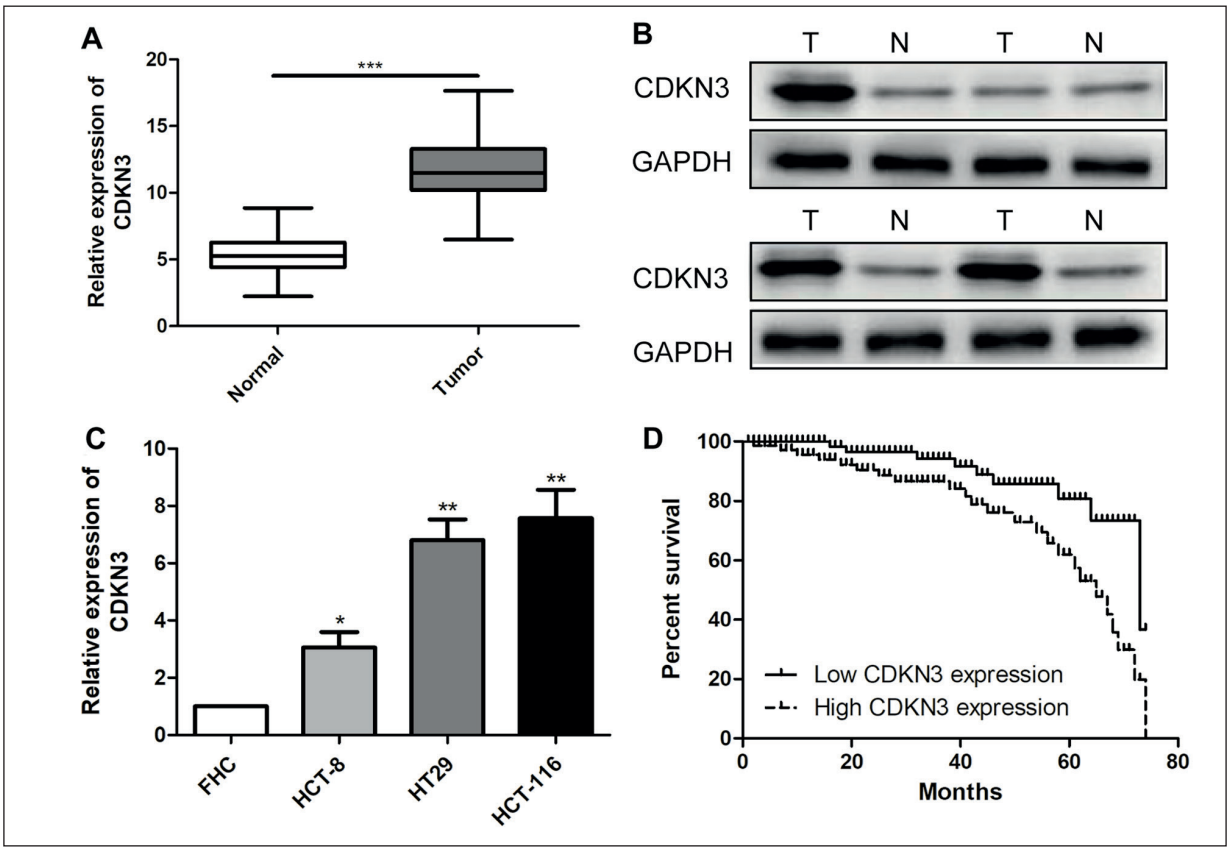

Figure 1. CDKN3 was highly expressed in CRC tissues and cell lines. **A,** QRT-PCR was used to detect the expression of CDKN3 in CRC tissues and adjacent tissues. **B,** Western blotting was used to detect the expression of CDKN3 in CRC tissues and adjacent tissues. **C,** QRT-PCR was used to detect the expression level of CDKN3 in CRC cell lines. **D,** Kaplan Meier survival curve of CRC patients based on CDKN3 expression was shown; the prognosis of patients with high expression was significantly worse than that of patients in low expression group. Data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

expression (p<0.05). These results indicated that CDKN3 might play a vital role in CRC.

