Abstract. The aim of the present study was to investigate the expression of cold-inducible RNA-binding protein (CIRP) in renal cell carcinoma (RCC) and to determine the effects of downregulation of CIRP on cell proliferation and chemosensitivity to gemcitabine. The expression of CIRP was detected by western blot analysis, quantitative polymerase chain reaction and immunohistochemistry (IHC) in 17 RCC and peri-cancerous tissue samples. Subsequently, the RCC 786-0 cell line was selected in order to investigate the function of CIRP using RNA interference (RNAi) technology, which was able to inhibit the expression of CIRP in vitro. Furthermore, the chemosensitivity to gemcitabine of each group [CIRP small interfering RNA (siCIRP), negative control small interfering RNA (siNC) and blank control] was compared. There were marked differences between the RCC and peri-cancerous tissues. IHC demonstrated that the CIRP expression in 13/17 (76.50%) tumor samples was markedly positive compared with that in the peri-cancerous tissues and the most common pathological type was clear cell RCC (92.30%). This observation was further confirmed through western blot analysis of protein expression levels. CIRP downregulation by RNAi in the RCC 786-0 cell line significantly decreased RCC proliferation. Additionally, when RNAi was coupled with gemcitabine treatment, there was a significant increase in apoptosis in the siCIRP group. CIRP was overexpressed in RCC tissues and in the 786-0 cell line. Downregulation of CIRP by siRNA inhibited the proliferation of the 786-0 cell line and enhanced the chemosensitivity of the cells to gemcitabine. Therefore, CIRP downregulation may provide a novel pathway for the treatment of metastatic RCC.

Introduction

The prognosis of patients affected by metastatic renal cell carcinoma (mRCC) has improved markedly with targeted therapies. However, 20-25% of these patients are refractory to treatment at the first response assessment and the majority of patients will acquire drug resistance during treatment. RCC was previously considered to be resistant to chemotherapy on the basis that the results of phase II trials had not always been promising. However, at present, chemotherapy may serve important roles in the development of novel treatment strategies that target different mechanisms of action, particularly in situations without other effective therapeutic options, including primary and secondary resistance to targeted therapy, tumors with poor prognoses and sarcomatoid histology (1).

CIRP is a highly conserved glycine-rich RNA-binding protein, which contains an amino-terminal consensus sequence RNA-binding domain and a carboxyl-terminal glycine-rich domain (2). CIRP may be induced by mild temperatures (32˚C), DNA damage, hypoxia and oxidative stress inducers (3). Upon stress induction, CIRP shuttles from the nucleus to the cytoplasm to stabilize target mRNAs (4,5). CIRP mediates suppression of cell proliferation with prolongation of the G1 phase and contributes to the suppression of apoptosis induced by tumor necrosis factor-α (6). CIRP also contributes to the maintenance of normal cellular function (7,8).

The precise function of CIRP in the cancer cell microenvironment remains unclear. Previous studies have identified CIRP as a novel oncogene that may facilitate the proliferation of tumor cells via its upregulated expression in various tumor cells and its further inhibition of oxidative-induced tissue damage, enabling it to exert a trophic role in oncogenesis (9). Ren et al (10) observed that CIRP was overexpressed in oral squamous cell carcinoma, which may be associated with the poor prognosis of this disease. Hamid et al (11) observed that CIRP participated in the cell cycle regulation of normal human endometrium and loss of its expression may be involved in endometrial carcinogenesis. Wang et al (12) observed that the expression of CIRP in pituitary adenoma was closely associated with tumor proliferation and invasion, and that its significantly elevated expression level indicates post-operative recurrence.
Another study revealed that CIRP may be involved in the occurrence of hepatocellular carcinoma (13). In vitro, the expression of CIRP was observed in Hep-G2, NC65, HeLa, T24 and NEC8 cell lines (14). Additionally, Zeng et al (15) demonstrated that downregulation of cold shock protein [which contains CIRP and RNA-binding motif protein 3 (RBM3)] impairs the proliferation and the enhances chemosensitivity of prostate cancer cells. However, the role of CIRP expression in RCC clinical tumor samples and in RCC cell lines remains unclear.