Knockdown of CDKN3 Inhibited Cell Proliferation

To explore the effects of CDKN3 on CRC cell function, CDKN3 knockdown expression model was first constructed. The transfection efficiency was verified by qRT-PCR and Western blotting (Figure 2A, 2B). Next, cell proliferation assays were performed in HT28 and HCT-116 cell lines, respectively. The results found that the proliferation of CRC cells significantly decreased in CDKN3 knockdown group compared with the sh-NC group, and the difference was statistically significant (p<0.05; Figure 2C). The colony formation assay showed that the cloning formation ability of CRC cells was remarkably reduced after knocking down CDKN3 when compared with the sh-NC group (p<0.05; Figure 2D). These results demonstrated that the knockdown of CDKN3 inhibited CRC cell proliferation.

CDKN3 Contributed to Cisplatin Resistance in CRC

To explore the effect of CDKN3 on cisplatin resistance in CRC patients, CDKN3 expression was first detected by qRT-PCR in patients with cisplatin resistance (Figure 3A). Compared with corresponding pro-cell lines, CRC cisplatin-resistant cell lines HT28R and HCT-116R were identified as cisplatin-resistant CRC cell lines by CCK-8 (Figure 3B). The differences in CDKN3 expression were examined in HT28 and HCT-116 cisplatin-resistant cell lines, respectively. It was found that CDKN3 was highly expressed in CRC cisplatin-resistant cell lines (p<0.05; Figure 3C). Besides, CCK-8 analysis showed that

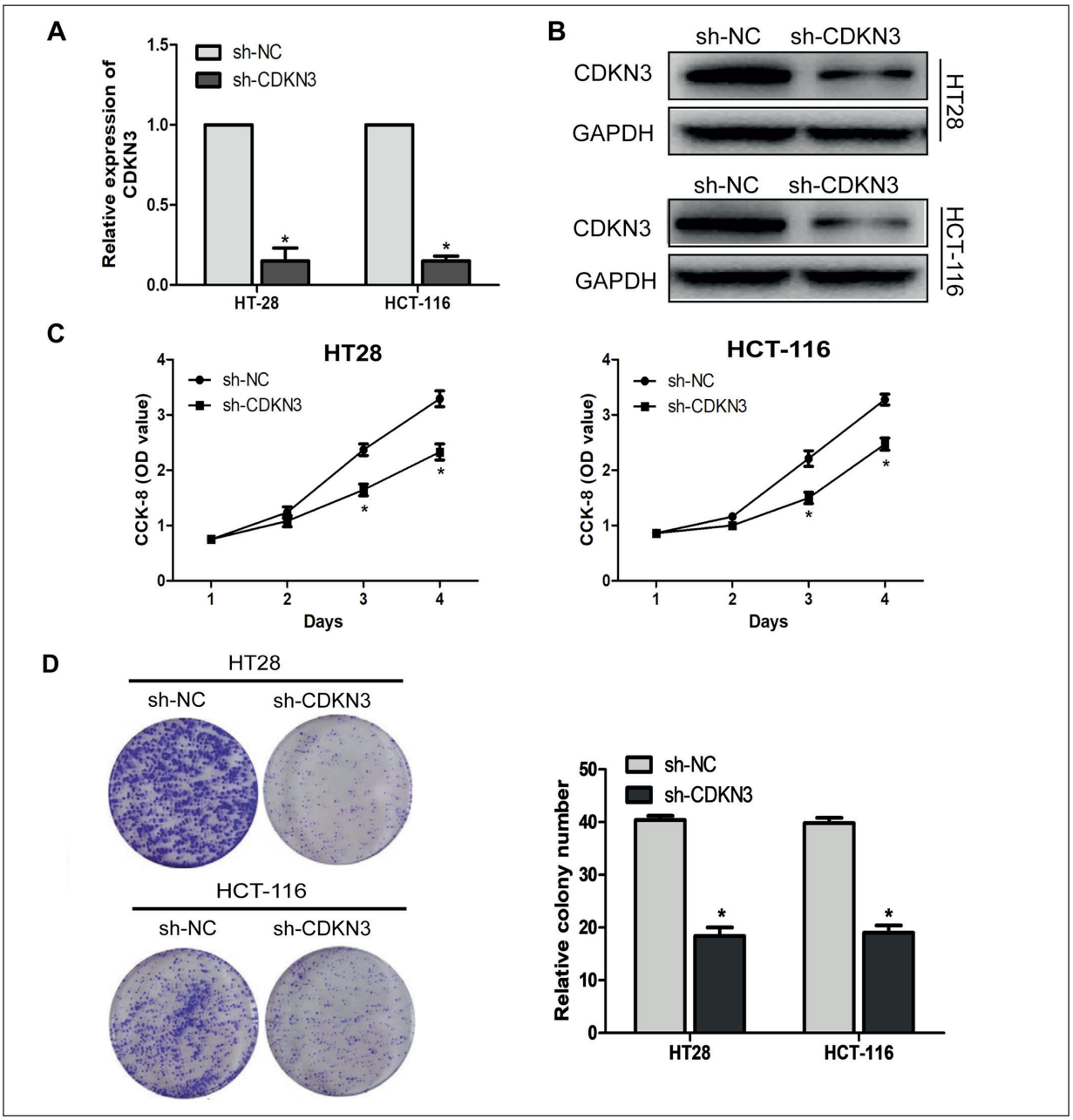

Figure 2. Silencing CDKN3 reduced the proliferation of CRC cells. **A,** QRT-PCR verified the interference efficiency of transfection of CDKN3 knockdown vectors in HT28 and HCT-116 cell lines. **B,** Western blotting verified the interference efficiency of transfection of CDKN3 knockdown vectors in HT28 and HCT-116 cell lines. **C,** CCK-8 assay detected the effects of CDKN3 knockdown on the proliferation of CRC cell lines. **D,** Plate cloning experiment examined the colony forming ability after transfection of CDKN3 knockdown vectors in HT28 and HCT-116 cell lines (magnification: 40×). Data were expressed as mean ± SD, *$p<0.05$.

the knockdown of CDKN3 remarkably reduced CRC cell viability ($p<0.05$; Figure 3D). The colony formation ability of HT28 and HCT-116 cisplatin-resistant cell lines was measured by plate cloning assay. The results demonstrated that the colony formation ability of CRC cells decreased remarkably after knocking down CDKN3 when compared with the sh-NC group ($p<0.05$; Figure 3E). All these results suggested that CDKN3 contributed to cisplatin resistance in CRC.