The present study investigated the expression of CIRP in RCC tissues and in a cell line in order to examine the potential chemosensitivity of the cells to gemcitabine. A siRNA approach was used to examine the proliferation of the 786-0 cell line in vitro. The results suggested a potential novel mechanistic pathway for RCC therapy.

Materials and methods

Patient clinical characteristics. RCC and peri-cancerous tissue samples from 17 patients were obtained from the Department of Urology at Ningbo Urology and Kidney Hospital (Ningbo, China) between January 2014 and December 2014. The patient population comprised 7 females and 10 males, aged between 31 and 77 years (mean, 54.6 years). The pathological types included clear cell RCC (n=14, 82.35%), chromophobe renal carcinoma (n=1), papillary carcinoma (n=1) and vascular leiomyoma (n=1). The present study was approved by the Ethics Committee of Ningbo Urology and Kidney Hospital and written informed consent was obtained from all participants.

Cell culture and treatment. The clear cell RCC 786-0 cell line was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured at 37°C in 5% CO₂ in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). 786-0 cells were treated with different concentrations (0.05, 0.10, 0.25, 0.75, 1.00, 5.00, 10.00, 20.00, 30.00 and 40.00 nM) of gemcitabine 72 h after transfection.

siRNA transfections. CIRP small interfering RNA (siCIRP) was obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China), with the sequences: Sense, 5’-CCAGAGAUUCUG GGGAUUUU-3', and antisense, 5’-PAAUCCGAGAC UCUGUUG-3’. RCC 786-0 cells were transfected with 33 nM siRNA at a total oligo concentration of 150 nM, using 10 µl Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). 786-0 cells were treated with different concentrations (0.05, 0.10, 0.25, 0.75, 1.00, 5.00, 10.00, 20.00, 30.00 and 40.00 nM) of gemcitabine 72 h after transfection.

Western blot analysis. Cells and tissues were lysed in a triple detergent RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China) containing a protease inhibitor cocktail. Protein (~40 µg, which was determined by BCA method) was separated by 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% skimmed milk at room temperature for 1 h, prior to being incubated with an anti-CIRP antibody (cat. no., 10209-2-AP; dilution, 1:1,000; ProteinTech Group, Inc., Chicago, IL, USA) and either an anti-GAPDH antibody (for the cells; dilution, 1:3,000; Bioworld Technology, Inc., St. Louis Park, MN, USA) or an anti-β-actin antibody (for the tissues; dilution, 1:100; cat. no. ab8226; Abcam, Cambridge, UK) at 4°C overnight. Following incubation with the secondary antibody (fluorescein-conjugated goat anti-mouse IgG H&L; cat ab6785; Abcam) at room temperature for 45 min, signals were visualized using enhanced chemiluminescence [cat.no. NEL103001EA; Western Lightning® Plus-ECL, Enhanced Chemiluminescence Substrate, PerkinElmer, Inc., Waltham, MA, USA].

Cell proliferation detection. A total of 5,000 cells/well were seeded onto 96-well plates. Next, 72 h after gemcitabine treatment (as aforementioned; 0.05-40 nM), 10 µl Cell Counting kit-8 (CCK-8; Boster Biological Technology, Pleasanton, CA, USA) was added to each well, according to the manufacturer's protocol. After 4 h incubation at 37°C, CCK-8 absorbance was measured at 450 nm.