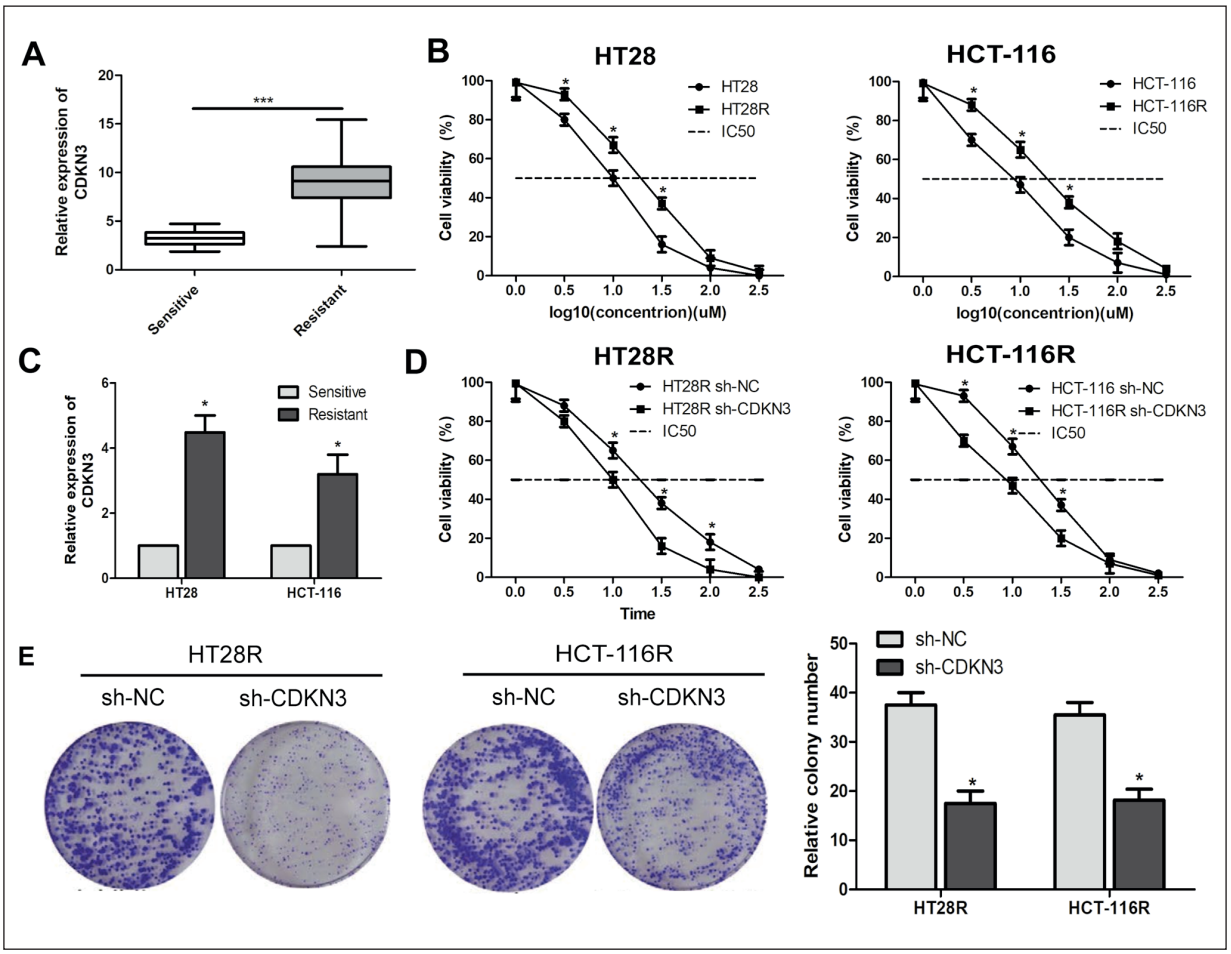

Figure 3. CDKN3 increased cisplatin resistance to CRC. **A,** QRT-PCR detected the expression of CDKN3 in cisplatin-resistant patients. **B,** CCK-8 analysis indicated successful establishment of CRC cisplatin-resistant cell lines. **C,** QRT-PCR was used to detect CDKN3 expression in CRC cisplatin-resistant cell lines. **D,** CCK-8 assay detected the effect of CDKN3 knockdown on CRC cell viability. **E,** Plate cloning experiment was performed to detect colony forming ability of cisplatin-resistant HT28R and HCT-116R cell lines (magnification: 40×). Data were expressed as mean ± SD, *p<0.05.

TIPE1 Was Lowly Expressed in CRC Tissues and Cell Lines

Bioinformatics predicted that CDKN3 and TIPE1 might have some association in CRC. In CRC cisplatin-resistant cell lines HT28R and HCT-116R, the expression of TIPE1 was also remarkably upregulated after knockdown of CDKN3 by Western blotting and qRT-PCR (p<0.05; Figures 4A, 4B). Similarly, TIPE1 expression was remarkably reduced in CRC tissues when compared with para-cancerous tissues (Figure 4C). Besides, qRT-PCR results indicated that CDKN3 expression was negatively correlated with TIPE1 expression in CRC cells (Figure 4D). The above findings suggested that there might be a mutual regulation between CDKN3 and TIPE1.

CDKN3 Modulated TIPE1 Expression in Human CRC Cells

To further explore the interaction of CDKN3 and TIPE1 in promoting malignant progression of CRC, CDKN3, and TIPE1 were co-transfected into CRC cisplatin-resistant cell lines HT28R and HCT-116R. QRT-PCR verified the transfection efficiency of CDKN3 *in vitro* (Figure 5A). Subsequent colony formation assay demonstrated that the knockdown of TIPE1 remarkably promoted the colony formation ability of CRC cells in silencing CDKN3 group, thereby counteracting the effect of CDKN3 on CRC cell proliferation (p<0.05; Figure 5B). In sum, CDKN3 regulated the progression of CRC *via* modulating TIPE1 expression.

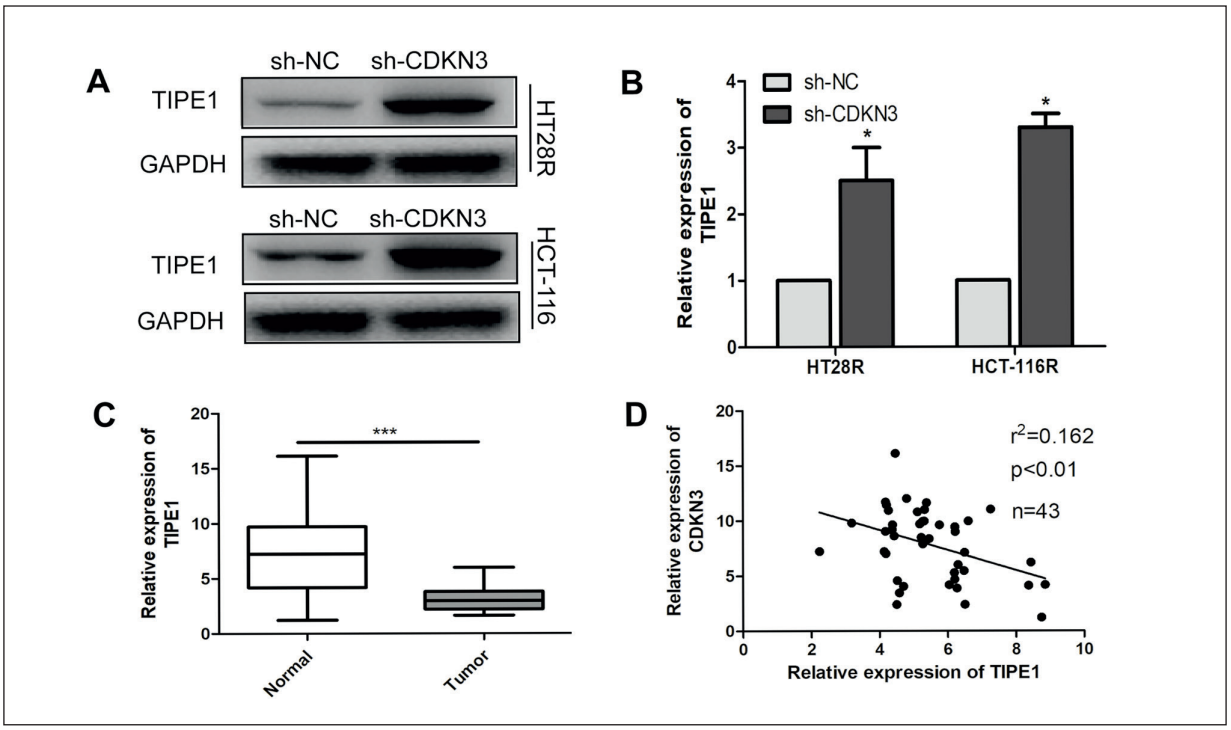

Figure 4. TIPE1 was lowly expressed in CRC tissues and cell lines. **A,** QRT-PCR verified the expression level of TIPE1 after transfection of CDKN3 knockdown vector in HT28R and HCT-116R cell lines. **B,** Western blotting verified the expression level of TIPE1 after transfection of CDKN3 knockdown vector in HT28R and HCT-116R cell lines. **C,** QRT-PCR was used to detect the expression of TIPE1 in CRC tissues and adjacent tissues. **D,** There was a significant negative correlation between the expression levels of CDKN3 and TIPE1 in CRC tissues. Data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Discussion

CRC is one of the most common malignant tumors that endanger human health worldwide. Its incidence rate ranks third among all malignant tumors, whose mortality rate ranks fourth[1-3]. The incidence of CRC has increased all over the world since the beginning of the 21st century, especially in the developing countries[2] and is thought to be related to changes in eating habits and reduced physical activity[3]. Due to advances in surgery, the overall survival rate of CRC patients has remarkably increased[3,4]. Currently, the molecular marker CEA has been used for follow-up of CRC. However, there is still no sensitive and specific index for the prognosis of CRC patients so far. Therefore, this paper aimed to find a new marker that could be used for accurate diagnosis and prognosis for CRC[5,6].