Immunohistochemistry. RCC specimens were fixed in 10% neutral-buffered formalin (Wuxi Zhanwan Chemicals, Yixing, China) at room temperature for 1 week prior to sectioning, and then embedded in paraffin and sectioned at a thickness of 3 µm. Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ in PBS at room temperature for 5-10 min, and a standard streptavidin/peroxidase (secondary antibody goat anti-Mouse IgG; cat ab6785, Abcam; incubated at 37°C for 15 min; dilution, 1:1,000) complex method (SP) was used for immunostaining. A rabbit anti-CIRBP polyclonal antibody (incubated at 37°C for 1.5 h; cat. no., 10209-2-AP; dilution, 1:100; ProteinTech Group, Inc.) was used to assess the presence of CIRP. Following counterstaining (37°C for 1 min) of the cell nucleus with hematoxylin, the slides were observed under an optical microscope (x100). One kidney peri-cancerous tissue obtained from surgery was used as a positive and negative control (without primary antibody). Immunopositivity criterion for CIRP was defined as the presence of ≥1 stained cell with a clearly outlined cytoplasm.
Flow cytometry. For cell cycle phase analysis, cells were fixed in 70% ethanol at room temperature and at 5% for 30 min at -20°C followed by washing with phosphate-buffered saline and centrifugation (4°C for 5 min at 200 x g). In order to label DNA, pellets were resuspended in Vindelöv solution (3.5 µM Tris-HCl (pH 7.6), 10 mM NaCl, 10 µg/ml propidium iodide (PI), 20 µg/ml RNase and 0.1% v/v NP40) and were incubated in the dark for 20 min on ice. Cell cycle analysis was performed by flow cytometry analysis using a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), counting in total 1x10^6 cells. Gating of G0/G1 and G2/M populations was performed manually using the CellQuest Pro software (version 5.1; BD Biosciences). Annexin V-fluorescein isothiocyanate (FITC) and PI (BD Pharmingen; BD Biosciences) double staining was performed to discriminate and quantify cell death induced by apoptosis or necrosis. Cell density was adjusted to 1x10^6/ml. After the cells had been resuspended twice in 100 µl binding buffer (Annexin V FITC Apoptosis Detection Kit; BD Pharmingen; BD Biosciences; cat no. 556547), 10 µl Annexin V-FITC was added with gentle mixing and the cells were incubated on 4˚C for 15 min. The labeled cells were then added with 5 µl PI in 300 µl binding buffer and were gently mixed prior to immediate analysis with a FACScan flow cytometer. A total of 10,000 cells were analyzed per sample. Data were recorded and processed using the CellQuest software.

Statistical analysis. Statistical analyses were performed using SPSS software 17.0 (SPSS, Inc., Chicago, IL, USA) and all data are presented as the mean ± standard deviation. One-way analysis of variance, followed by Tukey's post-hoc test, or Student's t-test was used to test for statistical significance. P<0.05 was considered to indicate a statistically significant difference.

Results

CIRP is overexpressed in RCC, particularly in clear cell RCC. CIRP was overexpressed in RCC tissues compared with expression in peri-cancerous tissues (Fig. 1), and that the majority of cases (92.30%) were clear cell RCC. Additionally, it was observed that CIRP was primarily located in the cell cytoplasm in RCC. To further explore the differential expression of CIRP between RCC and peri-cancerous tissues, western blotting was performed.

The difference in expression of CIRP mRNA between tumor samples and peri-cancerous tissues was compared using qPCR. The results demonstrated that CIRP was overexpressed in tumor samples (P<0.05; Fig. 2). A total of 11/14 (78.57%) clear cell RCC samples exhibited positive CIRP expression, and the total CIRP mRNA level in the tumor samples was significantly higher than that in the peri-cancerous tissues (P<0.05). Western blot analysis also demonstrated that the level of CIRP protein in the tumor samples was significantly higher than that in the peri-cancerous tissues (P<0.05; Fig. 3).

siRNA-mediated downregulation of CIRP impairs cell proliferation. The expression of CIRP was significantly decreased 3 days after transfection with 100 nM siRNA (total oligo concentration, 150 nM; P<0.05; Fig. 4). Additionally, knockdown of CIRP inhibited the proliferation of the RCC 786-0 cell line. Furthermore, a WST-1 assay was used to detect cell proliferation 1-5 days after transfection, and the results demonstrated that the cell proliferation of the siCIRP group was significantly decreased, compared with that of the siNC group (P<0.05; Fig. 5A).

Reducing the expression of CIRP enhances the chemosensitivity of 786-0 cells to gemcitabine. Cells were transfected with siRNAs (siCIRP/siNC) prior to being treated with gemcitabine for a further 72 h. Following transfection with siCIRP, the chemosensitivity of 786-0 cells to gemcitabine was enhanced compared with that of the cells transfected with siNC. When the concentration of gemcitabine was between 0.25 and 1.0 nM, this difference was significant (P<0.05) and when the concentration of gemcitabine was >5 nM, no differences were observed between the two groups (P>0.05; Fig. 5B).

CIRP may exhibit little impact on the cell cycle and cell apoptosis. Cells were transfected with siRNAs (siCIRP/siNC) and were cultured for 48 h. Flow cytometry was subsequently used to detect the impact of CIRP on the cell cycle and cell apoptosis. The cell cycle and cell apoptosis of the two groups (siCIRP/siNC) exhibited no significant differences (P>0.05; Table I; Fig. 6).
RCC accounts for ~3% of all adult malignancies. Despite advances in diagnosis, including improved imaging techniques, 20-30% of RCC patients are diagnosed with mRCC. Patients with mRCC exhibit a median survival time of ~13 months and the 5-year survival rate is <10% (17,18).

Immunotherapies (interleukin-2 and interferon-α) were considered to be the standard treatment for mRCC prior to 2005. Since 2005, targeted therapies have involved vascular endothelial growth factor and the mechanistic target of rapamycin in cases where patients with RCC are unable to undergo surgical therapy and these alternatives have been proven to be effective in the treatment of RCC, particularly in the treatment of mRCC. However, 20-25% of patients with metastatic disease, also known as ‘primary resistant’ disease, do not respond to any of the available treatments, leading to a rapidly evolving disease with a poor prognosis. These cases require further attention in order to identify novel effective treatment strategies. The present study aimed to provide novel information regarding adjuvant therapies for these aforementioned cases by studying the effect of CIRP on RCC in vitro.

Since CIRP was first isolated in 1997, a number of studies have demonstrated that it serves important roles in numerous physiological and pathological processes. CIRP may be induced by a number of factors, including mild/cold temperatures, DNA damage and hypoxia (3,19). CIRP may protect spermatogenesis against the DNA damage induced by ultraviolet irradiation (20,21). However, an increasing volume of evidence has indicated that CIRP may be involved in the development of tumors. CIRP was revealed to be overexpressed in oral squamous cell carcinoma (OSCC) and pituitary adenoma, and was demonstrated to be involved in the development of liver cancer (10,12,13).

The present study used RT-qPCR, IHC and western blot analysis to detect the expression of CIRP in RCC tissues. The results demonstrated that there were marked differences between expression levels in RCC and peri-cancerous tissues. A total of 13/17 (76.50%) tumor samples exhibited positive IHC staining for CIRP, compared with the peri-cancerous tissues (P<0.05). CIRP, cold-inducible RNA-binding protein; NC, negative control; s, standard deviation.

Table I. Cell cycle in two groups following transfection for 48 h (% ± s).

| Group    | G0/G1         | S            | G2/M         |
|----------|---------------|--------------|--------------|
| siCIRP   | 71.81±0.79    | 24.31±0.83   | 4.04±0.72    |
| siNC     | 70.34±1.36    | 24.65±1.70   | 5.02±0.73    |

There was no significant difference between the two groups (P>0.05). si, small interfering RNA; CIRP, cold-inducible RNA-binding protein; NC, negative control; s, standard deviation.

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The present study used RT-qPCR, IHC and western blot analysis to detect the expression of CIRP in RCC tissues. The results demonstrated that there were marked differences between expression levels in RCC and peri-cancerous tissues. A total of 13/17 (76.50%) tumor samples exhibited positive IHC staining for CIRP, compared with the peri-cancerous tissues (P<0.05) and the most common pathological type was clear cell RCC (92.30%). Furthermore, 11/14 clear cell RCC samples (11/14, 78.57%) exhibited positive CIRP mRNA levels. These observations were also confirmed in CIRP protein expression levels, as determined by western blot analysis. IHC staining indicated that CIRP was located in the cell cytoplasm. Previous studies have revealed that CIRP was located in the cell cytoplasm in OSCC and endometrial carcinoma (5), and in the cell cytoplasm and cell nucleus of healthy testicular cells (2). The fact that CIRP is located in different organs in different tumors may indicate its different functions. Solid tumors, including RCC, require a rich blood supply, which may result in an increase in the temperature of the tumor.
tissue and, in turn, the downregulation of CIRP. However, the microenvironment of every tumor cell was considered to be severely hypoxic, which may induce overexpression of CIRP. The results of the present study demonstrated that CIRP was overexpressed in RCC (particularly in clear cell RCC).