Cisplatin is the first generation of platinum-based drugs. It is a broad-spectrum anticancer drug widely used in cancer treatment. Cisplatin was approved by the US Food and Drug Administration (FDA) for cancer treatment in 1978[8,9]. The introduction of new drugs, such as cisplatin and other targeted therapies, has remarkably prolonged the median survival of patients with metastatic CRC[9-11]. During the treatment of CRC and its prognosis, the sensitivity to chemotherapy drugs is extremely important. However, cisplatin resistance is one of the main obstacles in clinical treatment. Furthermore, the specific mechanism has not been fully understood[12].

CDKN3 is a family of proteins containing a KRAB inhibitory domain. It has a synergistic inhibitory effect on zinc finger proteins 114q61[16,17]. CDKN3 can recruit and interact with many gene silencing complexes to inhibit the transcriptional activity of the targeted genes[17]. The most important biological function of CDKN3 is cell cycle control. Some studies[17,18] have found that it plays two opposite roles in different cells: facilitating cell cycle or inhibiting cell cycle transition. Al-

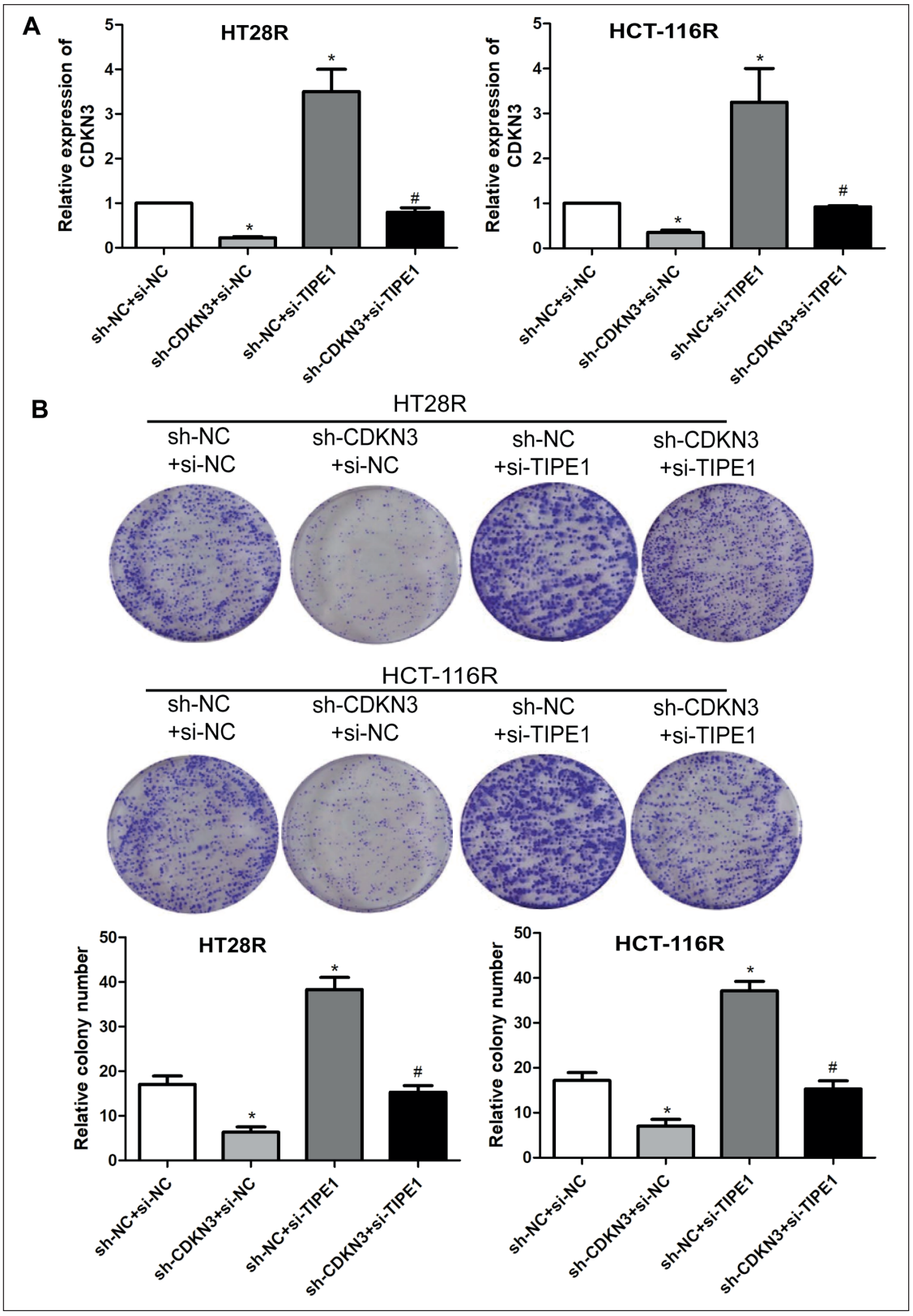

Figure 5. CDKN3 regulated the expression of TIPE1 in CRC cell lines. **A,** CDKN3 expression in CDKN3 and TIPE1 co-transfected cell lines was detected by qRT-PCR. **B,** Plate cloning assay was used to examine the effect of co-transfection of CDKN3 and TIPE1 in regulating colony-forming ability of CRC cells (magnification: 40×). Data were expressed as mean ± SD, $*^{\#}p<0.05$.

though CDKN3 has been extensively investigated in other tumors, its role in CRC has rarely been studied. Therefore, in this work, we focused on the effects of CDKN3 on the biology of CRC cells[16-19]. To explore the role of CDKN3 in the development and progression of CRC, qRT-PCR was used to detect CDKN3 level in 43 cases of CRC tissues and their para-cancerous tissues. The results showed that the expression of CDKN3 was remarkably upregulated in CRC tissues. In addition, CDKN3 expression was positively correlated with the prognosis of CRC patients. These results suggested that CDKN3 might play a cancer-promoting