CIRP mRNA has been revealed to be constitutively expressed in K562, Hep-G2, NC65, HeLa, T24, TERA2 and NEC8 cells (22), and to be involved in ovarian carcinoma (14), testicular carcinoma (23) and prostate cancer (15). In the present study, CIRP was revealed to be constitutively expressed in the clear cell RCC 786-0 cell line. Zeng et al (15) demonstrated that downregulation of cold shock protein genes (including CIRP and RBM3) impairs prostate cancer cell survival and enhances chemosensitivity. A similar phenomenon was observed in the 786-0 cell line in the present study. Furthermore, the present study demonstrated that knockdown of CIRP may inhibit the proliferation of the 786-0 cell line. Additionally, a WST-1 assay was used to detect the cell proliferation ability 1-5 days after transfection, and the results demonstrated that cell proliferation ability in the siCIRP group was significantly decreased (P<0.05), compared with that in the siNC group. In addition, the chemosensitivity of 786-0 cells to gemcitabine was enhanced in the siCIRP group compared with that in the siNC group. When the concentration of gemcitabine was >5 nM, no differences were observed between the two groups.

Due to the fact that the 786-0 cells had become damaged, continually increasing the drug concentration did not cause the cell survival rate to change significantly.

Xun et al (24) demonstrated that CIRP may regulate the cell cycle regulator cyclin E1, which is aberrantly expressed in a variety of human cancer types and is associated with a poor outcome in breast cancer. In the present study, CIRP had little impact on the cell cycle and cell apoptosis. Cells were transfected with siCIRP or siNC, and were cultured for 48 h, prior to flow cytometry being used to detect the impact of CIRP on the cell cycle and cell apoptosis. No significant differences were observed in the cell cycle or cell apoptosis between the two groups (siCIRP/siNC). Therefore, it was concluded that CIRP may inhibit cell proliferation through other signaling pathways, rather than by altering cell cycle or cell apoptosis.

Zeng et al (15) revealed that CIRP knockdown significantly inhibited the activation of p53-p21 proteins and increased γ-H2A.X expression under chemical stress in LNCaP cells, and also enhanced DNA damage and cytotoxic killing in PC-3 cells, in which p53 regulation is deficient. This suggested that CIRP inhibited cell proliferation through the p53-independent pathway. Lujan et al (25) observed decreased proliferation during the lactational switch in mammary glands, but CIRP did not affect apoptosis during mammary gland involution, suggesting a potential in vivo function in suppressing proliferation during a specific developmental transition.

In conclusion, the present study demonstrated that CIRP was overexpressed in RCC tissues and in the RCC 786-0 cell line. The knockdown of CIRP by siRNA inhibited the proliferation of the 786-0 cell line and enhanced the chemosensitivity of these cells to gemcitabine. These observations suggested a potential novel mechanistic pathway for RCC therapy. However, the precise mechanisms underpinning the effect of CIRP downregulation on the inhibition of cell proliferation remain unclear and require further elucidation.

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Availability of data and materials

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

Authors' contributions

GW designed the study. WZ collected clinical specimens. KZ and KJ performed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

Ethics statement and consent to participate

The present study was approved by the Ethics and Scientific Committee of Ningbo Urology and Kidney Hospital and written informed consent was obtained from all participants.

Consent for publication

All patients volunteered to participate in the study and agreed for the data to be used in academic exchange and publication.

Competing interests

The authors declare that they have no competing interests.

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