role in CRC. Later, we further explored the effect of CDKN3 on the biological function of CRC. CDKN3 knockdown expression model was first constructed *in vitro*. Cell proliferation assay and plate cloning showed that CDKN3 significantly promoted the proliferation of CRC. In addition, the knockdown of CDKN3 reduced the sensitivity of cisplatin to inhibit the proliferation of CRC cells. All these findings indicated that the knockdown of CDKN3 expression inhibited the sensitivity of cisplatin.

TIPE1 (short for TNFNP8L1) is an important member of the TNFAIP8 family, which is also highly homologous to TIPE2, TIPE3, and TNFmP8[20,21]. TNFAIP8 was once thought to belong to the FLIP family of regulatory cell death proteins. However, the amino-terminal of the open reading frame in its nucleic acid sequence structure is highly homologous to the sequence of death effector domain II in FILP[22,23]. TIPE1 plays an important role in a variety of tumors, including breast cancer, osteosarcoma, and colon cancer[20,21,24,25]. The expression of TIPE1 is related to the malignant degree of tumors. The stronger the tumor invasiveness indicates the lower expression rate. This suggests that TIPE1 gene has an inhibitory effect on the occurrence and development of a malignant tumor and is related to the prognosis of tumor[20,21,24,25]. To clarify the role and influence of CDKN3 and TIPE1 interaction on the development of CRC, we further verified that the knockdown of CDKN3 remarkably upregulated the mRNA and protein expression levels of TIPE1. Subsequent recovery experiment verified that the knockdown of TIPE1 remarkably enhanced the proliferation of CRC cells in CDKN3 silencing group, thereby counteracting the role of CDKN3. Therefore, CDKN3 affected cisplatin sensitivity and promoted the malignant progression of CRC by regulating TIPE1.

Conclusions

CDKN3 was highly expressed in CRC tissues, which was remarkably associated with the prognosis of patients. In addition, CDKN3 regulated cisplatin resistance *via* modulating TIPE1, thereby promoting the proliferation of CRC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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